

SOME PROPERTIES OF THE COMPONENTS OF GLUTATHIONE

REDOX SYSTEM IN RAT LIVER MITOCHONDRIA

by

HILDA TENDISA (MARIMA) MATARIRA, B.Sc., M.Sc.

DOCTOR OF PHILOSOPHY
UNIVERSITY OF EDINBURGH

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FOR

MY HUSBAND CAXTON

and

OUR CHILDREN

TARIRO and GAMUCHIRAI

A B S T R A C T

The removal of hydroperoxide (BuOOH) by rat liver mitochondria is investigated. The system requires high energy equivalents and contains catalytic amounts of GSH. Newly reported carboxylate carrier inhibitors are shown to inhibit both BuOOH reduction and GSH concentration.

The effect of carrier inhibitors was investigated on individual components of the GSH redox system. In the process, properties of the enzymes involved were elucidated. Mitochondrial and cytosolic GP and GR are insensitive to the carrier inhibitors, but show remarkably similar properties in their substrate affinities, suggesting a common origin.

The properties of TH reactions (energy and non-energy) are shown. The enzyme has similar K_m s for the substrates (NADH and $NADP^+$) for both reactions which are independent of pH. However, the two reactions show different pH dependency suggesting the involvement of ionisable groups in its mechanism.

Of importance to this study is the demonstration that carrier inhibitors stimulate the non-energy reaction suggesting an interaction of translocases with the TH protein. A conformational theory is put forward to explain the stimulations which are also obtained by Krebs acids the natural substrates for the carriers. Stimulation of the 'free' enzyme by the krebs acids particularly citrate and isocitrate suggests their involvement in regulating TH activity.

The inhibition of BuOOH reduction by uncouplers and carrier inhibitors is explained by the inhibition of the energy driven TH reactions. The energy dependent reaction is the principal reaction which provides $NADPH_2$ required in the GSH redox system. The inhibitors inhibit by interfering with the energy transfer system associated with the enzyme, ie ATPase complex and the respiratory chain.

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A C K N O W L E D G E M E N T S

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DECLARATION

The work described here was carried out by Hilda Tendisa (Marima) Matarira in the Department of Biochemistry, University of Edinburgh Medical School, between October 1978 and June 1981.

Signed: H. T. Matarira
HILDA TENDISA MATARIRA

ABBREVIATIONSCHEMICAL

ADH	Alcohol Dehydrogenase
ADP	Adenosine-5-diphosphoric acid
ANS	8-anilino-1-naphthalene-sulphonic acid
ANSA	1-Amino-2-naphthol-4-Sulphonic acid
APAD	Acetylpyridine adenine dinucleotide
ATP	Adenosine-5-Triphosphoric acid
ATPase	Adenosine Triphosphatase
BaCl ₂	Barium Chloride
BM	n-Butylmalonate
BSA	Bovine Serum Albumin
BT	1,2,3-Benzenetricarboxylate
BuOOH	Tert-Butyl hydroperoxide
CaCl ₂	Calcium Chloride
αCC	α-cyano-4-hydroxycinnamic acid
Cl ⁻	Chloride ions
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
CoASSG	Disulphide of coenzyme A and glutathione
CuOOH	Cumene hydroperoxide
Cys	Cysteine
DCPIP	Dichlorophenolindolphenol
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
EDTA	Ethylenediaminetetra-acetic acid
EGTA	Ethyleneglycolbis(amino ethylether)-tetra-acetic acid
ETOH	Ethanol
FAD	Flavine Adenine dinucleotide
FC	DL-Fluorocitrate
FCCP	Carbonylcyanide-p-trifluoromethoxyphenylhydrazone
Glu	Glutamate
Gly	Glycine
GP	Glutathione peroxidase
GR	Glutathione Reductase
GSH	Glutathione
GSSG	Oxidised Glutathione
GSSO ₃	Glutathione sulphite

CHEMICAL

HCl	Hydrochloric acid
H ₂ O ₂	Hydrogen Peroxide
KCl	Potassium Chloride
KCN	Potassium cyanide
KH ₂ PO ₄	Potassium dihydrogen phosphate
K ₂ HOP ₄	DiPotassium hydrogen phosphate
KSCN	Potassium thiocyanate
LDH	Lactic dehydrogenase
MB	p-hydroxymercuri-benzoate
MgCl ₂	Magnesium Chloride
NAD ⁺	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide, reduced
NADP ⁺	Nicotinamide Adenine Dinucleotide phosphate
NADPH	Nicotinamide Adenine Dinucleotide phosphate, reduced
NaCl	Sodium Chloride
NEM	N-ethylmaleimide
NaH ₂ PO ₄	Sodium dihydrogen phosphate
NaOH	Sodium hydroxide
O ₂	Oxygen molecule
O ₂ ⁻	Superoxide ion
OH ⁻	Hydroxide ion
OH•	Hydroxyl radical
PCoA	Palmityl Coenzyme A
PEP	Phosphoenol pyruvate
PhS	Phenylsuccinate
Pi	Inorganic phosphate
PP	β-Phenylpyruvate
PT	1,2,3-Propanetricarboxylate
QA	Quinacrine
ROH	Organic alcohol
ROOH	Organic hydroperoxide
Tris	Tris (hydroxymethyl aminomethane)
TH	Nicotinamide Nucleotide Transhydrogenase

ORGANISM

E. coli. Escherichia Coli

SYMBOL

$\Delta \psi$

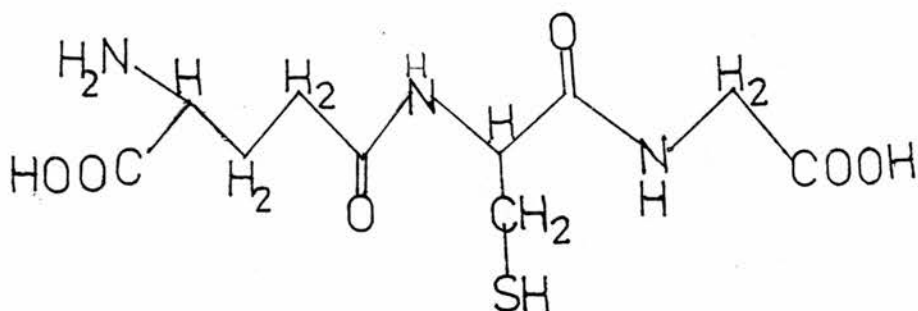
Membrane Potential

ΔpH

pH gradient

CHAPTER 1INTRODUCTION AND LITERATURE REVIEW1. GLUTATHIONE1.1 Distribution and Structure

Glutathione (GSH) a physiologically important tripeptide was isolated and found by Hopkins (1921-1929) in tissue extracts. It is a non-protein thiol with the following structure:



The molecule contains an SH group flanked on either side by two aminoacids. The structure of this tripeptide is unusual in that the amino terminal peptide bond utilizes the γ -carboxyl moiety of glutamate which makes it resistant to hydrolytic action of proteases and amino peptidases (McIntyre et al 1980).

GSH is a ubiquitous component of animal tissues and the most abundant sulphhydryl compound in cells. It is present in most animal cells' cytoplasm at a concentration of 2 to 10mM per cell (Metzler 1977) and (Moron et al 1979). Most recently it is reported to occur in extracellular fluids although in much smaller quantities (Grafström et al 1980) and (McIntyre et al 1980).

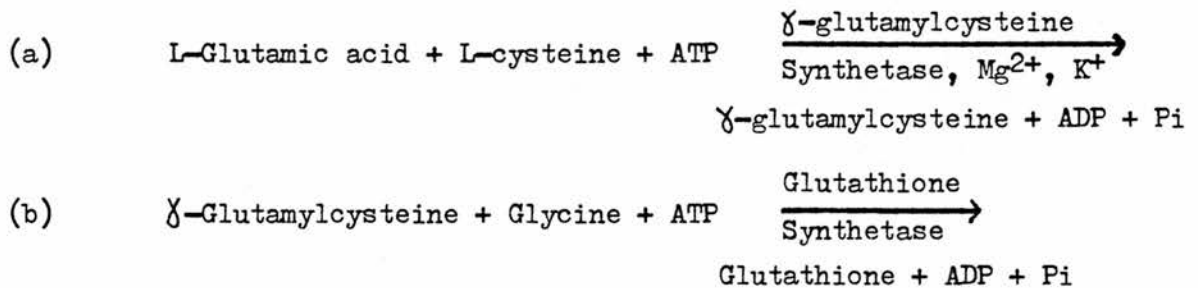
Glutathione is found not only as the free thiol (GSH), but also as the disulphide (GSSG) derived from it by dehydrogenation, and also as various mixed disulphides, especially those formed by combination with the SH groups of proteins (Protein-SSG). The proportions of glutathione in these various forms show diurnal variations (Jaeger et al 1973) and also depend on such factors as body weight (Massey et al 1965). It/

It is not surprising therefore to find data in the literature that is hardly comparable as they were seldom obtained under identical conditions.

Although the bulk of the non-protein thiol is found in cell cytoplasm, it is also found in mitochondria. GSH forms at least 70 to 90% of the mitochondrial thiol, with a GSH:GSSG of about 6:1 and in some cases, 20:1 (Jocelyn and Kamminga 1974) and (Jocelyn 1972).

1.2 Metabolism

Animals, plants and bacterial cells have the capability to synthesize glutathione in the cytoplasm, from free amino acids, as well as maintain it predominantly in the reduced form despite its tendency to oxidize (White et al 1973). The tripeptide is formed in two steps catalyzed by separate enzymes (Meister 1974);



Both steps require energy (ATP) to take place.

The turnover of glutathione in animals is high, especially in liver, kidney, pancreas and skeletal muscle (Griffith and Meister 1979). The half life of renal glutathione has been found to be about 30 minutes (Sekura and Meister 1974), while glutathione content of the liver is divided into two pools, one with a half life of 1.7 hours and the other of 28 hours (Tateishi and Higashi 1978). The route of GSH degradation is at present a subject of much controversy. GSH is largely degraded extracellularly by the kidney by the two enzymes, γ glutamyltranspeptidase and α peptidase to free amino acids (Section 1.3c). The amino acids are then re-absorbed and distributed by their respective transport mechanisms (Meister et al 1970) and (McIntyre and Curtloys 1980).

1.3/

1.3 Properties and Functions

GSH and GSSG participate in a wide variety of oxidation-reduction reactions extensively documented by Jocelyn (1972). This is made possible by the fact that GSH has a standard redox potential (E_0') of $-0.24V$ (Rost et al 1964) and E_0' of $-SH/SS$ -groups is generally in the range of $(-0.2$ to $-0.4)V$ (Jocelyn 1972). In principle disulphides are reduced by many intracellular substrates capable of reducing NAD^+ while the thiols on one hand can reduce substances of less negative redox potential, eg cytochromes and flavoproteins.

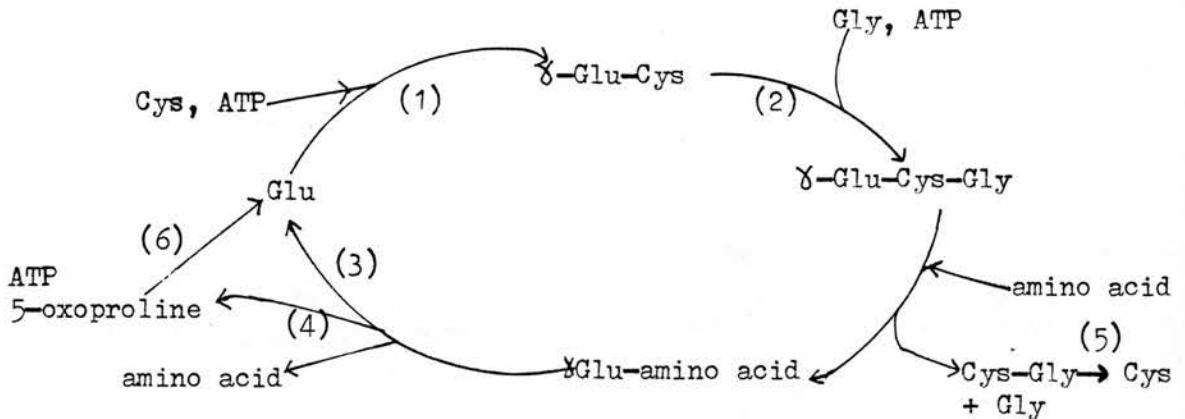
In addition, glutathione has a sulphur atom with a valency of 2 to 6. The sulphur centre can undergo different oxidation states and thus facilitates its involvement in many biological reactions.

The functions of GSH and GSSG depend largely upon the fact that they are involved in numerous reactions. These include alkylations by alkyl halides, additions to double bonds ($C=C$, $C=N$, $C=O$), acylations reactions to give more stable complexes (with metal ions) and affinity for free radicals. The following are some of the major functions of this GSH/GSSG couple in cells:

- (a) As stabiliser: In the oxidation-reduction reactions involving glutathione, it serves as a redox buffer by forming protein disulphides and thus protecting thiol groups of enzymes, membrane proteins, including hormones, which may be susceptible to oxidative attack by reactive metabolites rendering them inactive (Kosower and Kosower 1974), (Kinoshita and Masurat 1957) and (Jocelyn 1972). Consecutive disulphide exchange reactions with GSH or GSSG with proteins can serve to restore the active thiol or disulphide form, whichever is the case (Jocelyn 1972).
- (b) As cofactor: Certain enzymes, for example methylglyoxalase I, DDT dehydrochlorinase and formaldehyde dehydrogenase utilize GSH as a cofactor (Lochmann 1932), (Lipke and Kearns 1959) and (Stittmatter and Ball 1955), respectively. Hormones/

Hormones containing SS groups, eg insulin, vassopressin and oxytocin involve GSSG during their conversion to disulphide catalytic active forms (White et al 1973).

- (c) In amino acid transport: GSH is involved in amino acid transport by donating its γ -glutamyl group. This process known as the γ -glutamyl cycle, proposed by Meister et al (1970) is primarily based in the kidney. The reactions constituting this cycle are as follows:



The cycle involves six enzymes indicated as:

- 1 γ -glutamylcysteine Synthetase;
- 2 glutathione synthetase;
- 3 γ -glutamyl transpeptidase;
- 4 γ -glutamyl cyclotransferase;
- 5 peptidase; and
- 6 5-oxoprolinase (Meister et al 1970)

This sequence involves synthesis and degradation of glutathione coupled to uptake and release of amino acids, with γ -glutamyl transpeptidase functioning in translocation and glutathione as the carrier of the amino acids. The significance of this cycle is yet to be established considering the fact that three molecules of ATP are required per molecule of amino acid translocated (White et al 1973).

(d)/

- (d) As reductant/oxidant: GSH and GSSG have a special significance in that they are linked to the oxidation reduction of NADP(H₂) via the specific enzymes glutathione reductase (EC.1.6.4.2) found in both the cytoplasm and mitochondria (Flohe' and Schelgel 1971); the NADP⁺ linked isocitrate dehydrogenase (EC.1.1.1.42.) in the cytoplasm; the NADP⁺ linked glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase involved in glucose metabolism in the cytoplasm and also to the particulate enzyme pyridine dinucleotide transhydrogenase (EC1.6.1.1.). It is in connection with this property that the work described here was undertaken (see Section 5).
- (e) In detoxication: Glutathione plays a central role in detoxication of noxious substances, in particular removal of hydroperoxides and reactive xenobiotics. Detoxication of such dangerous metabolites involves a group of enzymes called glutathione-S-transferases and glutathione peroxidase which utilize organic hydroperoxides among other metabolites and GSH as substrates (see also Section 3 and introduction).
- In addition, SH groups have a capacity to 'collect' free radicals from other groups, a property which renders most SH enzymes susceptible to free radical inactivation. Glutathione plays an important role in removing free radicals in cells (Rink 1974) which would otherwise cause chain propagating, radical reactions and thus destroy the integrity of membranes and proteins, resulting in cell death. It is this function of glutathione in particular to its involvement in hydroperoxides removal that work described in this thesis was initiated (see also Section 5).

2. GLUTATHIONE PEROXIDASE

2.1 Occurrence and Properties

Glutathione peroxidase (EC.1.11.1.9) is widely distributed in most plants and animal cells. The/

The enzyme catalyzes the reduction of hydroperoxides in the reaction. $\text{ROOH} + 2\text{GSH} \longrightarrow \text{ROH} + \text{GSSG} + \text{H}_2\text{O}$ where ROOH can either be hydrogen peroxide (H_2O_2) or organic hydroperoxides. The most important hydroperoxides in cells being those of unsaturated fatty acids (Tappel 1978).

The enzyme was first isolated from bovine erythrocyte in 1957 by Mills. Ten years later, Little and O'Brien (1967) reported the rat liver enzyme. It was not until 1971 (Flohe) that the mitochondrial enzyme was reported. The enzyme was purified from bovine erythrocytes (Flohe 1971), human erythrocytes (Awasthi et al 1975), and from rat liver cytosol (Stults et al 1977) and it shows many similar properties. It is a selenium containing enzyme (Rostruck et al 1973), an element demonstrated by Schwartz in 1961 to be necessary in order to prevent liver necrosis. Selenium is bound to cysteine forming a selenocysteine moiety in the active site of the enzyme (Forström et al 1978). The enzyme has four identical subunits (Flohe 1976) and (Stults et al 1977) and an approximate molecular weight of 80,000.

In animal cells, glutathione peroxidase activity is high (75%) in the cytoplasm with 18% of the activity in the mitochondria (Stults et al 1977). Very low activities are found in nuclei, peroxisomes and microsomes (Chance et al 1978).

A non-selenium glutathione peroxidase activity also takes place in animal tissues. This activity is due to glutathione-S-transferases A, B and C (Prohaska et al 1977). This group of enzymes can only utilize organic hydroperoxides, but not hydrogen peroxide. Their contribution to hydroperoxides removal in mitochondria is relatively insignificant except maybe where glutathione peroxidase is deficient (Burk and Lawrence 1978).

2.2 Source of hydroperoxides

Hydroperoxides production in living organisms is a consequence of oxygen metabolism. Oxygen in its ground state has available to it two empty orbitals which can receive electrons upon excitation by energy from exergonic reactions that may take place in the cell or simply from radiation. As oxygen undergoes reduction (enzymic or non-enzymic) reactive intermediates for example the superoxide anion (O_2^-) or singlet oxygen (O_2^{I}) are formed. This/

This leads to the production of unstable and reactive hydroxyl radical (OH^\bullet) and hydrogen peroxide (H_2O_2) (Quagliariello et al 1974).

The hydroxyl radical is a very potent oxidant which reacts with cell constituents, forming chain propagating radical reactions which are incompatible with the delicate molecular fabric of the cells. The superoxide anion and hydrogen peroxide oxidize membrane lipids including those of mitochondria forming lipid hydroperoxides.

Oxygen-utilizing organisms must therefore face the threat posed by intracellular generation of these very reactive intermediates.

2.3 Removal of hydroperoxides and reactive oxygen intermediates

Enzymic defense mechanisms have evolved to deal with these reactive compounds:

- (a) Superoxide dismutase converts the superoxide radical to hydrogen peroxide by the reaction $\text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \longrightarrow \text{H}_2\text{O}_2 + \text{O}_2$. This enzyme is ubiquitous in aerobic cells, absent only in obligate anaerobes (White et al 1973).
- (b) Catalases plays an important role in controlling H_2O_2 levels chiefly in peroxisomes. The enzyme catalyzes the reaction: $\text{H}_2\text{O}_2 + \text{H}_2\text{O}_2 \longrightarrow \text{O}_2 + 2\text{H}_2\text{O}$. Catalase activity is present in nearly all animal cells and organs; the liver, erythrocytes and kidney being the rich sources (Chance et al 1978).
- (c) Glutathione peroxidase (see 2.1) together with catalase fulfill the important function that of maintaining H_2O_2 at low levels. Glutathione peroxidase is capable of utilizing both H_2O_2 and organic hydroperoxides in the cytoplasm and mitochondria matrix, (Flohe' and Schelgel 1971).

Compartmentation of catalase (in peroxisomes) and glutathione peroxidase (in cytoplasm and mitochondria) facilitates their effective control of hydrogen peroxide and organic hydroperoxides at those sites, (Poole 1975), (Boveris et al 1973) and (Section 5).

The/

The organisms that have not evolved these defenses against the potential toxicity of oxygen are restricted to anaerobic environment (Smith et al 1973).

2.4 Catalysis and Specificity of Glutathione Peroxidase

Glutathione peroxidase has been studied chiefly in erythrocytes (Flohe 1971) and liver cytosol (Stults et al 1977), but very little in liver mitochondria. It has the four selenocysteine groups in the active site (Stadman 1980). It is suggested by Flohe et al (1979) that the enzyme undergoes cyclic oxidation (by hydroperoxides) and reduction (by GSH) states which involve this selenocysteine moiety. From X-ray analyses, it is known that histidine and an arginine residue are near the selenocysteine residue (Stadman 1980).

The mechanism of catalysis of this enzyme is not yet fully understood. Several workers have suggested a ping pong mechanism, following their finding that plotting double reciprocal plots of $\frac{1}{V}$ versus $\frac{1}{S}$ where V is initial velocity and S the substrate concentration, parallel lines are obtained (Flohe et al 1972), Günzler et al 1972) and (Chill et al 1975).

2.5 Functions of Glutathione Peroxidase

As already mentioned (Section 2.3) glutathione peroxidase's main function is to control the levels of H_2O_2 and lipid hydroperoxides that may form in cell cytoplasm and membranes including those of mitochondria (Flohe and Zimmerman 1974), (Pryor 1973) and (Neubert et al 1962).

A broadly based function of glutathione peroxidase is its role in manipulating the oxidation-reduction potential of the cell by modulating GSH: GSSG and NADH: NADP(H) ratios and thus regulate the enzymes dependent on these substances for activity (see also Section 5).

3.0 /

3. GLUTATHIONE REDUCTASE

3.1 Distribution and Properties

A heat labile system capable of reducing GSSG in liver cytosol was discovered by Hopkins and Elliot in 1931. Mann in 1932 linked this GSSG reduction to glucose oxidation by NADPH₂ the product of the pentose phosphate pathway. Glutathione reductase (EC.1.6.4.2) is the enzyme involved in GSSG reduction by NADPH₂. It was demonstrated in plants in 1951 by Vennesland and Conn and by Mapson and Goddard. Rall and Lehninger (1952) demonstrated it too in rat liver cytosol. Flohe and Schelgel (1971) reported the presence of this enzyme in rat liver mitochondrial matrix constituting a third of the activities found with the cytosolic enzyme. Since then few workers have been concerned with the hepatic enzyme in particular the mitochondrial enzyme.

The rat liver cytosolic enzyme has been purified recently (Carlberg and Mannervik 1975). Some of the properties of the enzyme have been worked out. The enzyme is a dimer with identical subunits and a molecular weight of 100,000 to 125,000 (Zanetti 1979).

3.2 Catalysis

Glutathione reductase from different sources is a flavoprotein with an active disulphide centre. Human erythrocyte enzyme has been crystallized and X-ray diffraction analysis made, by Schul et al (1978). This group of workers has been able to show that the redox-active disulphide is in contact with the flavin molecule and that the C-terminal fragment contains a postulated catalytic histidine.

The enzyme has two FAD molecules per molecule. In catalysis, the FAD centre accepts two electrons from NADPH₂ and donates them to GSSG as it is instantaneously reoxidized (Williams 1976). From spectral studies, it has been possible to suggest that the principle features involved during catalysis are, the formation of complexes between the oxidized enzyme and the respective pyridine nucleotide as well as a charge transfer complex between a thiolate ion and a reoxidized flavin molecule (Williams 1976 for review).

In/

In addition to the active disulphide, there are essential thiol groups required in catalysis (Williams 1976).

The enzymes from E.Coli and yeast contain four to five thiol groups per FAD molecule (Williams 1976). These enzymes and that of the rat liver cytosol are inhibited by thiol reagents such as p-chloromercuri-benzoate (Colman and Black 1965).

3.3 Substrate Specificity

The specificity of glutathione reductase for the substrates has been investigated. The rat liver cytosolic enzyme is relatively much more specific for NADPH₂ than the erythrocyte enzyme having only 10% activity with NADH (Mize and Langdon 1962) compared to 20% with the later enzyme (Icen 1967). It is this NADPH₂ specificity of the liver enzyme which is of particular importance to the work described here (see Section 5).

The specificity of the cytosolic rat liver enzyme for the disulphide was also investigated. The enzyme is relatively specific for GSSG having about 15% activity with CoASSG and less than 1% with other disulphides (GSSO₃H or cystine). However, at pH below 7.0 the cytosolic rat liver enzyme has been reported to lose its specificity for both NADPH₂ and GSSG; showing an increase of activity with either NADH₂ or CoASSG (Carlberg and Mannervik 1975).

3.4 Kinetics

Glutathione reductase from rat liver, human serum yeast and E.Coli has an optimal activity around pH 7.0 to 8.0. The K_m for GSSG, ranges from 50 μM for the rat liver cytosolic enzyme (Mize and Langdon 1962) to that of 1400 μM in the E.Coli enzyme (Asnis 1954). The K_m for the NADPH₂ was reported to be 3 μM in the rat liver cytosolic enzyme to 37 μM in the bacterial enzyme by the workers mentioned above. The differences in the affinities of the enzyme from different sources maybe a reflection of their different properties.

3.5/

3.5 Functions

The main function of glutathione reductase in cells is to keep glutathione in its reduced state. In most conditions the ratio of GSH:GSSG is very high (Section 1.1). The functions of this enzyme, therefore, are linked with those of GSH (Section 1.3) and (Meister 1975).

Certain haematological diseases like haemolytic anaemia are correlated to GSH deficiency which maybe a consequence of a genetic defect resulting in glutathione reductase deficiency. Persons with such a defect also develop this disease when exposed to drugs that reduce its activity (Benöhr and Waller 1975).

4. THE PYRIDINE DINUCLEOTIDE TRANSHYDROGENASE

4.1 Isolation, Occurrence and Structure

Pyridine dinucleotide transhydrogenase (EC1.6.1.1) is widely distributed in bacteria, yeast and mammals. It is a particulate enzyme, bound in the inner side of the inner mammalian mitochondrial membrane, with its substrate binding sites facing the matrix (Lee and Ernster 1966). It was first reported by Colowick and co-workers (1952) who isolated it from bacteria. The first report of the mammalian enzyme was made by Kaplan and co-workers (1953).

The mitochondrial pyridine dinucleotide transhydrogenase catalyses the reversible transfer of hydride ions between the oxidized and reduced forms of intramitochondrial NADH and NADP in the reaction:

$$\text{NADH} + \text{NADP}^+ \rightleftharpoons \text{NAD}^+ + \text{NADPH} .$$

The enzyme is an integral protein requiring phospholipids for activity (Rydström et al 1976a) a fact that has made numerous attempts to purify it to homogeneity unsuccessful (Kaufman et al 1961), (Kaplan 1972), (Rydström et al 1975) and (Anderson and Fisher 1978). The beef heart enzyme has recently been purified to homogeneity (Rydström 1979) and (Earle et al 1980). It is a single polypeptide of molecular weight 90,000 to 120,000. The enzyme has no flavin or a metal prosthetic group (Rydström 1977).

4.2 Relationship with energy transfer

The enzyme from beef heart or rat liver has been shown to be energetically coupled to the respiratory chain and also to the ATPase (Rydström 1977 for review) and (Danielson and Ernster 1963). The purified transhydrogenase has now been incorporated into liposomes and reconstituted (Rydström 1979). Rydström (1979) has demonstrated that the beef heart enzyme is coupled to proton translocation during transhydrogenation between NADPH and NAD^+ and that between NADH and NADP^+ is driven by the proton gradient.

Transhydrogenation between NADH and NADP^+ is very slow in the absence of an energy source, it is accelerated about ten fold by addition of ATP or by succinate oxidation (Danielson and Ernster 1963) and (Galante et al 1980).

The equilibrium constant for the non-energy reaction of the beef heart, rat liver and other sources (Section 4.1) is approximately 1 (Kaplan et al 1953) and (Rydström 1977). Energization dramatically favours the forward reaction, ie $\text{NADH} + \text{NADP}^+ \longrightarrow \text{NAD}^+ + \text{NADPH}$ with an approximate equilibrium constant above 500 (Rydström 1977). Energization increases both the velocity and equilibrium constant of the reaction. It is with this reaction that I am primarily concerned with in this thesis.

The mechanism by which energy is transferred between the ATPase or the respiratory chain and the enzyme is not yet known. A number of hypotheses have been put forward and have been discussed below (a) to (d).

- (a) Chemical Hypothesis: This hypothesis postulated by Lipmann (1946) and Slater (1953) involves energized forms of substrates ($\text{NADH}\sim$ or $\text{NADP}\sim$) in the reaction. The limitations of this mechanism is that no such substrates have yet been isolated.
- (b) Conformational Hypothesis: Rydström and co-workers (1972) suggested that the transhydrogenase is a trans-membrane protein exposed both to the matrix and cytoplasmic sides of the inner mitochondrial membrane. But we know that the enzyme can not be assayed directly in mitochondria (Section 4.3). These/

These workers suggest that the enzyme undergoes conformational states coupled to the generation of proton gradient and membrane potential in the reverse reaction ($\text{NADPH} + \text{NAD}^+ \longrightarrow \text{NADH} + \text{NADP}^+$) and utilising electrochemical gradients from either ATP hydrolysis or succinate oxidation in the forward reaction. Rydström (1974 and 1979) has since demonstrated the involvement of electrochemical gradients during transhydrogenation.

- (c) Chemiosmotic Hypothesis: The theory was put forward by Mitchell (1966 and 1972) who proposed that the transhydrogenase enzyme reaction acts as a proton pump by carrying protonated species of the substrates across the inner mitochondrial membrane. This led to Skulachev (1970, 1971 and 1972) to investigate the involvement of electrochemical gradients in this reaction. He demonstrated that there are changes in membrane potential accompanying the transhydrogenase reaction. This has recently been demonstrated with the enzyme incorporated in Liposomes (Rydström 1979).
- (d) The Electromechano Chemical Hypothesis: This model was proposed by Green and Ji (1972) who suggested that the energy linked transhydrogenase involves a respiration or ATP-dependent polarization of the 'transhydrogenase Supermolecule', which is discharged in the course of the transhydrogenase reaction.

It is evident, therefore, that as yet, workers in this field are not in agreement over the nature of energy transfer between the transhydrogenase and respiration or ATP hydrolysis. This justifies more work in this field.

4.3 Transhydrogenase Preparations and Assays

It is well known that mitochondrial membranes are selectively permeable to various substance. The inner membrane, is impermeable to NADP(H) or NAD(H) although it quite readily takes up such metabolites as ATP, succinate, pyruvate and citrate among others. Since/

Since the transhydrogenase enzyme is bound in the inner membrane of the mammalian mitochondria (Section 4.1), it is difficult to measure with confidence the transhydrogenase reaction in whole mitochondria. The transhydrogenase reaction can only be estimated in intact mitochondria by measuring changes of intrinsic absorption by fluorescence of endogenous reduced nicotinamide nucleotides by determining them (nicotinamide nucleotides) in subsequently lysed aliquots (Klingenberg and Slenczka 1959) and (Estabrook et al 1963).

These types of assays are unsatisfactory due to the fact that no suitable substrate regenerating systems can be used with intact mitochondria and in addition various interfering dehydrogenation (NADH or NADP⁺ linked) also take place (Rydström 1976).

In order to circumvent this permeability barrier, mitochondrial membranes or cell walls in the case of bacteria have to be disrupted by either mechanical force (eg sonication) or by use of detergents (Kaplan et al 1953). Disruption of mitochondria by means of sonication has proved to be an excellent method for preparing submitochondrial particles mainly turned inside out and which carry out efficient respiration (Killey and Bronk 1958) and transhydrogenation (Rydström 1977).

Several assays of this enzyme have been reported. Identical methods are used to measure the reaction from different sources using either natural substrates or substrate analogs.

With the natural substrates, it is not possible to measure the reaction directly since both the reactants and products have identical absorption spectra (see methods, Chapter 2). Enzymic regenerating systems are available which keep one or both substrates constant and makes it possible to follow the rate of formation of the products (Kaplan et al 1953) and (Danielson et al 1963).

Nicotinamide nucleotide analogs are available which can substitute the reactants to give products that have different spectral properties, and thus make it easier to follow the reaction. Nicotinamide/

Nicotinamide analogs of NAD^+ , ie acetyl-pyridine- NAD^+ , and of NADP^+ , ie thio NADP^+ may be used in the absence of regenerating systems (Rydström 1977). (See methods, Chapter 2).

Energy transfer assays: There are several methods in the literature now available to study the interaction of the transhydrogenase enzyme and the energy pool of membrane fragments or reconstituted systems. Membrane potential of the fragments can be followed by fluorescence probes, for example 8-anilino-naphthalene-1-sulphonate which distributes itself in membranes depending on the magnitude of the membrane potential (Azzi et al 1969), (Lee 1971) and (Rydström 1974). Certain synthetic anions, eg phenyldicarbaundecaborane (PCB^-) also accumulate in submitochondrial particles in an energy dependent manner (Grinius et al 1970). Other fluorescence probes are available which are used to measure pH gradients, eg quinacrine (Lee 1971) and (Storey et al 1980). The use of pH microelectrode was employed by Earle and Fisher (1980b) in an attempt to measure directly proton translocation associated with the transhydrogenase.

In 1971, Van de Stadt and co-workers succeeded in demonstrating that the reduction of NAD^+ by NADPH (the reverse reaction) is accompanied by ATP synthesis. These workers made use of a high nicotinamide nucleotide potential ($[\text{NADPH}][\text{NAD}^+]/[\text{NADP}^+][\text{NADH}]$) made possible by regenerating both NAD^+ and NADPH . Other workers since then, were able to show that NAD^+ reduction is accompanied by generation of membrane potential (Dontsov 1972) and (Section 4.2).

4.4 Substrate and Stereospecificity

The enzyme has a broad specificity for its substrates and as a result it is possible to use substrate analogs to assay it (Section 4.3). Some of the substrates for this enzyme are as follows:

- (a) Natural substrates pair: NADH-NADP^+ ; $\text{NAD}^+-\text{NADPH}$ (Section 4.1)
- (b) Substrate-Analog pair: $\text{NADH-deamino NAD}^+$; NADPH-thio NADP^+ ; $\text{NADPH-acetyl-pyridine-NAD}^+$; NADPH-DCPIP and $\text{NADH-acetylpyridine-NAD}^+$ (Rydström et al 1976) and (Galante et al 1980). The use of substrate analogs is limited by the fact that there are other enzymes that may use them as substrates in impure preparations; for example, transhydrogenation between NADH and acetyl-pyridine- NAD^+ may be due to NADH dehydrogenase (Gremana et al 1965).

The enzyme is, however, stereospecific as regards the hydride ion of its natural substrates. Lee et al (1965) and Griffiths et al (1966) demonstrated that the hydride transfer between NAD(H) and NADP(H) occurred without exchange with the hydrogen atom of the surrounding water phase. The enzyme from certain bacteria, eg Pseudomonas fluorescens and Pseudomonas aeruginosa are BB specific; that is, they are specific for the 4B hydrogen of both NADH and NADPH (Hoek et al 1974). The mammalian enzyme, and that from E.Coli and Robustrum rubrum are stereospecific for 4A hydrogen of NADH and 4B hydrogen of NADPH thus AB specific (Rydström et al 1976b).

4.5 Mechanism and Kinetics

Much of the work described in the literature, on the enzyme's mechanism and kinetics, has been largely done on the beef heart enzyme. Rydström and co-workers (1971 and 1972) reported that the enzyme's reaction proceeds by a Theorell-Chance mechanism based on their findings that linear and convergent double reciprocal plots of initial velocities versus substrate concentration were obtained. From their product inhibition experiments, they came up with a conclusion that there are separate binding sites for NAD(H) and NADP(H) based on the findings that there was a competitive relationship between the oxidized and reduced form of the same nicotinamide nucleotide; but a noncompetitive relationship between NAD^+ and NADP^+ and NADH and NADPH. These workers assayed the enzyme activities using natural substrates, in the presence of a suitable regenerating system in order to keep one of the substrates constant.

Another group (Phelps et al 1980) proposed a random mechanism (Danziel 1958) first put forward by Hanson (1979) who suggested it for the E.Coli enzyme. These findings are based on the findings that non-linear Lineweaver-Burk plots were obtained. It may be important to note that Phelps' group (1980) was studying the transhydrogenase reaction not with natural substrates, but using substrate-analog pairs: NADH-APAD; NADH-thio NADP^+ ; NADPH-APAD and NADPH-thio NADP^+ .

At/

At neutral pH, it was reported that reduction of NADP^+ is about 5-fold slower than that of NAD^+ , in the transhydrogenase reaction (Rydström et al 1976b), with maximal activities at pH 5.5 and 7.0 respectively, for the non-energy reaction. Addition of an energy source (ATP or succinate) alters dramatically the maximal velocity, equilibrium constant and debatably the affinity for its substrates (Lee and Ernster 1964), (Rydström's review 1977) and (see also discussion Chapters 4 and 5).

Surprising enough only a few workers (see discussion Chapters 4 and 5) studied the liver enzyme. Many properties of this enzyme in rat liver have not been studied fully up to now. The liver enzyme has not yet been purified to homogeneity.

4.6 The Catalytic sites

A variety of specific and non-specific inhibitors of the transhydrogenase enzyme have been reported and reviewed (Rydstrom 1977). Unspecific inhibitors include lipid removing detergents, organic solvents and phospholipases (Kaplan 1953) and (Rydström 1976b). The enzyme is inhibited by metal ions such as manganese (Andreoli et al 1964) with magnesium preferentially inhibiting the non-energy reduction of NAD^+ by NADPH (Rydström et al 1970). Thiol reagents, eg p-chloromercuribenzoate and phenyl-mercuriacetate have been reported to inhibit both the energy and non-energy reactions (Humphrey 1957) and (O'Neal et al 1980)

Rhein, an inhibitor of several nicotinamide nucleotide enzymes was found to inhibit the transhydrogenase (Kean et al 1971). Uncouplers and energy transfer inhibitors have been shown to inhibit the energy dependent transhydrogenase selectively. Oligomycin inhibits the ATP driven reaction, but not the reaction supported by succinate oxidation while respiratory inhibitors like KCN inhibit the succinate driven reaction, but not the ATP driven reaction.

Of particular interest are site specific or group specific inhibitors of the transhydrogenase enzyme. Trypsin inhibits this enzyme irreversibly as demonstrated by several workers (Juntti et al 1961) and (Blazyk and Fisher 1975). Inhibition/

Inhibition of trypsin indicates the presence of an essential arginyl residue in the transhydrogenase protein. Djavadi-Chaniani and Hatefi (1975) found that the arginine binding reagent butanedione inhibited the beef heart enzyme. The enzyme from Rhodospirillum rubrum was inactivated by 2, 4-pentanedione and hydroxylamine treatment reversed the inactivation (Jacobs and Fisher 1979) suggesting modification of lysyl residues.

Site specific inhibitors for the beef heart enzyme have been reported by Rydström and co-workers (1971, 1972 and 1973). Of particular interest is the specific inhibitor, palmityl coenzyme A (PCoA) a competitive inhibitor of the NADP(H) site. This inhibitor was reported to increase its potency with increasing its hydrophobic chain, suggestive of a hydrophobic environment in or around the NADP(H) binding site. Other inhibitors reported by the same workers include the 2'- and 3'- substituted adenine nucleotides which showed competitive inhibition with NADP(H) while the adenine nucleotides without such substituents were competitive with respect to NAD(H) (Rydström 1977).

4.7 Functions

The transhydrogenase with particular emphasis on the energy linked reaction, can be regarded to be functionally related to intramitochondrial NADP-linked reactions in supplying reducing equivalents to the metabolism for example, of isocitrate and glutamate (Plaut 1970) and (Klingenberg 1961) for reviews. The enzyme is also linked to mono-oxygen reactions in mitochondria as a hydrogen donor for the 11 β -hydroxylation reaction (Klingenberg and Slenczka 1959). There is still controversy over the role of this enzyme in vivo due to much argument over the redox states of NAD^+ and NADP^+ and the related dehydrogenases.

Other workers regard the enzyme as a component of a fourth coupling site of the respiratory chain which may drive ATP synthesis (Van de Stadt 1971) or generate electrochemical gradients (Skulachev 1971) (Rydström 1979) and (Earle et al 1980b).

The/

The mitochondrial enzyme was suggested to have some function in fatty acid metabolism (Rydström 1972) due to its sensitivity to palmityl CoA. The isolation of a mitochondrial enoyl CoA reductase by Podack and Seubert (1972) was additional supporting evidence for the possible role of the enzyme in fatty acid elongation involving the enoyl CoA reductase.

The possibility that this enzyme maybe an important part of the glutathione redox system in rat liver mitochondria was investigated in this thesis. This was a suggestion put forward by Jocelyn (1978). The enzyme may be strongly linked to the overall mechanism involved in hydroperoxide detoxication in mitochondria.

5. INTRODUCTION TO MITOCHONDRIAL GLUTATHIONE REDOX SYSTEM

It has been found for several years that cells of animals, plants and bacteria contain powerful systems capable of detoxifying many dangerous injected substances or their metabolites.

The systems are comprised principally of the non-protein thiol glutathione (GSH), together with various enzymes which utilize it as substrate or generate it as product. There are many ways in which such systems may operate. They may substitute on the sulphhydryl group of GSH as shown in the general reaction:



catalyzed by a group of enzymes, ie Glutathione-S-transferases, for example, in drug conjugation where RX may be Bromobenzene epoxides (Orrenius et al 1978). Alternatively, the foreign metabolites may be reduced to harmless forms in the reaction:



In the process, GSH is converted to its oxidized form GSSG. The enzymes involved in catalyzing these reactions are glutathione-S-transferase or glutathione peroxidase where XO_2 are organic hydroperoxides. The latter enzyme also catalyzes the reaction when XO is H_2O_2 (see Section 2).

H_2O_2 /

H_2O_2 a harmful metabolic product, is the principal substance which can be reduced by GSH. It can be formed either directly or indirectly from H_2O via the operations of the enzyme superoxide dismutase (Section 2.2). More controversial is the possible reduction of hydroperoxides of lipids which are formed as by-products in oxidative metabolism. It is with this glutathione reductive system with which this thesis is chiefly concerned.

Clearly the system in addition to possible catalysis of the reductions themselves must contain a route for the regeneration of GSH and its oxidized form GSSG. Such a complete system (GSH redox system) was first studied in the cell cytoplasm, especially that of mammalian liver. In addition to GSH universally proposed in the cytoplasm, two other enzymes are involved, glutathione peroxidase (EC.1.11.1.9) and glutathione reductase (EC.1.6.4.2). The reductant for the later enzyme is NADPH, derived from glucose metabolism via the hexose monophosphate way.

Much more recently, it has been found that such a system also occurs in mitochondria, and it is this system about which little is known, which forms the subject of this thesis.

The presence of GSH in rat liver mitochondria was reported in 1972 (Jocelyn). At the same time two enzymes glutathione peroxidase and glutathione reductase were also found to be in the mitochondrial matrix (Flohe' and Schlegel 1971). These three components are separated by the impermeability of the mitochondrial membrane from those found in the cytoplasm, although they are functionally equivalent. A problem peculiar to mitochondria however, is the source of NADPH required for the reduction of GSSG since the NADPH formed in the cytoplasm is not accessible to mitochondria.

The proposed way in which NADPH is formed in mitochondria is by transhydrogenation from NADH via the mitochondrial enzyme nicotinamide nucleotide transhydrogenase (EC.1.6.1.1) (see figure 1). This enzyme was much studied in beef heart mitochondria, but has not been the subject of much attention in liver mitochondria. However, /

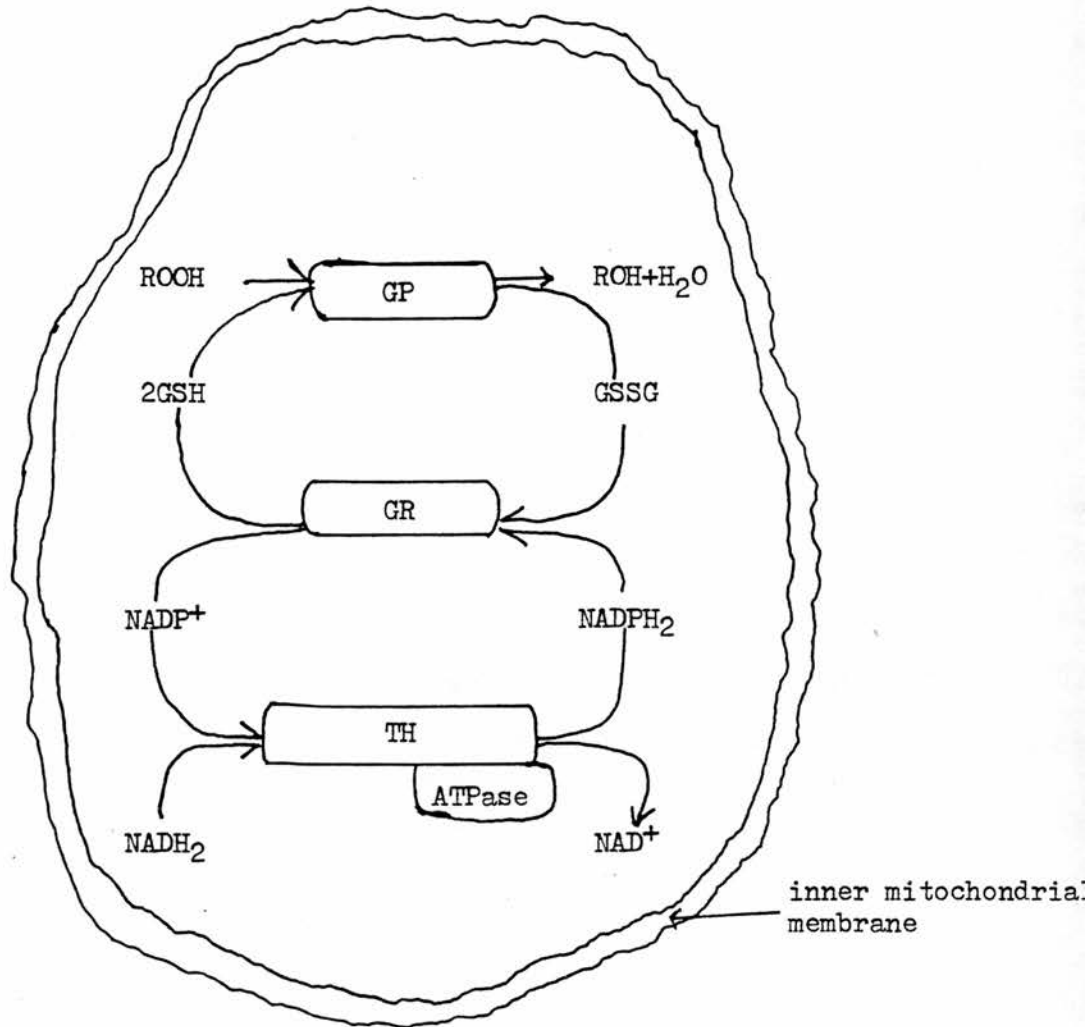


Fig 1: The Mitochondrial Glutathione Redox System

The diagram is a representation of the glutathione redox system in rat liver mitochondria, comprising Glutathione (GSH); Oxidized Glutathione (GSSG); Glutathione Peroxidase (GP); Glutathione Reductase (GR); and Pyridine Dinucleotide Transhydrogenase (TH) associated with the Adenosine Triphosphatase (ATPase) enzyme and the respiratory chain (not shown).

However, it clearly forms an essential component of the mitochondrial glutathione redox system. It is for this reason that I have studied it in some depth.

To examine the GSH redox system in whole mitochondria, it is necessary to stress it by providing a suitable substance to be reduced. The natural GSH oxidant H_2O_2 is not suitable for study due to high levels of catalase, but there are available other synthetic oxidants which can be used to stress the system. Among these are diamide and simple organic hydroperoxides (Oshino and Chance 1977), (Jocelyn 1978) and (Jocelyn and Dickson 1980). The commonly used organic hydroperoxides are cumene hydroperoxide (CuOOH) and tert-butyl hydroperoxide (BuOOH) which I have also used in this study.

It has been reported that the reduction of diamide or BuOOH is inhibited by various substances (Jocelyn 1978) and (Jocelyn and Dickson 1980) These inhibitors include the following:

- (a) Uncouplers, eg FCCP;
- (b) Thiol reagents, eg N-ethylmaleimide and p-hydroxymercuribenzoate
- (c) Specific inhibitors of mitochondrial transport of carboxylic acids. The carboxylic carrier inhibitors shown to inhibit the reductions are; phenyl-pyruvate and α -cyano-4-hydroxycinnamate, specific pyruvate (a monocarboxylate) transport inhibitors (Halestrap 1974); n-butylmalonate, a dicarboxylate carrier inhibitor ie, inhibitors of malate or succinate transport) (Robinson et al 1967); and tricarboxylate carrier inhibitors benzene-1, 2, 3-tricarboxylate and propane-1,2,3-tricarboxylate, ie inhibitors of citrate or isocitrate transport (Robinson et al 1972).

The inhibition of diamide or BuOOH by these inhibitors imply:

- (a) the involvement of energy dependent steps in the mechanism by the inhibition of uncouplers;
- (b)/

- (b) the presence of functional thiol group(s) in the enzyme(s) and or involvement of thiol components, (eg GSH) in the mechanism, by the inhibition of thiol reagents;
- (c) the involvement of carboxylate carrier proteins in the mechanism of reduction, may be by transporting carboxylic acid intermediates or by modulating any of the many reactions involved.

Jocelyn and Dickson (1980) have shown that succinate and 3-hydroxybutrate appreciably promote BuOOH reduction by the rat liver mitochondria. Isocitrate, malate and citrate being less effective in doing so.

It is my intention in this thesis to determine the 'levels' at which the above inhibitors work (see figure 1). Of particular interest are the mitochondrial carrier inhibitors and the carboxylic acid intermediates whose transport they inhibit. It is hoped that during the course of this study, some properties of the components of glutathione redox system in rat liver mitochondria will be elucidated. The study hopes to contribute to the understanding of the mechanism of control of this redox system in mitochondria and hopefully increase our insight into the mechanism of hydroperoxide detoxication.

CHAPTER 2MATERIALS AND METHODS1. MATERIALS1.1 Sources of Materials

Some of the specialised biochemicals used in this study are listed alphabetically under the names of the suppliers. More common laboratory reagents were either in stock or were purchased from the best analytical grade available. For abbreviations see page xiii.

- (a) Akzo Chemie (UK) Ltd: Cumene hydroperoxide and tert-butyl hydroperoxide.
- (b) Aldrich Chemical Co (UK): Benzene-1,2,2-tricarboxylic acid, α -cyano-4-hydroxycinnamate, 5,5'-Dithiobis-(2-nitrobenzoic acid) and phenyl-succinic acid.
- (c) B.D.H. Chemicals (UK): Acetone, Citric acid, Dichlorophenol indophenol, Digitonin, EDTA, Ethanol, Hydrogen peroxide, 3-Hydroxybutyric acid, Malic acid, Malonic acid, Mannitol, Tris, and Triton X-100 (Scintillation grade).
- (d) Boehringer Mannheim (West Germany and UK): Acetyl pyridine NAD^+ , ADP, Alcohol dehydrogenase (yeast enzyme), ATP, CCCP, FCCP, GSSG, Lactic dehydrogenase (rabbit muscle enzyme), NAD^+ , NADH, NADP^+ , NADPH, Oligomycin, Phosphoenol pyruvate and pyruvate kinase (rabbit muscle enzyme).
- (e) Sigma Chemical Co (UK): BSA, Coomassie Blue, EGTA, DL-Fluorocitric acid (Sodium Salt), Glucose-6-Phosphate dehydrogenase (yeast enzyme), GSH, Glutathione reductase (yeast enzyme), Hexokinase (yeast enzyme), p-hydroxy-mercuribenzoate, Lubrol-PX, Palmityl Coenzyme A, Phenyl-pyruvate, Rotenone and Sodium Isocitrate.

Butylmalonate/

Butylmalonate was a gift from Dr P. C. Jocelyn (University of Edinburgh) who prepared it as described by Vogel (1956) from diethylbutylmalonate.

1.2 Instruments Used

Specialised instruments available in the Department of Biochemistry, University of Edinburgh Medical School were the following:

Beckmann J2-21 (preparative centrifuge) Rotors JA14 & JA20;
Eppendorf Centrifuge 3200
MSE Sonicator (Rapidis 150)
Perkin Elmer Fluorescence Spectrophotometer;
Pye Unicam SP1800 Spectrophotometer

1.3 Animals

Female wistar rats approximately 3 months old (about 8g liver weight), were obtained from the University of Edinburgh Centre for Laboratory Animals at Penicuik. The animals were fed on normal diet (water and oils).

2. METHODS

2.1 Preparation of Reagents

All reagents unless otherwise stated were made up in once distilled water.

