XYLITOL METABOLISM AND OXALATE SYNTHESIS IN THE RAT

A Thesis submitted by

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STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief, this thesis contains no material that has been previously published or written by another person, except where due reference is made in the text.

ALLAN. M. ROFE.

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SUMMARY

The intravenous administration of xylitol to patients in the Royal Adelaide Hospital resulted in several adverse metabolic effects, including the increased production of lactic and uric acids, and the deposition of oxalate crystals in the kidneys and brains. The unexplained nature of this latter effect has culminated in this investigation, which has been conducted at the cellular level using the rat as an experimental model. This thesis contains three major areas of investigation:

1. The comparative metabolism of xylitol and sorbitol in isolated rat hepatocytes.

The hepatic metabolism of xylitol was compared with other carbohydrates, including sorbitol and fructose. In many respects the utilisation of xylitol was similar to that of sorbitol, with the initial dehydrogenation step being a major determinant in the degree of metabolism of both polyols. The increased NADH levels accompanying this step resulted in a shift in the cellular redox couples to a reduced state, leading to increased lactate and glycerophosphate production. This latter phenomenon was not observed in comparative studies with D-fructose and D-xylulose. The increased production of glycerophosphate during xylitol and sorbitol metabolism appeared to place greater emphasis on the glycerophosphate hydrogen shuttle, particularly with xylitol as substrate. A marked stimulation of polyol metabolism was observed in the presence of the artificial electron acceptor, phenazine methosulphate. Facilitation of hydrogen flux through the glycerophosphate shuttle was one postulated effect of this compound. Inhibition of the malate aspartate shuttle with the transaminase inhibitor, amino-oxyacetate indicated that this route of hydrogen transfer into the mitochrondria is also significant in polyol metabolism, with more hydrogen from sorbitol than xylitol being transferred by this mechanism. Although the differences in the metabolism of xylitol and sorbitol were not great, it can be suggested that as xylitol causes a greater accumulation of glycerophosphate than sorbitol, then the binding of intracellular phosphate, the decrease in adenine nucleotide levels, and the associated increase in uric acid production may be more pronounced during xylitol metabolism.

2. The mechanism of oxalate synthesis from the immediate precursors glycollate and glyoxylate.

Hepatocytes were found to effectively produce oxalate from the major precursors, glycollate and glyoxylate. Inhibition studies revealed that glycollate oxidase rather than lactate dehydrogenase was the most significant enzyme in hepatic oxalate synthesis. An oxidised cellular redox state was found to promote oxalate production from both precursors. The accepted oxidative

sequence of glycollate ⟶ glyoxylate ⟶ oxalate was not supported by studies with hydroxypyruvate and the transaminase inhibitor, amino-oxyacetate, for glycollate produced more oxalate than glyoxylate. Subcellular investigations provided further evidence that the peroxisomal enzyme, glycollate oxidase, is the major enzyme of hepatic oxalate synthesis. However, due to differences between physiological systems and purified enzymes, it is not possible to exclude the participation of lactate dehydrogenase in oxalate synthesis. A compartmentalised model involving both these enzymes in hepatic oxalate synthesis is proposed with peroxisomes as the major sight of hepatic oxalate synthesis. Experiments with clofibrate, a drug which causes peroxisomal proliferation, showed variable effects *in vivo* and *in vitro*, the most interesting being a reduction in oxalate synthesis from glycollate *in vitro*. This potentially useful experimental approach awaits further investigation.

3. Oxalate production from xylitol and other carbohydrates.

Labelled xylitol was shown to produce oxalate *in vivo* and *in vitro*, an effect which was enhanced by pyridoxine deficiency. In rat hepatocytes, a xylitol concentration of 1 mM was found to be optimal for oxalate synthesis. As seen with the major oxalate precursors, an oxidised cellular redox state facilitated oxalate synthesis from xylitol. This effect was demonstrated with the artificial electron acceptor phenazine methosulphate, which has the dual effect of enhancing xylitol metabolism, and increasing carbon flux through the oxalate biosynthetic pathway. Isotope dilution studies and *in vivo* labelling experiments have identified glycollate as a key intermediate in the pathway from xylitol to oxalate.

A major finding in these investigations was that at a concentration of 1 mM, in hepatocytes; xylitol produced more oxalate than any of the other carbohydrates examined. This finding has increased significance when viewed in light of the similarity in the metabolism of xylitol and sorbitol. Evidence for three mechanisms of oxalate synthesis from xylitol are discussed. The first involves the production of an oxalate precursor from a glycolaldehyde - thiamine pyrophosphate intermediate in the pentose phosphate pathway; the second postulates the action of aldolase on a fortuitously formed xylulose-1-phosphate and the third mechanism concerns reactions of the glucuronic acid cycle which are involved in oxalate synthesis from ascorbate. The first hypothesis remains the most tenable, for the second is untested and the third is not supported by data from isotope labelling experiments.

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NON STANDARD ABBREVIATIONS

AOA	Amino-oxyacetate
PCA	Perchloric acid
PMS	Phenazine methosulphate
DCIP	2:6 Dichlorophenol indophenol

CHAPTER ONE

GENERAL INTRODUCTION

INTRODUCTION

The intravenous administration of carbohydrates, lipids and amino acids, either separately or in mixtures, has become important in the management of acute medical and surgical illness. While glucose is the most commonly infused carbohydrate; fructose, sorbitol and xylitol have also been used.

The use of xylitol is of particular interest as a series of fatal, adverse reactions were observed by this laboratory in patients receiving large intravenous infusions of this carbohydrate. (Thomas et al, 1970, 1972a, 1972b, Evans et al 1972). These adverse reactions were characterised by a marked metabolic acidosis, hyperbilirubinemia, elevation of liver enzymes, hyperuricaemia and the deposition of calcium oxalate crystals in the kidney and brain. Other independent groups have also recorded metabolic acidosis, hyperuricaemia, hyperbilirubinaemia and oxalate crystal deposition in xylitol infused patients. (Schumer 1971, Donahoe and Powers 1970, 1974 and Schroeder et al 1974).

Hyperuricaemia at two times the upper normal limit was demonstrated by Donahoe and Powers (1970, 1974) following xylitol infusion in normal adult volunteers; an effect also recorded in patients by Thomas et al (1972a) and Schumer (1971). However, Forster et al (1970) found only transient hyperuricaemia in xylitol infusion studies. Elevated serum uric acid production after fructose loading is well established (Perheentupa and Ravio 1967). In addition, Forster (1974) has demonstrated that fructose, sorbitol and xylitol, but not glucose, produce elevated serum uric acid levels at equivalent dose rates, indicating a common mechanism by which uric acid synthesis is increased. For instance it is known that fructose, xylitol and glycerol are rapidly phosphorylated in the liver, with a consequent depletion of hepatic ATP, Pi and total adenine nucleotide levels (Woods et al 1970, Woods and Krebs 1973, Burch et al 1970). Two key enzymes in the degradative pathway from AMP to uric acid, namely AMP deaminase and 5'-nucleotidase, are normally inhibited by ATP and Pi. Woods and Krebs (1973) have suggested that activation of these enzymes, due to abnormally low ATP and Pi concentrations is the cause of the breakdown of AMP to uric acid during fructose, xylitol and glycerol metabolism.

While lactic acidosis has been reported after xylitol infusion into man and animals (Thomas et al 1972b, Schumer 1971, Thomas et al 1974), this

effect is not restricted to xylitol. Fructose infusions are known to induce lactic acidosis, (Cohen and Woods 1976), while investigations by Forster (1974) demonstrated that lactate release from isolated perfused rat liver was more rapid with fructose and sorbitol than with xylitol, a result reflected in experiments on patients. The acidosis seen in xylitol infused patients was particularly severe (Thomas et al 1972a, 1972b) and was not due to lactate alone, and thus suggested the presence of other organic acids.

The most outstanding and least understood adverse reaction to xylitol infusions is that of calcium oxalate deposition in the kidneys and brains of patients (Thomas et al 1972a, Evans et al 1972, Schroeder et al 1974). This phenomena appears to be a unique characteristic of xylitol administration though a recent report has disclosed renocerebral oxalosis in a patient receiving an intravenous infusion of glycerol (Krausz et al 1977). While certain pathological states, including uremia, neoplasia and hepatic disease are associated with oxalosis, (Brin and Miller 1974), the massive nature of the oxalate crystal deposition observed after xylitol infusions is usually seen only in two other conditions: primary hyperoxaluria, and ethylene glycol poisoning. The similarity of effects seen in a xylitol infused patient (Thomas et al 1972a), compared with a case of ethylene glycol poisoning (Parry and Wallach 1974) is striking. While one must be careful in drawing conclusions from such comparisons, these results do suggest that xylitol, like ethylene glycol, does give rise to oxalate precursors. Investigations by Hauschildt et al (1976a) and Chalmers et al (1975) have shown that the oxalate precursor, glycollic acid, is excreted in increased amounts from xylitol infused patients.

Experiments in this laboratory have shown that increased levels of oxalate are excreted by pyridoxine deficient rats infused with xylitol (Hannett et al 1977) However, attempts to demonstrate significant oxalate synthesis in rabbits using thiamine and pyridoxine antagonists have not been successful (Oshinsky et al 1977). It would appear that the type of animal employed and the degree of pyridoxine deficiency are two factors which may be important in the demonstration of oxalate synthesis from xylitol.

This was the setting under which this thesis commenced. More specific introductions to xylitol metabolism and oxalate biosynthesis are presented in chapters three and four.

AIMS OF THE THESIS

Following the detection of adverse metabolic effects in patients receiving intravenous xylitol, our group became interested in the metabolic behaviour of this sugar alcohol. Various studies using dogs as the experimental animals were undertaken (Thomas et al 1974). However, increased oxalate production and oxalate crystal deposition were not seen in these animals following the infusion of large quantities of xylitol, sorbitol, fructose and glucose. Further studies with rats demonstrated that pyridoxine deficiency caused the increased excretion of oxalate after xylitol infusions (Hannett et al 1977). Nevertheless, *in vivo* experiments yield limited information in the sense that urinary or serum parameters merely reflect the underlying metabolic events, and do not necessarily identify the metabolic lesions. Consequently this particular study was undertaken to examine more closely, at the cellular level, the metabolism of xylitol, with particular emphasis on the production of oxalate from this and other carbohydrates. To aid in this endeavour, oxalate synthesis from established precursors was also studied. Isolated rat hepatocytes were chosen as the experimental system for these studies.

The major aims of this thesis were:

1. To compare the hepatic metabolism of xylitol, D-xylulose, sorbitol and D-fructose in isolated rat hepatocytes and to investigate factors which regulate xylitol metabolism.

2. To investigate oxalate synthesis from the major oxalate precursors, glycollate and glyoxylate at the cellular and subcellular level in rat liver.

3. To investigate oxalate synthesis from xylitol and other carbohydrates in isolated rat hepatocytes.

CHAPTER TWO

MATERIALS AND METHODS

MATERIALS AND METHODS

1. MATERIALS

a) Chemicals

Most of the chemicals used in this study were purchased from Calbiochem.

Some exceptions were:

Oxamic acid	Sigma Chemical Co
DL phenyllactic acid	Sigma Chemical Co
NAD^+	Boehringer Mannheim Pty Ltd
Carboxy methoxylamine hemihydrochloride (Amino oxyacetate)	Aldrich Biochemical Co
Sodium pentobarbitone (Nembutal)	Abbott Laboratories Pty Ltd
Ketamine hydrochloride (Ketlar)	Parke Davis and Co
Sodium phenobarbitone (Phenobarbital)	F.H. Faulding and Co Ltd
Ethyl 2(4chlorophenoxy) 2 methyl propionate (Clofibrate)	I.C.I. Aust. Ltd
Heparin (mucous)	Glaxo Aust Ltd

b) Enzymes

Lactate dehydrogenase	Beef heart	Calbiochem
Hexokinase	Yeast	"
Glycerophosphate dehydrogenase	Rabbit muscle	"
Glucose 6 phosphate dehydrogenase	Yeast	"
Collagenase		Worthington
Hyaluronidase	Bovine testes	Sigma
Polyol dehydrogenase	Candida utilis	"
Oxalate decarboxylase	Collybia velutipes	"

c) Rats

Male Porton derived rats (150-300 gm) were used exclusively in these studies. These rats are a large white strain, which have been bred in this Institute for many years. All rats were fasted overnight prior to experimentation.

d) Radioisotopes

All radioisotopes were purchased from the Radio Chemical Centre, Amersham. Endogenous levels of oxalate present in carbon labelled substrates were measured with oxalate decarboxylase. Only glyoxylate had significant levels of radioactive oxalate, and this varied between batches. Uniformly labelled $|^{14}C|$ xylitol had less than 0.0014 ± 0.0003% of counts as enzymically detectable oxalate (n = 17).

METHODS

2. VITAMIN DEFICIENT RATS

Male Porton derived rats, weighing approximately 150 gm were fed a comprehensive synthetic diet (Paquet et al 1970) from which pyridoxine was omitted. The degree of pyridoxine deficiency was ascertained by measuring the aminotransferase activity (AST) in red blood cell haemolysates. This activity in pyridoxine depleted rats was reduced to half that observed in control fed rats after 5 weeks on the diet.

3. SYTHESIS OF D-XYLULOSE

D-xylulose was synthesised by the method of Touster (1962) whereby D-xylose was epimerised to D-xylulose in the presence of pyridine. D-xylulose was further purified by ion exchange chromatography (Khym and Zill 1952) and the purity was verified by descending paper chromatography (Touster 1962). The concentration of D-xylulose was determined both chemically (Ashwell and Hickman 1957) and enzymically (Bergmeyer 1963).

4. *In Vivo* OXALATE STUDIES

Male rats were fasted overnight and the following compounds were then administered intraperitoneally in 2 ml of saline: $|U\text{-}^{14}C|$ xylitol, sorbitol, 22 mmoles/kg body weight, 0.8 µCi/mmole, $|U\text{-}^{14}C|$ sodium glyoxylate or $|1\text{-}^{14}C|$ sodium glycollate, 3.3 µmoles/kg body weight, 2 µCi/µmole. The rats were restrained in cages and allowed free access to water. Urine was collected in acidified cylinders (0.5 ml of 6N HCl) at 10 hourly intervals. After adjusting the urine to pH 3.0, 0.5 ml aliquots were assayed for $|^{14}C|$ oxalate.

5. EXTRACTION AND ASSAY OF URINARY $|^{14}C|$ GLYCOLLIC ACID

To 2 ml of acidified urine (0.1 ml of 6N HCl) was added 2 µmoles of glycollic acid. Organic acids were then extracted from the urine with tri-n-butyl phosphate (water saturated) as described by McChesney et al (1971). This procedure resulted in greater than 70% recovery of glycollic acid. The extracted organic acids in ethanol were separated by ascending paper chromatography (Nordmann and Nordmann 1969) in ethanol: water: ammonia (16:3:1). This procedure gave good separation of glycollic acid (R_f = 0.45-0.55), glyoxylic, oxalic (R_f=0.3) lactic and pyruvic acids, glycolaldehyde (R_f = 0.65). Standards run concomitantly were located on both edges of the chromatogram using Nesslers reagent (Hawk et al 1951). Appropriate portions of the dried chromatogram containing $|^{14}C|$ glycollic acid were counted in 10 ml of toluene scintillant (0.4% PPO, 0.02% POPOP).

6. HEPATOCYTE INCUBATIONS

To ensure succintness in this section the preparation and characterisation of isolated rat hepatocytes is described in appendix 1. Hepatocytes were suspended in a medium containing 140mM NaCl, 5.4mM KCl, 0.8mM MgSO4, 0.4mM Na_2HPO4, 0.4mM KH_2PO4 and 2.0mM $CaCl_2$. In incubations where $|^{14}C|$ oxalate was to be assayed, 1.0mM instead of 2.0mM $CaCl_2$ was used. The cell suspension (5 x 10^6 viable cells/ml) was buffered to pH 7.2 - 7.4 with 1.3% $NaHCO_3$; the original buffer having been equilibrated with carbogen (95% oxygen/5% CO_2). The above preparation will in future be referred to as the cells. The only variation to the above recipe was the inclusion of 10mM sodium phosphate, pH 7.4 in the above medium, which was equilibrated with 100% oxygen prior to its use in cell suspensions. This preparation will be referred to as cells in phosphate buffer. Any variations of the following methods will be described where appropriate.

a) Carbohydrate metabolism

Two ml of cells were incubated with substrate in 2.5 x 8.0 cm plastic tubes, in a shaking water bath at 37^0C. After a 30 minute incubation, 1.0 ml of ice cold 5% perchloric acid (PCA) was added with rapid mixing. Following centrifugation at 5,000g for 5 minutes, the supernatant was removed, retained, the pellet mixed vigourously with a further 1 ml of PCA, and the process repeated. The combined supernatants were neutralised to pH 7.0 - 8.0 with 1 M KOH, left at 2^0C for 1 hour, centrifuged, and the

supernatant assayed immediately for metabolites.

b) Oxalate biosynthesis

Two ml of cells were incubated with 1mM sodium glycollate $|1\text{-}^{14}C|$, 0.5 µCi/Umole, or 1mM sodium glyoxylate, $|U\text{-}^{14}C|$, 0.5 µCi/Umole for 30 minutes. Incubations with xylitol or other carbohydrates were usually conducted for one hour. The reactions were terminated by transferring the incubation mixture into a glass test tube and boiling in a water bath for 2.0 minutes.

7. PREPARATION OF CELL FREE EXTRACTS FROM RAT LIVER.

Crude rat liver homogenates were prepared from starved male rats which were killed, bled, and the liver placed on ice. Ten grams of liver were minced briefly with sharp blades, washed three times with ice cold 0.25 M sucrose/10 mM sodium phosphate buffer, pH 7.5, which will subsequently be referred to as the buffer.

The tissue, in 4 volumes of buffer, was placed in the smooth walled homogenizer, and dispersed by one upward run with a rapidly rotating teflon pestle. Cell debris was removed by centrifuging the homogenate at 500g for 3 minutes. (the radial clearance in the homogeniser was 0.12 mm)

Further liver fractions were prepared from this 500g supernatant. After centrifuging at 15,000g for 10 minutes, the upper portion of the supernatant was retained and the remaining supernatant gently decanted and discarded, leaving the fluffy layer with the pellet. This fluffy pellet was then resuspended in buffer to give an absorbance of ≈1.0 at 280nm for a 1:100 dilution. The 15,000g supernatant from the initial centrifugation was centrifuged at 20,000g for 15 minutes and the supernatant retained.

Incubations using cell free extracts contained 1.0 ml of extract and either 5 mM glycollate (0.1µCi/umole) or 10 mM glyoxylate (0.05µCi/umole). Reactions were terminated by the addition of 1.5 ml of 1 M potassium citrate buffer, pH 3.0. One ml aliquots were then assayed for $^{14}CO_2$ and $|^{14}C|$ oxalate.

8. PURIFICATION OF OXALATE SYNTHESISING ACTIVITY FROM RAT LIVER

a) Ammonium sulphate fractionation

Five rat livers were minced with sharp blades, washed three times with cold 0.25 M sucrose/10 mM sodium phosphate buffer pH 7.0, and homogenised at high speed for 30 seconds in a Sorvall Onmimix to give 200 ml of homogenate. Centrifugation for 5 minutes at 1000g yielded supernatant 1, which was further homogenised in the cold for one minute, and then subjected to ultrasonication for 5 minutes using an M.S.E. Ultrasonic Disintegrator. Centrifugation for 10 minutes at 10,000g of extract yielded supernatant 2, which was adjusted to pH 4.8 with 2 N acetic acid and centrifuged at 15,000g for 10 minutes. The resulting supernatant was readjusted to pH 7.5 with sodium hydroxide, giving supernatant 3. Solid ammonium sulphate was added to this fraction to obtain 40%, 60% and 100% saturations. Centrifugation at 15,000g yielded the respective precipitates. These precipitates were suspended in 20 ml of 5 mM sodium phosphate buffer, pH 7.0 and dialysed against the same buffer. The 40-60% ammonium sulphate fraction, which contained the majority of the oxalate synthesising activity, was further purified by column chromatography.

b) Column Chromatography

DEAE - or CM - sephadex columns, 2 x 5 cm, were equilibrated with 5 mM sodium phosphate buffer, pH 7.0. Eight mls of the dialysed fraction was loaded on the column and eluted with the above buffer. With CM - sephadex, the majority of the lactate dehydrogenase activity (shown to be LD_5) was bound to the column and could be eluted with 5 mM sodium phosphate buffer, pH 7.0, containing 100 mM NaCl. The oxalate synthesising activity appeared in the protein peak associated with the initial eluate. Conversely, with DEAE sephadex, the lactate dehydrogenase activity appeared in the first eluate, whilst the oxalate synthesising activity was eluted with 200 mM NaCl in 5 mM phosphate buffer, pH 7.0.

c) Electrophoresis

Electrophoresis was performed on agarose, using 0.02 M barbitone buffer, pH 8.6. Samples of 10 µl were applied to the agarose and were run for 50 minutes at 240 volts and 23 milli-amps. Electrophoretograms were

developed in a reaction mixture containing 10 mM substrate, plus or minus 4 mM NAD, 100 µM tetranitro-blue tetrazolium and 10 uM phenazine methosulphate in 50 mM sodium phosphate buffer, pH 8.0.

9. ASSAY OF METABOLITES

a) Metabolites were assayed in neutralised PCA extracts using the enzymic methods of Bergmeyer(1963). The glucose determinations were performed with 0.05 M triethanolamine, pH 7.7 instead of Tris-buffer. Lactate and glycerphosphate were measured in the same test solution. A similar procedure was adopted when measuring dihydroxyacetone phosphate and pyruvate.

b) |^{14}C| Oxalate Assay

A discussion on the validity, and efficacy of this method appears in appendix 2.

|^{14}C| oxalate assays were carried out in standard glass scintillation vials, which contained 1.0 ml of 0.4 M potassium citrate buffer, pH 3.0, 0.5 ml of the extract being assayed and 0.05 units of oxalate decarboxylase. This enzyme had been previously prepared as a one unit per ml solution in 0.4M citrate buffer pH 4.5 and kept as frozen aliquots. One unit of oxalate decarboxylase will convert 1 µmole of oxalate to formate and CO_2 per minute, at pH 3.0 and 37^{0}C. To the incubation vial was added a small inner vial (1.0 x 4.5 cm) containing 0.2 ml of 1 M hyamine hydroxide in methanol. The large vial was capped and the reaction allowed to proceed for 4 hours. By placing the vials on the perspex lid covering a large water bath at 37^{0}C, a constant incubation temperature (approx 30^{0}C) was maintained and avoided any contamination of the hyamine with condensate. At the end of this incubation, the inner vial was removed, the exterior washed with distilled water and the radioactivity measured after the addition of 2.6 ml of toluene scintillant (0.4% PPO, 0.02% POPOP). For each assay a blank, containing all reactants except oxalate decarboxylase, was included to correct for endogenous $^{14}CO_2$ levels in the extract being measured. This step was found to effectively measure $^{14}CO_2$ derived from the substrates tested. Internal standards of |U-^{14}C| oxalate were included in each assay series to check recovery.

Preparation of extracts for the assay of $|^{14}C|$ oxalate

i) Hepatocytes

Cell incubations were terminated by boiling for 2 minutes in a water bath. When low levels of oxalate were to be measured, chips of solid carbon dioxide were added during the boiling procedure to dimish endogenous levels of labelled carbon dioxide. After centrifugation at 2,000g for 2 minutes, 0.5 ml of the supernatant was assayed for oxalate.

ii) Urine

Acidified urine was adjusted to pH 3.0 with KOH and 0.5 ml aliquots assayed as described above.

iii) Cell free extracts

Incubations in a volume of 1.0 ml were terminated with 1.5 ml of cold 1 M potassium citrate, pH 3.0, and 1.0 ml of the mixture was assayed directly for $|^{14}C|$ oxalate.

In most instances the results are expressed as the percentage conversion of substrate to oxalate during the incubation period. Corrections were made for endogenous levels of $|^{14}C|$ oxalate in the substrates. Only glyoxylate consistently required correction for this factor.

9c)

ENZYME ASSAYS

ENZYME	PHOSPHATE BUFFER	ACCEPTOR, WAVELENGTH (nm)	INITIATING SUBSTRATE
Lactate dehydrogenase (E.C. 1.1.1.27)	0.20 M pH 9.0	NAD^+, 4 mM, 340	L Lactate, 20 mM
Catalase (E.C.1.11.1.6)	0.05 M pH 7.5	H_2O_2, OD = 1.0, 240	H_2O_2, OD = 1.0
Succinate oxidase (E.C.1.3.99.1)	0.05 M pH 7.5	DCIP, OD = 1.0, 610	Succinate, 50 mM + KCN, 5 mM
*Glyoxylate reductase (E.C.1.1.1.26	0.05 M pH 7.5	NADH, 0.2 mM, 340	Glyoxylate, 10 mM
Pyruvate reductase (E.C.1.1.1.27)	0.05 M pH 7.5	NADH, 0.2 mM, 340	Pyruvate, 1 mM
*Glyoxylate dehydrogenase	0.20 M pH 8.5	NAD^+, 4 mM, 340	Glyoxylate, 10 mM
Glycollate oxidase	0.20 M pH 8.0	DCIP, OD = 1.0, 610	Glycollate, 5 mM
Xanthine oxidase (E.C.1.2.3.2.)	0.20 M pH 8.0	DCIP, OD = 1.0, 610	Hypoxanthine, 10 mM

The above assays are essentially those of Liao and Richardson (1972) and McGroarty et al (1974). Enzyme or extract was added in a volume of 50 µl, the dilution being adjusted for each extract. In assays using DCIP (2:6 dichlorophenol-indophenol) preincubation of the dye with the enzyme and an assessment of the endogenous rate of dye reduction is necessary, prior to the addition of the respective substrates. *Glyoxylate dehydrogenase and glyoxylate reductase are defined by the assay conditions, whereas the actual enzyme catalysing these reactions is probably lactate dehydrogenase. All assays were performed at 37^0C.

ENZYME ASSAYS

9

c) (cont.)

Aspartate:α ketoglutarate aminotransferase (AST) and alanine: α ketoglutarate aminotransferase (ALT) were measured by the method of Bergmeyer (1963). Alanine: glyoxylate aminotransferase was assayed by the method of Thompson and Richardson (1968), where excess Tris is added to the reaction mixture after termination with trichloro acetic acid. The excess Tris complexes unreacted glyoxylate which would otherwise interfere with the measurement of pyruvate with lactate dehydrogenase (Methods in Enzymolgy 17, 164).

d) Protein

Protein was determined by the method of Lowry et al (1951).

10. INDUCTION OF RATS WITH CLOFIBRATE OR PHENOBARBITONE

a) Clofibrate

Male Porton derived rats weighing 250→300 gm were given 300 mg/Kg body weight of clofibrate daily for 10 days. The drug was administered by oral gavage. The normal diet of food pellets was supplemented with 0.5% clofibrate. The rats used in the *in vitro* experiments were given subcutaneous clofibrate 500 mg/kg body weight/day for 10 days.

b) Phenobarbitone

Male rats were given 100 mg/kg body weight/day of sodium phenobarbitone in saline by intraperitoneal injection. After 5 days the hexobarbital sleeping time (125 mg/Kg body weight) of these rats was half that of control animals.

c) *In Vitro* Experiments

The fasted rats were stunned, bled, the livers removed, cooled on ice and weighed. Ten grams of liver were homogenised in 30 ml of cold 0.25 M sucrose, pH 7.0, for 20 seconds at full speed in a Sorvall Omnimix. The incubation mixtures contained 1 ml of homogenate and 5 mM glycollate (0.5 μCi $|1\text{-}^{14}C|$ glycollate or 10 mM glyoxylate (0.5 μCi $|U\text{-}^{14}C|$ glyoxylate.

The incubations were terminated with 1.5 ml of cold 1.0 M potassium citrate, pH 3.0, and assayed for labelled oxalate and CO_2 as described previously.

CHAPTER THREE

XYLITOL AND SORBITOL METABOLISM IN ISOLATED RAT HEPATOCYTES

INTRODUCTION

i) GENERAL ASPECTS OF XYLITOL METABOLISM

Xylitol, a pentitol, is an intermediate in the glucuronic acid oxidative pathway. Touster et al (1956) and Hollman and Touster (1957) prepared two xylitol dehydrogenases from guinea pig liver mitochondria; $NADP^+$ and NAD^+ linked, producing L and D-xylulose respectively. These enzymes have since been found in the cytosol (Arsenis and Touster 1969). In liver, xylitol is normally metabolised via the NAD^+ linked dehydrogenase to D-xylulose, which is then phosphorylated by D-xylulokinase (Hickman and Ashwell 1958) to D-xylulose-5-phosphate, a key intermediate of the pentose phosphate cycle. By a series of transketolase and trandaldolase reactions, D-xylulose-5-phosphate donates a two carbon fragment to either a four or five carbon sugar phosphate and thereby enters the glycolytic pathway at the hexose phosphate and triosephosphate levels (fig. 1). The high rate of xylitol utilisation in rat liver is remarkable as this compound is usually present in only trace amounts in natural diets. As discussed by Woods and Krebs (1973), this indicates that polyol dehydrogenase, D-xylulose kinase and the enzymes of the pentose phosphate pathway have a high capacity in rat liver.

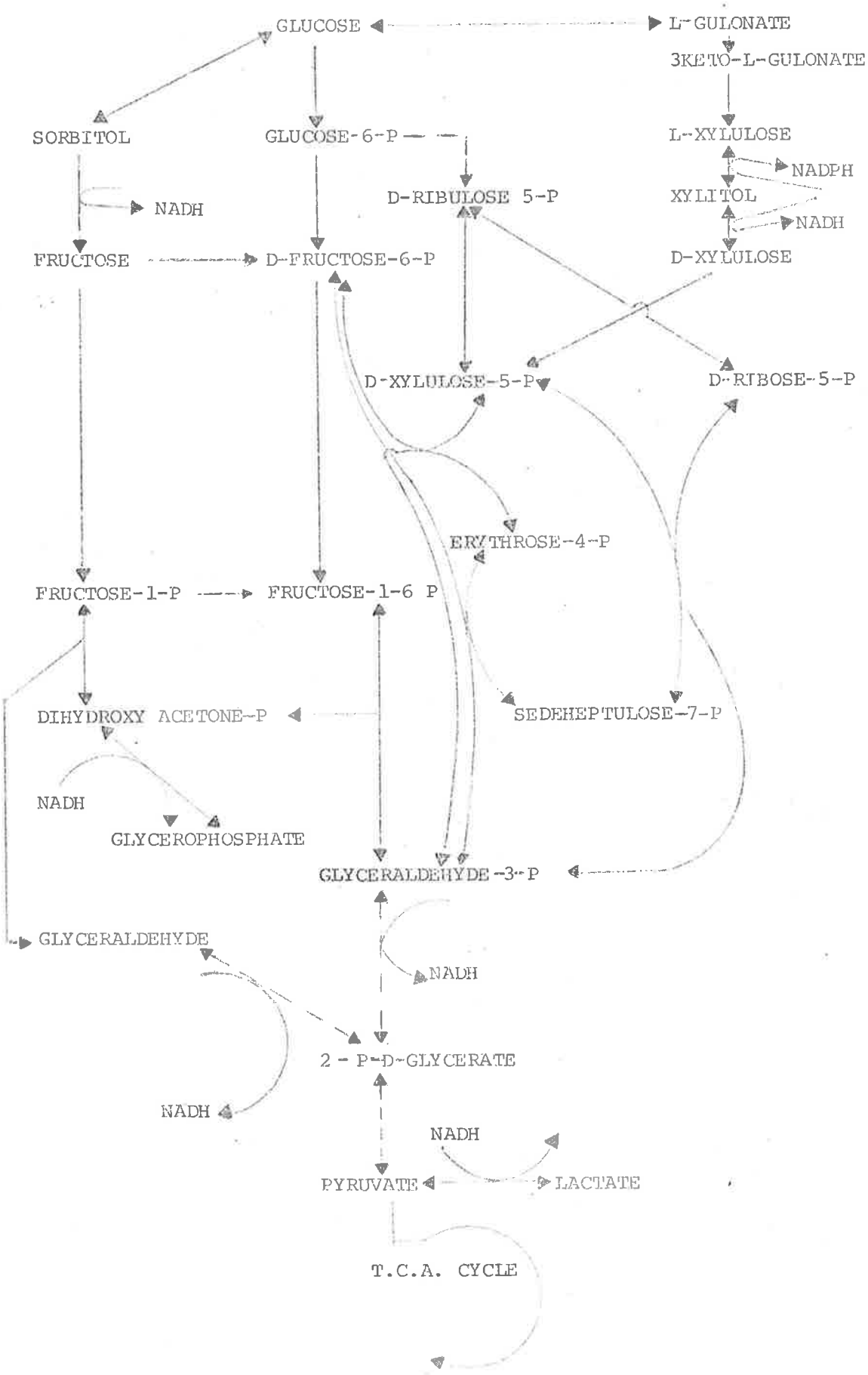
ii) XYLITOL AND SORBITOL METABOLISM IN ISOLATED RAT HEPATOCYTES

An important feature of hepatic xylitol metabolism is the initial dehydrogenation step, catalysed by the NAD^+ linked xylitol dehydrogenase. Hence the utilisation of xylitol, like that of sorbitol, glycerol and ethanol, can result in high cytosolic levels of NADH (Jakob et al 1971, Williamson et al 1971, Berry et al 1973, 1974a, Woods and Krebs 1973). These authors' experiments on the metabolism of xylitol in perfused rat liver and sorbitol in isolated rat hepatocytes have indicated that the rate of regeneration of NAD^+ is a major controlling factor in polyol metabolism

This factor is firstly dependent on the activity of the glycerophosphate and malate aspartate hydrogen shuttles and secondly on the rate of hydrogen flux to oxygen along the mitochondrial respiratory chain. Aspects of these controlling mechanisms are investigated in this section by comparing the metabolism of the redox pairs, xylitol - D xylulose and sorbitol - D fructose, in conjunction with the use of an artificial electron acceptor,

FIG. 1.

THE POSITION OF XYLITOL IN METABOLIC PATHWAYS



phenazine methosulphate (PMS). Specific enzyme inhibitors and metabolic intermediates are used to assess the difference between xylitol and sorbitol utilisation, particularly with respect to the hepatic accumulation of glycerophosphate. The significance of these results is discussed in relation to the role of the glycerophosphate and malate-aspartate hydrogen shuttles during xylitol and sorbitol metabolism.

RESULTS

Numerous investigators have shown that the isolated rat hepatocyte is an effective and valid model for studying carbohydrate metabolism. Nevertheless, comparative studies on the metabolism of xylitol, sorbitol, fructose and glucose were conducted in hepatocytes, the perfused liver and *in vivo*, to confirm the effectiveness of hepatocyte system, particularly with respect to xylitol metabolism. The trends in metabolite production from the above carbohydrates was indeed similar in all systems. The results of these experiments have not been included.

XYLITOL AND SORBITOL METABOLISM

1. EFFECT OF SUBSTRATE CONCENTRATION

An example of the effect of substrate concentration on the metabolism of xylitol and sorbitol in isolated rat hepatocytes is shown in fig. 2. The rate of glucose production from both substrates was similar. Sorbitol however produced more lactate than xylitol, the converse applying with respect to glycerophosphate production. It is interesting to note that the marked increase in glycerophosphate production was observed at a lower xylitol concentration (2 mM) than was seen with sorbitol (5 mM). Despite the different rates of metabolite production by xylitol and sorbitol, the overall rate of utilisation of these polyols is virtually identical (table 1). Double reciprocal plots of the results shown in the table gave apparent Kms of 0.8-1.0 mM for the hepatic utilisation of xylitol and sorbitol. Berry et al (1973) obtained a Km of 0.8 mM for sorbitol metabolism in isolated rat hepatocytes.

2. A COMPARISON OF XYLITOL, D-XYLULOSE, SORBITOL AND D-FRUCTOSE METABOLISM

The initial oxidation of xylitol and sorbitol in the liver generates stoichiometric amounts of NADH, D-xylulose and D-fructose respectively. Hence it can be suggested the major differences in metabolite production from the redox pairs can be attributed to this one enzymic step, whereby cytosolic NADH is produced. Glucose and lactate were the major products of xylitol, D-xylulose, sorbitol and D-fructose metabolism in the isolated rat hepatocytes (figs 3 and 4). D-xylulose and D-fructose produced 2 and 3 fold more lactate, and 7 and 25 fold more pyruvate than xylitol and

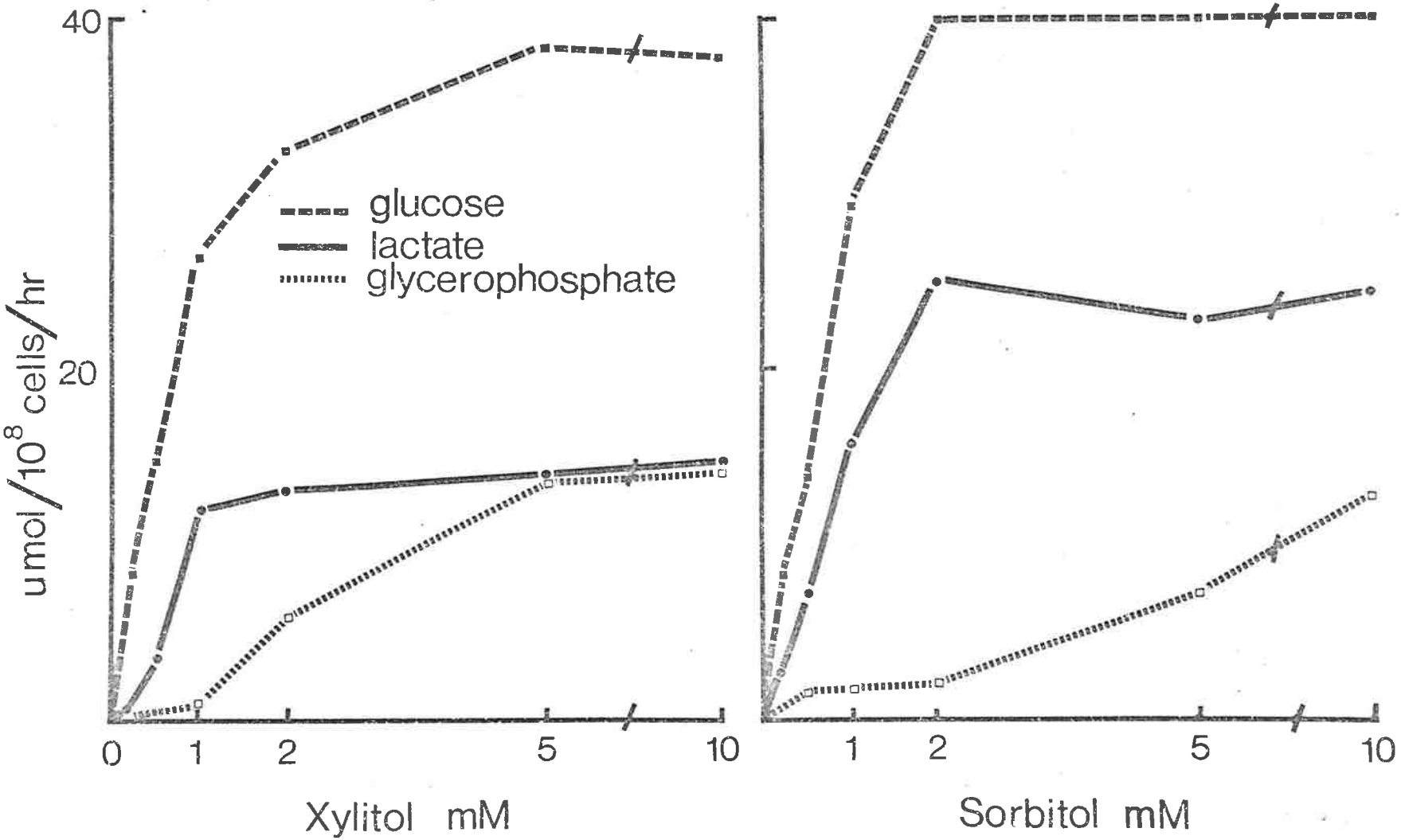


Fig. 2. The effect of substrate concentration on the metabolism of xylitol and sorbitol in isolated rat hepatocytes. Details of the incubations are given in the methods, chapter 2. The incubation time was 20 minutes. The results are from one liver cell preparation. These results were shown to be reproducable in other independent cell isolations.

