THE PENTOSE PHOSPHATE PATHWAY
AND
NADPH UTILIZATION IN RAT LIVER

A Thesis submitted by

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Doctor of Philosophy

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EGHAM
SURREY

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DEDICATION

To my late father and to my mother
ACKNOWLEDGEMENTS

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I am most grateful to the Beit Trust and my mother for financial support.

Last, but not least, my sincere and heart-felt gratitude goes to the Lord Jesus Christ, in whom this work was started and finished.
ABSTRACT

The role of the pentose phosphate pathway as a source of NADPH required for cytoplasmic processes such as lipogenesis and detoxification reactions has been examined. G6PDH and 6PGDH are known to be strongly inhibited by the high NADPH/NADP⁺ ratio which is thought to occur in the cytoplasm but no effector at physiological concentrations has yet been found which can overcome this inhibition.

Initially a possible role for F2,6P₂ as an activator of G6PDH, 6PGDH and FAS was investigated but no significant effect of this regulatory metabolite on any of these enzymes was discovered. An attempt was also made to demonstrate the reported reversal of the inhibition by GSSG and the cofactor reported by Eggleston and Krebs (1974). This too could not be demonstrated.

In the course of the work, a cytosolic NADPH-consuming reaction has been characterized. This has been shown to involve the reaction of a peptide-substrate with a cytoplasmic reductase specific for NADPH and a high affinity for the peptide. The physiological role of this reaction remains to be established, but it has been observed that the reaction exhibits a diurnal variation, the pattern of which is the reverse of that observed with lipogenesis.

The low molecular weight peptide, which appears to be distinct from glutathione, contains cystine residues which are apparently reduced in the presence of NADPH, resulting in the appearance of free thiol groups. The peptide may be phosphorylated but the nature of the linkage between the peptide and phosphate has not been established.

A possible role for this and other NADPH-dependent reactions in the regulation of the pentose phosphate pathway is discussed in this thesis.
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ABBREVIATIONS

absorption coefficient, molar \( \varepsilon \)
cyclic AMP or cAMP
adenosine 3', 5' - phosphate AMP
adenosine 5' - phosphate ADP
adenosine 5' - pyrophosphate ATP
adenosine 5' - triphosphate Tris
2-amino-2-hydroxymethyl propane 1,3-diol
biotin carboxylase BC
biotin carboxy carrier protein BCCP
(1,3-bis tris (hydroxymethyl)-methylamino propane) i.e. bis-tris propane
coenzyme A and its acyl derivatives CoA, acyl-CoA
cf.
concentrated conc.
concentration concn
constant, equilibrium Keq
counts per minute c.p.m.
deoxyribonucleic acid DNA
dibutryrly cyclic AMP \( \text{Bt}_2 \text{cAMP} \)
diethylaminoethyl cellulose DEAE-cellulose
d'hydroxyacetone DHA
5-dimethylaminonaphthalene-1-sulphonyl-chloride dansyl-chloride
disintegrations per minute d.p.m.
disulphide group S-S
5-5' - dithiobis (2-nitrobenzoic acid) DTNB
dithiothreitol DTT
ethylenediaminetetra-acetate EDTA
ethyleneglycol bis(amoethylether) tetraacetate EGTA
experiment(s) Expt(s)
fatty acid synthase FAS
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flavin adenine dinucleotide FAD
flavin mononucleotide FMN
fructose-1-phosphate F1P
fructose-6-phosphate F6P
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<td>glucose-1,6-bisphosphate</td>
<td>G1,6P₂</td>
</tr>
<tr>
<td>gram</td>
<td>g</td>
</tr>
<tr>
<td>gram-molecule</td>
<td>mol</td>
</tr>
<tr>
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<td>g</td>
</tr>
<tr>
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<td>cGMP</td>
</tr>
<tr>
<td>guanosine 5' -phosphate</td>
<td>GMP</td>
</tr>
<tr>
<td>guanosine 5' -pyrophosphate</td>
<td>GDP</td>
</tr>
<tr>
<td>guanosine 5' -triphosphate</td>
<td>GTP</td>
</tr>
<tr>
<td>hour</td>
<td>h</td>
</tr>
<tr>
<td>hydrogen ion concentration (-log)</td>
<td>pH</td>
</tr>
<tr>
<td>inhibitor constant</td>
<td>Kᵢ</td>
</tr>
<tr>
<td>Michaelis constant</td>
<td>Kₘ</td>
</tr>
<tr>
<td>messenger ribonucleic acid</td>
<td>mRNA</td>
</tr>
<tr>
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<td>min</td>
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<tr>
<td>molar (concn)</td>
<td>M or mol  l⁻¹</td>
</tr>
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<td>mole</td>
<td>mol</td>
</tr>
<tr>
<td>molecular weight</td>
<td>Mᵣ</td>
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<tr>
<td>nicotinamide-adenine dinucleotide, oxidised</td>
<td>NAD⁺</td>
</tr>
<tr>
<td>nicotinamide-adenine dinucleotide, reduced</td>
<td>NADH</td>
</tr>
<tr>
<td>nicotinamide-adenine dinucleotide phosphate oxidised</td>
<td>NADP⁺</td>
</tr>
<tr>
<td>nicotinamide-adenine dinucleotide phosphate reduced</td>
<td>NADPH</td>
</tr>
<tr>
<td>orthophosphate (inorganic)</td>
<td>Pᵢ</td>
</tr>
<tr>
<td>percent</td>
<td>%</td>
</tr>
<tr>
<td>perchlorate</td>
<td>HClO₄⁻</td>
</tr>
<tr>
<td>phosphofructokinase-1</td>
<td>PFK-1</td>
</tr>
<tr>
<td>phosphofructokinase-2</td>
<td>PFK-2</td>
</tr>
<tr>
<td>6-phosphategluconate</td>
<td>6PG</td>
</tr>
<tr>
<td>6-phosphogluconate dehydrogenase</td>
<td>6PGDH</td>
</tr>
<tr>
<td>Term</td>
<td>Abbreviation</td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>precipitate</td>
<td>ppt</td>
</tr>
<tr>
<td>probability of an event being due to chance alone</td>
<td>P</td>
</tr>
<tr>
<td>pyrophosphate (inorganic)</td>
<td>PP_i</td>
</tr>
<tr>
<td>ribonucleic acid</td>
<td>RNA</td>
</tr>
<tr>
<td>second</td>
<td>s</td>
</tr>
<tr>
<td>standard deviation</td>
<td>S.D.</td>
</tr>
<tr>
<td>standard error of mean</td>
<td>S.E.M.</td>
</tr>
<tr>
<td>substrate constant</td>
<td>K_s</td>
</tr>
<tr>
<td>sulphydryl or thiol</td>
<td>SH</td>
</tr>
<tr>
<td>sum</td>
<td>Σ</td>
</tr>
<tr>
<td>thin layer chromatography</td>
<td>t.l.c.</td>
</tr>
<tr>
<td>ultra violet</td>
<td>u.v.</td>
</tr>
<tr>
<td>velocity</td>
<td>v</td>
</tr>
<tr>
<td>volume</td>
<td>vol</td>
</tr>
<tr>
<td>volume per volume</td>
<td>v/v</td>
</tr>
<tr>
<td>wavelength</td>
<td>λ</td>
</tr>
<tr>
<td>weight</td>
<td>wt.</td>
</tr>
</tbody>
</table>
The regulation of the pentose phosphate pathway (PPP) has been the focus of a considerable amount of research. In particular, research workers have attempted to determine the regulatory mechanisms enabling glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH); the rate-limiting enzymes of the oxidative branch of the PPP, to function in the presence of the inhibitor NADP⁺/NADPH ratios found in the cell.