- (a) ANSA reagent: 1-amino-2-naphthol-4-sulphonic acid (20mg) were mixed with sodium sulphate (120mg) and sodium hydrogen sulphate (343 μ l) (35% W/V) and made up to 10ml.
- (b) Biuret reagent: Hydrated copper sulphate (3g) Potassium iodide (5g) and potassium sodium tartrate (9g) were dissolved and made up to one litre.
- (c)/

- (c) Coomassie Blue reagent (described by Bradford 1976): Coomassie brilliant blue (G250) (100mg) were dissolved in ethanol (50mls of 95% W/V) and then phosphoric acid (100ml of 85% W/V) were added and made up to 1 litre. The solution was filtered to remove the undissolved coomassie blue dye.
- (d) Digitonin: Aqueous solution of digitonin (100ml of 2% W/V) was brought to pH 8.0 with potassium hydroxide (1M). This was then neutralized to pH 7.0 with HCL (1M).
- (e) DTNB (Ellman's Reagent): A solution of 5,5'-dithiobis-(2-nitrobenzoic acid) (1mM) was made in sodium phosphate buffer (.5M) at pH 7.0.
- (f) Mannitol-Mops Buffer (used for preparation of mitochondria): The buffer was made by dissolving Mannitol (227.5g), 3-(N-Morpholino) propane sulphonic acid (MOPS) (10.5g) and EDTA (190.0mg) in 5 litres pH 7.0 (Jocelyn and Kamminga 1974), giving a final concentration of Mannitol (250mM), MOPS (10mM) and EDTA (.1mM). The buffer was kept at 4°C.
- (g) Tris-sucrose-HCl Buffer: The buffer containing Tris (100mM), Sucrose (500mM) and MgCl₂ (12mM) was made in 500ml pH 8.0 and kept at 4°C. For experiments on the Adenosine triphosphatase MgCl₂ (2mM) was present.
- (h) Tris-Sucrose-Acetate Buffer: The buffer containing Tris (100mM) Sucrose (500mM) and MgCl₂ (12mM) was made to pH 8.0 with acetic acid (10mM), unless otherwise stated and made up to 500ml.

2.2 Preparation of Particles

- (a) Mitochondria: Female wistar rats (3 months) were sacrificed by decapitation under anaesthetic influence of diethylether at room temperature (19°C to 22°C) in the mornings (between 10am and 11am) without prior starvation. Livers were removed and put in ice cold mannitol buffer pH 7.0. Six livers (approximately 50g) were excised individually and hand homogenized in a glass homogenizer with 10 full up and down strokes. The/

The homogenate was made up to 250ml (5 times the equivalent volume of the weight of livers).

Twice washed mitochondria were isolated by differential centrifugation at 2°C, removing cell debris at 4,000xg for 15 seconds and sedimenting mitochondria at 10,000xg as described by Jocelyn and Kamminga (1974). Mitochondria were resuspended in ice cold sucrose (.25M) at a concentration of about 30 to 40mg per ml.

The preparation was normally complete within 1½-2 hours and the concentrated suspension of mitochondria was used in experiments within a further 2 hours.

- (b) Submitochondrial Particles: Submitochondrial particles were prepared by sonication (as described by Kielley and Bronk 1958) in hypotonic sodium phosphate buffer (25mM) pH 7.0, unless otherwise stated. Mitochondria from livers (40g) were resuspended in the ice cold buffer (30ml) in an aluminium container in an ice bath. The suspension was sonicated by an MSE sonicator with the power control at number 10 and tuning control at number 5, for 45 seconds, unless otherwise stated. The sonicate was centrifuged for 20 minutes at 15,000xg collecting the supernatant which was centrifuged at 100,000xg in the Spinco or Beckmann ultracentrifuge rotor 40 or 50Ti.

The brownish red pellet was collected and suspended in sucrose (.25M) at a concentration of 15 to 20mg protein per ml and stored at -70°C. The time taken to prepare submitochondrial particles was >4 hours and <5½ hours.

2.3 Preparations with the solubilised Transhydrogenase Activity:

The nicotinamide nucleotide transhydrogenase enzyme was solubilised from submitochondrial particles prepared as in Section 2.2 using various detergents;

(a)/

- (a) Triton X-100 Preparation: The method used was described by Weinbach (1961) submitochondrial particles were put in an equal volume of ice cold triton X-100 (2% W/V) in potassium phosphate buffer (50mM) pH 7.4, containing EDTA (1mM). The mixture was left standing for an hour in an ice cold water bath (0°C). The soluble enzyme was collected in the supernatant after centrifuging for an hour at 100,000xg.
- (b) Tert-Amyl Alcohol Preparation: The method was modified from that used to solubilise the enzyme from mitochondria by Pesch (1964). An equal volume of tert-amyl alcohol (2% V/V) in sucrose (8.5% W/V) was added to submitochondrial particles and the mixture was left stirring for two hours at 0°C. The supernatant containing the transhydrogenase activity was collected after centrifuging for one hour at 100,000xg.
- (c) Digitonin or Lubrox-PX Preparation: This method was again modified from Pesch's (1964). An equal volume of either digitonin (2% W/V) (prepared as in Section 2.1) or Lubrol PX (2% W/V) at 0°C was added to submitochondrial particles. The mixture was mixed well over a vortex mixer then left to stand for 30 minutes in ice cold water. This was then incubated for 15 minutes at 37°C shaking vigorously over a vortex mixer at 3 minute intervals for 30 seconds.

The supernatant containing the transhydrogenase enzyme was collected at 100,000xg after centrifuging for 30 minutes.

2.4 Preparation of Glutathione Reductase

- (a) A glutathione reductase preparation from Mitochondria:

The method used was that described by Flohe' and Schlegel (1971). Washed mitochondria (Section 2.2a) from 40g of livers were suspended in sodium phosphate buffer (25mM) (30ml) pH 7.0. The/

The mitochondria were sonicated and submitochondrial particles removed (as in Section 2.2b) and the supernatant fraction collected after centrifugation at 100,000xg for 30 minutes at 4°C. This was dialysed in phosphate buffer (.01M)(2 litres) pH 8.0 for 8 hours with a one 2 litre change after four hours. Aliquots were stored at -18 °C.

(b) A Glutathione Reductase preparation from liver cytosol:

The liver homogenate in mannitol buffer (Section 2.2a) was centrifuged at 15,000xg for 20 minutes and the supernatant recentrifuged at 100,000xg for 30 minutes. The supernatant cytosolic fraction was dialysed in phosphate buffer (.01M) pH 8.0 as above. The aliquots were stored immediately at -18°C.

For specific activities see Chapter 3 (Section 3).

2.5 Preparation of Glutathione Peroxidase

(a) A Glutathione peroxidase preparation from Mitochondria:

The mitochondrial glutathione peroxidase was prepared as in Section 2.4a. The dialysed supernatant fraction was kept at -18°C.

(b) A Glutathione Peroxidase preparation from liver cytosol:

Cytosolic glutathione peroxidase was prepared as in Section 2.4b. Liver homogenate was centrifuged at 4,000xg to remove cell debris and at 15,000xg for 20 minutes to remove mitochondria. The cytoplasm was centrifuged for 30 minutes at 100,000xg before being dialysed as in Section 2.4b.

(c) Partially purified Glutathione Peroxidase: The method used was slightly modified from that described by Zakowski and Tappel (1978). To the dialysed glutathione peroxidase fraction (50ml) from (a) or (b) at 0°C, HCl (2N) was added to bring the pH to 5.0. This was allowed to stand for one hour at 4°C then centrifuged for 15 minutes at 15,000xg to remove non-glutathione peroxidase protein.

The supernatant was brought to pH 7.4 by NaOH (10% W/V) and then treated with acetone (50% W/V at -20°C). This was centrifuged immediately for 15 minutes at 15,000xg. The pellet was resuspended in 2ml Tris buffer (10mM) pH 7.6. The non soluble material was sedimented at 15,000xg after centrifugation for 15 minutes and discarded. The aliquots (soluble material) were immediately stored at -18°C .

For specific activities see Chapter 3 (Section2).

3.0 SIMPLE ASSAYS

3.1 Protein Assay

- (a) By Biuret Method: A routine protein assay was performed by a modified biuret assay (Jacobs et al 1956) using bovine serum albumin (BSA) as the standard.

The BSA standard solutions (0 to 25mg) or enzyme fractions were precipitated with perchloric acid (.5ml) (12% W/V) then made up to 6.0ml. The precipitate was sedimented at 100xg after 5 minutes centrifuging in an MSE bench centrifuge. This was resuspended in NaOH (2N) (0.1ml) and biuret reagent (Section 2.1b) (2.9ml). 30 minutes after, the solutions were centrifuged at 100xg for a further 5 minutes and the absorbance read at 540nm. A mixture of NaOH (2N) (0.1ml) and biuret reagent (2.9ml) was used as the blank. The BSA standard curve obtained is shown in figure 1.

In the case of enzyme fractions containing the solubilised transhydrogenase (100 μ l) NaOH (0.1ml of 2N) and biuret reagent (2.9ml) were added directly without acid precipitation. The absorbance was read 30 minutes after as above.

(b)/

(b) By Coomassie blue method: An attempt was made to estimate the amount of protein in submitochondrial particles by the Coomassie blue method as described by Bradford (1976) using the BSA as the standard.

The BSA standards (0 to 100mg) in water were either treated directly with the reagent or treated after precipitation with perchloric acid (0.5ml of 12% W/V) and redissolved in 1.5ml of either water, NaOH (10%) or sodium phosphate (.5M) buffer pH 7.0.

To the protein fractions (1.5ml), Coomassie reagent (3.5ml prepared as in section 2.1c) was added. This was mixed well and after 30 minutes standing at room temperature (19°C) the absorbances of aliquots (3.0ml) were read at 595nm. The BSA standard curves obtained are shown in figure 2.

The amount of protein in submitochondrial particles was determined as described above with prior perchloric acid (0.5ml of 12% W/V) precipitation. The results obtained were compared with those determined by the biuret method (a) (See Table 1).

Acid treatment abolishes the linearity of the BSA standard curve above 20µg and increases the absorbances at 595nm 6 to 8 fold depending on the media (Figure 2). In addition, Coomassie blue method grossly underestimates the amount of protein in the preparations (Table 1) hence, the biuret method was routinely used for protein determination.

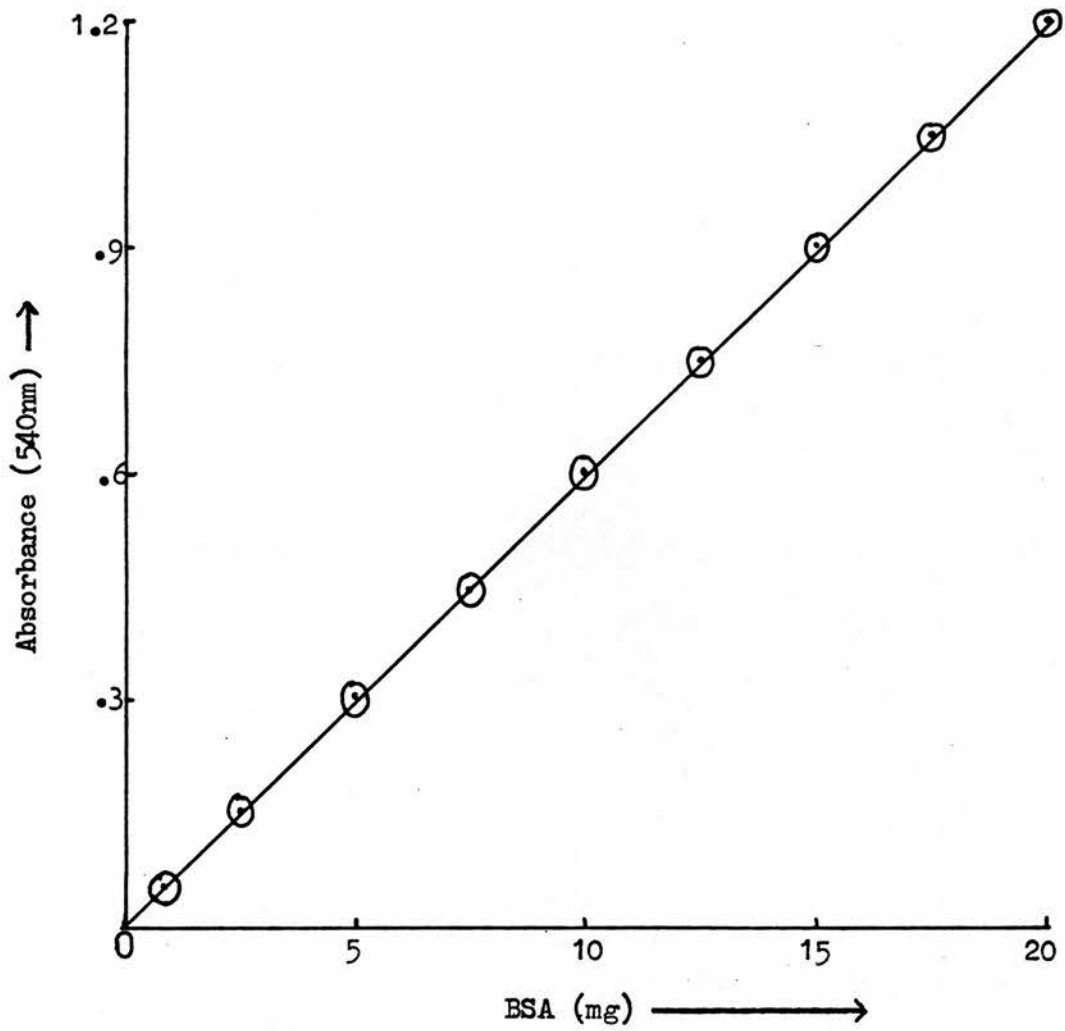


Fig 1: Variation of absorbance at 540nm with BSA Concentration

For conditions see Section 3.1 (a) (\pm S.D. \angle .05, $n=3$).

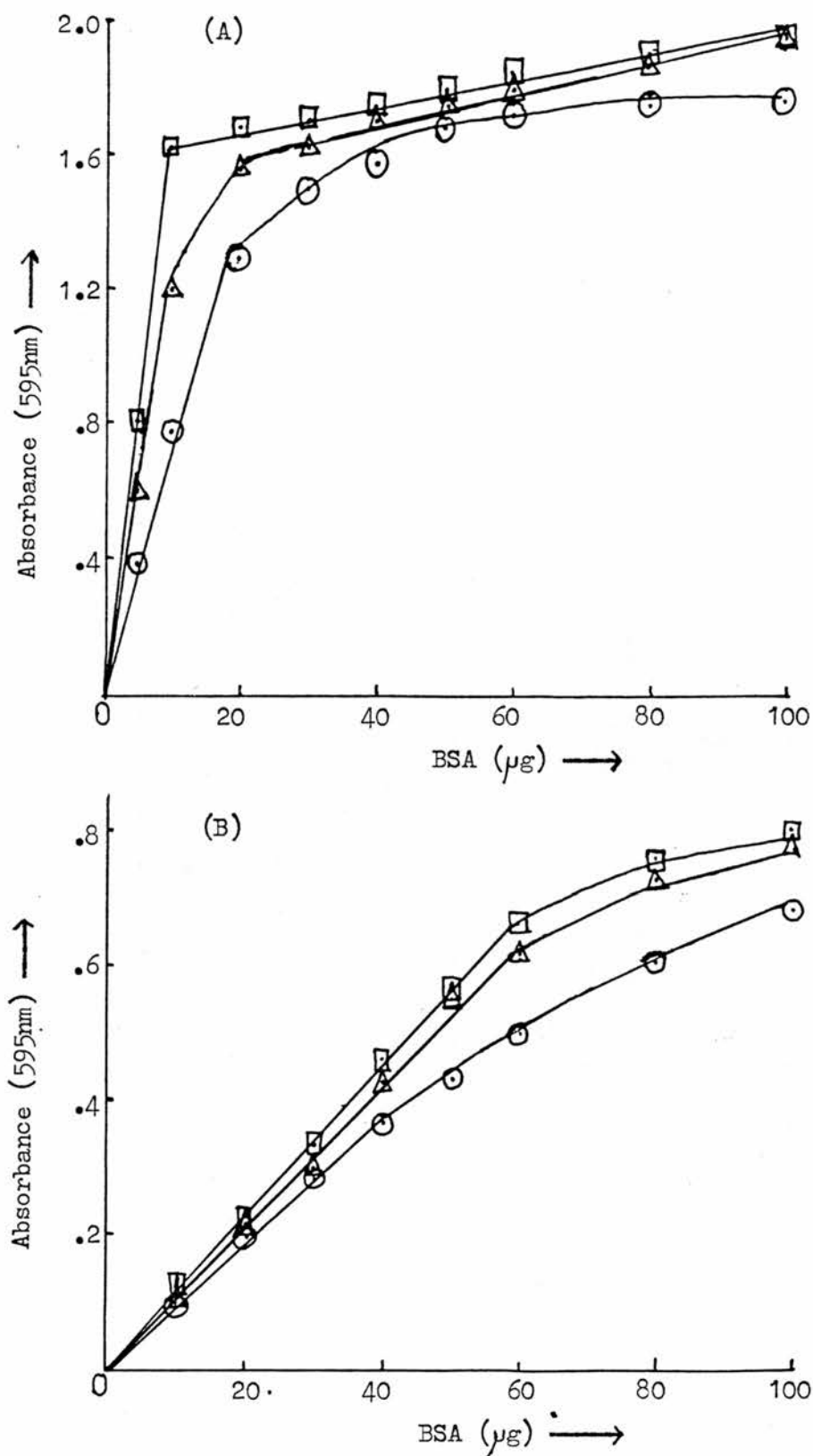


Fig 2: Effect of medium on the absorbance of BSA solutions treated with Coomassie blue.

The reagent was added to solutions dissolved in water (Δ); NaOH (10% W/V) (\circ); or sodium phosphate (.5M) buffer pH 7.0 (\square). In (A) BSA was acid treated and in (B) untreated (Section 3.1(b)) (\pm S.D. \angle .03, $n = 4$)

Table 1: Comparison of the amount of protein in submitochondrial particles determined by the biuret or coomassie blue method.

<u>Method</u>	<u>Assay Medium</u>	<u>mg protein per ml</u>
Biuret	Water	3.70 ± .03
Coomassie blue	Water	0.04 ± .01
Coomassie blue	NaOH (10%)	0.30 ± .04
Coomassie blue	Sodium Phosphate (.5M)	0.12 ± .02

Suspensions of submitochondrial particles (.1ml) were precipitated by perchloric acid (Section 3.1a and b) and the amount of protein present determined (Section 3.1), in three assays, from the BSA standard curves (figures 1 and 2) using the same batch of particles.

3.2 GSH Assay

Reduced glutathione was determined by Ellman's method (1959). Aliquots (.5ml) of reaction mixture treated with perchloric acid (50 μ l of 12% W/V) to stop the reaction, were centrifuged at 10,000xg for 2 minutes in a bench Eppendorf centrifuge to obtain the supernatants.

To the clear supernatant, DTNB (1mM) solution (.5ml) in phosphate buffer (.5M) pH 7.0 was added mixed and the absorbance read at 412nm after 2 minutes. A millimolar extinction coefficient of 13.6 cm⁻¹ was assumed in all GSH determinations.

4.0 ENZYME BASED ASSAYS

4.1 BuOOH Reduction

Tert-Butyl hydroperoxide reduction was determined in the reaction medium after incubating mitochondria according to the method described by Jocelyn and Dickson (1980). Immediately after sedimenting mitochondria, the supernatant (.25ml) was added to a solution of glutathione peroxidase (.06IU) of the dialysed cytosolic preparation (Section 2.5b), GSH (.3mM) and EDTA (30mM).
The/

The reaction was incubated for 20 minutes at 30°C then treated with perchloric acid (.25ml of 12% W/V). An allowance for GSH autoxidation was made by a control in which the hydroperoxide was present. Calculations of the amount of BuOOH present were deduced from the stoichiometry required by the equation:



which shows that the GSH lost in the reaction is equivalent to twice the amount of BuOOH present, after allowing for autoxidation.

4.2 Glutathione Peroxidase Assay

Glutathione peroxidase activity was determined by the method described by Pierce and Tappel (1978), in which the enzyme catalyses the reduction of hydroperoxides by GSH. The reaction is then coupled to GSSG reduction by NADPH₂ via the enzyme glutathione reductase.

To a 1ml cuvette in a carrier maintained at 30°C, by circulating water, GSH (.25mM), NADPH₂ (.1mM), commercially prepared glutathione reductase (11 U) from yeast, glutathione peroxidase preparation (0.5 to 2.0mg) and sodium phosphate buffer (50mM) pH 7.0 were added to give 0.9ml. After preincubating for 2 minutes, the reaction was started by adding 100µl of cumene hydroperoxide (.1mM) itself preincubated at 30°C.

The reaction was followed at 340nm by the recorder connected to the spectrophotometer (Unicam SP1800). The amount of NADPH₂ oxidized was determined from the slope of the linear (min) reaction assuming a millmolar extinction coefficient of 6.2cm⁻¹. This is equal to the amount of cumene hydroperoxide reduced.

4.3 Glutathione Reductase Assay

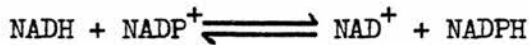
The method was as described by Carlberg and Mannervik (1975). The reaction is followed spectrophotometrically as NADPH₂ is oxidised by GSSG. To a 1ml cuvette at 30°C (as in Section 4.2) GSSG (.2mM), EDTA (.1mM), glutathione reductase preparation (.5 to 2.0mg) and sodium phosphate buffer (100mM) at pH 7.0 were added to give 0.9ml. After preincubating for 2 minutes, the reaction was started by adding 100µl of NADPH₂ (.1mM).

The/

The reaction was followed at 340nm by the recorder connected to the spectrophotometer (Unicam SP1800). The amount of NADPH₂ oxidized was determined from the slope of the initial (min.) linear reaction assuming a millimolar extinction coefficient of 6.2cm⁻¹.

4.4 Nicotinamide Nucleotide Transhydrogenase Assays

The enzyme catalyzes the reversible hydride transfer in the reaction:



(See Section 4, Chapter 1).

The reaction can not be assayed directly following the disappearance of the reactant or appearance of the product (NADH₂ and NADPH₂ respectively) since they have the same absorbance at 340nm. However, in the presence of sufficient regenerating systems, the optical density changes due to the enzyme activity can be obtained. This can be measured (A) continuously (spectrophotometric as in Sections 4.2 and 4.3) or (B) discontinuously (after stopping the reaction in aliquots with a protein precipitation agent).

A. Continuous Assays

- (i) With Regenerating NADP⁺: The method used was as described by Texeira da Cruz et al (1971). To a 1ml cuvette maintained at 30°C (as in Section 4.2), NADH (.25mM), NADP⁺ (.1mM) (unless otherwise stated), GSSG (1mM), KCN (2mM), rotenone (3.4µM) and Tris-sucrose-HCl buffer (Section 2.1g) containing Tris (50mM), sucrose (.25M) and MgCl₂ (6mM) pH 8.0 (unless otherwise stated) were added. For studies on the energy driven transhydrogenase, the mixture contained in addition, either ATP (1mM) or succinate (25mM). When succinate was the energy source, oligomycin (1µg/mg) was also present but KCN was omitted.

The reaction (final volume 1ml) was started by adding a mixture of the enzyme preparation (0.1 to 1.0mg) and 10µl of glutathione reductase (.45I U of commercial yeast enzyme), after 5 minutes preincubation. NADPH₂/

NADPH₂ formed was followed spectrophotometrically at 340nm (Section 4.3) indirectly, by measuring its oxidation by GSSG. The amount of NADPH₂ formed was determined from the slope of the initial linear part of the reaction, assuming a millimolar extinction coefficient of 6.2cm⁻¹.

- (ii) With Regenerating NADH: The method used was as described by Danielson and Ernster (1963).

To the 1ml cuvette maintained at 30°C (See i) NADP⁺ (.25mM), NADH (.25mM) (unless otherwise stated), ethanol (60mM), rotenone (3.4µM), KCN (2mM) and Tris-sucrose-HCl buffer (as in i) pH 8.0 were added. For studies on the energy driven reaction, ATP or succinate was added to the mixture (as in i).

After preincubating for 5 minutes, the reaction was started by adding a mixture of the enzyme preparation (0.1 to 1.0mg) and 10µl of alcohol dehydrogenase (.45I U of the commercial rabbit muscle enzyme). The reaction was followed at 340nm as in (i). Under these conditions NADH level remains constant and the observed increase in extinction at 340nm is due to the formation of NADPH. The amount of NADPH formed was determined from the slope of the initial linear part of the reaction, assuming a millimolar extinction coefficient of 6.2cm⁻¹.

- (iii) Acetyl pyridine NAD⁺ (APAD) Method: An attempt was made to measure the transhydrogenase activity between NADH and APAD as described by Stein et al (1959). The coenzyme analog APAD substitutes NADP⁺ in this reaction. APAD is reduced giving a different absorption maxima (at 375nm) from that of NADH (at 340nm).

The reaction mixture, in 1ml cuvette maintained at 30°C (see i), consisting of NADH (.15mM), KCN (2mM), rotenone (.5µM) and sodium phosphate buffer (80mM) pH 6.8 was preincubated for 5 minutes. The/

The reaction was initiated by adding APAD (.19mM) and submitochondrial particles (0.1 to 1.0mg) mixture, final volume 1.0ml.

The reaction was followed at 375nm by the recorder measuring the appearance of APADH₂. The amount of APADH₂ formed was determined from the slope of the initial part of the reaction assuming a millimolar extinction coefficient of 5.1.

- (iv) Dichlorophenol indolphenol (DCPIP) Method: Transhydrogenase is known to utilise DCPIP in place of NAD⁺ as substrate (Rydström et al 1973b). This method measures the reverse reaction (ie NADPH₂ + DCPIP → NADP⁺ + DCPIPH₂).

To a 1ml cuvette maintained at 30°C (as in iii) DCPIP (50mM), KCN (1mM), rotenone (2.4μM) and Tris-sucrose-acetate buffer (as Section 2.1h) pH 7.5 were added. After 5 minutes preincubation, the reaction was started by adding a mixture of submitochondrial particles (0.1 to 1.0mg) and NADPH₂ (.17mM), final volume 1.0ml.

The reaction was followed at 600nm by the recorder (as in iii) and DCPIPH₂ formed determined from the slope of the initial linear velocity, assuming a millimolar extinction coefficient of 16.1cm⁻¹.

B. Discontinuous Assays

- (i) Fluorometric Method: To aliquots (1ml) of the reaction mixture already described (A ii), .1ml of NaOH (.1M) was added at the required intervals. Samples (.3ml) of this alkaline solution were diluted to 3ml with more of the NaOH. The amount of NADPH₂ formed was determined fluorometrically at 340nm excitation wavelength and 450nm emission. Since the NADH in the reaction mixture was kept constant by alcohol dehydrogenase, fluorescence increases are due to the fluorescence of NADPH. The amount of NADPH₂ was obtained from the standard curve (figure 3).
The/

The results took into account the amount of NADH present initially.

- (ii) GSH Method (constant NADP⁺): A bulk solution was made up containing the following at the stated concentrations in Tris-sucrose-HCl, pH 8.0 or Tris-sucrose-acetate buffer made up as in Section 2.1 (g) and (h) at the required pH (between pH 5 and 9). NADP⁺ (.1mM), NADH (.25mM), GSSG (1mM) and rotenone (3.4µM).

For the energy dependent reaction ATP (1mM) or succinate (10mM) and oligomycin (1µg/mg) were also present.

Samples (445µl) of this solution were added to different Eppendorf tubes (capacity 1.5ml) and preincubated for 5 minutes at 30°C. The reaction was started by adding to each tube a mixture of the enzyme preparation (50µl, .1 to 1mg) and glutathione reductase (0.45I U, 5µl of the commercial yeast enzyme).

After incubating for the required time, perchloric acid (50µl of 12% W/V) was added.

The following controls were carried out: No NADP⁺ or no NADH₂ in the reaction mixture and the zero time control. The control without one of the substrates, was done by replacing the substrate with an equal volume of water and the procedure followed exactly as above. For the zero time control, perchloric acid (50µl of 12% W/V) was added to the reaction mixture prior to the addition of the enzyme preparation and glutathione reductase, then allowed to incubate for the required time. The control employed will be as stated in the results.

The amount of GSH formed was determined at 412nm in the supernatant after DTNB treatment (Section 3.2), assuming a millimolar extinction coefficient of 13.6cm⁻¹.

The/

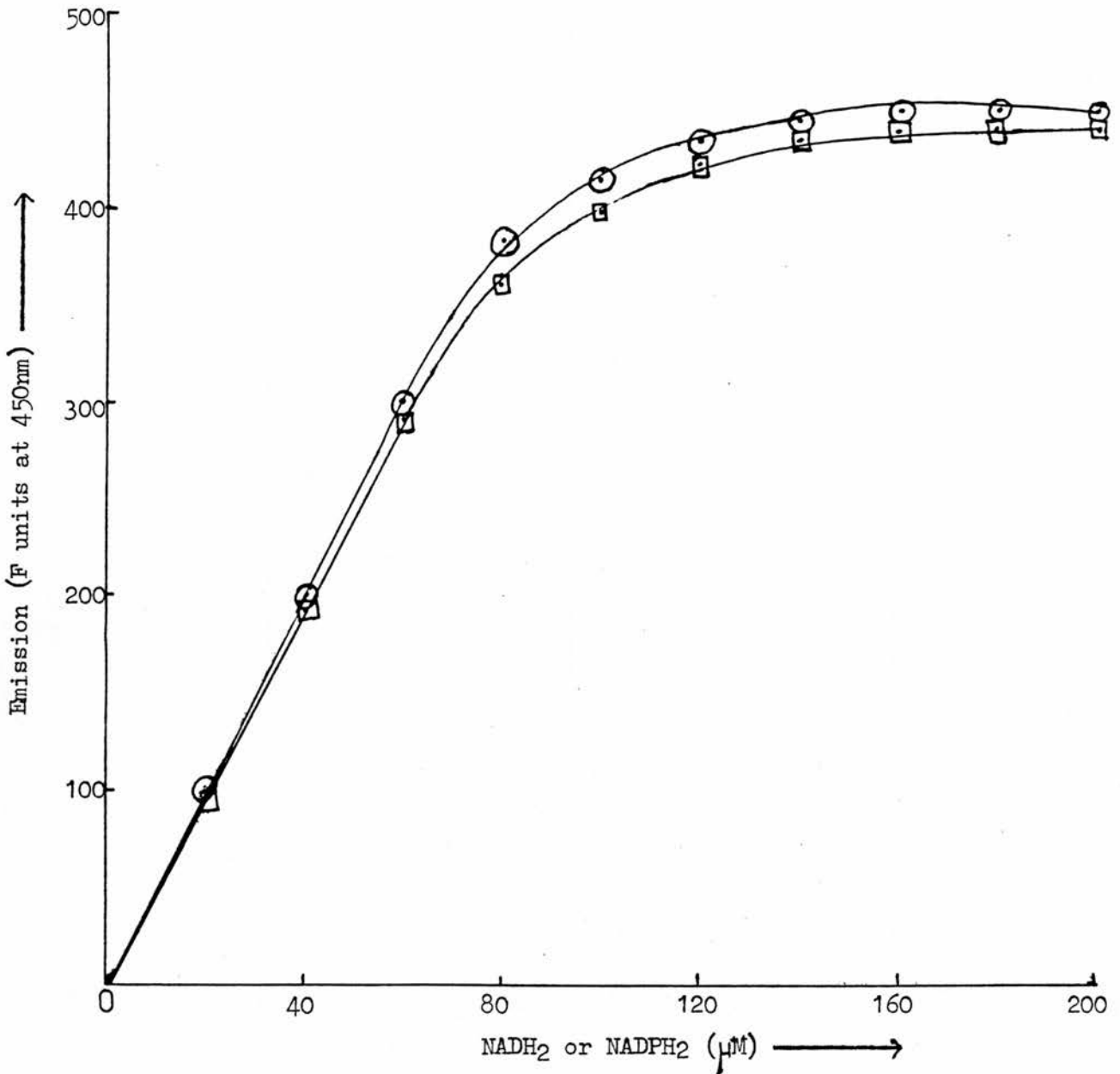


Fig 3: Effect of NADH₂ (○) and NADPH₂ (◻) Concentration on the fluorescence

To 2.7ml of NaOH (.1M) in quartz optical cell (Hitachi) capacity 4ml at room temperature (20°C), NADH₂ or NADPH₂ was added (0 to 0.2mM). The fluorescence was obtained in a fluoremeter (Section 1.2) at 340nm excitation and 450nm emission wavelengths, with sensitivity control at number 7 and selectivity control at number 1. Values are means of 4 determinations (\pm S.D. \angle 5F units).

The value obtained is equal to half the amount of NADH oxidized or NADPH formed in the reaction.

- (iii) GSH Method (constant NADP⁺ and NADH): The method differs from the previous one(ii) only in that in the bulk solution there was also present ethanol (60mM) and alcohol dehydrogenase (5 μ l, .45I U) ~~was present~~ was present together with enzyme preparation and glutathione reductase (see ii). The controls were done as in (ii).

4.5 ATP Hydrolysis and ATP Synthesis Assays

- (i) ATP Hydrolysis (ATPase): The method for assaying ATPase activity was as described by Pougéois et al (1978 and 1979) and by Pullman (1960) with minor modifications. The reaction was coupled to NADH oxidation and followed spectrophotometrically at 340nm.

To 1ml cuvette maintained at 30°C (as Section 4.2) phosphoenol pyruvate (2mM), NADH (.2mM), ATP (10mM) (unless otherwise stated) KCl (10mM), NaHCO₃ (10mM) and Tris-sucrose-HCl buffer pH 8.0 (Section 2.1g) except for MgCl₂ (1mM) were added to give .9ml. After preincubating for 5 minutes, the reaction was started by adding a mixture of enzyme preparation (80 μ l, 0.1 to 1.0mg), pyruvate kinase (1I U of the commercial muscle enzyme) and lactic dehydrogenase (1I U of the commercial rabbit muscle enzyme).

The reaction was followed at 340nm by the recorder connected to the spectrophotometer (Section 1.2). The amount of NADH oxidized was determined from the slope of the linear initial velocity assuming a millimolar extinction coefficient of 6.2cm⁻¹. This is equal to the amount of ATP hydrolysed.

Phosphate/

Phosphate Determination: An attempt was made to measure the rate of ATP hydrolysis alternatively by measuring inorganic phosphate release of the above reaction (except for the absence of NADH and lactic dehydrogenase) after terminating the reaction with perchloric acid (.3M).

First an inorganic phosphate standard curve was determined by a method as described by Fiske and Subbarow (1925). To a glass test tube thoroughly washed and oven dried, potassium dihydrogen phosphate (0 to 400nmol) were added to give 1.0ml. To this water (.7ml), ammonium molybdate (0.2ml of 2.5% W/V) in sulphuric acid (5N) were added mixed and then ANSA, the reducing agent, was added. The mixture was left standing at room temperature (20°C) for ten minutes then the optical density at 600nm was obtained.

The method was rendered unsatisfactory as a result of very high blank values and poor reproducibility (results not shown).

- (ii) ATP Synthesis: The method was as described by (Bergmeyer 1965) in which the reaction is coupled to NADP^+ reduction via the enzymes hexokinase and glucose-6-phosphate dehydrogenase.

To 1ml cuvette equilibrating at 30°C (see i), glucose (100mM), NADP^+ (.1mM), inorganic phosphate (NaH_2PO_4) (2.5mM), ADP (5mM), sucrose (250mM), succinate (2mM), Hexokinase (5I U of the commercial yeast enzyme), glucose-6-phosphate dehydrogenase (5I U of the commercial yeast enzyme) and Tris-HCl buffer (50mM) pH 8.0 were added.

After/

After 2 minutes preincubation, the reaction was started by adding submitochondrial particles (0.2 to 2.0mg), final volume 1.0ml. The reaction was followed at 340nm by the recorder (as in i). The amount of NADP^+ reduction was determined from the slope of the initial linear velocity assuming a millimolar extinction coefficient of 6.2cm^{-1} .

The correction for myokinase activity in the particles was determined as above except for the fact that inorganic phosphate was replaced by water in the reaction mixture.

An alternative method to determine ATP synthesis in whole mitochondria and submitochondria will be discussed under Section 4.6, measuring oxygen consumption.

4.6 Respiration Rates

A polarographic method was used to determine respiration rates in intact mitochondria and submitochondrial particles as described by Kielley and Bronk (1958).

To a Clark type oxygen electrode chamber (capacity 6ml) mitochondria or submitochondrial particles (2 to 8mg) and Tris-sucrose-HCl buffer pH 8.0 (Section 2.1g) were added, FCCP (1mM) was either present or omitted and the reaction preincubated for 2 minutes at 30°C . The reaction was started by adding 100 μl succinate (2mM) made in the buffer (Tris-sucrose-HCl), final volume 2.0ml.

Oxygen uptake was followed by the recorder connected to the oxygen electrode. The amount of oxygen consumed was determined from initial slopes recorded, assuming that 100% of oxygen consumption was equivalent to 445n atoms/ml at 30°C (Chance and Williams 1955).

Oxidative phosphorylation (ATP synthesis) was determined as above, but FCCP was omitted in the reaction mixture. After adding succinate (2mM), the reaction was followed for 1 minute, then a mixture of ADP (5mM) and inorganic phosphate (NaH_2PO_4) (1.25mM) was later added. The/

The reaction was followed in the same way as before and oxygen consumption due to phosphorylation was determined taking into account the succinate oxidation without phosphorylation.

5. MEMBRANE POTENTIAL AND PROTON GRADIENT

5.1 Determination of Membrane Potential

The method was as described by Azzi et al (1969) using a fluorescence probe 8-anilino-1-naphthalene, sulphonic acid (ANS, a negatively charged ion).

To a quartz optical cell (Hitachi) capacity 4ml, rotenone (3.4 μ M), ANS (5 μ M), sucrose (.25M), MgCl₂ (3mM), and Tris-acetate buffer (50mM) pH 8.0 were added to give 2.7ml, at room temperature (19 to 22°C). Mitochondria or submitochondrial particles (150 μ l, 0.5 to 2.0mg) were added, mixed and the fluorescence read after 1 minute (as specified below). ATP (1mM) or succinate (5mM) and oligomycin (1 μ g/mg) were then added, final volume 3.0ml, mixed and 15 sec. after the ANS fluorescence again taken at 366nm for excitation and 470nm for emission in a Perkin Elmer fluorimeter (Section 1.2) with sensitivity control switch at number 6 and selectivity control switch at number 1.

5.2 The Transhydrogenase as Proton Pump

The transhydrogenase enzyme is currently being regarded as an additional energy coupling site in mitochondria (Rydström 1979). This property was investigated according to the method described by Rydström (1974) where, at low energy levels (suppressed succinate oxidation), energy dependent ANS fluorescence is a sensitive tool for estimating relative energy requirement of the enzyme.

To/

To the quartz optical cell (Section 6.1) submitochondrial particles (1mg) oligomycin (1 μ g/mg), ANS (5 μ M) and Tris-acetate buffer (50mM) pH 8.0 containing sucrose (.25M) and MgCl₂ (3mM) were added then mixed and the fluorescence read at room temperature (19 to 22°C) at 405nm excitation and 480nm emission and sensitivity control at 4 and selectivity control at number 1. Malonate (1mM) and succinate (5mM) were then added, the further increase in the ANS fluorescence was recorded (as above). Either NADH (200 μ M) or NADP⁺ (20 μ M) was then added to the mixture without any fluorescence change, but when both the two substances were present together, a fluorescence decrease was read after 15sec.

An attempt was made to measure ANS fluorescence changes in submitochondrial particles in the absence of succinate and malonate and in the presence of NAD⁺ (200 μ M) and NADPH (100 μ M) (reverse transhydrogenase reaction) without much success (results not shown).

5.3 Determination of Proton Gradient (Δ pH)

A positively charged quinacrine (QA), (9-4-diethylamino-1-methylbutylamino-3-chloro-7-methoxyacridine) at low concentration can be used as a probe for changes in proton (H⁺) concentration as described by Lee (1971).

To a quartz optical cell (Section 1.2), rotenone (3.4 μ M) quinacrine (3 μ M), KSCN (2mM), MgCl₂ (3mM), sucrose (.25M), Tris-acetate buffer (50mM) pH 8.0 and mitochondria or submitochondrial particles (0.5 to 2.0mg) were added and mixed well. The QA fluorescence was taken after 15 sec. at room temperature (19 to 22°C) (see below). ATP or succinate (5mM) with oligomycin (1 μ g/mg) were added and mixed then the fluorescence read again at 420nm excitation and 500nm emission with sensitivity control at number 7 and selectivity control at number 1.

QA fluorescence quenching was also determined in the presence of specified substances, indicated in the results.

RESULTS AND DISCUSSION

The aim of the investigation was to examine the glutathione redox system of the rat liver mitochondria. The approach to this study was to look at the effect of inhibitors on the complete system in intact mitochondria, then to investigate their action on each of the components of this system. I shall therefore, begin by describing the effect on intact mitochondria (Chapter 3, Section 1.1 and 1.2).

The system comprises GSH, Glutathione Reductase (GR), Glutathione Peroxidase (GP) and Pyridine Dinucleotide Transhydrogenase (TH) (see introduction). The effect of inhibitors on mitochondrial GSH are, therefore, next described (Chapter 3, Section 1.3). The first of the two enzymes GR and GP follow (Chapter 3, Sections 2 and 3).

Most of the work has, however, concentrated on the properties of mitochondrial TH since the behaviour of this enzyme proved to be of greatest interest. For this reason, the work with TH is described in two separate Chapters (Chapters 4 and 5).

Chapter 4 is concerned with the non-energy dependent TH and Chapter 5 with the energy-dependent TH.

CHAPTER 3GLUTATHIONE DEPENDENT REACTIONS1. SOME EXPERIMENTS WITH INTACT MITOCHONDRIA1.1 Stressing the System

The GSH redox system is normally quiescent and in order to be studied, it requires to be stressed by an oxidant (see introduction). A suitable oxidant which was used in the present work is tert-butyl hydroperoxide (BuOOH).

This substance was added to mitochondria and its rate of loss by reduction determined by periodic assay. Reducing equivalents were provided by succinate as shown in figure 1. There is substantial loss of the oxidant which was found to be linear with time up to 5 minutes, but if an uncoupler is also added, the rate of BuOOH reduction is greatly reduced by about 40% after 4 minutes (figure 1).

1.2 Inhibitor Effects

A number of inhibitors of BuOOH reduction by mitochondria are described in the literature. It has been claimed that uncouplers largely inhibit this reduction and as shown (figure 1), I have confirmed this result.

However, the main interest was to investigate new inhibitors and of most interest has been the demonstration that substances which inhibit the transport of those carboxylic acids which are of importance in mitochondrial metabolism, are also potent inhibitors. This finding is of special interest since the substances have hitherto been reported as specific inhibitors of the transport processes (Chapter 1, Section 5).

I used the following substances, phenyl pyruvate (PP) (inhibitor of pyruvate transport), n-butylmalonate (BM), phenylsuccinate (PhS) (inhibitors of succinate or malate transport), benzene-1,2,3-tricarboxylate (BT) and propane-1,2,3-tricarboxylate (PT) (inhibitors of citrate transport).

The/

The results obtained (figure 2A) show that the carboxylate carrier inhibitors do inhibit the reduction of BuOOH by mitochondria in a concentration dependent manner, ie the inhibition increases with increasing inhibitor concentration. The transport inhibitors, however, inhibit the loss of BuOOH to a lesser extent, in the presence of FCCP (figure 2B).

1.3 Mitochondrial GSH

The reduction of hydroperoxides has been shown to involve mitochondrial GSH (Jocelyn and Dickson 1980). The effect of these carboxylate carrier inhibitors on the concentration of this substance was therefore studied.

Mitochondrial GSH concentration was determined in sediments obtained after BuOOH reduction in the presence of the transport inhibitors (Table 1).

The results (Table 1) show that these carboxylate carrier inhibitors like the uncoupler (FCCP) do greatly reduce mitochondrial GSH concentration (by about 70%).

Table 1: Mitochondrial GSH concentration after incubating with BuOOH and succinate plus the indicated inhibitor (10mM except FCCP, 0.5µM)

<u>Inhibitor</u>	<u>GSH (nmol mg⁻¹)</u>
Nil	4.2 ± 1.1
FCCP	1.3 ± 0.3
PP	0.9 ± 0.2
BM	1.5 ± 0.4
PhS	1.5 ± 0.4
BT	1.3 ± 0.3
PT	0.9 ± 0.3

The conditions for BuOOH reduction were as in figure 1. The reaction was stopped by sedimenting mitochondrial pellets (3.3mg) at 100xg, for 2 minutes. Mitochondrial pellets were treated with perchloric acid (0.5ml of 2.4% W/V), shaking vigorously and after 2 hours centrifuging for 2 minutes at 100xg. The acid extract was assayed for GSH as in methods (Section 3.2). Values in the table are means from two assays ± S.D.

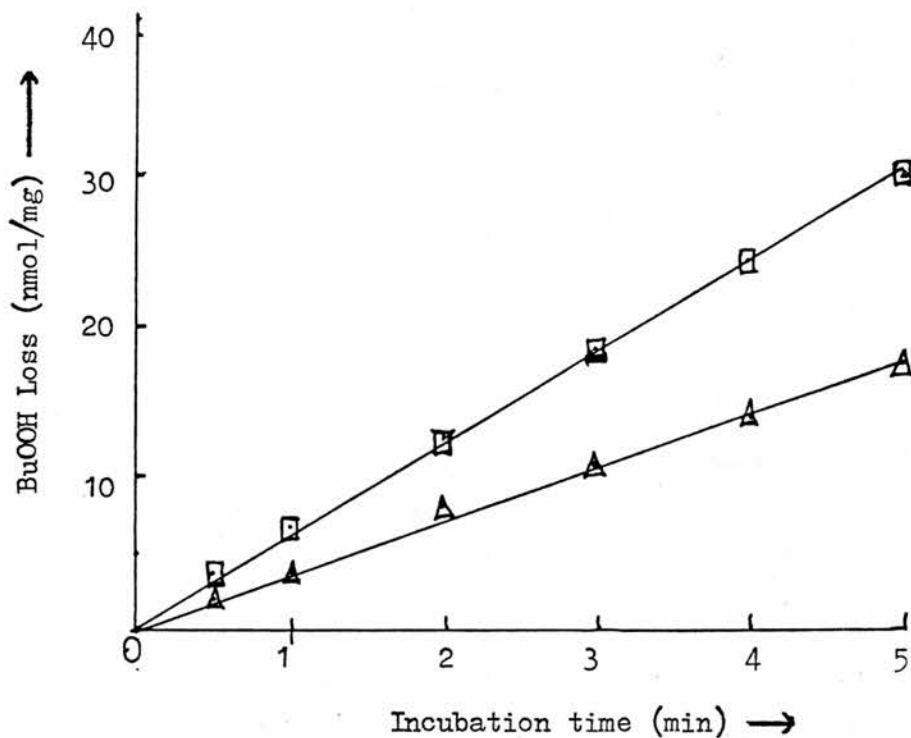


Fig 1: The time course of BuOOH reduction in the presence of mitochondria and succinate (□) or succinate with FCCP (△).

BuOOH ($.5\mu\text{M}$) was added to mitochondria (3.3mg) suspended in a medium at 30°C (methods, Section 4.1) containing succinate (50mM) and FCCP ($.5\mu\text{M}$). The reaction was terminated with perchloric acid ($.25\text{ml}$ of 12% W/V) at the times indicated above and BuOOH loss calculated (Section 4.1 in methods). Values plotted are the means of 3 assays ($\pm \text{SD}$, 3nmol mg^{-1}).

BuOOH reduced with succinate alone was $24 \pm 4\text{nmol mg}^{-1}$ and with succinate plus FCCP was $14\text{nmol mg}^{-1} \pm 3$, after 4 minutes.

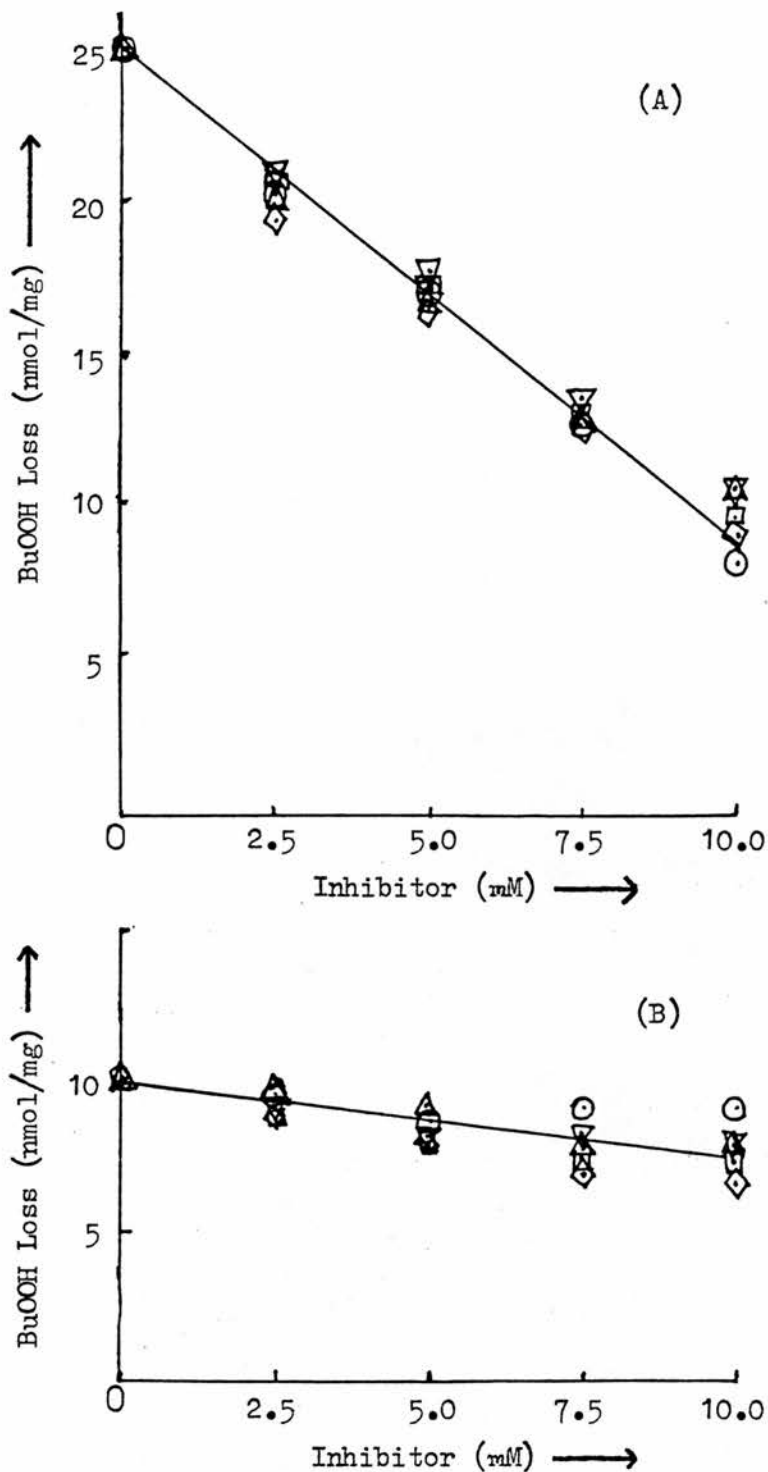


Fig 2: Mitochondrial reduction of BuOOH by succinate (A) or by succinate plus FCCP (B) with no addition or in the presence of PP (○), BM (△), PhS (▽), BT (□) and PT (◇).

The reactions were incubated at 30°C for 4 minutes as in figure 1. Values plotted are the means from 3 assays (\pm S.D. < 2 nmol/mg).

2. GLUTATHIONE PEROXIDASE (GP)

One component of the GSH redox system is GP. The enzyme is present in the mitochondria and as a separate entity in the cytosol. Preparations of both enzymes were studied with regard to their susceptibility to inhibition by the inhibitors of hydroperoxides used in intact mitochondria.

2.1 Hydroperoxide Reduction

GP catalyzes the reduction of synthetic organic hydroperoxides with GSH as the hydrogen donor. A time course of the reaction, linear for 2 minutes, was obtained (figure 3), with the dialysed enzyme preparation (see methods, Section 2), assaying GSSG formed spectrophotometrically by following NADPH₂ oxidation at 340nm in the presence of yeast glutathione reductase.

I used cumene hydroperoxide (CuOOH) as the oxidant (substrate) which was a slightly better substrate than tert-butyl hydroperoxide (BuOOH) (results not shown). The natural substrate hydrogen peroxide (H₂O₂) for this enzyme was found unsuitable due to the contamination of my preparations with catalase.

The specific activity of the cytosolic enzyme was found to be about 2-fold higher than the mitochondrial enzyme (figure 3).

2.2 GP activity varying substrate concentration

The Kms of the mitochondrial and cytosolic enzymes for the substrates, GSH and CuOOH were determined by separately varying the concentration of one with the other held high.

The data are presented by the method ~~$\frac{S}{V}$~~ versus **S** now widely accepted (figure 4). This form of presentation (**S** against $\frac{S}{V}$) is used throughout this thesis since it has been shown (Cornish-Bowden 1976) to be superior to the most widely used double reciprocal plot ($\frac{1}{V}$ against $\frac{1}{S}$) (Lineweaver-Burk 1934).