TABLE 1

THE EFFECT OF SUBSTRATE CONCENTRATION ON XYLITOL AND SORBITOL METABOLISM IN RAT HEPATOCYTES

XYLITOL M(mM)	μ moles metabolised	percent metabolised	Km
1	38.7	98	
2	50.5	64	0.8--1.0 mM
5	61.7	31	
10	62.7	16	
SORBITOL (mM)			
1	38.0	95	
2	52.5	66	0.8--1.0 mM
5	55.2	28	
10	58.6	15	

Experimental details are given in the methods, chapter 2. The incubation time was 20 minutes. The results are expressed as μ moles of substrate metabolised / 10^8 cells/h which was calculated from the following expression.

μ moles metabolised = glucose + 0.5 (lactate + glycerophosphate)

As xylitol is a 5 carbon sugar, the above expression is multiplied by a factor of 1.2 with xylitol as substrate.

The apparent Km values were determined from reciprocal plots of 1/S versus 1/V, S and V being the substrate concentration and the rate of metabolism respectively.

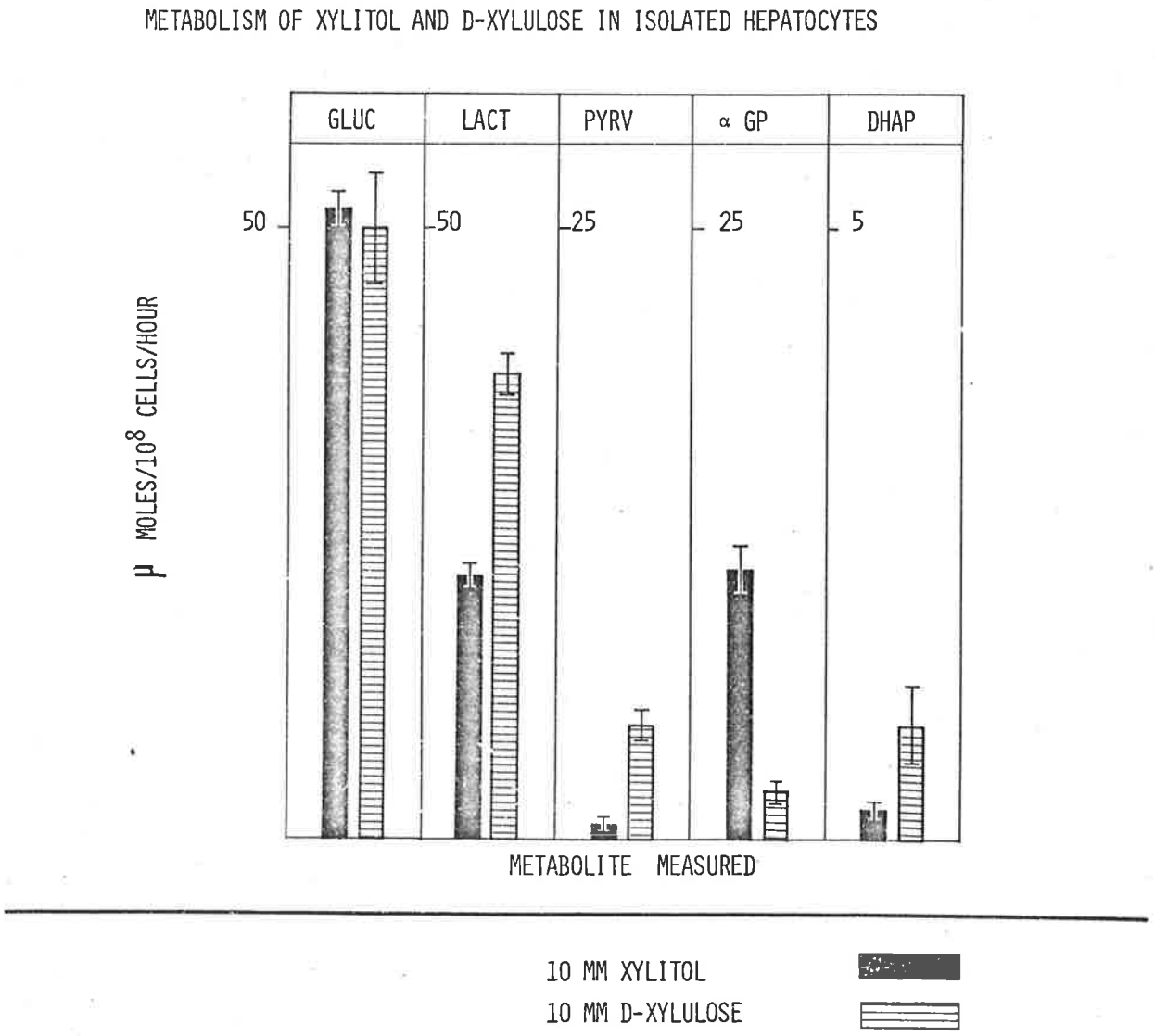


Fig. 3. Details of the incubations are given in the methods, chapter 2. The concentration of xylitol and D-xylulose was 10 mM, and the incubation time was 30 minutes. The results represent the mean ± S.E.M. of 3 independent hepatocyte isolations.

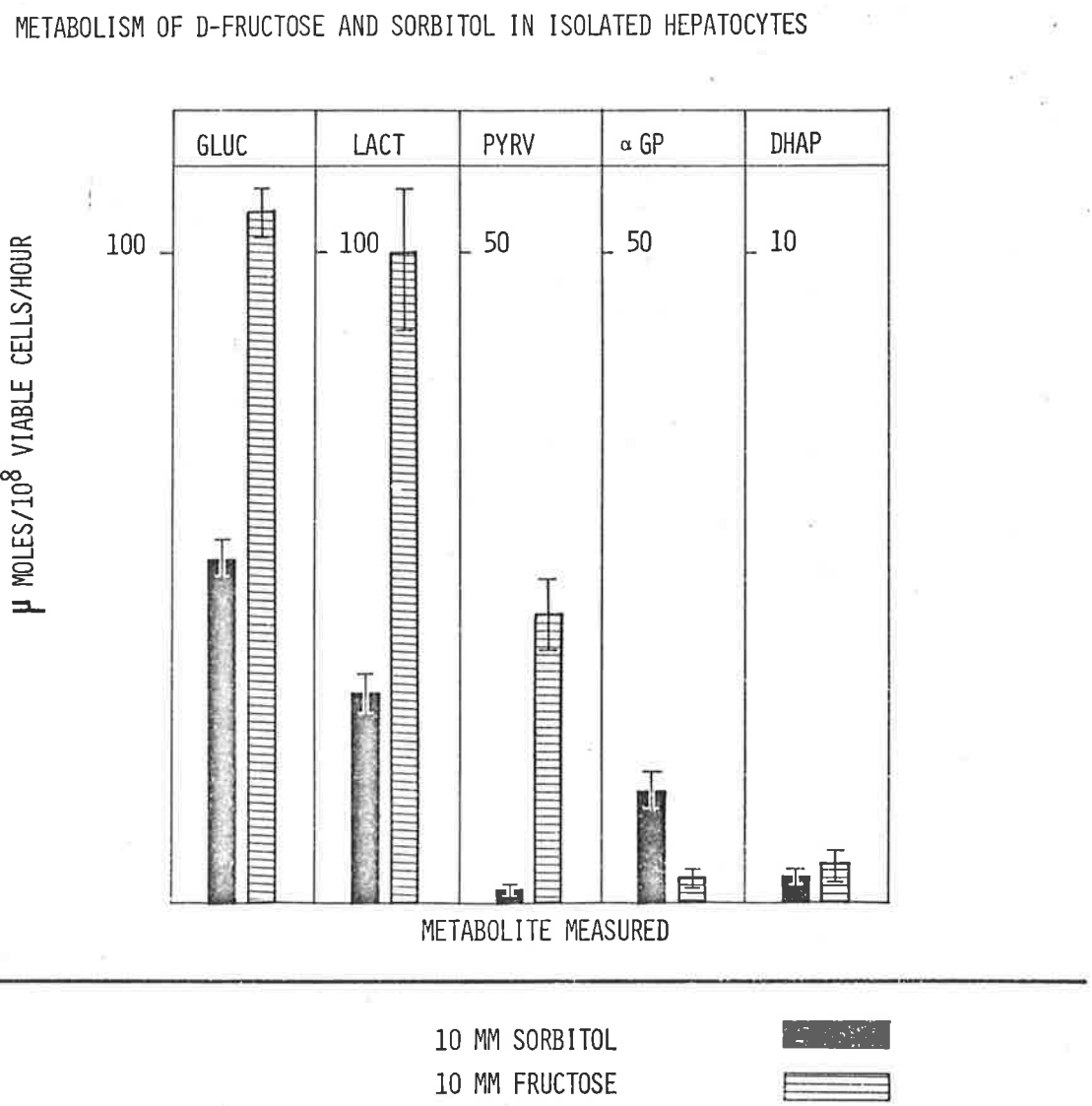


Fig. 4. Details of the incubations are given in the methods, chapter 2. The concentration of sorbitol and D-fructose was 10 mM, and the incubation time was 30 minutes. The results represent the mean ± S.E.M. of 3 independent hepatocyte isolations.

sorbitol respectively. As observed here and elsewhere (Woods and Krebs 1973, Berry et al 1973), the hepatic metabolism of xylitol and sorbitol resulted in a marked increase in glycerophosphate levels. Since this phenomena was not seen during the breakdown of D-xylulose and D-fructose it would appear that high cytosolic NADH levels, in conjunction with the supply of dihydroxyacetone phosphate are the prime causative factors in glycerophosphate accumulation during xylitol and sorbitol metabolism. The presence of high NADH levels is substantiated by the reduced state of the cytosolic NADH / NAD^+ redox couples, as indicated in table 2 by the elevated lactate / pyruvate and glycerophosphate / dihydroxyacetone phosphate ratios.

3. THE EFFECT OF PHENAZINE METHOSULPHATE (PMS) ON CARBOHYDRATE METABOLISM

Physiological hydrogen acceptors, such as pyruvate have been used to facilitate the regeneration of cytosolic NAD^+ during polyol metabolism (Werner and Berry 1974). However, the use of such acceptors makes calculation of appropriate redox couples difficult. Artificial hydrogen acceptors offer a more desirable alternative in this situation and have been used previously to study hepatic polyol metabolism (Williamson et al 1971, Berry et al 1973). Initial experiments showed PMS to be most effective in stimulating polyol metabolism, with a optimum concentration of 10μM for the stimulation of gluconeogenesis. Ferricyanide, methylene blue and 2:6: dichlorophenol-indohpenol had little effect at a concentration of 25 μM (results not included).

The results in figs 5 and 6 demonstrate the effect of 10μM PMS on xylitol and sorbitol metabolism, where hepatocytes accumulated higher levels of all metabolites except glycerophosphate, the production of which was decreased, compared to the control. By comparison, PMS had little effect on D-xylulose and D-fructose utilisation, causing a decrease in glucose and lactate production and an increase in pyruvate production. As shown in table 2, the overall metabolism of xylitol and sorbitol was increased by 40-50% while that of D-xylulose was slightly diminished. Presumably, the major action of PMS is the facilitation of hydrogen flow to oxygen and hence an increase in NAD^+ regeneration. This action is consistent with the more oxidised NADH/NAD^+ redox couples in the presence of PMS. However, while there was increased carbon flow from xylitol to pyruvate and lactate, the lactate/pyruvate ratio remained elevated, despite the decrease in the

TABLE 2

THE EFFECT OF PHENAZINE METHOSULPHATE (PMS) ON THE REDOX STATE OF RAT HEPATOCYTES DURING CARBOHYDRATE METABOLISM

SUBSTRATE	LACTATE / PYRUVATE	GLYCEROPHOSPHATE / DI-HYDROXYACETONE PHOSPHATE	µMOLES METABOLISED /10^8 cells/h
XYLITOL	38 ± 8	47 ± 12	84
+PMS	44 ± 7	9.9 ± 2.2	119
D-XYLULOSE	8.3 ± 0.7	2.2 ± 0.5	88
+PMS	5.9 ± 1.2	2.2 ± 0.4	83
SORBITOL	43 ± 12	27 ± 9	74
+PMS	24 ± 4	5.3 ± 1.2	111
D-FRUCTOSE	4.5 ± 0.6	3.4 ± 0.4	168
+PMS	1.7 ± 0.3	1.4 ± 0.3	146

Incubations contained 10 mM substrate and 10 µM PMS. The incubation time was 30 minutes. The calculation of µ moles metabolised is outlined in table 1. Further experimental details appear in figs. 5 and 6 and in the methods, chapter 2. The results represent the mean ± S.E.M. of 3 independent cell isolations.

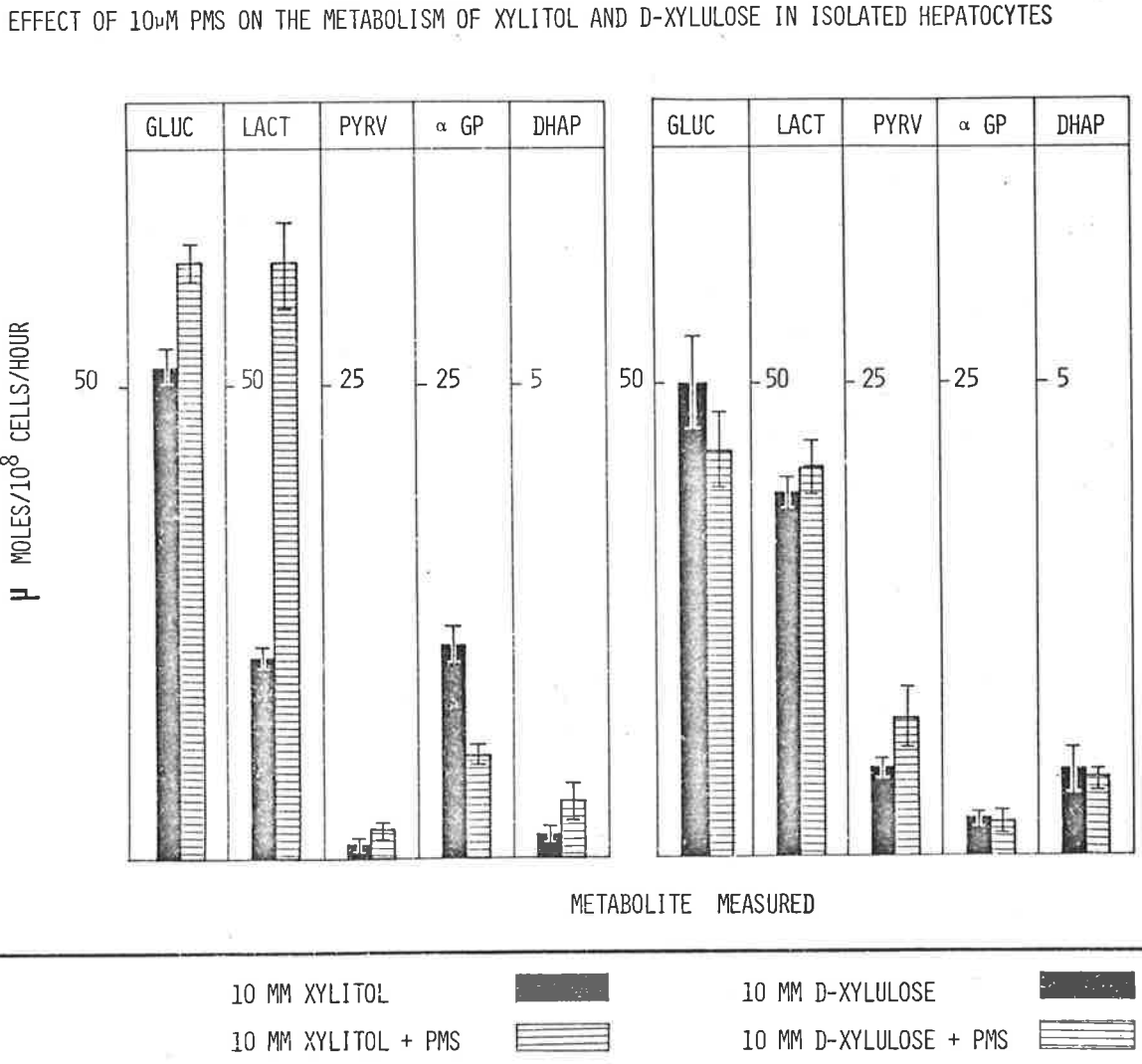


Fig. 5. Details of the incubations are given in the methods, chapter 2. The concentration of xylitol and D-xylulose was 10 mM, and phenazine methosulphate (PMS), 10 uM. The incubation time was 30 minutes. The results represent the mean ± S.E.M. of 3 independent hepatocyte isolations.

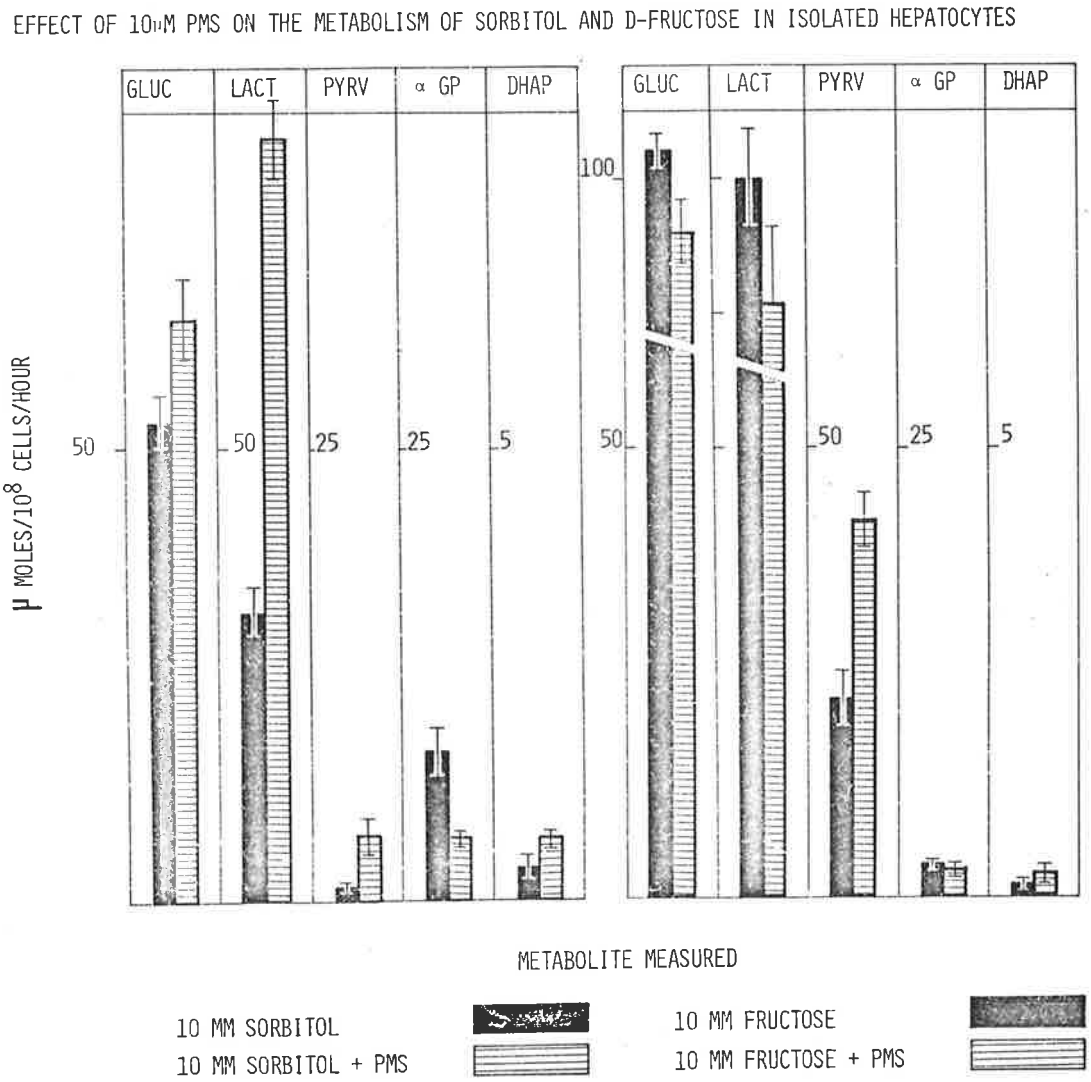


Fig. 6. Details of the incubations are given in the methods, chapter 2. The concentration of sorbitol and D-fructose was 10 mM and phenazine methosulphate (PMS), 10 uM. The incubation time was 30 minutes. The results represent the mean ± S.E.M. of 3 independent hepatocyte isolations.

glycerophosphate : dihydroxyacetonephosphate ratio (table 2). It is apparent that these two cytosolic redox couples are not in equilibrium in the presence of PMS, which may indicate a localised site of action for PMS.

4. THE EFFECT OF TRICARBOXYLIC ACID INTERMEDIATES, AND RESPIRATORY INHIBITORS ON THE METABOLISM OF XYLITOL AND SORBITOL

The similarity in the effects of the above compounds on xylitol and sorbitol metabolism is demonstrated in Fig. 7. In terms of the effect on the overall metabolism, only rotenone and amino-oxyacetate (AOA) demonstrated any differences between xylitol and sorbitol breakdown. Ethanol, succinate, malate and βhydroxybutyrate were included in the experiment as producers of either cytosolic or mitochondrial reducing equivalents, these compounds being the reduced substrates for their respective dehydrogenases. It has been reported (Ross et al 1967) that some of the above intermediates do not freely cross the outer liver cell membrane. However, Dubinsky and Cockrell (1974) found that succinate, β-hydroxybutyrate , and malate entered and were respired by isolated rat hepatocytes, a result which is in accord with the observations presented in fig. 7.

The inhibitory effect of malate and ethanol addition on xylitol and sorbitol utilisation can be attributed to cytosolic NADH production, and hence competition for reducing equivalent translocation into the mitochondria.

Contrary to the results of Williamson et al, (1971) β-hydroxybutyrate had no effect on the metabolism of either polyol, a finding which is directly supported by the work of Grunnet (1974) on xylitol with a reconstituted mitochondrial system, and indirectly by the work of Berry et al 1974b, who found that acetoacetate did not enhance sorbitol or glycerol uptake by isolated liver cells. The mitochondrial location of β hydroxybutyrate dehydrogenase indicates that both the mitochondrial NADH redox state, and electron transport from the NADH level of the mitochondria are not rate limiting. However, the inhibition of xylitol and sorbitol metabolism by succinate implies competition within the mitochondria for reducing equivalent oxidation at the flavoprotein level, i.e. at the glycerophosphate and succinate dehydrogenase level of the respiratory chain. The

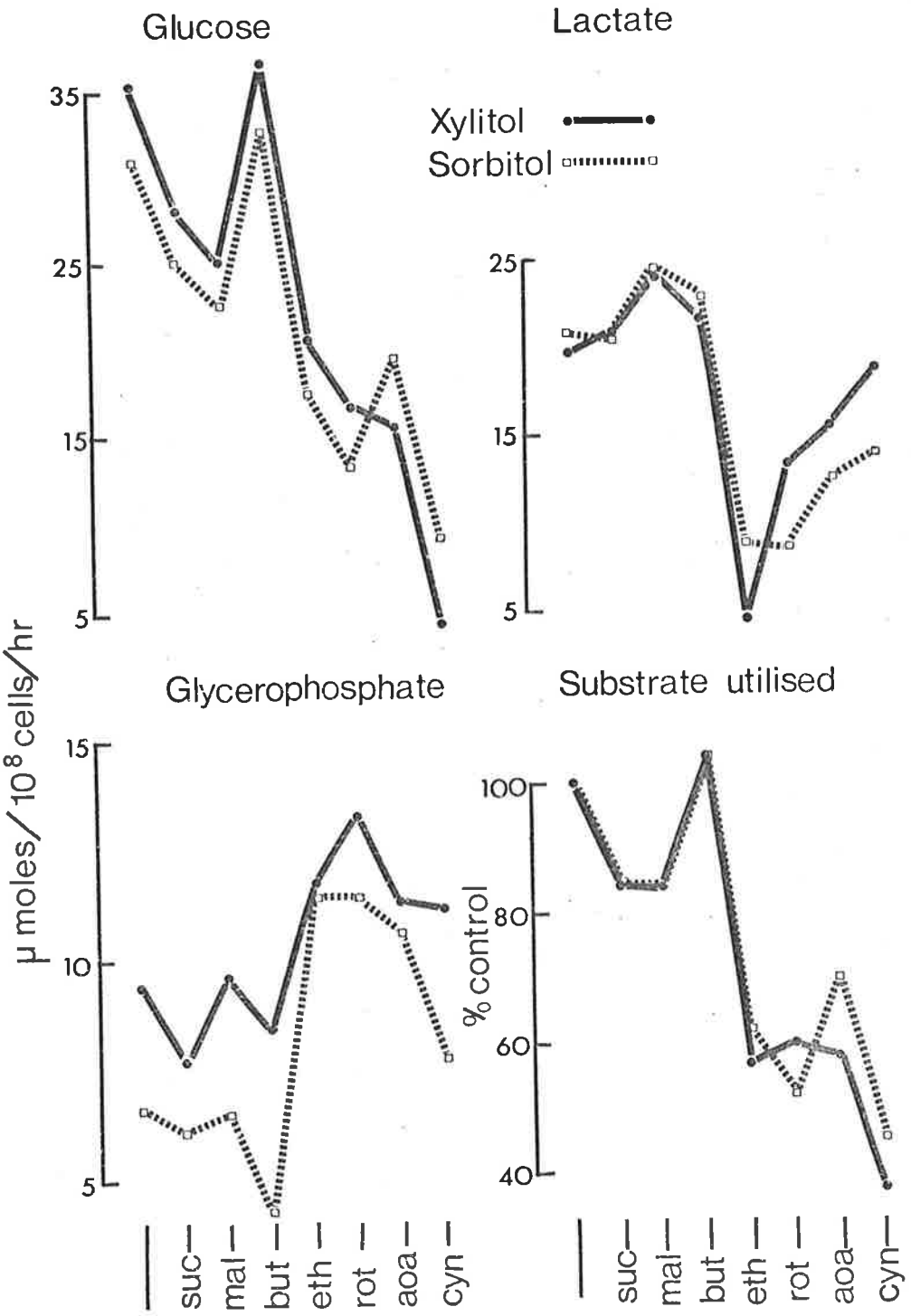


Fig. 7. The effect of metabolic intermediates and respiratory inhibitors on the metabolism of xylitol and sorbitol in isolated hepatocytes. The incubation conditions are given in the methods, chapter 2. The concentrations used were, xylitol and sorbitol, 10 mM, succinate (suc), malate (mal) and hydroxybutyrate (but) 5 mM, ethanol (eth) 10 mM, rotenone (rot) 50 uM, and amino oxyacetate (aoa) and potassium cyanide (cyn), 1 mM. The incubation time was 30 minutes. Inhibitors were added to the incubation at the same time was the substrate. The calculation of the amount of substrate utilised is outlined in table 1.

observation that rotenone had a greater inhibitory effect on sorbitol utilisation than xylitol suggests that more reducing equivalents from sorbitol are entering the respiratory chain at the NADH level, compared with xylitol. This result, however, contradicts the effect of amino-oxyacetate as xylitol metabolism was affected more than sorbitol. In these experiments it is assumed that by reducing transaminase activity with AOA, the effectiveness of the malate-aspartate hydrogen shuttle to transfer reducing equivalents into the mitochondria at the NADH level is diminished.

The inhibition by cyanide of both xylitol and sorbitol metabolism demonstrates that hydrogen flux to oxygen via the mitochondrial respiratory chain is necessary for polyol metabolism. However, as this inhibition was not complete, it can be suggested that some of the excess NADH generated in the cytosol may be oxidised by the microsomal or peroxisomal electron transport systems (Williamson et al 1971, deDuve and Baudhuin 1966).

5. THE EFFECT OF AMINO-OXYACETATE ON XYLITOL AND SORBITOL METABOLISM

Williamson et al (1971) have demonstrated that AOA, a transaminase inhibitor would decrease glucose and lactate production from xylitol. The effect is shown in fig. 7. The present finding of an increase in glycerophosphate production in the presence of the transaminase inhibitor (fig. 7.) was investigated at increasing xylitol and sorbitol concentrations (fig. 8). While xylitol caused a more marked increase in glycerophosphate production, compared with sorbitol, the addition of 1 mm AOA caused a greater stimulation of glycerophosphate production from sorbitol compared to the control. The reciprocal plots of the results show that in the absence of AOA, both substrates have the same Km of 5 mM for glycerophosphate production, while xylitol has the higher V max, of 20 µmoles / 10^8 cells / hr, compared to 12 µmoles / 10^8 cells / hr for sorbitol. However, by inhibiting the malate - aspartate hydrogen shuttle with AOA, differences between glycerophosphate production from xylitol and sorbitol are immediately seen. AOA lowers the Km from 5 to 3 mM but does not alter the Vmax for glycerophosphate production from xylitol. On the other hand, AOA increases the Vmax for glycerophosphate production from sorbitol from 12 to 20 µmoles/10^8 cells/hr but does not alter the Km of 5 mM for this substrate. It would appear that glycerophosphate production is maximal with xylitol at saturating substrate concentrations,

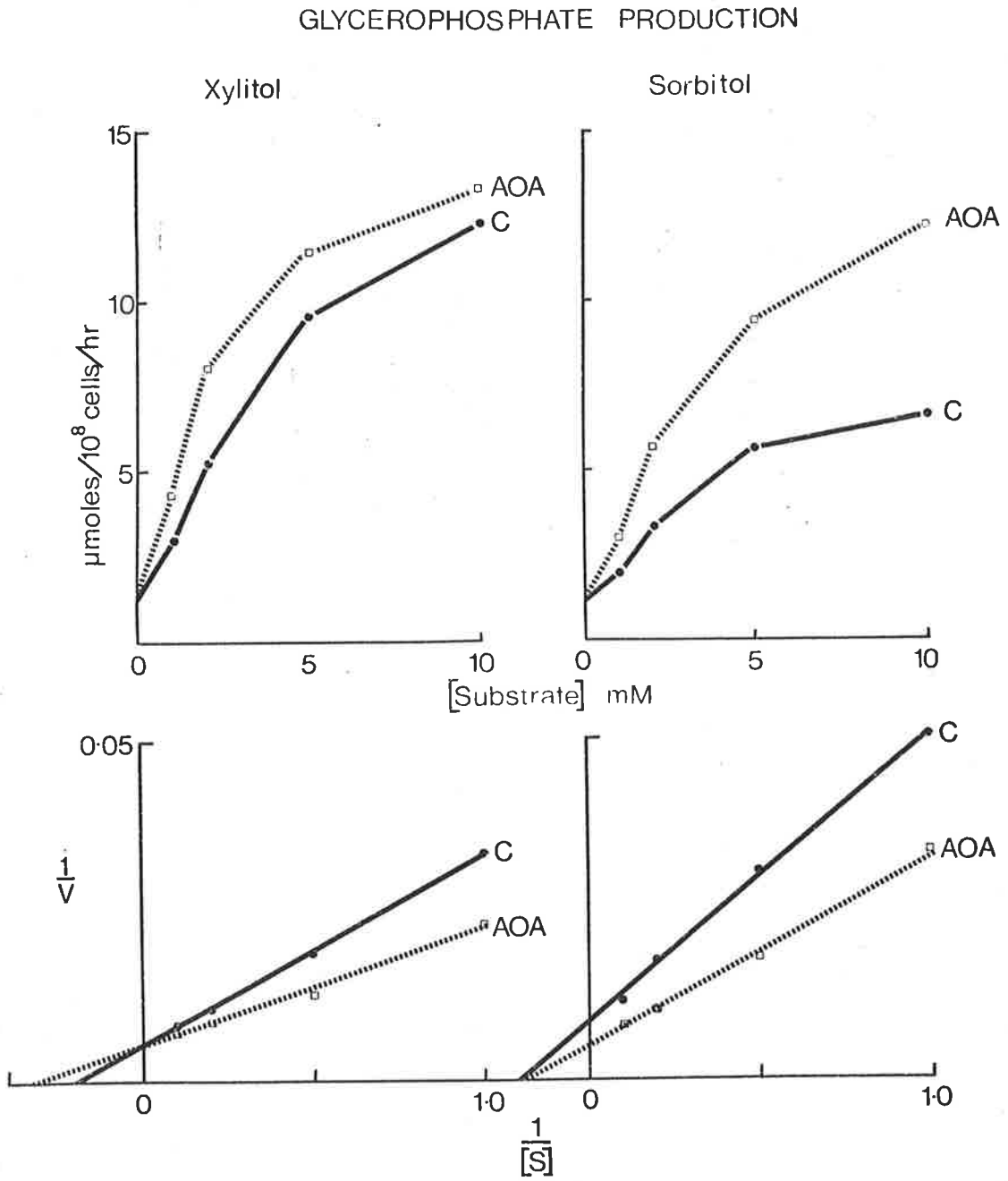


Fig. 8. The effect of 1 mM amino oxyacetate (AOA) on glycerophosphate production from xylitol and sorbitol in hepatocytes at increasing substrate concentrations. The incubation conditions are given in the methods, chapter 2.

V = µmoles glycerophosphate producted /10^8 cells/hr

S = concentration of xylitol and sorbitol.

and submaximal with sorbitol. By inhibiting hydrogen flux through the malate-aspartate shuttle, greater emphasis may be placed on the glycerophosphate shuttle. It would appear that this increased use of the glycerophosphate shuttle is not possible with xylitol as substrate, due to the system operating at close to maximum under normal conditions of saturating xylitol concentrations. While the validity of representing the results in fig. 8 as reciprocal plots may be questioned on the grounds that this is not a one enzyme system, the fact that the results can be plotted in this manner indicates that one enzyme is the rate limiting step in this glycerophosphate production.

DISCUSSION

Suspensions of isolated rat liver parenchymal cells from fasted rats rapidly metabolised 10 mM xylitol, D-xylulose, sorbitol and D-fructose. Though similar trends were observed in the utilisation of D-fructose and D-xylulose in the isolated liver cells, fructose was metabolised at more than twofold the rate observed with the pentose. One explanation of this finding is that the initial breakdown of fructose is mediated by very active fructokinase and aldolase enzymes whereas xylulose is initiated by xylulokinase, a less active enzyme, though as demonstrated here and by other workers, (Woods & Krebs 1973) the pentose phosphate pathway has considerable capacity in the rat liver. In light of the findings with fructose and xylulose, and considering the different enzymic steps by which hexoses and pentoses enter the glycolytic and gluconeogenic pathways, the quantitative similarity in the comparison between the metabolism of sorbitol and xylitol is of particular interest. The similarity in the pattern of intermediary metabolites seen in cell incubations, together with almost identical Km values for their utilisation, indicate that a common control mechanism is operating in the hepatic handling of xylitol and sorbitol.

Other workers have found that the metabolism of xylitol (Williamson et al 1971), and sorbitol and glycerol (Berry et al 1973), is regulated by the rate of reducing equivalent transfer into the mitochondria and the rate of electron flow to oxygen via the mitochondrial respiratory chain. In support of their findings, the present experiments with PMS show that increasing the flow of reducing equivalents to oxygen greatly enhanced the hepatic utilisation of both xylitol and sorbitol. The mechanism by which PMS mediates its action in these experiments is unclear. While PMS may directly accept hydrogen from NADH in the cytosol, with subsequent reoxidation by molecular oxygen, the low concentrations of PMS used suggests a catalytic role in the mitochondria. While PMS appears to have limited entry into the mitochoncria in the absence of Ca^{++} (Arrigoni and Singer 1962), an adequate Ca^{++} concentration was present in these studies. It has been demonstrated that with various flavoprotein dehydrogenases, notably the mitochondrial succinate and glycerophosphate dehydrogenases, PMS is the most effective artificial electron acceptor (Takemor and King 1964, Arrigoni and Singer 1962). From the marked fall in glycerophosphate levels on addition of PMS to cells metabolising xylitol, it can be postulated that PMS stiumulates the mitochondrial

linked glycerophosphate dehydrogenase, thus increasing hydrogen flux through this hydrogen shuttle. An interesting experiment in this area would be to test the effect of tri-iodothyronine on xylitol metabolism as this treatment appears to increase the activity of the mitochondrial glycerophosphate dehydrogenase and should therefore increase xylitol metabolism (Rognstad, 1977).

Rotenone is known to inhibit respiratory linked NADH dehydrogenases without affecting the mitochondrial glycerophosphate dehydrogenase (Bianchi et al 1964). Hence the greater sensitivity of sorbitol metabolism to this inhibitor compared with xylitol implies that there are differences in the shuttle systems involved in the hepatic handling of these two substrates. It appears that more hydrogen from sorbitol may enter the respiratory chain via the NAD^+ linked malate aspartate shuttle, rather than via the FAD^+ linked glycerophosphate shuttle, with the converse applying to hydrogen transfer from xylitol. This is further borne out by the more rapid accumulation of glycerophosphate in cells incubated with xylitol compared with sorbitol. This difference in the degree to which hydrogen from xylitol and sorbitol is translocated by the different shuttles is obviously small but may be important.

It appears that the glycerophosphate shuttle has low activity in the mammalian liver due to the low affinity of the mitochondrial glycerophosphate dehydrogenase for its substrate (Meijer et al, 1975) However it seems that during xylitol metabolism, the high rate of glycerophosphate production is sufficient to produce Vmax flux through this enzyme system. With sorbitol as substrate, the flux of glycerophosphate through the mitochondrial glycerophosphate dehydrogense would appear to be less than maximal as shown in fig. 8. Only in the presence of AOA, which effectively inhibits the malate aspartate shuttle (Williamson et al 1974), does the rate of glycerophosphate production from sorbitol approach that produced from xylitol in the absence of this inhibitor. The accumulation of glycerophosphate within the cell occurred at a lower xylitol concentration (2 mM) than was observed by Woods and Krebs (1973) in the perfused rat liver (xylitol 10 mM). It is possible that the effective intracellular concentration of xylitol is higher in the isolated hepatocytes due to the increased surface area available for the uptake of substrates.