Linear/



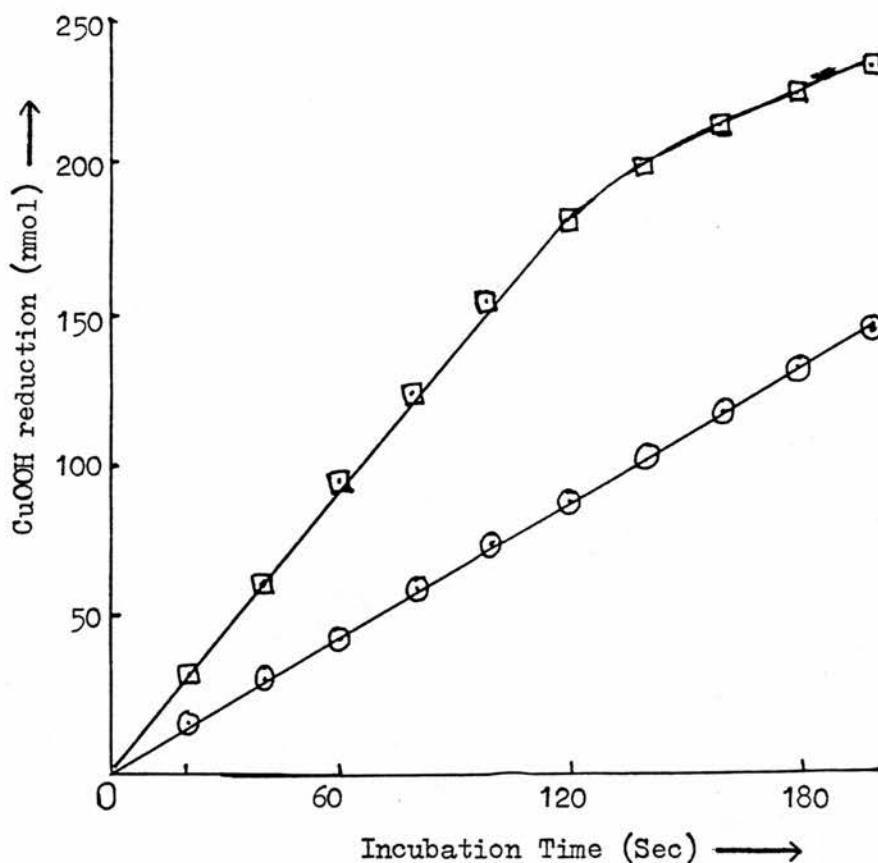


Fig.3: Time Course of Glutathione Peroxidase (GP).

The reaction was started by adding CuOOH (.1mM) to the reaction mixture (final volume 1ml) preincubating with mitochondrial enzyme (.9mg, \ominus), or cytosolic enzyme preparation (.7mg, \oplus) at 30°C. Data plotted are means from 4 assays (\pm S.D. \angle .3nmol).

The specific activity of mitochondrial GP was 0.05I U, and that of the cytosolic enzyme 0.08I U.

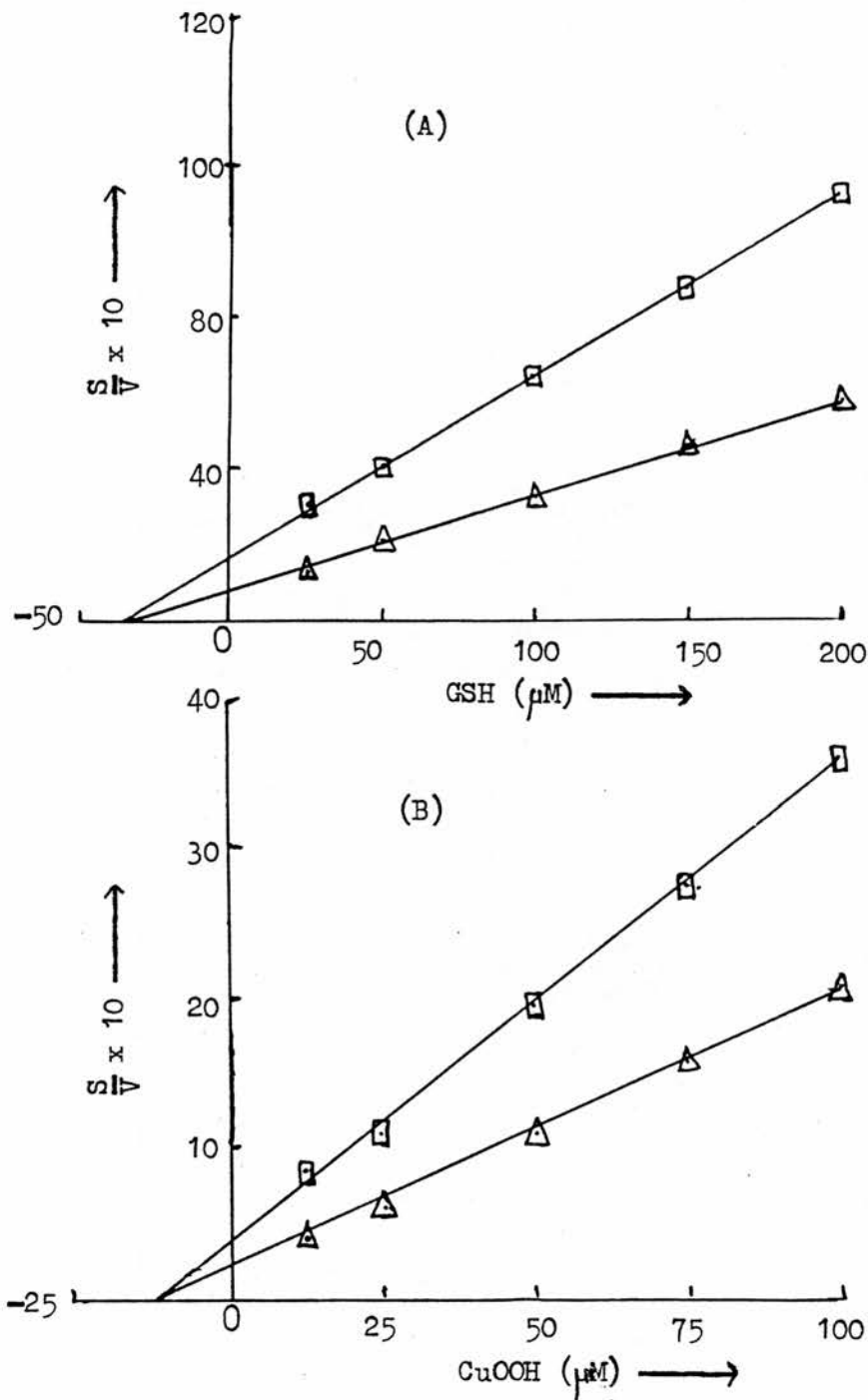


Fig. 4: Variation of mitochondrial (\square) and cytosolic (\triangle) GP activities with (A) GSH and (B) CuOOH concentration.

This method is described in methods, (Section 4.2), when GSH was varied CuOOH was at 0.1 mM ; and when CuOOH was varied GSH was held constant at (0.25 mM). The assays had mitochondrial (1.5 mg) and cytosolic (0.9 mg) preparations. Data plotted are means from 4 assays (\pm S.D. of $\sqrt{L \cdot 5 \text{ nmol/min}}$, $K_m \text{ GSH} = 35.5 \pm 3.4 \mu\text{M}$ and $K_m \text{ CuOOH} = 11.0 \pm 2.5 \mu\text{M}$

Linear $\frac{S}{V}$ versus S plots were obtained for both the mitochondrial and cytosolic enzymes which remarkably showed same affinities for the two substrates (figure 4), with stronger affinity for CuOOH ($K_m = 11\mu M$) than GSH ($K_m = 35\mu M$).

2.3 The effect of carboxylate carrier inhibitors

I proceeded to investigate the effect of carboxylate carrier inhibitors on both the mitochondrial and cytosolic GP.

These substances inhibited both enzymes when GSH was varied (figure 5), but did not inhibit either enzyme when CuOOH was varied (figure 6).

The inhibitions were non-competitive with respect to GSH (figure 5) since the K_m s were unchanged. The data was replotted according to the method of Dixon (1953), a method for estimating K_i ($\frac{1}{V}$ against i) (the plots not shown) which gave straight lines intersecting on the i -axis, confirming the non-competitive nature of the carrier inhibitors with propane-1,2,3-tricarboxylate (PT) being the strongest inhibitor than n-butylmalonate (BM) or phenyl pyruvate (PP) (figure 5).

2.4 Inhibitor effect on partially purified GP

The discovery that the inhibitors affect GP activity in crude preparations was clearly important and so it was decided to partially purify the enzyme from mitochondria, to study it further.

Purification of the mitochondrial enzyme has been described (Zakowski and Tappel 1978, see also methods, Section 2) in which the dialysed preparation is subjected to acid treatment followed by acetone extraction. The resulting preparation (purification achieved about 3-fold, figure 7) was then used to repeat the inhibition experiments already described (Section 2.3).

The results (figure 7) show that the K_m s of the enzyme are not changed by the purification procedure, but that there is now no inhibition by the carboxylate carrier inhibitors as GSH is varied. There is as before (figure 6) no inhibition on the partially pure enzyme as CuOOH is varied.

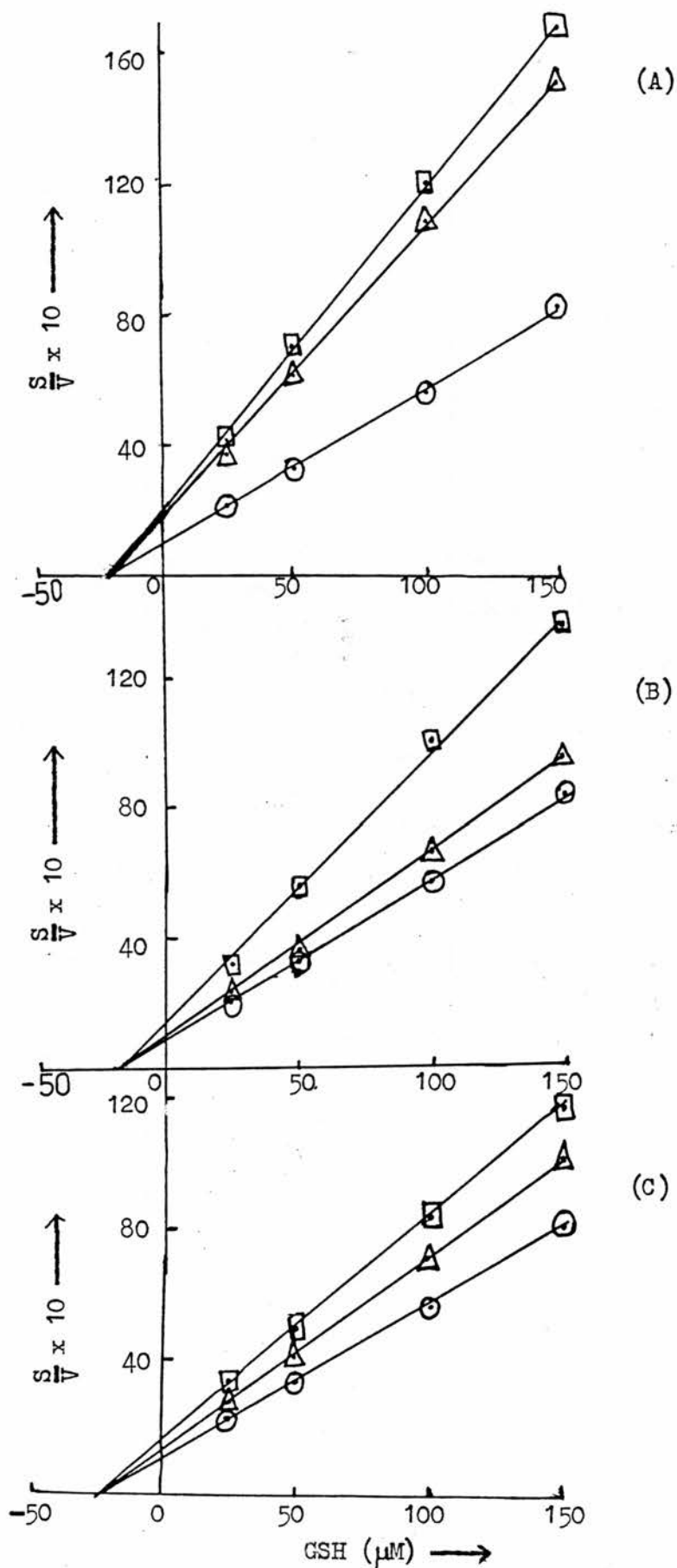


Fig 5: Effect of carboxylate carrier inhibitors on GP activity with varying GSH; PT (A and D); BM (B and E) and PP (C and F): Mitochondrial enzyme (A, B and C)(1.2mg). Cytosolic enzyme (.7mg)(D, E and F). Continued on page 56).

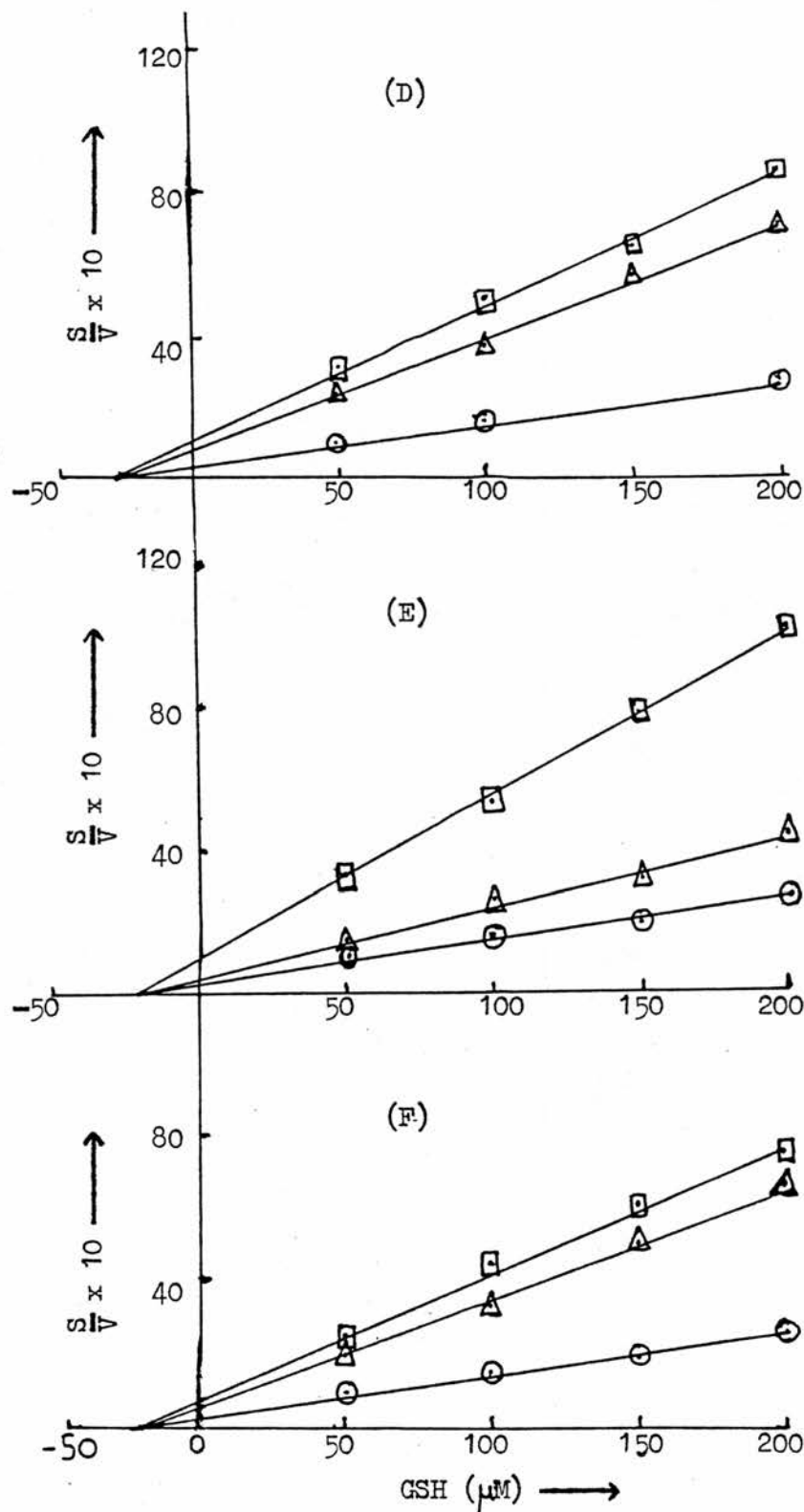


Fig 5: The method is as described for figure 4 with CuOOH at 0.1mM . The inhibitor additions were: Nil (\circ), 2.0mM (\triangle) and 5mM (\square).

Data are means from 3 assays (\pm S.D. of $\sqrt{2}$ $.8\text{nmol}/\text{min}$).

The data replotted according to Dixon method gave the following values for K_i (mM) with mitochondrial enzyme given first: ie PP ($1.28 \pm .08$, $1.15 \pm .07$), BM ($1.2 \pm .05$, $1.18 \pm .04$); and PT ($.35 \pm .01$, $.35 \pm .01$)

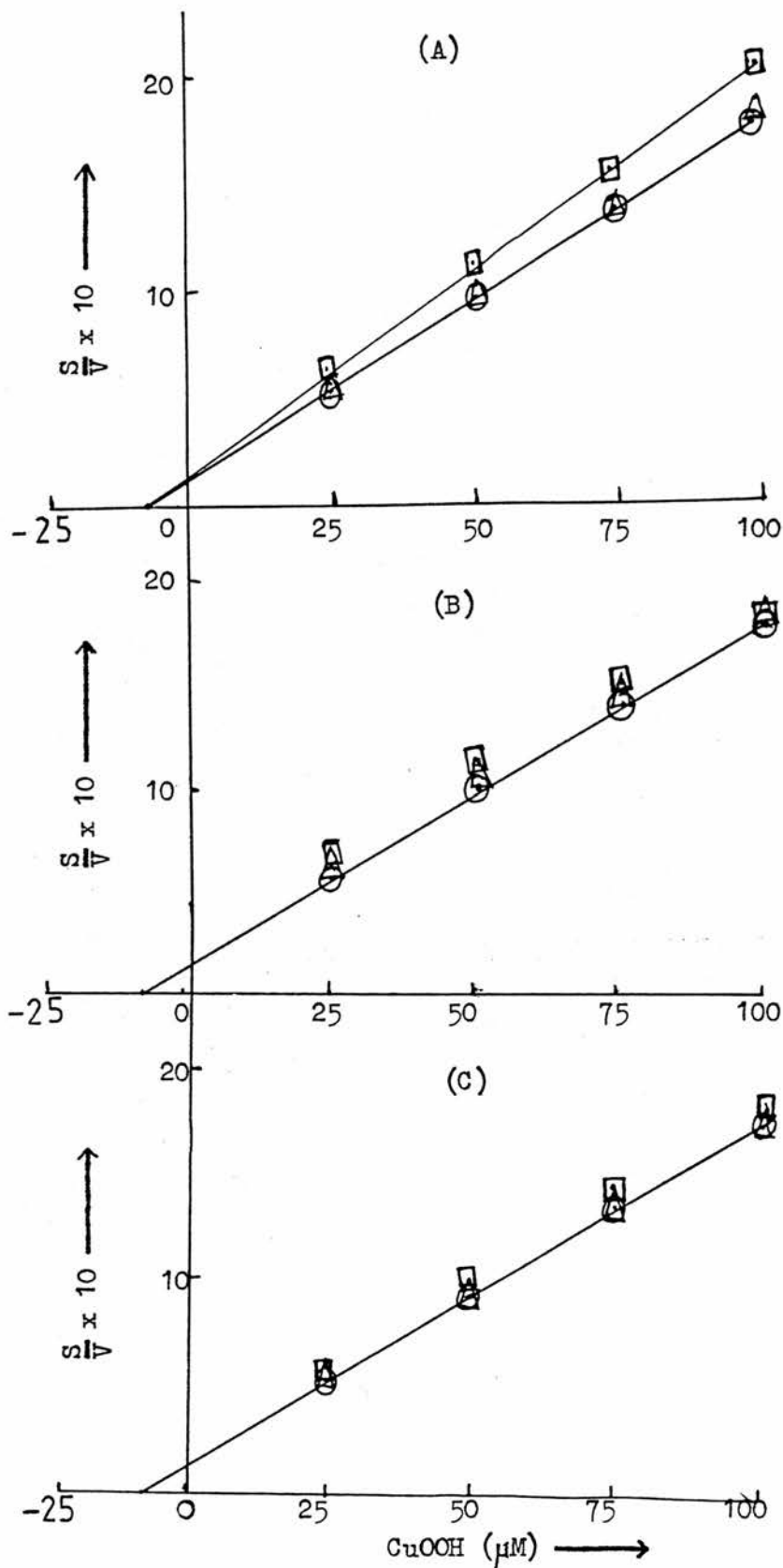


Fig 6: Effect of carboxylate carrier inhibitors on GP activity with varying CuOOH; PP (A and D); BM (B and E); PT (C and D).

All the other conditions as described in figure 5.

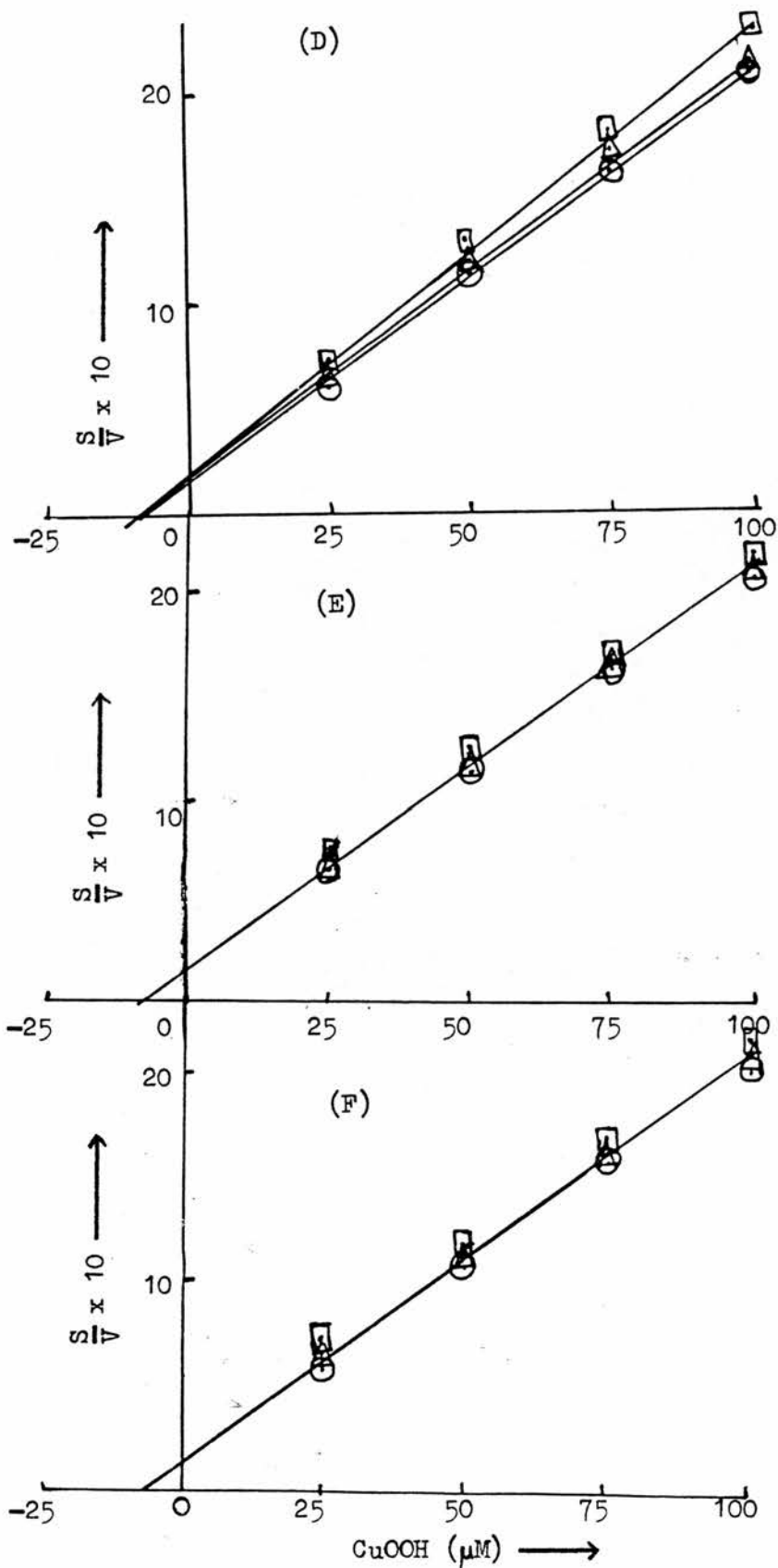


Fig 6: (Continued from p 57)

All other conditions as described in figure 5, except for GSH at (.25mM).

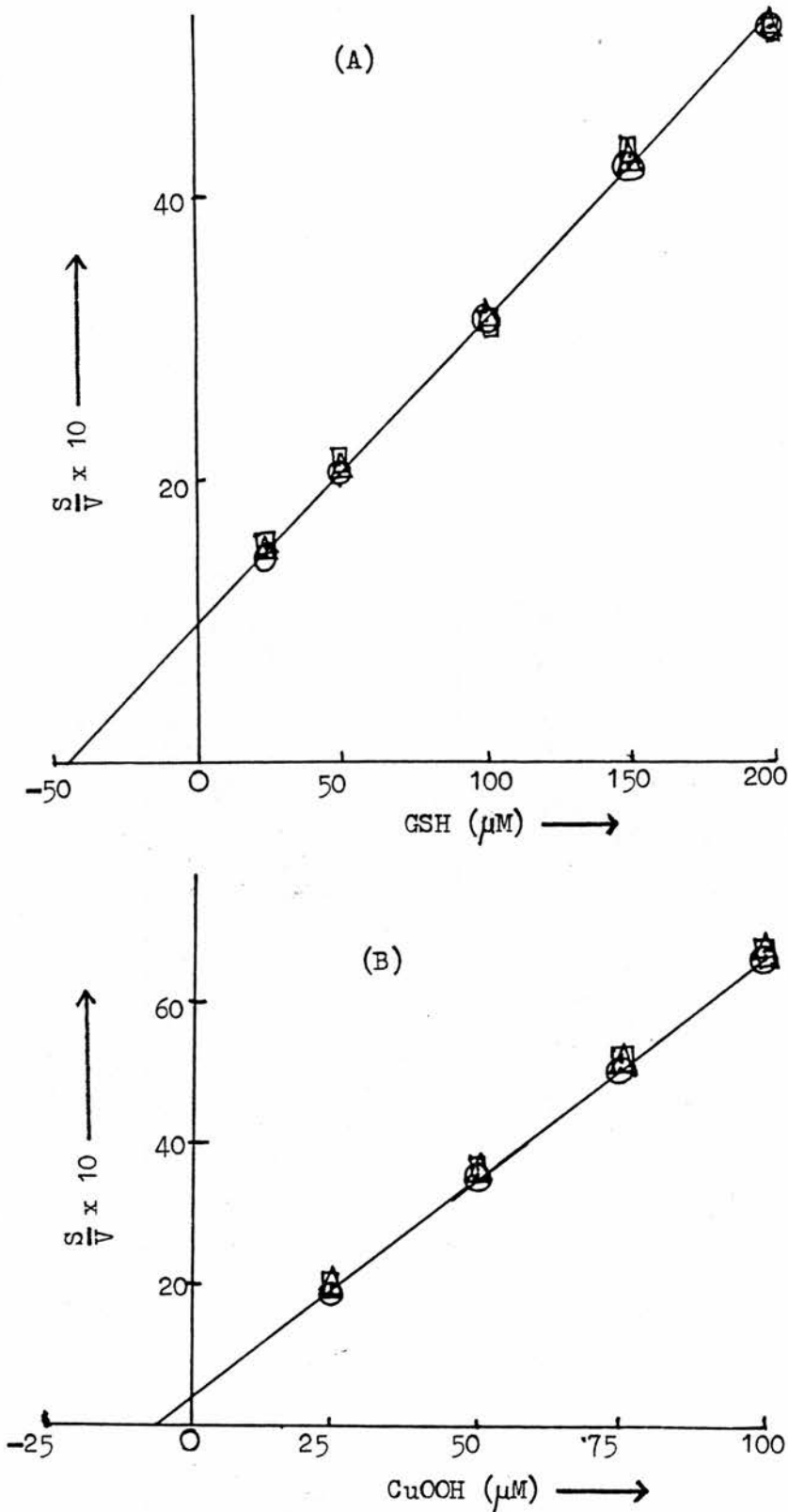


Fig 7: The effect of carboxylate carrier inhibitors on partially purified GP activity with varying GSH (A) and varying CuOOH (B).

The method and conditions were as described in figure 4. The inhibitors (PP, BM, BT and PT) were present at concentrations (mM); Zero (\ominus), 2.5 (\triangle) and 5.0 (\boxplus). Data plotted are means from three assays (\pm SD. of $V \leq 5 \text{ nmol/min}$). The specific activity for mitochondrial enzyme was .096IU.

3.0 GLUTATHIONE REDUCTASE (GR)

The finding that mitochondrial GP was not directly inhibitable by inhibitors such as carboxylate carrier inhibitors, led me to pass on to consider their effect on GR. Any inhibition of this enzyme would account for low GSH levels found on treating mitochondria with inhibitors in the presence of the hydroperoxide substrate (Section 1.2).

3.1 GR Activity

GR catalyses the oxidation of NADPH_2 by GSSG (see Chapter 1). Like GP, GR is also present as both a cytosolic and a mitochondrial enzyme. Investigations were done on both enzymes, to see if there were any obvious differences.

Both enzymes, from the cytosol and mitochondria, were studied in crude preparations following NADPH_2 oxidation at 340nm. A time course for GR reaction was determined (figure 8) and showed to be linear for 2 minutes for both enzymes.

The specific activity of the cytosolic enzyme was much higher (3-fold) than the mitochondrial enzyme (figure 8).

The K_m s of the mitochondrial and cytosolic enzymes for the substrates NADPH_2 and GSSG were determined by varying the concentration of one with the other held high as previously described for the GP enzyme (Section 2.2).

The $\frac{S}{V}$ versus S plots obtained (figure 9) show that both the mitochondrial and cytosolic enzymes have same affinities for their substrates, with much stronger affinity for NADPH_2 ($K_m = 2.5\mu\text{M}$) than GSSG ($K_m = 50\mu\text{M}$).

3.2 The effect of carboxylate carrier inhibitors

The effect of carboxylate carrier inhibitors was investigated on both the mitochondrial (figure 10) and cytosolic (figure 11) crude dialysed preparations (see methods, Section 2); at different substrate concentrations.

These/

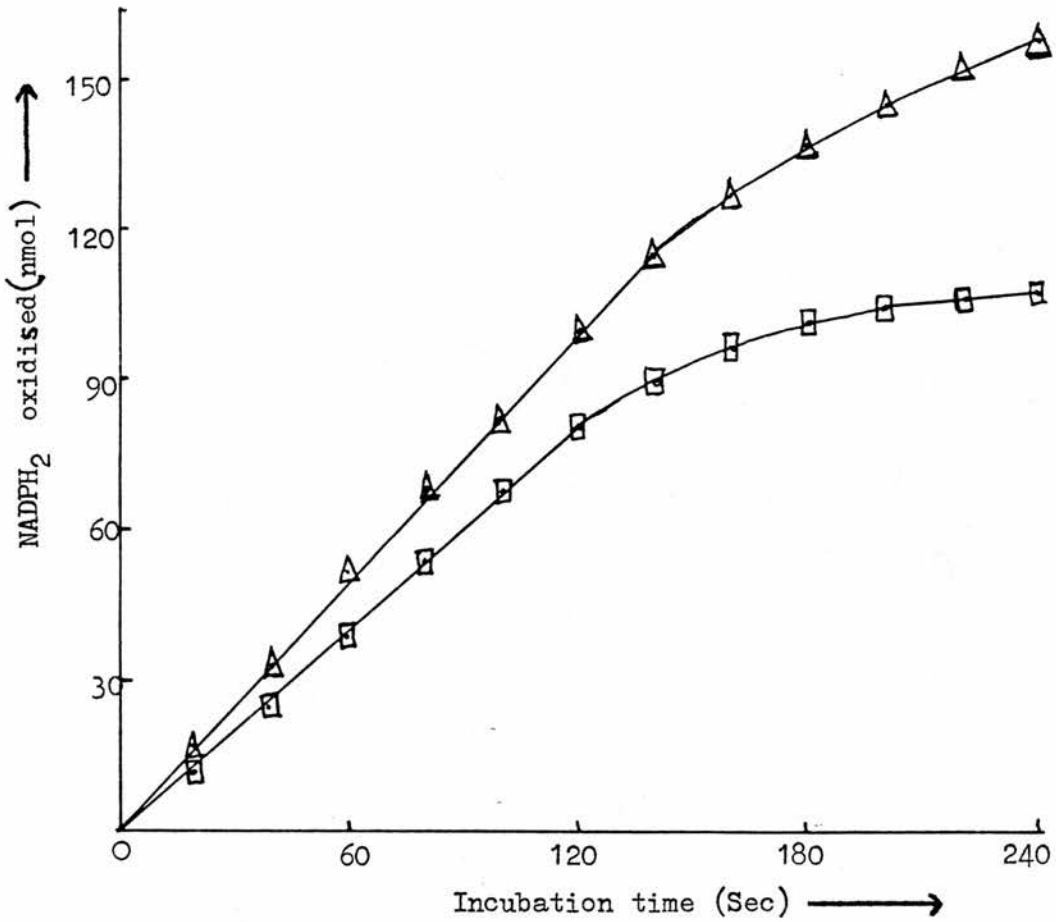


Fig.8: Time course of GR activity by the mitochondrial (◻) and cytosolic (Δ) enzyme preparations

The reaction was carried out as described in Section 4.3 (in methods), with GSSG (200 μ M) and NADPH₂ (100 μ M) in the reaction mixture. The reaction mixture contained mitochondrial (1.2mg) and cytosolic (0.6mg) enzyme preparations. The values plotted are means from two determinations (\pm SD. \angle .6nmol/min).

The specific activities for the mitochondrial and cytosolic preparations were (.033IU) and (.091IU), respectively.

These substances had no effect on the enzymes when either NADPH_2 or GSSG was varied.

There was, therefore, no need to investigate the inhibitions observed in hydroperoxide reduction (Section 1) on GR any further.

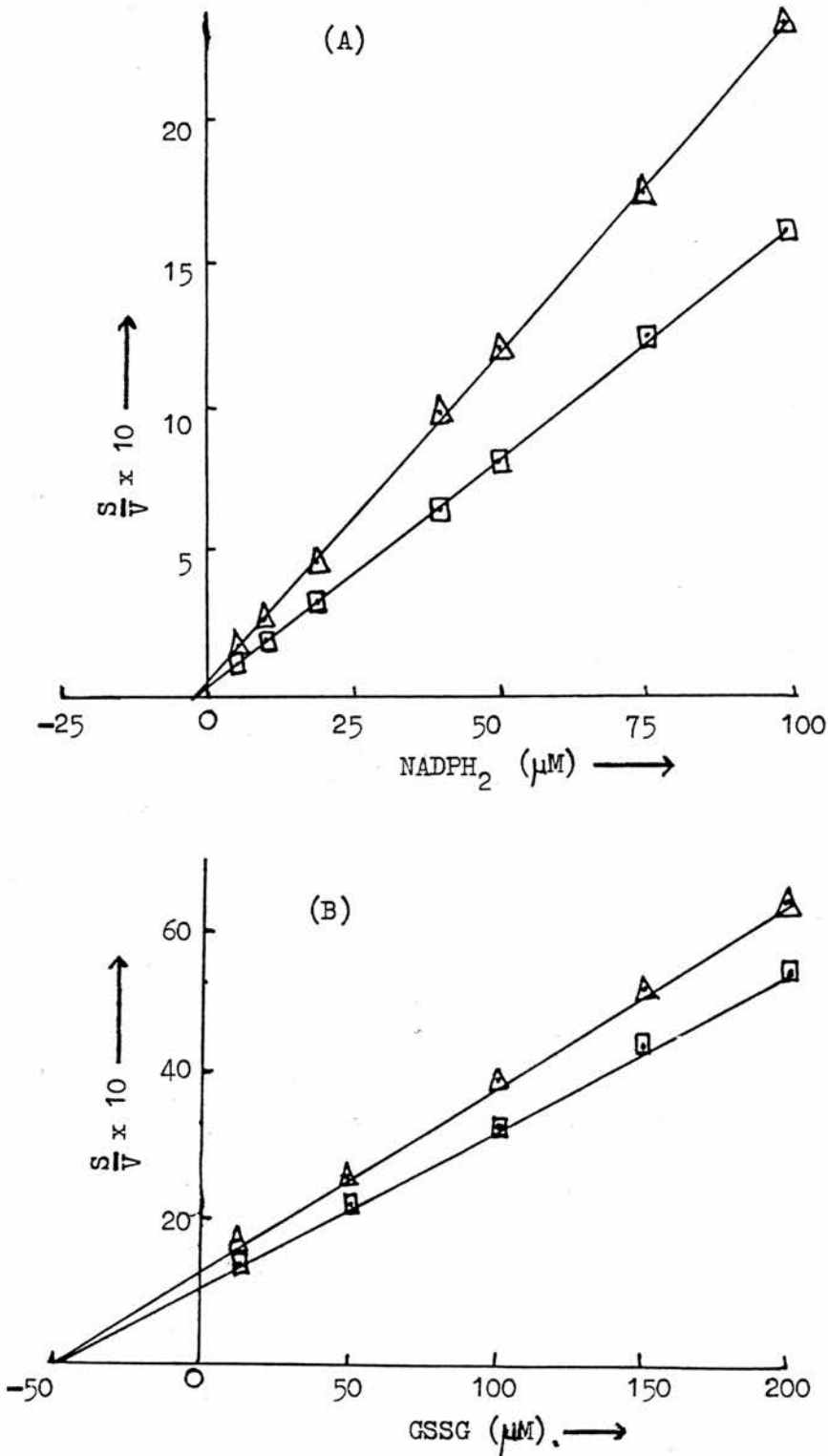


Fig 9: Variation of mitochondrial (\square) and cytosolic (Δ) GR activities with (A) NADPH_2 and (B) GSSG concentration.

The method is as described (methods, Section 4.3), when NADPH_2 was varied GSSG was at $.2\text{mM}$; and when GSSG was varied NADPH_2 was at $.1\text{mM}$. The amount of protein was mitochondrial (2.0mg) and cytosolic ($.6\text{mg}$). Initial velocities per minute were obtained at 340nm . Data plotted are means from 4 assays (\pm SD. of $V < .8\text{nmol/min}$).

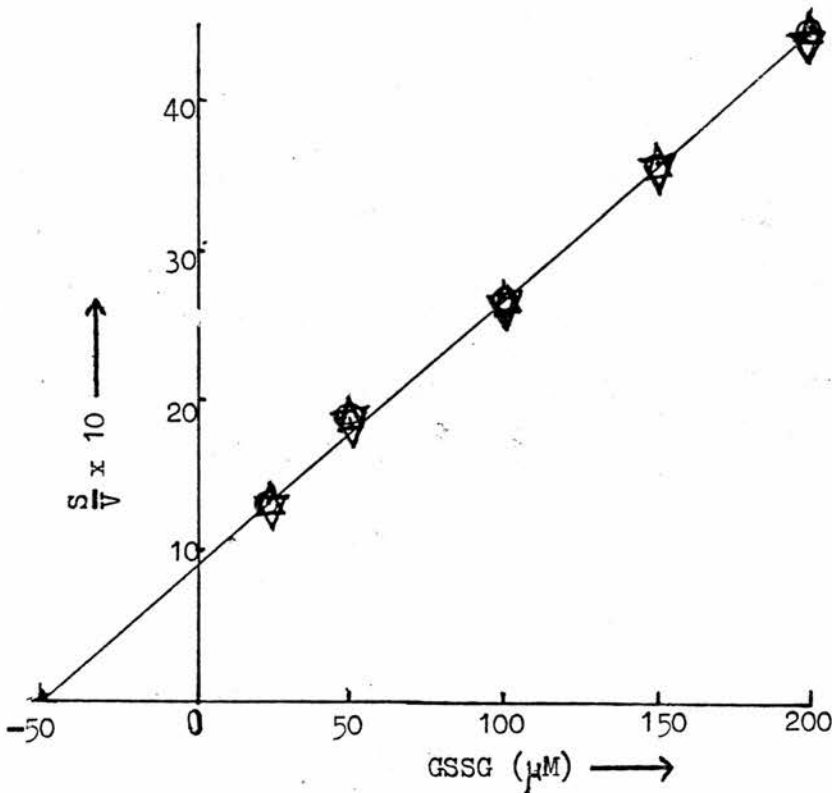
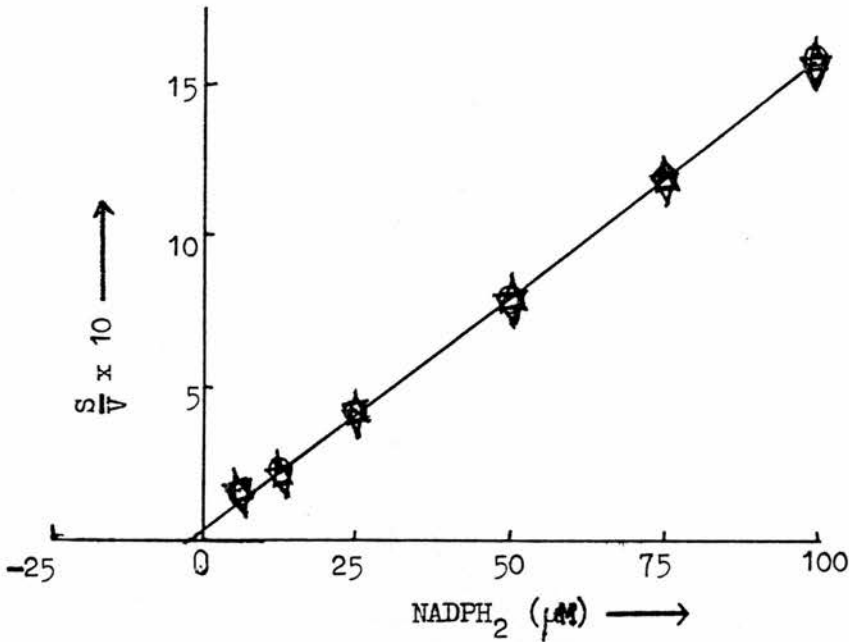


Fig 10: Effect of carrier inhibitors on mitochondrial GR activity with varying NADPH₂ (A) and with varying GSSG (B).

The reaction was carried out as in methods, Section 4.3; when NADPH₂ was varied GSSG was at (.2mM) and when GSSG was varied NADPH₂ was at (.1mM) in the presence of the enzyme preparation (2mg). The inhibitors (PP, BM, BT and PT) were present at 0mM (○); 2.5mM (△) and 5mM (▽) in both (A) and (B). Data plotted are means of two assays (\pm S.D. of $V_L \cdot 7n$ mol/min).

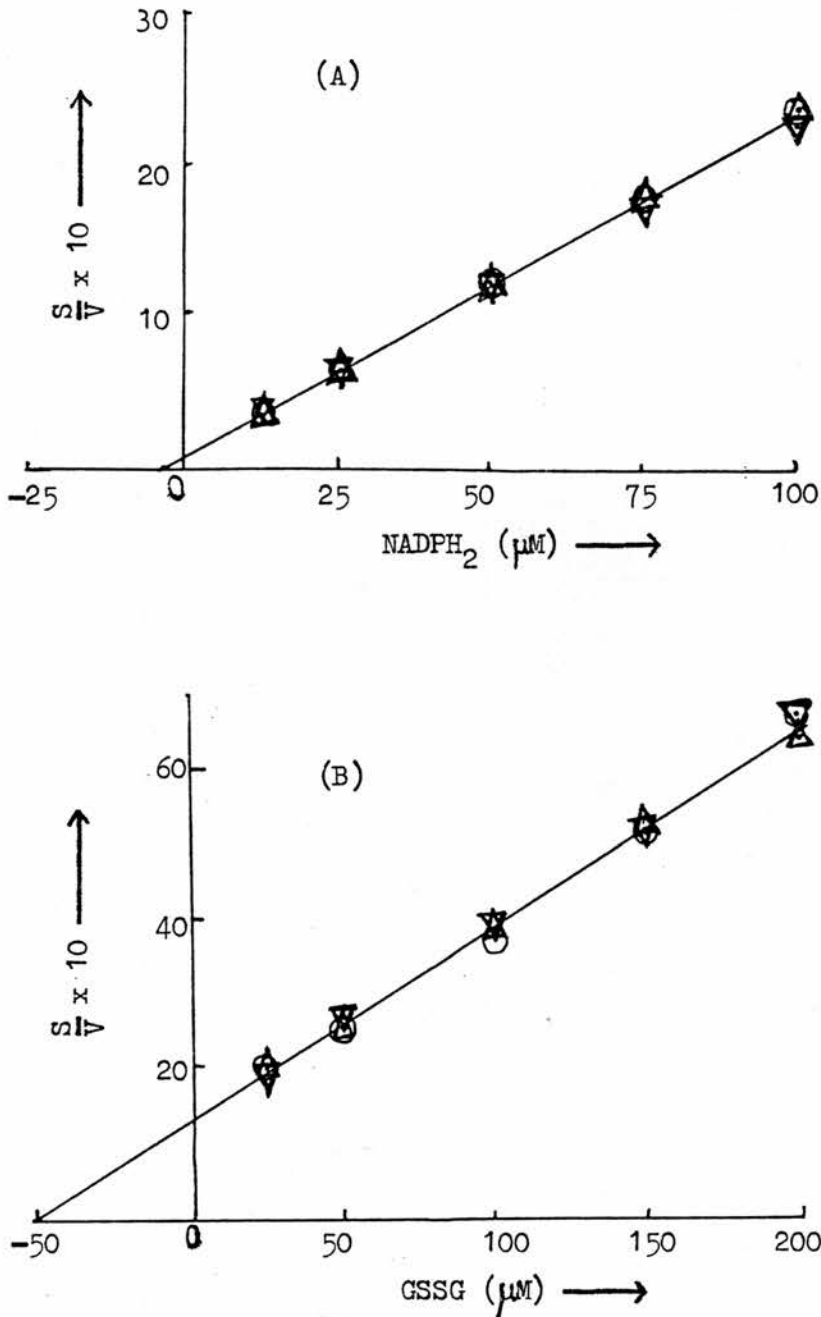


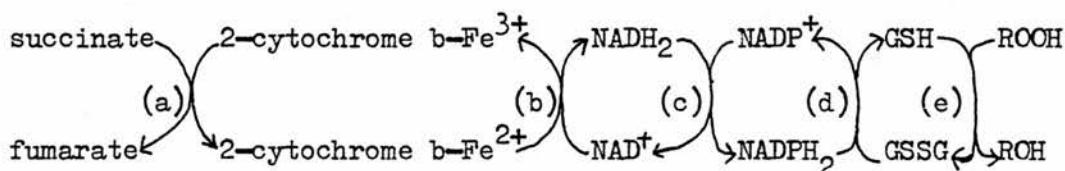
Fig 11: Effect of carrier inhibitors on cytosolic GR activity with varying NADPH_2 (A) and varying GSSG (B)

The reaction was carried out as in figure 10, under the same conditions. The carrier inhibitors, (PP, BM, BT and PT) were all present in (A) and (B) at the following concentrations; 0mM (\ominus), 2.5mM (\triangle) and 5mM (∇); and the amount of protein was (.7mg). Data plotted are means of two assays (\pm SD. of $V \pm .8 \text{ nmol/min}$).

4.0 DISCUSSION OF CHAPTER 3 (1 - 3)4.1 BuOOH reduction by mitochondria

Rat liver mitochondria are capable of reducing BuOOH at a rate ($25\text{nmol min}^{-1} \text{mg}^{-1}$) far higher than the amount of reducing equivalents available, i.e. GSH (4nmol mg^{-1}) (see Sections 1.1 and 1.3). It is this finding, first reported by Jocelyn (1978) using diamide as the oxidant and later by Jocelyn and Dixon (1980) using BuOOH as the oxidant, which makes the glutathione redox system (Chapter 1, Section 5) relevant to the subject of hydroperoxides detoxification.

The following pathway for the reduction of hydroperoxides supported by succinate has been proposed by Oshino and Chance (1977) and Jocelyn and Dickson (1980).



One of the features of this scheme is the essential role of GSH. The involvement of GSH is maybe catalytic and the role of the rest of the pathway above is to regenerate GSH continuously from GSSG.

It is proposed that succinate is oxidized at (a) via the reverse electron transfer (Ernster and Lee 1967), and NAD⁺ is reduced to NADH₂ at (b). Indirectly, NADH₂ which is produced is oxidised by the ATP supported nicotinamide nucleotide transhydrogenase at (c) forming NADPH₂ required at (d) by the NADP(H) dependent glutathione reductase (Chapter 1, Section 2) to reduce GSSG to GSH. At step (e) GSH is utilised by glutathione peroxidase (Chapter 1, Section 3) in the presence of hydroperoxides giving corresponding alcohols and in the case of hydrogen peroxide giving water.

There/

There is direct evidence for energy involvement at step (a) reported by Jocelyn and Dixon (1980) in which antimycin A inhibited BuOOH reduction by rat liver mitochondria and ATP relieved the inhibition.

The evidence for an energy dependent step (c) is reported in Chapter 5 of this thesis. The involvement of energy at step (e) cannot be ruled out since high energy equivalents may be required to transport oxidising equivalents from the hydroperoxides. The source of necessary high energy equivalents is uncertain, for instance Jocelyn and Dickson (1980) reported that cyanide at levels which inhibit O_2 consumption by 95% does not inhibit BuOOH reduction.

The evidence for energy involvement at step (e) is the striking inhibition of BuOOH reduction by FCCP (see Section 1.2). This was also observed by Jocelyn and Dickson (1980) who reported that FCCP however, does not inhibit glutathione peroxidase in lysates. This finding is compatible with the fact that FCCP does also diminish GSH levels in mitochondria (Section 1.3). FCCP in addition to possible inhibition of the entry of oxidants into mitochondria, is more likely to dissipate high energy equivalents required at steps (a) and (c).

What is of particular importance to this study is the inhibition of hydroperoxides reduction by carboxylate carrier inhibitors. In Section 1.2 I have confirmed Jocelyn and Dickson's (1980) findings that the following specific carboxylate carrier inhibitors; PP (mono-); BM (di-); BT and PT (tri-) (carboxylate carrier inhibitors) inhibit BuOOH reduction. An additional dicarboxylate carrier inhibitor PhS was introduced and it does inhibit the reduction too.

These carrier inhibitors inhibit the BuOOH reduction in a concentration dependent manner (figure 2); having approximately 50% inhibition at around 10 μ M. It may be important to point out that the carrier inhibitors inhibit specifically the transport of corresponding carboxylic acids (Chapter 1, Section 5). The/

The fact that mitochondria preloaded with succinate were used in BuOOH reduction and that all carrier inhibitors inhibit the reduction imply the involvement of carrier proteins in the mechanism of reduction (p66). Much more important is the fact that these carboxylate carrier inhibitors inhibit BuOOH reductions at concentrations which they normally inhibit the specific proteins in cytoplasm and mitochondria (see literature, Chapter 1).

The carrier proteins may be involved in the direct transport of oxidising equivalents (hydroperoxides) into mitochondria (Jocelyn 1978) or may be involved in modulating the reaction(s) involved in the redox system. Direct evidence for the latter, is the fact that the carrier inhibitors also inhibit GSH concentration in intact mitochondria (Section 1.3). This can be explained if they inhibit at either of the steps (a), (b), (c) or (d), i.e. a low GSH implies that inhibition is on its regeneration rather than on GP. Depletion of mitochondrial GSH by mono- and tricarboxylate carrier inhibitors and to a lesser extent by dicarboxylate carrier inhibitor (8M) was also reported by Jocelyn and Dickson (1980).

4.2 Glutathione Peroxidase

Crude mitochondrial and cytosolic GP activities were inhibited non competitively only when GSH was being varied (Section 2.3), but not when CuOOH was varied. However, upon partial purification of both the cytosolic and mitochondrial GP, the inhibitions were lost (Section 2.4).

A number of suggestions could explain this observation: (A) There is a protein factor in the crude preparations which may bind to the enzyme maybe near the GSH binding site and makes the enzyme susceptible to inhibition by carrier inhibitors. (B) There is a protein factor which may modify in some way the inhibitor molecule (either by catalysing metabolism, eg oxidation) or by binding them and so making the carrier inhibitors accessible to the peroxidase. (C)/

(C) The third possibility is that, GP activity with the substrates used (CuOOH or BuOOH) is found not only with the true selenium containing enzyme (GP), but also by the non-specific glutathione-S-transferases (Burk and Lawrence 1978). It is possible that this latter enzyme is inhibitable by carboxylate carrier inhibitors. Upon partial purification, the glutathione-S-transferase enzyme activity was lost and therefore, no inhibition could be observed.

However, although glutathione-S-transferase is abundant in cytosol, it is said to be low in concentration compared to GP in mitochondria (Burk and Lawrence 1978), and thus (C) is unlikely to be able to account for the inhibition in crude mitochondrial preparations.

The most likely explanation is point (A) as (B) implies that the enzyme is capable of interacting with synthetic substances (Carrier inhibitors) which do not occur naturally. The effect of carboxylate carrier inhibitors may be significant when dealing with intact mitochondria due to above considerations, but do not seem to be direct inhibitors of GP.

One other observation made out of this study is the fact that there is a close resemblance between the cytosolic and mitochondrial GP enzymes in relation to their substrates affinities (Section 2.2). The cytosolic enzyme activity however, is much higher than the mitochondrial one, as has been reported by various workers (see literature, Chapter 1). It is possible that the two enzymes from the cytosol and mitochondria are one, only separated into different compartments.

4.3 Glutathione Reductase: Crude mitochondrial and cytosolic glutathione reductase showed marked similarity in their affinities for their two substrates (GSSG and NADPH₂, Section 3, of 50 μ M and 2.5 μ M, respectively). The Kms are remarkably similar to Mize and Langdom's finding in 1962 with the purified cytosolic GR enzyme. However, it is the cytosolic enzyme which has the higher activity. Again, here, the similarity in their Kms imply that the enzymes have the same origin, but distributed into different compartments for localized functions.

It/

It was surprising, however, to find that the mitochondrial glutathione reductase was not inhibited by carboxylate carrier inhibitors since it was one of the good candidates for such inhibitions, in view of the fact that these substances inhibited GSH levels (Section 1.3). GR is the enzyme directly involved in GSH regeneration.

The possibility that carboxylate carrier inhibitors were inhibiting BuOOH reduction in intact mitochondria by affecting step (d) has therefore, been ruled out. It is at this point that I decided to investigate the inhibitions by these carboxylate carrier inhibitors further, at step (c).

CHAPTER 4NON-ENERGY DRIVEN PYRIDINE DINUCLEOTIDE TRANSHYDROGENASE

The mammalian nicotinamide nucleotide transhydrogenase is a membrane bound enzyme, bound to the inner mitochondrial membrane (Chapter 1, Section 4). It catalyses the transfer of hydride ions when NADH_2 and NADP^+ are brought together (ie non-energy reaction). But there is a several fold increase if ATP or high energy intermediates are also present (ie the energy reaction). The non-energy dependent reaction is the subject of my discussion in this Chapter.

The natural substrates for this enzyme (NAD(H) and NADP(H)) are impermeable to the inner mitochondrial membrane, and for this reason, one cannot use whole mitochondria to study it. Instead it is necessary to use submitochondrial particles in which the membrane is predominantly turned inside out, thus exposing the enzyme to its substrates.

The transhydrogenase enzyme is a very labile enzyme and especially that from rat liver mitochondria. First of all, I had to establish the most suitable method for preparing submitochondrial particles with a high enzyme activity. Next it was necessary to select suitable assay conditions and methods.

The difficulty here is that the reaction can only be followed indirectly since there is no change in optical density at 340nm, from the transhydrogenation itself (Literature, Chapter 1).

The work reported in this Chapter and Chapter 5 was, whenever possible, carried out in parallel with the same stock solutions and same batches of enzyme preparations. The results have been separated into two chapters to make them easier to follow.

A/

A number of controls were done for both reactions (non-energy and energy driven) and this would be specified in the results. These include the use of a specific transhydrogenase inhibitor (PCoA) (Rydström 1977); omitting one of the substrates, ie the no NADP⁺ or no NADH control; and finally the zero time control where the reaction was stopped by acid or base treatment (see results) before it even started.

1. DETERMINING CONDITIONS FOR THE NON-ENERGY REACTION

1.1 The Sonication Medium

Submitochondrial particles were prepared in various media (see methods, Section 2.2). A time course for each preparation was determined by the discontinuous GSH method (constant NADP⁺) as described in methods, Section 4.4B(ii) (see figure 1).

The non-energy transhydrogenase reaction was linear for about 5 minutes in the particles prepared in all the media, but preparing particles in phosphate and Tris-HCl buffers lengthened their stability by improving the linear time course for up to 10 minutes. In addition, enzyme activity was highest in particles prepared in phosphate medium (.0071 U) followed by those prepared in Tris-HCl medium (.0041 U) and least in particles prepared in sucrose (.0021 U).

1.2 The Storage Medium

The minimum time required to prepare submitochondrial particles for the transhydrogenase assay was $4\frac{1}{2}$ hours. There was therefore, a need to store the particles to obviate their daily preparation. A satisfactory storage medium had to be established.

This was achieved by resuspending the particle pellet collected at 100,000xg (methods, Section 2.2) in different media storing them immediately at -70°C and assaying the enzyme activity at intervals by the GSH method (constant NADP⁺) (methods, Section 4.4B(ii)).

The/

The results (figure 2) show that the transhydrogenase activity was relatively more stable in a non-ionic medium, sucrose. But even then the enzyme is very unstable, it loses 50% of its activity in 14 days in sucrose medium and more than 75% in particles in the other media. Freezing the particles had no effect on enzyme activity since the activities on day (0) and day (1) were the same.

1.3 The Suitable solubilising detergent

In the study of the non-energy transhydrogenase reaction, it was critically necessary to solubilise the enzyme from the membrane. A suitable solubilising detergent had to be determined.

The enzyme was solubilised from submitochondrial particles prepared in the usual way (methods, Section 2.2) by various detergents (Table 1) as described in methods (Section 2.3). The non-energy transhydrogenase activity was determined immediately in unfrozen preparations by the GSH method (constant NADP^+ and NADH) see methods, Section 4.4B(iii).

The results (in Table 1) show that digitonin followed by tert-amyl alcohol achieved about 2-fold purification although the recovery was about 30%. There was no significant purification with Lubrol PX or Triton X-100. Digitonin was rendered the satisfactory solubilising detergent among the others, and was used routinely in the preparation of the membrane free transhydrogenase enzyme.

A time course of the digitonin transhydrogenase fraction was determined by the same method (GSH constant NADP^+ and NADH), and was found to be linear for about 10 minutes (results not shown). The transhydrogenase enzyme activity in submitochondrial particles and in solubilised preparation is however, unstable, losing its linearity at about 10 minutes even when both the substrates (NADH and NADP^+) are regenerated (results not shown).

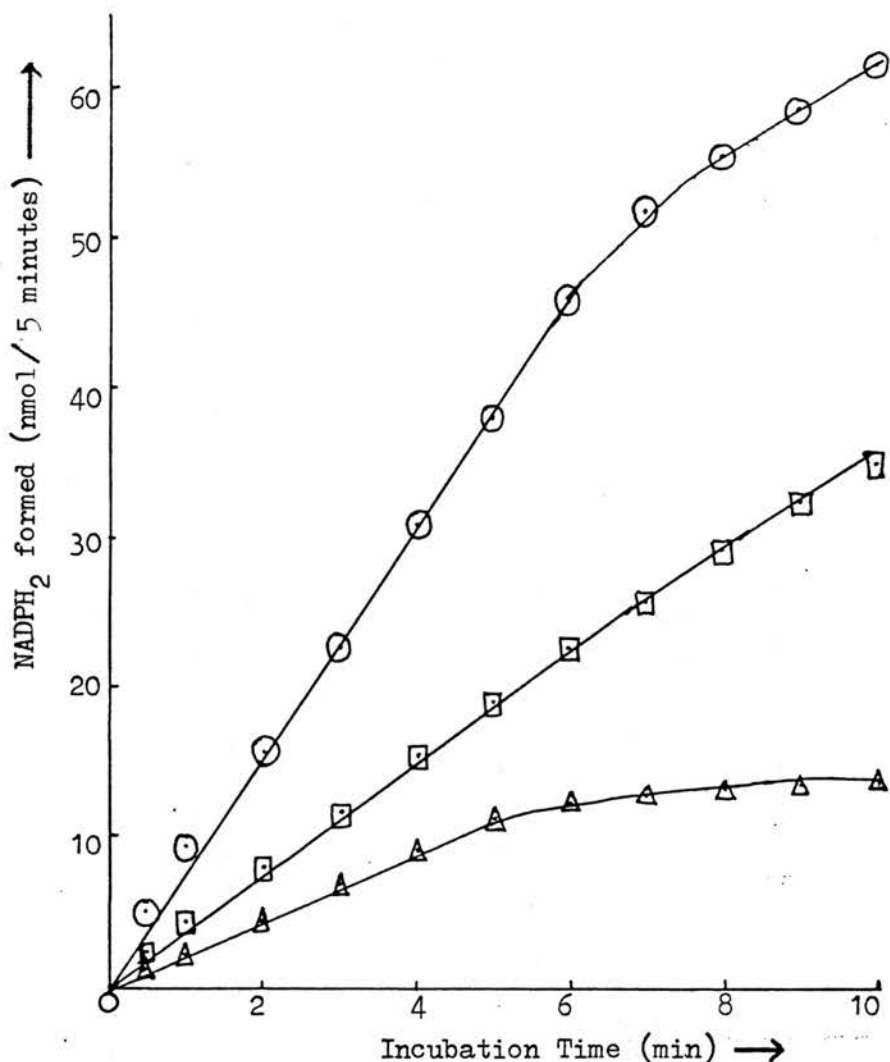


Fig 1: Time course for the non-energy transhydrogenase reaction in particles prepared in different media

To reaction mixture (445 μ l) prepared as in methods (Section 4.4ii) in Tris-sucrose-HCl buffer pH 8.0 preincubating at 30°C in Eppendorf tube a mixture (55 μ l) of submitochondrial particles (1mg) sonicated (see methods 2.2) in sucrose (.25M) (A), Tris-HCl (.033M) pH 7.0 (B) and sodium phosphate (.025M) pH 7.0 and glutathione reductase (.45I U) of commercial yeast enzyme) was added to start the reaction. 5 minutes after incubation, the reaction was terminated with perchloric acid (50 μ l of 12% W/V) and GSH formed determined as in methods (Section 3.2). A zero time control was taken into account, determined in the same way except that perchloric acid was added prior to the addition of enzymes.

Data plotted are means from 4 assays each from different mitochondria preparation and unfrozen particles (0 day old), (\pm S.D. \angle 1nmol).

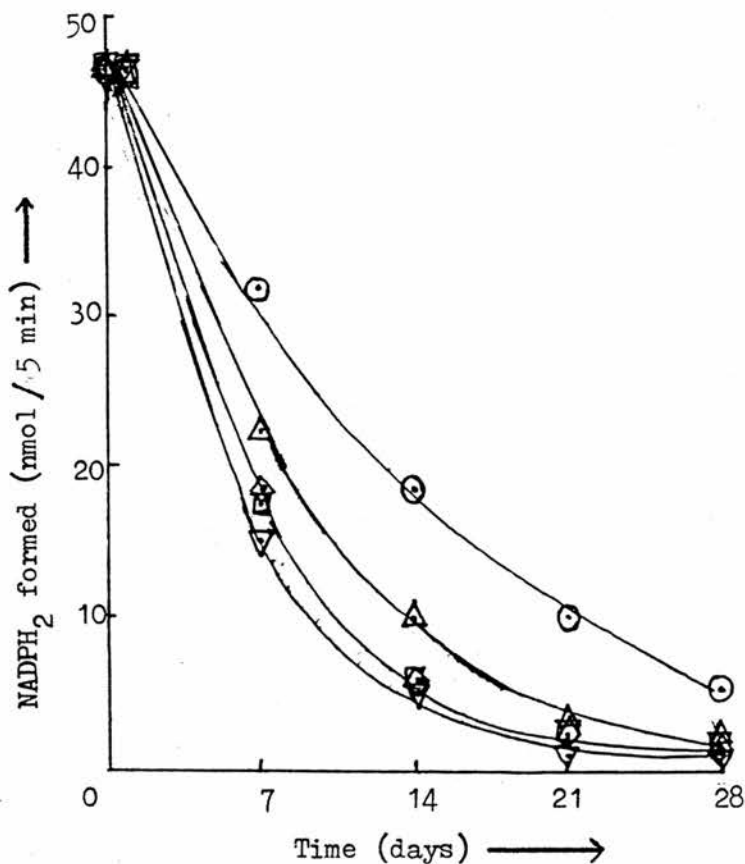


Fig 2: Variation of the non-energy transhydrogenase activity with time in different storage media.

To the reaction mixture (445 μ l) preincubating at 30°C a mixture (55 μ l) of glutathione reductase (.45I U of commercial yeast enzyme) and submitochondrial particles (.9mg) prepared as in methods (Section 2.2) and resuspended in sucrose (.25M) (○), sucrose (.25M) and MgCl₂ (.01M) (△), sucrose (.25M) and BSA (2% W/V) (▽), Tris-HCl (.25M) pH 7.0 (◻), and water (once distilled (◊)); was added. After 5 minutes the reaction was terminated and GSH determined (as in figure 1). Unfrozen particles were assayed at day (0). The particles were prepared from the same mitochondria batches and stored at -70°C (with slow cooling).

Data plotted are means from 2 assays (\pm S.D. \angle .5nmol) taking into account the zero time control (as in figure 1).

Table 1: Solubilisation of the non-energy transhydrogenase activity in various media

<u>Detergent</u>	<u>Percentage Recovery</u>	<u>Specific Activity (I U)</u>
Nil (particles)	100	.006
Triton X-100	76 \pm 4	.008
Lubrol PX	50 \pm 6	.008
Tert-Amyl alcohol	29 \pm 5	.011
Digitonin	34 \pm 4	.013

To the reaction mixture (440 μ l) containing ethanol (60mM) in Tris-sucrose-HCl pH 8.0 preincubating (5 min) at 30°C (as in figure 2), a mixture (60 μ l) of ADH (.45I U of commercial yeast enzyme), GR (.45I U from yeast) and enzyme preparation (.5mg) i.e. submitochondrial particles (Nil) or solubilised by various detergents (2% W/V) in the ratio 1:1 particle volume : detergent (see methods, Section 2.3); was added. After 5 minutes, the reaction was terminated with perchloric acid (50 μ l of 12% W/V) and GSH formed determined (as in figure 2). Values shown in the table are from 3 assays of 3 different batches of particles (\pm S.D. \angle 0.0001I U) taking into account the zero time control determined as in figure 2).

1.4 pH dependency of activity

The non-energy transhydrogenase activity was determined in Tris-sucrose-acetate buffer prepared as described (methods, Section 2.1) at different pH's (figure 3). This buffer was chosen to study the pH effect since it was used by most workers (Orneal and Fisher 1980) (see also literature, Chapter 1) and was readily available. The reaction was assayed by the GSH method (constant NADP⁺ and NADH) (methods, Section 4.4B(iii)) in submitochondrial particles and solubilised enzyme preparations (methods, Section 2.2 and 2.3).

Results (figure 3) show that the enzyme activity is lowest at pH 9.0 and increases continuously as the pH is lowered to a maximum at pH 6.0 and then falls again. Solubilising the enzyme had no effect on this trend. The/

The implications of this large increase in activity with decreasing pH are discussed later (Section 6), however, as pH of the natural environment of the enzyme (ie mitochondrial matrix) is likely to be above 7, enzyme preparations were chiefly studied at pH 8.0, to a lesser extent at pH 6.0 where enzyme activity was optimal.

As previously mentioned (Section 1.3), the specific activity of the solubilised enzyme was higher (2-fold) than that of the enzyme in particles, across the pH range (pH 5 to 9).

1.5 Reversibility of pH effect

Since non-energy driven transhydrogenase activity at pH 6 was highest, it was of interest to know whether the lower activity at higher pH values was due to partial inactivation of the enzyme (presumably irreversible) or to a requirement for protons in the catalysis. In the latter case, the rate would increase again when the pH of the medium was also increased.

Hence, submitochondrial particles were left without substrates for a time in Tris-sucrose acetate buffer at the required pH (Table 2) and then brought to the different pH (ie. pH 6 to pH 8, [8*] or pH 8 to pH 6, [6*]). Substrates were then added and the reaction rate then measured as before [GSH method constant (NADP⁺ and NADH)].

The results (Table 2) show that the pH effect is reversible and so it appears, the enzyme requires protons in catalysis (see later, discussion, Section 6).

1.6 Variation of activity with protein concentration

Non-energy driven transhydrogenase activities in submitochondrial particles and solubilised enzyme preparations were determined by the GSH method (constant NADP⁺ and NADH) (methods, Section 4.4B(iii)) using different amounts of the enzyme preparations (figure 4).

The/

The results are shown in figure 4 and show that, whether the assays (for both enzyme preparations) are carried out at pH 6.0 or at pH 8.0, there is as expected a linear increase in the activities up to 1mg protein. Thereafter, there are serious departures from linearity. Enzyme preparations up to 1mg were routinely used in assays.

1.7 Temperature profile

Non-energy transhydrogenase activity in submitochondrial particles was studied to determine the effect of temperature on the reaction at both pH 8.0 and pH 6.0.

Results (figure 5) are plotted by the Arrhenius method (1889). They show the expected linear response up to 40°C at both pH's. Above this temperature however, the activity at pH 6 dramatically falls presumably due to inactivation of the enzyme. The activity at pH 8 does not greatly change on increasing the temperature above 40°C. From the slopes over the range 20 to 40°C, the activation energy calculated at pH 6 (21 KJmol⁻¹) was 2-fold higher than at pH 8 (10.5 KJmol⁻¹). For the discussion of these results see Section 6.

2. THE NON-ENERGY (TRANSHYDROGENASE) REACTION KINETICS

In this section I studied the effect of varying the substrate concentrations on the activity of the enzyme to measure the rate of the reaction using a variety of methods.

2.1 Determination of Km

The transhydrogenase (TH) has at least two separate sites, one for NADP⁺ and another for NADH (considering the forward reaction). It was of interest for the subsequent studies to determine Km values for each site when the other was saturated with substrate. These/

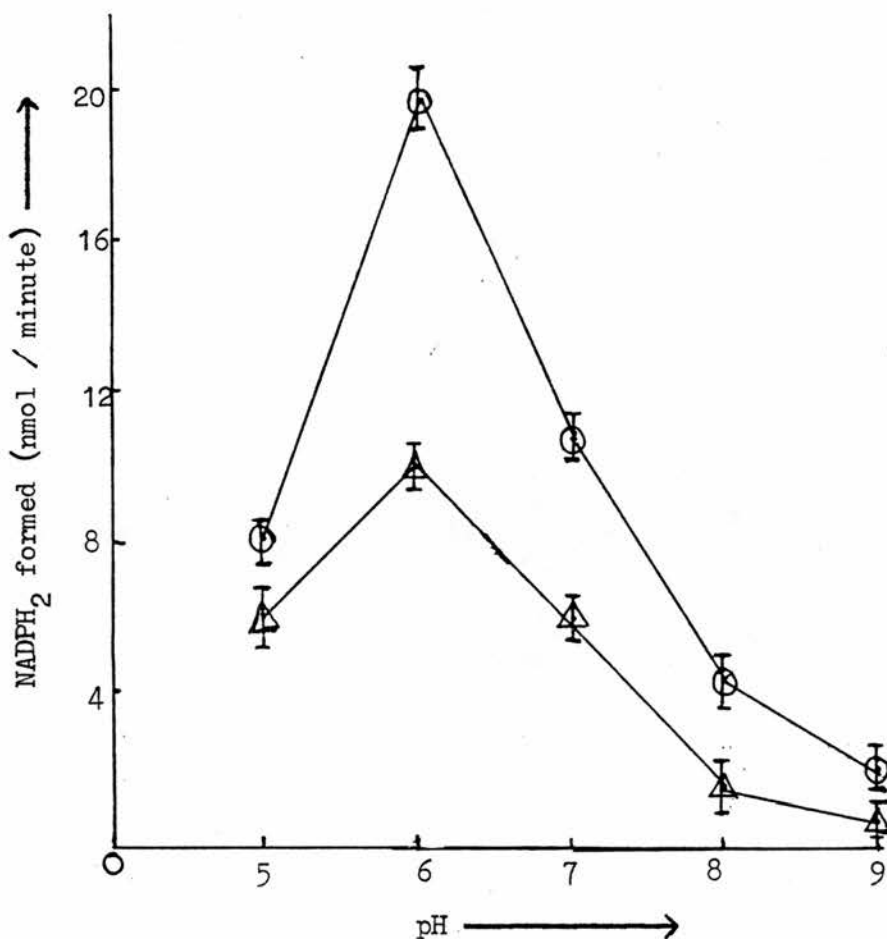


Fig 3: pH profile of the non-energy transhydrogenase activity.

To the reaction mixture (440 μ l) (as in table 1) in Tris-sucrose-acetate buffer (pH 5 to 9) preincubating for 5 minutes at 30°C, a mixture (60 μ l) of ADH (.45I U), GR (.45I U) (as in Table 1 and submitochondrial particles (.5mg) (O) or solubilised preparation (.1mg) (Δ) was added to start the reaction. GSH formed was determined after 5 minutes (see methods, Section 3.2).

Data plotted are means of 3 assays \pm S.D. taking into account the zero time control determined as in figure 1.

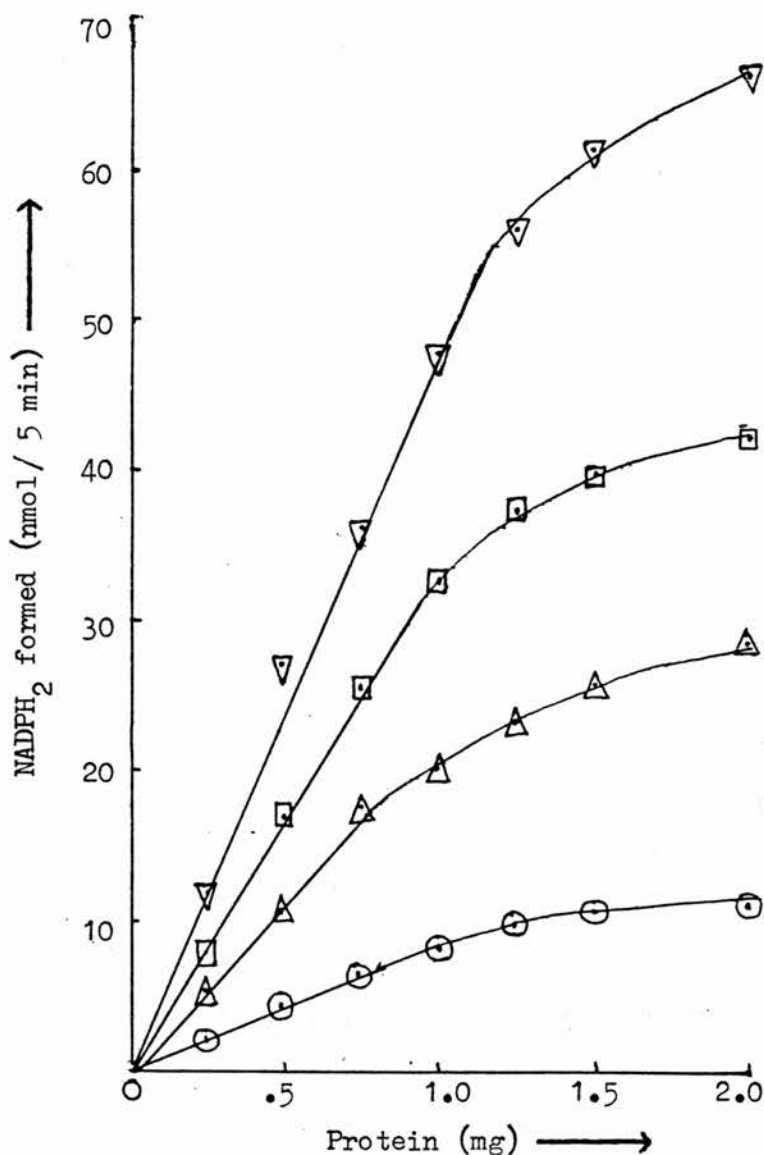


Fig 4: Variation of activity with amount of protein

The method [GSH (constant NADP⁺ and NADH)] was as described in figure 3 using the indicated amounts of enzyme. The reaction was incubated for 5 minutes, terminated and GSH formed determined (methods, Section 3.2). Enzyme activity in submitochondrial particles was determined at pH 8 (⊖) and pH 6 (△) and that from solubilised preparation also at pH 8 (⊞) and pH 6 (▽). Values plotted are means of 3 assays (+ S.D. < 1nmol) taking into account the zero time control (as in figure 1).

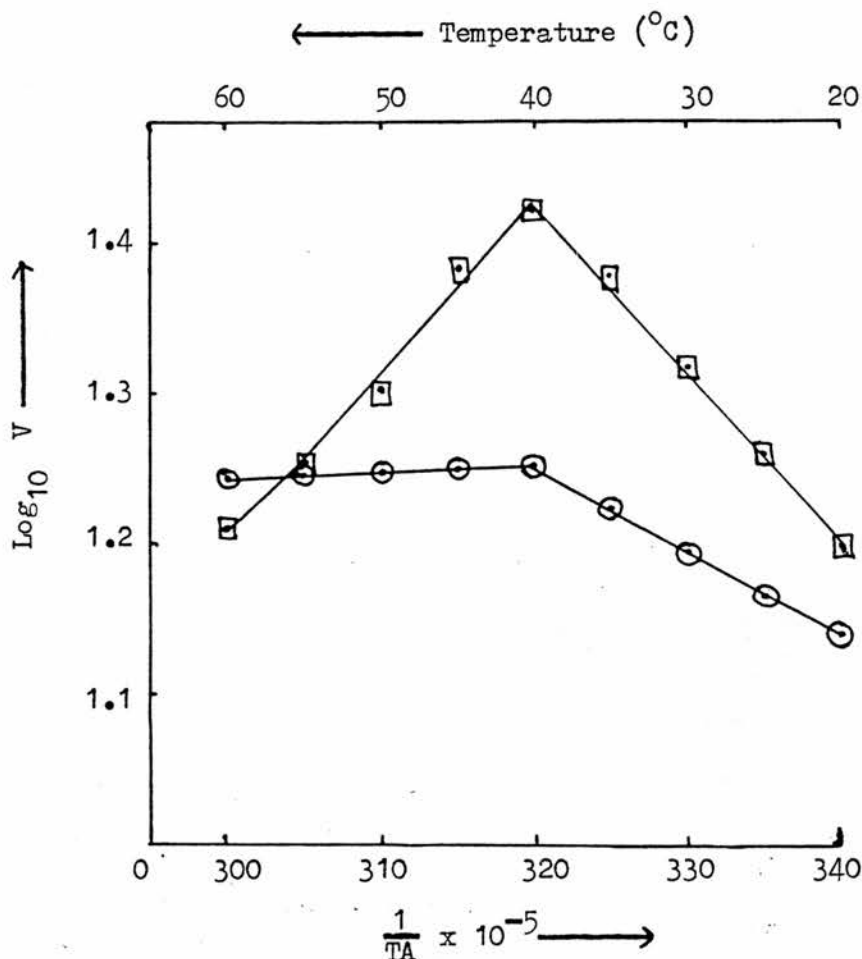


Fig 5: Arrhenius plot of the non-energy transhydrogenase activity

The method [GSH (constant NADP^+ and NADH)] was as described (figure 4) before, preincubating (5 minutes) and assaying the enzyme activity in particles (.3mg) at the above indicated temperature. The enzyme activity was determined at pH 8 (\ominus) and pH 6.0 (\square) after 2 minutes (determining GSH formed at 412nm as in figure 4). Data plotted (ie $\log_{10} V$ versus $\frac{1}{T}$ absolute where V is NADPH_2 formed in 2 minutes) are means of 4 assays taking into account the zero time control (\pm SD, $<.01$).

These studies were therefore, carried out under various conditions in particular by using not only submitochondrial particles, but also the enzyme solubilised by detergent treatment (figures 6 and 8). Because of the large difference in activity at pH8 and pH6, the values were determined at these pH's.

The values for NADP were determined using the GSH method with or without regeneration of NADH. Because of the high initial concentration of NADH however, the absence of the regeneration system (Ethanol and ADH) did not affect the result and an excellent linear relationship was obtained in the S versus $\frac{S}{V}$ plot (figure 6).

It has been reported that the substrate APAD can replace NADP⁺. From the point of view of the assay, this has the advantage that the reduced form (APADH) which is the product of the reaction has an absorption maxima (375nm) away from that of NADH (340nm). Results using this substitute are shown in (figure 7). The enzyme has poor affinity for substrate analog (APAD) (86 μ M) despite the fact that there is similar enzyme activity (around pH7) as with natural substrates.

When NADH was varied, with NADP⁺ held (figure 8) constant, the GSH method with NADH regeneration was used, but, particularly at low initial NADH concentrations, it was convenient to compare the results obtained with a method in which the product (NADPH) was measured indirectly by means of a fluorometric assay (figure 8). Again good linearity was found in the plotted results.

The K_m and V_{max} values obtained by some of the methods are shown in Table 2 at pH8 and pH6.

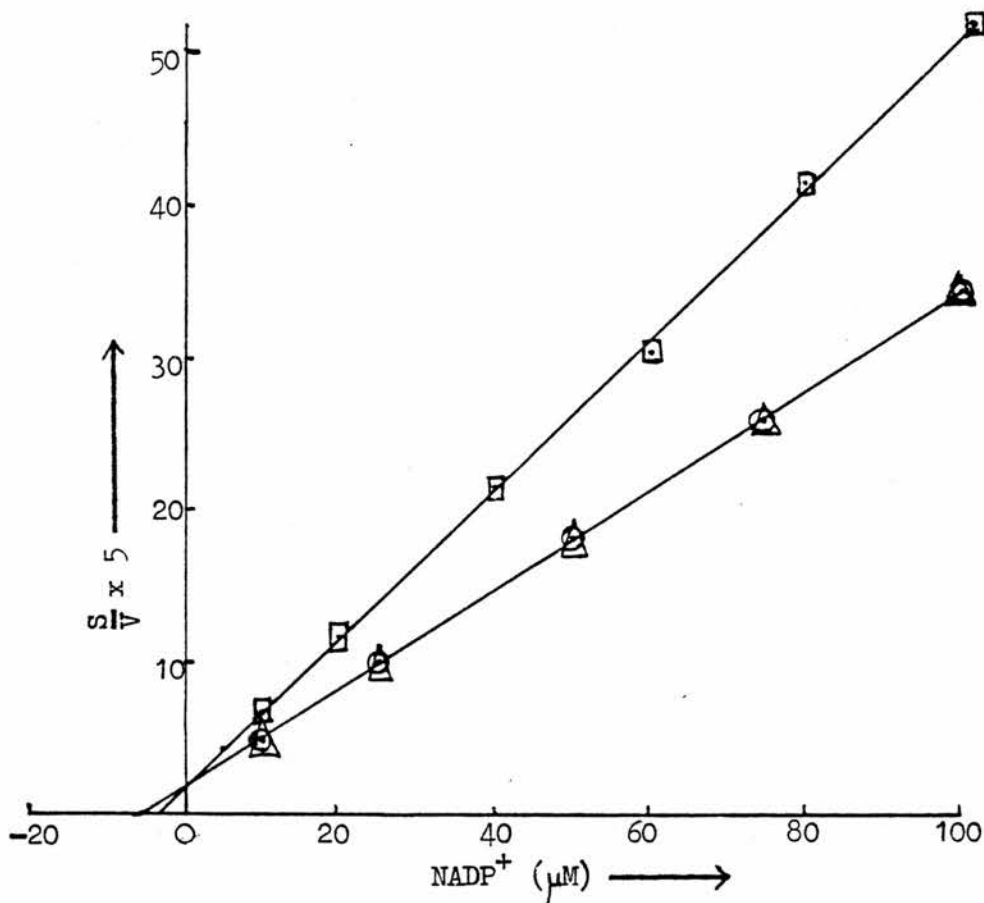


Fig 6: Variation of enzyme activity with NADP⁺ concentration

To the reaction mixture (440μl) at 30°C in Tris-sucrose-HCl buffer pH 8.0 containing ethanol (60mM) NADH (250μM) and NADP⁺ as specified above, a mixture of GR (.45I U), ADH (.45I U) and the soluble enzyme preparation (.2mg) (□); or SMP (.4mg) (○); or just a mixture of GR (.45I U) and SMP (.4mg) (△) (with ethanol omitted from the reaction mixture) was added to start the reaction in a final volume of .5ml.

The mixture was incubated for 5 minutes and GSH formed determined (see methods, Section 4.4Bii and iii). Data plotted in the plot of $\frac{S}{V}$ against $S(NADP^+)$ and V is absorbance at 412nm, are means from 4 assay (\pm S.D. of $V < .03$ OD. units/min) taking into account the minus NADP⁺ control. The control was run exactly as above except, NADP⁺ was omitted. The K_m NADP⁺ for SMP ($6.0 \pm \mu M$) and for the soluble enzyme ($3.7 \pm .6 \mu M$).

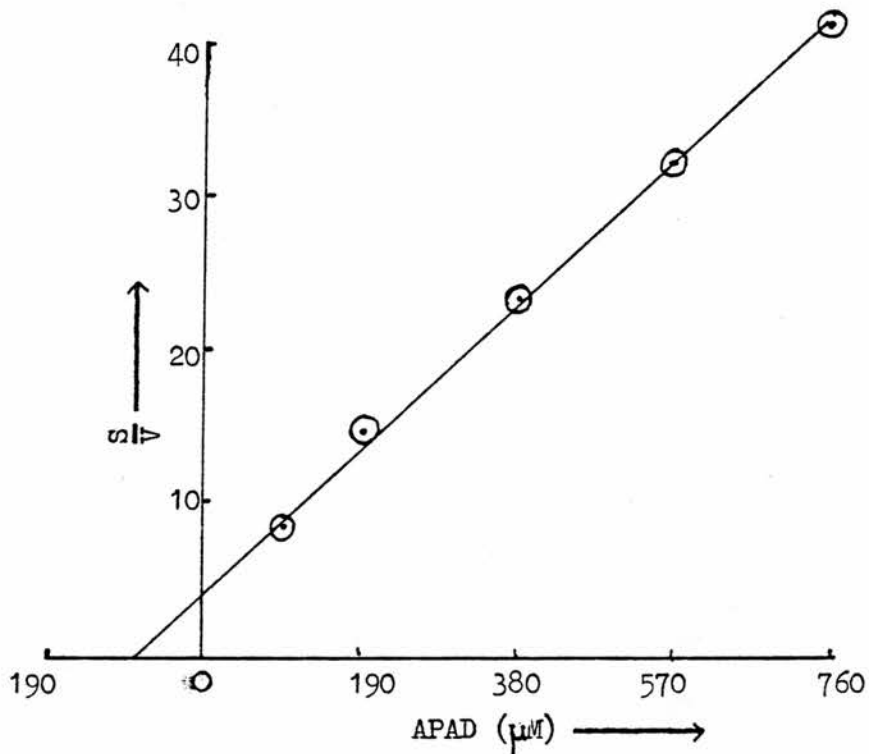


Fig 7: Variation of enzyme activity with APAD concentration.

To the reaction mixture (.9ml) preincubating at 30°C in sodium phosphate buffer pH 6.8 containing NADH (150mM) and APAD (as specified) SMP (.1ml and .4mg) were added to start the reaction which was followed at 375nm. Data plotted $\frac{S}{V}$ against S (APAD) and V (optical density change at 375nm per minute) are means from two assays (\pm S.D. of $\sqrt{4.02}$ OD units/min) taking into account the minus APAD control. The control was determined as described previously, omitting APAD in the reaction mixture.

$$K_m \text{ for APAD} = 85.5 \pm 5 \mu\text{M}.$$

$$V \text{ max for the reaction} = 23.5 \pm 3.8 \text{ nmol NADH oxidised/min/mg}.$$

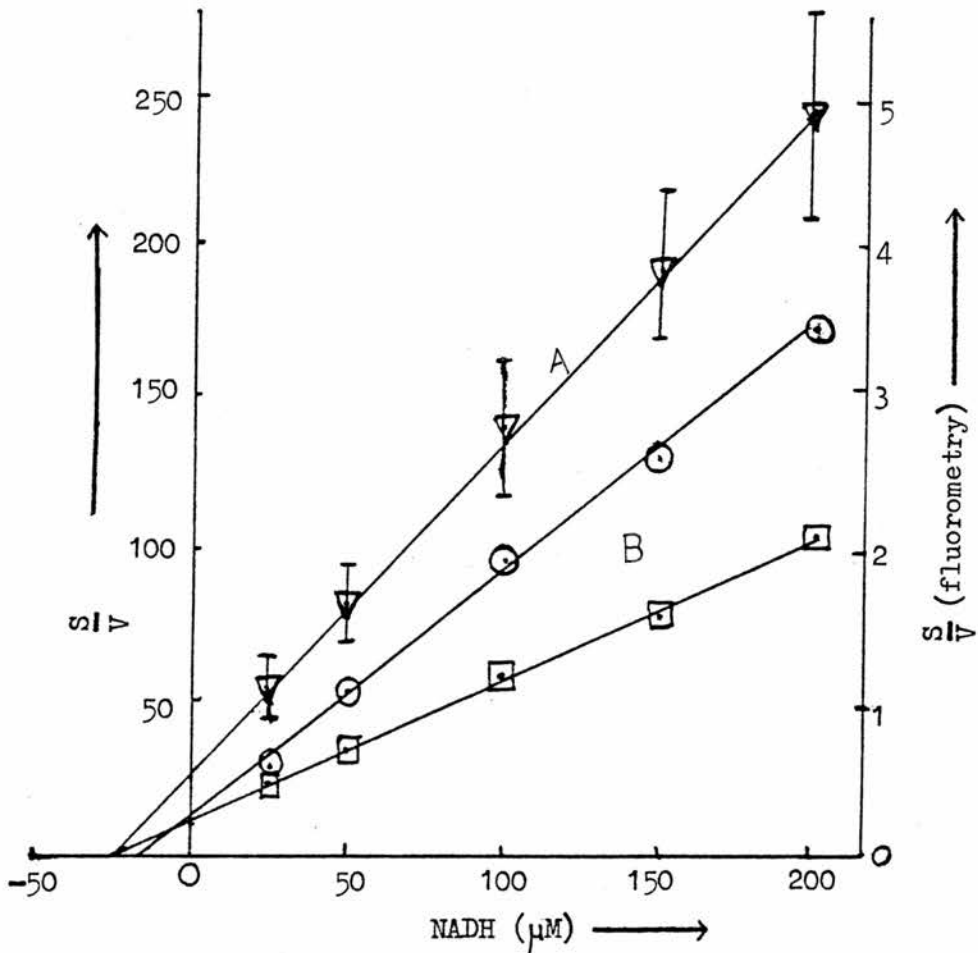


Fig 8: Variation of enzyme activity with NADH concentration

- (A) To the reaction mixture (.9ml) preincubating at 30°C in Tris-sucrose-HCl buffer pH 8.0 containing NADP^{+} ($100\mu\text{M}$), ethanol (60mM) and NADH (as indicated), a mixture ($100\mu\text{l}$) of ADH (.45I U) and SMP (1mg) was added to start the reaction. After 2 minutes incubation samples (.3ml) were assayed fluorometrically (∇) for NADPH_2 formed as in methods (Section 4.4Bi). Data plotted are means from 3 assays \pm S.D.
- (B) To the reaction mixture ($440\mu\text{l}$) at 30°C in the same buffer (A) and NADP^{+} ($100\mu\text{M}$), ethanol (60mM) and NADH (as indicated) a mixture of GR (.45I U), ADH (.45I U) and SMP (.57mg) (\square) or soluble preparation (\odot) was (1mg) added. After 5 minutes incubation GSH formed was determined (as in figure 6). Data plotted are means from 5 assays taking into account the minus NADH (omitted in the reaction mixture) (\pm S.D. ± 4.03 OD units/5min). The K_m NADH = $22.5 \pm 5.7\mu\text{M}$ (A) and $25 \pm 1.3\mu\text{M}$ (B).

Table 2: K_m and V_{max} values of TH in different preparations by different methods.

<u>Preparation</u>	<u>Method</u>	K_m NADP ⁺ (μ M)	K_m NADH (μ M)	V_{max} (IU)	<u>pH</u>
Particles	GSH	6.0 \pm .5	25.0 \pm 1.3	.007* \pm .001	8 or 8*
"	GSH	5.0 \pm .6	26.5 \pm 2.7	.023* \pm .003	6 or 6*
"	Fluorometry	-	22.5 \pm 2.8	.024 \pm .004	8
Solubilised	GSH	3.7 \pm .4	15.0 \pm 2.6	.013 \pm .001	8
"	GSH	2.5 \pm .5	18.0 \pm 3.3	.040 \pm .003	6
Teixeira da Cruz et. al.	(1971)	40.0	9.0	.038	7.5

For methods and conditions, see figures 6 and 8 (also methods, Section 4).

The enzyme has much greater affinity for NADP⁺ than it has for NADH. Decreasing the pH from pH8 to pH6 has no significant effect on the K_m s for both the substrates, but as already stated (Section 1), there is considerable increase of the enzyme's activity.

As described earlier (Section 1), solubilisation of the transhydrogenase greatly increases the specific activity of the enzyme. It was possible that this could be due to an effect on K_m values rather than V_{max} and consequently these constants were determined using the GSH method (constant NADP⁺ and NADH) as for submitochondrial particles (Table 2).

In order to understand more the great increase in enzyme activity found when the pH is decreased (see figure 3), the K_m values were also determined by the same GSH method at pH6 as well as at pH8.

Again, pH had no significant effect on K_m values in the solubilised enzyme as it was with the submitochondrial enzyme preparations. But as expected there is an increase in the affinity for both NADP⁺ and NADH. This is discussed later (Section 6).

3. EFFECT OF VARIOUS SUBSTANCES ON THE ENZYME

3.1 The effect of various substances

A number of substances, including inhibitors of hydroperoxide reduction by intact mitochondria reported in Chapter 3 were tested on the non-energy transhydrogenase activity.

The different assay methods previously described were all used to follow the effects obtained. In each case, a known specific transhydrogenase inhibitor, palmityl coenzyme A (PCoA) was also tested as an inhibitor control monitoring the responsiveness of the enzyme preparation used. PCoA is an established competitive inhibitor of the beef heart and also the rat liver transhydrogenase (see Chapter 1, Section 4) with respect to the NADP^+ site, but does not affect the NADH site.

The results are presented in Table 3. It is to be noted that PCoA inhibited the enzyme as determined by all assay methods; however, the inhibition using APAD was slight and in general none of the substances used affected the enzyme activity measured by this method to any marked extent. The DCPIP method which measured the reverse reaction gave results which were opposite to those of the remaining and more authentic method. Since the enzymatic basis for APAD and DCPIP reduction is in some doubt (see discussion) results obtained by these methods will not be further described in this section.

Turning to the GSH (principally used throughout this section) method a remarkable result is seen. The enzyme activity is unaffected by an uncoupler but except for the monocarboxylate carrier inhibitor PP, there is a large increase in activity (up to three-fold the control value) when either of the other carboxylate carrier inhibitors (BM, PhS, BT, PT or FC) is present.

A substance (FC) which binds irreversibly to one of these carriers (the tricarboxylate) also has some stimulant effect though small (fig 10) compared to the other (reversible) tricarboxylate carrier inhibitors BT and PT.

The/

Table 3: Effect of substances on TH activity by different methods

Addition	Activity (% Control) by assay method:		
	APAD	DCPIP	GSH
PCoA	90	66	57
FCCP	100	100	115
PP	100	56	96
BM	110	83	301
PhS	-	-	318
BT	120	89	291
PT	120	94	320

The methods (APAD and DCPIP) were as described in methods (Section 4.4A). The reactions were carried out at 30°C and followed for 2 minutes at 375nm and 600nm for the APAD and DCPIP method; respectively in the presence of submitochondrial particles (.4mg).

The GSH method was as described in methods (Section 4.4B), regenerating either NADP⁺ alone or NADP⁺ and NADH. The reaction was started at 30°C by adding a mixture of submitochondrial particles (.3mg) and GR (.45IU) ± ADH (.45IU). After 2 minutes incubation the reaction was terminated and GSH formed determined (as in figure 8).

The additions were carboxylate carrier inhibitors (5mM), PCoA (10µM) and FCCP (5µM). Data shown are means from 2 assays for APAD and DCPIP methods and from 4 assays for the GSH methods (which showed no differences) (+ SD. < 5%) taking into account the zero time control in each case.

The following specific activities were obtained for the control values; APAD (.018IU), DCPIP (.020IU) and GSH method (.006IU).

The substances described above however, had no effect on the activities of the commercial enzymes GR and ADH used in the regenerating systems of NADP^+ and NADH respectively, as determined by the conventional assays of measuring changes in optical densities at 340nm and also by the GSH method for the former enzyme.

3.2 Carboxylate carrier inhibitor concentration on activity

The effect of varying the amount of carboxylate carrier inhibitors was determined on TH. The results of this study are shown in figure 9.

Except for the monocarboxylate carrier inhibitor (PP) which was slightly inhibitory at higher concentrations, the two dicarboxylate carrier inhibitors (BM and PhS) had similar effects i.e. of stimulating the reaction in a concentration dependent manner. On the other hand, the tricarboxylate carrier inhibitors (BT and PT) which also stimulated the reaction, reached maxima (at 5mM) above which there was a decrease in the stimulation.

3.3 Effect of aging on the carboxylate carrier inhibitors' effects

Since I had to store submitochondrial particles for most of the experiments with the inhibitors, it was necessary to determine first the effect of aging on the sensitivity of the non-energy trans-hydrogenase activity to carboxylate carrier inhibitors. This was achieved by assaying the enzyme activity at intervals.

The results (figure 10) show what has already been mentioned, that, there is considerable loss in activity of the enzyme with time (Section 1). However, the slight inhibitory effect of the monocarboxylate carrier inhibitor (PP) remains unchanged. Similarly there is no significant effect on the stimulations by BM and PhS (Dicarboxylates) and BT, PT and FC (tricarboxylates) carrier inhibitors. I could, therefore, study the effects of these substances without worrying about the age factor, so long as the enzyme activity was adequate.

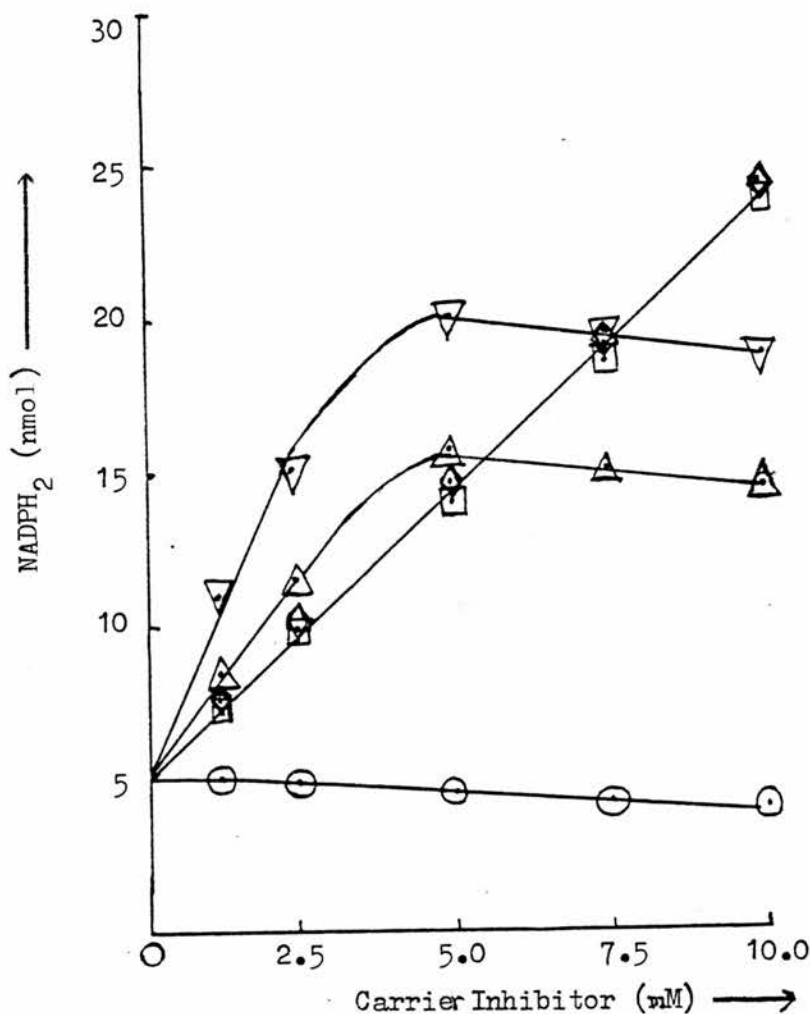


Fig 9: Variation of TH activity with carboxylate carrier inhibitor concentration

The reaction mixture (440 μ l) in Tris-sucrose-HCl buffer pH 8.0 consisting of NADP⁺ (100 μ M), NADH (250 μ M) and carrier inhibitors PP (\ominus); BM (\square); PhS (\diamond), BT (\triangle) and PT (∇) (as specified above) was preincubated for 5 minutes at 30°C. The reaction was started by adding a mixture (60 μ l) of GR (.45I U), ADH (.45I U) and SMP (.5mg), incubated for 2 minutes and GSH determined as in figure 8. Means of 3 assays obtained are plotted minus the zero time control (see figure 1) (\pm S.D. \angle .9 nmol/2 min).

The zero time control was the same as minus NADH control (figures 6 and 8). The specific activity of the enzyme was .005I U

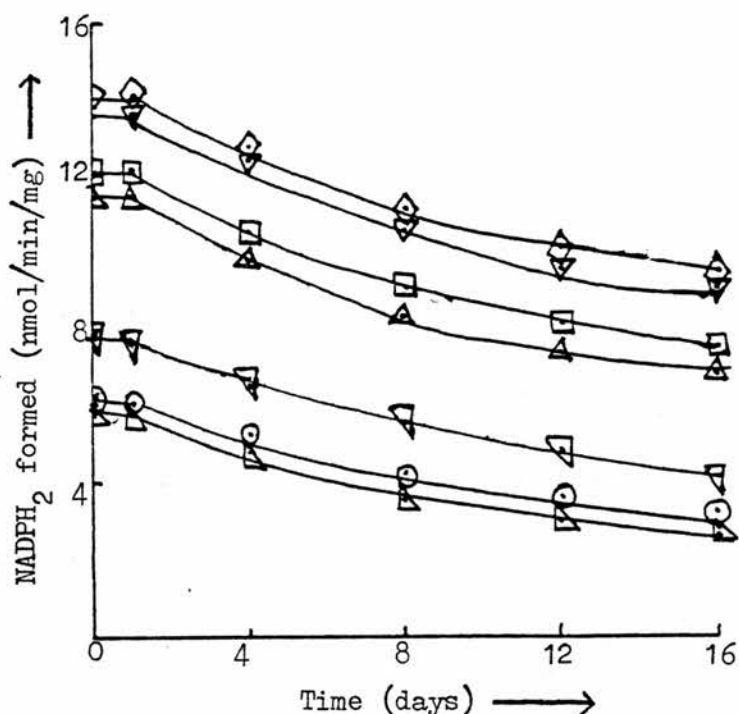


Fig 10: The effect of carrier inhibitors on TH activity with age.

To the reaction mixture as in (figure 9) containing the following carrier inhibitors (5mM) PP (▲), BM (△) PhS (▼), BT (◻), PT (◆), FC (◻) and no addition (○) and preincubating at 30°C, a mixture of GR (.45I U) ADH (.45I U) and SMP (.6mg) was added to start the reaction. After 2 minutes the reaction was terminated and GSH determined (methods, Section 4.4Biii).

Data plotted are means from 3 assays minus the zero time control (figure 9) (\pm SD. \angle .9nmol/2min) taken at the indicated time intervals.

Unfrozen SMP were used at day (0). All the other preparations used were frozen only once (on the day of preparation).

4. INHIBITOR KINETICS

An analysis of possible sites of action of each of the substances previously described was undertaken by varying NADP^+ or NADH concentrations with the substances present.

In the first case, the data relate to effects on the NADP^+ binding site of the enzyme (Section 4.1) and in the second to the NADH site (Section 4.2) and for this reason they are presented in separate sections.

4.1 NADP^+ Varied(a) PCoA

The substance has been found to be a competitive inhibitor of the NADP^+ binding site both in the beef heart (Rydström et al 1971) and rat liver (Blazyk and Fisher 1975) enzymes.

This type of inhibition yields parallel lines when slopes of S against $\frac{S}{V}$ are found in the presence of the inhibitor (Cornish-Bowden 1976). Conversely for the non-competitive inhibition, they converge on the substrate (S) axis. K_i values are conveniently obtained from the Dixon plot (1953) [inhibitor concentration (i) against $\frac{1}{V}$] which gives an intersection point for different substrate values above the inhibitor (i) axis (competitive). This is in contrast to the non-competitive inhibition where the intersection occurs on the inhibitor axis.

As shown in Figure 11 in which the data is presented by both methods, I was able to confirm this result by varying NADP^+ concentration in the presence of the coenzyme.

(b)/

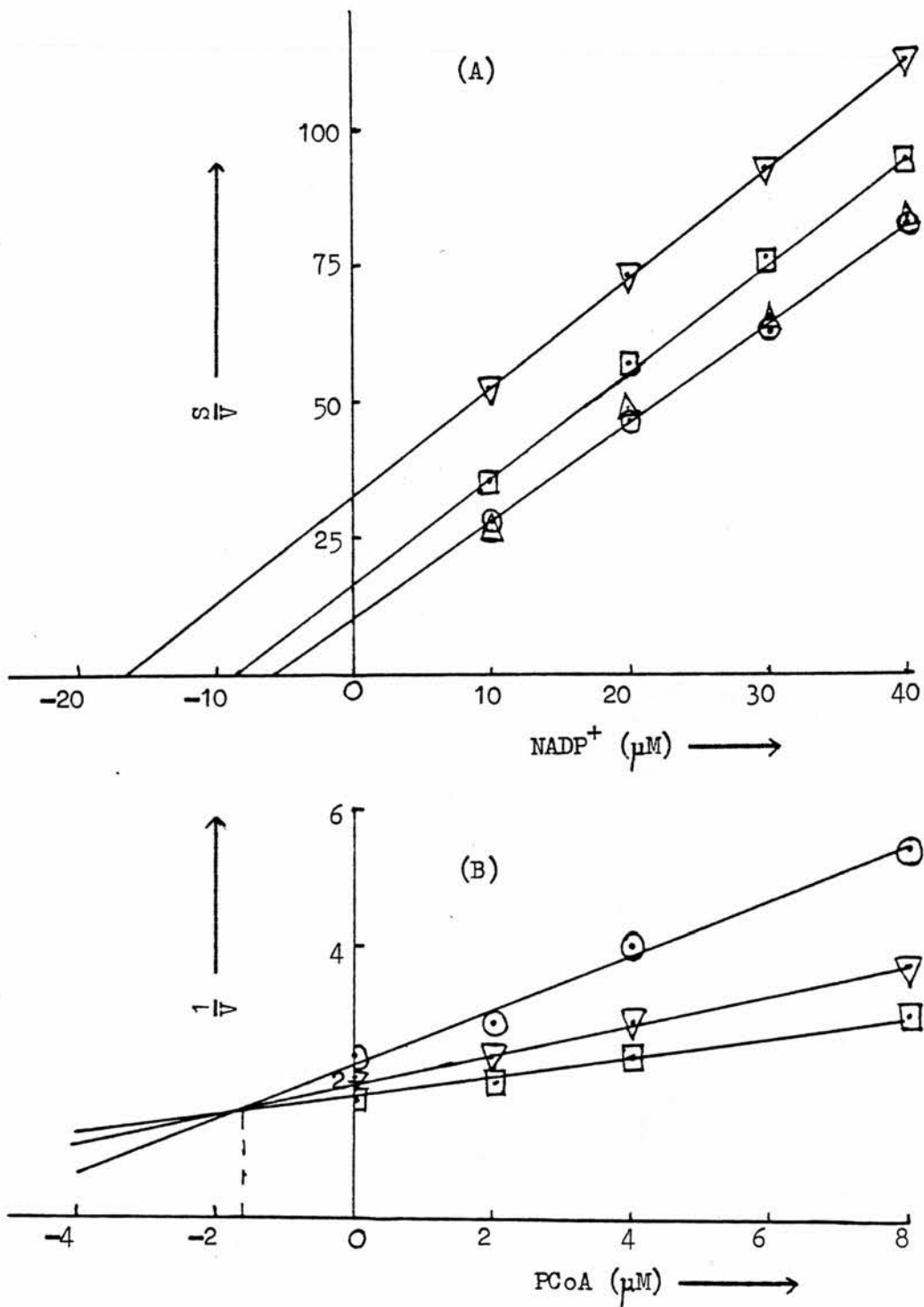


Fig 11: The effect of PCoA on TH activity at different fixed $NADP^+$ concentrations.