Woods and Krebs (1973) have indicated that the rapid phosphorylation of D-xylulose to D-xylulose 5 phosphate, together with the accumulation of glycerophosphate is probably the cause of the decrease in inorganic phosphate, ATP and total adenine nucleotide levels observed during xylitol breakdown and may directly lead to increased uric acid production from a stimulation of the adenosine degrading enzymes. This problem may be further compounded by hydrogen from xylitol entering the respiratory chain at the flavin level (glycerophosphate dehydrogenase), which would yield only 2 molecules of ATP per hydrogen, compared to 3 ATP produced from the NADH level (malate-aspartate shuttle).

The cellular energy charge (ATP/ADP/AMP), and the redox state are important, interrelated, factors in metabolic control. The decrease in ATP and total adenine nucleotide levels, together with the marked elevation in NADH levels are the most important consequences of xylitol metabolism in the liver. It can be argued that the overriding event in polyol metabolism is the generation of large amounts of cytosolic NADH. That this NADH is generated in the initial dehydrogenation of xylitol and sorbitol is amply demonstrated in comparative experiments with the respective redox pairs D-xylulose and D-fructose. A consequence of this rapid NADH production is that the rate of regeneration of NAD^+ regulates the further metabolism of the polyols, as shown here and by other workers (Williamson et al 1971, Berry et al 1973).

One consistent difference between xylitol and sorbitol breakdown in hepatocytes was that sorbitol produced more lactate and less glycerophosphate than xylitol at comparable concentrations. This difference may arise from the pathways by which carbon from these polyols enters the glycolytic pathway (fig. 1). More carbon from xylitol than sorbitol must pass through the triose phosphate level, as sorbitol has the alternate route through glyceraldehyde. Hence, the small but consistent difference between xylitol and sorbitol breakdown may reflect differences in the activity, of the glyceraldehyde and the glyceraldehyde 3 - phosphate dehydrogenase enzymes, in conjunction with the degree to which the glycerophosphate and malate-aspartate shuttles are utilised.

This factor, and the ability to accelerate NAD^+ regeneration with artificial electron acceptors, has an important bearing on studies into oxalate production from xylitol. The marked similarity in the metabolism of xylitol and sorbitol makes these compounds ideal for comparative studies

on oxalate synthesis.

This chapter has described in detail, at the cellular level, aspects of xylitol metabolism which have not previously been fully elucidated. The influence that the intracellular production of high levels of NADH has on polyol metabolism was amply demonstrated by comparing the utilisation of D-xylulose with that of xylitol. The phenomena of glycerophosphate accumulation during polyol metabolism was investigated, with one conclusion being that the activity of the mitochondrial glycerphosphate dehydrogenase is an important controlling factor in sorbitol and particularly xylitol utilisation. Experimental concepts used by other investigators, using different systems, have been reapplied here to describe both the overall similarity in, and the minor differences between the hepatic metabolism of xylitol and sorbitol. These findings, in isolated rat hepatocytes, have important implications in later experiments on oxalate synthesis from these polyols.

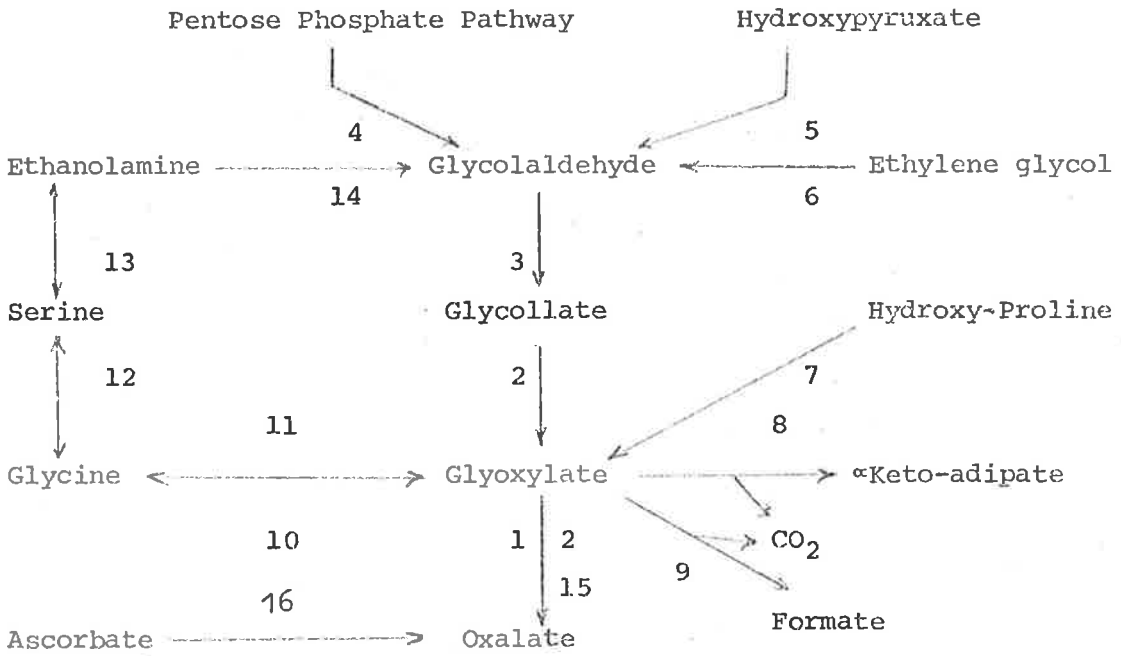
CHAPTER FOUR

THE HEPATIC SYNTHESIS OF OXALATE

INTRODUCTION

i) General Aspects of Oxalate Biosynthesis

Oxalate, in mammals, is an inert metabolic end product. Ingested oxalate is usually poorly absorbed from the gastrointestinal tract and hence urinary oxalate is largely of metabolic origin. The major pathways leading to oxalate synthesis have been reviewed by Watts (1973) and Williams and Smith (1972) and are shown below.



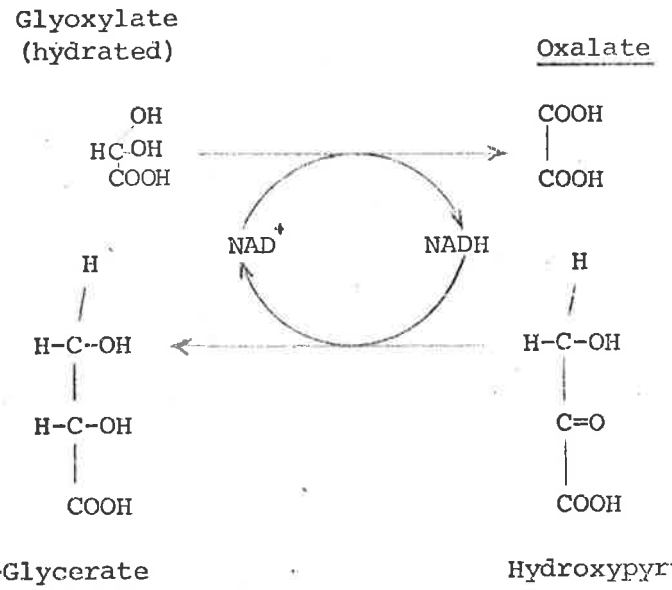
1. Lactate dehydrogenase
2. Glycollate oxidase
3. Aldehyde dehydrogenase
4. Transketolase
5. Pyruvate oxidase
6. Alcohol dehydrogenase
7. ∝-Keto-∝hydroxyglutarate dehydrogenase
8. Glyoxylate —- ∝ketoglutarate carboligase
9. H_2O_2 decarboxylation
10. Transaminases
11. D-amino acid oxidase
12. Serine hydroxymethyl transferase
13. Transaminase
14. traminase
15. Xanthine Oxidase
16. L-ascorbate oxidase

ASCORBIC ACID

Studies using radioisotopes have established that ascorbate is an oxalate precursor in man, rats and guinea pigs; accounting for up to 50% of the daily oxalate excretion in man (Curtin and King 1955, Baker et al 1966, Hellman and Burns 1958). However the injestion of large doses of ascorbate did not appear to have any adverse effect, nor did it significantly raise the urinary oxalate levels, which indicates that this pathway of oxalate synthesis is operating at a maximal rate under normal conditions (Tachenouchi et al 1966). Recently, the practice of consuming large quantities of ascorbate has become increasingly common, and some cases of oxaluria associated with this practice have been seen (Briggs 1976). Nevertheless, ascorbate consumption does not appear to lead to the pathological states observed with xylitol, ethylene glycol or glycollate administration.

GLYOXYLIC ACID PATHWAY

Glyoxylate is the major precursor of oxalate in most biological systems. At least 17 reactions involving glyoxylate are known (Williams and Smith 1972), with the major reactions in mammalian oxalate biosynthesis being outlined on the preceding page. These reactions include reduction to glycollate, transamination to glycine, decarboxylation to carbon dioxide and oxidation to oxalate. This latter reaction is usually the least significant quantitatively. However, under conditions where the enzymes of transamination or carboligation have reduced activity (i.e. associated with pyridoxine or thiamine deficiency respectively, Gershoff 1964, Liang 1962), then oxalate production from glyoxylate becomes of increasing quantitative and pathological importance. Similarly, inborn errors can increase oxalate biosynthesis. Carboligase may be lacking completely, as in primary hyperoxaluria type I, (Kock et al 1967), or the variant, type II, where the absence of D-glycerate dehydrogenase causes the increased formation of L-glycerate from hydroxypyruvate. It is postulated that this reductive step facilitates the oxidation of glyoxylate to oxalate catalysed by lactate dehydrogenase and NAD^+ (Williams and Smith 1971). This relationship is depicted in the following diagram.



This example suggests that the intracellular redox state can be a significant factor in oxalate biosynthesis. This point has increased relevance when considering the association between xylitol and oxalate, for xylitol is known to perturb the intracellular $NADH/NAD^+$ ratio.

GLYCINE AND HYDROXYPROLINE

In man, glycine is an important source of urinary oxalate. Glycine can be converted to glyoxylate by D-amino acid oxidase, which has high activity in mammalian kidney (Niems and Hellerman 1962). The equilibria of enzymes which transaminate glyoxylate to glycine do not favour glyoxylate production (Rowsell et al 1972) hence enzymic transamination of glycine to glyoxylate seems to be of little significance in oxalate production. An alternative pathway exists from glycine to oxalate, involving serine, ethanolamine, glycolaldehyde and glycollic acid. The differential conversion of $|1\text{-}^{13}C|$ glycine and $|2\text{-}^{13}C|$ glycine to oxalate in a primary hyperoxaluric patient corresponds with the two suggested pathways (Dean et al 1967). Furthermore, in rat liver, $|3\text{-}^{14}C|$ serine was a better precursor of $|^{14}C|$ oxalate than $|1\text{-}^{14}C|$ serine, suggesting that the major metabolic pathway from serine to oxalate does not involve glycine as an intermediate (Liao and Richardson 1972). As observed with ascorbate, the administration of large doses of glycine to normal and hyperoxaluric patients did not increase oxalate excretion or cause crystal formation (Archer et al 1958). This evidence would indicate that this metabolic route is not significant in the production of oxalate from xylitol. Similar considerations hold for hydroxyproline, which can be converted to oxalate by α ketohydroxyglutarate aldolase. However this enzyme has low activity in human tissue, which suggests a

low capacity for this pathway (Payes and Laties 1963). Furthermore, administration of hydroxyproline to hyperoxaluric subjects failed to increase oxalate excretion (Williams and Smith 1972), though it could be suggested that the oxalate biosynthetic pathway was already sarurated in these patients.

GLYCOLLIC ACID

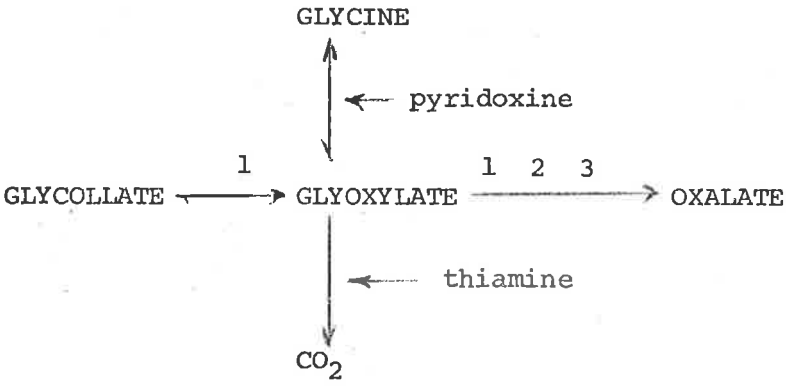
Whilst glycine and hydroxyproline are non-toxic, the administration of glycollic acid to rats at relatively low levels (1-2%) can result in oxalosis and death (Silbergeld and Carter 1959). The only established reaction of glycollate in mammals is oxidation to glyoxylate which is catalysed by a flavin linked glycollate oxidase (Kun et al 1954, Schuman and Massey 1971, Liao and Richardson 1973). While lactate dehydrogenase can reduce glyoxylate to glycollate the oxidative step is essentially inoperative (Warren 1970). Apart from glyoxylate, glycolaldehyde is the only other known precursor of glycollate, being produced from ethanolamine, hydroxypyruvate, ethylene glycol, (Kun et al 1954, Fonseca-Wollheim 1962, Parry and Wallach 1974) or via a glycolaldehyde—thiamine pyrophosphate intermediate of the transketolase reaction (Holzer et al 1962, Datta and Racker 1959, Prochoroff et al 1962).

ii) THE HEPATIC SYNTHESIS OF OXALATE FROM GLYCOLLATE AND GLYOXYLATE

Glycollate and glyoxylate are the major oxalate precursors in man and animals. Glycollate has special significance in this study as increased levels of this compound have been detected in the urine of patients receiving intravenous xylitol (Chalmers et al 1975 , Hauschildt et al 1976a)

Some controversy still surrounds the relative contributions of glycollate oxidase, xanthine oxidase and lactate dehydrogenase to oxalate synthesis. That glycollate oxidase or related L-αhydroxy acid oxidases catalyse the oxidation of glycollate to glyoxylate appears well established (Ushijima 1973, McGroarty et al 1974, Liao and Richardson 1972). The further oxidation of glyoxylate however, can be catalysed by glycollate oxidase, xanthine oxidase or lactate dehydrogenase. Gibbs and Watts (1966) have demonstrated that xanthine oxidase is of minor importance in oxalate synthesis in man and the rat. In contrast, lactate dehydrogenase has often been suggested as the major enzyme of oxalate synthesis (Williams and Smith 1972, Gibbs and Watts 1973, Gibbs et al 1977). Liao and Richardson (1973), however, have presented evidence that it is glycollate oxidase rather than lactate dehydrogenase which is the key enzyme in the hepatic production of oxalate from glyoxylate. It is probable that these differing conclusions have arisen from the use of varying experimental systems, ie rat liver or blood supernatant fractions versus perfused rat livers, respectively. The question can also be raised as to the physiological significance of a reaction such as the lactate dehydrogenase catalysed oxidation of glyoxylate, which is only demonstrable in the presence of high concentrations of substrate and cofactor, and in the absence of likely competing substrates.

Pyridoxine deficiency will increase oxalate synthesis *in vivo* (see Watts 1973) yet the mechanism of this effect is supposed rather than demonstrated. For instance, it is presumed that this deficiency causes a reduction in the transamination of glyoxylate to glycine and thereby increases the level of glyoxylate available for oxidation to oxalate. However, whilst glyoxylate gave rise to more oxalate than glycollate in normal rats, this trend was reversed during pyridoxine deficiency (Runyan and Gershoff 1965, Coryell et al 1961). These findings are not consistent with the following generally accepted scheme.



1. GLYCOLLATE OXIDASE
2. LACTATE DEHYDROGENASE
3. XANTHINE OXIDASE

If transamination of glyoxylate is diminished, one would expect oxalate synthesis from this precursor to be increased to at least the same extent as that seen with glycollate. This was not observed in the above studies. Another observation that is not consistent with the above scheme is that at low substrate concentrations, glycollate is a more effective oxalate precursor than glyoxylate (Liao and Richardson 1972).

In this study, the hepatic synthesis of oxalate from glycollate and glyoxylate will be investigated with particular emphasis on the controversial areas described above.

A OXALATE SYNTHESIS FROM GLYCOLLATE AND GLYOXYLATE IN RAT HEPATOCYTES

INTRODUCTION

The liver has been identified as a major site of oxalate synthesis in the rat (Liao and Richardson 1972, Richardson 1973). Numerous studies on hepatic oxalate synthesis have either used high speed supernatant fractions (Smith et al 1972, Williams and Smith 1971) or particulate fractions (Crawhall and Watts 1962). The perfused rat liver is possibly the most physiological system used to date in this area of investigation (Liao and Richardson 1972) and therefore raises the possibility that isolated rat hepatocytes may be an equally effective, and perhaps a more easily manipulated experimental system for studying oxalate biosynthesis.

This section describes oxalate synthesis from glyoxylate and glycollate in isolated rat hepatocytes and includes investigations of the effects of enzyme inhibitors, vitamin antagonists and altered cellular redox states.

RESULTS

1. THE EFFECT OF SUBSTRATE CONCENTRATION

Isolated rat hepatocytes produced oxalate from glycollate and glyoxylate at rates comparable to those reported by Liao and Richardson (1972) for perfused rat liver. Maximal oxalate production occurred at substrate concentrations of 1 mM glycollate and 15 mM glyoxylate (fig 9). Double reciprocal plots of the results from a similar experiment gave apparent Km values of 0.7 and 10 mM for oxalate production from glycollate and glyoxylate respectively. It should be noted that the apparent Km values seen here may be influenced by the rate of entry into the cell of glyoxylate and glycollate. Liao and Richardson (1972) found that the rate of glycollate uptake by the liver was less than that observed with glyoxylate.

Whilst glyoxylate was many times more effective than glycollate as an oxalate precursor at high substrate concentrations (>5 mM), this difference was only two fold at 1 mM substrate concentration (fig 10). Glyoxylate remained the most effective oxalate precursor at concentrations as low as 0.2 mM. Glycine at 1 and 2 mM was a poor oxalate precursor in hepatocytes, with less than 0.05% of the added radioactivity being recovered as oxalate in 30 minutes (results not included). Liao and Richardson (1972) have described a similar low rate of oxalate production from glycine in the perfused rat liver.

A significant correlation was observed between $|^{14}C|$ oxalate and $^{14}CO_2$ production from $|1\text{-}^{14}C|$ glycollate but not from $|U\text{-}^{14}C|$ glyoxylate (Figure 10). In control experiments, it was observed that significant quantities of $^{14}CO_2$ were produced from $|U\text{-}^{14}C|$ glyoxylate in the presence of boiled cell extracts. This non-enzymic, or heat stable $^{14}CO_2$ production was not due to any of the preparative procedures, but was due to a factor in the cells. As this effect sometimes accounted for the total $^{14}CO_2$ produced by viable cells, non enzymically produced $^{14}CO_2$ from $|U\text{-}^{14}C|$ glyoxylate has been included in the results. This effect, possibly due to hydrogen peroxide, undoubtedly contributed to the lack of correlation between CO_2 and oxalate produced from this precursor. Non enzymic degradation of glycollate was not observed, indicating that an initial enzymic step is a prerequisite for this decarboxylation process.

All results have been corrected for endogenous $|^{14}C|$ oxalate present in

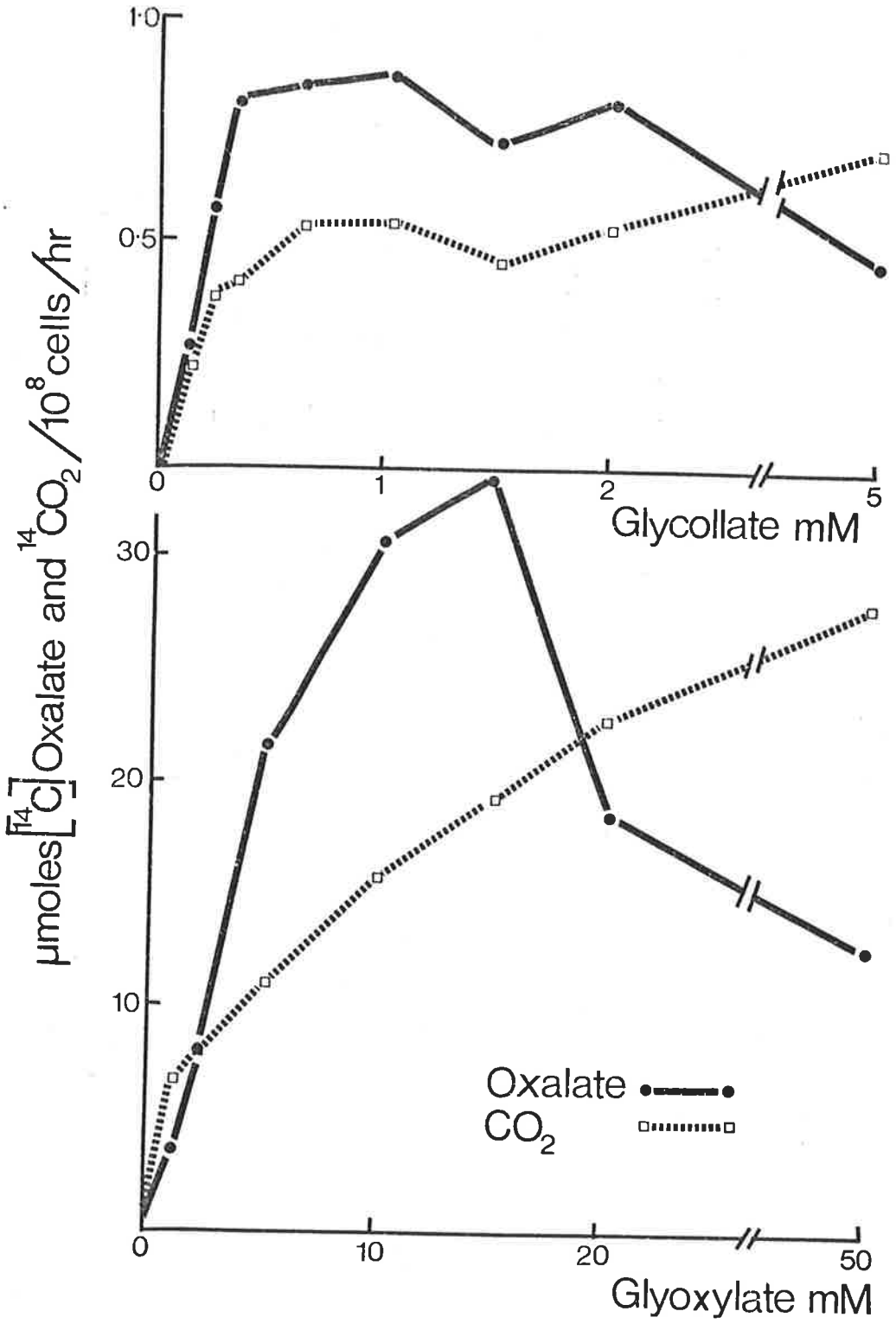


Fig. 9. The effect of varying the glycollate and glyoxylate concentrations on oxalate synthesis in isolated rat hepatocytes. Details of the incubation appear in the methods, chapter 2. The incubation time was 20 minutes.

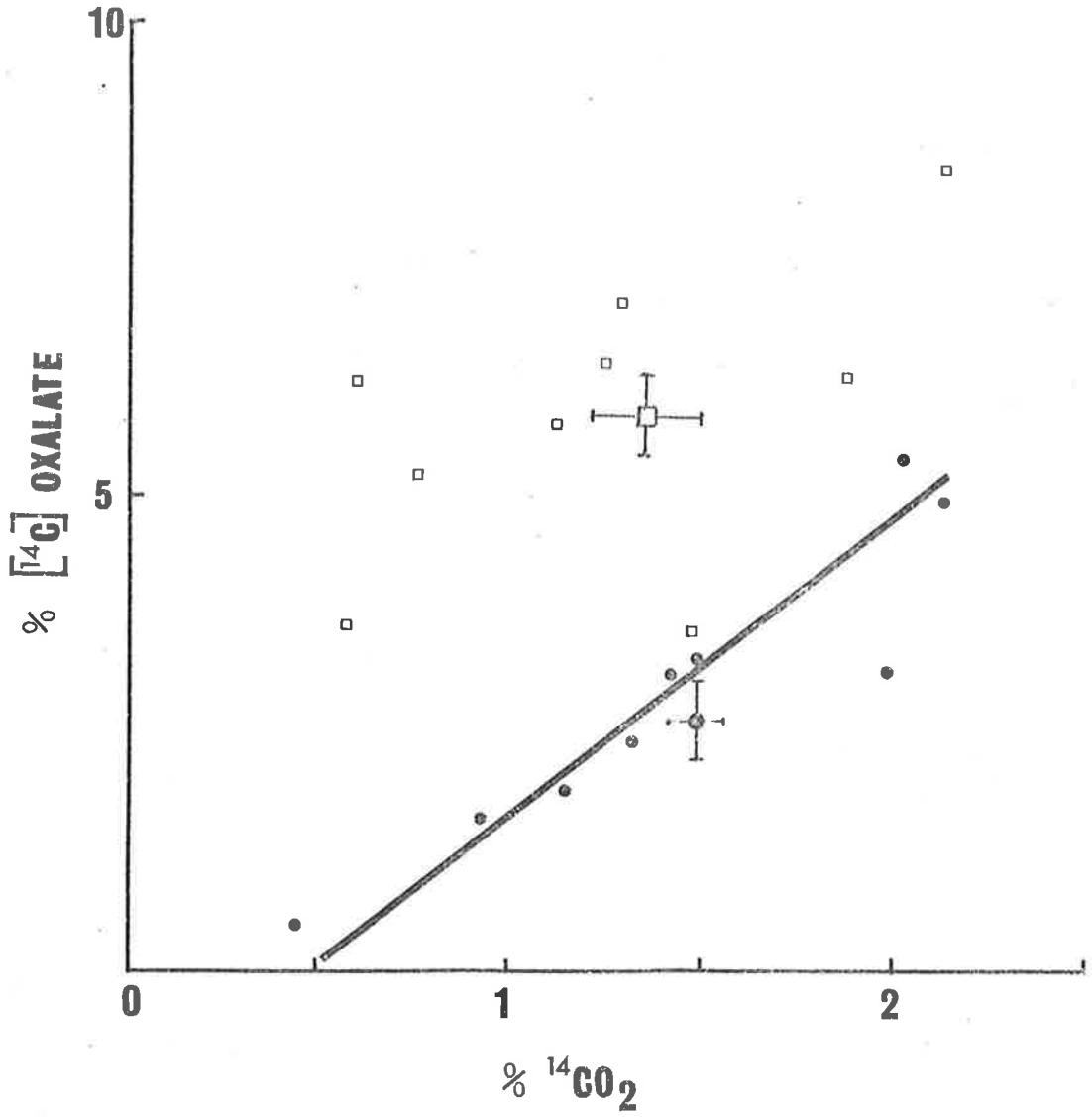


Fig. 10. Correlation between oxalate and CO_2 production from glyoxylate (open squares, r = 0.548, not significant) and glycollate (closed circles, r = 0.926, p 0.001). The large symbols represent the mean ± S.E.M. of 9 independent hepatocyte isolations.
The glyoxylate and glycollate concentrations were 1 mM and the incubation time was 30 minutes. Further details are given in the methods, chapter 2.

commercial $|U\text{-}^{14}C|$ glyoxylate and $|1\text{-}^{14}C|$ glycollate preparations. The brief boiling procedure used to lyse the cells did not oxidise or decarboxylate labelled glycollate, glyoxylate or oxalate. Recovery of $|U\text{-}^{14}C|$ oxalate and residual $^{14}CO_2$ was greater than 90% and 85% respectively, using the assay system described. It should be stressed that the measurement of labelled CO_2 does not take into account any losses that occur during the hepatocyte incubation. The data, while being reproducable, is actually a semiquantitative measure of residual CO_2 and represents less than half that actually produced metabolically by the hepatocyte. Whilst considerable variation in $|^{14}C|$ oxalate and $^{14}CO_2$ production can occur between different cell preparations (fig 10) the reproducibility within the one preparation was 6% and 15% for $|^{14}C|$ oxalate and $^{14}CO_2$ determinations respectively. Therefore, comparative experiments were usually conducted on the one liver cell preparation.

2. THE EFFECT OF METABOLIC INTERMEDIATES ON OXALATE SYNTHESIS

Various metabolic intermediates appear to be involved in oxalate biosynthesis. L-alanine participates in transamination reactions with glyoxylate (Rowsell et al 1972), α-ketoglutarate is involved in the decarboxylation of glyoxylate (Koch et al 1967), DL-phenyllactate is a competitive inhibitor of glycollate oxidase (Liao and Richardson 1973), and hydroxypyruvate increases oxalate synthesis from glyoxylate in cell free systems (Williams and Smith 1971). When incubated with isolated hepatocytes in the presence of labelled glyoxylate and glycollate, these compounds markedly effected oxalate production from $|1\text{-}^{14}C|$ glycollate but not from $|U\text{-}^{14}C|$ glyoxylate, as shown in Table 3. The stimulation of oxalate production from glycollate but not glyoxylate by the addition of 5 mm hydroxypyruvate, conflicts with the evidence of Williams and Smith (1971). This effect will be further investigated as it implies both a differential effect of redox and independent routes for glycollate and glyoxylate oxidation. Hydroxypyruvate may be acting as a physiological hydrogen acceptor in addition to being a competitive substrate in reactions involving glyoxylate. DL-phenylalanine may, like DL-phenyllactate, act as a competitive inhibitor of glycollate oxidase.

3. THE EFFECT OF OXAMATE AND DL-PHENYLLACTATE ON OXALATE SYNTHESIS

Glycollate oxidase and lactate dehydrogenase are key enzymes in oxalate

TABLE 3

THE EFFECT OF METABOLIC INTERMEDIATES ON $|^{14}C|$ OXALATE PRODUCTION FROM $|1\text{-}^{14}C|$ GLYCOLLATE AND $|U\text{-}^{14}C|$ GLYOXYLATE IN ISOLATED RAT HEPATOCYTES

		% $|^{14}C|$ Oxalate from	
	mM	$|U\text{-}^{14}C|$ Glyoxylate	$|1\text{-}^{14}C|$ Glycollate
Control	-	8.5	5.5
α-ketoglutarate	1.0	6.0	5.0
L-alanine	1.0	5.4	3.1
DL-phenylalanine	1.0	5.4	1.3
DL-phenyllactate	12.5	5.0	1.6
Hydroxypyruvate	5.0	6.5	21.7

The hepatocyte incubations contained 1 mM glycollate or glyoxylate and 5 mM glucose. All additions were made simultaneously, and the incubation time was 30 minutes. Further details appear in the methods, chapter 2.

synthesis. Both glycollate and glyoxylate are oxidised by the flavin linked glycollate oxidase, a reaction which is inhibited competitively by DL-phenyllactate (Liao and Richardson 1973). Glyoxylate is also oxidised by lactate dehydrogenase a reaction which is inhibited by oxamate. This compound has no effect on glycollate oxidase *in vitro* (Smith et al 1972). DL-phenyllactate on the other hand does not influence the lactate dehydrogenase catalysed oxidation of glyoxylate (Liao and Richardson 1973). The effect of these inhibitors on oxalate synthesis in hepatocytes is shown in fig 11. DL-phenyllactate is clearly the most effective inhibitor of oxalate synthesis from both precursors. This result supports the conclusions of Liao and Richardson (1973) that glycollate oxidase is the most important enzyme in hepatic oxalate synthesis. Other aspects of the effect of these inhibitors will be investigated more fully in subcellular investigations as it is possible that oxamate may have limited entry into the cell.

4. THE EFFECT OF AMINO-OXYACETATE ON TRANSAMINATION AND OXALATE PRODUCTION

Amino-oxyacetate (AOA) is an inhibitor of mammalian transaminases, the inhibition being competitive with respect to the amino acid and uncompetitive with the keto acid. Alanine ketoglutarate aminotransferase (ALT) is 100 fold more sensitive to inhibition by AOA than aspartate ketoglutarate amino transferase (AST) (Hopper and Segal 1964). Alanine glyoxylate amino transferase (GLT) and ornithine glyoxylate aminotransferase may control oxalate synthesis by regulating the level of glyoxylate available for oxidation to oxalate. GLT may be the most important enzyme in this context as its activity in mammalian liver is many fold that of the other aminotransferases (Thompson and Richardson 1967, Rowsell et al 1972).

Prior to the use of amino-oxyacetate (AOA) in hepatocyte preparations, the sensitivity of transaminases to inhibition by AOA was investigated (Table 4). The order of sensitivity was alanine : glyoxylate aminotransferase (GLT) > alanine : ketoglutarate aminotransferase (ALT) > aspartate : ketoglutarate aminotransferase (AST). The degree of sensitivity with respect to the AOA concentration required to give 90% inhibition was 10,000 : 700 : 1 / GLT : ALT:AST. This difference in susceptibility of ALT and AST to inhibition by AOA agrees with the results of Hopper and Segal (1964). The extreme sensitivity of GLT to inhibition by AOA has, to my knowledge, not been reported previously. Clearly then, the use of this inhibitor has possible

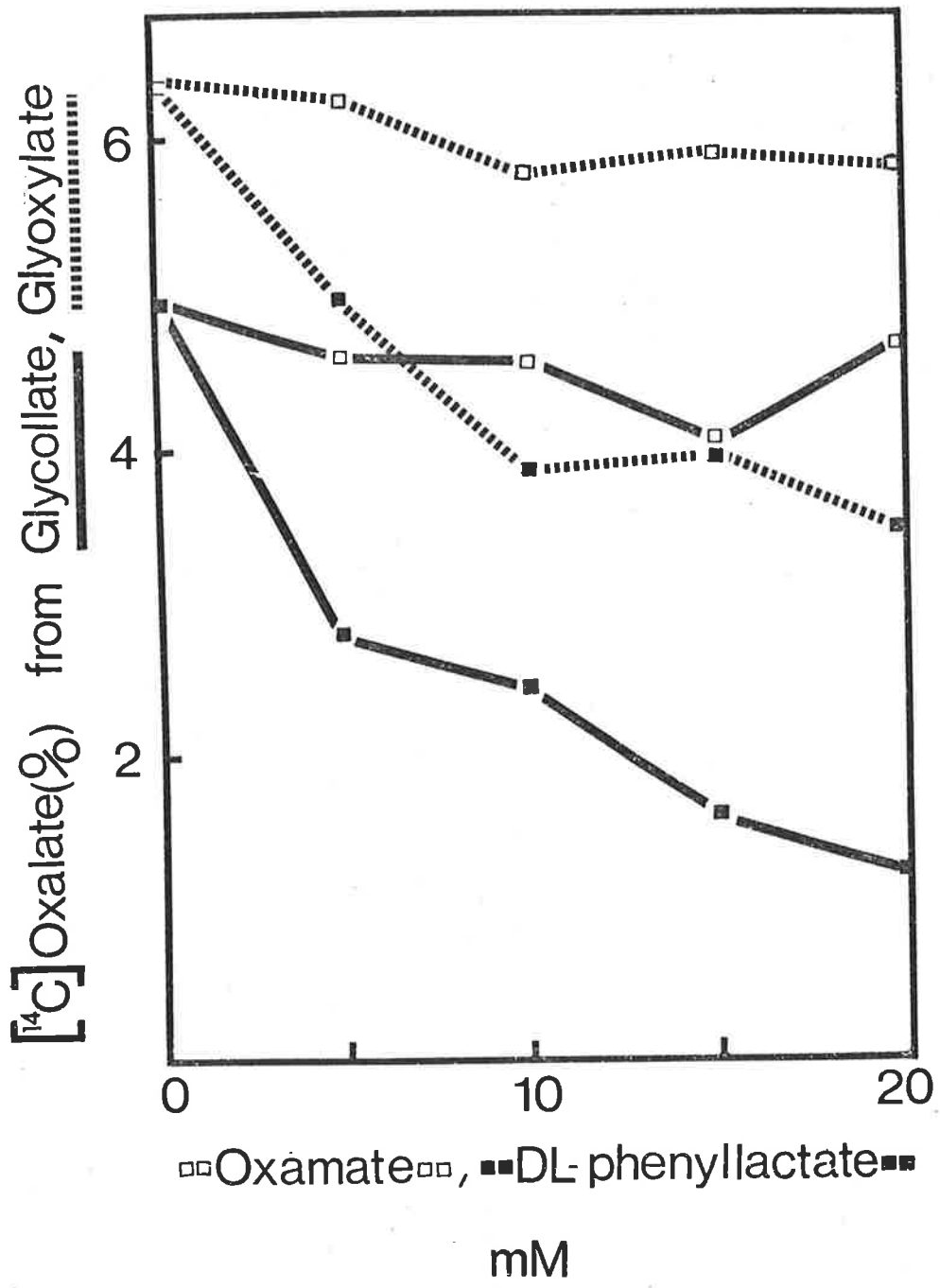


Fig. 11. Effect of oxamate and DL-phenyllactate on oxalate synthesis from labelled glyoxylate and glycollate in isolated rat hepatocytes. The concentration of glyoxylate and glycollate was 1 mM, and the incubation time was 30 minutes. Details of the incubation appear in the methods, chapter 2.

TABLE 4

THE EFFECT OF AMINO OXYACETATE ON LIVER TRANSAMINATION REACTIONS

AMINO TRANSFERASE		AMINO OXYACETATE CONCENTRATION(M) FOR 50% INHIBITION	90% INHIBITION
Aspartate : αKetoglutarate	AST	1.7×10^{-4}	1.0×10^{-3}
Alanine : αKetoglutarate	ALT	5.0×10^{-7}	1.5×10^{-6}
Alanine : Glyoxylate	GLT	1.5×10^{-8}	1.0×10^{-7}

Homogenised rat hepatocytes were used as the source of enzyme. Details of the enzyme assays appear in the methods, chapter 2.

advantages over general pyridoxine deficiency.

The effect of AOA on hepatic oxalate synthesis from glycollate and glyoxylate in hepatocytes is shown in fig. 12. Oxalate synthesis from glycollate was stimulated by 1 mM AOA whereas that from glyoxylate was inhibited. It is significant that 10 μM AOA had an effect on the oxidation of glycollate to oxalate, whereas there was no observable effect on oxalate production from glyoxylate at this concentration. This low level of AOA would inhibit ALT and GLT, but not AST. It should be pointed out that AOA can react non enzymically with aldehydes (Smith et al 1977). This reaction, if it is significant, would have little effect when the substrate concentrations are 100 fold those of the inhibitor.

Alanine and glutamate have been identified as contributing substrates in transamination reactions involving glyoxylate (Nakada 1964, Rowsell et al 1972). The effect of AOA on oxalate and CO_2 production from glycollate and glyoxylate in the presence of alanine and glutamate is shown in Table 5. As indicated before, oxalate production from glycollate but not glyoxylate was markedly stimulated by 1 mM AOA. This effect was still apparent, though diminished, in the presence of alanine or glutamate. The production of CO_2 from both glycollate and glyoxylate was inhibited by 1 mM AOA. Alanine appeared to enhance the decarboxylation of both substrates, except in the presence of AOA. It is possible that alanine is transaminated to pyruvate which may facilitate the oxidation of glycollate to oxalate and CO_2 in an analogous manner to that seen with hydroxy-pyruvate (chapter 4, section A5).

Inhibition of the decarboxylation of both substrates by AOA may result from inhibition of transamination or a direct inhibition involving the binding of glyoxylate, the presumed substrate of decarboxylation. Glyoxylate is thought to be decarboxylated via a thiamine pyrophosphate dependent reaction (Stewart and Quayle 1967). However, when pyridoxal phosphate was added to hepatocytes prepared from a pyridoxine deficient rat, (Table 6), the opposite effect of AOA was observed. In this case, decarboxylation of both oxalate precursors was stimulated, whilst oxalate production was decreased. This latter effect is consistent with increased transamination of glyoxylate in the presence of pyridoxal phosphate, but is not consistent with known mechanisms of decarboxylation. These observations suggest that pyridoxine deficiency may mediate its effect on oxalate synthesis by means other than diminished transamination.

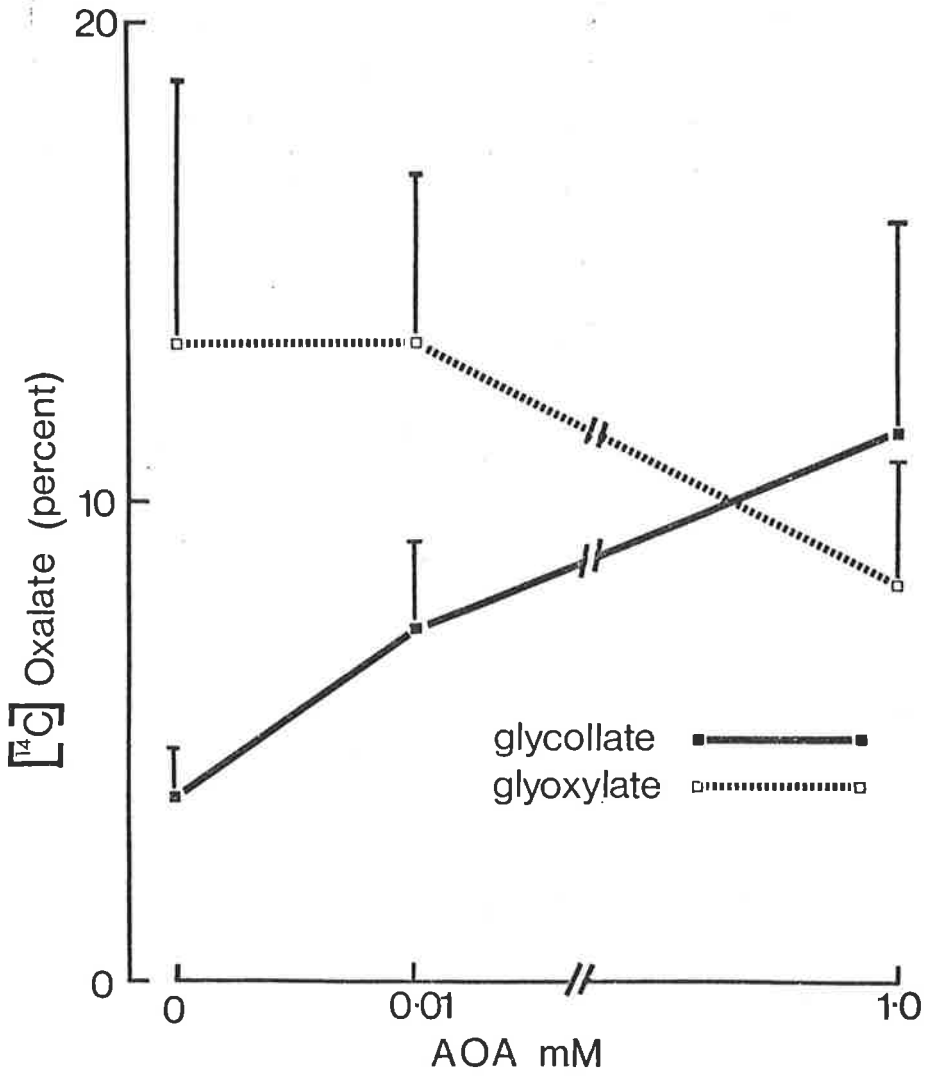


Fig. 12. The effect of aminooxyacetate (AOA) on oxalate synthesis from glycollate and glyoxylate in isolated rat hepatocytes. The concentration of glyoxylate and glycollate was 1 mM and the incubation time was 30 minutes. The results represent the mean ± S.E.M. of 5 independent hepatocyte isolations. Further details appear in the methods, chapter 2.

TABLE 5

THE EFFECT OF AMINOOXYACETATE ON OXALATE AND CO_2 PRODUCTION FROM GLYCOLLATE AND GLYOXYLATE IN HEPATOCYTES

SUBSTRATE	ADDITION	PERCENTAGE CONVERSION TO |^{14}C| OXALATE	$^{14}CO_2$
|U-^{14}C| GLYOXYLATE	-	5.6	3.3
" "	AOA	5.2	1.6
" "	L - Alanine	5.7	5.6
" "	L - Alanine + AOA	3.5	1.2
" "	L - Glutamate	5.6	3.8
" "	L - Glutamate+AOA	3.5	1.3
|1-^{14}C| GLYCOLLATE	-	1.8	6.2
" "	AOA	28.7	3.2
" "	L-Alanine	5.9	10.3
" "	L-Alanine+AOA	15.2	1.7
" "	L-Glutamate	7.5	3.9
" "	L-Glutamate+AOA	10.4	2.1

The concentrations used were; glyoxylate, glycollate and AOA, 1 mM., and alanine and glutamate, 5 mM. Alanine, glutamate or AOA were added to the incubations immediately after the addition of glyoxylate or glycollate. The incubation time was 30 minutes. Further experimental details are given in the methods.

TABLE 6

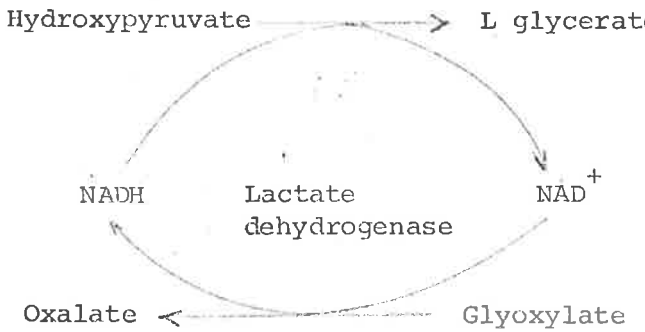
THE EFFECT OF PYRIDOXAL PHOSPHATE ON OXALATE AND CO_2 PRODUCTION FROM GLYCOLLATE AND GLYOXYLATE IN HEPATOCYTES FROM A PYRIDOXINE DEFICIENT RAT

SUBSTRATE	ADDITION	PERCENTAGE CONVERSION TO $\|^{14}C\|$ OXALATE	$^{14}CO_2$
$\|U\text{-}^{14}C\|$ Glyoxylate	-	5.27 ± 0.05	0.67 ± 0.10
$\|U\text{-}^{14}C\|$ Glyoxylate	Pyridoxal Phosphate	5.05 ± 0.31	3.01 ± 0.31
$\|1\text{-}^{14}C\|$ Glycollate	-	3.24 ± 0.16	1.30 ± 0.11
$\|1\text{-}^{14}C\|$ Glycollate	Pyridoxal Phosphate	2.16 ± 0.06	3.79 ± 0.05

Incubations contained 1 mM glycollate, glyoxylate and pyridoxal phosphate, and 5 mM glucose. The incubation time was 30 minutes. The results represent the average of duplicate experiments on the one hepatocyte preparation. Further details appear in the methods, chapter 2.

5. EFFECT OF HYDOXYPYRUVATE ON OXALATE PRODUCTION

Hydroxypyruvate has been implicated as a causative factor in type II hyperoxaluria which is characterised by hyperglyceric aciduria (Williams and Smith 1971). It is postulated that hydroxypyruvate accumulates due to a deficiency in D-glycerate dehydrogenase and that this compound can act as the hydrogen acceptor in a coupled reaction involving lactate dehydrogenase and glyoxylate as shown below



Williams and Smith (1971) have shown that this coupled reaction occurs in rat liver supernatant, with hydroxypyruvate stimulating oxalate synthesis from glyoxylate in the presence of NADH. With these findings in mind it was decided to test the effect of hydroxypyruvate on oxalate synthesis in a physiological system, such as used here.

Contrary to the above findings stimulation of oxalate synthesis from glyoxylate by rat hepatocytes was not observed in the presence of hydroxypyruvate. As shown in fig. 13, inhibition of oxalate synthesis from this precursor occurred when the concentration of hydroxypyruvate was raised above 5 mM. Oxalate synthesis from glycollate by hepatocytes was stimulated by hydroxypyruvate; the critical concentration for this effect apparently being between 2.5 and 5.0 mM hydroxypyruvate (fig. 14).. The hepatic production of $^{14}CO_2$ from labelled glycollate and glyocylate was dramatically increased by the addition of unlabelled hydroxypyruvate (fig. 13). This effect was observed using hydroxypyruvate concentrations as low as 1 mM (fig. 14).

Preliminary studies with rat liver homogenates indicate that hydroxypyruvate does not stimulate oxalate synthesis from glycollate, which suggests that maintainance of the cellular integrity is essential for the demonstration of the above phenomena. Almost complete inhibition of oxalate synthesis from glycollate occurred with 5 mM hydroxypyruvate in cell free extracts

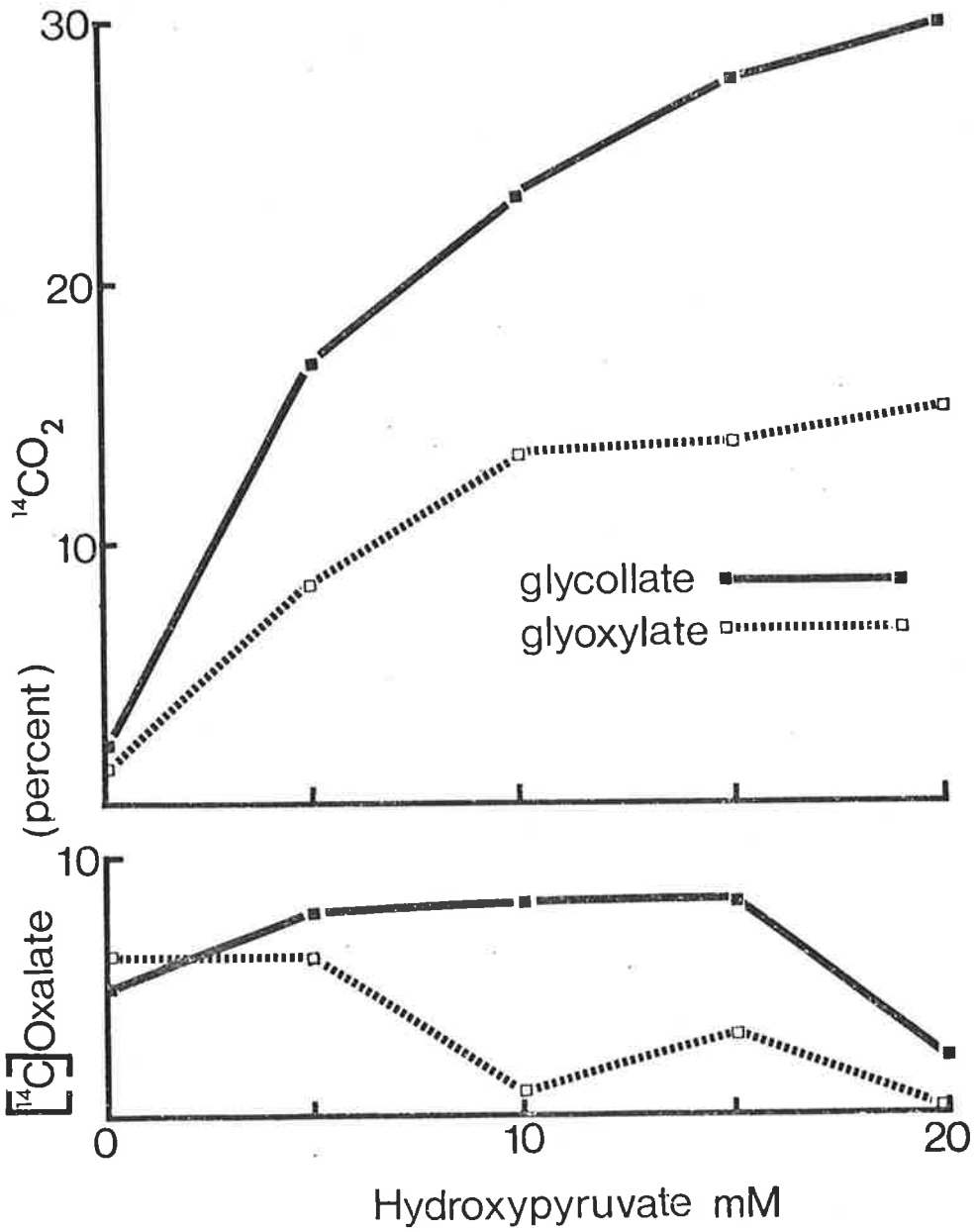


Fig. 13. The effect of hydroxypyruvate on oxalate and CO_2 production from glycollate and glyoxylate in rat hepatocytes. The concentration of glyoxylate and glycollate was 1 mM and the incubation time was 30 minutes. Further details appear in the methods, chapter 2.

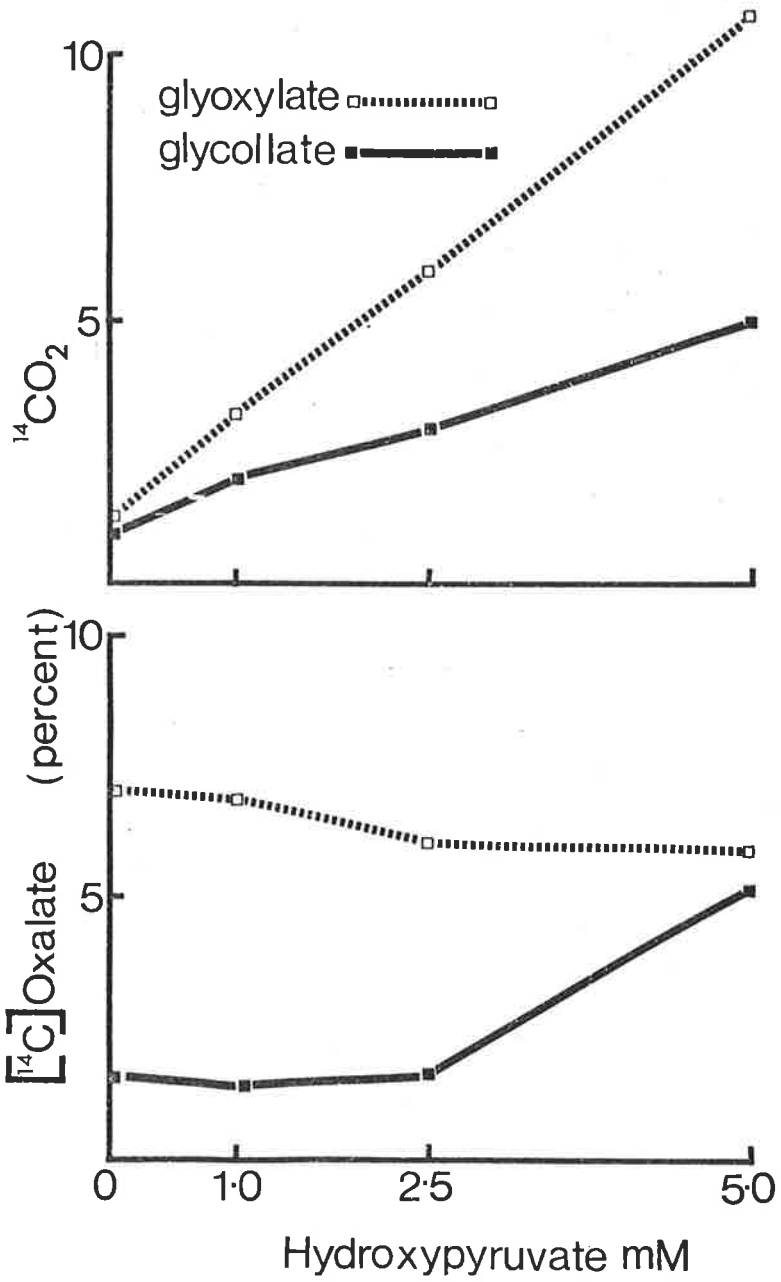


Fig. 14. The effect of hydroxypyruvate on oxalate and CO_2 production from glycollate and glyoxylate in rat hepatocytes. The concentration of glyoxylate and glycollate was 1 mM, and the incubation time was 30 minutes. Further details appear in the methods, chapter 2.

from rat liver, which is contrary to the effects observed in hepatocytes. However, the effect of hydroxypyruvate on oxalate and CO_2 production from glyoxylate in these extracts was consistent with those observed with hepatocytes (results not included).

6. COMPETITION BETWEEN GLYCOLLATE AND GLYOXYLATE IN OXALATE SYNTHESIS

The effect of unlabelled glycollate on $|^{14}C|$ oxalate synthesis from $|U^{14}C|$ glyoxylate, and vice versa, is shown in table 7. The inhibition of $|^{14}C|$ oxalate synthesis from $|U^{14}C|$ glyoxylate by unlabelled glycollate may be due to competition between the two oxalate precursors. The assay of $|^{14}C|$ oxalate using oxalate decarboxylase is unaffected by an increase in unlabelled oxalate so the results seen are not due to an excess of oxalate. It is also unlikely that unlabelled glyoxylate produced from glycollate is the reason for the inhibitory effect of glycollate on oxalate synthesis from labelled glyoxylate, as glyoxylate is not saturating at this concentration (1 mM).

As glyoxylate is presumed to be the intermediate in the oxidation of glycollate to oxalate, it was thought that the addition of unlabelled glyoxylate may have diminished $|^{14}C|$ oxalate produced from $|^{14}C|$ glycollate. The finding of a stimulation in this experiment may be explained by the enhancement of the conversion of the small portion of labelled glyoxylate intermediate to oxalate. Alternatively, the glyoxylate produced as an intermediate in the oxidation of glycollate may remain enzyme bound, or be produced in a cellular compartment which is not readily accessible to added glyoxylate.

7. EFFECT OF ALTERED INTRACELLULAR REDOX STATES ON OXALATE SYNTHESIS

Sorbitol has been shown to cause a marked elevation in the intracellular $NADH/NAD^+$ ratio in isolated hepatocytes (Berry et al 1973), an effect which can be reversed by the addition of phenazine methosulphate (PMS), an artificial electron acceptor (table 8). The effect of this alteration in redox state on oxalate production from glycollate and glyoxylate is shown in Table 9. Phenazine methosulphate stimulated the hepatic production of $|^{14}C|$ oxalate from both precursors, whilst sorbitol produced the opposite effect. These results reflect lowered and raised $NADH/NAD^+$ ratios. Whereas the effect of sorbitol on glyoxylate oxidation to oxalate was completely

TABLE 7

EFFECT OF COMPETING SUBSTRATES ON OXALATE PRODUCTION IN HEPATOCYTES

SUBSTRATE	ADDITION mM		$\lvert^{14}C\rvert$ OXALATE (%)
$\lvert U\text{-}^{14}C\rvert$ GLYOXYLATE 1 mM	-		6.2
	GLYCOLLATE	0.5	5.5
	"	1.0	4.7
	"	2.0	2.8
$\lvert 1\text{-}^{14}C\rvert$ GLYXOLLATE 1 mM	-		4.9
	GLYOXYLATE	0.5	4.4
	"	1.0	5.3
	"	2.0	6.9

Details of the incubations appear in the methods, chapter 2. Incubation time was 30 minutes. Additions to the incubation were made at the same time as that of the labelled substrate.

TABLE 8

THE EFFECT OF GLUCOSE, SORBITOL AND PHENAZINE METHOSULPHATE (PMS) ON THE REDOX STATE OF ISOLATED RAT HEPATOCYTES

SUBSTRATE	TREATMENT	LACTATE / PYRUVATE	αGLYCEROPHOSPHATE / DI HYDROXYACETONE PHOSPHATE
Glucose	-	18.0 ± 5.5	5.0 ± 1.4
	+ PMS	5.7 ± 1.0	2.4 ± 0.6
Sorbitol	-	43.0 ± 12.0	27.0 ± 9.0
	+ PMS	24.0 ± 4.0	5.3 ± 1.2

The concentrations used were, glucose and sorbitol, 10 mM and PMS, 10 uM. The incubation time was 30 minutes. The results are expressed as the mean ± SEM of 3 independent cell isolations. Further details are given in the methods, chapter 2.

TABLE 9

THE EFFECT OF SORBITOL AND PHENAZINE METHOSULPHATE (PMS) ON THE CONVERSION OF $|U\text{-}^{14}C|$ GLYOXYLATE AND $|1\text{-}^{14}C|$ GLYCOLLATE TO $|^{14}C|$ OXALATE

SUBSTRATE	% $|^{14}C|$ OXALATE			
	CONTROL	+PMS	+ SORBITOL	+ PMS + SORBITOL
$|U\text{-}^{14}C|$ Glyoxylate	13.0	19.0	6.8	19.3
$|1\text{-}^{14}C|$ Glycollate	3.8	16.8	1.1	1.6

The concentrations used were, sorbitol 5 mM, PMS 20 µM, and glyoxylate and glycollate 1 mM. The incubation time was 30 minutes. Further details are given in the methods, chapter 2. Incubations not containing sorbitol, contained 5 mM glucose.

reversed by 20 μM PMS, only a partial reversal was seen with glycollate as precursor, indicating that the effect of sorbitol on glycollate oxidation is not entirely due to an elevated $NADH/NAD^+$ ratio. In this latter case the increased levels of lactate or other metabolites produced from sorbitol in the presence of PMS, may inhibit oxalate production from glycollate.

Phenazine methosulphate is known to accept electrons from flavo-proteins (Takemor and King 1964). Glycollate oxidase is a flavin linked enzyme with a higher affinity for glycollate than glyoxylate (Km values 0.24 and 1.41 mM respectively Ushijima 1973). It can therefore be suggested that the greater stimulation by PMS of oxalate production from glycollate, compared with glyoxylate, is a reflection of an enhanced activity of this enzyme. A similar explanation may hold for the marked reduction in the glycerophosphate/dihydroxyacetone phosphate ratio, in the presence of PMS (Table 8). In this case, the mitochondrial glycerophosphate dehydrogenase is possibly the enzyme affected. It should be noted however, that PMS can also accept hydrogen from NAD^+ linked enzymes.

8. THE EFFECT OF SOME AROMATIC COMPOUNDS ON OXALATE SYNTHESIS FROM GLYCOLLATE

Tryptophan, tyrosine and phenylalanine can contribute to urinary oxalate in the rat, though this contribution, in the case of tryptophan is estimated to be less than 3% of the total oxalate excreted by the normal rat (Cook and Henderson 1969, Faragalla and Gershoff 1963, Gambardella and Richardson 1976). Some evidence suggests that tyrosine and phenylalanine are metabolised to oxalate via glycollate, whereas oxalate synthesis from tryptophan only involves glyoxylate as an intermediate (Gambardella and Richardson 1976). These authors used labelled tyrosine, tryptophan and phenylalanine and unlabelled glycollate and glyoxylate in *in vivo* experiments.

In this study, I have briefly examined the effect of some unlabelled aromatic compounds on the conversion of labelled glycollate to oxalate in rat hepatocytes. An additional reason for including this experiment was to illustrate the potential use of hepatocytes to screen inhibitors of oxalate biosynthesis, especially those which will inhibit glycollate oxidase. The results in table 10 show that L-tryptophan and the D-isomers of phenylalanine and phenyllactate were potent inhibitors of oxalate synthesis from glycollate in rat hepatocytes. It is unlikely that the level of unlabelled glycollate

TABLE 10

THE EFFECT OF AROMATIC COMPOUNDS ON OXALATE SYNTHESIS FROM GLYCOLLATE IN HEPATOCYTES

	$\lvert U\text{-}^{14}C \rvert$ GLYCOLLATE	$\lvert ^{14}C \rvert$ OXALATE
ADDITION	PERCENT CONVERSION TO OXALATE	% INHIBITION
-	3.99	0
L-tyrosine	1.11	72
L-tryptophan	0.87	78
L-hydroxyproline	2.19	45
L-phenylalanine	1.70	57
DL-phenylalanine	0.84	79
L-phenyllactate	2.59	35
DL-phenyllactate	0.97	76

The glycollate concentration and all additions were 1 mM. Glucose, 5 mM, was included in all the incubations and the reaction time was 30 minutes. Further experimental details appear in the methods, chapter 2.

produced from these aromatic compounds would cause this effect and so it can be suggested that these compounds are specifically inhibiting glycollate oxidase.

In man, tyrosine has been shown to diminish urinary oxalate (Zinsser and Karp 1973) while DL- phenyllactate will reduce the toxicity of ethylene glycol in rats, as well as inhibiting glycollate oxidase *in vitro* (Liao and Richardson 1973). This approach to the regulation of endogenous oxalate synthesis is interesting in light of the observed correlation between lowered urinary oxalate levels and raised urinary phenyllactate levels in patients with phenylketonuria (Chernoff 1975).

DISCUSSION

Some of the areas of investigation presented here, including the effects of substrate concentrations, the inhibitory effect of oxamate and DL phenyllactate, and the contribution of glycollate oxidase and lactate dehydrogenase to hepatic oxalate biosynthesis will be examined in more detail in the next section.

The present discussion will largely centre on the effects of altered redox states, hydroxypyruvate and aminooxyacetate on hepatic oxalate synthesis, as these effects could only be demonstrated in isolated rat hepatocytes. This observation in itself implies that the maintainance of the cellular integrity is an important factor in understanding the mechanisms involved in oxalate biosynthesis.

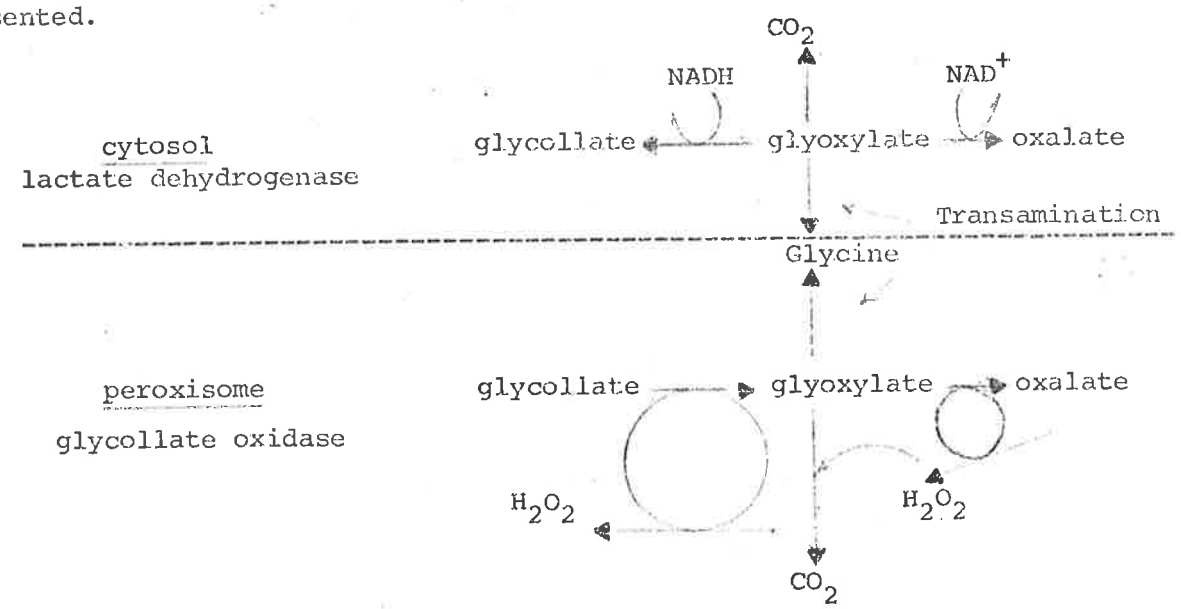
It has been suggested that the intracellular redox state may be important in the control of oxalate biosynthesis (Smith et al 1972). In this present study, this factor was clearly demonstrated, with an oxidised redox state facilitating, and a reduced redox state inhibiting oxalate synthesis from glycollate and glyoxylate. This finding is particularly important with respect to oxalate synthesis from xylitol; an area which will be examined in chapter 5.

The pyridoxine antagonist, aminoxyacetate (AOA) has been used in a number of applications, including the inhibition of gluconeogenesis and urea synthesis in isolated rat hepatocytes (Rognstad and Clarke 1974, Meijer et al 1975). In this investigation it has been demonstrated that AOA may be successfully used to modify hepatic oxalate synthesis. A major drawback in the use of this compound is that it can complex ketones and aldehydes, including pyruvate, acetaldehyde (Smith et al 1977) and most probably glyoxylate. While this is unfortunate, it may in fact reinforce some of the conclusions drawn from these studies. For example, transamination in the oxalate biosynthetic pathway is assumed to occur via glyoxylate. This compound is also presumed to be the intermediate in the oxidation of glycollate to oxalate. However, in the presence of 1 mM AOA, oxalate production from glyoxylate was inhibited, whereas that from glycollate was increased. The inhibition of oxalate synthesis from glyoxylate may be attributed to the complexing of this aldehyde by AOA.

Nevertheless, if glyoxylate is a free intermediate in the oxidation of

glycollate, inhibition of oxalate production from glycollate should also have occurred in the presence of AOA. Clearly this situation does not hold as demonstrated in fig.12 and table 5.

A similar conclusion can be reached when considering the differential effects of hydroxypyruvate on oxalate production from glycollate and glyoxylate. That is, if glyoxylate is a product of glycollate oxidation, then at high concentrations of hydroxypyruvate, inhibition of oxalate synthesis from glycollate, as seen with glyoxylate, should have occurred instead of the observed stimulation. In an attempt to explain these findings, the following compartmentalised scheme for hepatic oxalate synthesis is presented.



This scheme, which will be expounded further in the discussion on subcellular studies (Chapt. 4B and fig. 20), is based on the following observations. Lactate dehydrogenase is a cytosolic enzyme whereas glycollate oxidase, the other major enzyme in oxalate biosynthesis, is located in the peroxisomes (McGroarty et al 1974, Hand 1975). These organelles are also the major site of catalase, D-amino acid oxidase, urate oxidase and some α-hydroxy acid oxidases of which glycollate oxidase is an example (de Duve and Baudhuin 1966). It has also been shown that peroxisomes contain specific aminotransferases which are capable of forming glycine from glyoxylate (Vandor and Tolbert 1970, Hsieh and Tolbert 1976). Furthermore, peroxisomes are also a major site of hydrogen peroxide generation (de Duve and Baudhuin 1966) a factor which is important in the decarboxylation of glyoxylate and glycollate (Halliwell and Butt 1974). A recent report by Gibbs et al (1977) supports the concept of the peroxisome as a site of oxalate synthesis. From this proposed scheme,

a number of suggestions can be put forward to explain the results presented here.

If glyoxylate is produced as an intermediate in glycollate oxidation, but remains enzyme or membrane associated within the peroxisome, then this glyoxylate will probably be metabolised in a different manner than glyoxylate added externally to the hepatocyte. This could explain the differential effect of AOA on oxalate production from glycollate and glyoxylate. The glyoxylate added to the cell may be more easily complexed with AOA than the glyoxylate produced from glycollate in a coupled oxidative sequence within the peroxisome.

A similar consideration may hold for the effect of hydroxypyruvate in these experiments. While hydroxypyruvate can be transaminated to serine, it is unlikely that the small amount of oxalate produced from serine would contribute to the effects seen here (Liao and Richardson 1972). Furthermore, Williams and Smith (1971) report that $|^{14}C|$ hydroxypyruvate was not incorporated into urinary oxalate in a patient with L-glyceric aciduria. Hydroxypyruvate may act as a physiological hydrogen acceptor within the cell and stimulate oxalate synthesis by causing a more oxidised cellular redox state. However, hydroxypyruvate, like glyoxylate, is a substrate for lactate dehydrogenase and thus may compete with added glyoxylate resulting in less conversion of this precursor to oxalate. Therefore the effects of hydroxypyruvate on oxalate synthesis may be explained as being a redox effect with glycollate as substrate, and competitive inhibition with glyoxylate as substrate.

Whilst the differential effect of AOA and hydroxypyruvate on oxalate synthesis from the two oxalate precursors necessitated the postulate of an enzyme or peroxisomal associated "glyoxylate" intermediate, the same postulate is not necessary to explain the effects of AOA and hydroxypyruvate on CO_2 production. As demonstrated in this communication, AOA inhibits CO_2 production from both glycollate and glyoxylate, whereas hydroxypyruvate caused a reverse effect. These results can be explained if one assumes that decarboxylation proceeds via free glyoxylate. The inhibition of CO_2 production by AOA could be a result of complex formation between glyoxylate and AOA (Smith et al 1977). The stimulation of decarboxylation by hydroxypyruvate may arise in several different ways:
(a) competition with glyoxylate for lactate dehydrogenase and thereby increasing the level of glyoxylate available for decarboxylation; (b) the

production of a metabolite from hydroxypyruvate which aids in the synergistic decarboxylation of glyoxylate; (c) the increased production of hydrogen peroxide via the flavin linked oxidation of L-glycerate which is the product of NADH catalysed reduction of hydroxypyruvate by lactate dehydrogenase. These possibilities can only be suggested at this stage.

It is clear that the effect of hydroxypyruvate is more complicated than a mere coupled redox effect on glyoxylate oxidation, an effect which was not demonstratable in this study. The inability to show increased oxalate synthesis from glyoxylate in the presence of hydroxypyruvate can be explained in terms of competition between glyoxylate and hydroxypyruvate in both the reductive and oxidative steps. Preliminary experiments with cell free extracts indicate that, in the absence of added cofactors, hydroxypyruvate will stimulate CO_2 but not oxalate production from glyoxylate. It is not clear whether this stimulation of CO_2 production was recognised by Williams and Smith (1971). If blank assays (minus oxalate decarboxylase) are not performed for each individual oxalate assay then the amount of oxalate formed will be grossly overestimated.
In purified lactate dehydrogenase or erythrocyte preparations with added NADH, a redox coupling of hydroxypyruvate reduction and glyoxylate oxidation does occur, as no decarboxylation would be expected in these fractions. That a similar phenomenum exists in the liver is clearly disputed. From these results it could be speculated that the mechanism of hyperoxaluria, associated with increased L-glycericaciduria, may involve glycollate, not glyoxylate, as the key oxalate precursor.

B. SUBCELLULAR STUDIES ON OXALATE SYNTHESIS FROM GLYCOLLATE AND GLYOXYLATE IN RAT LIVER

INTRODUCTION

A major problem with the use of hepatocytes is that substrates, cofactors and inhibitors may have limited entry into the cell. *In vitro* cell free systems, as well as allowing easier access of these factors to catalytic sites, also permit the isolation and characterisation of specific enzyme activities. However, as will be discussed later, the physiological importance of such observations must be weighed carefully in terms of the conditions employed.

This section examines in detail the relative importance of glycollate oxidase and lactate dehydrogenase in hepatic oxalate synthesis. The former enzyme, together with xanthine oxidase constitute the major NAD^+ independent pathway of oxalate synthesis, while lactate dehydrogenase is the major NAD^+ dependent route (Gibbs and Watts 1973, Gibbs et al 1977).

Investigations by Liao and Richardson (1973) demonstrated that oxalate synthesis from glyoxylate and glycollate in the perfused rat liver was almost completely inhibited by DL phenyllactate, a competitive inhibitor of glycollate oxidase. This inhibitor was shown to have little effect on the oxidation of glyoxylate by lactate dehydrogenase and xanthine oxidase. There is, other evidence, however, which indicates that lactate dehydrogenase is the most significant enzyme involved in oxalate synthesis in the liver and other tissues. It has been clearly shown that lactate dehydrogenase will catalyse the oxidation and reduction of glyoxylate *in vitro* (Warren 1970, Romano and Cerra 1969, Williams and Smith 1971). Other investigations with liver supernatants from rat and man have clearly shown the major enzymic oxidation of glyoxylate is NAD^+ dependent.

Intensive studies have often centred on liver supernatant preparations, despite the demonstration that both the soluble and particulate fractions of liver will catalyse oxalate production from glyoxylate (Crawhall and Watts 1962, Smith et al 1972, Gibbs and Watts 1967, Gibbs et al 1977).

In this section the subcellular location of oxalate synthesising activity is examined in conjunction with the distribution of the key enzymes of oxalate synthesis.

RESULTS

1. EFFECT OF SUBSTRATE CONCENTRATION

Maximum oxalate production from glycollate and glyoxylate occurred at substrate concentrations of 5 and 20 mM respectively (fig. 15), compared to 1 and 15 mM in the hepatocyte study. In addition to the higher substrate concentrations required here, the rate of oxalate production, particularly with glyoxylate as substrate was less than that observed in hepatocytes. The disruption of the cellular integrity is possibly a major reason for this difference.

2. EFFECT OF pH

Oxalate synthesis from glyoxylate had a broad pH optimum of 7.5 while that with glycollate as substrate was 8.5 (fig. 16). Optimal decarboxylation of both substrates occurred at pH 7.5 in phosphate buffer. Inhibition of oxalate synthesis sometimes occurred using 50 mM potassium phosphate buffer but not at a concentration of 10 or 20 mM. This effect has not been investigated further.

3. EFFECT OF OXAMATE AND DL-PHENYLLACTATE

As observed previously with hepatocytes, DL-phenyllactate was a more potent inhibitor than oxamate with respect to oxalate production from both substrates (fig. 17). DL-phenyllactate also caused a parallel decrease in CO_2 production from both substrates. Whilst this result is consistent with glycollate being oxidised via glycollate oxidase before decarboxylation, the inhibition of glyoxylate decarboxylation implies that either some glyoxylate is decarboxylated via glycollate or that DL phenyllactate interferes with the enzymes of decarboxylation. Oxalate and CO_2 production from 10 mM glyoxylate was linear for 60 minutes. With 5 mM glycollate as substrate, however, a distinct lag period of up to 30 minutes was observed for both CO_2 and oxalate production. A similar finding was recorded for glycollate decarboxylation in leaf peroxisomes (Halliwell and Butt 1974) and may indicate a two step reaction.