The reaction was done at $30^\circ C$ as described in methods (Section 4.4Bii) in Tris-sucrose-HCl buffer pH 8.0, containing NADH ($250\mu M$), $NADP^+$ and PCoA as specified. To start the reaction, a mixture of GR (.45IU) and SMP (.23mg) was added and incubated for 5 minutes then GSH determined. The data plotted are means from 3 assays minus the no $NADP^+$ control (see figure 6) (\pm S.D. of $V_{L.020}$ units/5min). S versus $1/S$ plot is shown in (A) and Dixon plot in (B). The K_i for PCoA is $1.6 \pm .4\mu M$.

(A) PCoA (μM) was nil (\circ), 2 (Δ), 4 (\square) and 10 (∇)

(B) $NADP^+$ (μM) was 10 (\circ), 20 (∇) and 40 (\square)

(b) Carboxylate carrier inhibitors

Each of the substances was used in place of PCoA in the kinetic experiments described in (a) and the data obtained was plotted by the $\frac{S}{V}$ versus S method (figure 12).

As expected from Section 3, phenylpyruvate (PP) still inhibits with varying NADP^+ concentration, but unlike PCoA, the inhibition is non-competitive. The data similarly replotted (Dixon plot) gave the inhibitor constant ($K_i = 14\text{mM}$).

Similar linear results are also obtained in the Eadie-Hofstee plots by the other carrier inhibitors BM, BT, PT and FC (result not shown) with the important difference that the slopes decrease when the inhibitor concentration is increased.

The significant result is that stimulation by these inhibitors does not affect the value for K_m which is unchanged throughout.

It is the values for V_{max} only which are changed (see also figure 10). The values for V_{max} (figure 12B) were plotted against the inhibitor concentration giving linear parallel lines whose slopes increased with inhibitor concentration (figure 13) at different substrate concentrations. A similar pattern was obtained with the tricarboxylate carrier inhibitors.

4.2 NADH Varied(a) PCoA

As shown in figure 14 and in agreement with the literature (Rydström 1977) there is no inhibition by this coenzyme when in the presence of saturating amounts of NADP^+ , the concentration of NADH is varied. These results were obtained by the fluorometric assay method (methods, Section 4.4Bi).

(b)/

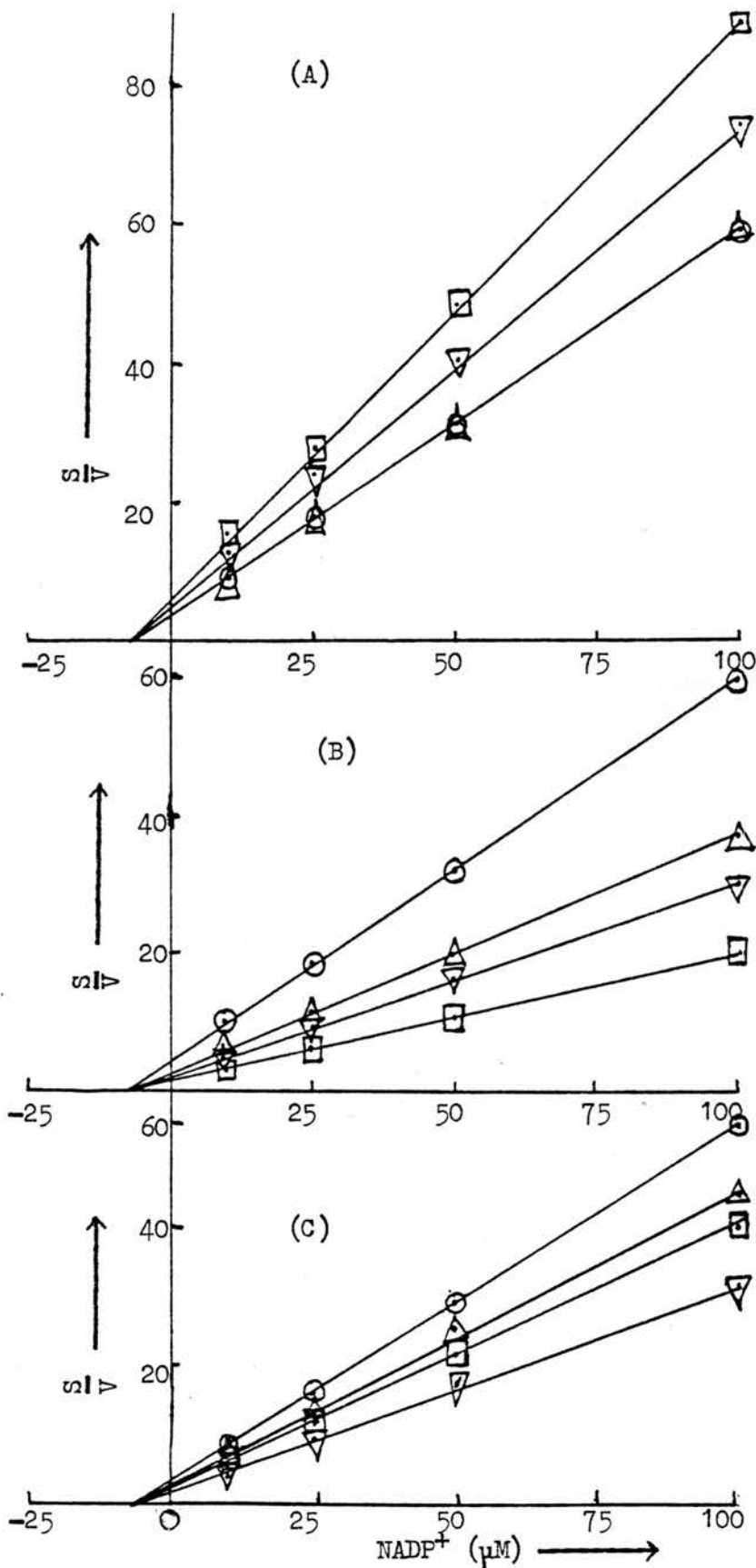


Fig 12: The effect of carrier inhibitors on TH activity at different fixed NADP⁺ concentrations.

The reaction was carried out at 30°C in Tris-sucrose-acetate buffer pH 8.0 containing NADH (250μM), NADP⁺ varied as indicated and PP(A) BM (B), BT or PT(C) at (○) (0mM) 2.5mM(△); 5.0mM(▽) and 10mM (□) as described by the GSH method (constant NADP⁺ and NADH) (see methods, Section 4.4Biii). The reaction was incubated for 5 minutes in the presence of SMP (.23mg), GR (.45IU) and ADH (.45IU) means from 5 assays are plotted (as in figure 11A) (± SD, of V.L.02 OD units/5min).

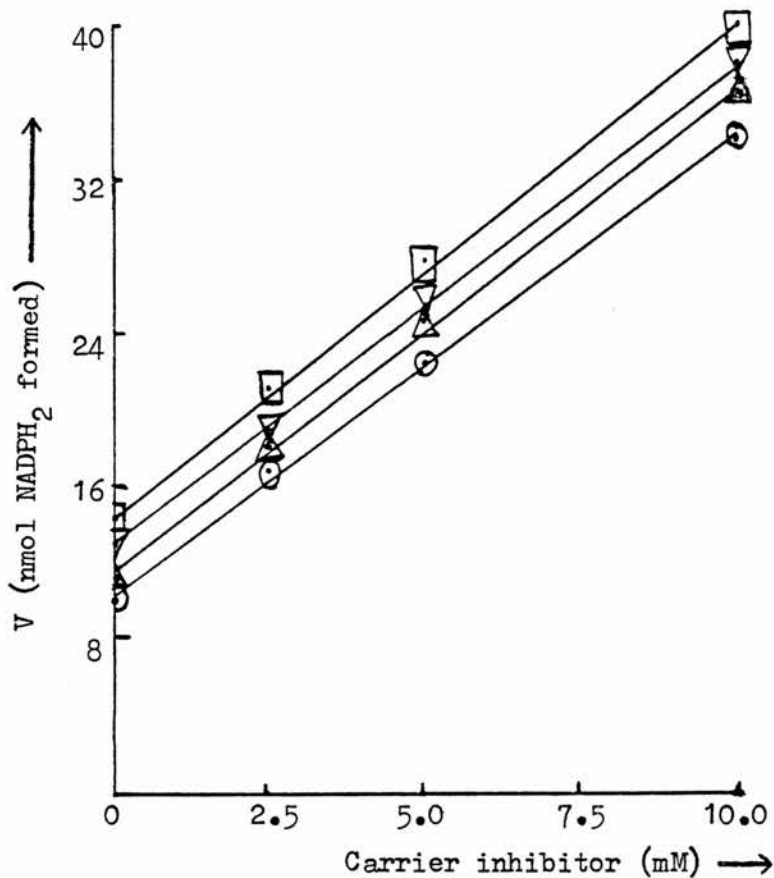


Fig 13: Variation of V_{max} with inhibitor concentration at different fixed $NADP^+$ concentrations

The data for figure 12 (B) was replotted as shown above, i.e. the rate (V) of the TH reaction against the carrier inhibitor (BM) concentration at the following $NADP^+$ concentrations
 ○ (25 μ M), △ (25 μ M), ▽ (50 μ M), and ◻ (100 μ M).

A non-linear relationship was obtained for figure 12 (C) above 5mM concentration of the carrier inhibitors (results not shown, but see also figure 9).

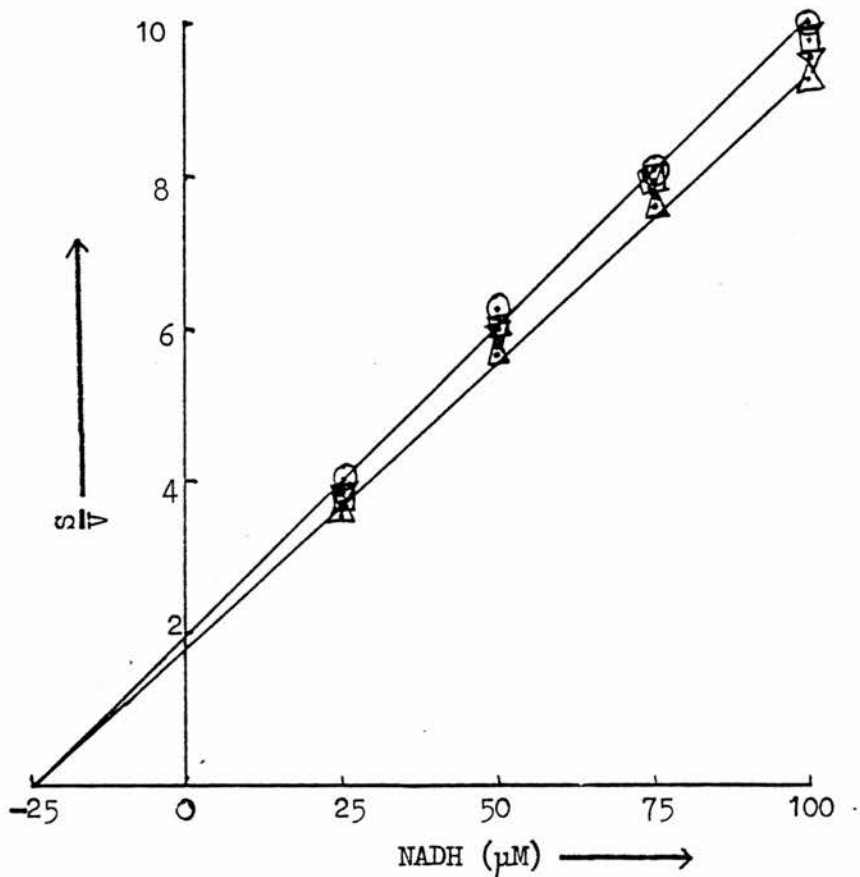


Fig 14: The effect of PCoA on TH activity at different fixed NADH concentrations.

To the reaction mixture (.9ml) containing NADP^+ ($100\mu\text{M}$) NADH as indicated and PCoA \ominus ($0\mu\text{M}$), \triangle ($33\mu\text{M}$), \square ($13.2\mu\text{M}$) in Tris-sucrose-HCl buffer pH 8.0 preincubating at 30°C , a mixture (.1ml) of ADH (.45IU) and SMP (.67mg) was added to start the reaction. After 5 minutes incubation, fixed samples (.3ml) were assayed fluorometrically (methods, Section 4.4Bi) for the NADPH_2 formed taking into account the no NADH control and initial amounts of NADH present in the mixture. Data plotted (by Eadie-Hofstee method) are means from 2 assays ($\pm \text{SD}$) ($2\text{nmol}/5\text{min}$).

PCoA has no significant effect on the reaction at different NADH concentrations.

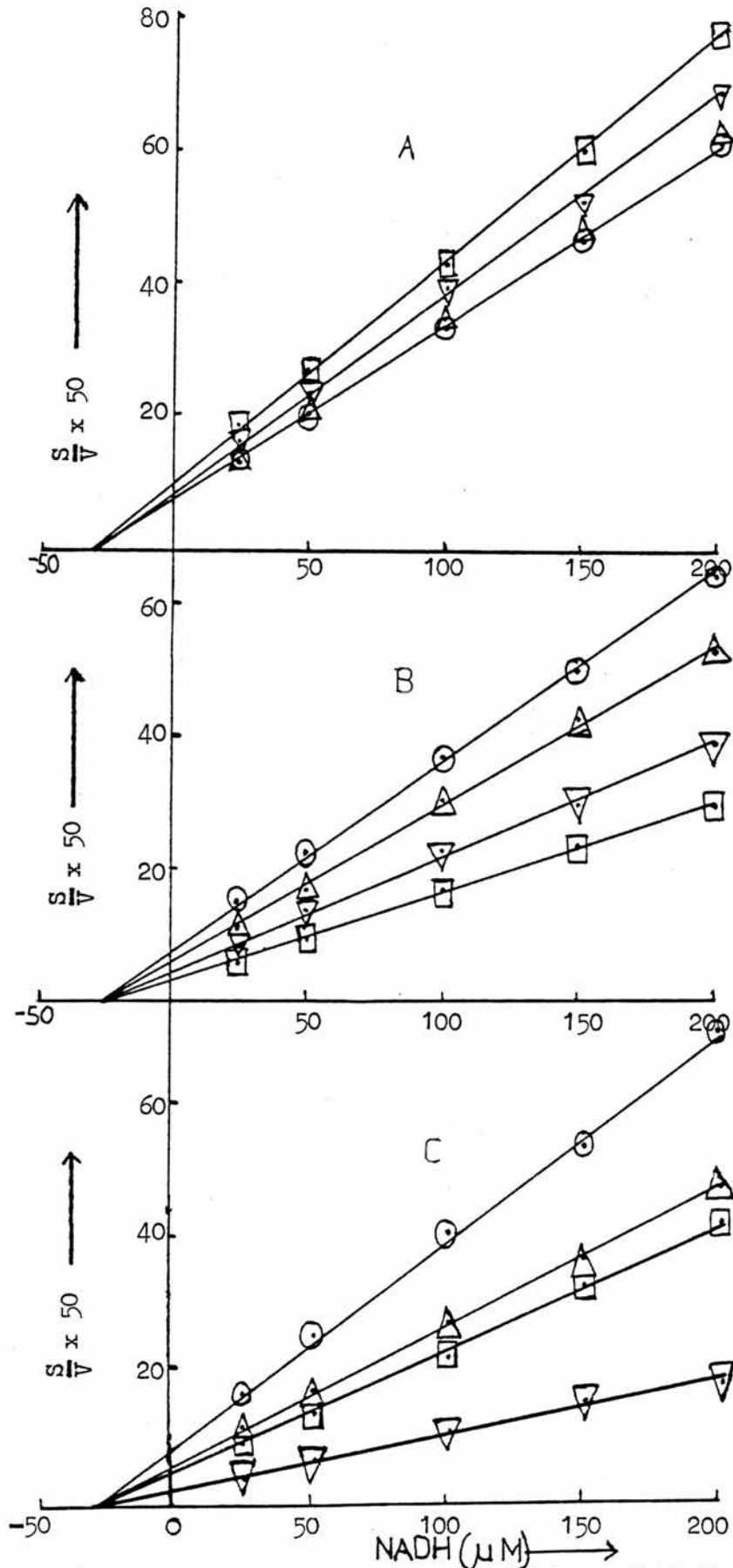


Fig 15: The effect of carrier inhibitors at different fixed NADH concentrations.

The reaction was carried out for 5 minutes at 30°C as described in figure 12 except that NADP^+ (100 μM) and NADH was varied; PP(A) BM(B) and BT and PT(C) at \ominus (0mM), \triangle (2.5mM), ∇ (5mM) and \square (10mM) in the presence of SMP (.2mg). Means from 4 assay are plotted (\pm SD) (V.L.0200 units/5min) minus no NADH control.

(b) Carboxylate Carrier inhibitors

The effect of varying the NADH concentration in the presence of saturating amounts of NADP^+ is shown for each of the carboxylate carrier inhibitors in figure 15.

Once again phenylpyruvate (PP) show non-competitive kinetics. The other carrier inhibitors BM (dicarboxylate) BT, PT and FC (result not shown) (tricarboxylates), continue to stimulate at all concentrations of NADH used, but also do not affect the K_m values. The result obtained by the fluorometric and GSH methods is identical to that obtained in the last section (4.1) and suggests that the inhibitors act at site(s), remote from the binding site for either NADP^+ or NADH.

The values for V_{max} were plotted against the inhibitor concentration (i) as in figure 13. The Dixon plot gave linear parallel lines (results not shown) in the previous section (for BM, BT and PT) at different substrate concentrations suggesting that although the inhibitor binding site(s), may affect the enzyme remotely, there is a definite linear relationship with either NADP^+ or NADH binding sites.

4.3 Effect of carrier inhibitors at pH6

The inhibitor kinetics were once again determined at pH6 where the enzyme activity was maximal (figure 3). The effects observed by these inhibitors on the enzyme (ie. no change in K_m of either substrate) in the previous sections (4.1 and 4.2) are qualitatively similar to those obtained with hydrogen ions (ie. by decreasing pH (Section 1.5)) raising the possibility that these substances may bind at or near a proton binding site presumably responsible for the pH dependent stimulation. If this is so, it might be expected that these carboxylate carrier inhibitors would be less effective or ineffective at pH6, whereas if their stimulant action were by quite a different mechanism, they would continue to stimulate at lower pH.

It/

It was mainly for this reason that the kinetic experiments reported in the last two sections were repeated at pH6.

As before no change in K_m at either site results from the action of these carrier inhibitors. I plotted the data by Eadie-Hofstee plot (figure 16). The profiles are to be compared with those obtained at pH8 (figures 11 and 12). They show a linear response when either $NADP^+$ (figure 16) or $NADH$ (figure 17) is varied, but the carboxylate carrier inhibitors have lost the inhibitory (as for PP) and most of their stimulation (as for BM, BT, PT and FC) effects at pH6, which were very much apparent at pH8.

4.4 Effect of Carrier inhibitors on solubilised TH

The theory behind the effects of carboxylate carrier inhibitors on the transhydrogenase reaction is that a conformational change occurs as a result of the binding of the inhibitor to the carrier protein in the membrane which is then relayed to the enzyme. The stimulants (dicarboxylate and tricarboxylate carrier inhibitors) provide a favourable conformation change for the TH reaction whereas the inhibitor (monocarboxylate) the opposite, as a result of such binding (see also discussion).

The second possibility is that the carrier inhibitors do affect the enzyme directly, not necessarily because of the above property.

In/

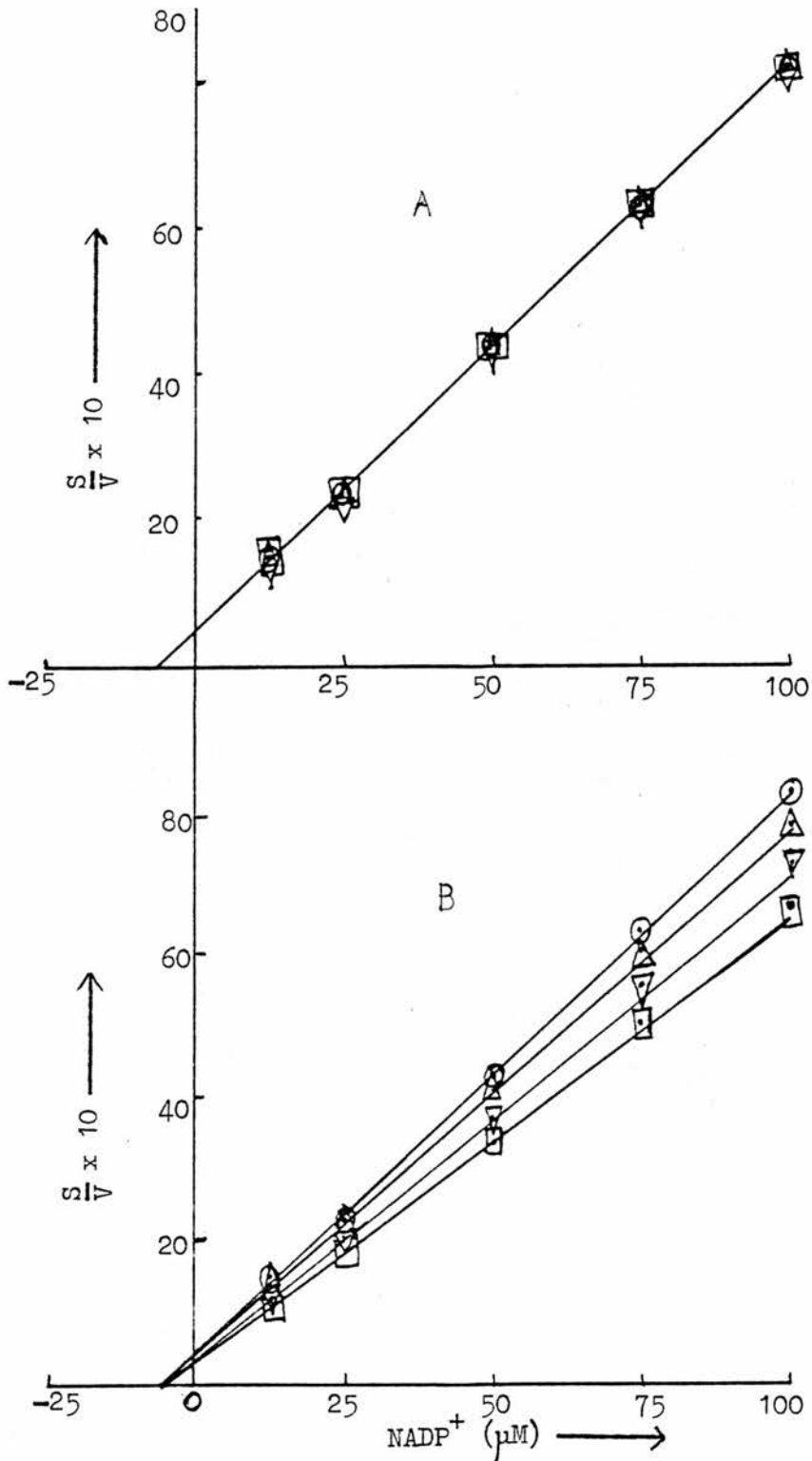


Fig 16: The effect of carrier inhibitors on TH activity at different fixed $NADP^+$ concentrations at pH6.

The reaction was carried out in Tris-sucrose acetate buffer pH6 as described in figure 12, containing PP(A), BM, BT, PT and (B) at the following concentrations \circ (0mM), \triangle (2.5mM), ∇ (5mM) and \square (10mM) except FC (0, 10, 20, 40 μM , respectively) in the presence of SMP (.1mg) (7 days old). Means from 3 assays are plotted (\pm SD of 1.03 OD units/5min) minus no $NADP^+$ control. Specific activity of TH (.004IU).

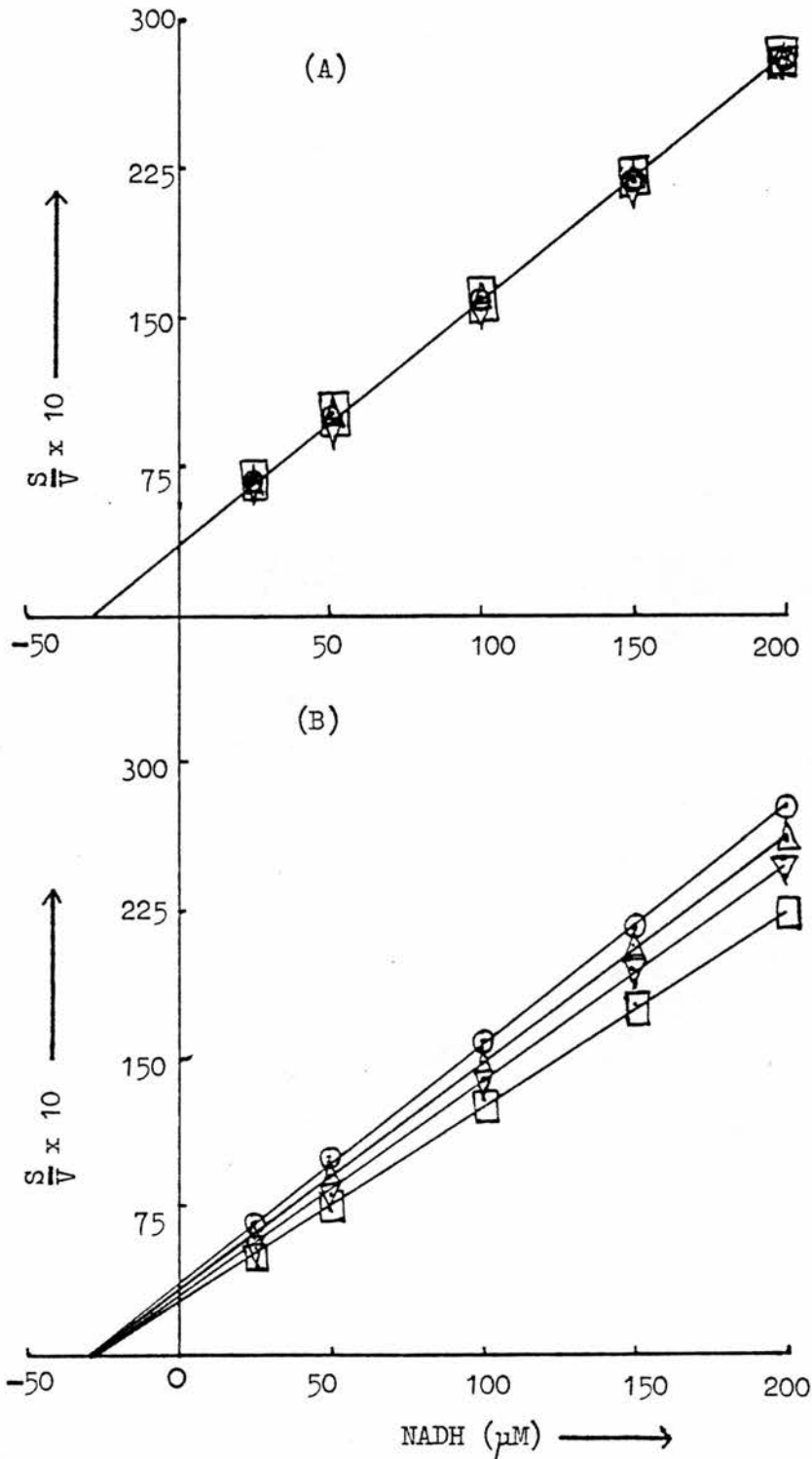


Fig 17: The effect of carrier inhibitors on TH activity at different fixed NADH concentrations at pH6.

The reaction was as described for figure 15, carried out in the buffer figure 16, with PP in (A) and BM, BT, PT and FC in (B) at \circ (0mM) \triangle (2.5mM) ∇ (5mM) and \square (10mM) except FC (10, 20 and 40 μM respectively). The mixture contained SMP (.1mg) [8 days] of specific activity .0035IU. Means plotted are from 3 assays minus no NADH control (\pm SD of 4.0300, Units/5min).

In order to test these possibilities, it was critically necessary to isolate the enzyme from the membrane, which was done by solubilising it (with digitonin) and testing the effects of the inhibitors.

Another way of looking at this problem was to examine the effects of the natural substrates for the carrier proteins (carboxylic acids) on the particulate enzyme and also the solubilised form as above. These experiments will be described later (section 5).

The experiments on the effect of carrier inhibitors on the solubilised enzyme will now follow. The effects of the inhibitors were determined at both pH8 and pH6 as was with the particulate enzyme at low and saturating initial concentrations of either NADP^+ or NADH. Figure 18 shows the effects of the inhibitors at pH8 (A) and pH6 (B) at saturating substrates concentrations. Similar results (not shown) were obtained at unsaturating levels of either substrate. The results mean that; the carboxylate carrier inhibitors have no direct effect on the transhydrogenase enzyme.

Much more important was the fact that the effects of all the carrier inhibitors were lost upon solubilising the enzyme. This strongly suggests that it is by the former possibility (ie, binding to the carrier proteins) that these inhibitors affect the membrane bound TH.

4.5 Effect of thiol reagents

Since carboxylate carriers are SH dependent (Passarella et al 1976) it is possible that some inhibitors could block SH group(s). It was therefore, desirable to see the effect of the authentic SH reagents on the transhydrogenase activity.

The effects of n-ethylmaleimide (NEM) and p-hydroxy-mercuribenzoate (MB) were determined on the enzyme's activity at both pH8 and pH6.

The results in Table 4 show that the thiol reagents (NEM and MB) inhibit the enzyme activity at both pH8 and pH6 and NEM being the stronger inhibitor. Less/

Less inhibition (by about 30%) was observed when there was no prior incubation of the enzyme with the thiol reagents (results not shown). This result suggests that although SH group(s) on TH are required for the enzyme activity, they are not easily accessible to the reagents. Excess amounts of the regenerating enzymes (GR and ADH) were used in these experiments, since they themselves were slightly inhibited (20%) by the thiol reagents at the highest concentration.

A kinetic analysis was done in the presence of the stronger inhibitor NEM. This is shown in figure 19 (Dixon plot) where it shows non-competitive inhibition when either NADP^+ or NADH is varied at pH8. There was no significant difference in the K_i ($30\mu\text{M}$) at pH6 (results not shown).

Table 4s The effect of NEM and MB on TH activity

Inhibitor	Concentration (μM)	Activity (% of Control)	
		pH8	pH6
Nil	-	100	100
NEM	10	106	81
"	20	63	60
"	40	23	43
MB	10	112	95
"	20	106	65
"	40	63	45

To the incubation mixture containing NADH ($250\mu\text{M}$) and NADP^+ ($100\mu\text{M}$) in Tris-sucrose-acetate, pH8 and pH6, a mixture of GR (.9IU) and ADH (.9IU) was added followed by SMP (.16mg) preincubated for 5 minutes with either NEM or MB at 0°C to start the reaction (see methods, Section 4.4Biii). The reaction was incubated for 5 minutes and GSH formed determined. Data presented are means from 2 assays (\pm SD. $\angle 8\%$).

The enzyme activity for the control (100%) was .008IU at pH8 and .23IU at pH6.

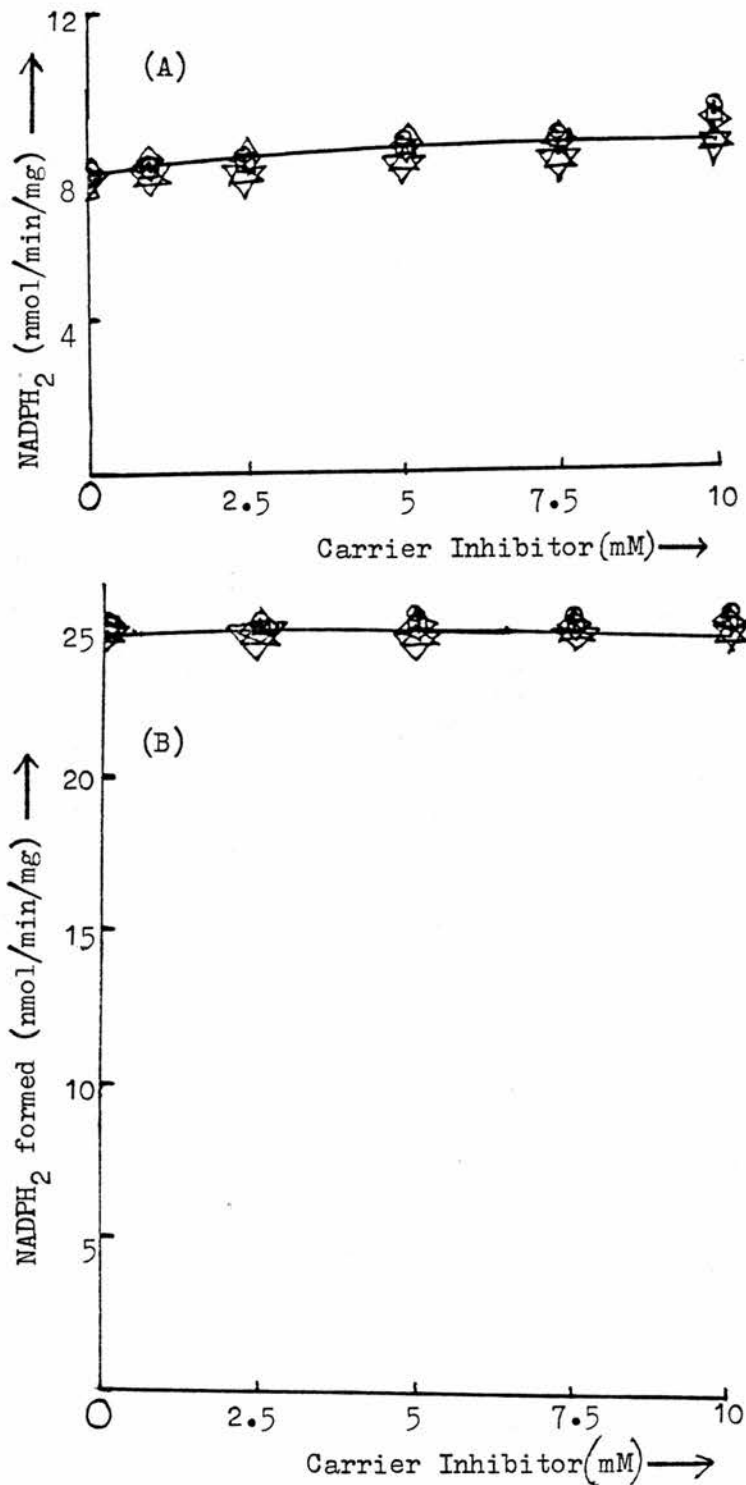


Fig 18: Effect of carrier inhibitors on solubilised TH activity.

The reaction was carried out in Tris-sucrose-acetate buffer at pH8 (A) and pH6 (B) containing NADH (250 μ M), NADP⁺ (100 μ M) and varying amounts of carrier inhibitors, PP (\triangle), BM (\square), PhS (\diamond), BT (\blacktriangle) and PT (∇), as indicated above and as described in methods (Section 4.4Biii) by the GSH method (constant NADP⁺ and NADH). SMP (3mg) were present and 5 minutes after incubation, GSH was determined. Data are means from 3 assays (\pm SD. < 1.3 nmol/min/mg).

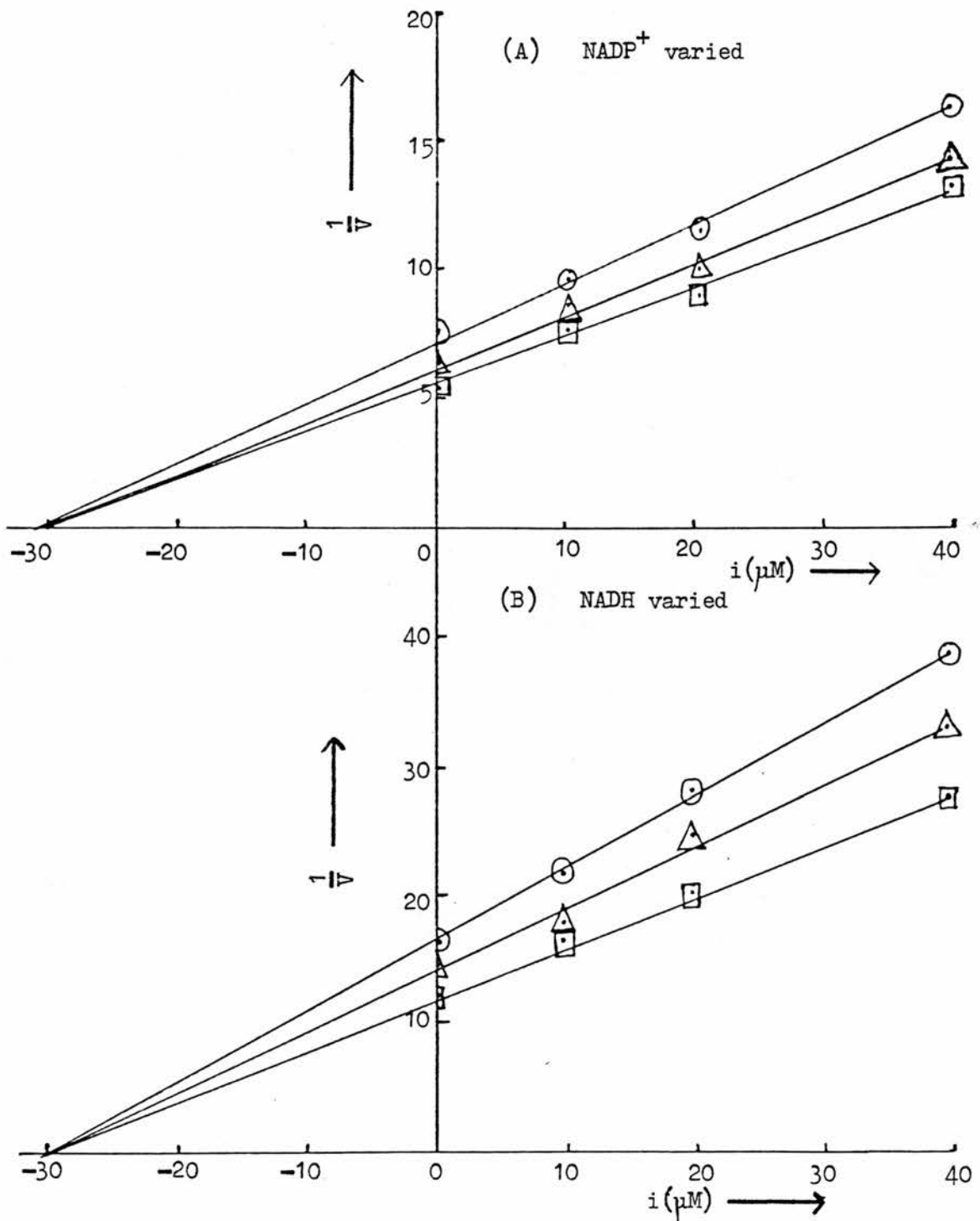


Fig 19: Variation of TH activity with concentration of thiol reagent at different fixed NADP^+ (A) or NADH (B) concentrations.

The reaction was carried out at 30°C as described for Table 5, except for the varying amounts of either NADP^+ or NADH as above with one of them high. After a mixture of GR (.9IU) and ADH (.9IU) was added SMP (.2mg) preincubated at 0°C for 5 minutes with NEM were added to start the reaction. Data are means from 2 assays plotted by Dixon method taking into account the no NADP^+ or no NADH control. (\pm SD. $< 1.7\text{nmol}/5\text{min}$). (K_i for NEM = $30 \pm 2 \mu\text{M}$ at both sites).

5. EXPERIMENTS WITH CARBOXYLIC ACIDS INTERMEDIATES

The function of carrier proteins in mitochondria is to translocate the carboxylic acid intermediates. Of particular importance to this study are the monocarboxylates (pyruvate and 3-hydroxybutyrate), dicarboxylates (malate and succinate) and tricarboxylates (citrate and isocitrate). It is their transport across the mitochondrial membrane which is specifically inhibited by the carrier inhibitors which have been studied so far (see literature).

It was important to examine the effects of the carboxylates on both the membrane bound and the solubilised enzyme since they are the natural substrates for the carrier proteins in question.

5.1 Effect of Krebs Acids

The same experiments described for the carrier inhibitors were carried out with the krebs acids. What I had to establish first, was whether the carboxylates had an effect on the membrane bound transhydrogenase reaction. Table 5 shows that (except for 3-hydroxybutyrate) all the krebs acids tested significantly stimulated the activity of the enzyme. Succinate in particular did stimulate the TH activity even in the presence of antimycin A (a respiratory inhibitor).

Experiments with pyruvate were inconclusive since it greatly depletes NADH concentrations much more so at pH6 than at pH8. This was possibly due to lactic dehydrogenase contamination which is known to be a difficult enzyme to get rid of in particular, in crude preparations like submitochondrial preparations.

There was however, no significant 'malic' enzyme activity in these preparations since a control following NADPH_2 disappearance in the presence of pyruvate did not show any NADPH_2 oxidation.

Table 5: Effect of krebs acids (5mM) on TH activity.

<u>Krebs Acid</u>	<u>Activity (% control)</u>
Nil	100
3-hydroxybutyrate	125 \pm 10
Malate	150 \pm 10
Succinate	300 \pm 15
Citrate	250 \pm 9
Isocitrate	350 \pm 18

The reaction was as described in methods (Section 4.4Biii) by the GSH method. To the reaction mixture in Tris-sucrose-HCl pH 8.0 buffer, containing the above carboxylic acids (see figure 20), a mixture of GR (.45IU), ADH (.45IU) and SMP (.54mg) was added to start the reaction. GSH was determined 5 minutes after incubation. Data presented are means from 3 assays \pm SD. The control activity (100%) was (.006IU).

The controls on GR and ADH enzymes showed no effects by the krebs acids, neither was there a depletion of the TH substrates or products in their presence. This was monitored (as in Section 3) by following changes at 340nm in the presence of either GR or ADH and their substrates and also with SMP plus TH substrates or products at either pH8 or pH6 (see also Section 3.1). The other carboxylates however, had no effects on either NADH depletion or NADP⁺ reduction, ie there was no 3-hydroxybutyrate dehydrogenase or appreciable isocitrate dehydrogenase in these preparations. It may be important to mention that a potent aconitase inhibitor fluorocitrate was present at a concentration which had no effect on the TH whenever citrate or isocitrate was being tested.

The interesting finding here is the fact that dicarboxylates and tricarboxylates stimulate the enzyme activity like their corresponding carrier inhibitors to more or less the same level (2 to 3-fold). This suggests the involvement of carrier proteins in the mechanism of the TH reaction.

5.2 The effect of pH on the behaviour of carboxylic acids

We have seen in Section 4 that the amount of protons determine the extent of the stimulations of the TH activity by di- and tri-carboxylate carrier inhibitors. It is at higher pH where there is marked stimulation of the enzyme. It was of interest to study the effect of pH on the carboxylic acids stimulations as well. This was done over a wide pH range (pH5 to pH9).

The results in figure 20, show that, indeed the pH of the reaction medium determines the extent of carboxylic acids stimulations of the TH activity.

It was interesting to note that the profile of the TH reaction in the absence or presence of 3-hydroxybutyrate and malate was very similar with an optimum around pH 6 and it is these substances that gave less stimulations above pH 6. The other dicarboxylate (succinate) however, and the tricarboxylates (citrate and isocitrate) stimulated the TH activity with increasing pH.

5.3 Carboxylic acids kinetics

The next experiments which followed were the analysis of the stimulations observed by the krebs acids. It was of interest to determine whether the carboxylic acids stimulated the enzyme in a now familiar non-competitive fashion exhibited by their corresponding carrier inhibitors.

This was indeed so at pH8 as shown in figure 21 and 22 (~~$\frac{S}{V}$~~ versus S plots). Again 3-hydroxybutyrate and malate have little but non-competitive stimulation when either NADP^+ or NADH is varied. Succinate, citrate and isocitrate all stimulate the activity of the enzyme likewise, non-competitively, with respect to both NADH and NADP^+ sites. However, these stimulations were abolished at pH6 (result not shown).

The/

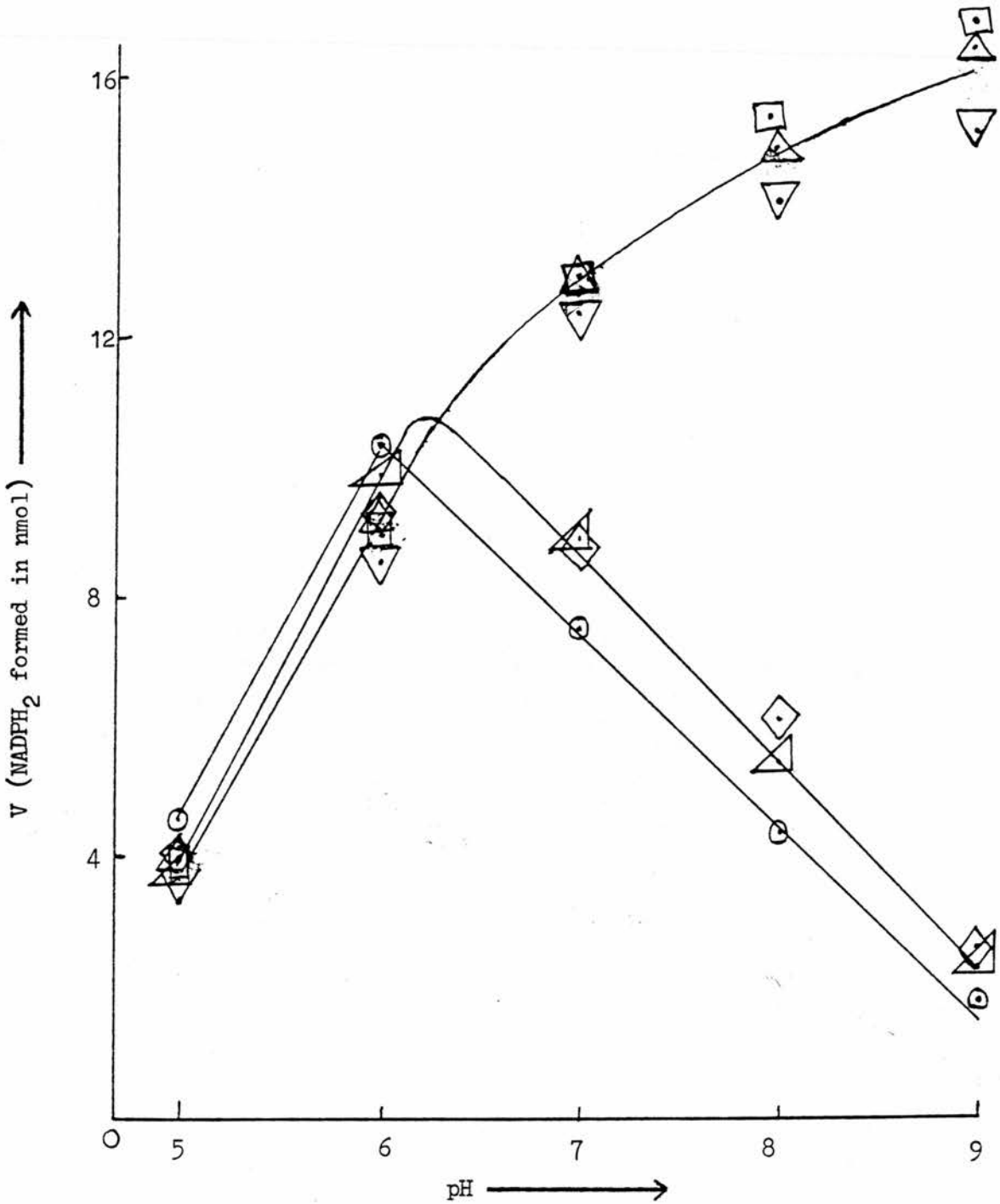


Fig 20: The effect of Krebs acids on TH activity at different pH.

The reaction was carried out as previously described (methods, Section 4.4Biii) in Tris-sucrose acetate buffer at the indicated pH containing no acid (\ominus), 3-hydroxybutyrate (\triangle), Malate (\diamond), Succinate with antimycin A (44ng) (\triangle), Citrate with FC ($5\mu\text{M}$) (∇) and Isocitrate with FC ($5\mu\text{M}$) (\boxplus) all at 5mM. The mixture of GR, ADH and SMP (.1mg) started the reaction. Data are means from two assays (\pm SD. $\angle .5$ nmol).

The results closely resemble the findings with carrier inhibitors, suggesting that the acids also affect the enzyme in a similar fashion (most likely by binding to the carrier proteins (see Section 4.4). However, the results also show that the carboxylic acids saturate the sites at lower concentration (2.5mM) compared to the carrier inhibitors (above 5mM or more). One explanation for this may be that since the krebs acids are antiporters, ie only bind to the carrier inhibitors in exchange for another substrate (see p123) there may be a limited amount of endogenous substrates for the carriers in submitochondrial preparations which allow limited amounts of the krebs acids to bind, and stimulate the TH activity. It is also possible that the sites on the carriers are saturated at around that concentration (2.5mM).

5.4 Effect of krebs acids on solubilised TH activity

In order to test the theory, ie that carboxylic acids stimulate TH activity by their binding to the carriers (see figure 24) it was necessary to study their effects on the solubilised system at both pH8 and pH6 as with the carrier inhibitors. Figure 23 shows an unexpected finding.

Although the stimulations are abolished at pH6 (B) as expected (see figure 18B), the dicarboxylates succinate, to a lesser extent malate and the tricarboxylates citrate and isocitrate significantly stimulated the TH activity at pH8 (A). The stimulation by both dicarboxylates however, is much reduced in the solubilised enzyme, especially by succinate.

There are problems in interpreting the effects of succinate in submitochondrial preparations, since it can also be used as the energy source for the TH (Chapter 5). The presence of antimycin A however, does not rule out the possibility of succinate oxidation via the respiratory chain.

However/

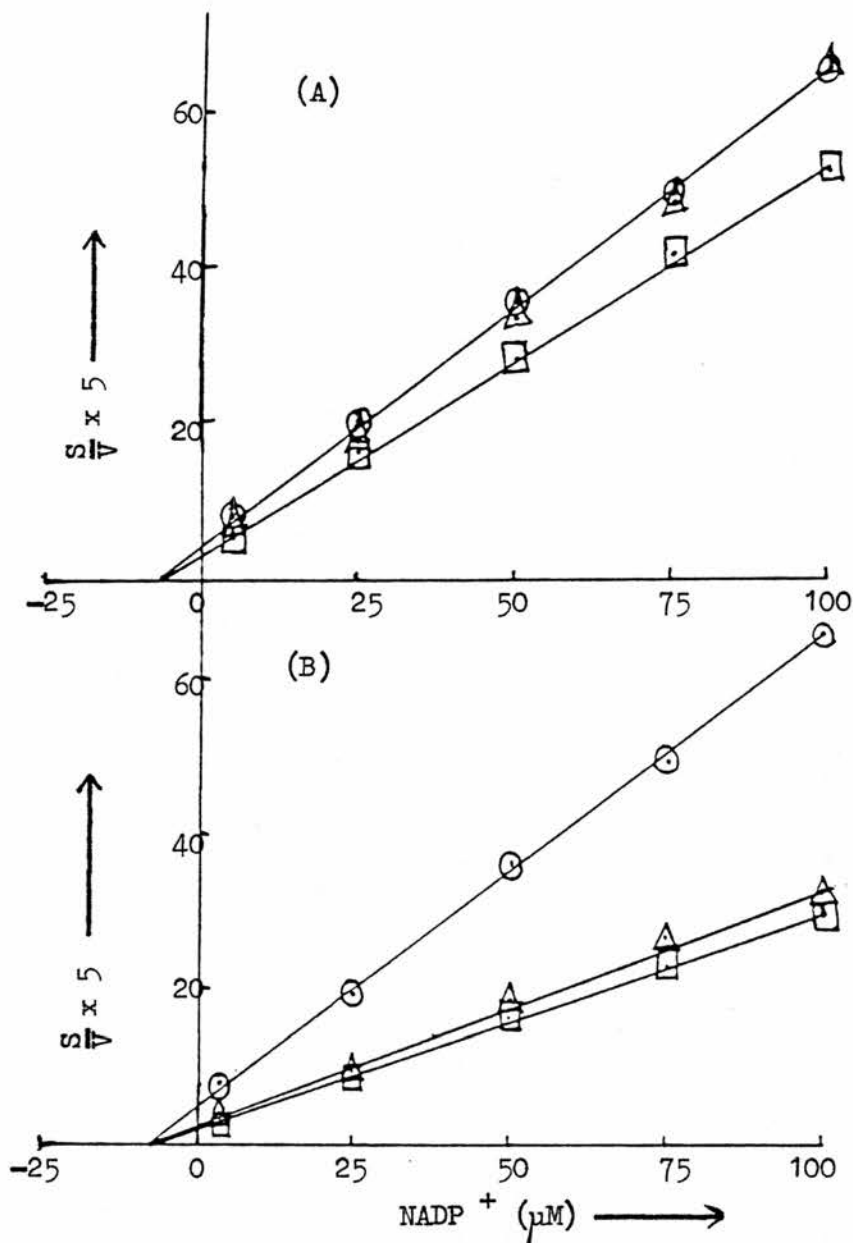


Fig 21: Effect of Krebs Acids on TH activity at different fixed NADP^+ concentrations.

The transhydrogenase reaction was determined in Tris-sucrose-acetate buffer pH 8.0 containing 3-Hydroxybutyrate or Malate (A); Succinate or Citrate or Isocitrate (as in figure 20) (B) at the following concentration ○ (Nil), △ (2.5mM) and □ (5mM); NADH (250µM) and varied amounts of NADP^+ as indicated. To start the reaction a mixture of GR, ADH and SMP (.4mg) was added. After 5 minutes the reaction was terminated and GSH determined (see method 4.4B). Data plotted (Eadie-Hofstee plot) are means from 3 assays minus the no NADP^+ control (figure 6) (\pm S.D. of $\sqrt{0.03}$ OD Units/5 min).

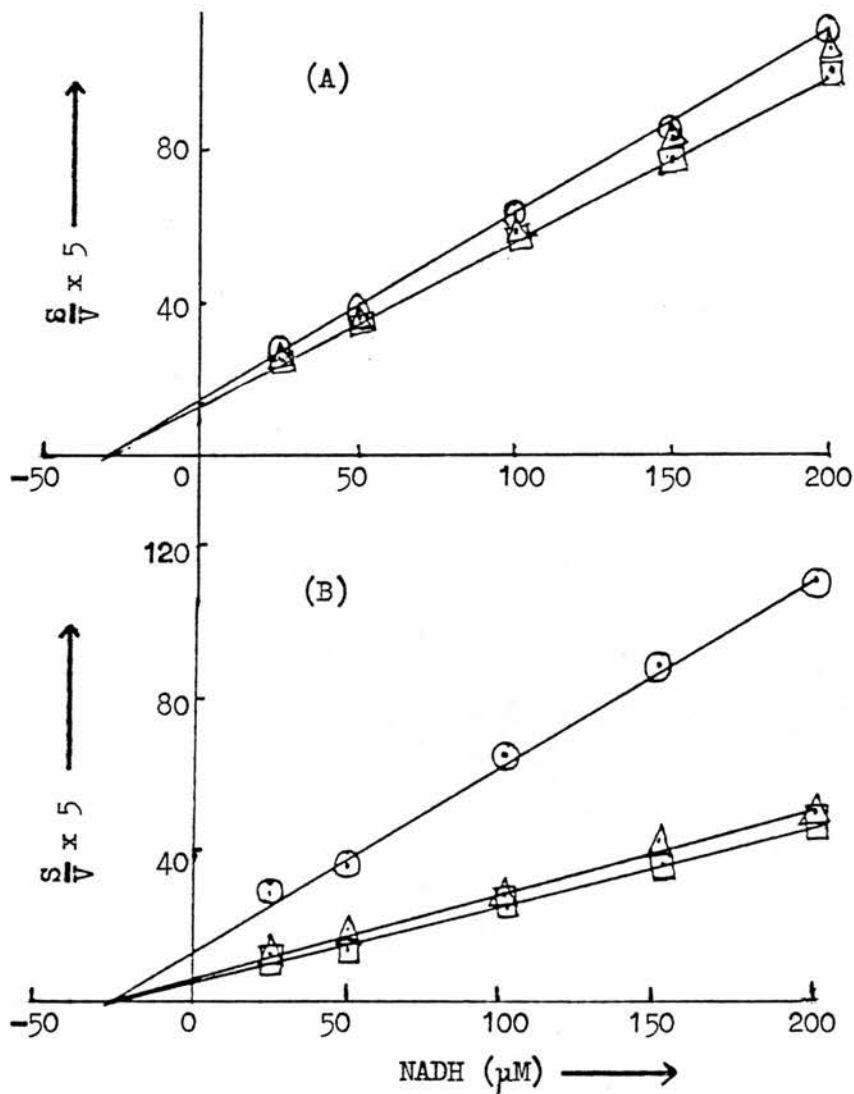


Fig 22: Effect of Krebs Acids on TH activity at different fixed NADH concentrations.

The reaction was determined at pH6 exactly as in figure 21, except high NADP^+ ($100\mu\text{M}$) and varied amounts of NADH.

(A) contained 3-hydroxybutyrate or Malate

(B) contained succinate or citrate or Isocitrate (as in figure 21) at the following concentrations nil

(\circ), 2.5mM (\triangle); and 5mM (\square).

The reaction was started by adding GR, ADH and SMP (.4mg), incubated for 5 minutes and GSH determined. Data plotted are means from 3 assays minus the no NADH control (see figure 8 and \pm SD. of $\sqrt{.03}$ OD Units/5min).

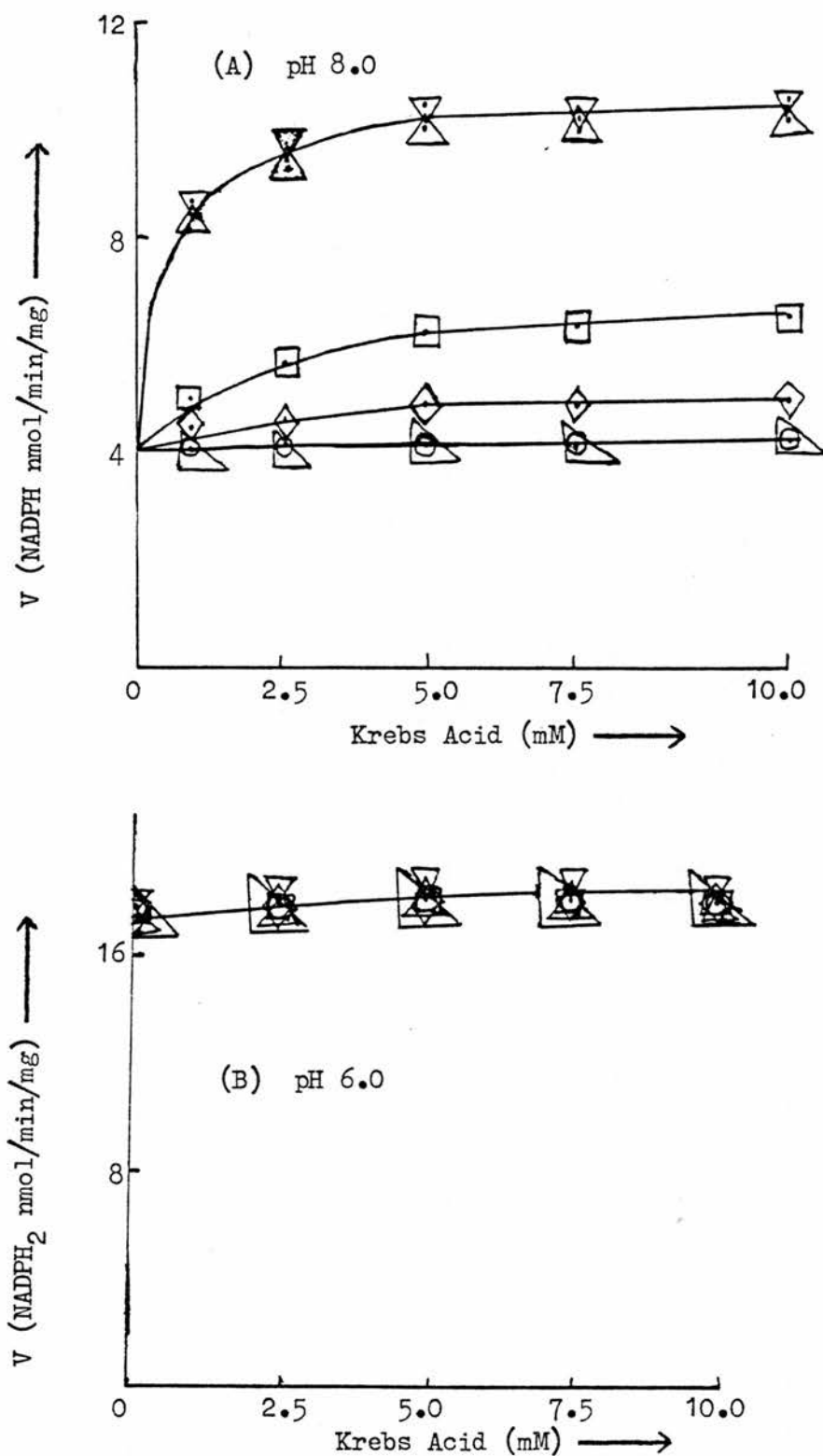


Fig 23: Effect of Krebs Acids on solubilised TH activity

The reaction was carried out at 30°C as described previously (figure 18), at $\text{pH } 8$ (A) and $\text{pH } 6$ (B) in the presence of varying amounts of carboxylic acids, pyruvate (\ominus); 3-hydroxybutyrate (∇); Malate (\diamond); Succinate (\square) with antimycin A (44ng); Citrate (\triangle) and Isocitrate (∇) both with FC ($5\mu\text{M}$). GR, ADH and SMP ($.3\text{mg}$) started the reaction which was incubated for 5 minutes. Data are means from 3 assays (\pm SD. $\angle .4\text{nmol}$).

However, the fact that the activity of the soluble enzyme is stimulated by succinate, citrate and isocitrate suggests the possibility that these substances also affect the enzyme directly (see discussion). The effects of citrate and isocitrate in particular which are not much diminished upon solubilising the enzyme strongly support this possibility.

The direct metabolic control of the transhydrogenase by carboxylic acid intermediates in particular citrate and isocitrate is suggested.

6 DISCUSSION OF THE NON-ENERGY LINKED REACTION

6.1 The non-energy linked enzyme activity

Mitochondrial transhydrogenase (TH) has not been greatly studied in rat liver. The bulk of the investigations of the enzyme have been done using beef heart as the source (of the 152 references cited in a recent review by Rydström in 1977, less than 10 refer to the rat liver enzyme). It is known that the enzyme is unstable and this is specially true for the rat liver enzyme.

I found that a linear reaction rate decreased with time after about 5 to 10 minutes at 30°C and this was probably due to its denaturation. In accordance with this observation, the enzyme loses much activity even when stored at a low temperature (-70°C), unlike the beef heart enzyme which maintains unchanged activity for 2 weeks stored at (-15°C) (Teixeira da Cruz 1974). There are two obvious variables which I studied in order to achieve maximum activity and longevity. One is the sonication medium and the other the storage medium. These led empirically to the use of an ionic medium (ie hypotonic phosphate buffer) for the former and a non-ionic one (sucrose) for the latter. It is of interest that the beef heart enzyme is usually prepared in a sucrose medium and sonicated 3 times longer to give approximately the same yield (about 20% of mitochondrial protein) (Löw and Vallin 1963).

The/

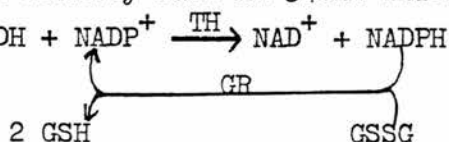
The enzyme cannot be assayed directly using the natural substrates since there is no change in optical density at 340nm nor of fluorescence intensity due to the reaction: $\text{NADH} + \text{NADP}^+ \rightleftharpoons \text{NAD}^+ + \text{NADPH}$. There are two kinds of methods used to obviate this difficulty:

- (a) to use either NAD^+ or NADPH generated to drive some dehydrogenase reaction specific for one of these substrates. Alternatively one can use two dehydrogenase reactions together, one specific for NAD^+ and one specific for NADPH ;
- (b) to substitute for either NADH or NADP^+ with a synthetic substrate or alternatively in the back reaction for NAD^+ or NADPH . Method (b) suffers from the disadvantage that the substrate may not be specific for the transhydrogenase.

I used in some of my work two synthetic substrates APAD and DCPIP. These on reduction, give optical changes away from 340nm. DCPIP was used to substitute for NAD^+ in the back reaction, but while this work was in progress, a report appeared (Anderson and Fisher 1978) stating that it could not be a substrate for the reverse TH reaction only, especially in impure submitochondrial particles which may have NADPH dehydrogenase activity using DCPIP as an electron acceptor.

Similarly the reduction of APAD by NADH is now known to be catalysed by NAD(H) dehydrogenase (Gutman et al 1970). The work I had started on it, was abandoned since a more satisfactory method (GSH method) was worked out.

Method (a) involves adding a suitable dehydrogenase and substrate. I used GR + GSSG to remove NADPH and regenerate NADP^+ as most other workers have also done. This depends on the proven specificity of the yeast GR enzyme for NADPH . However while many study the optical density fall at 340nm which results from the overall reaction: (ie $\text{NADH} + \text{NADP}^+ \xrightarrow{\text{TH}} \text{NAD}^+ + \text{NADPH}$)



I measured the amount of GSH produced which is of course, equivalent to half the amount of NADPH lost. NAD^+ can be removed by the reaction with ADH in the presence of ethanol and so regenerate NADH. By using both systems together, the initial concentrations of NADH and NADP^+ are maintained throughout the incubation period. This was the method I adopted in the main part of the study.

Mitochondrial TH is a difficult enzyme to purify and this has only recently been achieved for the beef heart enzyme. I applied four methods for the solubilisation of the rat liver enzyme and found that one of them, digitonin, gave better results than the others (tert-amyl alcohol, Triton X-100 and Lubrol PX). However, although the former method gave an enzyme preparation with a higher specific activity (Table 1) than that present in the submitochondrial particles, the total protein recovery was only 30%, and further purification was thus impracticable. However, working with this soluble enzyme, I was able to compare its behaviour with that of the membrane bound transhydrogenase.

In both cases the transhydrogenase activity between NADH and NADP^+ is very much affected by the pH (Table 2). There is a remarkable and reversible increase in activity as the pH of the medium is decreased with an optimum at pH 6.0. This has already been observed for the rat heart enzyme (Humprey 1957) and for the beef heart enzyme (Anderson and Fisher 1977) and (Earl et al 1978) and the bacterial enzyme (Kaplan and coworkers 1952) and recently studied in depth (Galante et al 1980) in the beef heart enzyme.

The concept that there is a group on the enzyme which requires protonation to give maximum activity, which emerges from this work has implications for the mechanism of the energy dependent reaction (see Chapter 5). It is to be noted that the activity at pH 6 which I observed is roughly equivalent to the rate at pH 8 obtained in the presence of succinate or ATP. Galante et al (1980) suggested the possible involvement of a 2'-phosphoryl group known to have a $\text{pK}_a = 6.1$. They suggested that protonation of this group (G in figure 24) may be a significant factor in increasing the reaction rate, with reactions involving hydride ion transfer to NADP^+ being more significantly affected than those involving NAD^+ . One/

One would expect however, that both reactions are affected to the same extent since both NADP^+ and NADPH involved in the forward and reverse reaction respectively possess the 2'-phosphoryl group.

It is now known that the enzyme from both the beef heart and the rat liver have an essential arginyl residue (Djavadi-Chaniane 1975) and (Blazyk and Fisher 1976) respectively. An essential Lysyl residue in Rhodospirillum rubrum was demonstrated by Jacobs and Fisher (1979). Electrostatic interaction with these groups at or near the active site(s) is a possible way by which the enzyme's reaction rate could be modified by protons.

Apparent high activation energy at the lower pH suggests that a conformational change may also occur as a result of such protonation (see general discussion).

The finding that the enzyme is more thermolabile at pH6 than at pH8 also suggests that maybe catalytic group(s) are exposed more at pH6 and become more susceptible to heat inactivation.

6.2 Kinetic Studies

K_m values for the non-energy transhydrogenase reaction obtained by varying either substrate and holding the other high and constant have been obtained (see Table 2).

There is a marked difference between the K_m s and V_{max} of the rat liver and beef heart enzymes. I found that the rat liver enzyme has higher affinity for NADP^+ ($K_m = 6\mu\text{M}$) than for NADH ($K_m = 25\mu\text{M}$) contrary to Teixeira da Cruz et al's report (see Table 2) with the beef heart enzyme. The disparity may be due to different properties of the enzymes from these sources or a consequence of using different assay methods. Teixeira da Cruz and coworkers (1974) used fluorescence and optical density changes at 340nm to measure the TH reaction rates, which I found to be less precise and unsatisfactory, especially in kinetic analysis. There is again a significant difference in the V_{max} of the two enzymes with the beef heart enzyme several (six) fold higher than the rat liver enzyme. This/

This, maybe explains why most workers are not keen to study the rat liver enzyme. The few who do, prefer to use substrate analogs which makes it impossible to compare my findings with theirs directly. The use of substrate analogs is also becoming widespread, particularly in studying the beef heart enzyme.

The K_m s of the solubilised and particulate enzyme were also compared at pH8 and pH6 where their activities are very different. To my surprise, K_m values were virtually unchanged at the two pHs, thus any conformational change resulting from the pH shift, does not affect the two K_m s and binding sites of the enzyme. This is compatible with the finding that energization of the TH from rat liver by either ATP or succinate also has no effect on the K_m s (see Chapter 5).

From the work with substrate analog (APAD), I obtained the K_m for this substrate ($86\mu\text{M}$) which agrees closely with that (about $90\mu\text{M}$) reported by Galante et al (1980) measuring the reverse TH reaction between NADPH and APAD. But, as already mentioned (Section 6.1) the specificity for APAD by TH is in doubt.

6.3 Effect of Inhibitors

A Miscellaneous Inhibitors: Inhibition of the TH by PCoA is well established, both for the rat liver and the beef heart enzyme. It was used in this work as a control and I verified that the inhibition is competitive and restricted to the NADP^+ site. It is to be noted that this is the only instance of competitive inhibition which I encountered at the NADP^+ site throughout this work.

Since thiol agents inhibit the GSH redox system of rat liver mitochondria, they were also tested and found to inhibit non-competitively both the NADP^+ and NADH sites.

As/

As expected, uncouplers were ineffective. This means that the preparations were not in any way coupled to residual energy producing mechanisms (ie in the sub-mitochondrial particles). For this reason there was no need to add the uncoupler routinely to the assays.

B Carboxylate Carrier Inhibitors: The chief interest in this study of the non-energy transhydrogenase, was to examine the effect of these inhibitors on the TH and the surprising observation emerged. Some of the carrier inhibitors (dicarboxylates and tricarboxylates) considerably stimulate enzyme activity in submitochondrial particles.

The monocarboxylate carrier inhibitor used (phenyl-pyruvate) was the exception. It proved to be inhibitory. Although it was desirable to try another monocarboxylate carrier inhibitor (α -cyano-4-hydroxycinnamate) too, to see if the inhibition was general or a specific property of the inhibitor. α -cyano-4-hydroxycinnamate (α CC), however absorbs at 412nm and therefore interferes with the assay and could not give accurate results. Such indications as were available, do however suggest that it too is an inhibitor.

The dicarboxylate carrier inhibitors used were n-butyl-malonate (BM) and phenylsuccinate (PhS). Both had the same effect, ie that of stimulation of the TH which increased with the concentration of the inhibitor. It is of interest that the concentration range where such stimulation was obtained is that one which the inhibitors are normally used for work in intact mitochondria (Halestrap 1978), (Palmieri et al 1974) and (Stipani et al 1981).

Also stimulating TH activity were the tricarboxylate carrier inhibitors propane-1,2,3-tricarboxylate (PT) and benzene-1,2,3-tricarboxylate (BT). Their/

Their behaviour was different from that of the dicarboxylate carrier inhibitors because they showed a stimulation which increased with the inhibitor concentration to a maximum (5mM) and then decreased again.

Since the pairs of substances mentioned have in common only their known ability to bind to the mono, di, or tri carboxylate carriers of mitochondria, the above observations suggest strongly that they are exerting their effects on TH as a result of such binding. This concept would be rationalised by postulating that their binding to these carrier sites perturbs the membrane bound TH, producing in it a conformational change which conduces a higher enzyme activity (see figure 24).

I examined the effect of all these inhibitors on the kinetics of the TH reaction. The most interesting finding was that one could apply Michaelis-Menten kinetics to both the stimulated or inhibited reaction which showed the normal linear response to the Eadie-Hofstee plot. The conclusion is that none of the inhibitors tested altered the K_m for either substrate. The effect was entirely on the V_{max} of the reactions which increased up to 3-fold upon stimulation.

Another interesting finding was that the stimulant effect of the porter inhibitors on the bound enzyme was largely eliminated after solubilisation. Thus the soluble enzyme appeared unresponsive to the presence of the inhibitors. This is, of course, in accordance with the theory outlined above (figure 24). The soluble enzyme would be freed from the membrane and so no longer subject to changes relayed by it.

Another result which has bearing on the mechanism of inhibition is that, the stimulation by the carrier inhibitors of the membrane bound TH is also much reduced on decreasing the pH. It will be remembered that this itself increases activity without affecting K_m . One/

One explanation for this lack of responsiveness at lower pH is that the inhibitors have the same function as protons themselves, namely they promote changes in enzyme structure which allows group(s) which normally accept proton(s) only at pH6 to do so at pH8. This would presumably operate by changing the pKa of the suitably dissociating group(s). Another explanation is that at the lower pH the binding of the inhibitor to the carboxylate carrier for which it is specific is itself inhibited again due to ionic changes, but this time on the carrier protein itself (for review see LoNoue et al 1979).

6.4 Effect of Krebs Acids

The carboxylate carriers are required in mitochondria to carry metabolites across the membrane. The chief metabolites relevant to this study are pyruvate and B-hydroxybutyrate (monocarboxylates), succinate and malate (dicarboxylates) and citrate and isocitrate (tricarboxylates).

It was therefore, interesting to note that they themselves also had effects on the TH activity.

The effect of pyruvate could not be studied accurately because it rapidly depletes NADH in the absence of NADP^+ , perhaps because of the presence of contaminating lactate dehydrogenase. It is generally accepted that this enzyme is very difficult to eliminate from submitochondrial preparations.

Pyruvate could also react with NADPH formed in the reaction using the 'Malic' enzyme although this was found to be insignificant in my preparation.

The effect of succinate in the presence of a respiratory inhibitor antimycin A and malate however, has been shown to be stimulating the enzyme's activity and this is also the case with isocitrate and citrate. Moreover/

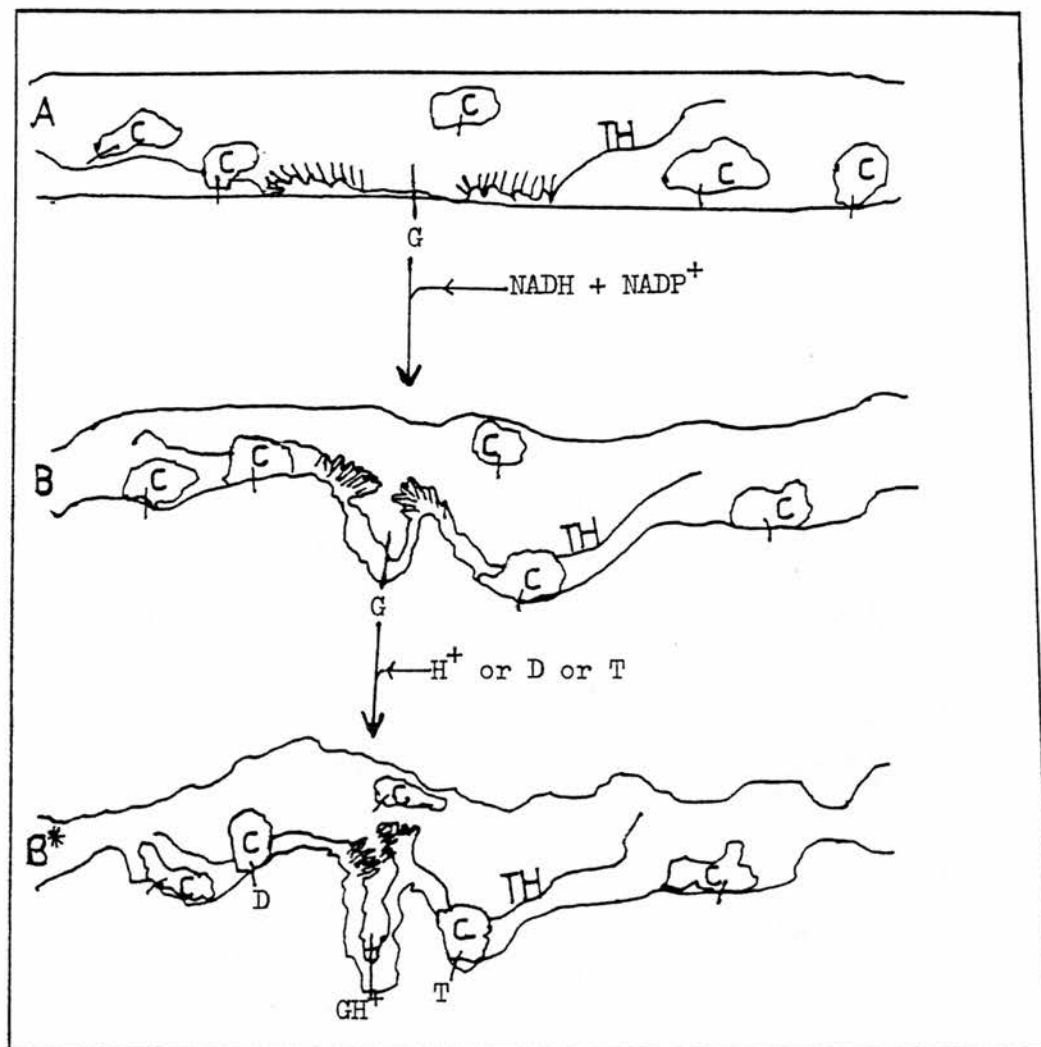


Fig 24: Proposed mechanism of TH activation by protons or carboxylate carrier inhibitors.

A conformational hypothesis is proposed for the TH activation by either protons (H^+) or substances (D) or (T), ie the dicarboxylates or tricarboxylates carrier inhibitors, respectively which bind to carrier proteins (C). It is suggested that the enzyme is in a resting (inactive) state at (A) in the inner mitochondrial membrane but upon addition of NADP^+ and NADH , it is converted to an active form (B) by binding the substrates. A more active conformation (B)* is achieved by either protonation of group(s) G on the enzyme exposed to the reaction medium or binding to (C) by D or T. The (B)* conformation thus achieved promotes the catalysis without affecting the affinity for the substrates.

Moreover, as^{with} the carrier inhibitors, these acids are not effective at pH6. At first sight these results are consistent with the idea that they act by the same mechanism as the carrier inhibitors (figure 24). However this cannot be maintained for the following reason. On solubilising the enzyme, the stimulant effect of the carboxylic acid substrates is still apparent, suggesting that they act directly on the enzyme.

This has not been reported in the literature so far. The implication of the metabolic control of the transhydrogenase enzyme by Krebs acids in particular the tricarboxylates (citrate and isocitrate) is of considerable interest. The stimulation by these tricarboxylates, could not have been due to isocitrate dehydrogenase contaminating my preparations since its activity was negligible. One other reason that could explain citrate stimulation is its conversion to isocitrate by the aconitase. However, a potent inhibitor of this enzyme fluorocitrate was always present together with the two tricarboxylates.

The possibility that the carboxylates stimulate the enzyme directly in addition to their effect relayed via their carriers clearly cannot be pursued. It should perhaps be pointed out however, that these carriers are antiporters, they do not carry and may not bind an acid on one side of the membrane if another is not present on the other side, eg succinate \rightleftharpoons malate. In this respect they differ from the inhibitors used which bind in the absence of the substrates. Experiments in which acids were mixed would have been difficult to interpret.

CHAPTER 5THE ENERGY DRIVEN NICOTINAMIDE NUCLEOTIDE TRANSHYDROGENASE

As already mentioned in Chapter 4, mitochondrial TH catalyses an energy-driven hydride transfer between NAD(H) and NADP(H) as well as the non-energy dependent reaction.

Two methods have been used to follow the energy dependent reaction, namely (i) using ATP as the energy source, and (ii) using succinate to generate high energy equivalents by electron transport.

1 SOME PROPERTIES OF THE REACTIONS

In order to study the energy-driven TH reactions (supported by either ATP or succinate) in detail, I had first to characterize the conditions most suitable for both enzyme preparation and assay (see p 71) as I did for the non-energy reaction.

1.1 Effect of omitting substances in the energy-driven TH assay

In Table 1, the effect of omitting each of the components of the reaction in turn is shown on the reaction rate studied in this case by the fluorimetric method, ie by measuring the increase in NADPH₂ and maintaining NADH₂ constant with alcohol dehydrogenase (ADH) as in methods, Section 4.4B(i).

Clearly, omission of the energy source greatly decreases the rate (by 5 to 7 times) giving the non-energy reaction rate (see p 72). Omission of either substrate (NADP⁺ or NADH) give a negligible rate. These two omissions were mainly used as no NADP⁺ or no NADH control (indicated accordingly in the results). However, omitting ADH or ethanol at saturating levels of NADH markedly decreased the reaction rate, which would be expected in the fluorimetric assay method.

1.2/

Table 1: Effect of omitting components of the energy-driven TH

Omission	V (with ATP) nmol NADPH ₂ formed/min/mg	V (with Succinate) nmol NADPH ₂ formed/min/mg
Nil	66.2	45.2
NADP ⁺	1.4	1.3
NADH	1.7	1.6
ADH	0.7	0.6
ETOH	0.9	0.8
ATP	9.4	-
Succinate	-	8.4

The reaction was carried out at 30°C in Tris-Sucrose-HCl buffer pH 8.0 as described in methods (Section 4.4Bi) in the presence of SMP (1mg), NADP⁺ (100µM) and NADH (250µM) or omitted as shown. Water was put in place of each substance omitted. The reaction was assayed for NADPH₂ formed with or without ATP (1mM) or succinate (25mM) fluorometrically in aliquots (3ml) after 2 minutes incubation. Data presented are means from two assays (\pm SD \angle 2nmol).

1.2 Time course of energy dependent TH

Time courses of either ATP or succinate driven TH were determined by both continuous and discontinuous assays (methods, Section 4.4). Results obtained using the GSH method (constant NADP⁺ and NADH) are shown in figure 1.

Using ATP and particles prepared in hypotonic ionic phosphate medium, the rate is greater than when the particles have been prepared in sucrose and also remain linear with time for longer. Particles prepared in Tris-HCl, although they have a similar time course with those prepared in phosphate, are however, less active (result not shown).

Using/

Using succinate as the energy source and preventing endogenous ATP formation by the addition of oligomycin, rates with the (phosphate) particles are considerably lower than with ATP (figure 1).

As with the non energy reaction, rates vary linearly with the amount of protein in the particles up to a limit (1mg protein/.5ml) as shown for both ATP and succinate driven reactions (figure 2).

1.3 Effect of storage in different media

I have already described the effect on the non-energy reaction, of storing particles in different media at -70°C , on their activity when subsequently thawed and assayed (p 72). As shown in figure 3, similar results are also obtained with the ATP dependent reaction. The succinate driven reaction gave very similar results (not shown).

1.4 Temperature and pH profiles

The variation of TH activity with temperature (figure 4) was also determined. The temperature profile of the ATP driven TH reaction at pH8 and pH6, follow a pattern like that of the non-energy reaction at pH6, except that here the activation energy is very much higher ($26-28 \text{ KJmol}^{-1}$) than that of the non-energy reaction at both pH8 and pH6 (see p 78).

When the pH profile of the energy driven TH reaction was determined, a quite different picture was obtained from that of the non-energy reaction (figure 5). Whereas the non-energy reaction rate increases with decreasing pH with a maximum at pH6, the opposite is true for ATP driven TH. From pH8 downwards the rate decreases with decreasing pH so that at pH6, the rate of the energy and non-energy reactions are not greatly different. This finding is also obtained for the succinate driven TH. An explanation for this decline maybe is that there is increasing difficulty for the TH to pump out protons against an increasing external proton concentration (see discussion).

1.5/

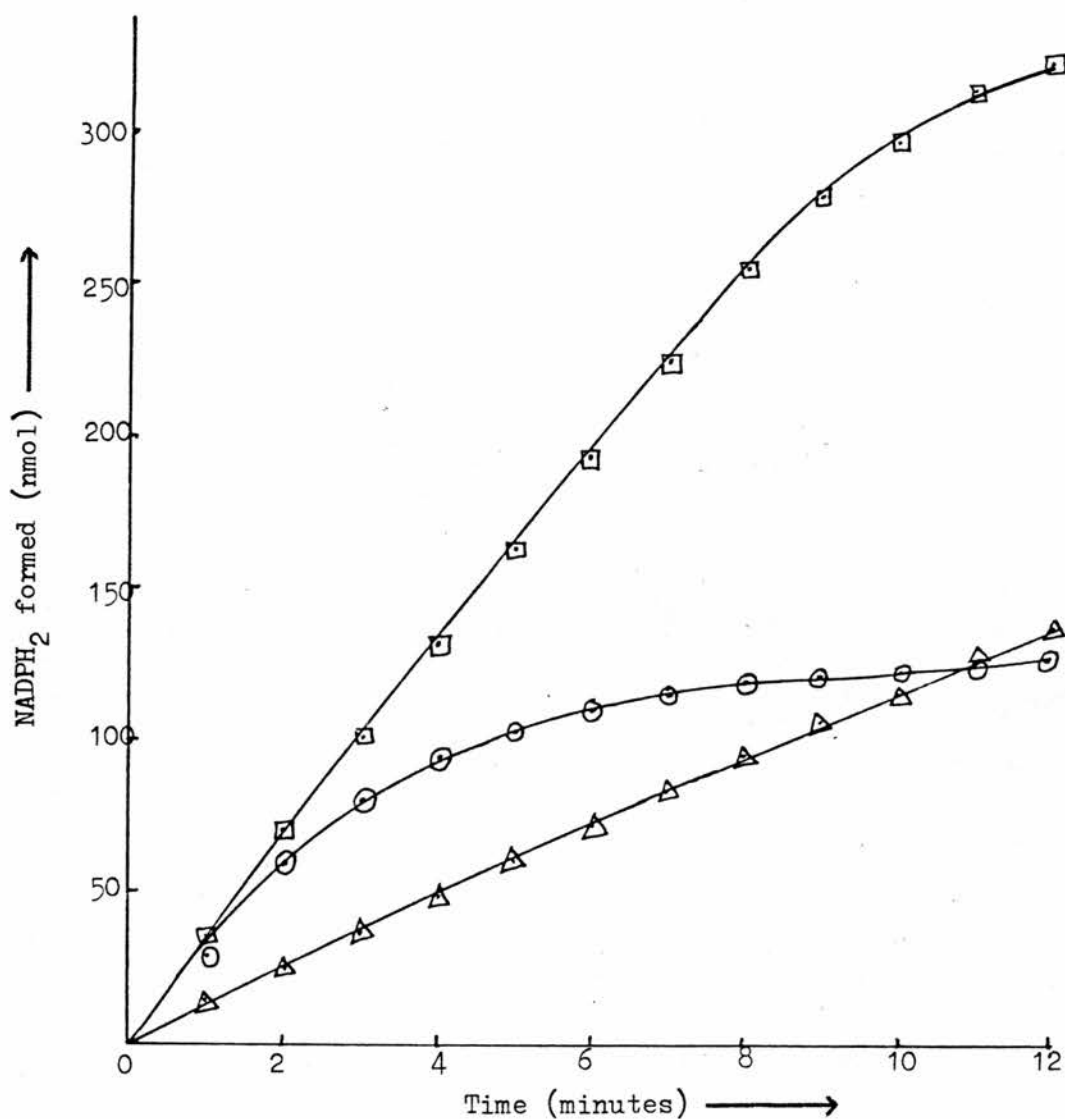


Fig 1: Variation of energy driven TH with incubation time.

To the reaction mixture as for Table 1, containing ATP (1mM) (□) and (○) and succinate (25mM) with oligomycin (1μg)(△), a mixture of GR (.45IU), ADH (.45IU) and SMP (.5mg) prepared either in phosphate (.025M) (□), (△) or in sucrose (.25M) (○); was added. After incubation for the required time (indicated above) at 30°C, the GSH formed was determined (see methods, Section 3.2). Data shown are means from five assays minus the zero time control (\pm SD. \angle 1.3 n mole), from particles prepared from different mitochondrial preparations.

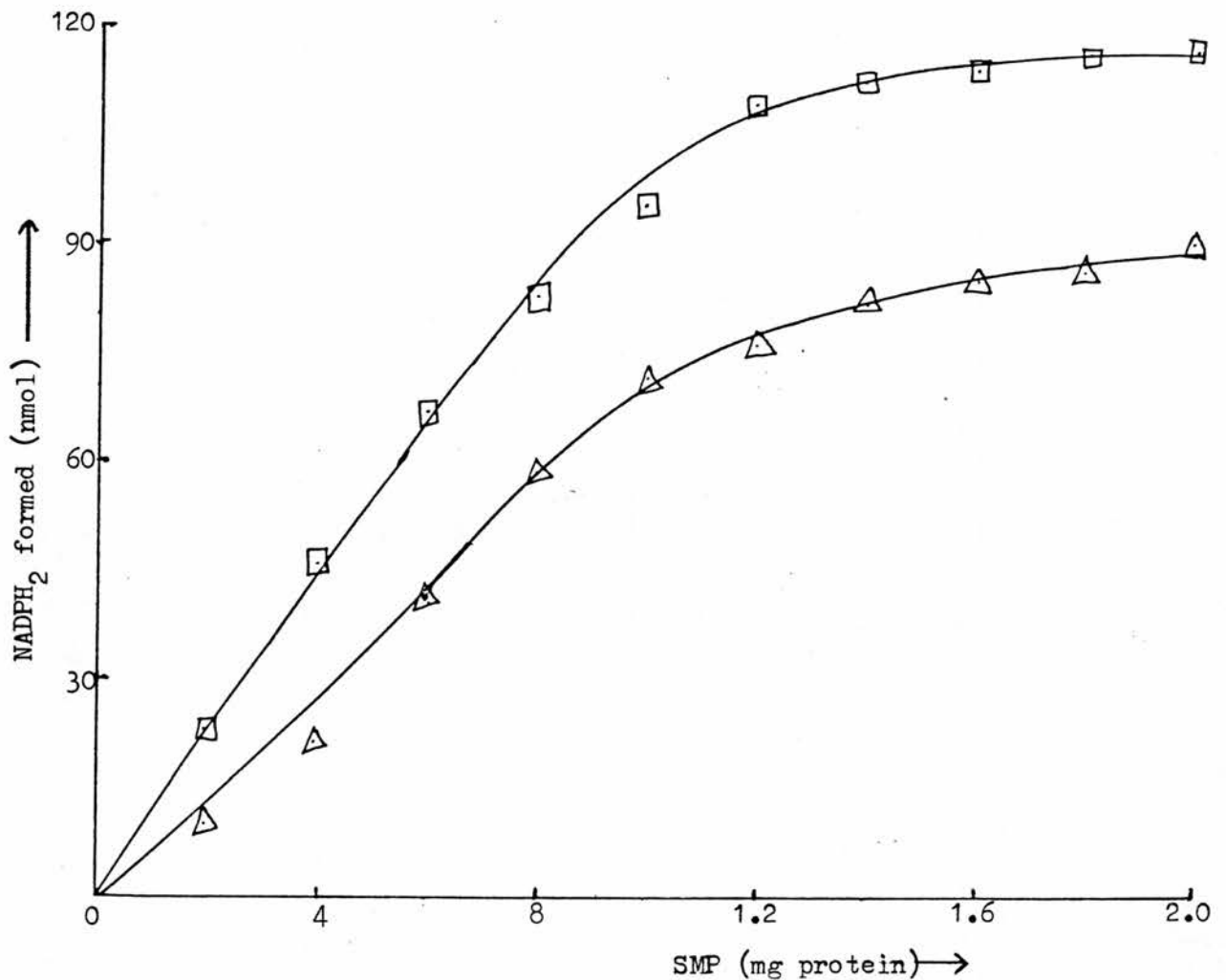


Fig 2: Variation of energy dependent TH with amount of protein.

To the reaction mixture as for figure 1 containing ATP (1mM) (\square) or succinate (25mM) (\triangle), preincubating at 30°C pH 8.0 in Tris-sucrose-HCl, a mixture of GR, ADH and SMP, from different preparations at protein concentration indicated above, was added to start the reaction. After 2 minutes incubation, the reaction was terminated and GSH determined as in figure 1.

Mean values plotted were obtained from three assays taking into account the zero time control, (\pm SD. $\Delta 1.7$ nmol).

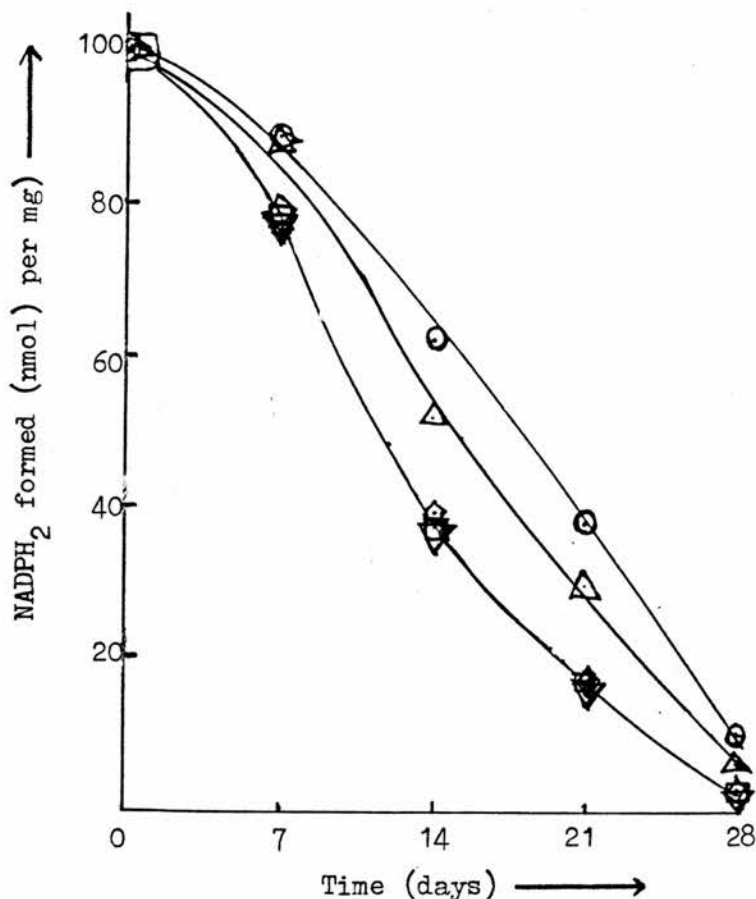


Fig 3: Variation of ATP driven TH with storage in different media

To the reaction mixture containing saturating NADP^+ ($100\mu\text{M}$) NADH ($250\mu\text{M}$) in Tris-sucrose-HCl buffer pH 8.0 as in figure 2, a mixture of GR (.45IU) and SMP (.52mg) stored in Sucrose (.25M) (\ominus); Sucrose (.25M) + MgCl_2 (10mM) (\triangle); sucrose (.25M) + BSA (.2% W/V) (∇); Tris (.25M)-HCl pH 7.0 (\square); and once distilled water (\diamond); was added. After 2 minutes incubation, GSH formed was determined as before (see methods, Section 3.2). Mean values of two assays minus the zero time control are shown above (\pm SD. $< 1.3\text{nmol/mg}$).

TH activity of unfrozen particles at day (0) was the same as in the frozen at day (1), ie (.05IU).

1.5 Kms of energy driven TH for NADP⁺ and NADH

It has been reported that using the beef heart enzyme the K_m for NADH₂ and NADP⁺ are each different when the TH reaction is energy linked (Rydström 1977). Kinetic studies were therefore, performed and the results are given in figure 6, using the GSH method (constant NADP⁺ and NADH). Like the non-energy reaction, the energy dependent reaction follows Michaelis-Menten kinetics as shown by the linearity obtained in the $\frac{S}{V}$ versus S plots.

Whether the reaction is driven by ATP or by succinate, there is no significant difference obtained for the K_m values of either substrate. In view of the different specific activities obtained with these two energy sources, this is an interesting finding and strengthens the conclusion that the K_m does indeed measure the affinity of the substrates for the transhydrogenase enzyme protein. The mean values obtained from these plots were K_m NADP⁺ (6.25 μ M) and K_m NADH (25 μ M). These are to be compared with those obtained for the non-energy reaction (see p 86), ie K_m NADP⁺ (6 μ M) and K_m NADH (25 μ M). The same result was obtained with particles prepared in either sucrose or Tris-HCl (not shown).

It can be seen that for both the energy and non-energy reactions, the enzyme has a higher affinity for NADP⁺ than it has for NADH.

2 THE EFFECT OF INHIBITORS

2.1 Miscellaneous Inhibitors

As for the non-energy reaction, various substances were tested on the energy driven reaction. In either case palmityl coenzyme A (PCoA) the specific TH inhibitor was tested as the control. Again PCoA inhibits the ATP and succinate driven reactions with respect to the NADP⁺ site in a competitive manner, (Dixon plot, K_i 1.6 μ M), but does not inhibit the NADH site (figure 10) as indeed it did with the non-energy reaction (p92). The two methods previously described, ie $\frac{S}{V}$ versus S and Dixon plots, (see p 92) were used in the kinetic analyses of most inhibitors.

Thiol/

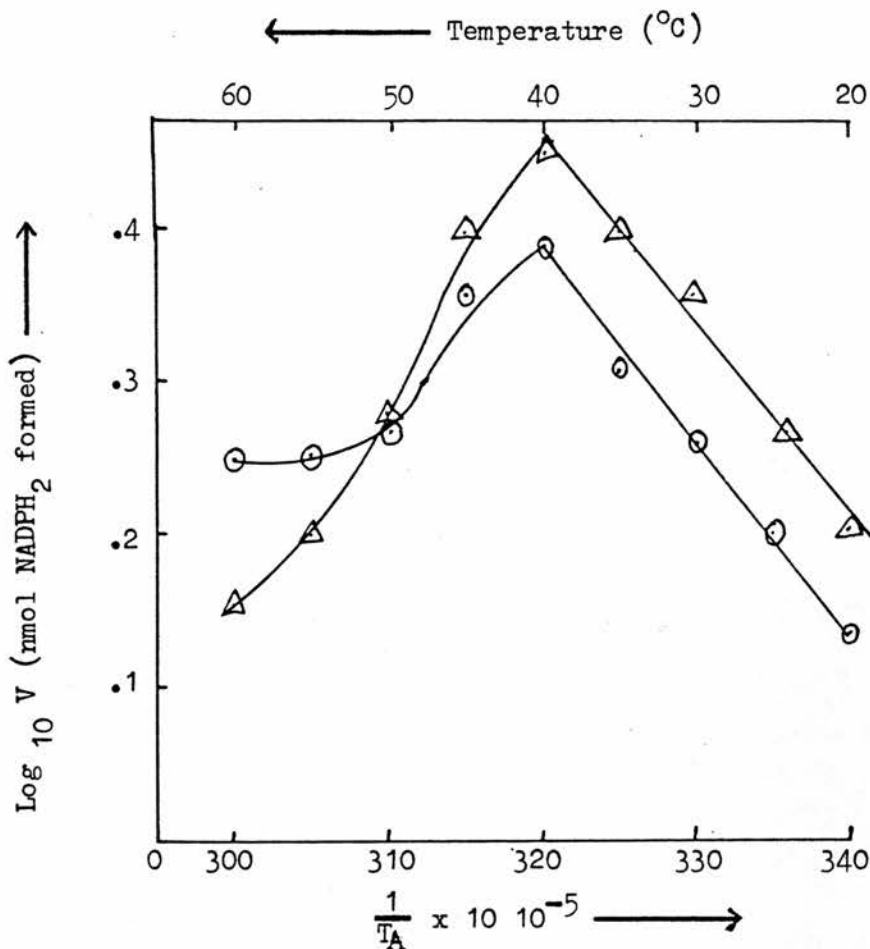


Fig 4: Arrhenius Plot of the ATP driven TH activity

To the reaction mixture containing NADP^+ ($100\mu\text{M}$), NADH ($250\mu\text{M}$) in Tris-acetate buffer pH 8 (Δ) and pH 6 (\ominus) preincubating at the above specified temperature, a mixture of GR ($.45\text{IU}$) and SMP ($.28\text{mg}$) was added to start the reaction. After 2 minutes incubation at the desired temperature, the reaction was terminated and GSH determined as in methods, Section 4.4B(ii).

Data from two assays are plotted in the above Arrhenius plot ($\text{Log}_{10} V$ against $\frac{1}{T}$ absolute) ($\pm \text{SD} < 1.5 \text{nmol NADPH}_2$).

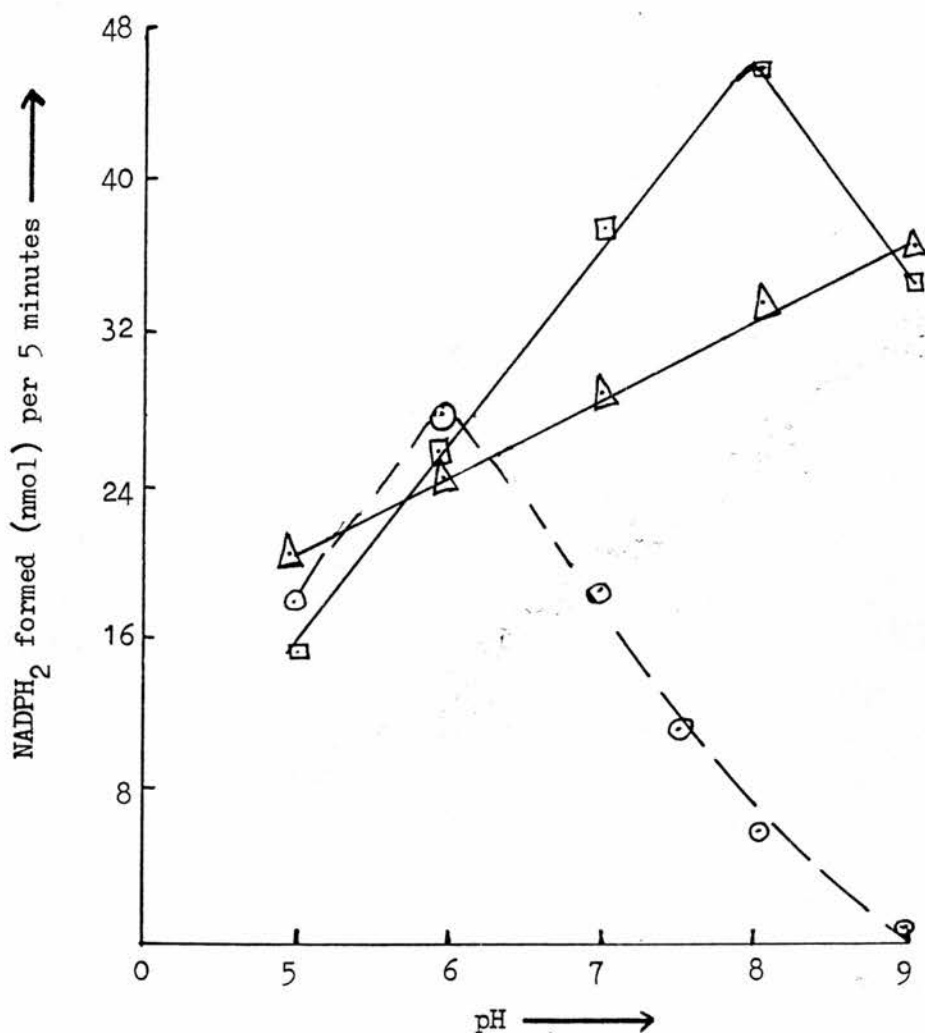


Fig 5: Variation of TH activity with pH

To the reaction mixture containing NADP^+ ($100\mu\text{M}$), NADH ($250\mu\text{M}$) either no energy source ($-\text{O}-$), or ATP (1mM) (◻) or succinate (25mM) with oligomycin ($1\mu\text{g}/\text{mg}$) (△) preincubating at 30°C in Tris-sucrose-acetate buffer at the specified pH (see above); a mixture of GR ($.45\text{IU}$), ADH ($.45\text{IU}$) and SMP ($.25\text{mg}$) stored for 8 days, was added to start the reaction.

After 5 minutes, the reaction was terminated and GSH formed determined, see methods, Section 4.4B(iii). Data plotted are means from 3 assays from same batches of particles minus the zero time control ($\pm \text{SD. } < 1.3 \text{ nmol}/5 \text{ min}$). The specific activities (in IU) of TH showing pH6 first then pH8 were nonenergy ($.022$, $.005$); + ATP ($.020$, $.037$); + succinate ($.019$, and $.026$).