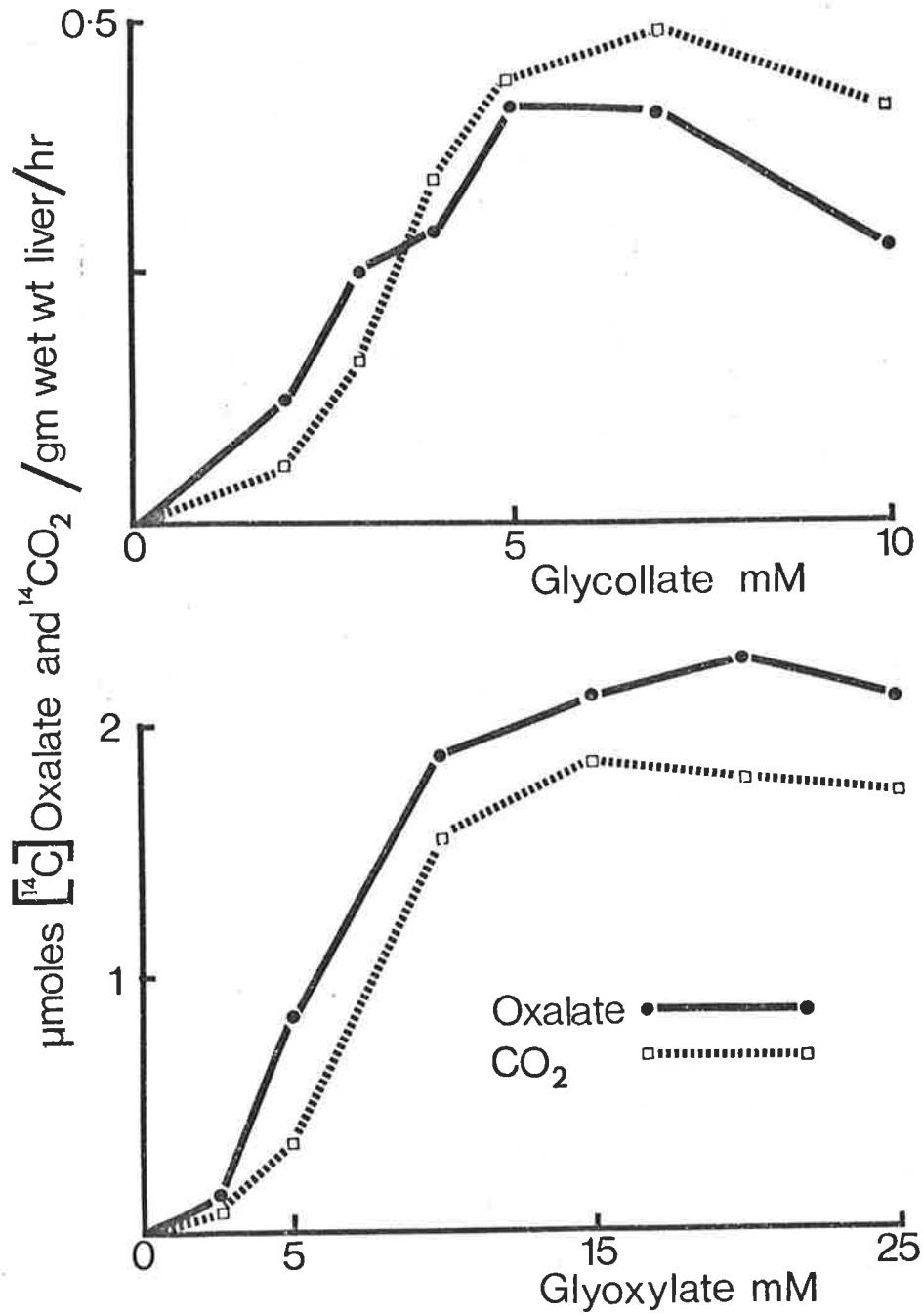


Fig. 15. The effect of the concentration of glycollate and glyoxylate on oxalate and CO_2 production in cell free extracts (500g supernatant) from rat liver. Incubations contained |1-^{14}C|glycollate (0.1 µCi/umol) or |U^{14}C|glyoxylate (0.05 µCi/umol) and extract in a volume of 1.0 ml. The incubations were performed in standard glass scintillation vials at 37^{0}C for 1.0 hour. Further details are given in the methods, chapter 2.

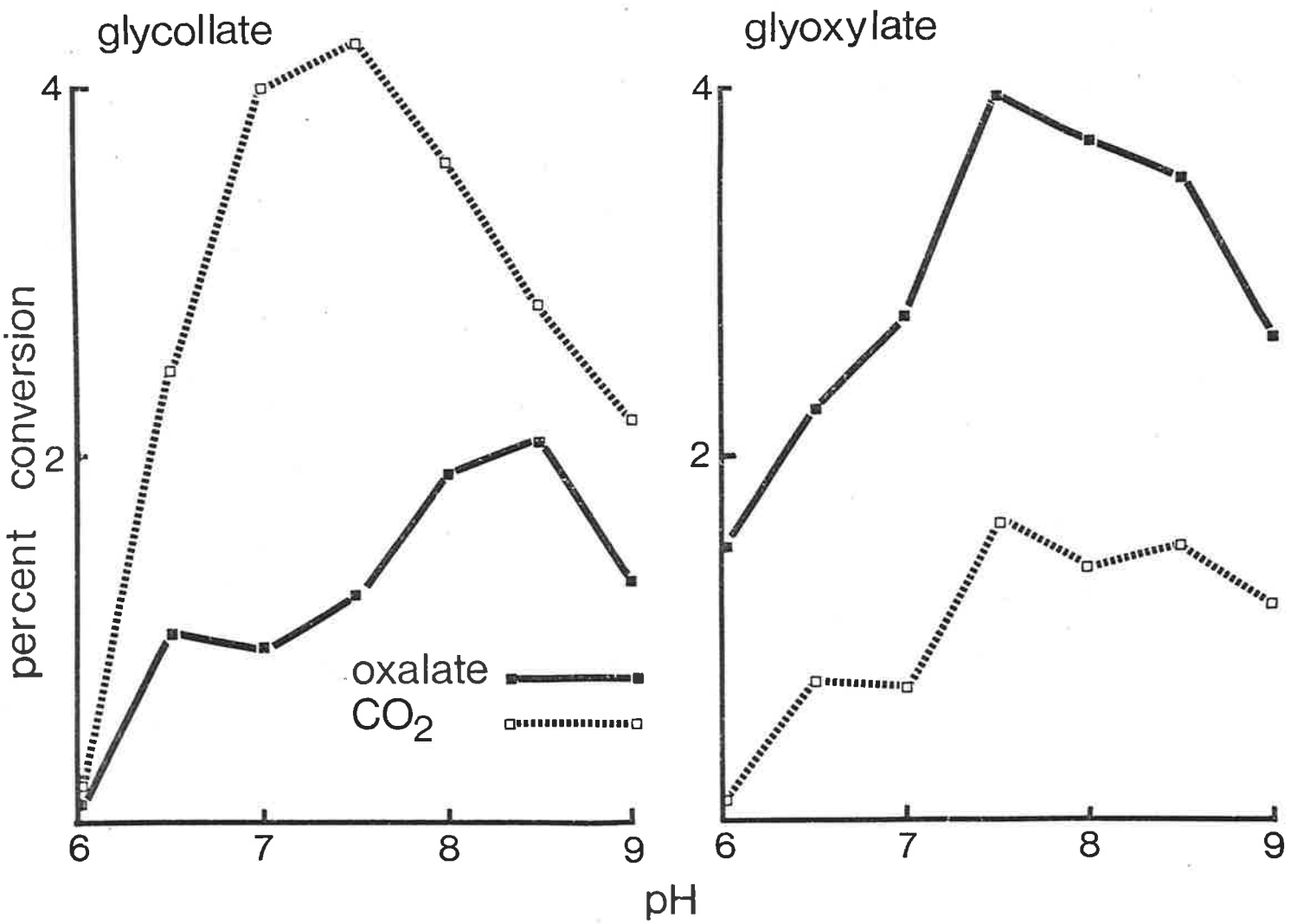


Fig. 16. The effect of pH on oxalate and Co_2 production from glycollate and glyoxylate in cell free extracts (500 g supernatant) from rat liver. Potassium phosphate buffer (50 mM) was used in this experiment. The incubations contained 5 mM glycollate or 10 mM glyoxylate. The incubation time was 1.0 hour. Further details are given in the legend to fig. 15 and in the methods, chapter 2.

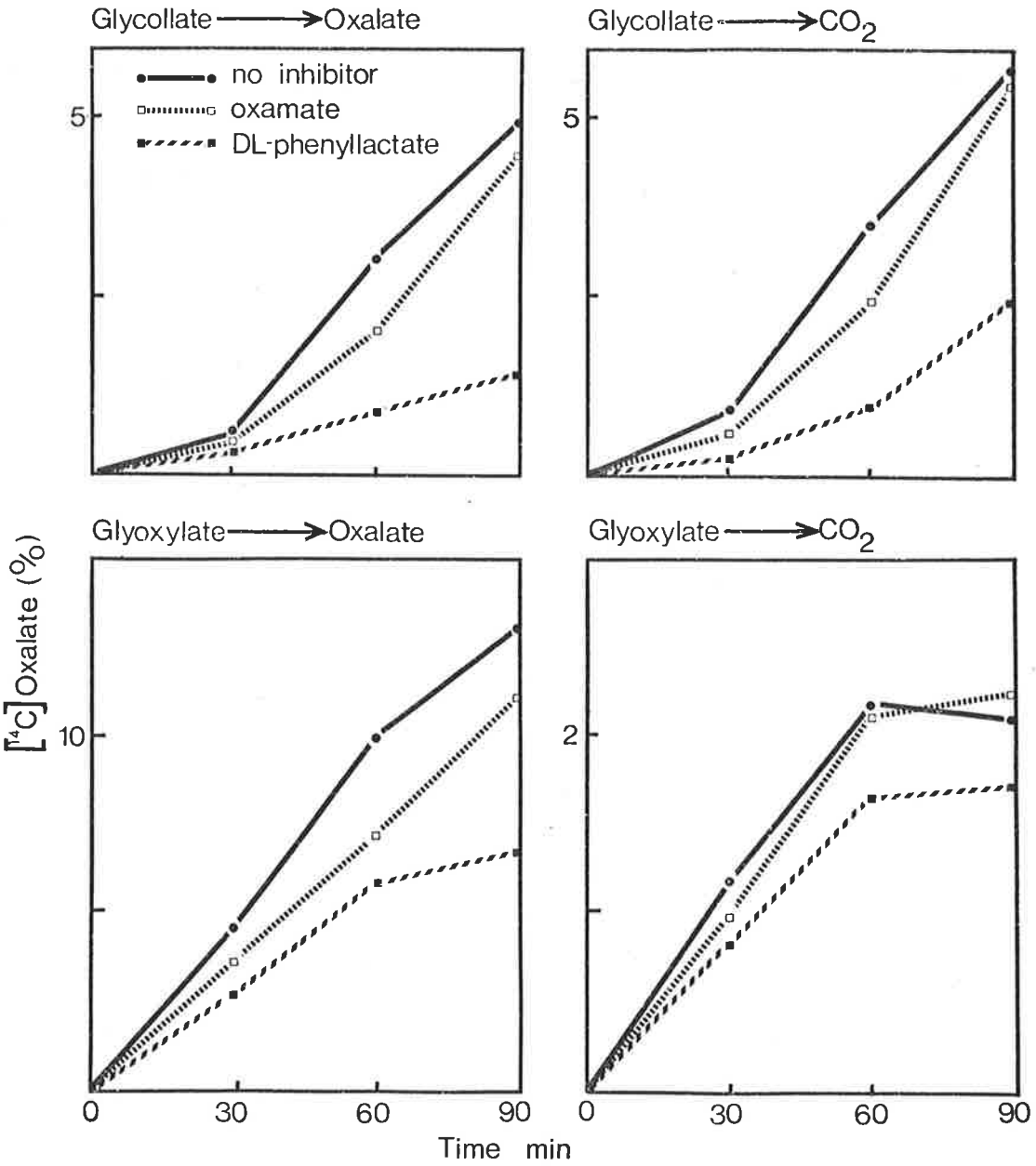


Fig. 17. The effect of oxamate and DL-phenyllactate on oxalate and CO_2 production from glycollate and glyoxylate in cell free extracts (500g supernatant) from rat liver. The concentrations used were, glycollate 5 mM, and glyoxylate, oxamate and DL-phenyllactate, 10 mM. The inhibitors were added to the incubations at the same time as the substrate. Further details are given in fig. 15 and in the methods, chapter 2.

4. THE SUBCELLULAR DISTRIBUTION OF KEY ENZYMES IN OXALATE BIOSYNTHESIS

In order to verify the efficacy of oxamate and DL phenyllactate as inhibitors, an investigation of enzyme activities in subcellular fractions was under taken (table 11). Only 3 of the 5 postulated reactions shown in that table could be demonstrated spectrophotometrically. Glycollate oxidase activity was mainly present in the particulate fraction. DL phenyllactate was an effective inhibitor of this activity at concentrations above 10 mM; oxamate having little effect on this reaction. Conversely the oxidation and reduction of glyoxylate in the presence of NAD^+ and NADH was largely catalysed by the supernatant. Oxamate was a potent inhibitor of these NAD^+ linked steps with DL phenyllactate having little effect. These results agree with the reported specificity of these inhibitors (Smith et al 1972, Liao and Richardson 1973).

Glyoxylate is known to be oxidised by rat liver glycollate oxidase (Ushijima 1973 Richardson and Tolbert 1961) but not surprisingly, this step was not demonstrable in crude homogenates. However, using dialysed liver supernatant or partially purified glycollate oxidase the oxidation of glyoxylate in the presence of DCIP was demonstrable (table 12). The low velocity and high substrate concentration required to sustain this activity compared with glycollate as substrate, is in agreement with published reports (Richardson and Tolbert 1961, Ushijima 1973). Unlike the studies of Liao and Richardson (1973) DL phenyllactate was not a good inhibitor of glyoxylate oxidation by glycollate oxidase. The possibility that other hydroxy acid oxidases are contributing to glyoxylate oxidation cannot be excluded (deDuve and Baudhin 1966, Mcgroarty et al 1974).

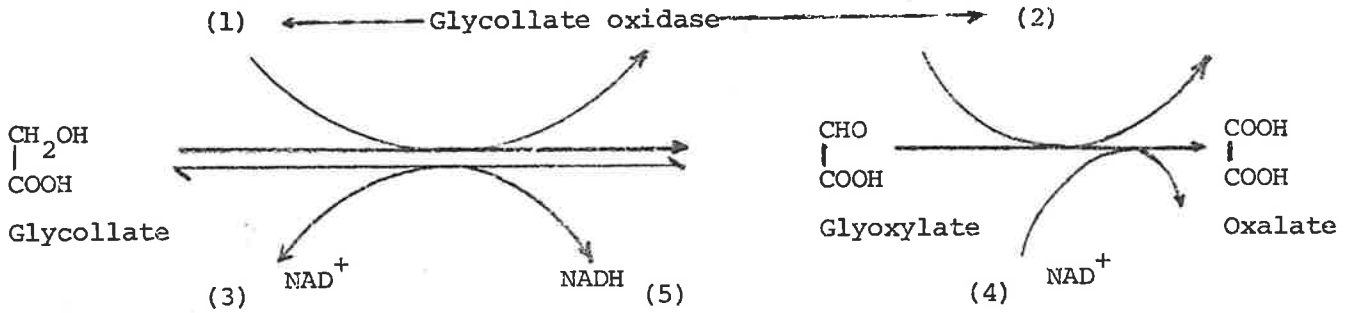
5. SUBCELLULAR DISTRIBUTION OF OXALATE SYNTHESISING ACTIVITY

Analysis of the subcellular fractions as outlined in table 13 for their ability to produce oxalate from glycollate and glyoxylate clearly showed that the particulate fraction was a major site of oxalate synthesis. This fraction was mainly composed of mitochonria, peroxisomes and some lysosomes (Boveris et al 1972). As seen previously, oxamate had little effect on oxalate synthesis from either substrate, whilst DL phenyllactate inhibited oxalate synthesis from glycollate and glyoxylate by 75% and 50% respectively, in these fractions.

TABLE 11

A SPECTROPHOTOMETRIC INVESTIGATION OF GLYCOLLATE OXIDATION AND GLYOXYLATE OXIDATION AND REDUCTION IN RAT LIVER EXTRACTS

REACTION	SUBSTRATE	HYDROGEN DONOR/ ACCEPTOR	VELOCITY RAT LIVER PELLET	VELOCITY RAT LIVER SUPERNATANT	Km mM	% INHIBITION DL-PHENYL-LACTATE (mM) 5	10	20	% INHIBITION OXAMATE (mM) 5	10	20
1	Glycollate	DCIP	0.19	0.05	2	35	60	90	-	-	10
4	Glyoxylate	NAD^+	0.60	13	7	-	-	0	60	75	85
5	Glyoxylate	NADH	40	257	20	-	-	33	90	90	95



Velocity is expressed as μmoles substrate oxidised or reduced/minute/ml of extract. Details of the assays and rat liver fractionation are given in the methods, chapter 2. Reaction (2) was of very low activity, and reaction (3) not detectable.

TABLE 12

THE OXIDATION OF GLYOXYLATE AND GLYCOLLATE BY RAT LIVER GLYCOLLATE OXIDASE

ENZYME SOURCE	SUBSTRATE	VELOCITY	Km (mM)	%INHIBITION BY DL PHENYLLACTATE	%INHIBITION BY OXAMATE
Rat liver Supernatant	Glycollate	17	2.5	90	10
	Glyoxylate	5	7.0	0	0
Partially purified Glycollate oxidase	Glycollate	69	0.7	85	0
	Glyoxylate	6	5.0	24	0

Rat liver supernatant was obtained from 20,000 g centrifugation of homogenised rat liver (sorvall omnimix) in 0.2 m phosphate buffer pH 8.0 dialysed overnight. Glycollate oxidase was partially purified by the method of Liao and Richardson (1973). The concentration of DL-phenyllactate and oxamate was 20 M mM. The velocity is expressed as nmols oxidised/min/mg protein. Further details are given in the methods, chapter 2.

TABLE 13

THE EFFECT OF OXAMATE AND DL-PHENYLLACTATE ON OXALATE SYNTHESIS IN SUBCELLULAR FRACTIONS OF RAT LIVER

		% OXALATE from			
		GLYCOLLATE		GLYOXYLATE	
ADDITION		PELLET	SUPERNATANT	PELLET	SUPERNATANT
-		4.4	1.7	12.8	5.8
Oxamate	10mM	3.8	1.2	11.7	5.4
DL-Phenyllactate	10mM	1.1	0.4	6.8	2.7

Incubations contained 0.5 ml of either liver pellet or supernatant made up to 1 ml with buffer. The concentrations used were glycollate, 5 mM and glyocylate 10 mM. The incubation time was 60 minutes at 37^0C. Further experimental details are given in the methods, chapter 2.

It should be stressed that the distribution of enzyme and oxalate synthesising activities shown in tables 11 and 13 is best demonstrated using gentle homogenisation techniques. Vigorous homogenisation results in solubilisation of the oxalate synthesising activity (table 14). Reports that glycollate oxidase is readily lost from peroxisomes are consistent with this observation (Hayashi et al 1971).

6. THE EFFECT OF NAD^+, NADH AND $NADP^+$ ON OXALATE SYNTHESIS

The addition of NAD^+ or NADH to homogenates resulted in a 4 fold stimulation of oxalate production from glyoxylate, whereas that from glycollate was inhibited (table 15). The NAD^+ dependent oxalate synthesising activity is clearly shown to be located in the supernatant fraction, though some stimulation of oxalate synthesis from glyoxylate in the presence of NAD^+ was also seen in the pellet fraction which still contained considerable lactate dehydrogenase activity. It was interesting to note that $NADP^+$ had a similar effect to NAD^+. Whilst the presence of NAD^+ in the $NADP^+$ or transhydrogenation reactions cannot be excluded, this result could also indicate that enzymes other than lactate dehydrogenase are operative in glyoxylate oxidation. Glycerate dehydrogenase, which will reduce glyoxylate in the presence of NADPH (Dawkins and Dickins 1965), may oxidise glyoxylate in the presence of high levels of $NADP^+$. The stimulation of oxalate synthesis from glyoxylate by NADH and NAD^+ supports the conclusions of Romano and Cerra (1969), who claim that glyoxylate and lactate dehydrogenase constitute a true redox couple, though much more oxalate should have been produced in the presence of NAD^+ compared to NADH, than was observed here.

7. CHROMATOGRAPHIC SEPARATION OF NAD^+ DEPENDENT AND NAD^+ INDEPENDENT OXIDATION OF GLYOXYLATE TO OXALATE.

A once washed pellet fraction containing considerable lactate dehydrogenase activity was chromatographed on a CM-sephadex column (fig 18). The major NAD^+ dependent oxalate synthesising activity was associated with the lactate dehydrogenase peak (fig. 18). This lactate dehydrogenase which was eluted with 100 mM NaCl and was shown to be predominantly lactate dehydrogenase 5 (LD_5). The NAD^+ independent oxalate synthesising activity was coincident with the initial protein peak which contained the glycollate oxidase activity and some LD_2 and LD_5. At this stage, the possibility that LD_2 as reported by Skilleter and Kun (1972) contributes to oxalate synthesis

TABLE 14

EFFECT OF HOMOGENISATION METHOD ON THE DISTRIBUTION OF OXALATE SYNTHESISING ACTIVITY IN SUBCELLULAR FRACTIONS OF RAT LIVER

METHOD OF HOMOGENISATION	FRACTION	% OXALATE FROM		% CO_2 FROM		LD	GO
		Glycollate	Glyoxylate	Glycollate	Glyoxylate		
Teflon pestle	Pellet	2.2	6.3	1.1	0.6	50	3.0
	Supernatant	0.6	2.2	0.2	0.2	1400	0.6
High speed blade (Omni mix)	Pellet	0.4	1.1	0.4	0.3	50	0.4
	Supernatant	1.5	4.3	2.5	0.6	1280	0.8

Details of the preparation of the rat liver fractions and of the enzyme assays are given in the methods, Chapter 2. All activities are expressed per ml of fraction. Lactate dehydrogenase and glycollate oxidase are expressed as arbitrary units (optical density change). Incubations for oxalate and CO_2 determination contained 5 mM glycollate or 10 mM glyoxylate in 1.0 ml of extract. The incubation time was 1.0 hour. Further experimental details appear in the methods, chapter 2.

TABLE 15

THE EFFECT OF NAD^+, NADH AND $NADP^+$ ON OXALATE SYNTHESIS FROM GLYOXYLATE AND GLYCOLLATE IN RAT LIVER FRACTIONS

FRACTION	SUBSTRATE	COFACTOR	PERCENTAGE CONVERSION TO OXALATE
HOMOGENATE	GLYCOLLATE	-----	1.7
" S 0.5	"	NAD^+	0.8
"	"	NADH	0.8
"	GLYOXYLATE	----	4.9
"	"	NAD^+	17.2
"	"	NADH	17.0
PELLET	GLYOXYLATE		5.1
" P 15	"	NAD^+	8.2
"	"	$NADP^+$	10.6
SUPERNATANT	"		1.2
" S 15	"	NAD^+	22.0
"	"	$NADP^+$	15.0

Details of the preparation of the homogenate (500g supernatant), pellet and supernatant fractions, as well as details of the incubations are given in the methods, chapter 2. The concentrations used were glycollate 5 mM, glyoxylate 10 mM, NAD^+ and $NADP^+$ 4 mM, and NADH 1 mM. The incubation time was 30 minutes at 37^0C.

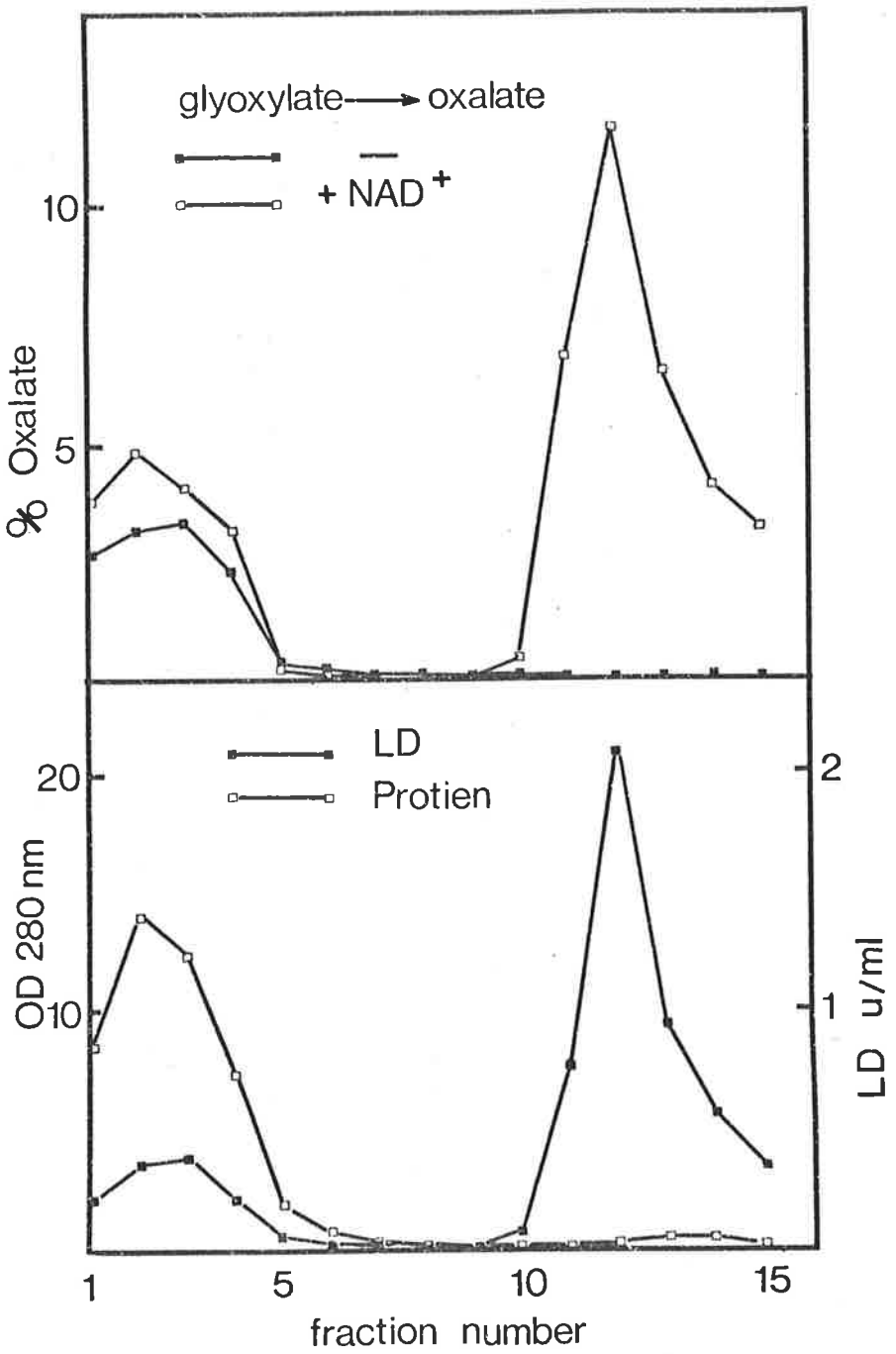


Fig. 18. The chromatographic separation of the NAD^+ dependent and NAD^+ independent oxidation of glyoxylate to oxalate from a 15,000g pellet fraction from rat liver. Eight mls of the pellet containing 0.5% nonidet, 0.12 M sucrose and 5 mM sodium phosphate buffer, pH 7.0 (1:1 dilution of the original extract) were loaded on a 2 x 6 cm CM-sephadex (Pharmacia, C-50) column which had been equilibrated with 5 mM sodium phosphate, pH 7.0. Elution was commenced with this phosphate buffer and 4 ml fractions were collected. When the absorbance at 280 nm was less than 0.2, the eluting buffer was changed to one containing 100 mM NaCl in 5 mM sodium phosphate buffer, pH 7.0(after fraction 8). Oxalate production from 10 mM glyoxylate, plus or minus 4 mM NAD^+, was determined in 1.0 ml aliquots of these fractions, as described for cell free extracts (methods, chapter 2).

from glyoxylate, cannot be excluded.

8. AN ELECTROPHORETIC INVESTIGATION OF THE PARTICULATE FRACTION OF RAT LIVER

The once washed 15,000 g pellet fraction was found to contain considerable lactate dehydrogenase activity, with LD_5 being the predominant isoenzyme. Enzyme activities corresponding to LD isoenzymes 2,3 and 4 were also detected if the electrophoretogram was allowed to develop for one to two hours (table 16). A band, at the position of glycollate oxidase, was detected with lactate plus NAD^+ but not in the absence of NAD^+. While lactate is known to be a substrate for glycollate oxidase, (McGroarty et al 1974) this stimulation in the presence of NAD^+ has not been recorded previously. Glyoxylate, in the presence of NAD^+, demonstrated activity at all of the positions shown with lactate as substrate. Unlike the results observed with lactate, the reactivity of glyoxylate with glycollate oxidase and lactate dehydrogenase 2 was not dependent on NAD^+. For convenience this latter activity will be termed glyoxylate dehydrogenase as neither lactate nor glycerate were substrates for this activity.

Xanthine oxidase activity was not detected in this particulate preparation, but was particularly active in the supernatant. Its electrophoretic mobility was between that of LD_3 and LD_4, which is quite distinct from glycollate oxidase activity which appears between LD_2 and LD_3. Glycollate oxidase activity, with both glycollate and glyoxylate as substrate, was greatly diminished by heating the fractions for 15 minutes at 56^0C. The glyoxylate dehydrogenase activity, however, was little affected by this treatment.

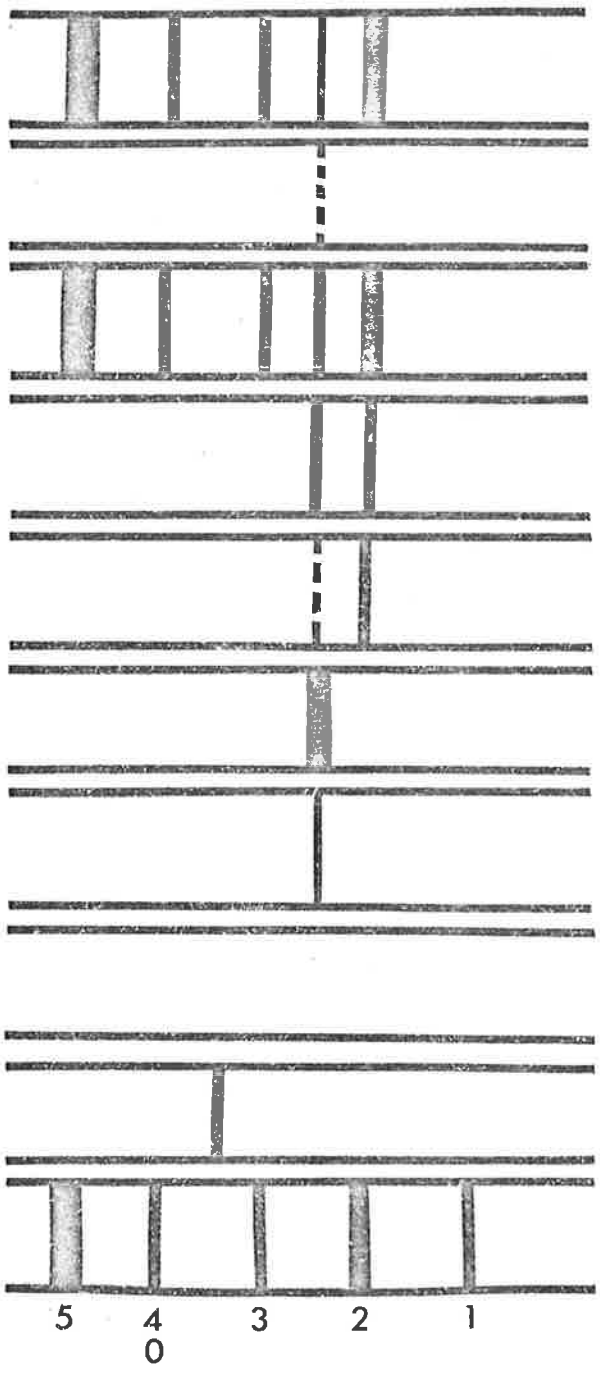
In summary, the pellet fraction contained lactate dehydrogenase isoenzymes 2,3,4 and 5, with lactate dehydrogenase 5 predominating, a heat stable glyoxylate dehydrogenase activity, and a heat labile glycollate oxidase activity. It should be pointed out that the lactate dehydrogenase activity shown here represents a very small portion of the total liver activity, the majority of which is found in the supernatant fraction. However this lactate dehydrogenase activity in the pellet fraction is still significant in comparison with the activity of glycollate oxidase.

TABLE 16

ELECTROPHORETIC SEPARATION OF A PELLET FRACTION FROM RAT LIVER

FRACTION	SUBSTRATE
Pellet	L-lactate + NAD^+
Pellet	L-lactate
Pellett	Glyoxylate + NAD^+
Pellet	Glyoxylate
Pellet 56^0/15 min	Glyoxylate
Pellet	Glycollate
Pellet 56^0/15 min	Glycollate
Pellet	DL-glycerate
Supernatant	Hypoxanthine
Heart, liver standard homogenate	L-lactate + NAD^+

ELECTROPHORETIC PATTERN



A once washed 15,000g pellet fraction (Methods, chapter 2), containing 0.2% nonidet was used for this separation. Heating was performed in a water bath for the prescribed period. The rat heart and liver preparation was included to show the position of the respective lactate dehydrogenase isoenzymes. The origin is denoted by 0. Details of the electrophoresis are given in the methods, chapter 2.

9. AN INVESTIGATION OF THE GLYOXYLATE DEHYDROGENASE ACTIVITY

The glyoxylate "dehydrogenase activity" observed in the pellet was not readily washed from this fraction except by using sonication or detergent (nonidet). This finding corresponds with the results of Skilleter and Kun (1972) who reported that rat liver LD_2 contained bound NAD^+. As this activity may contribute to the mitochondrial oxalate synthesis from glyoxylate, this particulate fraction was further purified and investigated. The results of this procedure are shown in table 17. As shown previously, the unpurified pellet fraction contained two distinct bands of activity with glyoxylate as substrate in the absence of added NAD^+. Only one band of activity was seen with glycollate as substrate. After further purification, however, the glyoxylate dehydrogenase activity was precipitated with 40% ammonium sulphate, whereas glycollate oxidase activity appeared in the 40-60% fraction. As this latter fraction also contained the major oxalate synthesising activity with both glycollate and glyoxylate as substrates, it must be concluded that the glyoxylate dehydrogenase activity, in this type of preparation, is not contributing to hepatic oxalate synthesis. Furthermore, this activity, was lost after one day at 4^0C, whereas glycollate oxidase activity and the major oxalate synthesising activity from the 40-60% ammonium sulphate fraction, was stable for at least a week at 4^0C in 5 mM sodium phosphate buffer, pH 7.0.

A point for further discussion is that whilst glycollate produced a more intense electrophoretic band than glyoxylate, this latter compound remained, in these fractions, the most effective oxalate precursor.

10. PURIFICATION OF OXALATE SYNTHESISING ACTIVITY FROM RAT LIVER

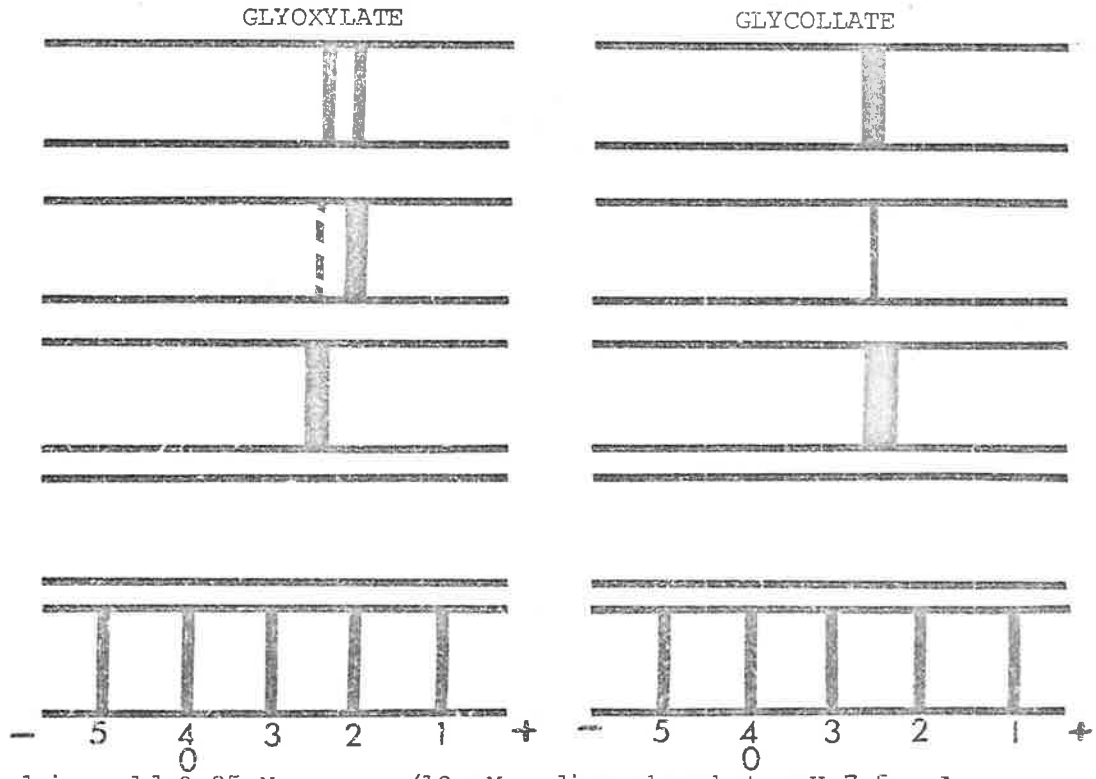
The majority of the oxalate synthesising activity with both glyoxylate and glycollate as substrates was recovered in the 40-60% ammonium sulphate fraction, which also contained most of the glycollate oxidase, lactate dehydrogenase and xanthine oxidase activity (table 18). Further purification of this fraction (table 18, fig. 19) using DEAE or CM sephadex indicated that the oxalate synthesising activity was associated with glycollate oxidase or xanthine oxidase. Electrophoresis of the DEAE fraction (table 19) showed that, with glycollate and glyoxylate as substrates, only one distinct band of activity was present, conincident with glycollate oxidase. This electrophoretic mobility was between that of LD_2 and LD_3. Xanthine oxidase activity on the other hand occurred at a position between

TABLE 17

GLYOXYLATE DEHYDROGENASE ACTIVITY IN THE PARTICULATE FRACTION OF RAT LIVER

FRACTION	PROTEIN mg/ml	% OXALATE from GLYOXYLATE	% OXALATE from GLYCOLLATE
Pellet	25	5.5	0.7
Ammonium Sulphate 0 - 40%	6	1.1	0.8
40→60%	16	10.8	3.7
60→100%	2	1.2	0.6

Heart and liver std, lactate + NAD



Four livers from male rats were minced with sharp blades and washed in cold 0.25 M sucrose/10 mM sodium phosphate pH 7.5. A 15,000 g pellet fraction (200 ml) was prepared as described in the methods, and was further purified as described for supernatant 1 in the purification of oxalate synthesising activity in the methods, chapter 2 and also table 18. Oxalate production was determined in 1.0 ml incubations containing 5 mM glycollate or 10 mM glyoxylate. The incubation time was 1.0 hour at 37^0C. Details of the electrophoresis appear in the methods, chapter 2. The origin is denoted by 0, and the numbers refer to the isoenzymes of lactate dehydrogenase.

TABLE 18

PURIFICATION OF OXALATE SYNTHESISING ACTIVITY FROM RAT LIVER

FRACTION						$\|^{14}C\|$ OXALATE					
Fraction	Vol ml	PROTEIN	LD	GO	XO	$\|1\text{-}^{14}C\|$ Glyoxylate			$\|1\text{-}^{14}C\|$ Glycollate		
			activity/ml			nmol/ml hr	nmol/mg protein/hr	Purification (fold)	nmol/ml hr	nmol/mg protein/hr	Purification (fold)
Homogenate	200	23	123			1000	43	1	30	1.3	1
S1	200	16	63			680	42		30	1.9	
S2	200	12	62	12	14	980	82		30	2.5	
S3	200	7	47	8	11	1280	183	4	140	20	15
0-40% AS	20	8	8	4	5	120	15		10	1.2	
40-60% AS	20	12	225	25	35	1740	145	3	480	40	30
60-100%AS	20	9	5	2	1	140	15		0	0	
CM Sephadex	10	3	1	21	30	1660	553	13	265	88	68
DEAE	10	3	1	18	27	1060	353	8	290	96	74

LD, lactate dehydrogenase. GO glycollate oxidase, XO xanthine oxidase, AS ammonium sulphate. Details of the purification are described in the methods, chapter 2. The enzyme activity is in arbitrary units. Oxalate synthesis was measured in 1.0 ml of the fractions, using 5 mM glycollate or 10 mM glyoxylate. The incubation time was 1.0 hour at 37^0C.

Fig. 19. The chromatographic separation of lactate dehydrogenase and oxalate synthesising activity from a 40→60% ammonium sulphate fraction from rat liver. Experimental details are given in the methods, chapter 2 and in table 18. Oxalate synthesis was determined in 1.0 ml of the respective 4.0 ml fractions, using 5 mM glycollate or 10 mM glyoxylate. The incubation time was 1.0 hour, as described for liver extracts.

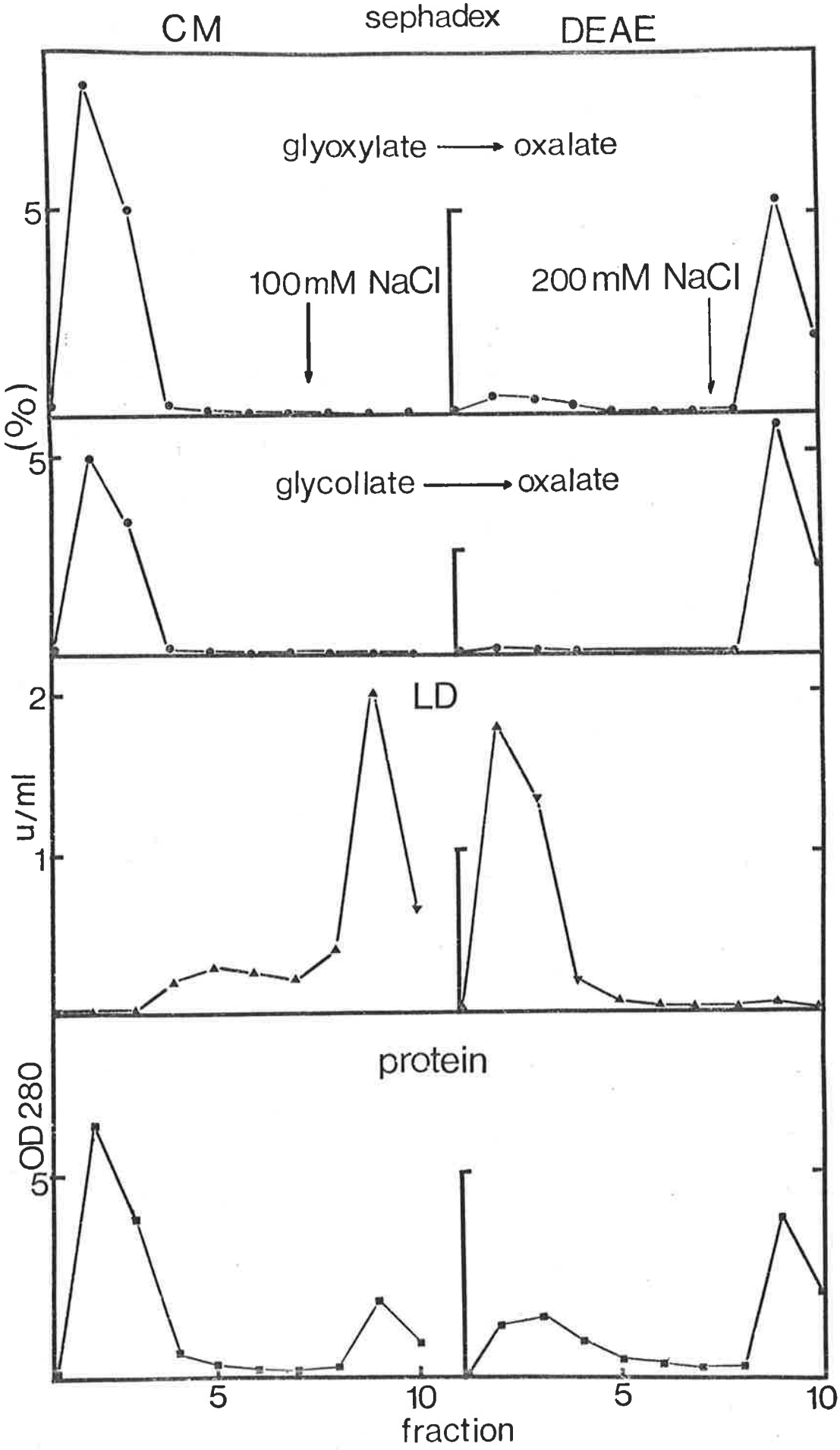
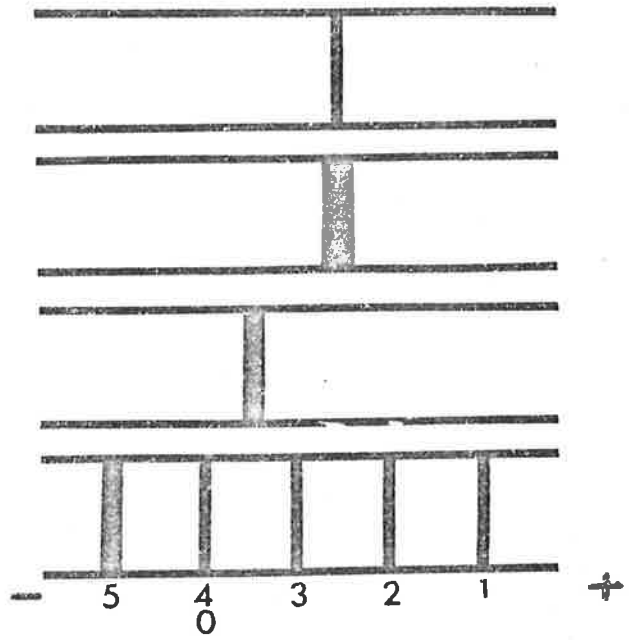

CM
sephadex
DEAE
glyoxylate → oxalate
100mM NaCl
200mM NaCl
5
(%)
5
glycollate → oxalate
2
LD
u/ml
1
protein
OD 280
5
5
10
5
10
fraction

TABLE 19

ELECTROPHORESIS OF DEAE SEPHADEX FRACTION 9

FRACTION	SUBSTRATE
9	GLYOXYLATE
9	GLYCOLLATE
9	HYPOXANTHINE
Heart Liver standard	LACTATE + NAD^+



Details of the electrophoresis appear in the methods, chapter 2. The preparation and description of fraction 9 is given in the methods and also in table 18 and fig. 19. The origin is denoted by 0, and the numbers refer to the respective isoenzymes of lactate dehydrogenase.

that of LD_3 and LD_4. It can therefore be concluded that xanthine oxidase does not contribute significantly, if at all, to oxalate synthesis in this fraction. While the intensity of the electrophoretic band, and the spectrophotometrically measured activity of glycollate oxidase was always greatest with glycollate as substrate, glyoxylate remained the most effective oxalate precursor in these situations.

The effect of inhibitors and heat on oxalate and CO_2 synthesis from both substrates is shown in table 20. The oxidation of glyoxylate to oxalate by the purified glycollate oxidase showed some sensitivity to heating at 56^0C as did oxalate synthesis from glycollate in this purified fraction, whereas in crude extract this latter activity was abolished after heating at 56^0C for 15 minutes (table 21). The sensitivity of the purified fraction to the inhibitors, DL-phenyllactate and oxamate, paralleled those results obtained with crude homogenates, which supports the major involvement of glycollate oxidase in hepatic oxalate synthesis. Glycollate and glyoxylate were competitive substrates during oxalate synthesis in this purified fraction.

The majority of the decarboxylating activity exhibited with both glycollate and glyoxylate as substrates was lost after the first step in the purification (S1). If a pellet fraction was first prepared (15,000 g) decarboxylating activity was not lost until the acidification step with acetic acid (S3), though some residual activity was seen in the 60-100% ammonium sulphate fraction. While the 40-60% ammonium sulphate fraction contained the major oxalate synthesising activity, this fraction showed neglibible decarboxylating activity with either substrate. However, significant CO_2 production did occur if this fraction was heated for 15 minutes at 56^0C or treated with azide (table 20). As both these treatments will inhibit catalase activity, which was present in this fraction, it can be suggested that this enzyme is an important component in the mechanism of oxalate production from glycollate and glyoxylate, catalysed by the hydrogen peroxide producing glycollate oxidase.

11. THE EFFECT OF HEAT FRACTIONATION AND DIALYSIS ON THE OXIDATION OF GLYCOLLATE TO CO_2 AND OXALATE IN RAT LIVER FRACTIONS

Whilst the effectiveness of glyoxylate and glycollate as oxalate precursors was demonstratable in purified liver fractions, certain effects seen with

TABLE 20

EFFECT OF INHIBITORS ON OXALATE AND CO_2 PRODUCTION FROM GLYCOLLATE AND GLYOXYLATE BY PARTIALLY PURIFIED GLYCOLLATE OXIDASE

Addition	OXALATE % from Glyoxylate	OXALATE % from Glycollate	CO_2 % from Glyoxylate	CO_2 % from Glycollate
-----	7.7	3.5	0.1	0.1
DL-phenyllactate	5.4	1.8	0.1	0.1
Oxamate	7.0	3.5	0.1	0.1
Azide	6.4	2.6	1.6	1.8
Heat extract 56^0C	5.8	2.1	1.4	2.7
Glycollate	3.7		0.4	
Glyoxylate		0.9		0.1

The enzyme source was the glycollate oxidase peak from the CM sephadex separation of the 40 60% ammonium sulphate fraction. Glycollate 5 mM, Glyoxylate 10 m mM either as substrates (labelled) or as competitive substrates. DL-phenyllactate and oxamate 10 mM, azide 1 mM and heating of the extract was for 15 minutes at 56^0 . Incubations were performed with 1 ml of undiluted enzyme (fraction 2 CM sephadex, fig 19) in 10 mM sodium phosphate buffer, pH 7.0 for 1 hour.

crude subcellular fractions indicate that important components of the oxalate biosynthetic mechanism are removed or modified during the purification procedure. In fact, separation of the crude extract into pellet and supernatant fractions constitutes a major modification, as shown with glycollate as substrate in table 21, where the addition of pellet and supernatant fractions resulted in more CO_2 production from glycollate than would be expected from a purely additive effect. This synergistic effect was not influenced by dialysing the pellet or heating the supernatant at 56 or 100^0C for 15 minutes, but was greatly diminished by heating the pellet at 56 or 100^0C for 15 minutes or by dialysing the supernatant. Therefore, this synergistic decarboxylation of glycollate is probably due to enzymic components in the pellet, which are stimulated by a heat stable, dialysable factor in the supernatant.

The effect of dialysis and heat on oxalate production from glycollate are different from those observed for CO_2 production (table 21). Dialysis had little effect on oxalate production from the pellet and supernatant, either alone or in combination. Heating either fraction at 56^0C for 15 minutes, almost abolished oxalate biosynthesis, but both these fractions retained the ability to stimulate oxalate synthesis from the untreated pellet or supernatant. This effect was not observed if the respective fractions were heated at 100^0C for 15 minutes. It would appear that an enzymic activity other than glycollate oxidase can contribute to oxalate synthesis from glycollate. An important observation noted during these experiments was that catalase activity was completely abolished from these fractions by heating at 56^0C for 15 minutes. The role of catalase in oxalate production will be referred to in the discussion. In this context, it was interesting to observe that the catalase inhibitor azide, caused a marked decrease in oxalate production and an increase in CO_2 synthesis from glycollate in these extracts (table 22).

Preparation of the pellet and supernatant fractions are described in the methods, chapter 2. Heating was performed in a water bath, the fractions being placed in a bath of cold water after treatment. Dialysis was performed with for 3.0 hours using a 500 fold excess of buffer (0.25 M sucrose/10mM sodium phosphate buffer pH 7.5). Incubations contained 0.5 ml of fraction, made up to 1.0 ml with buffer when not in combination. The glycollate concentration was 5mM and the incubation time was 90 minutes at 37^0C.

TABLE 21

EFFECT OF DIALYSIS AND HEATING ON CO_2 AND OXALATE SYNTHESIS FROM GLYCOLLATE IN RAT LIVER SUBCELLULAR FRACTIONS

	Percentage conversion to	
EFFECT OF DIALYSIS	CO_2	OXALATE
P = pellet	0.72	1.65
S= supernatant	0.35	1.63
P + S	3.84	4.74
P + S dialysed	1.35	4.10
P dialysed + S	4.86	4.10
P dialysed + S dialysed	1.25	5.06
EFFECT OF HEATING AT 56^0 for 15 MIN		
P + S	3.84	4.74
P + S 56	5.47	6.32
P 56 + S	0.64	3.83
P 56 + S 56	4.65	0.00
EFFECT OF HEATING AT 100^0 for 15 min		
P + S	3.84	4.74
P + S 100	3.54	1.58
P 100 + S	0.29	0.92
P 100 + S 100	0.00	0.00
CONTROLS		
P	0.72	1.65
S	0.35	1.63
P dialysed	0.45	1.38
S dialysed	0.21	1.66
P 56	4.85	0.30
S 56	3.00	0.20
P 100	0.02	0.00
S 100	0.00	0.00

TABLE 22

THE EFFECT OF AZIDE ON OXALATE AND CO_2 PRODUCTION FROM GLYCOLLATE

	PELLET	SUPERNATANT	PELLET SUPERNATANT
		% OXALATE	
----	3.5	0.9	4.5
Azide	2.7	0.3	2.9
		% CO_2	
----	0.5	0.2	1.4
Azide	1.6	1.8	4.4

The preparation of the pellet and supernatant fractions is given in the methods, chapter 2. The incubations contained 0.5 ml of fraction, made up to 1.0 ml with buffer when not in combination. The concentrations used were glycollate 5 mM, azide 1 mM, with an incubation time of 90 minutes at 37^0C.

DISCUSSION

Numerous studies have clearly demonstrated that oxalate production from glyoxylate is stimulated by NAD^+ (Smith et al 1972, Gibbs et al 1977). It is also clearly evident that this NAD^+ dependent step is catalysed by lactate dehydrogenase in the supernatant fraction of liver, as shown here and by other workers (Gibbs et al 1977). However, the significance of this reaction *in vivo* is difficult to ascertain. In the absence of added NAD^+ the majority of the oxalate synthesising activity occurred in a pellet fraction, where lactate dehydrogenase activity was low but glycollate oxidase activity high, compared to the supernatant fraction. The high level of glycollate oxidase in the pellet fraction corresponds with this fraction being mainly composed of mitochondria and peroxisomes; glycollate oxidase being a peroxisomal enzyme (McGroarty et al 1974, Hand 1975) Fractionation of the pellet fraction on CM-sephadex supplied further evidence against the involvement of lactate dehydrogenase in glyoxylate oxidation in the absence of NAD^+. This experiment also confirmed that the major NAD^+ dependent oxidation is catalysed by LD_5, the predominant liver isoenzyme, which is in agreement with the results of Gibbs and Watts (1973). Whilst the addition of NAD^+ to subcellular fractions will stimulate oxalate synthesis, the concentration of NAD^+ required to demonstrate such effects casts doubt on the physiological importance of lactate dehydrogenase catalysed oxalate synthesis.

Glyoxylate reductases have been reported in rat liver, but there appears to be no catalytic activity in the oxidative direction (Vandor and Tolbert 1970, Suzuki, et al 1973). McGroarty et al (1974) have pointed out that it is unlikely that lactate dehydrogenase reduces glyoxylate in the liver under physiological conditions. Therefore it would seem most unlikely that the less thermodynamically favoured oxidative step has physiological significance. The presence in crude homogenates of substrates such as lactate and pyruvate which have greater affinities for lactate dehydrogenase and which are normally in higher concentration than glyoxylate, would strengthen the view that lactate dehydrogenase has a minor role in oxalate biosynthesis.

The apparent Km values for oxalate production from glycollate and glyoxylate in isolated hepatocytes (0.7 mM and 10 mM respectively) are of the same order as published values for the affinity of glycollate oxidase for these substrates (Ushijima 1973, McGroarty et al 1974).

However, this correlation is not conclusive evidence in support of a major involvement of glycollate oxidase in the oxidation of glyoxylate as high concentrations of glyoxylate are also required to demonstrate lactate dehydrogenase catalysed oxalate synthesis (Warren 1970, Williams and Smith 1971). More conclusive evidence, against the involvement of lactate dehydorgenase in hepatic oxalate synthesis is seen in the inhibitor studies. The potent lactate dehydrogenase and glyoxylate dehydrogenase inhibitor, oxamate did not have any marked influence on oxalate synthesis from glyoxylate, whereas DL - phenyllactate was an effective inhibitor of oxalate synthesis from both glyoxylate and glycollate. This evidence supports the findings of Liao and Richardson (1973), although in their experiments DL - phenyllactate caused complete inhibition of oxalate synthesis from glyoxylate in perfused rat liver.

The possibility that other enzymes in the mitochondrial and peroxisomal pellet fraction, may contribute to oxalate synthesis from glyoxylate cannot be excluded. Although some xanthine oxidase activity occurs in the unwashed pellet fraction, the predominant xanthine oxidase activity occurs in the supernatant (Gibbs and Watts 1973) which suggests that it plays a minor role in hepatic oxalate biosynthesis.

A peroxisomal glyoxylate dehydrogenase (Vandor and Tolbert 1970) and a mitochondrial LD_2 (Skilleter and Kun 1972) have been reported, though at least one investigator has suggested that these are lactate dehydrogenase artifacts arising from isolation procedures (McGroarty et al 1974). The possibility that the oxidation of glyoxylate is being catalysed by glycerate dehydrogenase also warrants further investigation (Dawkins and Dickens 1965). However, from the evidence presented in this study using both cellular and subcellular liver experiments it can be concluded that glycollate oxidase is the major enzyme of hepatic oxalate synthesis thereby implicating the hepatic peroxisomes as the major site of oxalate synthesis.

A recent report by Gibbs et al (1977) has indicated that this is indeed the case as the NAD^+ independent synthesis of oxalate from glyoxylate was catalysed by liver, but not kidney peroxisomes. These organelles contain most of the glycollate oxidase activity

Other enzymes similar to glycollate oxidase have been identified in the rat liver. These include L -αhydroxy acid oxidases which have specific affinities for long and short chain hydroxy acids (deDuve and Baudhuin 1966,

McGroarty et al 1974), D -α-hydroxy acid oxidases (Tubbs and Greville 1961) and hydroxyacid oxidases which lack stereospecificity (Schafer and Lamprecht 1961, 1962). These enzyme activities may in fact be identical with glycollate oxidase (Ushijima 1973). In all cases reported glycollate is the preferred substrate for these enzymes, with glyoxylate, if a substrate at all, being poorly metabolised. This pattern was observed here in the electrophoretic investigations and in the spectrophotometric assays of glycollate oxidase. Glyoxylate, however, remained the most effective oxalate precursor with partially purified glycollate oxidase. A possible explanation of this finding is that whereas the oxidation of glyoxylate by glycollate oxidase yields oxalate, this is not necessarily the case with glycollate as substrate. This compound can give rise to glyoxylate which in turn can be metabolised to glycine, CO_2 or oxalate. It is therefore not surprising that a correlation between the spectrophotometrically determined glycollate oxidase activity, and the isotopic production of oxalate from glyoxylate and glycollate by this enzyme was not observed.

Catalase, like glycollate oxidase is a peroxisomal enzyme, yet this former enzyme has received little attention with respect to the role it may play in oxalate biosynthesis. In this section it was shown that the catalase inhibitor, azide, would decrease oxalate synthesis and increase CO_2 production from glycollate in rat liver extracts. A similar effect could be achieved by heating extracts for 15 minutes at 56^0C, a treatment which completely inhibits catalase activity. It is known that the generation of H_2O_2 by plant peroxisomes is a key factor in the decarboxylation of glycollate and glyoxylate (Halliwell and Butt 1974). Hence a reduction in catalase activity should result in the increased accumulation of H_2O_2 with a concommitant increase in the rate of glyoxylate decarboxylation at the expense of oxalate synthesis. Richardson and Tolbert (1961) demonstrated that the production of oxalate from glyoxylate by spinach leaf glycollate oxidase was stimulated by the addition of catalase. Carbon dioxide production was at the same time inhibited. Similarly, Robinson et al (1962) have shown that pig kidney glycollate oxidase produced more glyoxylate from glycollate in the presence of catalase. With this in mind, any proposed mechanism of hepatic oxalate synthesis should incorporate catalase activity as an important factor. Whilst catalase may be important in the peroxisomal decarboxylation of glycollate and glyoxylate, one cannot ignore the role of the mitochondrial ketoglutarate – glyoxylate carboligase in decarboxylation (Ofallan and Brosemer 1977). This enzyme together with the amino transferases, whether mitochondrial, peroxisomal or cytosolic

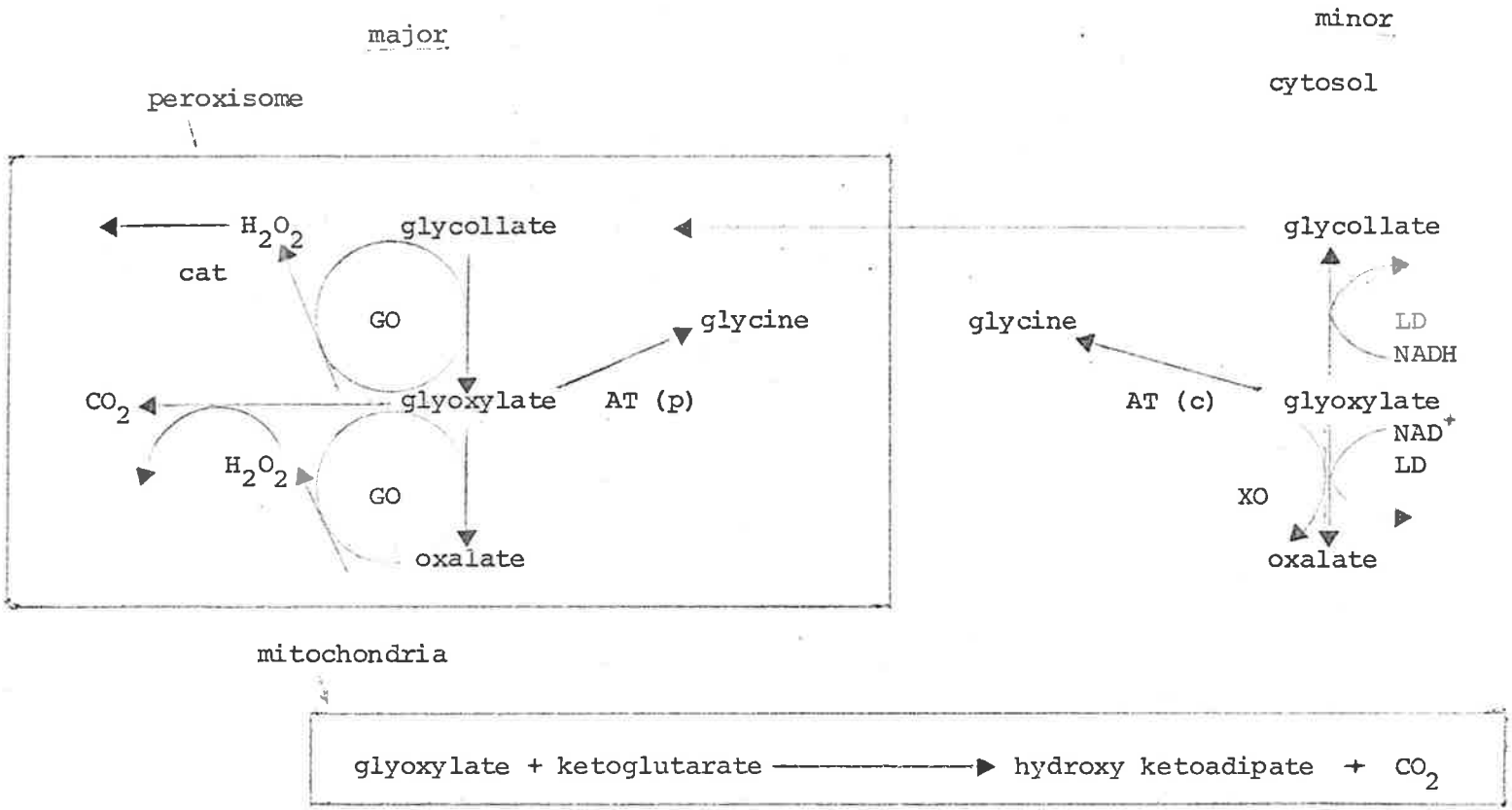
will serve to regulate the concentration of glyoxylate available for oxidation to oxalate.

A proposed scheme for hepatic oxalate synthesis is outlined in figure 20 and includes some of the areas discussed above. Lactate dehydrogenase has been incorporated in this scheme, despite some of the evidence presented here, as there is some uncertainty regarding the physiological conditions under which this reaction may become significant. From this scheme, it can be suggested that substrates in the cytosol, like urate, may facilitate the production of intraperoxisomal H_2O_2, which in turn would increase the rate of CO_2 production from glycollate and glyoxylate (Boveris et al 1972, Halliwell and Butt 1974). This is one possible explanation for the synergistic decarboxylation of glycollate which was observed when the pellet and supernatant fractions were combined (table 21). Inhibition of catalase would enhance this effect. Therefore, catalase, like the pyridoxine dependent transaminases, may be of major importance in regulating the level of glyoxylate available for oxidation to oxalate. An important aspect of this compartmentalised model is that glycollate, being oxidised within the peroxisome, may produce oxalate via glyoxylate, with little of this glyoxylate entering the cytosolic pool. Hence different mechanisms will possibly control the decarboxylation and transamination of glyoxylate derived from glycollate, compared to the glyoxylate added to the cell.

A number of studies have aimed at reducing the *in vivo* and *in vitro* synthesis of oxalate by inhibiting specific enzymes involved in oxalate; namely lactate dehydrogenase (O'Keeffe et al 1973) and glycollate oxidase (Liao and Richardson 1973). The latter authors showed that DL phenyllactate would inhibit oxalate synthesis from glycollate and glyoxylate in the perfused rat liver and would also reduce the toxicity of ethylene glycol *in vivo*. This approach, aimed at regulating the activity of a specific liver enzyme (glycollate oxidase), would appear to have more potential than trying to limit the activity of the non specific and ubiquitous lactate dehydrogenase. An interesting finding in support of this concept is that patients with phenyl-ketonuria show a correlation between increased phenyllactate excretion and decreased oxalate excretion (Chernoff, 1975).

FIG. 20

SCHEME FOR HEPATIC OXALATE SYNTHESIS



This scheme incorporates three pools of glyoxylate within the cell; ie peroxisomal, cytosolic and mitochondrial. Only one site of glycollate metabolism is envisaged (peroxisomal). GO, glycollate oxidase, LD lactate dehydrogenase, cat, catalase, AT, aminotransferases (p, peroxisomal, c, cytosolic), XO, xanthine oxidase

C. PEROXISOMES AND OXALATE SYNTHESIS: THE EFFECT OF CLOFIBRATE

INTRODUCTION

Glycollate oxidase, a key enzyme in oxalate synthesis, is located in the hepatic peroxisomes of the rat, and in the renal and hepatic peroxisomes of the pig (McGroarty et al 1974, deDuve and Baudhuin 1966). My hypothesis that peroxisomes are a major site of oxalate synthesis has recently been supported by the work of Gibbs et al (1977), who found that these organelles contain the major NAD^+ independent oxalate synthesising activity in rat liver. Therefore the observation that the hypolipodaemic drug, clofibrate (ethyl 2, (4 chlorophenoxy), 2 methyl propionate) will cause hepatomegally in the rat, characterised by proliferation of peroxisomes, is of special interest (Hess et al 1965, Hayashi et al 1975).

In this section, the effect of clofibrate on the activity of peroxisomal enzymes is examined in relation to oxalate synthesis *in vivo* and *in vitro*. The effect of phenobarbitone on oxalate synthesis *in vivo* is also briefly investigated.

RESULTS

1. *IN VITRO* EXPERIMENTS

Liver extracts from clofibrate treated rats converted less glycollate to oxalate per gram wet weight of liver than did the controls (table 23). This result corresponded with decreased glycollate oxidase activity in these tissues. Oxalate synthesis from glyoxylate however, did not follow this trend, being slightly increased in liver tissue from clofibrate treated rats. This result corresponded with a rise in lactate dehydrogenase activity. Whilst the administration of clofibrate resulted in a 50% increase in liver weight, the activities of the peroxisomal enzymes glycollate oxidase and catalase were not increased. The reduced activity of these enzymes, as well as that of alanine aminotransferase would appear to be a result of the increase in liver weight, without a corresponding increase in catalytic activity, ie some dilution of the original (control) activity has occurred. Lactate dehydrogenase activity, however, appears to have been induced by clofibrate treatment.

The decarboxylation of both glycollate and glyoxylate was less in livers from clofibrate treated animals compared to the controls. Furthermore a distinct lag phase which was apparent in the *in vitro* oxidation of glycollate to oxalate by cell free extracts was accentuated by clofibrate pretreatment (fig. 21), an effect not seen with glyoxylate as substrate. This result possibly is a reflection of altered catalase and glycollate oxidase activities. For example, if less glycollate is metabolised, then there will be less H_2O_2 produced. This should then result in less CO_2 production. In addition, the reduced catalase activity, coupled with the lower aminotransferase activity, could lead to increased decarboxylation of glycollate during the prolonged incubations.

2. *IN VIVO* EXPERIMENTS

As clofibrate administration had a significant effect on glycollate metabolism *in vitro*, the effect of this drug *in vivo* was investigated. Phenobarbitone induction was also examined, partly as an example of another compound which causes hepatomegaly, though characterised in this instance by microsomal induction. The results of this experiment, shown in table 24 demonstrated that phenobarbitone induction caused a 2 to 3 fold increase in oxalate excretion after glycollate administration.

TABLE 23

EFFECT OF CLOFIBRATE INDUCTION ON OXALATE AND CO_2 PRODUCTION BY RAT LIVER HOMOGENATES

		CONTROL	CLOFIBRATE TREATED	
Glyoxylate ⟶ oxalate	%	9.7 ± 0.2	10.8 ± 0.7	
Glycollate ⟶ oxalate	%	0.27 ± 0.07	0.12 ± 0.03	*
Glyoxylate ⟶ CO_2	%	2.6 ± 0.2	1.7 ± 0.2	**
Glycollate ⟶ CO_2	%	1.2 ± 0.3	0.15 ± 0.02	**
Lactate dehydrogenase		1270 ± 50	2060 ± 70	*
Glycollate oxidase		3.2 ± 0.2	1.6 ± 0.3	**
Catalase		1380 ± 190	1140 ± 100	
Succinate oxidase		42 ± 2	50 ± 1	
Alanine amino transferase		125 ± 8	81 ± 2	**
Protein	mg/ml	152 ± 5	177 ± 7	**
Liver weight	gm/100g body wt	3.16 ± 0.08	4.53 ± 0.26	**

Details of the induction of rats with clofibrate, and the preparation of liver homogenates, the composition of incubation mixtures and the enzyme assays are given in the methods, chapter 2. All activities refer to 1.0 ml of homogenate. Enzyme activities are expressed as OD/min/ml for the respective assays. Results are expressed as the mean ± SEM of 5 livers $p < 0.01$ ** $p < 0.05$ * Student's t test.

Oxalate and CO_2 synthesis from glycollate and glyoxylate was determined in 45 minute incubations at 37^0C.

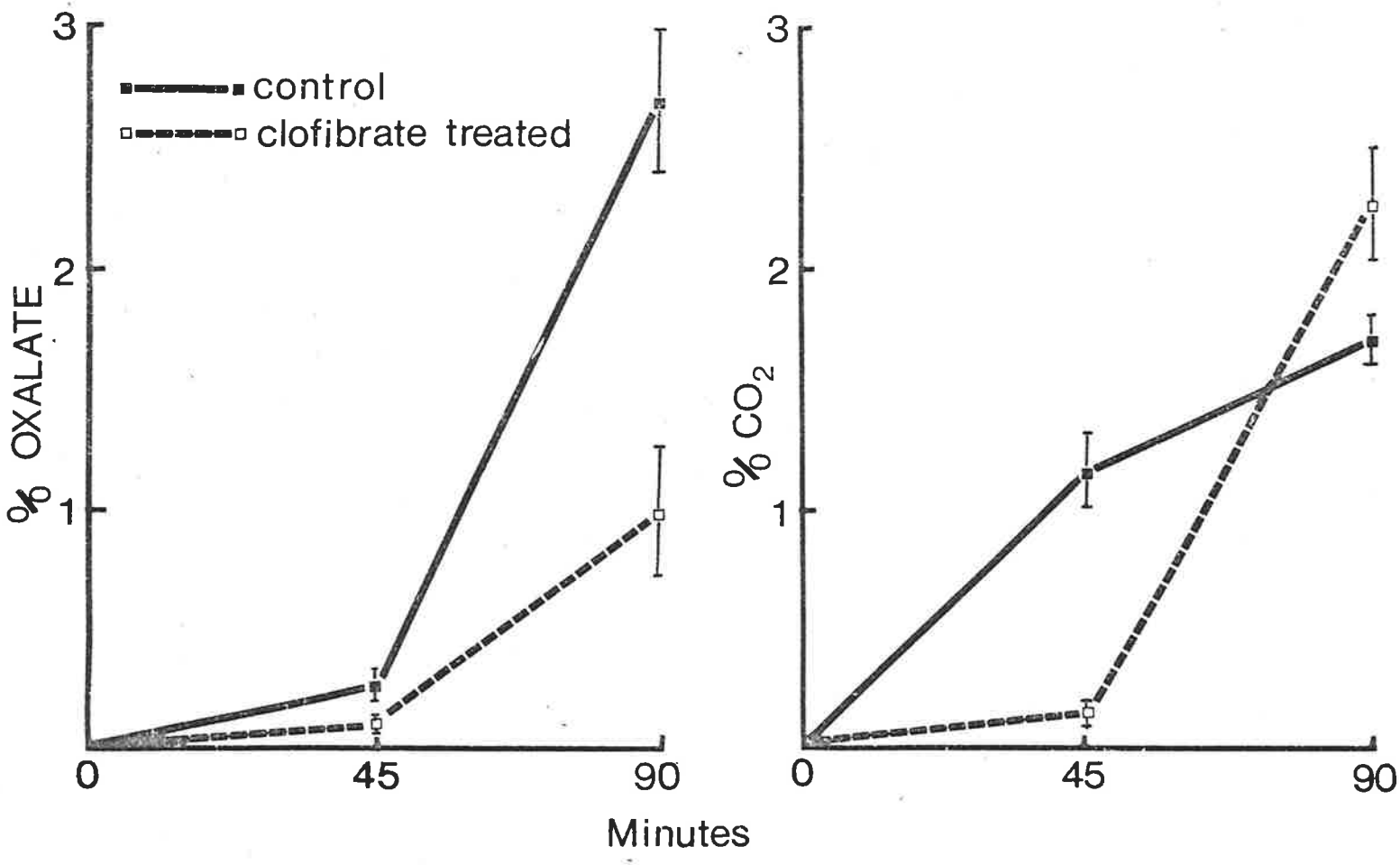


Fig. 21. Oxalate and CO_2 production from glycollate in liver homogenates from clofibrate treated rats. The preparation of the rats and the homogenates is described in the methods. Incubations were performed with 5 mM labelled glycollate, as described for cell free liver extracts in the methods, chapter 2. N = 5.

TABLE 24

THE EFFECT OF CLOFIBRATE AND PHENOBARBITONE TREATMENT ON OXALATE PRODUCTION FROM GLYCOLLATE *IN VIVO*

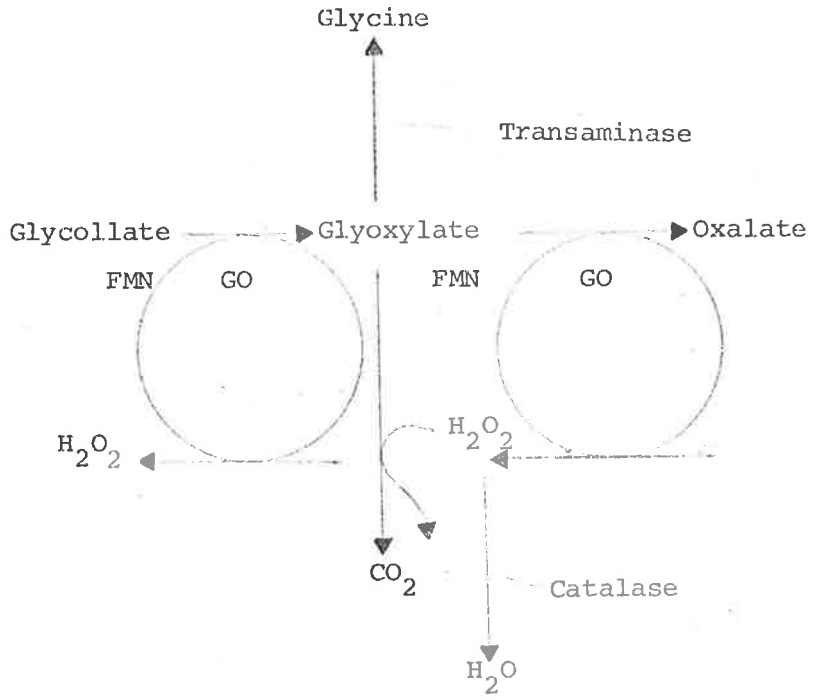
Treatment	Urine vol/ml	Total CPM/ml x 10^3	% of dose excreted	Creatinine u moles/20 hrs	$\lvert^{14}C\rvert$ OXALATE % of dose	CPM/ml urine x10^3	CPM/umol creatinine
none	23.0 ± 6.7	8.9 ± 3.1	7.30 ± 0.42	95 ± 5	0.78 ± 0.23	0.68 ± 0.24	163 ± 53
Phenobarbitone	21.5 ± 0.7	7.6 ± 1.0	7.40 ± 0.50	72 ± 6 *	1.44 ± 0.14 *	1.45 ± 0.18 **	412 ± 61 ***
Clofibrate	6.6 ± 0.7 *	16.4 ± 2.3 *	5.23 ± 0.50 **	44 ± 5 ***	0.88 ± 0.20	2.70 ± 0.54 ***	472 ± 146 *

Sodium glycollate (10 µmoles/2µCi $\lvert 1\text{-}^{14}C\rvert$ glycollate) was administered intraperitoneally in 2 mls of saline to male rats which had been fasted overnight. Urine was collected in acidified cylinders (0.5 ml of 6N HCl) for 20 hours, the rats being held in individual cages with free access to water. Details of the clofibrate or phenobarbitone treatments are given in the methods, chapter 2. Four rats were used in each treatment, the results representing the mean ± S.E.M. Significance levels are denoted by *$p<0.05$, **$p<0.01$ and *** $p<0.005$ Students t test.