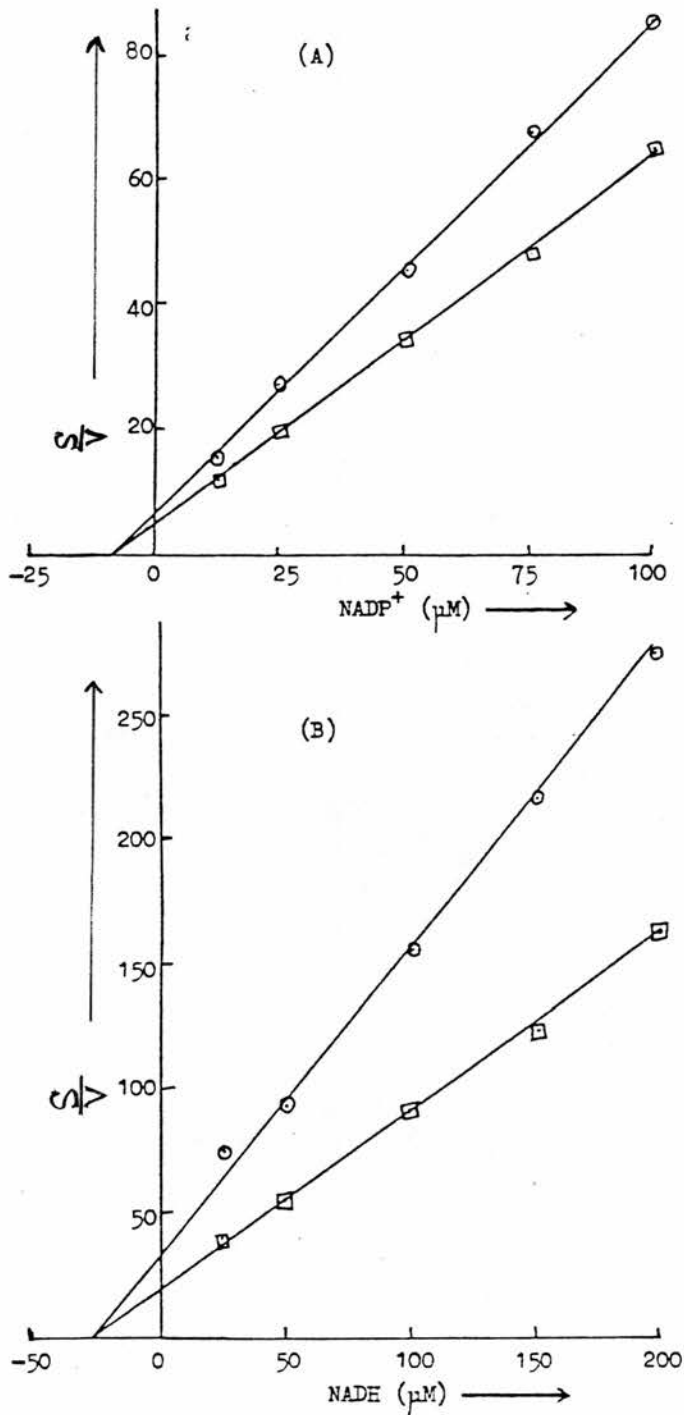


Fig 6: Variation of energy driven TE with either NADP⁺ (A) or NADH (B) concentrations.

To the reaction mixture in Tris-sucrose-HCl/acetate buffer pH 8.0 containing NADP⁺ varied and NADH (250µM) (A) or NADP⁺ (100µM) and NADH varied (B) with ATP (1mM) (◻) or succinate 25mM (○), a mixture of GR, ADH and SMP (.24mg) was added to start the reaction. After 5 minutes incubation at 30°C, GSH formed was determined as in

methods, Section 4.4B(iii). Mean values of eight assays minus no NADP⁺ (A) or no NADH (B) control (\pm S.D. of $\sqrt{4.04}$ OD Units/5min). K_m NADP⁺ = $6.25 \pm .6\mu\text{M}$ and K_m NADH = $25.0 \pm 1.5\mu\text{M}$ for both reactions for frozen or unfrozen SMP.

Thiol reagents behave in a similar fashion showing varying degrees of inhibition of the energy driven reaction (figure 7) as indeed it was for the non-energy reaction (p 135). After a short preincubation *n*-ethylmaleimide (NEM) and *p*-hydroxymercuribenzoate (MB) inhibited at concentrations below 50 μ M, NEM being the more effective. The inhibitors are also more pronounced when the reaction is driven by ATP in keeping with the suggested thiol dependency of the F_1F_0 -ATPase (Gould 1978). The SH group(s) blocked by the thiol reagents are not very reactive since the inhibition is not obtained unless the preincubation is first done.

The most important inhibitors of the energy driven reaction are of course, uncouplers. Thus FCCP (.5 μ M) was found to reduce the rate of the reaction driven by either ATP or succinate to the level of the non-energy reaction (results not shown).

Other substances were also tested on the energy driven reactions. These include:

- (i) Cations (up to 5mM) viz, Ca^{2+} , Ba^{2+} , K^+ and Na^+ ;
 - (ii) chelators (below 1mM), ie EDTA and EGTA; and
 - (iii) anions (up to .1mM), ie OH^- , Cl^- , SO_4^{2-} and acetate;
- suggesting that the enzyme is relatively insensitive to various ions with respect to the forward TH reaction (results not shown). The exception was Mg^{2+} (6mM) which inhibited the non-energy (by 35%) and succinate driven (by 10%), but not the ATP driven reaction (results not shown).

2.2 Carboxylate Carrier Inhibitors

The most pertinent to my interest however, was the effect of the carboxylate carrier inhibitors on the reaction and because of the interesting effects observed by these substances on the non-energy reaction (p89), this was studied in depth. With saturating concentrations of both substrates, it was initially found that these carrier inhibitors all inhibited the TH reaction. The inhibitions increased with increasing inhibitor concentration until a maximal inhibition of about 50% was achieved. This was true for either succinate or ATP supported TH (figure 8).

In/

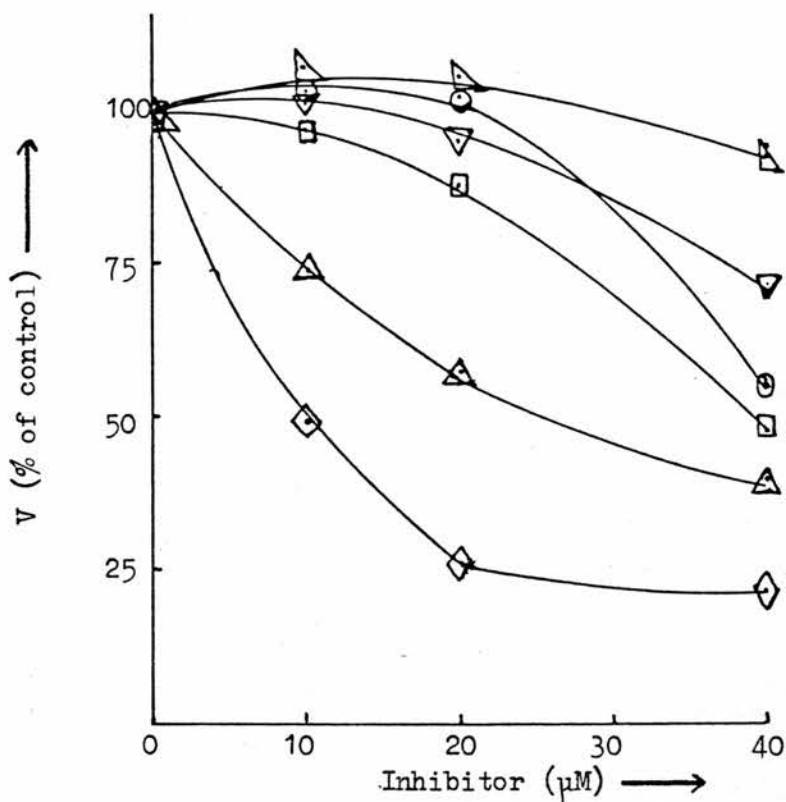


Fig 7: Effect of thiol reagents on energy driven TH

To the reaction mixture (400μl) containing NADP^+ (100μM), NADH (250μM) in Tris-sucrose-HCl buffer pH8 preincubating at 30°C, a mixture (10μl) of GR (.45IU), ADH (.45IU) was added then followed by a mixture (90μl) of SMP (.1mg) and the thiol reagent (at specified concentration above) preincubating at 0°C for 5 minutes, to start the reaction. For the succinate driven reaction, NEM (⊖) or MB (▽) was present and for the ATP driven reaction NEM (◇) or MB (△) was present.

The ATP driven reaction was also determined without prior incubation of SMP with the thiol reagent and NEM (⊖) or MB (▽) was present.

After 5 minutes incubation; GSH formed was determined as in methods, Section 4.4B (iii). The data plotted % V (NADPH_2 formed) against the inhibitor concentration are means from two assays \pm SD. $\leq 3\%$. The control (100%) activity for ATP driven reaction was (.033IU) and for succinate driven reaction (.022IU).

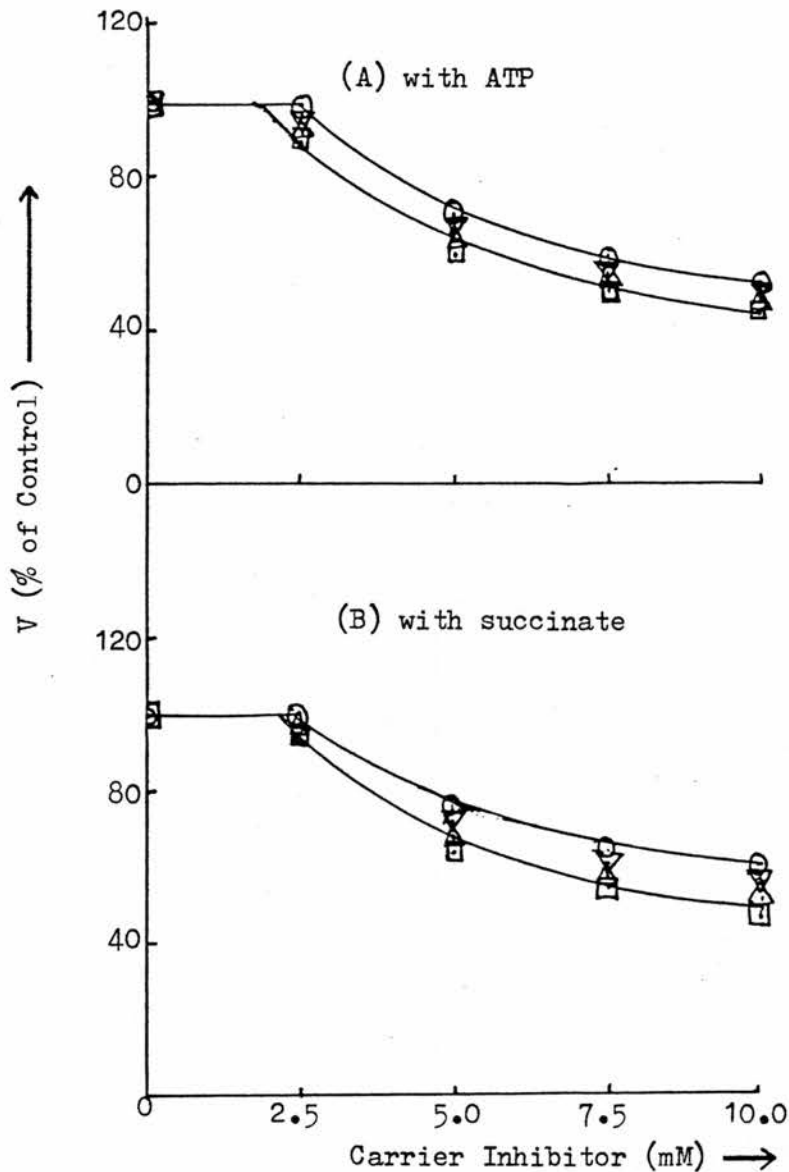


Fig 8: Effect of carrier inhibitors on energy driven TH activity

To the reaction mixture (440 μ l) containing NADP⁺ (100 μ M) NADH (250 μ M), ATP(A)succinate (25mM)(B) and the following carrier inhibitors (at specified concentrations) PP (\ominus); BM (∇); BT (\triangle); and PT (\square); preincubating at 30 $^{\circ}$ C in Tris-sucrose-HCl (or acetate) at pH8, a mixture of GR, ADH and SMP (.3mg) (6 days old) was added to start the reaction. After 5 minutes the reaction was terminated and GSH determined as described in methods, Section 4.4B (iii). Data plotted, ie V (% of control) against inhibitor concentration are means from two assays (\pm SD. \angle .04 OD Units at 412nm). The control (100%) activity for A (.04IU) and for B (.03IU).

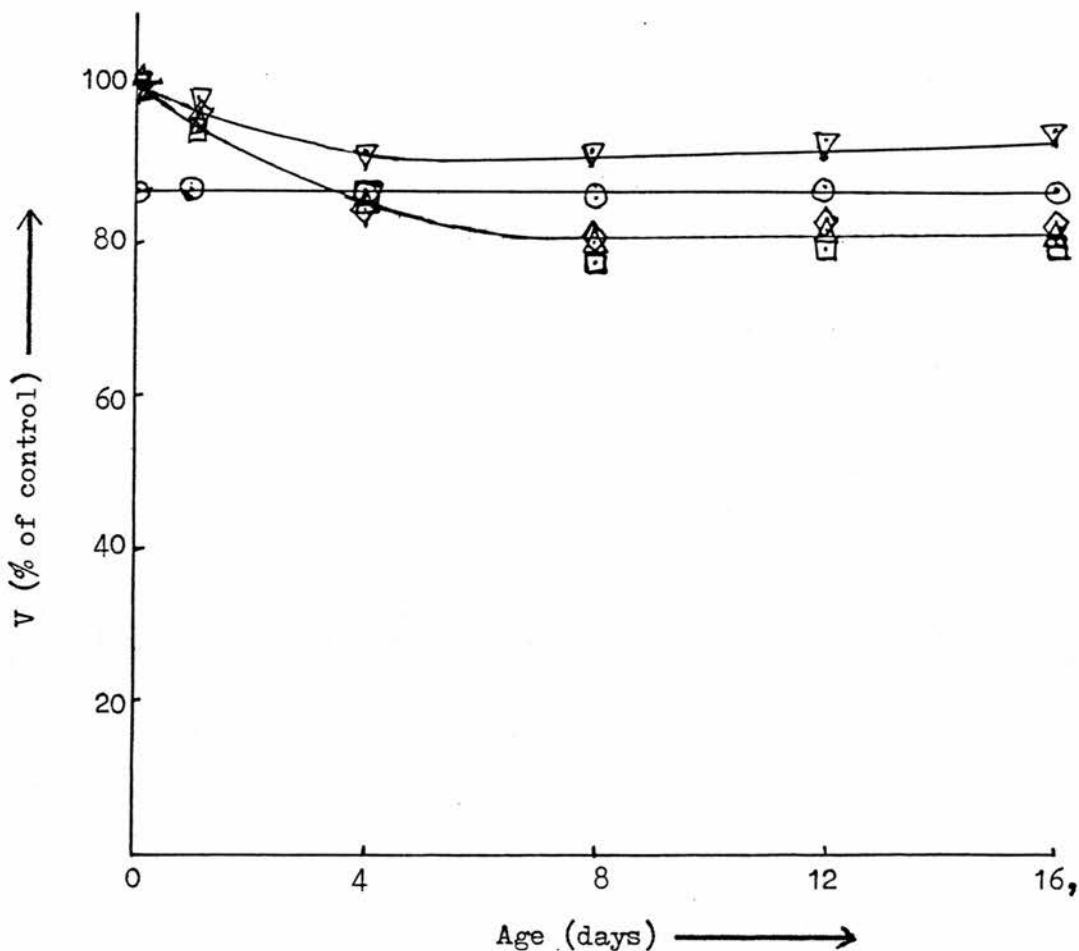


Fig 9: Effect of carrier inhibitors on ATP driven TH activity with aging

The method and conditions are as described for figure 8 with ATP (1mM) and carrier inhibitors (5mM) present in the reaction mixture. A mixture of GR, ADH and SMP (.23mg) was added to start the reaction. Unfrozen freshly prepared SMP were used at day (0) and once frozen SMP, at subsequent intervals.

After 2 minutes incubation, the reaction was terminated and GSH determined at 412nm. Data plotted are means from two determinations using same batches of SMP minus the zero time control \pm S.D. (.04 OD Units). The control activity was (100%) for each determination with a specific activity of .046IU on day (0) which declined accordingly (see figure 3).

In later investigations, as a result of discordant results, the effect of aging on the action of these inhibitors was investigated. On freshly prepared particles, there is minimal inhibition (about 20% at most) of the reaction at the highest carrier inhibitor concentration (10mM). However, after storage at -70°C , the inhibitions are more marked. This is true for both the succinate and ATP driven reactions. Further aging of particles had no significant effect on the inhibitions (figure 9).

Of the substances tested, only thiol reagents slightly inhibited (20%) the two regenerating enzymes (GR and ADH) used in the TH assay (see also the non-energy reaction, p 107). This was compensated by increasing GR and ADH two-fold.

2.3 Carboxylate carrier inhibitor kinetics

The inhibitions by all the carrier inhibitors in particular, di- and tri-carboxylate carrier inhibitors on the energy-driven reaction, was very interesting since the non-energy reaction was itself remarkably stimulated (Chapter 4).

A kinetic analysis of the inhibitions was performed on both the ATP and succinate driven reactions as with the non-energy reaction (p 140 to 142). The data obtained when the inhibitor concentration is varied, in the presence of different concentrations of one of the substrates with the other held constant, is shown for one inhibitor BT (in figure 11). The other inhibitors show the same pattern, the most striking common result being that there was no change in the value for K_m for either NADP^+ or NADH . The carrier inhibitors (as with the non-energy reaction) therefore, affected V_{max} and operate at sites which are not the binding sites for NADH and NADP^+ .

The results for each inhibitor are shown as Dixon plots in figure 12. From these graphs and other graphs not shown, the K_i for each inhibitor were obtained (Table 2) for either NADP^+ or NADH site.

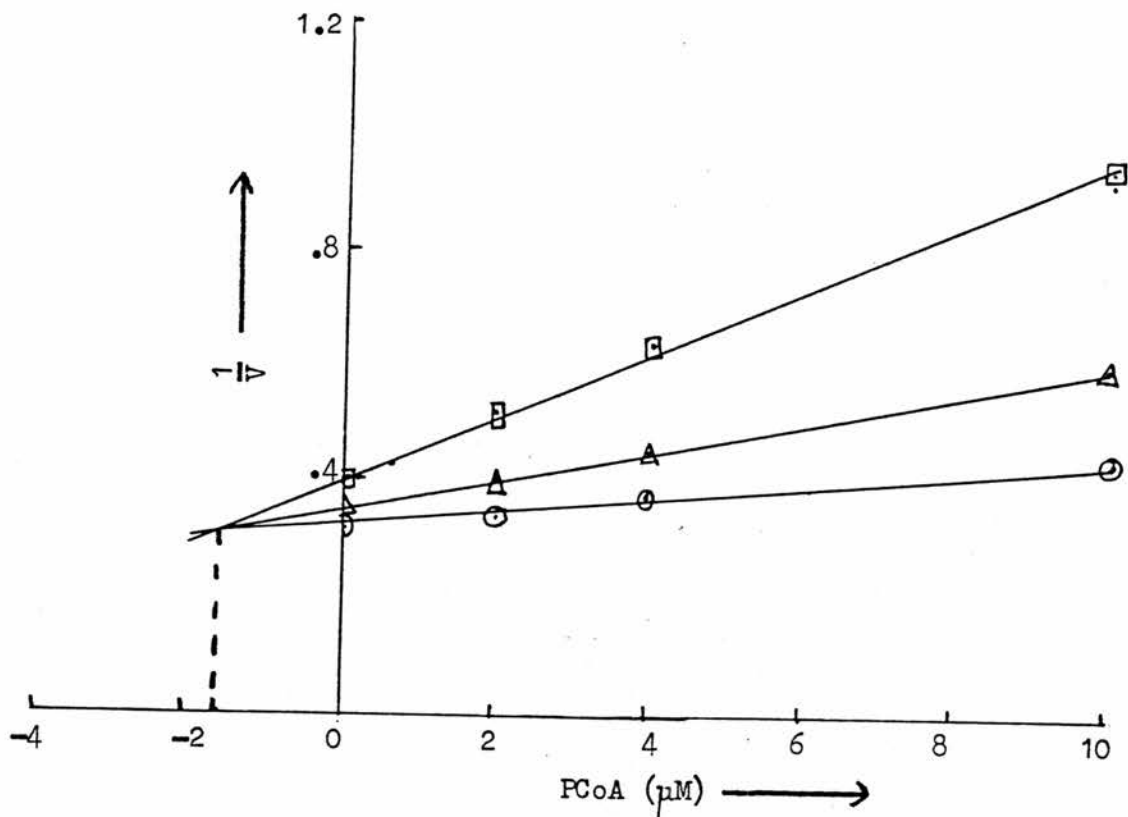


Fig 10: Effect of PCoA on ATP driven TH at different fixed NADP^+ concentrations

The ATP driven reaction was carried out as described in figure 9, in the presence of NADH ($250\mu\text{M}$), PCoA (varied as indicated above), NADP^+ ($10\mu\text{M}$) (□); $20\mu\text{M}$ (△) and $50\mu\text{M}$ (○). The reaction was started by adding a mixture of GR, ADH and SMP ($.23\text{mg}$). After 5 minutes incubation, GSH formed was determined as before (see figure 9). Mean values of 3 assays minus the no NADP^+ control are plotted (Dixon plot) ($\pm \text{SD}, \angle .03$) where V is NADPH_2 formed.

K_i for PCoA = $1.6 \pm .2\mu\text{M}$.

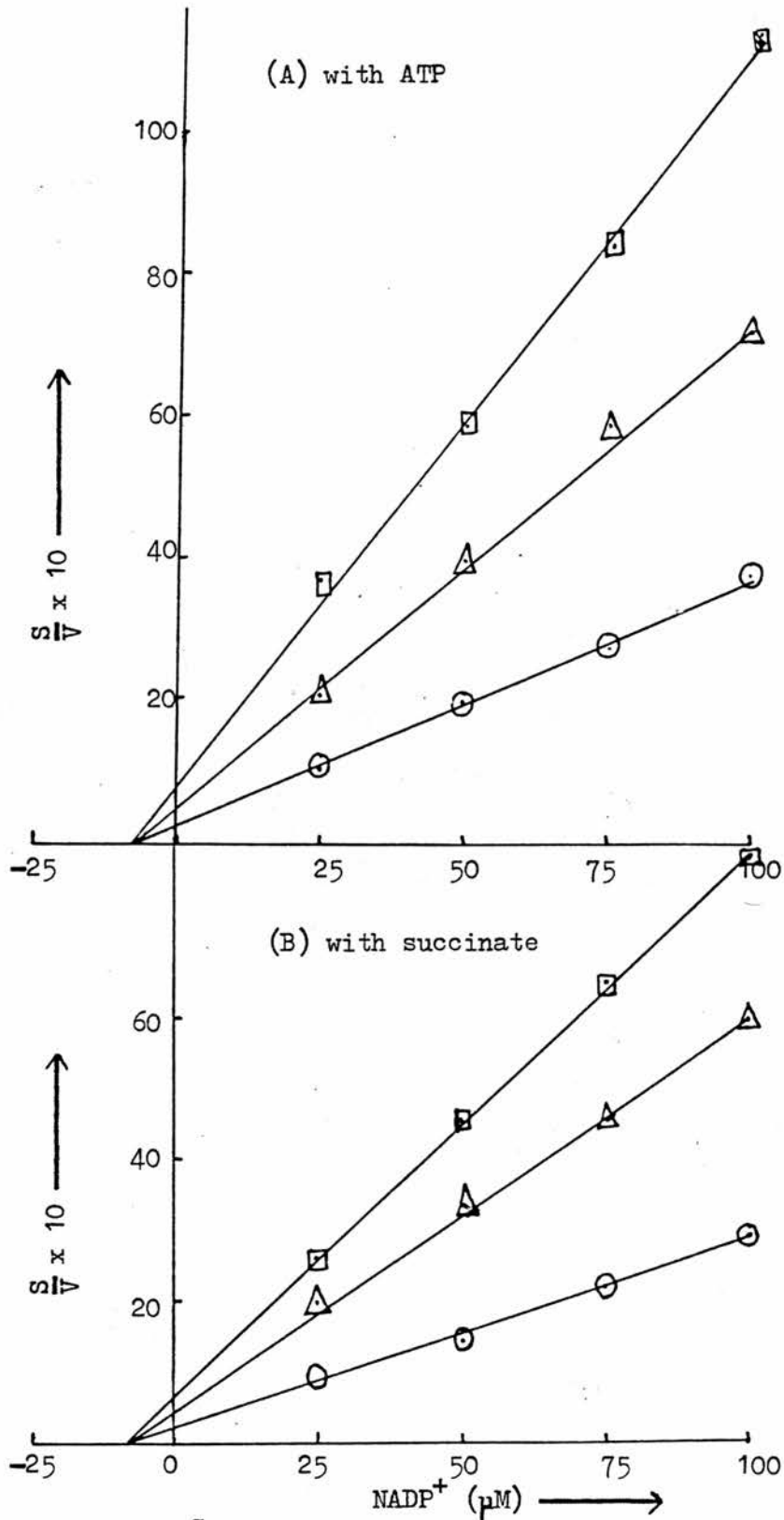


Fig 11: Variation of $\frac{S}{V}$ against substrate concentration of the ATP (A and C) or succinate (B and D) driven TH activity.

The reaction is as described for figure 10, at varied NADP⁺ concentration and NADH (250 μM) for (A and B), or varied amount of NADH and NADP⁺ (100 μM) for (C and D), ATP (1mM) was in A and C and succinate (25mM) in B and D. A mixture of GR, ADH and SMP (.19mg) started the reaction

(cont. p41)/

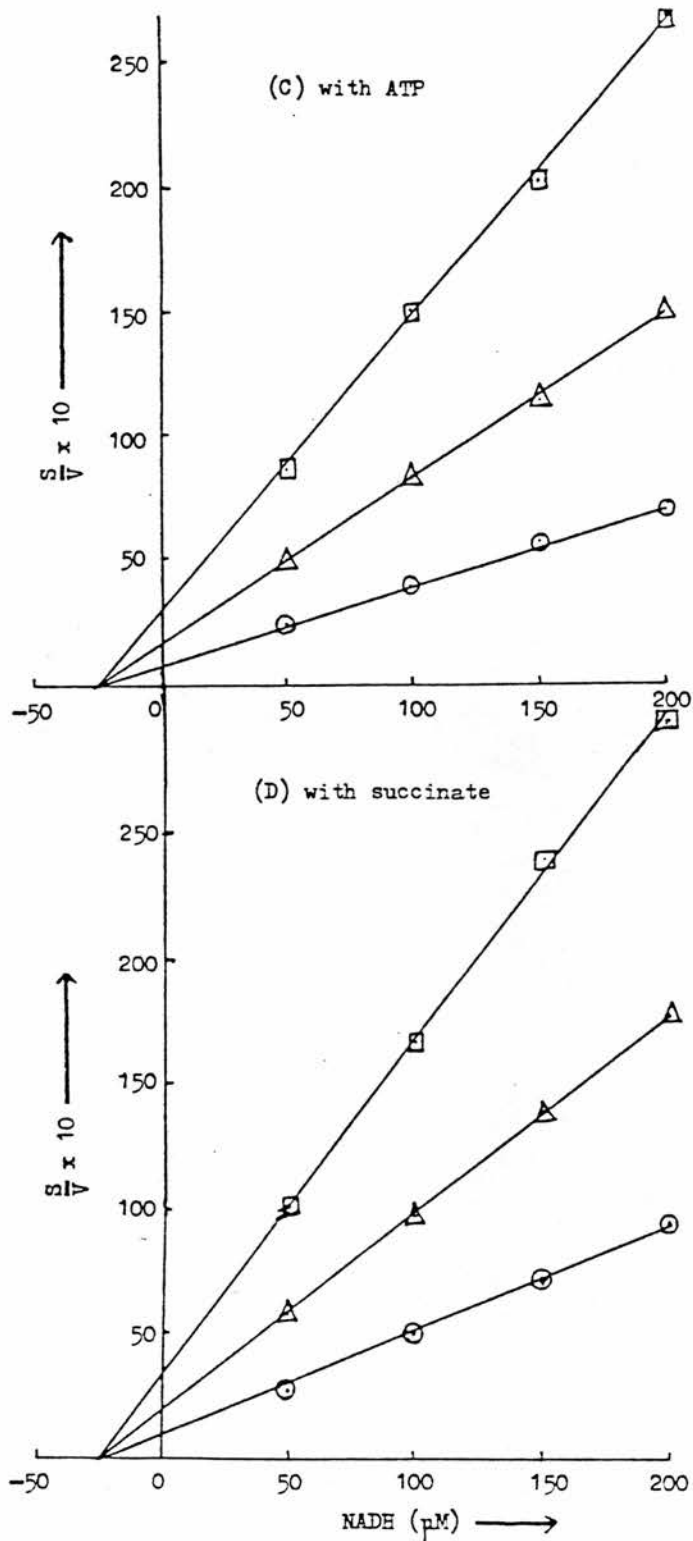


Fig 11

The reaction was incubated in the presence of the carrier inhibitor BT 0mM (○); 5mM (△); and 10mM (◻) for 5 minutes and then GSH determined as before (see figure 10). Data plotted are means from 4 assays minus the controls (\pm SD of 0.04 OD Units) where V is absorbance at 412nm.

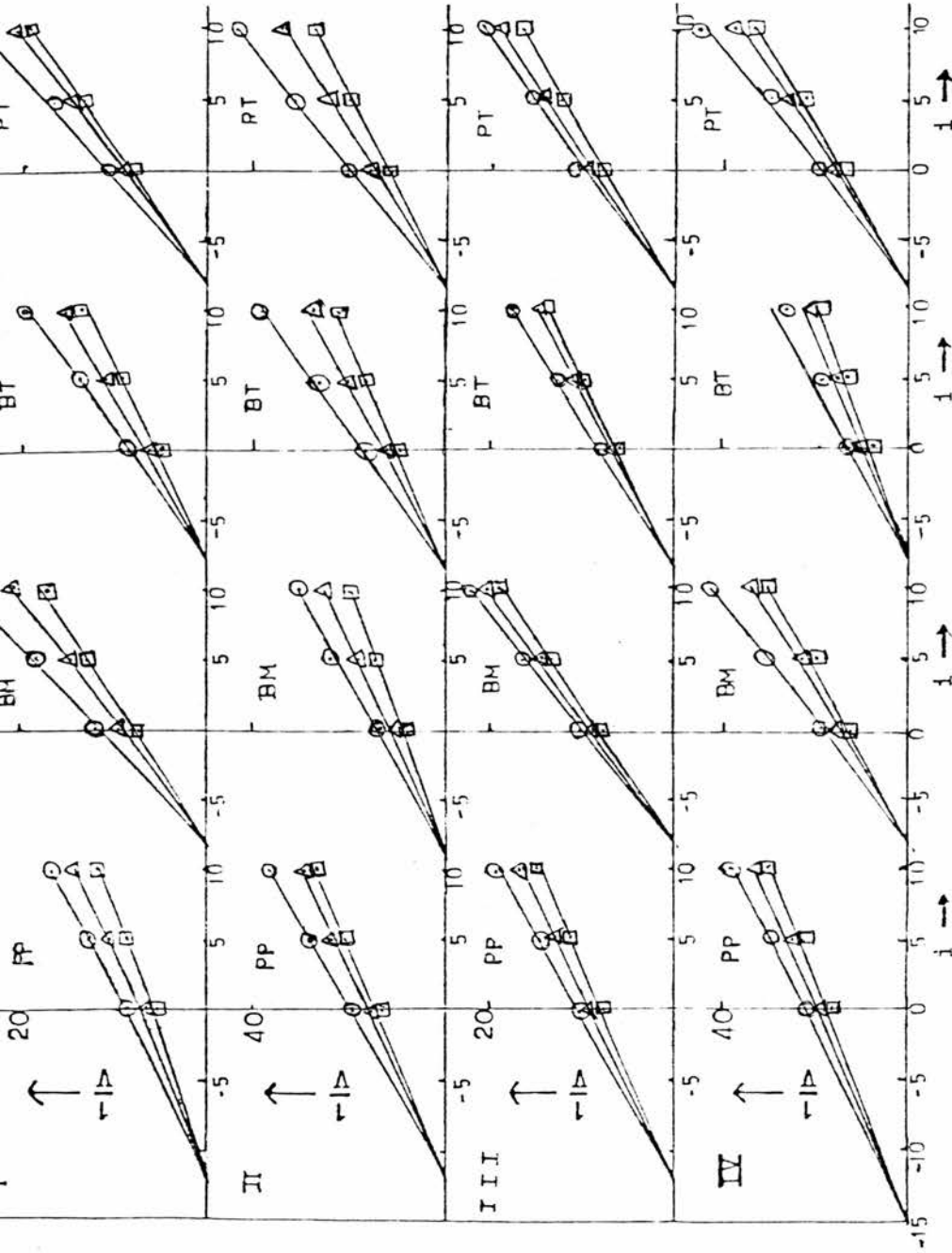


Fig. 12: Effect of carrier inhibitors on energy driven III activity at different substrate concentrations. The reactions for each of the carrier inhibitors tested varied as specified above on the ATP (1mM) I and II or succinate (25mM) III and IV driven reaction with either NADP⁺ varied and NADH (250μM) I and III or NADH varied and NADP⁺ (100μM) II and IV was as described for figure 11. To the reaction mixture (440μl) preincubating at 30°C at NADP⁺ or NADH concentration (μM) in that order: 12.5 or 25(⊖); 25 or 50 (⊕) and 50 or 100 (⊖), a mixture (60μl) of GR (.45IU); ADH (.45IU) and SMP (.3mg) 6 days old, was added to start the reaction. After 5 minutes GSH was determined (see figure 11). Data plotted (Dixon method) are means from at least 3 assays (± SD) 2.03 OD Units at 412nm taking into account the no NADP⁺ or no NADH control

where (V) is GSH absorbance at 412nm and (i) the carrier inhibitor concentration (mM).

Table 2: The K_i s of carrier inhibitors of the energy driven TH activity.

(A) $NADP^+$ VARIED

Inhibitor	Ki (with ATP) (mM)		Ki (with Succinate) (mM)	
	day 1	day 6	day 1	day 6
PP	13.5	12.5	13.7	14.2
BM	17.5	9.0	16.0	8.5
BT	17.5	8.4	14.0	8.5
PT	14.0	8.2	15.5	8.0

(B) $NADH$ VARIED

Inhibitor	Ki (with ATP) (mM)		Ki (with Succinate (mM)	
	day 1	day 6	day 1	day 6
PP	15.6	14.5	15.2	15.0
BM	17.9	8.0	18.3	8.8
BT	18.7	7.5	17.9	7.5
PT	15.6	7.5	17.5	7.5

The method and conditions for the experiments with carrier inhibitors have already been described in figure 12. The kinetic analysis was performed on 1 day and 6 days old particles. The experiments on day (0) (unfrozen SMP) are typical of day (1) shown above.

The same trend in the results was confirmed (not shown) by (a) GSH method (without ADH and ethanol regeneration system), (b) the fluorometric method, (see methods, Section 4.4B). The data presented are means from 3 assays (\pm SD. ± 1 mM).

The/

The results (Table 2) show that K_i for PP, a monocarboxylate carrier inhibitor does not significantly change with age of SMP and that it is the poorer inhibitor at day 6. This may be due to the difference in the mechanism of inhibition by this inhibitor. It should be borne in mind that again PP was the only inhibitor of the non-energy reaction (Chapter 4).

The other three inhibitors, dicarboxylate (BM) and tricarboxylates BT and PT decrease their K_i s with the aging of particles. This is consistent with the finding (figure 9) where the inhibition increases with aging up to a constant at about 6 days. All the inhibitors have relatively similar K_i s with PT and BT slightly lower than for BM.

All the inhibitors however, do not discriminate between the two reactions (ATP or succinate driven TH). They inhibit these reactions more or less to the same extent.

2.4 Effect of Carboxylic acids on energy driven TH

It has been shown that the carboxylic acids themselves affect the non-energy TH (p106) and therefore, their action on the energy driven TH was examined.

The results (figure 13) show that citrate and isocitrate both stimulate the reaction, especially the latter. The activity of citrate, is not due to its isomerisation to isocitrate by the aconitase present in the SMP, because fluorocitrate a powerful inhibitor of this enzyme does not prevent the stimulation. Nor was the stimulation by isocitrate due to the isocitrate dehydrogenase in these preparations, since controls showed that there was negligible contamination by this activity, and the little activity that was present was taken into account in the results.

Among other acids investigated, malate and 3-hydroxy-butyrates have little effect. Investigations with pyruvate were inconclusive since it rapidly depleted NADH in SMP (see also p107). I could not test the effect of succinate in these experiments since itself is an energy supplier to the TH reaction (one of the reactions being studied).

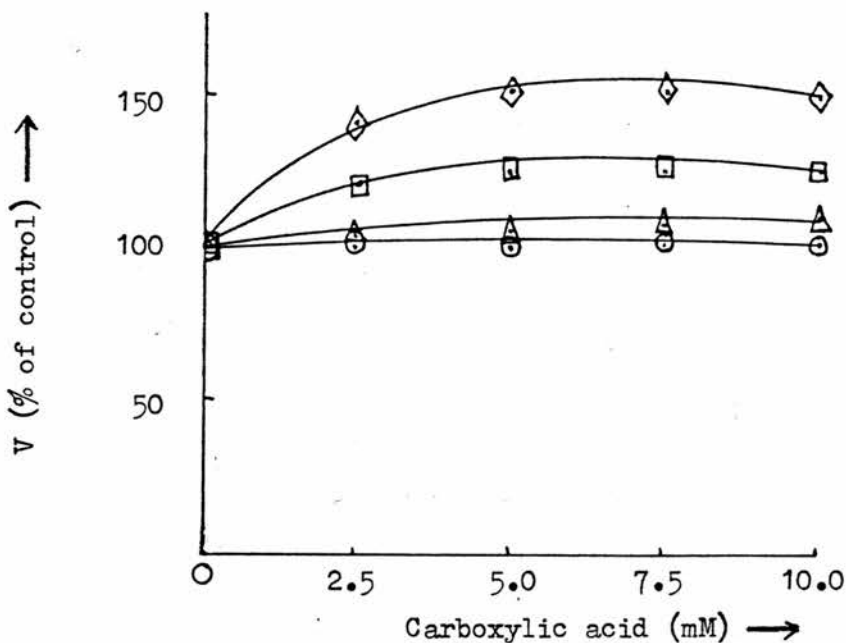


Fig 13: Effect of carboxylic acids on ATP driven TH activity.

To the reaction mixture consisting of ATP (1mM), NADP^+ (100 μM), NADH (250 μM) and the following carboxylic acids (5mM): 3-hydroxybutyrate (\ominus), Malate (\triangle), Citrate + FC (5 μM) (\square), Isocitrate + FC (5 μM) (\diamond); a mixture of GR, ADH and SMP (.17mg) was added to start the reaction as described in methods, Section 4.4B (iii). GSH formed was determined after 5 minutes incubation. Data presented are means from three assays taking into account the zero time control in the presence of the acid (\pm SD. \angle 5%).

The control activity (100%) was (0.031IU).

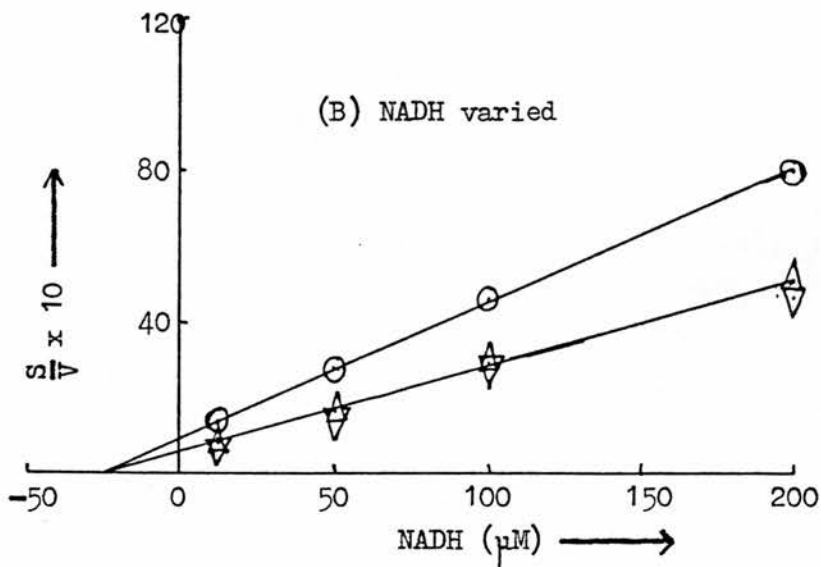
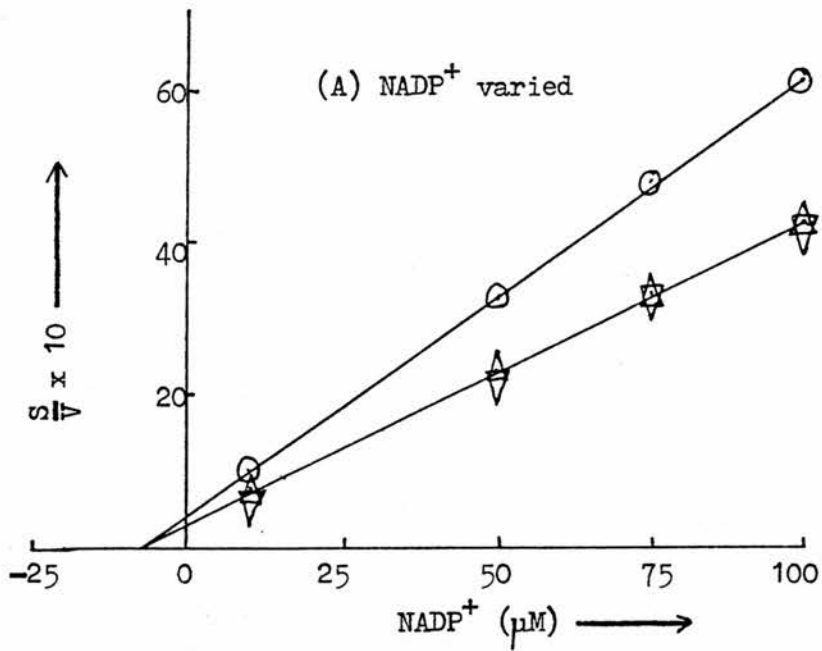


Fig 14: The effect of citrate on ATP driven TH reaction at different fixed substrate concentrations.

The reaction was as described for figure 13, ATP (1mM) with varied amount of NADP^+ and NADH (250 μM) in (A) and varied amount of NADH with NADP^+ (100 μM) in (B) and citrate 2.5mM (\triangle) and 5.0mM (∇) in the reaction mixture. A mixture of GR, ADH and SMP (.2mg) started the reaction. Data plotted as $\frac{S}{V}$ against substrate concentration (where V is absorbance at 412nm after 5 minutes incubation) are means of two assays minus the controls (\pm SD. of $\frac{1}{4}$ OD Units/5min).

Kinetic analysis of these data are shown in figure 14 for the tricarboxylic acid, citrate. The same pattern was obtained with isocitrate. With 3-hydroxybutyrate and malate the results are not shown, however, they both had no significant effect on the enzyme's activity when either substrate was varied.

Clearly these results do not support the idea that the effects observed on TH activity are due to the binding at translocase sites. Thus the stimulation observed with citrate and isocitrate contradicts with the inhibition found with the corresponding tricarboxylate carrier inhibitors (BT and PT).

It would seem that other or additional, effects are operating and this led to the examination of the other energy dependent parameters.

3 OTHER ENERGY DEPENDENT FACTORS

It is known that the energy dependent TH driven by ATP involves the enzyme F_1 -ATPase. During the reaction, 1ATP is hydrolysed to ADP and Pi for each transfer of the reducing equivalents from $NADH_2$ to $NADP^+$. The effect of carboxylate carrier inhibitors on this enzyme may account for their inhibition of the ATP-driven reaction. However, it cannot account for the inhibition of the succinate supported reaction. It was therefore, necessary to investigate each of the components involved, namely the ATPase and the respiratory chain.

3.1 The ATPase

The activity of enzyme complex (F_1 -ATPase) measuring ATP hydrolysis is high in SMP, with a fast initial velocity (linear for about 40 sec) (results not shown); followed by coupling ADP to NADH oxidation using the commercial enzymes pyruvate kinase and lactic dehydrogenase.

The/

The effect of carboxylate carrier inhibitors was determined on the rate of ATP hydrolysis. Figure 15 shows that indeed the carrier inhibitors inhibit this activity in a concentration dependent manner, 50% of the inhibition is achieved with the tricarboxylate carrier inhibitor (PT) at the highest concentration (10mM).

A kinetic study of the inhibition pattern by one of these inhibitors (PT) is presented in figure 16. It shows that there is a competitive type inhibition with respect to ATP. It is not easy to interpret this finding, but it is possible that in addition to binding to carrier proteins the tricarboxylate carrier inhibitor can also bind to sites on or near the ATPase enzyme (see discussion). This led me to investigate further the effect of this inhibitor on the free (from membrane) ATPase activity. This was done by repeating the same experiments on the oligomycin insensitive ATPase, which was 23% of total activity, and no inhibition was observed on this activity.

An attempt was made to determine the effects of these carrier inhibitors on the reverse ATPase activity that is on the ATP synthesis measuring NADP^+ reduction in the presence of hexokinase and glucose-6-phosphate dehydrogenase. No appreciable rates could be obtained with SMP preparations (results not shown), but there were indications that this process was also inhibited.

The inhibitors however, had no effects on the commercial enzymes used in the two assays measuring either ATP hydrolysis or synthesis, as determined by the conventional optical density measurements at 340nm following NADH oxidation for the former and NADP^+ reduction for the latter enzymes.

3.2 Succinate Oxidation

The succinate driven TH derives its energy from succinate oxidation (Danielson and Ernster 1963), through the respiratory chain coupled to proton transport. It was therefore, logical to investigate the effect of these carrier inhibitors on succinate oxidation in coupled SMP.

The/

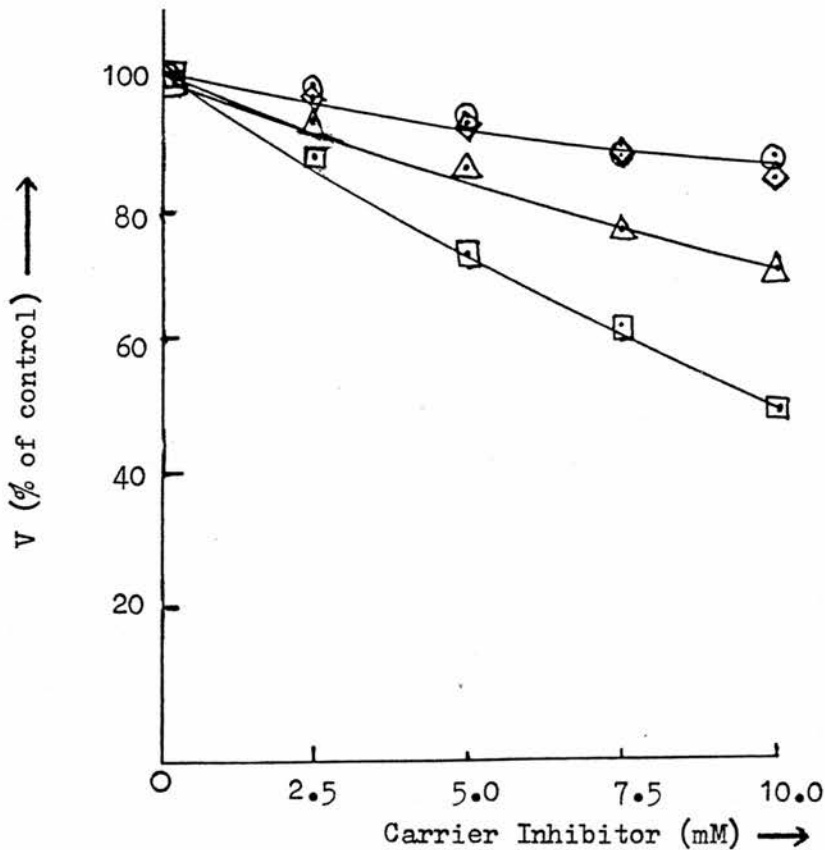


Fig 15: The effect of carboxylate carrier inhibitors on ATPase activity

To the reaction mixture (.9ml) consisting of NADH ($200\mu\text{M}$), PEP (2mM), ATP (10mM), inhibitor (5mM) PP (\ominus), BM (\triangle), BT (\diamond) and PT (\square) in Tris-sucrose-HCl buffer pH 8.0 containing MgCl_2 (1mM), a mixture (20 μl) of pyruvate Kinase (1IU) + Lactic dehydrogenase (1IU) was added immediately followed by 80 μl of SMP (.7mg) to start the reaction. The reaction was followed at 340nm. The initial velocity (V) was obtained from the linear part of the slope (see methods, Section 4.5). Data plotted are means from two assays (\pm SD. $\angle 6\%$).

The control (100%) activity was .039IU.

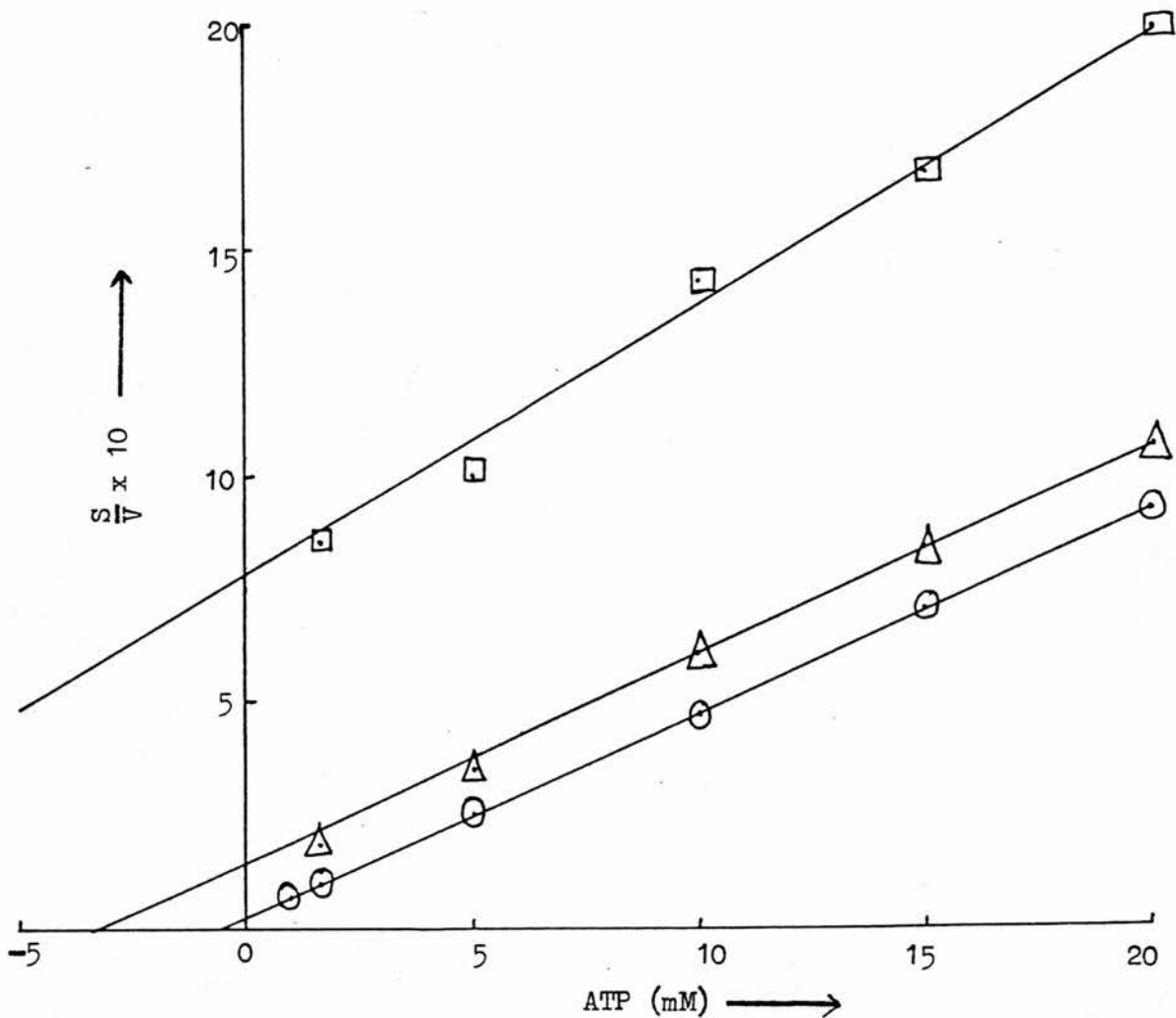


Fig 16: Effect of PT on the ATPase activity at different ATP concentrations.

The reaction was carried out as described in figure 15 with varying amounts of ATP and the carrier inhibitor PT: 0mM (\ominus) 5mM (\triangle) and 10mM (\square) in the reaction mixture. SMP (.6mg) started the reaction. Data plotted ($\frac{S}{V}$ versus S method, where V is absorbance change at 340nm per 30 sec., and S, ATP) are means from two assays (\pm S.D. of $\frac{S}{V}$ 0.030D Units/30sec.).

The K_m for ATP was $333.3 \pm 23\mu\text{M}$.

K_i for PT (Dixon plot) was $3.75 \pm .3\text{mM}$.