Phenobarbitone did not effect urine output or the percentage of the dose excreted. Clofibrate induced rats, however, excreted an average of 7 mls of urine over 20 hours, compared to over 20 mls by the control or phenobarbitone treated animals. Less of the administered dose was excreted by the clofibrate treated animals and the creatinine output over the 20 hour period was less than half that of the control rats. While the oxalate excretion was significantly elevated, if calculated per µmole of creatinine or per ml of urine, the percentage of the administered dose that was converted to oxalate was no different to the controls. It would appear likely that clofibrate administration has an effect on the kidney as well as the liver, resulting in some impairment of renal function, as indicated by the creatinine output.

DISCUSSION

An outline of a possible mechanism for oxalate synthesis, but only incorporating enzymes of peroxisomal origin, is shown below.



GO, glycollate oxidase

Whereas clofibrate administration can result in a wide spectrum of effects with respect to liver enzyme activities, (Zakim et al 1970) this discussion will centre only on the major enzymes of oxalate synthesis and of hydrogen peroxide generation and degradation. With the identification of peroxisomes as a major site of NAD^+ independent synthesis (Gibbs et al 1977) the role of catalase in this area has special significance.

The hepatomegalic effects of clofibrate were clearly shown here, and agree with the results of Hess et al (1965). However, while these authors reported an increase in the specific activity of catalase in the livers from clofibrate treated rats, this effect was not evident here, with glycollate oxidase and catalase activity per gm wet weight of liver being diminished after clofibrate treatment. Goldenberg et al (1976) indicated that proliferated peroxisomes are not enzymically or morphologically normal, while Federico et al (1975), using a drug with similar effects to clofibrate, found the activity of hydroxy acid oxidase and particularly D-amino acid oxidase to be highly diluted in proliferated peroxisomes. The peroxisomal core enzyme, urate oxidase, was also diluted by clofibrate treatment in the studies of Hess et al (1965). Therefore, it would appear

that peroxisomal catalytic activity is not necessarily induced by this drug.

The observed decrease in the activity of glycollate oxidase and catalase in clofibrate treated livers may be important with respect to the control of oxalate production in the liver. The majority of the hepatic hydrogen peroxide is generated within the peroxisomes with little leaving these organelles (Boveris et al 1972, Poole 1975). The addition of substrates like urate or glycollate will facilitate hydrogen peroxide accumulation in peroxisomes (Boveris et al 1972, Thurman and McKenna 1974). This accumulation could enhance the decarboxylation of glycollate and glyoxylate (Halliwell and Butt 1974), an effect which may be further stimulated by the addition of azide with inhibits catalase. As indicated in the previous section, (B), any increase in the decarboxylation of glycollate by way of catalase inhibition, results in decreased oxalate synthesis from this precursor *in vitro* as discussed previously in chapter 6, Section B.

The observation of this present study that the *in vitro* activity of catalase was diminished by clofibrate treatment should have been associated with increased decarboxylation of both glycollate and glyoxylate. That this was not seen with glycollate as substrate may reflect the reduced activity of glycollate oxidase.

Whereas earlier *in vitro* studies indicated that glycollate oxidase is the most significant enzyme in oxalate production from both precursors, there was no correlation between glycollate oxidase activity and oxalate synthesis from glyoxylate in this study. In fact, oxalate synthesis from glyoxylate was slightly increased *in vitro* after clofibrate treatment, which corresponds with an increase in lactate dehydrogenase activity. There is evidence that the activity of mitochondrial enzymes are increased by clofibrate treatment (Hess et al 1961) as shown here with succinate oxidase. However, while early experiments by Crawhall and Watts (1962) indicated that glyoxylate was metabolised to oxalate in liver mitochondria of the rat, recent studies by Gibbs et al (1977) have shown that the oxidation of glyoxylate to oxalate by subcellular fractions from rat and kidney and liver is not associated with mitochondria. Therefore the results here would support the conclusion of the above authors that lactate dehydrogenase is influencing the oxidation of glyoxylate to oxalate.

From the effects of clofibrate on peroxisomes, and the association between glycollate oxidase activity and oxalate synthesis from glycollate, it

is possible to make some interesting comparisons between what may appear to be unrelated experiments. Richardson (1967) showed that the livers of male rats contained more glycollate oxidase than those of females. Testosterone maintained glycollate oxidase levels in castrated males or ovarectomised females. The correlation between maleness, glycollate oxidase activity and oxalate excretion is supported by the observations of Sibergeld and Carter (1959) who found glycollic acid to be more toxic in male rats, Hayashi et al (1973) has shown that the livers of male rats contain more of the peroxisomemal enzymes, catalase and hydroxy acid oxidase than do the livers of female rats. Svoboda et al (1969) found that clofibrate was only effective in inducing peroxisomal proliferation in male rats. However if females were first treated with testosterone, peroxisomal proliferation could be induced with clofibrate. Hence there would appear to be a correlation between endocrine control, glycollate oxidase activity and peroxisomal proliferation.

Other experiments by Richardson (1973) have shown a significant reduction in oxalate production from, and the toxicity of, glycollate, in rats after partial hepatectomy. In other experiments Goldenberg et al (1975) showed that partial hepatectomy reduced the specific activity of α hydroxy acid oxidase and catalase in the liver, concommitant with peroxisomal proliferation. The proliferation was apparently due to fragmentation of large peroxisomes resulting in dilution of the activity of the above peroxisomal enzymes. This finding then, adds an additional facet to the results of Richardson.

While clofibrate administration greatly reduced the *in vitro* synthesis of oxalate from glycollate, this result was not reflected in *in vivo* experiments. There was, however, some evidence of renal complication in clofibrate treated rats compared to control or phenobarbitone treated animals. Phenobarbitone treated rats were included in this study partly as an example of another drug which can cause hepatomegaly. The results, however, were more clear cut than with clofibrate. Rats induced with phenobarbitone excreted more oxalate after glycollate administration than control animals. There was no evidence of renal impairment in this instance and hence it can be suggested that the mixed function microsomal oxidases may participate in the oxidation of glycollate. It has been reported that phenobarbitone pretreatment of rats results in increased oxalate production from methoxyflourane (Son et al 1972). Furthermore the toxicity of methoxyflourane was also increased by this treatment, due

most probably to the increased levels of oxalate or its precursors.

This study has shown the effects of two commonly administered drugs on oxalate synthesis in the rat. While these experiments are of a preliminary nature, the results obtained do indicate that the effect of these drugs on hepatic oxalate biosynthesis is worthy of more intensive investigation.

CHAPTER FIVE

OXALATE SYNTHESIS FROM XYLITOL AND OTHER CARBOHYDRATES

INTRODUCTION

General aspects of oxalate biosynthesis were reviewed in chapter four, where mention was made of the possible intermediates in the pathway from xylitol to oxalate. With the development of a highly sensitive assay for the detection of low levels of $|^{14}C|$ oxalate, and with a better understanding of the metabolic behaviour of xylitol and the immediate oxalate precursors, glycollate and glyoxylate, an attempt can now be made to elucidate the mechanism of oxalate synthesis from xylitol.

Investigations by Hauschildt and Watts (1976b) revealed little difference in the urinary excretion of $|^{14}C|$ oxalate from normal rats infused with $|U\text{-}^{14}C|$ xylitol, sorbitol, fructose and glucose. However, similar studies in this laboratory have demonstrated that pyridoxine deficient rats, infused with $|U\text{-}^{14}C|$ xylitol, excreted significantly more $|^{14}C|$ oxalate when compared with the other labelled infusates (Hannett et al 1977). Recently, Hauschildt *et al* (1976) and Chalmers *et al* (1975) have shown an increased urinary excretion of glycollic acid in patients infused with xylitol, but they found no evidence of increased levels of oxalate in the urine. Current evidence therefore suggests that, while xylitol breakdown may generate oxalate precursors, the eventual oxidation of these precursors to oxalate appears to be dependent on special conditions. In an endeavour to define some of these conditions I have investigated the metabolism of xylitol to oxalate, both *in vivo* and *in vitro*. Isolated rat hepatocytes were used in this section, for, as demonstrated previously, these cells will readily produce oxalate from glycollate and glyoxylate and will also metabolise xylitol and other carbohydrates.

This section describes some of the factors affecting oxalate production from xylitol, including the effects of pyridoxine deficiency, substrate concentration and altered intracellular redox states.

RESULTS

1. DETECTION OF OXALATE IN TISSUES OF THE RAT AFTER THE INJECTION OF |U-^{14}C| XYLITOL

This experiment was prompted by the clinical observation of calcium oxalate crystals in the lungs, kidneys and brains of patients who had received intravenous xylitol. Only three rats were used in this study due to the amount of |U-^{14}C| xylitol required. The results in table 25 show that the rat killed at 1.5 hours had substantially more |^{14}C| oxalate in its tissues than one killed 6 hours after the injection of xylitol. The accumulation of oxalate was greatest in the kidneys with the urinary level of oxalate being greatest after 6 hours. The simplest interpretation of these results is that oxalate production from xylitol occurs rapidly within the first hour after injection followed by clearance from the body. From this preliminary data, it cannot be concluded that oxalate is accumulated to any extent in the kidneys or brains of rats.

2. OXALATE AND GLYCOLLATE PRODUCTION IN VIVO

|^{14}C| oxalate was excreted in the urine from rats which had been injected with |U-^{14}C| xylitol, |U-^{14}C| sorbitol, |1-^{14}C| glycollate and |U-^{14}C| glyoxylate (table 26). Pyridoxine deficiency increased this excretion in all treatments. Perhaps the most important observation is that control animals excreted 12-fold more labelled oxalate after xylitol injection, compared with the sorbitol treatment. Hauschildt and Watts (1976) were unable to show this difference. One possible explanation is that the enzymic oxalate assay used here is the only method capable of reproducibly detecting the low levels of |^{14}C| oxalate generated from these carbohydrates.

The stimulating effect of pyridoxine deficiency on oxalate excretion from glycollate (5-fold increase) compared with glyoxylate (1.5-fold increase) has previously been recorded by Runyan and Gershoff (1965). In this respect, the results obtained with xylitol parallel those observed after glycollate administration. Between 80% and 90% of the total |^{14}C| oxalate excreted was recovered in the first 10 hours and hence only the results for this period were included.

TABLE 25

$|^{14}C|$ OXALATE IN THE TISSUES OF PYRIDOXINE DEFICIENT RATS AFTER $|U^{14}C|$ XYLITOL INJECTIONS.

TISSUE	$|^{14}C|$ OXALATE (cpm/gm wet wt tissue) HOURS AFTER INJECTION		
	1.5	3	6
Liver	107	89	10
Kidney	398	213	70
Brain	70	120	45
Blood	82	24	0
Urine	0	56	1900

Three pyridoxine deficient rats were injected intraperitoneally with 0.5 gm of xylitol, 10 µCi $|U^{14}C|$xylitol in 2.0 ml of normal saline. The rats were held in individual cages with free access to water and were killed at 1.5, 3 and 6 hours after the injection of xylitol.

Tissues (0.5 gm) were homogenised in 2.5 ml of 0.4 M potassium citrate buffer pH3.0, using sharp blades and a glass homogeniser. One ml of the homogenate was assayed for oxalate, using oxalate decarboxylase. Recovery of $|^{14}C|$ oxalate from tissues was more than 60% as determined in control experiments where $|^{14}C|$ oxalate was injected into rats. The results are expressed as counts per minute of oxalate per gm wet weight of tissue.

TABLE 26

OXALATE PRODUCTION IN VIVO AND IN ISOLATED HEPATOCYTES FROM NORMAL AND PYRIDOXINE DEFICIENT RATS.

SUBSTRATE	Percentage $\|^{14}C\|$ Oxalate			
	URINE		HEPATOCYTES	
	CONTROL	PYRIDOXINE DEFICIENT	CONTROL	PYRIDOXINE DEFICIENT
$\|U\text{-}^{14}C\|$ Xylitol	0.072 ± 0.010	0.350 ± 0.010	0.013 ± 0.002	0.046 ± 0.016
$\|U\text{-}^{14}C\|$ Sorbitol	0.006 ± 0.003	0.044 ± 0.013	0.007 ± 0.004	0.014 ± 0.008
$\|1\text{-}^{14}C\|$ Glycollate	1.29 ± 0.15	6.13 ± 3.20	1.27 ± 0.18	1.72 ± 0.74
$\|U\text{-}^{14}C\|$ Glyoxylate	12.9 ± 1.90	17.5 ± 3.20	7.29 ± 0.96	7.37 ± 1.44

Experimental details are described in the methods, chapter 2. The urinary results represent the first 10 hour collection. The results for the hepatocytes represent the percentage conversion of substrate to oxalate in 30 minutes. In all cases, the results represent the mean ± S E M of either 3 rats, or 3 separate liver cell isolations. Aspartate amino transferase activity of the pyridoxine deficient rats was 54% ± 6% of control values, as determined in washed red blood cell haemolysates.

Rats treated with |U-^{14}C| xylitol excreted more |^{14}C| glycollate in the urine than those treated with |U-^{14}C| sorbitol (Table 27). There was, however, no difference between control and pyridoxine deficient rats, indicating that pyridoxine deficiency does not cause an increase in precursor production but merely enhances oxalate production from such precursors.

3. OXALATE PRODUCTION IN HEPATOCYTES

Oxalate production from xylitol, sorbitol, glycollate and glyoxylate in hepatocytes demonstrated similar trends to those observed *in vivo* (Table 26). As the liver has been identified as a major site of oxalate synthesis in the rat (Richardson 1973, Liao and Richardson 1972), this correlation between the hepatic production and urinary excretion of oxalate from these precursors was not unexpected. The diminished effect of pyridoxine deficiency on oxalate production in hepatocytes compared to the *in vivo* study may relate to the different experimental times employed. A further comparison of |^{14}C| oxalate production from various carbon labelled carbohydrates in hepatocytes isolated from pyridoxine deficient rats is shown in table 28. In preliminary experiments under these conditions, it was found that 1 mM xylitol, sorbitol, fructose and glycerol were almost completely utilised, as determined by the production of glucose, lactate, pyruvate and α-glycerophosphate. |U-^{14}C| xylitol produced 3-fold more |^{14}C| oxalate than |U-^{14}C| fructose, the next most effective precursor.

4. EFFECT OF XYLITOL CONCENTRATION AND REDOX STATE

Investigations using increasing concentrations of |U-^{14}C| xylitol indicated an optimum concentration of 1 mM for maximal conversion of xylitol to oxalate in 1 hour (Fig. 22). Metabolic investigations (table 29) have revealed that at xylitol concentrations above 1 mM, the apparent intracellular NADH/NAD$^+$ ratio increases markedly, due to NADH generated in the dehydrogenation of xylitol. This factor alone will inhibit oxalate synthesis from glycollate and glyoxylate as shown previously with sorbitol in chapter four, and may explain why increased oxalate synthesis was not observed at xylitol concentrations above 1 mM.

TABLE 27

|^{14}C| GLYCOLLATE PRODUCTION FROM |U-^{14}C| XYLITOL AND |U-^{14}C| SORBITOL IN VIVO

	Percentage |^{14}C| Glycollate in the Urine	
SUBSTRATE INJECTED	CONTROL	PYRIDOXINE DEFICIENT
|U-^{14}C| Xylitol	0.38 ± 0.02	0.39 ± 0.03
|U-^{14}C| Sorbitol	0.20 ± 0.04	0.19 ± 0.01

|^{14}C| glycollate is expressed as a percentage of the radioactivity of the substrate added, and represents the first 10 hour urine collection. The results are expressed as the mean ± SEM of 3 rats. Experimental details are given in the methods, chapter 2 and in table 26.

TABLE 28

|^{14}C| OXALATE PRODUCTION FROM |^{14}C| LABELLED CARBOHYDRATES IN ISOLATED HEPATOCYTES

SUBSTRATE		CONVERSION TO |^{14}C| OXALATE	
		%	n moles/10^8 cells/hr
Xylitol	|U-^{14}C|	0.210	42
Sorbitol	"	0.040	8
Glucose	"	0.003	1
Fructose	"	0.066	13
Glycerol	"	0.046	9

The reaction contained 1mM substrate, 1 µCi/µmole. Further details are given in the methods, chapter 2. The reaction time was 1 hour with the amount of |^{14}C| oxalate formed being expressed as a percentage of the initial substrate added. Hepatocytes were isolated from a pyridoxine deficient rat. The limit of detectability using the enzymic oxalate assay is 0.001% conversion.

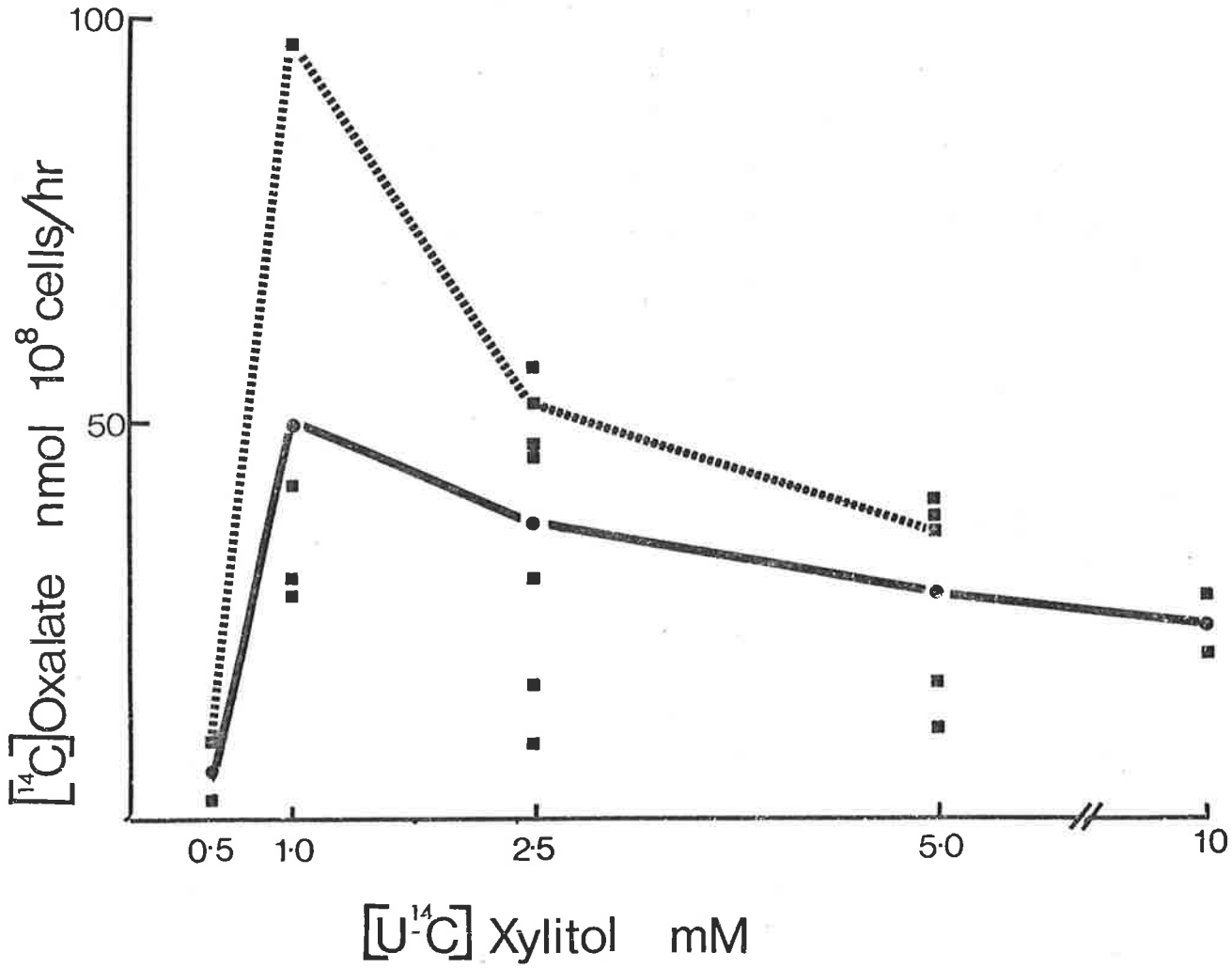


Fig. 22. The effect of xylitol concentration on oxalate production in isolated hepatocytes. Experimental conditions are given in the methods, chapter 2. |U-^{14}C| xylitol was used at a specific activity of 0.4 µCi/µmole. The broken line represents the results from one cell isolation, and the solid line is the average of all experiments. Pyridoxine deficient hepatocytes were used. The incubation period was 1.0 hour.

TABLE 29

THE EFFECT OF INCREASING XYLITOL CONCENTRATIONS ON CYTOSOLIC NADH/NAD^+ COUPLES IN RAT HEPATOCYTES

Xylitol concentration mM	Lactate / Pyruvate	Glycerophosphate / Dihydroxyacetone-phosphate	Xylitol metabolised µmoles/10^8cells/hr
0	12	1	-
1	11	6	42
2	25	12	52
5	22	39	59
10	32	31	60

Details of the reaction are given in the methods, chapter 2. The reaction time was 30 minutes. The amount of xylitol metabolised was calculated from the metabolites produced, i.e. xylitol metabolised = $^6/_5$ |Glucose + $^1/_2$ (Lactate + Pyruvate + Glycerophosphate + Dihydroxyacetone-phosphate)|

5. THE EFFECT OF PHENAZINE METHOSULPHATE

This effect of high intracellular NADH levels on both xylitol utilization and oxalate synthesis from glycollate and glyoxylate can be relieved by using an artificial electron acceptor, such as phenazine methosulphate (PMS) which facilitates NAD^+ regeneration within the hepatocyte. The effect of increasing concentrations of PMS on the metabolism of xylitol is shown in Figure 23. In this experiment, 5 mM xylitol was used so that the substrate concentration would not be limiting, whilst at the same time the intracellular $NADH/NAD^+$ ratio would initially be elevated. This electron acceptor stimulated glucose, lactate and oxalate production and dramatically lowered glycerophosphate synthesis. Whilst the maximum effect on glucose and glycerophosphate production occurred at 20 µM PMS, the increase in lactate and oxalate production was sustained up to 50 and 100 µM PMS respectively.

The ability of PMS to stimulate oxalate synthesis from glycollate and glyoxylate was used in an attempt to elucidate the pathway by which xylitol is metabolised to oxalate. Table 30 shows the effect of unlabelled xylitol on the oxidation of $|1\text{-}^{14}C|$ glycollate and $|U\text{-}^{14}C|$ glyoxylate to $|^{14}C|$ oxalate. The presence of xylitol diminished oxalate production from both precursors, a result which is similar to the effect of sorbitol (chapter four). This result indicates that there is either an inhibitory effect of the NADH generated during polyol metabolism, or that a metabolite of xylitol is competing with the labelled oxalate precursors. The observation that PMS would not reverse but actually increased this inhibition of $|^{14}C|$ oxalate production from $|1\text{-}^{14}C|$ glycollate indicates the latter possibility may be true. However, partial reversal of this inhibition by PMS with $|U\text{-}^{14}C|$ glyoxylate as substrate would suggest a redox effect is responsible in this instance. Hence this experiment implies that a metabolite is being produced by xylitol which interferes with oxalate synthesis, particularly from $|1\text{-}^{14}C|$ glycollate.

6. ISOTOPE DILUTION STUDIES

The addition of unlabelled carbohydrates to hepatocytes in the presence of labelled glycollate and glyoxylate resulted in decreased $|^{14}C|$ oxalate synthesis (table 31). Studies with the enzymic oxalate assay employed here, did not reveal any loss of efficiency in the presence of increased amounts of unlabelled oxalate. Therefore these results are not

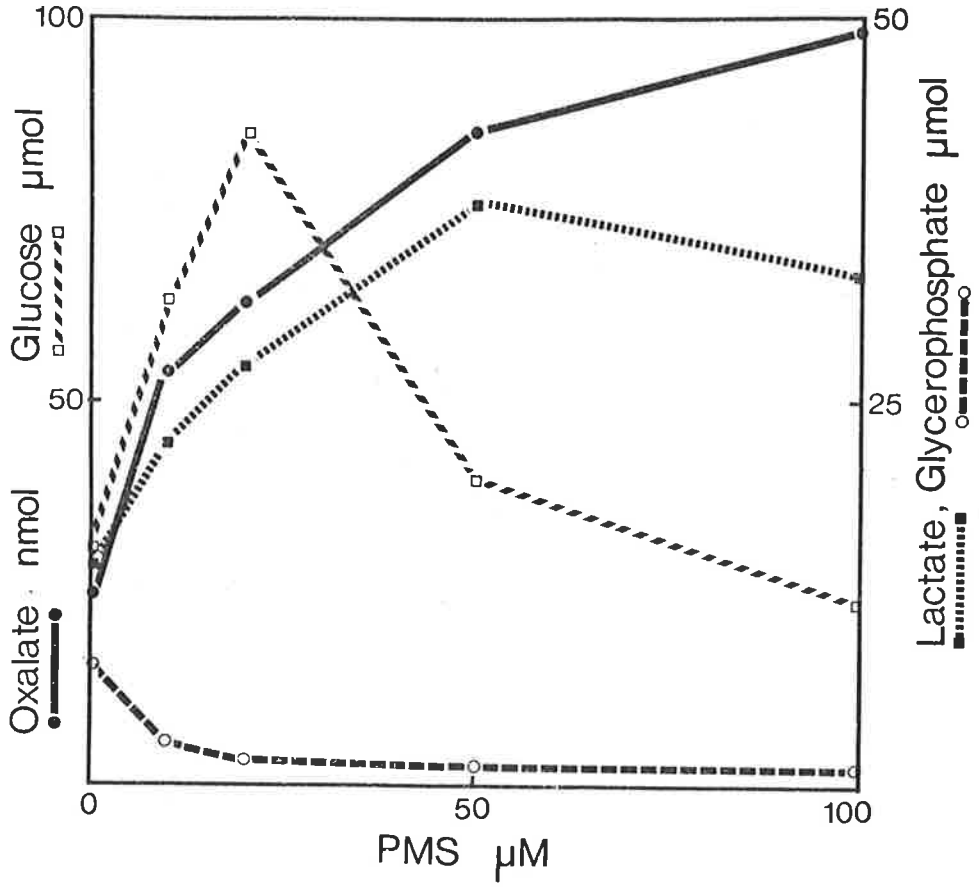


Fig. 23. The effect of phenazine methosulphate (PMS) on the metabolism of xylitol in isolated rat hepatocytes. Hepatocytes were prepared from a pyridoxine deficient rat and incubated with 5 mM xylitol (0.4 µCi/µmole) in oxygenated phosphate buffer for 30 mins. Results are expressed per 10^8 cells/hr. Experimental details are given in the methods, chapter 2.

TABLE 30

THE EFFECT OF XYLITOL AND PHENAZINE METHOSULPHATE (PMS) ON THE CONVERSION OF |U-^{14}C| GLYOXYLATE AND |1-^{14}C| GLYCOLLATE TO |^{14}C| OXALATE IN RAT HEPATOCYTES

	|^{14}C| OXALATE (%)			
SUBSTRATE	CONTROL	PMS	XYLITOL	PMS AND XYLITOL
|U-^{14}C| Glyoxylate	12.7	19.3	7.1	8.4
|1-^{14}C| Glycollate	4.8	13.9	2.1	0.8

Experimental conditions are given in the methods, chapter 2. The concentration of xylitol was 5mM and PMS, 10 µM. Hepatocytes were isolated from a pyridoxine deficient rat. The incubation time was 30 minutes.

TABLE 31

EFFECT OF UNLABELLED CARBOHYDRATES ON OXALATE SYNTHESIS FROM LABELLED GLYCOLLATE AND GLYOXYLATE IN RAT HEPATOCYTES

	PERCENTAGE OF $\lvert^{14}C\rvert$ OXALATE FROM	
ADDITION	$\lvert U\text{-}^{14}C\rvert$ GLYOXYLATE	$\lvert 1\text{-}^{14}C\rvert$ GLYCOLLATE
----	16.3	5.1
D-glucose	13.9	3.8
Sorbitol	6.8	1.1
D-xylulose	8.2	1.3
Xylitol	4.9	0.7

The incubation conditions are given in the methods, chapter 2. The incubations contained 1mM glycollate or glyoxylate and 5mM carbohydrate. The reaction time was 30 minutes.

influenced by any dilution of $|^{14}C|$ oxalate with unlabelled oxalate produced from the carbohydrates. It would appear that the carbohydrates are producing metabolites which compete with glycollate or glyoxylate in the oxalate biosynthetic pathway, or, in the case of xylitol and sorbitol, the change in the cellular redox state has caused a decrease in the oxidation of glycollate and glyoxylate. This latter effect would explain the greater inhibitory effect of xylitol compared to D-xylulose. Whilst it can be speculated that glycollate produced during the metabolism of xylitol or D-xylulose is the cause of the reduced conversion of glycollate to oxalate, the presence of other competing substrates, particularly lactate cannot be ignored. As the difference between the effects of xylitol and sorbitol are small, it can be concluded that the results observed here are largely the result of a more reduced intracellular redox state, and the production of competing substrates not related to the oxalate biosynthetic pathway.

An alternative approach to the previous experiment is shown in table 32. Here, the effect of unlabelled oxalate precursors on $|^{14}C|$ oxalate synthesis from $|U\text{-}^{14}C|$ xylitol in hepatocytes is examined. Surprisingly, the major oxalate precursors, glycollate and glyoxylate increased rather than decreased the amount of oxalate produced from xylitol. L-ascorbate was the only compound which significantly diminished oxalate synthesis from xylitol, which indicates that L-ascorbate either inhibits the metabolism of xylitol, or has a specific effect on oxalate biosynthesis.

The effect of another intermediate of the uronic acid cycle is shown in fig. 24. Like L-ascorbate, D-glucuronolactone also inhibits oxalate synthesis from xylitol, whereas 1 mM glycollate caused a 3 fold increase in $|^{14}C|$ oxalate synthesis from $|U\text{-}^{14}C|$ xylitol. From the results obtained in chapter four regarding the affinity of glycollate oxidase for glycollate (Km 0.7 mM) it can be suggested that this increase in oxalate production is due to a stimulation of glycollate oxidase activity which would normally be low, assuming that low levels of glycollate are produced from xylitol.

The effect of these compounds on general xylitol metabolism is shown in table 33. Glucuronolactone appeared to decrease glucose production from xylitol and also limited the amount of glycerophosphate formed. This latter effect was also seen in the presence of glycollate. However, while the metabolism of xylitol is decreased by these compounds the effects would not appear to be great enough to explain the large change in oxalate

TABLE 32

THE EFFECT OF UNLABELLED OXALATE PRECURSORS ON |^{14}C| OXALATE SYNTHESIS FROM |U-^{14}C| XYLITOL IN HEPATOCYTES

ADDITION (0.5mM)	PERCENTAGE |^{14}C| OXALATE FROM |U-^{14}C| XYLITOL
-----	0.060
Glycolaldehyde	0.056
Glycollate	0.084
Glyoxylate	0.086
Glycine	0.058
DL-Serine	0.070
L-Hydroxy proline	0.068
L-Ascorbate	0.004
Boiled cells	0.004

Hepatocytes were prepared from a pyridoxine deficient rat. Incubations contained 2.5mM xylitol, (0.4µCi/µmol). The incubation time was 1.0 hour. Further details appear in the methods, chapter 2.

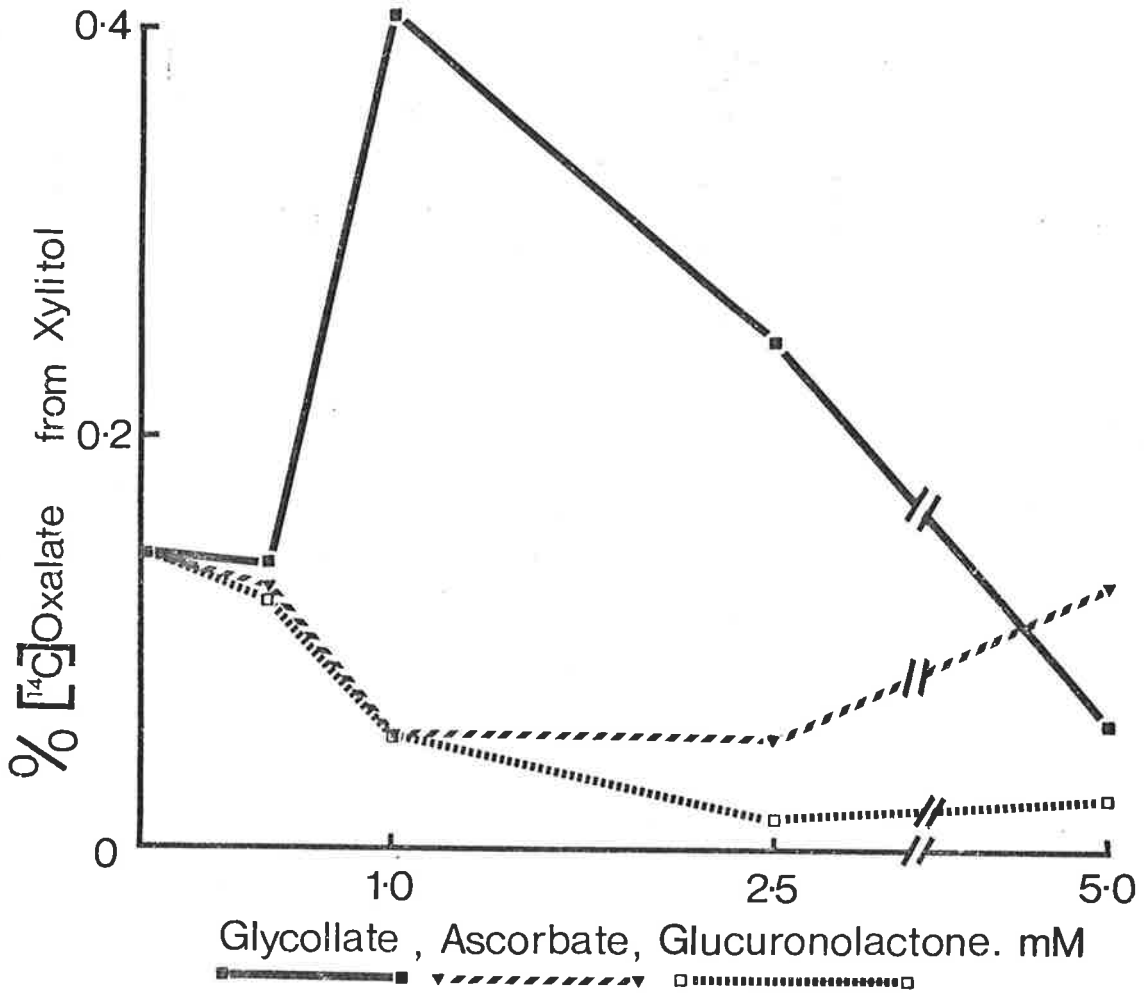


Fig. 24. The effect of glycollate, ascorbate and glucuronolactone on $|^{14}C|$ oxalate synthesis from $|U\text{-}^{14}C|$ xylitol in pyridoxine deficient hepatocytes. The xylitol concentration was 2.5 mM, 0.4 µCi/µmole and the incubation time was 1.0 hour. Further details are given in the methods, chapter 2.

TABLE 33

THE EFFECT OF OXALATE PRECURSORS ON XYLITOL METABOLISM IN HEPATOCYTES

ADDITION	CONCENTRATION (mM)	METABOLITE GLUCOSE	METABOLITE LACTATE	METABOLITE GLYCEROPHOSPHATE
-----		17.6	10.6	5.3
Glyoxylate	1.0	19.0	10.4	4.8
Glycollate	1.0	16.8	6.3	1.4
L-Ascorbate	1.0	16.2	6.5	4.1
D-Glucuronolactone	1.0	13.6	3.4	0.3

Incubations contained 2.5mM xylitol and 10^7 cells in 2.0 ml. Hepatocytes were prepared from a pyridoxine deficient rat. The reaction time was 30 minutes. Results are expressed as µmoles produced/10^8 cells/hr and have been corrected for the appropriate controls. The addition of the precursors was made simultaneously with xylitol. Further details appear in the methods, chapter 2.

synthesis shown in fig. 24. The mechanism of this latter effect would appear to be specifically related to the pathway from xylitol to oxalate, rather than a general inhibition of xylitol breakdown.

7. OXALATE PRODUCTION FROM DIFFERENTIALLY LABELLED CARBOHYDRATES

The limitations of using labelled compounds in the investigation of complicated metabolic pathways is recognised. However, as hepatocytes will metabolise a number of carbohydrates, oxalate synthesis from various labelled compounds was investigated. Phenazine methosulphate was included in incubations to facilitate flux through the oxalate biosynthetic pathway. The results in table 34 indicate that $|2\text{-}^{14}C|$ glucose is a better oxalate precursor than either $|U\text{-}^{14}C|$ or $|6\text{-}^{14}C|$ glucose, a result which does not support the involvement or the uronic acid cycle in oxalate biosynthesis as the carbon 1 position of ascorbate is derived from carbon 6 of glucose. The observation that $|U\text{-}^{14}C|$ fructose is superior to $|1\text{-}^{14}C|$ fructose as an oxalate precursor, together with the results obtained using labelled glucose, suggest that the 2,3 position of glucose is a possible source of two carbon oxalate precursors. This 2,3 position of glucose or fructose will give rise to either the carbon atoms 2 and 3 of glycerophosphate or 1 and 2 of xylulose-5-phosphate in the pentose phosphate cycle, as suggested by Runyan and Gershoff (1965). Unfortunately, insufficient oxalate was produced from labelled ribose to fully test this hypothesis.

8. COMPETITION BETWEEN XYLITOL AND FRUCTOSE IN HEPATOCYTES

If the mechanism of oxalate production from xylitol is common to other carbohydrates, then some competition would be expected between the most effective oxalate precursors, xylitol and fructose. This in fact does occur as shown in table 35 for oxalate synthesis and in table 36 for general metabolism. The addition of unlabelled xylitol to hepatocytes reduced the level of $|^{14}C|$ oxalate produced from $|U\text{-}^{14}C|$ fructose, and vice versa. The pattern of metabolite production from these carbohydrates, in combination, was also markedly different to that obtained if xylitol or fructose were added alone. The elevated glycerophosphate levels seen during xylitol metabolism were reduced by more than 70% in the presence of fructose, whilst the levels of pyruvate in the presence of xylitol and fructose were less than 30% of those observed with fructose as the sole substrate. Competition for glucose production was also apparent. While these results give no clue to the origin of oxalate precursors in general

TABLE 34

$|^{14}C|$ OXALATE SYNTHESIS FROM $|^{14}C|$ LABELLED CARBOHYDRATES IN HEPATOCYTES

SUBSTRATE		PERCENTAGE $|^{14}C|$ OXALATE ----	+ PMS
$|U^{14}C|$	D-Glucose	0.003	0.007
$|2^{14}C|$	"	0.009	0.013
$|6^{14}C|$	"	0.003	0.003
$|U^{14}C|$	D-Fructose	0.051	0.088
$|1^{14}C|$	"	0.001	0.004
$|U^{14}C|$	Dribose	0.000	0.009
$|1^{14}C|$	"	0.000	0.007
$|U^{14}C|$	Xylitol	0.027	0.095
$|1^{14}C|$	L-Arabinose	0.000	0.000
$|U^{14}C|$	D-Xylose	0.000	0.000

Hepatocytes were isolated from a pyridoxine deficient rat. The incubation time was 1.0 hour. All substrates were 5mM, 0.5μCi/μmole and (PMS) phenazine methosulphate was 50μM. $|^{14}C|$ oxalate was detectable to 0.001% conversion.

TABLE 35

COMPETITION BETWEEN XYLITOL AND FRUCTOSE FOR OXALATE PRODUCTION IN HEPATOCYTES

		$\lvert^{14}C\rvert$ OXALATE from			
		$\lvert U^{14}C\rvert$ Fructose	$\lvert U^{14}C\rvert$ Fructose Xylitol (unlabelled)	Fructose (unlabelled) $\lvert U^{14}C\rvert$ Xylitol	$\lvert U^{14}C\rvert$ Fructose $\lvert U^{14}C\rvert$ Xylitol
Fructose (mM)	0	-	-	20	20
	2.5	36	13	17	30
	5.0	39	14	8	36
	10.0	73	49	9	61

The results are expressed as nmoles $\lvert^{14}C\rvert$ oxalate produced/10^8 cells/hour. Details of the incubation procedure appear in the methods, chapter 2. The xylitol concentration was 5mM, 0.4µCi/µmole. Two µCi of fructose were used per incubation. The reaction time was 1.0 hour. Hepatocytes were prepared from a pyridoxine deficient rat.

TABLE 36

COMPETITION BETWEEN XYLITOL AND FRUCTOSE METABOLISM IN RAT HEPATOCYTES

		METABOLITES PRODUCED							
		GLUCOSE		LACTATE		PYRUVATE		GLYCEROPHOSPHATE	
		fructose	fructose + xylitol	fructose	fructose +xylitol	fructose	fructose +xylitol	fructose	fructose +xylitol
Fructose (mM)	0	-	67	-	23	-	0.55	-	9.4
	2.5	74	122	15	45	2.70	0.55	1.4	9.0
	5.0	141	156	51	78	6.15	1.35	2.1	6.8
	10.0	114	132	100	90	6.90	1.90	2.4	4.8

The results are expressed as μmoles produced/10^8cells/hour. Details of the incubation procedure appear in the methods, chapter 2. The xylitol concentration was 5mM and the reaction time was 30 minutes. Hepatocytes were prepared from a pyridoxine deficient rat.

metabolic pathways, it would nevertheless seem that the pathway of oxalate production from xylitol is shared, at least in part, by fructose.

One aspect of these results, which has not been fully investigated is that at high substrate concentrations, fructose appears to be a more effective producer of oxalate than xylitol. However, it should be pointed out that at a substrate concentration of 10 mM, the rate of fructose breakdown is 2-3 fold that of xylitol. Therefore, in this respect, sorbitol is the more useful compound for comparative studies with xylitol, as the rate of utilisation of these polyols is similar as are the factors which regulate their metabolism.

DISCUSSION

The administration of $|U\text{-}^{14}C|$ xylitol to the rat *in vivo*, or to hepatocytes *in vitro*, resulted in the production of $|^{14}C|$ oxalate. This phenomena was readily demonstrated using intraperitoneal injections of xylitol, rather than the more complicated infusion technique used previously (Hannett et al 1977).

Xylitol produced 12 and 8 fold more urinary oxalate than sorbitol in normal and pyridoxine deficient rats respectively. This marked difference in oxalate production from xylitol and sorbitol is of particular interest, as the hepatic metabolism of both carbohydrates, both *in vivo* and *in vitro*, results in the production of similar levels of the major metabolites, glucose, lactate and α-glycerophosphate. Therefore, the production of an oxalate precursor from xylitol most likely occurs before the triosephosphate level of glycolysis, thus implicating the reactions of the pentose phosphate or uronic acid cycles in oxalate synthesis from xylitol.

The role of pyridoxine in oxalate synthesis from glycollate and glyoxylate has been discussed previously. In this section, the *in vivo* experiments demonstrated that pyridoxine deficient rats excreted 5-fold more oxalate than control animals, when injected with xylitol or glycollate, but only 1.5-fold more when glyoxylate was administered. Similar studies by Runyan and Gershoff (1965) demonstrated an even greater difference between glycollate and glyoxylate; pyridoxine deficient rats excreting 18-fold and 1.5-fold more oxalate respectively, compared with normal animals. The implication from these experiments is that glycollate rather than glyoxylate may be the key intermediate in oxalate synthesis from xylitol. A similar inference can be made from the isotope dilution experiments with labelled glycollate and glyoxylate, and unlabelled xylitol, in the presence of PMS. Other evidence which supports this concept is the actual demonstration, both here and by Chalmers et al (1975) and Hauschildt et al (1976a), of glycollate as a metabolite of xylitol metabolism. Apart from glyoxylate, glycolaldehyde is the only established source of glycollate in mammalian systems.

Speculation on the origin of oxalate from xylitol has largely centred on a mechanism whereby glycolaldehyde is produced, either free, or bound to thiamine pyrophosphate, during the transketolase reaction (Hauschildt et al 1976a Hannet et al 1977). Evidence from *in vitro* experiments with

mammalian transketolase supports this speculation (Datta and Racker 1959, Holzer et al 1962). Furthermore, in bacterial and plant systems it has been established that glycollic acid can be produced from pentose phosphates by the action of transketolase or phosphoketolase (Shain and Gibbs 1971, Holzer and Schroeter 1962). However, other possibilities, including the involvement of the uronic acid pathway in oxalate synthesis, cannot be excluded, particularly in view of the gas chromatographic evidence that xylitol infusions into patients resulted in the increased excretion of some uronic acids (Hauschildt et al 1976a)

The decarboxylation of 3-keto L-gulonic acid to form L-xylulose is thought to be an irreversible reaction. However, Bublitz and York (1961) have reported the production of small amounts of a 6 carbon compound, presumed to be 3-keto L-gulonic acid, by a gulonate dehydrogenase - decarboxylase complex in the presence of L-xylulose, CO_2 and high levels of NADH. 3-Keto L-gulonic acid is only one oxidative step from 2,3-diketo L-gulonic acid, the immediate precursor of oxalate in the uronic acid cycle. (Baker et al 1966, and also figure 25). In the present study, attempts were made to show the incorporation of labelled CO_2 into oxalate during xylitol metabolism in hepatocytes, with negative results.

Threonic acid, a product of 2,3-diketo L-gulonic acid metabolism is present in increased amounts in the urine of xylitol infused patients (Chalmers et al 1975, Hauschildt et al 1976a). These authors suggest that the source of threonic acid, in this instance, is D-erythrulose from the pentose phosphate pathway. These xylitol infused patients exibited more glucuronate in their urine than patients receiving glucose. This finding, together with the demonstration here, that L-ascorbate and D-glucuronolactone inhibited $|^{14}C|$ oxalate synthesis from $|U-\ ^{14}C|$ xylitol in rat hepatocytes, shows that this area of metabolism requires further attention. Metabolic studies with guinea pig liver mitochondria have shown that glucuronolactone inhibits the utilisation of L-xylulose (Touster et al 1956). Hence while attention is usually focused on the further metabolism of D-xylulose, the possibility that L-xylulose may be involved in oxalate synthesis cannot be ignored, though the lack of pathological effects in patients with pentosuria is evidence against such an involvement.

There is other evidence which does not support the involvement of the

uronic acid cycle in the pathway of oxalate synthesis from xylitol. Oxalate is derived from carbon atoms 1 and 2 of ascorbate, which in turn come from carbon atoms 5 and 6 of glucose, yet as shown here and by Runyan and Gershoff (1965), $|2\text{-}^{14}C|$ glucose is a better oxalate precursor than $|6\text{-}^{14}C|$ glucose, a finding which supports the pentose phosphate-transketolase hypothesis for the generation of two carbon oxalate precursors.

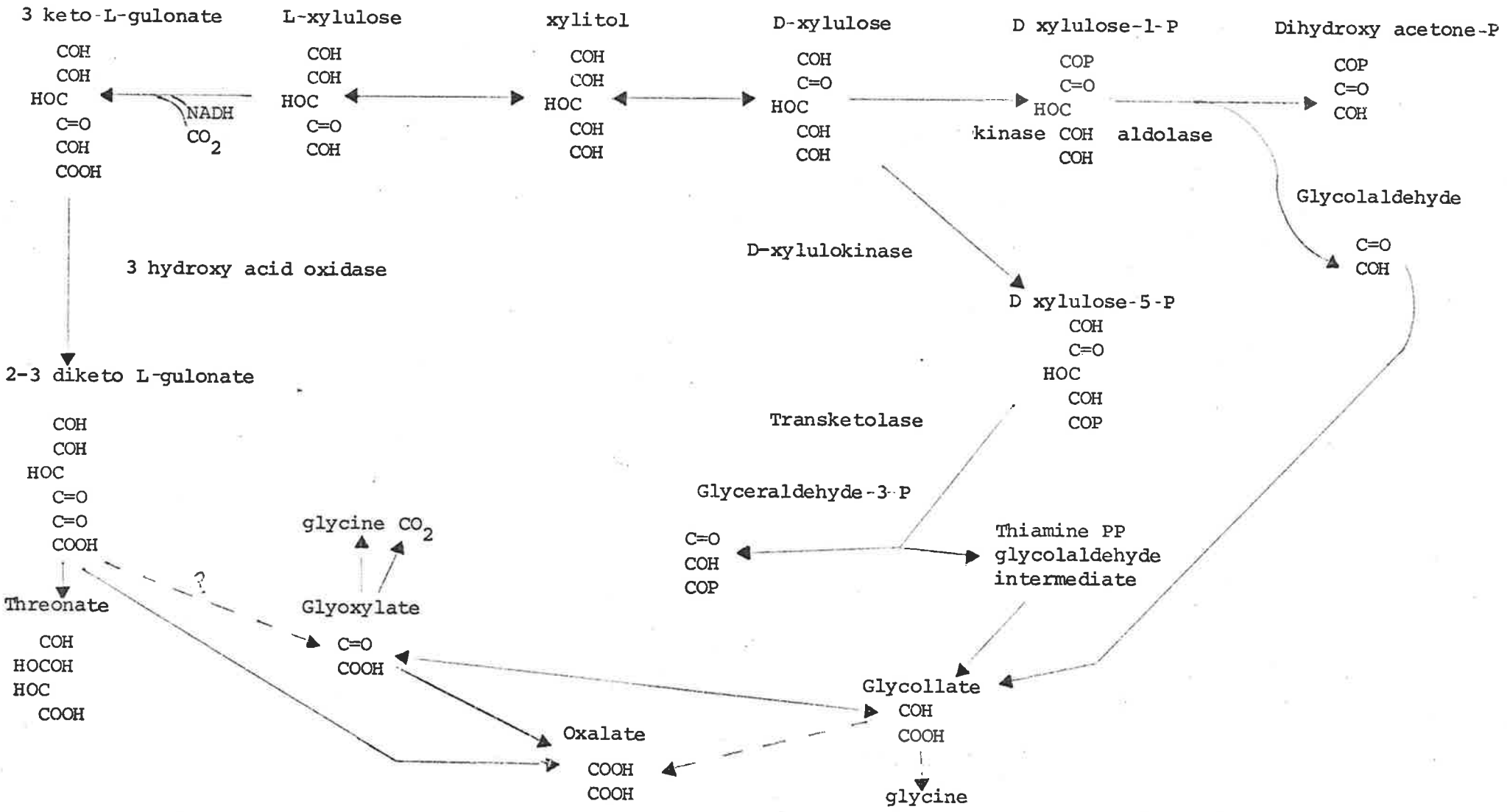
In a paper published over 25 years ago, (Dische and Borenfreund 1951), it was mentioned that xyloketose-1-phosphate, presumably D-xylulose-1-phosphate, was prepared by condensing dihydroxy-acetone phosphate and glycolaldehyde in the presence of aldolase. It was suggested during recent discussions with Dr M.G.Clarke that if xylulose-1-phosphate were produced as a metabolic error, catalysed possibly by ketohexokinase, then fructose-1-phosphate aldolase could produce the oxalate precursor, glycolaldehyde.

As the enzymic oxalate assay can detect low levels of $|^{14}C|$ oxalate, the use of specifically labelled compounds would appear to be the most viable approach to the elucidation of the pathways of oxalate synthesis from carbohydrates. Experiments with differentially labelled D-xylulose would be most enlightening, as this compound appears to be metabolised at the same rate as xylitol in the rat, without the accompanying perturbation in the cellular redox couples. Some possible mechanisms of oxalate synthesis are outlined in Figure 25.

With the demonstration of glycollate as a product of xylitol metabolism the effect of altered intracellular redox states on oxalate synthesis from xylitol are more easily understood. Any reduction in the cellular redox state (i.e. high NADH levels) does not favour oxalate synthesis, from glycollate or glyoxylate, as demonstrated previously (Chapter four). The elevation of the apparent $NADH/NAD^+$ ratio, at a xylitol concentration above 1 mM, possibly explains why oxalate production does not parallel the increase in xylitol concentration. By facilitating the regeneration of NAD^+ with PMS, and thus producing an oxidised cellular redox state, the generation of oxalate from xylitol, glycollate and glyoxylate was stimulated. There would appear to be dual effect of PMS on oxalate production from xylitol, with PMS causing both increased xylitol breakdown, and increased flux through the oxalate biosynthetic pathway. Whilst increased oxalate synthesis from xylitol in the presence of PMS is consistent with an oxidised redox state, the parallel increase in lactate

FIG. 25

POSSIBLE PATHWAYS OF OXALATE SYNTHESIS FROM XYLITOL



also observed (Figure 23) appears to be largely a result of increased pyruvate supply; as an oxidised redox state would favour lactate oxidation, rather than its production.

It is known that PMS will facilitate a more oxidised cellular redox state, but this artificial electron acceptor probably has more than one mode of action. The regeneration of NAD^+ is an established effect, but PMS probably also stimulates oxidations catalysed by flavin linked enzymes, namely the mitochondrial glycerophosphate dehydrogenase, and the peroxisomal glycollate oxidase, which would facilitate xylitol utilisation and oxalate production respectively. Hauschildt and Brand (1977) have reported increased $|^{14}C|$ oxalate production from $|U\text{-}^{14}C|$ xylitol by rat liver homogenates, in the presence of oxidants like NAD^+ and pyruvate. Precise details of their experiments are not yet available, but the report appears to support the findings obtained here with PMS.

The association between an oxidised cellular redox state and increased oxalate production from xylitol has important clinical implications. It can be suggested that the infusion of intermittant doses of xylitol are more liekly to favour the production of oxalate than if xylitol were infused continuously. In the latter instance, the redox state within the liver would be more reduced over a longer time period and hence would not favour oxalate production.

The question may be asked as to whether the low rate of oxalate production from xylitol is of any pathological importance. While it is dangerous to extrapolate from animal models to the human situation, an answer may be seen in the toxic effects of ethylene glycol. Only 2% of an ingested dose of ethylene glycol appears as oxalate, yet this low rate of conversion is still sufficient to cause massive renal deposits of oxalate (Gessner et al 1961, Parry and Wallach 1974). Therefore the 0.2% conversion of xylitol to oxalate observed here cannot be dismissed lightly.

While the pyridoxine deficient rat is a useful model for demonstrating oxalate synthesis from xylitol, it is not suggested that the patients that died after the infusion of large amounts of xylitol were pyridoxine deficient. It is likely that deficient nutritional states are only one of a number of possible precipitating factors, as suggested by Hauschildt et al (1976a), who found only slight evidence of pyridoxine deficiency in patients receiving xylitol. These authors suggest renal insufficiency may be an

important factor in oxalate deposition, resulting in poor clearance of glycollate with subsequent oxalate production, particularly if calcium ion concentrations are high. Other predisposing factors, such as the effect of drugs on the activity of the uronic acid cycle can also be suggested. The association between high urinary uric acid levels and oxalate stone formation (Rose 1977) is interesting as hyperuricaemia is a major side effect of xylitol infusions (Thomas et al 1972a, Donahoe and Powers 1970).

This study has described some of the characteristics of hepatic oxalate production from xylitol in the rat. Glycollate has been further implicated as a key intermediate in this pathway, and the effect of the intracellular redox states, pyridoxine deficiency and isotope dilution on oxalate synthesis from xylitol, is discussed. Sufficient time was not available to examine a number of areas, the insight into which was gained from the experiments presented in chapter four. These include the effect of specific enzyme inhibitors like oxamate, DL phenyllactate and amino-oxyacetate, the use of physiological hydrogen acceptors, (hydroxypyruvate and pyruvate), and the effect of phenobarbitone and clofibrate on oxalate synthesis from xylitol. With the unequivocal demonstration of oxalate production from xylitol in rat liver, it can be stated that isolated rat hepatocytes will remain a most useful *in vitro* system for future studies in this area.

CONCLUDING REMARKS

CONCLUDING REMARKS

This thesis has described *in vitro* investigations relating to the problem of oxalate crystal formation in patients following xylitol infusions. Isolated rat hepatocytes have been presented as a viable system for examining both xylitol metabolism and oxalate biosynthesis. As hepatocytes had not been used previously in these areas, some of the studies presented are of an extensive, definitive nature. Hopefully, these investigations will provide direction for more specific studies, some ideas for which are summarised below:

OXALATE BIOSYNTHESIS

1. The hepatic peroxisomes as a major site of oxalate synthesis.
2. The effect of drugs, such as clofibrate and phenobarbital on the peroxisomal and microsomal involvement in oxalate synthesis.
3. The induction of enzymes involved in oxalate synthesis
   a) Endocrine control
   b) Vitamin depletion, particularly of riboflavin (glycollate oxidase)
4. A further assessment of the role of lactate dehydrogenase in the oxidation of glyoxylate, in the presence of competing substrates and under physiological conditions. This area could include a assessment of glyoxylate as a substrate for lactate dehydrogenase after heat fractionation, as well as an examination of the labile "glyoxylate dehydrogenase" activity reported in chapter four (B), tables 16 and 17.
5. The potential use of aromatic compounds like tyrosine, phenylalanine and phenyllactate in the regulation of oxalate synthesis, *in vivo* and *in vitro*.

XYLITOL METABOLISM AND OXALATE SYNTHESIS

6. An examination of the regulatory role of the mitochondrial glycerophosphate dehydrogenase in xylitol metabolism using
   a) Tri-iodothyronine treated rats
   b) Riboflavin deficiency
7. The use of specifically labelled D-xylulose to further examine the route of oxalate synthesis from xylitol.
8. The possibility of forming xylulose-1-phosphate from the action of ketohexokinase on D-or L-xylulose, and the subsequent degradation of the xylulose-1-phosphate to dihydroxyacetone phosphate and

glycolaldehyde with fructose-1-phosphate aldolase.

9. An *in vivo* evaluation of continuous versus intermittant infusions of xylitol, with respect to changes in the redox state of the liver, and the effect this has on oxalate synthesis.
10. The association between hyperuriceamia and oxalate crystal deposition during carbohydrate administration in animals.

By using isolated rat hepatocytes as an model *in vitro* system, results have been obtained which have furthered understanding of xylitol metabolism, particularly with respect to the phenomena of oxalate synthesis. While the potential hazards of xylitol infusion into patients is acknowledged, this compound would appear to be a valuable tool for future *in vitro* investigations into mechanisms of hydrogen transfer via hydrogen shuttle systems, and also in the area of oxalate synthesis from carbohydrates.

APPENDIX ONE

ISOLATED RAT LIVER PARENCHYMAL CELLS. ISOLATION AND CHARACTERISATION.

Isolated rat liver cells offer numerous experimental advantages over the perfused rat liver and liver slice systems (Schimasseck, et al 1974, Krebs et al 1974). Suspensions of these hepatocytes are an easily manipulated, homogeneous suspension, which allows a variety of concurrent experiments to be conducted on the one liver preparation. Problems of tissue heterogeneity and localised variations in substrate concentrations encountered in the perfused liver are obviated (Gorensky, 1974).

Early attempts to prepare rat hepatacytes by mechanical means, resulted in a high percentage of damaged cells. A major technical advance was achieved by Howard et al (1967) who introduced the collagenase-hyaluronidase dispersion of rat liver slices. Berry and Friend (1969) further refined and improved this technique by perfusing rat livers "in situ" with collagenase and hyaluronidase, thus enabling the isolation of high yields of intact metabolically active hepatocytes. Many minor variations of this method have since been published, including prior perfusion of the liver with EDTA, and the addition of erythrocytes or albumin to the perfusion medium. However, the original method (Berry and Friend 1969), as later modified by Berry and Kun (1972) remains the simplest and most successful technique and was the method of choice in these investigations.

This section describes the isolation of liver cells from rats, together with an assessment of viability, as determined by exclusion of trypan blue, leakage of enzymes and gluconeogenesis from lactate.

METHODS

PREPARATION OF ISOLATED RAT HEPATOCYTES

Hepatocytes were prepared by the method of Berry and Kun (1972). Any variations to this method will be mentioned where appropriate.

BUFFERS

BICARBONATE BUFFER

This buffer contained 140 mM NaCl, 5.4 mM KCl, 2.0 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.4 mM Na_2HPO_4 and 0.4 mM KH_2PO_4. The buffer was equilibrated with carbogen (95% oxygen, 5% CO_2), and the pH was maintained at 7.2-7.4 (phenol red indicator) by the addition of 1.3% $NaHCO_3$. The buffer was preheated to 37^0C prior to use in the cell isolation.

PHOSPHATE BUFFER

This buffer varied from the one above in that it contained, 10 mM sodium phosphate pH 7.4 and was equilibrated with 100% oxygen.

PREPARATION OF THE RAT

Male Porton derived rats (200-300 g) were starved overnight to deplete hepatic glycogen. The animals were anaesthetised with 15 mg of nembutal and given 100 i.u. of heparin via the femoral vein. The abdominal cavity was opened and the inferior hepatic portal vein cannulated with a 17 gauge stainless steel cannula. The chest was then opened and the inferior vena cava cannulated with a 16 gauge plastic cannula. The inferior vena cava was ligatured above the junction of the renal veins and the liver, in situ, was attached to the perfusion apparatus in a constant temperature cabinet maintained at 37-40^0C.

LIVER PERFUSION AND ISOLATION OF HEPATOCYTES

The liver was perfused in situ via the hepatic portal vein with bicarbonate buffer, minus calcium, at a flow rate of 30-40 ml per minute. Rapid blanching of the liver was indicative of a good perfusion. After allowing 100 mls of perfusate to flow to waste, collagenase (0.05%) and

hyaluronidase (0.10%) were introduced, the volume of buffer in the reservoir adjusted to 80 mls, and the flow rate reduced to 20-30 mls minute. The pH was maintained at approximately 7.4 by the addition of 1.3% sodium bicarbonate. It was essential to maintain a hydrostatic head of 30 cm on the outflow to prevent liver distension. Covering the liver with plastic film decreased leakage during the latter stages of the perfusion. When excessive fluid loss occurred and the hepatic structure could be visibily disturbed by gentle stroking, the liver was removed and placed in a beaker containing 1-2 ml of 0.05% collagenase, 0.10% hyaluronidase in bicarbonate (minus calcium) buffer. Thirty ml of buffer was then added (37^0C) and the liver dispersed by gentle dissection with small forceps. This suspension, after being oscillated gently for one minute, was, in most cases suitable for immediate filtering. In some instances, it was necessary to shake gently in a water bath at 37^0C for 5 minutes to further separate the cells. However, this treatment invariably led to a poor preparation, compared to suspensions which were immediately filtered through muslin, and centrifuged at 100g for 1 minute. After washing twice with calcium free bicarbonate buffer, the hepatocytes were resuspended in either bicarbonate or phosphate buffer containing calcium, to a concentration of 5×10^6 viable cells per ml and used immediately for metabolic studies. Cells were maintained in suspension by gentle oscillation at 37^0C in a water bath.

RESULTS

VIABILITY

A number of criteria have been applied to assess the viability of hepatocyte suspensions. These include:

(1) Microscopic appearance of cells.

(2) Oxygen uptake, both endogenous or with added substrate.

(3) Membrane permeability to trypan blue, or leakage of cell contents.

(4) Ability to carry out metabolic activities, i. e. gluconeogenesis, protein or urea synthesis.

The first two criteria are not necessarily a true indication of viability as electron micrographs can demonstrate intactness, yet at the same time there may be gross leakage of cell components and lack of metabolic integrity. Oxygen uptake signifies mitochondrial integrity, not cell integrity. Therefore an intact cellular membrane, determined histochemically and biochemically, together with the ability to perform complex metabolic functions, including gluconeogenesis, are arguably the best measures of viability.

VIABILITY ASSESSED BY TRYPAN BLUE STAINING

MAINTENANCE OF pH, OXYGENATION AND TEMPERATURE

More than 90% of hepatocytes prepared by the above procedure excluded 0.2% trypan blue. Rigid maintenance of pH 7.4 did not appear to be necessary factor. The viability of hepatocyte suspensions which were allowed to fall to pH 6.6 after a 1.0 hour incubation with 10 mM xylitol or sorbitol did not differ markedly from suspensions where the pH was maintained at 7.4.

Oxygenation of the initial suspension medium, followed by incubations of less than 5 mm depth (exposed to air) was found to be adequate for the incubation times and cell concentrations used in these experiments. Cells were used immediately for metabolic studies, thus obviating the need for cooling to 4^0C, and hence avoiding the swelling which occurs with this practice (Krebs et al 1974). Perhaps the most important aspect of maintaining the cells in suspension is that of sustained gentle shaking. Lack of movement, in a well oxygenated medium at adequate pH and temperature appeared to be more detrimental to cell viability than anoxia in a well

shaken medium. This phenomena may relate to localised accumulation of lactic acid in and around cells which are not kept in motion.

GLUCONEO GENESIS FROM LACTATE

Isolated hepatocytes produced glucose from 10 mM lactate at a rate of 20 µmols/10^8 cells/hr) which is comparable to those reported by other workers (Table 37). Cells from fasted rats must be used to observe this effect, as cells from fed rats, in the absence of lactate, rapidly produced glucose from endogenous glycogen. The data depicted in Table 37 illustrates the diversity in representation of results. For this reason, 10^8 cells, which represent 1 gm wet weight of packed cells has been chosen as a convenient standard.

LOSS OF INTRACELLULAR ENZYMES

The levels of lactate dehydrogenase and alanine α-ketoglutarate aminotransferase (ALT) found in cells and supernatant are comparable to those recorded by Berry and Friend (1969) (table 38). In fact the loss of these enzymes, particularly ALT, into the first supernatant was considerably less than that reported by these authors. While some enzymic activity is expected in the third supernatant due to incomplete washing and loss from non viable cells, an increase in this proportion of activity over a 1 hour period was not seen, indicating a maintenance of cell membrane integrity.

EFFECT OF HIGH CARBOHYDRATE CONCENTRATION

Incubation of hepatocytes with concentrations of carbohydrates (fructose, glucose, xylitol, sorbitol) up to 50 mM, did decrease cell viability, particularly with the polyols after one hour of incubation. This effect did not appear to be related to lactate concentration, or the pH of the medium. Incubations of 1.0 hour or less using less than 20 mM xylitol, sorbitol or fructose, did not appear to effect the cell viability as determined with trypan blue.

TABLE 37

A COMPARISON OF RATES OF GLUCOSE PRODUCTION FROM LACTATE IN ISOLATED RAT HEPATOCYTES

RAT	INCUBATION vol (ml)	CELLS ml ($x10^5$)	INCUBATION Time (min)	recorded GLUCOSE Production	corrected GLUCOSE PRODUCTION µmol/10^8 cells/hr	REFERENCE
fasted	4	4	40	1.9 - 2.5 umol/g dry wt min	40	Berry and Friend (1969)
fasted	0.4	5	60	30 umol/g wet wt	30	Seglen (1974)
fasted	2.5	10	60	1.3 nmol/mg prot/min	30	Quistorff et al (1973)
fasted	2 or 5	5	60		20	This study
fasted	-	7.4	120	26 ug/10^6 cells	10	Johnson (1972)
fasted	2.5	5	30	2 umol/g wet wt /30 min	4	Veneziale and Lohmar (1973)

The corrected glucose conversion is based on 10^8 cells = 1 gm wet wt liver = 0.27 dry wt liver = 0.14 g protein

The lactate concentration was 10 mM in all incubations.

TABLE 38

ENZYME RELEASE FROM ISOLATED RAT HEPATOCYTES

	units/ml x 10^3 Lactate dehydrogenase		Alanine amino transferase	
Dispersion	47.5	(58.3)	15.4	(7.1)
1st supernatant	11.7	(43.9)	2.5	(5.7)
2nd supernatant	3.6	(11.5)	0.8	(1.0)
3rd supernatant	1.6	(1.7)	0.4	(0.2)
4th supernatant	1.4		0.3	
Cells	25.0	(39.6)	6.0	(4.43)

The figures in parenthesis are from Berry and Friend (1969). The initial dispersion volume was 40 mls. The supernatants refer to subsequent washings after centrifugation (Berry and Friend 1969). The enzyme assays are given in the methods chapter 2.

DISCUSSION

The isolation of rat hepatocytes, requires a degree of skill and practice to successfully prepare high yields of intact, metabolically active cells. Critical aspects of the preparation have been discussed elsewhere (Krebs et al 1974, Berry and Werner, 1974) and will only be briefly reiterated here.

The initial perfusion is of paramount importance in the preparation. Rapid clearance of blood from the liver is essential, with uniform blanching occurring in the first five seconds. Patchiness of the liver indicates poor perfusion, and almost always results in low yields. This step was aided by adequate heparinisation and speed during the surgical procedure. A syphon head of 30 cm on the out flow of the liver facilitated rapid clearance of blood from the liver, and prevented undue liver distension. The pH, and temperature of the perfusion medium did not appear to be critical if maintained within acceptable limits (pH 7.1 - 7.4, temp, 35-37^{0}C), which indicates that preparation need not necessarily involve the use of a constant temperature cabinet providing the buffer temperature is maintained. Berry has commented (lecture) that hepatocytes, once isolated, should be treated with care, and not subjected to unnecessary shaking, centrifugation, and pipetting forces.

Hepatocytes were successfully isolated from rats with 40-50% yield and >90% viability. Enzyme loss and rates of gluconeogenesis were comparable to those recorded in the literature. High levels of carbohydrates did not affect viability during the first hour of incubation, though longer time periods demonstrated a deterioration in the percentage of viable cells, particularly with high levels of xylitol and sorbitol (20-50 mM).

APPENDIX TWO

THE ASSAY OF $|^{14}C|$ OXALATE IN BIOLOGICAL FLUIDS

INTRODUCTION

Shimazono and Hayashi (1957) first described a highly specific oxalate decarboxylase obtained from the wood fungus, Collybia velutipes. This enzyme has been successfully used to assay $|^{14}C|$ oxalate in urine (Okeefe et al 1973), blood (Smith et al 1971) and liver perfusates (Liao and Richardson 1973). In the present study, this method was adapted and simplified for the detection of low levels of $|^{14}C|$ oxalate in biological samples.

RESULTS AND DISCUSSION

The essential details of the assay appear in the methods, chapter 2. The experimentation which provided that detail is outlined below.
Oxalate decarboxylase catalyses the following reaction:

$$\begin{matrix} {}^{14}COOH \\ | \\ {}^{14}COOH \end{matrix} \longrightarrow {}^{14}CO_2 + H^{14}COOH$$

In the assay, $^{14}CO_2$ released during the reaction is trapped in hyamine hydroxide and the radioactivity is measured by scintillation counting. Under certain conditions, a stoichiometric conversion of oxalate to CO_2 occurred after a 4 hour incubation period. The ability of this enzyme to detect low levels of labelled oxalate is shown in figure 26. While the assay conditions described here were designed for the most economical use of oxalate decarboxylase, additional enzyme (0.05 units per assay) was necessary to achieve stoichiometric $^{14}CO_2$ production from $|^{14}C|$ oxalate in certain extracts, particularly urine. Whereas increasing the temperature from 20^0C to 30^0C did increase the reaction rate, (20%) there was some loss of sensitivity at low oxalate concentrations due to condensate contaminating the hyamine hydroxide. When greater than 10^3 cpm of oxalate per 0.5 ml of extract was present, higher temperatures ($30\text{-}37^0C$) could be used to hasten the reaction without any loss in sensitivity.

Using hepatocytes in conjunction with the assay described, $|^{14}C|$ oxalate was measured (reproducibility 6%) after the metabolism of labelled glyoxylate glycollate, glycine and various carbohydrates. When assaying substrates with low percentage conversion to oxalate, it was necessary to add chips

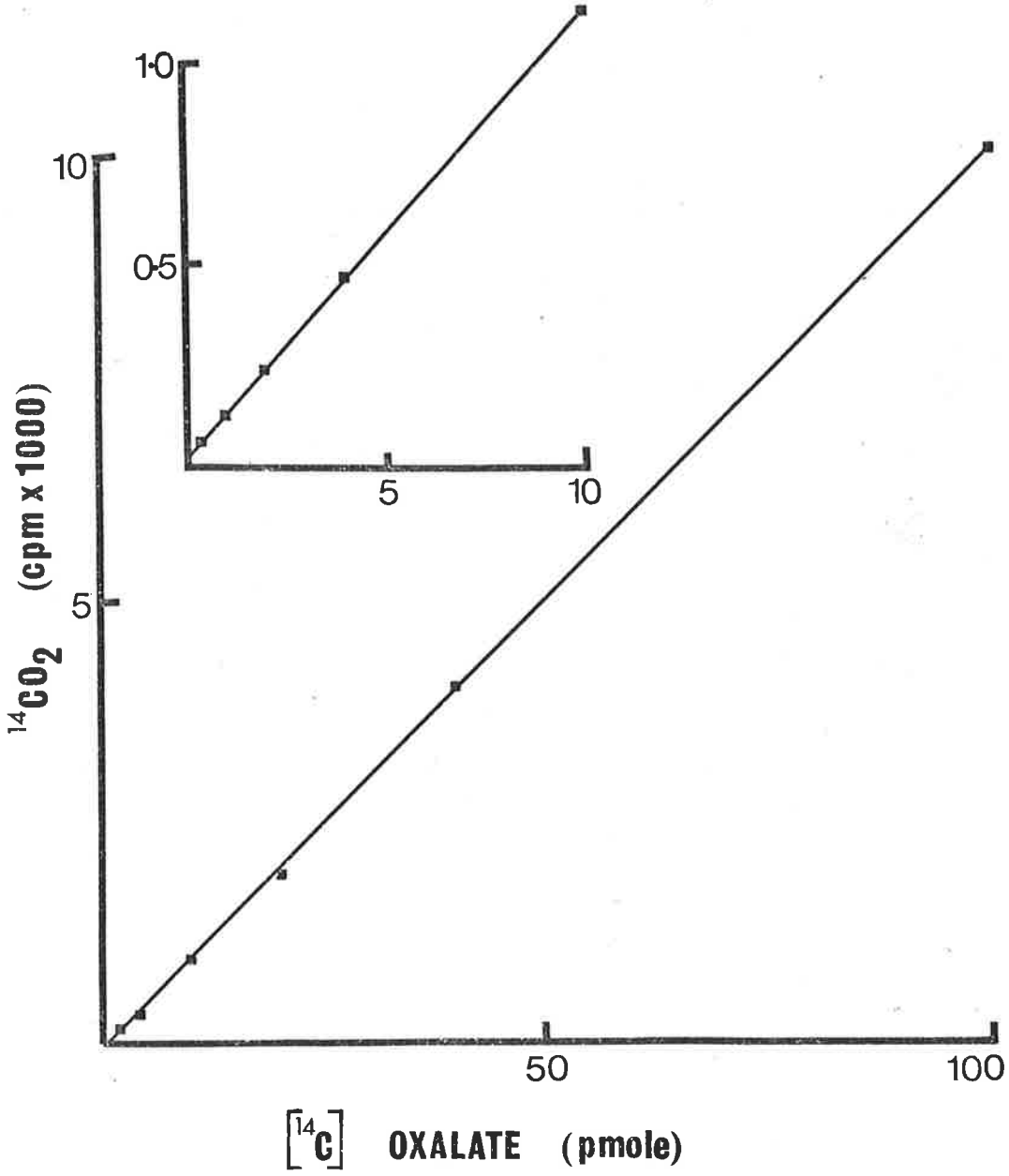


Fig. 26. Assay of $|^{14}C|$ oxalate using oxalate decarboxylase. The incubation mixture contained 1.0 ml of 0.4 M potassium citrate, pH 3.0, 0.5 ml of $|^{14}C|$ oxalate (100 cpm/pmole) and 0.02 units of oxalate decarboxylase. The reaction time was 4.0 hours with trapping of $^{14}CO_2$ in 0.2 ml of 1M hyamine hydroxide in methanol.

of solid carbon dioxide during the 2 minute boiling procedure. This effectively removed excess $^{14}CO_2$, and improved the sensitivity of the assay at low oxalate concentrations.

The production of $^{14}CO_2$ from various precursors by isolated hepatocytes can be measured using this assay. The addition of extract to citrate buffer, pH 3.0, resulted in 85% recovery of $^{14}CO_2$, compared with a duplicate procedure using 2M HCl in place of citrate. It should be re-emphasised here that there are two determinations of CO_2 for each extract. The first assay, minus oxalate decarboxylase, measures CO_2 produced during hepatocyte incubations, while the second assay, plus oxalate decarboxylase, measures $^{14}CO_2$ produced from $|^{14}C|$ oxalate as well. Hence, the expression total $^{14}CO_2$ recovered (+ enzyme) - endogenous $^{14}CO_2$ (- enzyme) multiplied by 2, gives the amount of oxalate (in cpm) present in the extract.

The short boiling procedure described in the method did not affect oxalate, glycollate or glyoxylate in water, buffer or boiled cell controls. Introductio of appropriate boiled cell and buffer controls into the experimental procedure was necessary to correct for endogenous oxalate in the substrates and for non enzymic $|^{14}C|$ oxalate or $^{14}CO_2$ production. The recovery of $|^{14}C|$ oxalate added to hepatocytes was greater than 90%. Increasing the calcium concentration from 0 to 5 mM, had little effect on the recovery of low levels of labelled oxalate with a maximum of 15% reduction in recoverable counts occurring at 5 mM $CaCl_2$.

The validity and effectiveness of the oxalate decarboxylase in this assay has been previously demonstrated (Smith et al 1971, 1972). High levels of oxamate and DL-phenyllactate did not adversely effect the oxalate decarboxylase. Appropriate use of internal standards allowed correction for any minor inhibitions, which, once recognised, could usually be avoided by using additional enzyme in the assay. Routinely, 0.05 units of oxalate decarboxylase per assay was found to be the most effective and economical concentration.

While various techniques have been previously used to assay oxalate in biological fluids (Eswara Dutt and Mottala, 1974) the enzymic assay described in this section is a convenient and effective method for the rapid determination of low levels of $|^{14}C|$ oxalate in a variety of biological fluids.

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