The investigation was carried out by measuring oxygen consumption with or without the inhibitors present. The results (Table 3) show no significant inhibition of succinate oxidation. However, the rate of succinate oxidation by SMP is low and the inhibitions are less than 10% and could not reliably be detected. Moreover, I found out coupled and uncoupled rates of succinate oxidation in SMP do not greatly differ (results not shown) as it is generally accepted. For this reason, the experiment was repeated using intact mitochondria and in this case too, there was little inhibition (see Table 3) of less than 15%.

These experiments were also repeated in the presence of ADP and Pi again this time, there was no substantial inhibition with mitochondria. The results with SMP were also inconclusive. The inhibition in this case was however, to be expected because of the already demonstrated inhibition of the ATPase.

Although I have failed to demonstrate in this way an inhibition of energy transduction with succinate, later results (see p 159) studying proton transport directly, do give a satisfactory explanation.

3.3 Effect of carrier inhibitors on membrane potential ($\Delta\psi$)

It is now well established that the membrane bound F_1 -ATPase is a proton pump. Succinate oxidation is similarly coupled to proton pumping and clearly the inhibitory effect of the carboxylic acid carrier inhibitors on either ATP or succinate driven transhydrogenase could be due to some general effect they might have on proton pumps in general. This concept has therefore, been examined by more direct studies on proton movement as measured by membrane potential and proton gradients. The experiments studying the effects of these inhibitors on the membrane potential created by either succinate oxidation or ATP hydrolysis will now be described.

The membrane potential ($\Delta\psi$) is the electrical component of the proton motive force (ΔP), which together with the proton gradient ΔpH , make up this proton motive force of a given system. Mitochondria/

Table 3: Effect of carrier inhibitors (10mM) on oxygen consumption due to succinate oxidation

Addition	Oxygen consumption (% of Control)			
	(A) SMP		(B) Mitochondria	
	No ADP + Pi	With ADP + Pi	No ADP + Pi	With ADP + Pi
Nil	100	100	100	100
PP	98	94	96	88
BM	96	95	88	85
BT	94	96	85	75
PT	93	86	90	78

To the reaction mixture (1.7ml) containing SMP (4mg) and mitochondria (4mg) in Tris-sucrose-HCl buffer pH 8.0 preincubating in an equilibrated (30°C) Clark type oxygen electrode chamber connected to the recorder, 100 μ l succinate (50mM) was added to start the reaction which was followed for a minute. ADP (5mM) and Pi (2.5mM) was then added and the reaction followed for a further two minutes in a final volume of 2.0ml.

The linear portions of the two slopes were taken as oxygen consumed. Data presented above are means from two assays taking into account oxygen consumption without the substrates and myokinase activity (\pm SD, \angle 10%).

Oxygen consumption (n atoms/min/mg) in controls (100%) were SMP (2.9) without and (7.2) with ADP and Pi and mitochondria (6.7) without and (43.4) with ADP and Pi assuming a 100% oxygen consumption of 445n atoms/ml at 30°C.

Mitochondria and submitochondrial particles (SMP) are capable of accumulating synthetic ionized compounds in response to the magnitude of $\Delta \psi$.

Mitochondria which is normally negatively charged (in the matrix) accumulate lipophilic cations whereas SMP which are mainly turned inside out and therefore, positively charged inside, accumulate the negatively charged anions. 8-anilino-1-naphthalene-sulphonic acid (ANS) and safranin O are commonly used cations used to measure $\Delta \psi$. This is made possible due to the spectral properties of these compounds.

ANS fluoresces in a hydrophobic environment, such as that found in membranes, in response to $\Delta \psi$, and similarly safranin O, a dye, absorbs light depending on the size of $\Delta \psi$. I used ANS in these experiments because it was readily available and most workers use it too.

ANS can be employed to study the $\Delta \psi$ in mitochondria too, except that mitochondria drives the dye outwards with concomitant decrease in fluorescence. The opposite is, of course, true for SMP (Azzi et al 1969).

ANS when excited in the region 360 to 390nm, emits light in a hydrophobic environment that can be measured in the range 470 to 520nm. I started these experiments by characterizing the technique. First, a standard curve of ANS fluorescence was determined with or without membranes (figure 17) and found to increase with increasing ANS up to a maximum value (40 μ M) followed by a slow decline. Without membranes, there was a low background fluorescence which was almost independent of ANS. This could have been due to small amounts of ethanol added with rotenone in the buffer. This was taken into account in later experiments.

ANS/

ANS fluorescence enhances proportionally with the increasing amount of membrane (figure 18). This is expected in view of the fact that it requires a hydrophobic environment (which increases with amount of membranes) to fluoresce. Levels of ANS and membrane protein were therefore, chosen to give low initial readings.

Under these conditions, addition of either ATP or succinate to SMP considerably enhance the ANS fluorescence. ~~(Eq. 10)~~ The evidence that this does indeed represent the membrane potential was obtained by repeating the measurements using mitochondria, and the opposite result, a fall in fluorescence was obtained (results not shown).

It was not possible to determine the time course of the energization process (figure 19A) because the first reading obtained 15 seconds after adding ATP or succinate did not change after a further 10 minutes. Thus equilibration is extremely rapid in agreement with the findings of other workers (Gains and Dawson 1976).

The effect of carrier inhibitors on this system in SMP was now studied (Table 4). As expected, FCCP greatly inhibits the fluorescence increase whether induced by ATP or succinate. The effect of the carboxylate carrier inhibitors is also clear cut, with the ATP induced fluorescence increase.

Three of the inhibitors (BM, BT and PT) also inhibit the increase (by about 80%) while the monocarboxylate carrier inhibitor (PP) has only small inhibition (about 15%) at the highest concentration (5mM) used.

With the succinate induced fluorescence increase however, there is surprisingly little effect (10 to 25% inhibition) by all the inhibitors at these concentration. It was my intention to compare the two processes, that is why I did not use the highest concentration (10mM) used in the transhydrogenase assays since I had achieved even more inhibition at much lower concentration (5mM) with the ATP system.

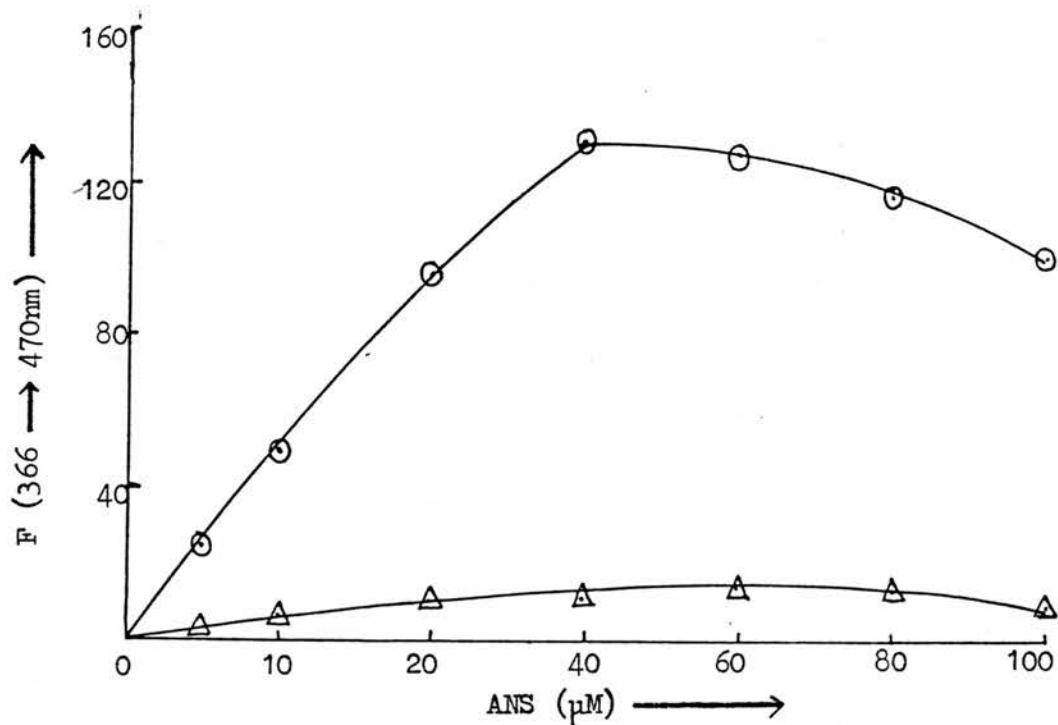


Fig 17: ANS Fluorescence with and without membranes

ANS fluorescence was determined at room temperature (19 to 22°C) in Tris-sucrose-acetate buffer pH8 containing NaHCO_3 (10mM), KCl (10mM), with SMP (.1mg) (\ominus) or without SMP (\triangle) and varied amounts of ANS were added (specified above) and the fluorescence read in a final volume of 3.0ml as described in methods (Section 5.1). Data presented are means of two assays (\pm SD, $\angle 5$ F units).

Fluorescence (F) units are arbitrary.

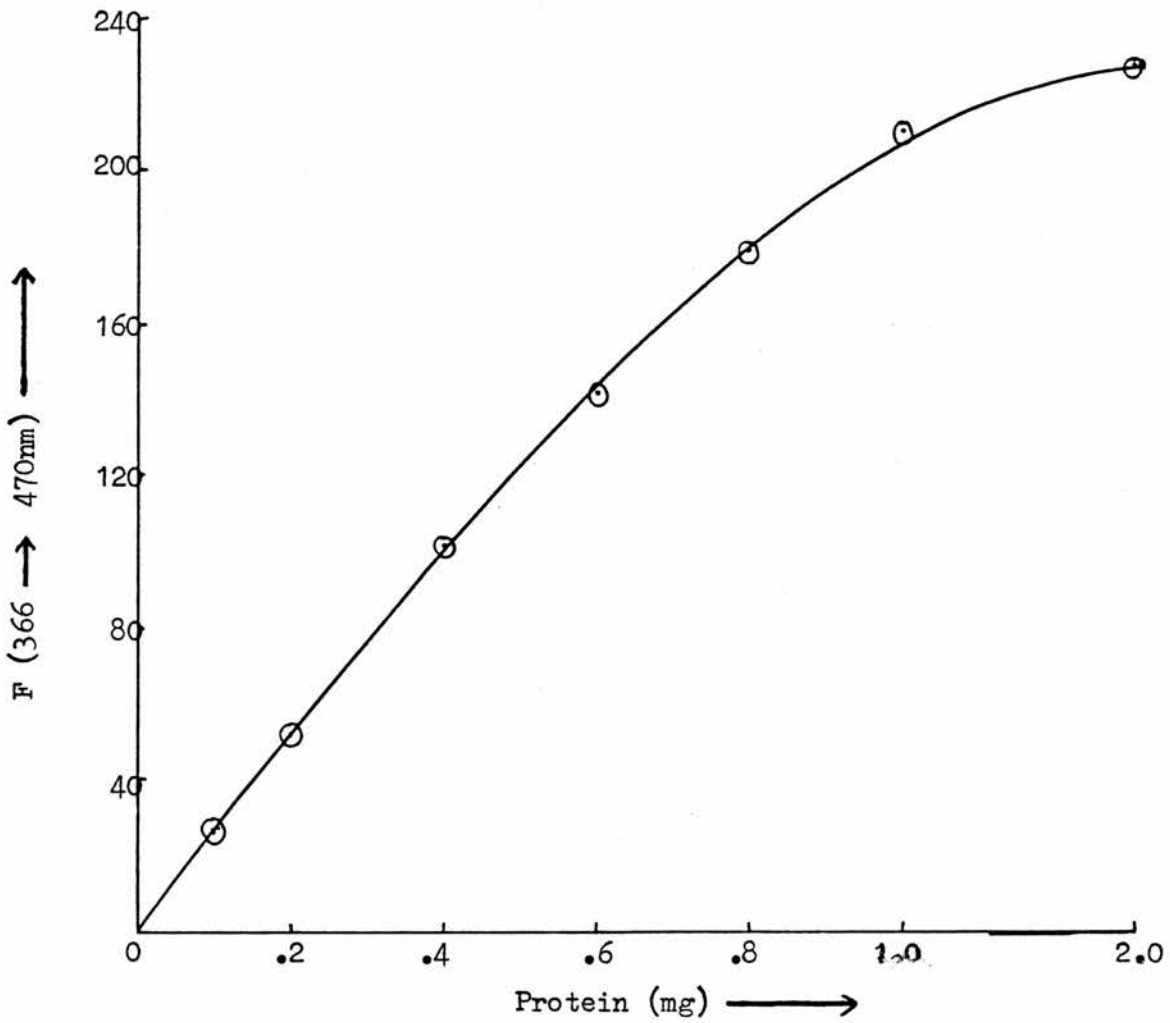


Fig 18: Variation of ANS fluorescence with amount of protein

To the buffer described in figure 17, containing ANS ($5\mu\text{M}$), SMP (at different protein concentrations specified above) were added, mixed and the fluorescence taken 15 seconds after.

Data plotted are means from two assays (\pm SD. $\angle 5$ F units).

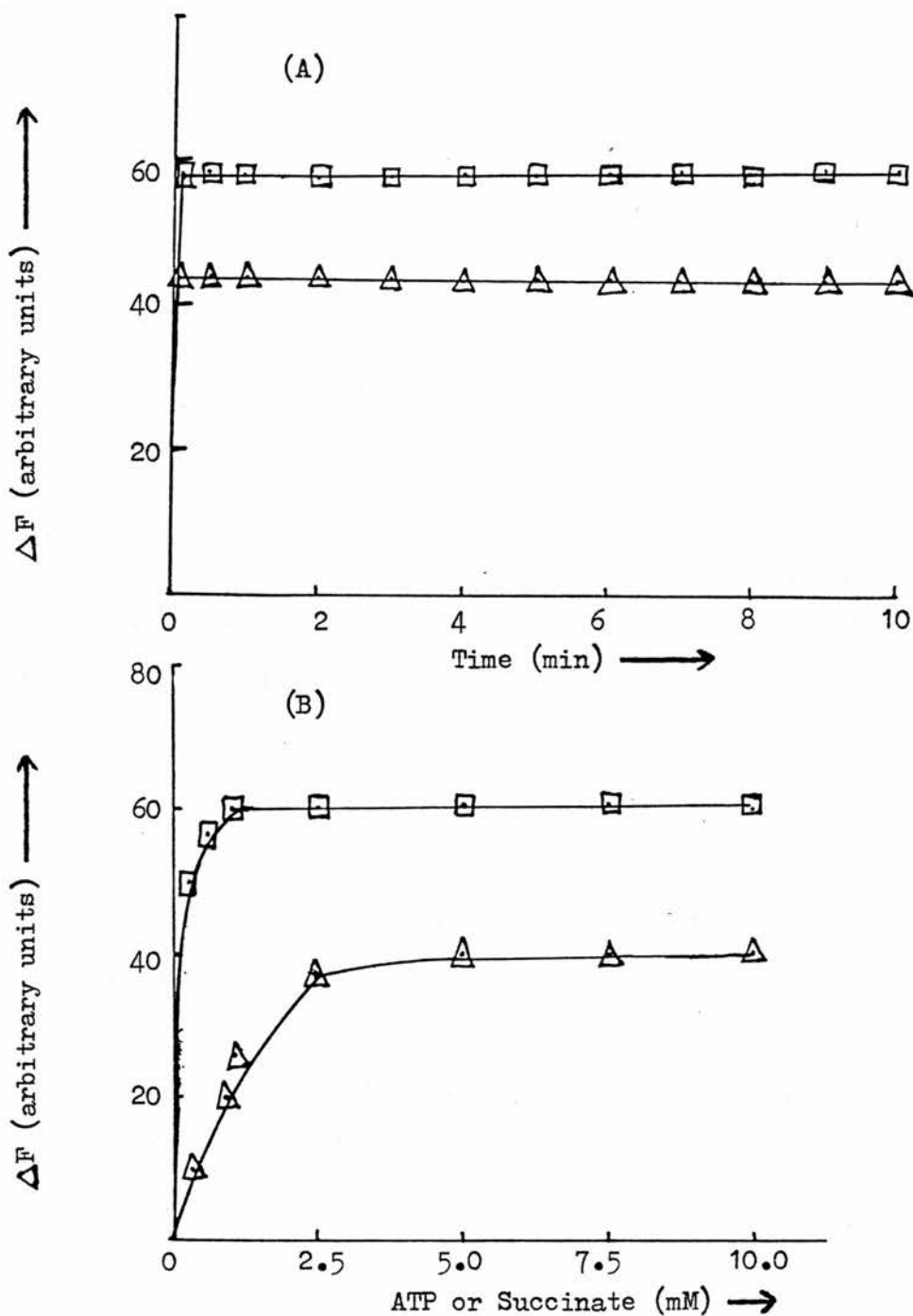


Fig 19: Time course; and effect of ATP or succinate concentrations on $\Delta\psi$.

- (A) The time course of energising SMP (.6mg) at room temperature (19 to 20°C) with ATP (1mM) (□) and succinate (10mM) (△) was determined as in figure 18. The last addition was the energy source. Data are from 2 assays (\pm SD \angle 4 F units) minus ANS fluorescence without an energy source.
- (B) The effect of varying ATP (□) or succinate (△) was determined on SMP (.6mg) as described above. Data are means from 3 assays \pm SD, \angle 5 F units.

Table 4: The effect of carrier inhibitors and FCCP on $\Delta \Psi$ induced by ATP or succinate

Addition	Concentration	ΔF (as % of Control)	
		A (with ATP)	B (With Succinate)
Nil	-	100	100
FCCP	3 (μM)	30	42
PP	2.5 (mM)	90	92
	5.0 "	85	75
BM	2.5 "	48	87
	5.0 "	20	80
BT	2.5 "	40	90
	5.0 "	20	86
PT	2.5 "	27	92
	5.0 "	7	88

The reaction was carried out in the presence of the inhibitors (specified above) at room temperature (19 to 22°C) with ATP (1mM) or succinate (10mM) as described in figure 19A. The change in fluorescence was determined 15 seconds after addition of the energy source (ATP or succinate) (see also methods, Section 5). Data presented are means from 3 assays with different batches of SMP (\pm SD. $\pm 8\%$).

The control (100%) ΔF with ATP was (59 F units) and with succinate (38 F units).

3.4 Effect of carrier inhibitors on pH gradient (ΔpH)

Because of the uncertainties in the interpretation of the ANS experiments, a different technique was also investigated, namely the indirect measurement of ΔpH .

Submitochondrial particles and mitochondria, are capable of accumulating ions in response to ΔpH ; and weak bases or weak acids distribute themselves respectively in these components, according to the magnitude of ΔpH . Direct measurements of this distribution requires the use of expensive radiolabelled substances (Nicholls 1977).

However, a fluorescent technique using quinacrine (QA, ie 9-4-diethylamino-1-methylbutyl amino-6-chloro-2-methoxyacridine) as a probe can also be used satisfactorily to measure qualitatively ΔpH in SMP (Lee 1971). Energization of particles results in QA fluorescence decrease which is potentiated by thiocyanate (SCN^-) ions. This was the method used in the experiments which are now to be described.

Quinacrine when excited in the region 405-436nm, emits fluorescent light around 500 to 520nm. Addition of SMP or mitochondrial membranes (figure 20) has no effect over this fluorescence. Addition of ATP or succinate has little effect on the fluorescence, but in the presence of thiocyanate ions, there is a decrease (Table 5).

The effect of the carrier inhibitors on this system is shown in Table 5. It is evident from this table that all the carrier inhibitors except PP (the monocarboxylate) considerably inhibit fluorescence quenching induced by either ATP or succinate, even greater than that obtained by the uncoupler (FCCP). As found with the measurements (Section 3.3), the monocarboxylate carrier inhibitor PP, gives little inhibition with either energization agent. The two methods thus shown yield concordant results agreeing particularly in showing that only PP, does not inhibit significantly the proton pump.

4.5/

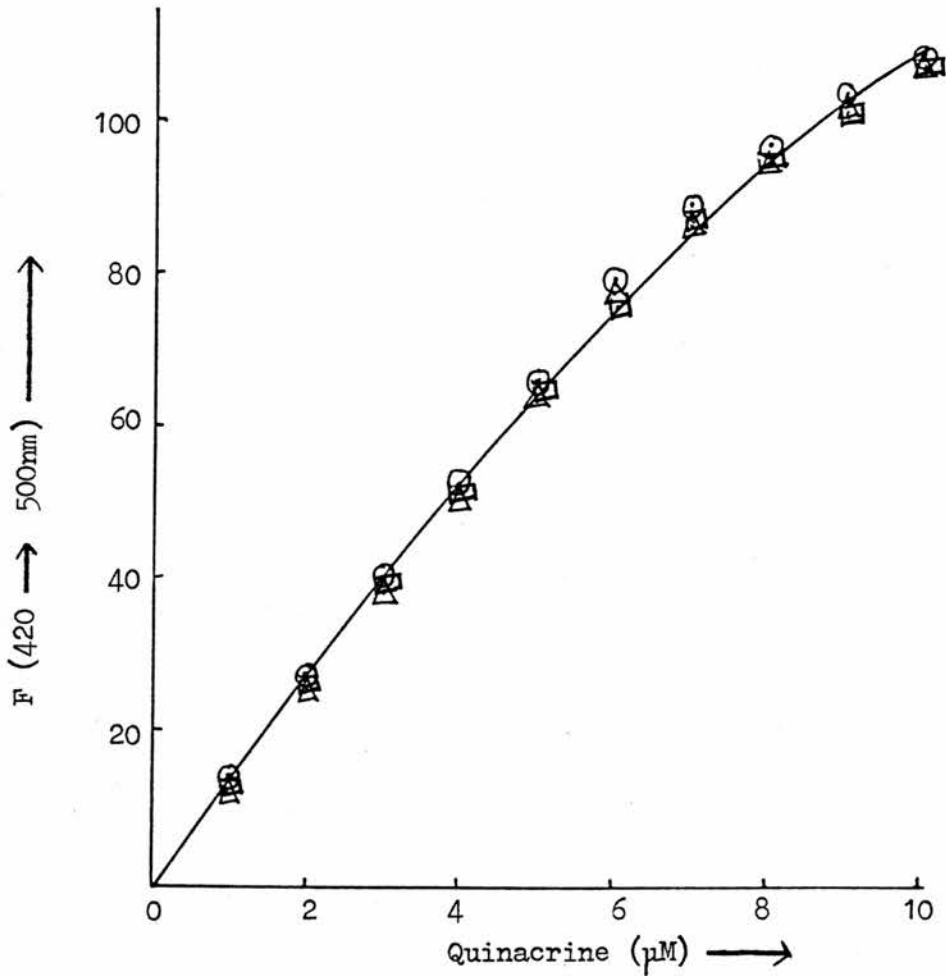


Fig 20: The standard curve of Quinacrine fluorescence with and without membranes

To the reaction mixture containing rotenone ($3.4\mu\text{M}$) KSCN (4mM) in Tris-sucrose-HCl buffer pH 8.0 and varied amounts of Quinacrine (as specified above) at room temperature (19 to 22°C) membranes nil(\ominus)(water instead), mitochondrial (1mg) (\boxminus) and SMP (1mg)(\triangle) were added to a final volume of 3.0ml . This was mixed and 15 sec. later the fluorescence read as specified in methods, Section 5.3. Data are means of two determinations (\pm SD, $\angle 4$ F arbitrary units).

Table 5: Effect of carrier inhibitors and FCCP on Δ pH induced by ATP or succinate

Addition	Concentration	QA Fluorescence Quenching (% of Control) (in arbitrary units, ie ΔF)	
		A (with ATP)	B (with succinate)
Nil	-	100	100
FCCP	3 μ M	25	50
PP	2.5 mM	85	93
	5.0 "	82	87
BM	2.5 "	54	44
	5.0 "	13	31
BT	2.5 "	41	56
	5.0 "	13	44
PT	2.5 "	22	18
	5.0 "	13	11

Quinacrine (3 μ M) fluorescence quenching was determined in the presence of the above specified substances, SMP (.8mg), KSCN (4mM) at room temperature (as for figure 20) upon addition of either ATP (1mM) or succinate (10mM). The fluorescence fall recorded (ΔF) is after the addition of an energy source.

Data presented are means from 2 assays (\pm SD. $\angle 4$ F units).

The controls (100%) with ATP was (19 F units) and with succinate 15 (F units).

3.5 Effect of Carrier inhibitors on TH proton pump

The finding that the carboxylate carrier inhibitors inhibit the proton pumps driven by either succinate or ATP leads one to consider the proton pump apparently associated with the trans-hydrogenase (TH) itself.

The enzyme is itself known to pump protons in the opposite direction from that of the two other systems above (Rydström 1974). Thus in SMP, TH pumps protons out while the ATPase or electron transfer-dependent pumps, pump them back in again. These facts have been recently confirmed using the purified enzyme incorporated into liposomes (Rydström 1979).

In the present system, the change in $\Delta\psi$ due to the TH reaction can be studied by means of ANS fluorescence. In this case, one is interested in changes when in the presence of an energy source and either NADH_2 or NADP^+ is added, followed by a decrease in fluorescence upon adding the second substrate (again either NADP^+ or NADH). A profile of ANS fluorescence changes as a result of such additions is shown in figure 21.

Addition of one of the TH substrates has no effect on $\Delta\psi$. However, upon the addition of the second substrate, there is a fall in ANS fluorescence indicating the fall in $\Delta\psi$. This fall is attributed to the TH reaction.

Unfortunately, the changes obtained as a result of this last addition were found to be rather low in two out of three such experiments. However, the addition of the carrier inhibitors did not make any difference to the values obtained.

Thus, there is no evidence that carrier inhibitors inhibit the proton pump of the TH enzyme. However, this is not decisive since it may be that the susceptible part of the channel system requires accessibility to the part of the enzyme located within the organelle. Since the inhibitors are probably unable to enter SMP, this possibility could not be tested.

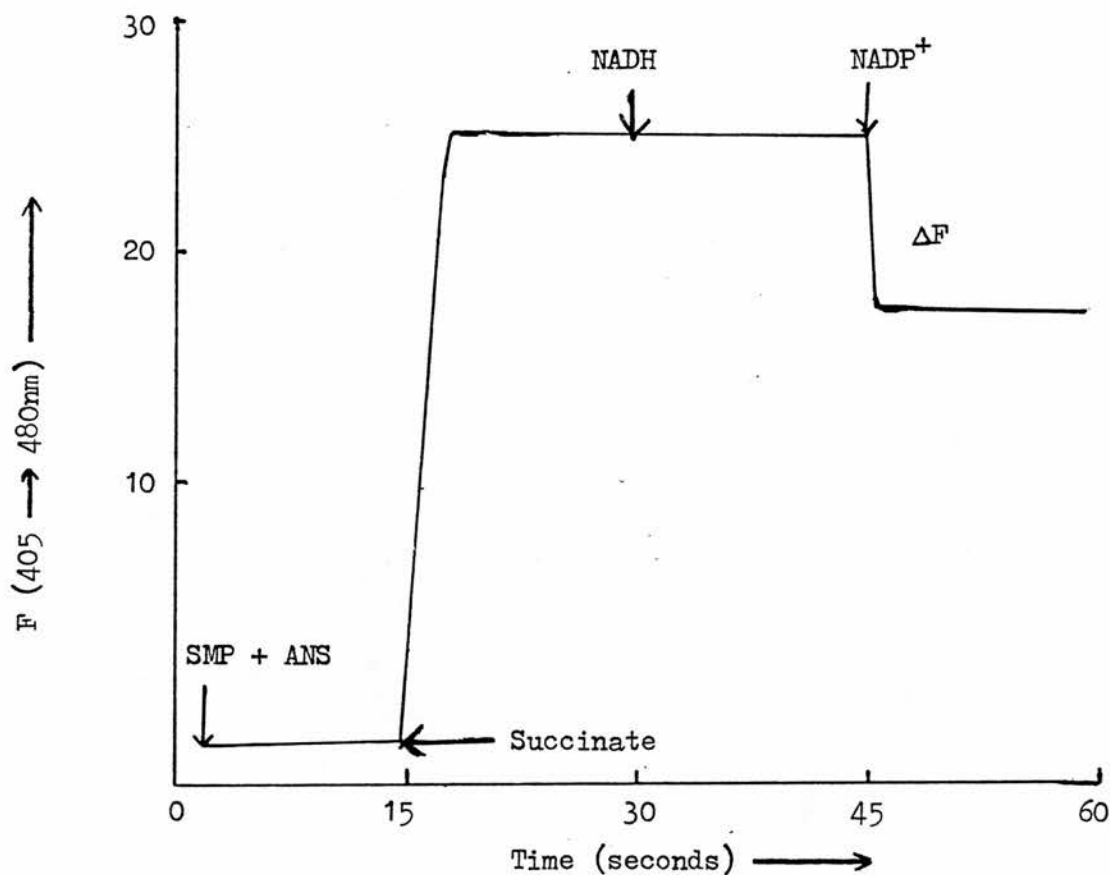


Fig 21: A profile of ANS fluorescence with TH reaction.

To the reaction mixture consisting of ANS ($5\mu\text{M}$), in Tris-sucrose-HCl buffer pH 8.0, SMP (1mg) preincubating at room temperature (19 to 20°C), succinate (10mM) was added, mixed and the fluorescence read as described in methods (Section 5.3). NADH ($200\mu\text{M}$) was added then 15 seconds later NADH ($20\mu\text{M}$) was also added and the fluorescence drop (ΔF) recorded in these experiments.

Table 6: Effect of carrier inhibitors and FCCP on the TH proton pump.

Addition	Concentration	F (ANS) (as % of control)
Nil	-	100
FCCP	3 μ M	96
PP	2.5 mM	100
	5.0 "	93
BM	2.5 "	100
	5.0 "	94
BT	2.5 "	100
	5.0 "	97
PT	2.5 "	100
	5.0 "	95

The reaction was carried out exactly as described in figure 21. The ΔF measurements were taken upon the addition of NADP^+ (20 μ M). Data shown are means from 3 assays (\pm SD. 45%)

The control (100%) was 8 ± 3 F (arbitrary) units.

Submitochondrial particles from rat liver, are capable of catalyzing the energy-linked transhydrogenase in addition to the non-energy linked reaction discussed in Chapter 4. The energy dependent reduction of NADP^+ by NADH , can be supported by either succinate oxidation or ATP hydrolysis (Danielson and Ernster 1963).

4.1 Energy-Driven TH Activity

This reaction like the non-energy reaction has been greatly studied in beef heart mitochondria. Addition of ATP or succinate to SMP increases the TH reaction rate several (5 to 12) fold (Rydström 1977). I confirmed these findings with SMP. The succinate driven reaction was often 4 to 6 fold and the ATP driven reaction 5 to 12 fold higher than the non-energy reaction. The variations could have been a result of the extent of damage during the preparation of SMP, of the respiratory chain components or the ATPase enzyme complex.

The problems of assaying the TH reaction are not unique to the non-energy reaction only. The energy driven reaction too, can not be assayed directly since the ultra-violet absorption spectra of NADPH_2 and NADH_2 are the same. Several methods previously described (Chapter 4) were at first used to study this system.

One of them, namely, fluorometry was later abandoned as a result of poor reproducibility. As with the non-energy reaction, the GSH method with the regeneration of NADP^+ from NADPH_2 by glutathione reductase and GSSG (GSH method, constant NADP^+) proved satisfactory provided the initial concentrations of the two substrates were both high. However, at low NADH concentrations, it was necessary to regenerate NADH_2 from the NAD produced using ethanol and alcohol dehydrogenase. This method (GSH method, constant NADP^+ and NADH), was superior since it could be used at any concentrations of NADP^+ or NADH .

As/

As has been reported for the non-energy reaction, the energy driven TH activity increases proportionally with increasing protein concentration. Since the activity of this reaction was much higher than the non-energy reaction less protein (5 times less) was routinely used in these assays.

The energy driven TH activity from rat liver is just as unstable as the non-energy activity. Thus the activity of the enzyme is much depleted upon storage at -70°C . About half the activity is lost in about 2 weeks as was the case with the non-energy reaction.

Since the ATPase is known to be quite stable upon storage under these conditions, this result suggests that it is the trans-hydrogenase enzyme protein which limits the rate.

Another indication of this is that, as with the non-energy reaction, the rate of the energy-linked reaction remains linear with time, longer in particles prepared in phosphate or Tris-HCl buffer than in those prepared in sucrose.

The energy driven reaction however, differs from the non-energy reaction in that it has an apparent high activation energy ($26-28\text{KJ mol}^{-1}$) which is independent of pH (between pH6 and 8). The non-energy reaction was shown (p78) to be pH dependent, rising from 10.5 KJ mol^{-1} at pH8 to 21KJ mol^{-1} at pH6.

It is not easy to explain the high activation energy of the energy dependent TH. One possibility is that the micro-environment around the enzyme is changed by energisation in a way very similar to that obtained by decreasing pH (since the non-energy reaction at pH6 has a similar value). One could envisage that various ionisable group(s) become charged (p116) either by local pH change induced by the proton pumping of the ATPase or by a bulk pH change. Evidence that there are such groups in the TH itself has been given (p116) (Galante et al 1980). The whole subject of activation energy is however, complex: thus it has been shown by (Blazyk et al 1981) that the value may itself be temperature dependent. Using/

Using beef heart particles and APAD⁺ as a substrate, he found a sharp increase in the value of activation energy at 20°C for the membrane bound but not for the solubilised enzyme which he attributed to bulk phase changes of the membrane lipids ('melting').

The most remarkable difference between the energy and the non-energy linked reactions has been the effect of pH on their rates. The non-energy linked reaction has a pH optimum in the acidic region (pH6) whereas the energy driven reaction above pH7. There is an additional difference between the two energy linked reactions, ATP driven TH has an optimum at pH8 whereas the succinate driven TH activity increases without an optimum up to pH9. This maybe due to the properties of the systems involved, ie the ATPase complex and the respiratory chain. For instance, the ATPase is known to have an optimum activity at pH around 8 (Bergmeyer 1965).

Thus Galante et al (1980) consider that the activation of the non-energy TH by lowering the pH mimics that of the energy reaction. They believe that the ionisation of a critical 2-phosphoryl group on the enzyme is a rate limiting step (see also p116).

Superimposed on this in the case of the energy driven TH is the response of the proton pumps activated by ATP hydrolysis or succinate oxidation.

The decline in TH activity with decreasing pH maybe related to its own property as a proton pump (Rydström 1979), ie the pumping of protons on the reverse direction to that of the ATPase or succinate oxidation. It is conceivable that in order to pump protons more easily, the external medium should have less proton concentration.

4.2 Energy-dependent TH Kinetics

The Km values for NADP⁺ and NADH of both the ATP and succinate driven reactions were obtained. Identical Km values for NADP⁺ (6.25µM) and NADH (25µM) were obtained for each of the energy linked reactions which were also identical to the values obtained in the non-energy reaction. The/

The K_m value for NADP^+ was also similar to that of the beef heart enzyme ($K_m = 6.5\mu\text{M}$) driven by succinate and reported by Rydstrom and coworkers (1971). The same group of workers however, reported a lower K_m value for NADH ($12.6\mu\text{M}$) than that which I found.

The identical K_m s which I found for either substrate for the non-energy and energy linked TH reactions are consistent with the finding that lowering pH of the non-energy reaction (to pH6), has no effect on the K_m s. This consolidates the suggestion that the mechanisms of activation of the TH enzyme by ATP, succinate or protons maybe closely related.

4.3 Effect of Inhibitors

A Miscellaneous Inhibitors

The most important inhibitors of either the ATP or succinate driven TH reaction are uncouplers. This is expected since uncouplers dissipate high energy equivalents required to drive the reaction. FCCP inhibited the reactions to the level of the non-energy reaction. Again in accordance with theory, I found that oligomycin inhibits the ATP driven reaction (to the non-energy reaction level), but not the succinate driven reaction.

The specific inhibitor palmitoyl coenzyme A inhibited competitively both the ATP and succinate driven reactions with an identical K_i ($1.6\mu\text{M}$) with respect to the NADP^+ site as was the case with the non-energy reaction.

Sulphydryl reagents (NEM and MB) were tested on the energy driven reaction, and found to inhibit more the ATP driven reaction than the succinate driven reaction. The inhibition of the beef heart TH by thiol reagents has also been reported by others (O'Neal et al 1980). It/

It appears that TH possesses SH groups which are not very susceptible to blockage in SMP (Earle et al 1978b), possibly because they are located within the particles (Sweetman et al 1974). Thus it is necessary to preincubate for effective inhibition. The greater susceptibility of the ATP driven reaction to thiol reagents is perhaps due to a similar vulnerability of the ATPase to SH blockage (as shown by Godinot et al 1980), the SH dependency of succinate dehydrogenase (Singer et al 1973) is much less sensitive.

Some laboratory reagents were also tested on the energy driven reaction. Most monovalent and divalent anions and cations do not affect this reaction, neither do metal chelating agents (EDTA or EGTA). However, Mg^{2+} showed small inhibition (10%) of the succinate driven reaction less than the inhibition found of the non-energy reaction (of 35%). The effect of Mg^{2+} was not studied with the ATP driven TH reaction since the ATP-Mg complex is the natural substrate for the enzyme (Santiago et al 1979). The ATP driven reaction too, is insensitive to the anions used.

B Carboxylate Carrier Inhibitors

The effect of carboxylate carrier inhibitors, PP, BM, BT and PT was of particular interest to this study. All the carrier inhibitors inhibited both the ATP and succinate driven reactions. I studied the inhibitor Kinetics and found that they followed Michaelis-Menten kinetics as shown by linear $\frac{S}{V}$ against S plots. None of the inhibitors however, altered the K_m for either substrate, as found with the non-energy reaction. Their effect was simply to alter V_{max} and thus they all inhibited non-competitively.

Considering/

Considering the complexity of the system, this clear cut result is surprising. However, even more remarkable was the finding that Dixon plots also gave linear profiles from which values for K_i could be calculated.

The K_i s for each inhibitor were obtained and the monocarboxylate (PP) was the weakest inhibitor. The tricarboxylates (PT and BT) appear to be stronger inhibitors than the dicarboxylate inhibitor (BM).

A closer look at these inhibitions revealed that on fresh SMP preparations, K_i s were higher than on older preparations, and after about six days from the day of preparation, the inhibitions were constant.

The effect of di- and tri-carboxylate carrier inhibitors is thus in contrast with the stimulations observed on the non-energy reaction. The simplest explanation for this difference is clearly that the inhibitors were exerting their effect on sites (or a site) different from those involved in the non-energy reaction. Presumably they were still binding in these latter sites, but also in addition, at sites required only for the energy linked TH reaction.

Of the Krebs acids investigated on the non-energy dependent TH reaction (p 121) monocarboxylate (3-hydroxybutyrate) and the dicarboxylate (malate) have no significant effect on the ATP driven reaction. The finding makes it less likely that the corresponding carrier inhibitors for these acids were exerting their effect by binding to the translocases.

However/

However, this cannot be excluded since the acids themselves can be transported whereas the inhibitors bind only to the exterior of the membrane. In addition, krebs acids bind to their translocases only in the presence of another substrate unlike the inhibitors (see p175). For these reasons, mixing carrier inhibitors with Krebs acids would not have made the interpretation of the results any easier.

The most interesting finding with the carboxylic acids was the stimulation of the energy dependent TH reaction by citrate and isocitrate. It will be recalled that these acids also stimulate the non-energy reaction and the finding that they also stimulate the energy linked reaction, suggests that they do indeed interact directly with the TH enzyme protein itself. Relevant in this connection is that, these stimulators gave linear $\frac{S}{V}$ against S plots.

The extent of maximal stimulation of the energy linked reaction by citrate was only about 30% compared to 200% for the non-energy reaction. However, it has been reported that the ATPase is itself inhibited by citrate (Santiago et al 1979). This maybe an explanation for the discrepancy.

4.4 Effect of carrier inhibitors on ATPase and Respiratory Chain

The obvious possibility for the inhibitions by the carrier inhibitors on the energy driven TH reaction was the inhibition of the systems associated with the energy transfer to the enzyme. One of these systems is the mitochondrial ATPase which links proton translocation with ATP hydrolysis (or synthesis).

The/

The effect of inhibitors on the ATPase activity directly was studied by measuring the rate of hydrolysis of added ATP in the absence of other substrates. The reaction was indeed inhibited and as with the inhibition of TH the stronger inhibitor was PT and the least effective PP.

A kinetic analysis of PT inhibition on ATPase activity was performed and found to inhibit competitively with respect to ATP. Santiago et al (1979) reported that citrate, a tricarboxylic acid whose carrier PT inhibits, also inhibits the rat liver mitochondrial ATPase activity competitively. These workers suggested the presence of a citrate binding site on the enzyme which coincides with that of ATP. This may be the same site PT binds, since it is structurally similar to citrate.

The ATPase consists of two parts, an ATP hydrolysis part and an associated proton channel. Thus, it can be inhibited by direct blockage of either function.

For this reason I went on to see if the ATP driven movement of protons was inhibited by the carriers. It is difficult to measure this movement and the resultant membrane potential directly by ion movement in SMP (because of their small internal volume), but indirect methods have come into use. Using either the ANS method (which measures $\Delta\psi$) or the quinacrine method (which measures ΔpH). I found that indeed the inhibitors did have the expected effect on proton movements. Of course, the primary inhibition could still be ATP hydrolysis itself. However, this could not explain why the inhibitors also inhibit when TH is driven by succinate.

I, therefore, turned to this aspect of the problem. Initial experiments measuring oxygen consumption in coupled SMP showed small inhibitions with or without ADP and Pi. The problem with SMP was mainly the smallness of the readings. I then went on to examine oxygen consumption in intact mitochondria. The inhibitors here too showed no significant effect on the uncoupled mitochondrial respiration, and small inhibitions on the coupled mitochondrial respiration rate, with or without ADP and Pi. Although/

Although the carrier inhibitors do not enhance oxygen consumption of mitochondria as uncouplers do. This possibility cannot be ruled out entirely since the inhibitors bind only outside the membrane.

The effect of the inhibitors on the succinate driven proton movement was investigated and as with the ATPase, there was inhibition as shown by the failure to develop ΔpH in particular and to a lesser extent $\Delta\psi$ in the presence of the inhibitors. Here again, the best inhibitor was PT and the least effective PP.

This result taken with the previous one suggests that these substances may be general inhibitors of proton pumps and this is how they inhibit energy driven TH activity.

Since according to most recent work, TH is itself a proton pump, the same technique was applied to the TH enzyme, namely comparing $\Delta\psi$ and ΔpH in the absence and in the presence of the two substrates together, ie NADP^+ and NADH . I could not find any significant effect of the carrier inhibitors on this system. There were problems of poor sensitivity of the method to detect what appears to be low levels of proton pumping in this system.

However, assuming that the inhibitors indeed have no effect, it is, of course, possible that the inhibitors cannot gain access to the appropriate site to enable them to block H^+ transport; since H^+ are pumped by TH in the opposite direction to that of the other proton pumps. The failure of the inhibitors to enter the SMP might be a sufficient explanation to account for their apparent inactivity.

5 GENERAL DISCUSSION AND CONCLUSION

5.1 GENERAL DISCUSSION

Hydroperoxides (H_2O_2 or Lipid hydroperoxides) are harmful metabolic products chiefly formed in the cell cytosol and mitochondria among other organelles as a result of oxygen metabolism. Living organisms have evolved mechanisms of removing these harmful metabolites.

The/

The glutathion redox system is the main pathway for detoxifying hydroperoxides in liver mitochondria via the matrix enzyme glutathione peroxidase (GP) (p6). GP, together with GSH, GR and TH form the main components of this (glutathione redox) system.

I investigated the capacity of intact mitochondria to reduce BuOOH. Mitochondria have indeed the capability to reduce BuOOH in the presence of succinate at an appreciable rate (of 25nmol/min/mg protein) in agreement with the report of Jocelyn and Dickson (1980).

However, mitochondria have small amounts of GSH (4nmol/mg) the reducing equivalents required for the reduction of BuOOH, in agreement with the findings of Jocelyn (1978). Direct evidence for the requirement of GSH in BuOOH reduction is described below.

The main interest to my investigations was to determine the sites or 'levels' at which the newly reported inhibitors of hydroperoxide reduction by liver mitochondria (Jocelyn and Dickson 1980) inhibit. These substances are carboxylate carrier inhibitors namely, phenylpyruvate (PP), an inhibitor of pyruvate transport (Parvin and Pande 1978), n-butyl malonate (BM) (Saint-Macary 1980) and phenylsuccinate (PhS) (Robinson et al 1967) and (Le Noue et al 1979) inhibitors of dicarboxylates malate and succinate; 1,2,3-benzenetricarboxylate (BT) and 1,2,3-propane Tricarboxylate (PT) the inhibitors of citrate transport (Robinson et al 1970) which specifically inhibit the uptake of these important carboxylic acid intermediates by mitochondria.

I was able to confirm the finding of Jocelyn and Dickson (1980) that all the carrier inhibitors do indeed inhibit BuOOH reduction by liver mitochondria. They inhibit by the same percentage (about 60%) as does the uncoupler (FCCP). I also showed the direct evidence that GSH is required in the reduction of BuOOH by the findings that the carrier inhibitors and FCCP also inhibit mitochondrial GSH concentration by about the same percentage (60-80%).

These carrier inhibitors have one property in common, that is, their ability to bind specifically and reversibly the carboxylate carriers known to translocate the above mentioned krebs acids across the inner mitochondrial membrane.

The/

The fact that the carrier inhibitors strongly inhibit BuOOH reduction in mitochondria imply the involvement of translocases in the mechanism of the hydroperoxide reduction. The possibility that it was the entry of BuOOH which is inhibited can not be ruled out entirely at present, but it seems a less likely explanation since the inhibitors are known to inhibit specifically only the carboxylic acid transport, and most important to this study, they also inhibited GSH concentration in mitochondria.

The effect of these inhibitors were then studied on individual enzymes involved in the mechanism of hydroperoxide reduction. In the process of the investigations, the properties of these enzymes were elucidated.

Starting with GP, I found that there was strong similarity between the cytosolic and mitochondrial enzyme in their affinities for the substrates (GSH and CuOOH) and insensitivity to the carrier inhibitors of partially pure preparations. The crude enzyme preparations however, were inhibited non-competitively with respect to the GSH site, to the same extent by the inhibitors. The tricarboxylate carrier inhibitors PT and BT were the best inhibitors and BM and PP, the weaker inhibitors. The inhibition of GP can be explained by the fact that GSH was being depleted too in Mitochondria by the inhibitors. One would expect an accumulation of steady state GSH concentration if the inhibitions were entirely on GP.

The obvious possibility was that the inhibitors were inhibiting GR the enzyme involved in GSH regeneration. Again the properties of the mitochondrial and cytosolic enzymes were obtained and found to be similar in their affinities for the substrates (NADPH₂ and GSSG). However, the enzymes were not inhibited by the carrier inhibitors.

Failure to inhibit either GP or GR directly meant that the third possibility, ie TH, was the most likely candidate. TH a mitochondrial membrane bound enzyme, catalyzes the hydride transfer between NAD(H) and NADP(H). The relevant reaction to this study is the production of reducing equivalent NADPH₂ in what is known here as the forward TH reaction. NADPH₂/

NADPH₂ production is of crucial importance since it is required by GR in the regeneration of GSH. The TH enzyme catalyzes two reactions involved in NADPH₂ formation, ie the non-energy and energy dependent reactions. The properties of these two reactions have been discussed in detail in the previous Section 4.

Of particular interest are the effects of the carrier inhibitors and the corresponding Krebs acids. The carrier inhibitors had an unexpected effect on the non-energy reaction. All but PP (the monocarboxylate) stimulated non-competitively the TH reaction by altering only the V_{max}, but not the K_ms. The way in which the inhibitors may stimulate is shown on p122, ie by combining with the carrier which they are the inhibitor and causing membrane conformation which upon transmission to the TH protein stimulates the enzyme. The demonstration that upon solubilising the TH from the membrane, these stimulations by the carrier inhibitors disappear, consolidated this theory. Another way of testing this theory further was to determine the effects of the structurally similar carboxylic acids, which are natural substrates for the carriers, on the TH activity. Here too the dicarboxylates (malate and succinate) and tricarboxylate (citrate and isocitrate) stimulate the enzyme activity. The stimulations of TH by these acids may be a general phenomenon since these carriers have broad substrate specificity (Lo Noue et al 1979). The results with the monocarboxylate pyruvate carrier which is relatively specific for pyruvate transport were indecisive due to high dehydrogenase activity which depleted the TH substrate (NADH). The lack of stimulation by 3-hydroxybutyrate, a monocarboxylate which crosses the membrane in a non-carrier mediated diffusion probably as a free acid (Halestrap 1978) suggests the involvement of the carrier proteins in modulating TH activity.

However, upon solubilizing the enzyme, the stimulations particularly by citrate and isocitrate were maintained, suggest a physiological role of these substances in regulating the TH directly, in addition to the indirect effects on the enzyme.

Coming/

Coming back to the effects of carrier inhibitors on the TH enzyme and in relation to the reduction of hydroperoxides by intact mitochondria. It is not easy to explain why no stimulations were obtained of the BuOOH reduction in the presence of FCCP (p47) since the di- and tri-carboxylate carrier inhibitors (at 5mM) stimulate the TH reaction (200 to 300%) (p87). The fact that no reversal of FCCP inhibition was observed could be explained by the fact that these inhibitors bind to the external^{surface} of the membranes (Robinson et al 1970 and 1971) and therefore, have limited influence on the TH enzyme whose catalytic site is known to be facing the matrix (p17).

The significance of the non-energy TH reaction above pH7 the physiological condition of mitochondrial matrix, appears to be relatively small to the overall GSH redox system. It has been shown that GP has a far higher capacity to reduce BuOOH (30nmol/min/mg) than the amounts of GSH available in mitochondria. However, the GR enzyme has adequate (98nmol GSH/min/mg) capability to provide the reducing equivalent GSH to GP. The non-energy TH reaction however, provides far less the required amounts of NADPH₂ (7nmol/mg), ie about 30% of the total requirement.

It was necessary therefore, to investigate the energy driven TH reaction. Indeed, it is the energy dependent TH reaction which provides, in excess, the required NADPH₂ to sustain the GSH redox system at full capacity. This reaction is much faster (5 to 12 fold) when driven by either succinate oxidation or ATP hydrolysis.

Most important was the finding that it is the energy driven TH reactions which is inhibited by both the uncoupler and the carrier inhibitors. The inhibition by carrier inhibitors was (50%) at most and by the uncoupler (about 80%) for both the reactions. The carrier inhibitors were used at the concentrations normally accepted and used in mitochondria to study the inhibitions of carboxylic acids' transport.

The/

The non competitive inhibition of the two energy (ATP or succinate) driven reactions with similar K_{is} suggests that the inhibitors were affecting the energy transfer part of this system. This was indeed the case since the inhibitors clearly inhibited ATPase activity (ATP hydrolysis) and the membrane potential ($\Delta\psi$) as well as proton gradient (ΔpH) generated by both ATP hydrolysis and succinate oxidation.

It appears, however, that it is mainly the interaction of inhibitors with carrier proteins which affect the TH activity in particular, as a result of interference with the associated ATPase and respiratory chain systems by blocking somehow the formation of the required driving force (ΔpH) for the energy driven reaction. Similarly, the activation of the non-energy TH reaction is a result of such interaction, but this time in favour of a conformation that promotes the reaction. The non-competitive kinetics obtained throughout this study support this form of interaction.

5.2 CONCLUSION

The glutathione redox system comprising principally of GP, GR, GSH and TH has been studied in rat liver mitochondria. Intact mitochondria are capable of reducing BuOOH supported by high energy intermediates as shown by the inhibition of the succinate supported reduction by the uncoupler (FCCP). The reduction is also dependent on the continuous supply of GSH as demonstrated by the finding that substances which diminish mitochondrial GSH also inhibited the reduction rate.

The enzymes GP, directly involved in hydroperoxide removal, and GR, responsible for GSH regeneration are sufficiently active to account for the observed hydroperoxide reduction in mitochondria. The properties of these enzymes both from the mitochondria and cytosol were investigated, and found to be remarkably similar in the affinities (K_m s) for their substrates. However, the cytosolic enzymes were much more active (2 to 3 fold) than the mitochondrial.

Of/

Of particular interest was the investigation of newly reported inhibitors of mitochondrial hydroperoxide reduction. These are the mono-, di- and tri-carboxylate carrier inhibitors. By a process of elimination, I have shown that these inhibitors do not inhibit the two enzymes GP and GR.

However, the carrier inhibitors have contrasting effects on the two reactions (non-energy and energy) by TH. The non-energy reaction is stimulated at pH8, while the energy driven reaction is inhibited under the same conditions by inhibiting the energy transfer processes. However, what emerged from this study is the great significance of the energy-driven TH to the GSH redox system. It provides 2 to 3 times the required reducing equivalents (NADPH_2) for hydroperoxide reduction.

The effect of these carrier inhibitors on TH have important implications on its mechanism. It is suggested that the enzyme (TH) interacts with membrane proteins (eg, translocases) by responding to the conformational changes resulting from the binding of such proteins. The enzyme is very sensitive to its environment as depicted in a number of properties in particular, the pH dependency of the two reactions. By identifying the nature of the enzyme's micro-environment, it is hoped that more information on the mechanism can be obtained.

What was of particular importance is the fact that the enzyme has remarkably identical affinities for the two substrates NADH and NADP^+ for the three reactions studied (non-energy, ATP or succinate driven) irrespective of the pH of the medium. In this case, it may not be the substrate binding which is thus affected, but the mechanism of catalysis itself.

Results with natural substrates for the carrier proteins were, however partially confirmatory of this trend. But, as shown with the solubilised enzyme, these substances have additional effects on the TH activity, suggesting a regulatory role of the enzyme, particularly by the tricarboxylates, citrate and isocitrate.

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