Two regulatory mechanisms for de-inhibition have been proposed: one is the possible existence of a de-inhibitor functioning in conjunction with oxidised glutathiones (Babayan and Ara, 1974; Rodriguez-Segade, et al., 1975, 1979, 1980), and the other is the "FULL" hypothesis (Tepperman and Tepperman, 1981; Bonnigton and Cintora, 1979; Taketa et al., 1979); which state the de-inhibition of the PPP dehydrogenases is the oxidation of NADPH, (the inhibitor) by NADPH requiring processes, with consequent production of NADP⁺ (the activator). This thesis is primarily concerned with the short-term regulation of G6PDH and 6PGDH.

I. INTRODUCTION

1. The Pentose Phosphate Pathway

1. Distribution and Function

The PPP is widely distributed in nature and has been shown to exist in mammals, plants, micro-organisms and yeast (Babayan and Gershon, 1976; Holzmann and Levy, 1956; Miller, 1960; Pon, 1964; Levy, 1979). This metabolic route represents an alternative mode of glucose oxidation coupled with the production of NADPH (Gibson and McLean, 1927; Bonnigton and Cintora, 1979).
The regulation of the pentose phosphate pathway (PPP) has been the focus of a considerable amount of research. In particular, research workers have attempted to determine the regulatory mechanisms enabling glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH), the rate limiting enzymes of the oxidative branch of the PPP, to function in the presence of the inhibitory NADPH / NADP+ ratios found in the cell.

Two regulatory mechanisms for de-inhibition have been proposed: one is the possible existence of a de-inhibitor functioning in conjunction with oxidised glutathione (Eggleston and Krebs, 1974, Rodriguez-Segade, et al., 1978, 1979, 1980), and the other is the "PULL" hypothesis (Tepperman and Tepperman, 1963; Bonsignore and DeFlora, 1972; Taketa et al., 1970), which states that the primary event in the de-inhibition of the PPP dehydrogenases is the oxidation of NADPH (the inhibitor) by NADPH requiring processes, with consequent production of NADP+ (the activator). This thesis is primarily concerned with the short-term regulation of G6PDH and 6PGDH.

A. THE PENTOSE PHOSPHATE PATHWAY

1. Distribution and function

The PPP is widely distributed in nature and has been shown to exist in mammals, plants, micro-organisms and yeast (Negelein and Gerischer, 1936, Noltmann and Kuby, 1963; Müller, 1960; Pon, 1964; Levy, 1979). This metabolic route represents an alternative mode of glucose oxidation coupled with the production of NADPH (Glock and McLean, 1953; Bonsignore and DeFlora, 1972).
The NADPH generated by the oxidative branch of the PPP is used for reductive biosyntheses, notably lipogenesis (Abraham and Chaikoff, 1959; Tepperman and Tepperman, 1961; 1963; Kather et al., 1972a; Kelley and Kletzien, 1984) in hepatic and adipose tissue and for the reduction of glutathione (Bonsignore and DeFlora, 1972; Puente et al., 1981). The PPP also provides pentose for nucleotide synthesis and triose phosphates which can enter the glycolytic pathway and can therefore be used for energy production (Gumaa and McLean, 1969; Yoshimoto et al., 1983). It has been postulated, however, that the limiting function of the PPP is the supply of reducing equivalents for reductive biosynthesis (Bonsignore and DeFlora, 1972; Raivio et al., 1981). Fig. 1.1 summarises the functions of the PPP.

G6PDH deficiency has been associated with increased susceptibility to haemolytic anaemia and the aging of the erythrocyte cell (Marks, 1964; Fornaini, 1967; Bonsignore and DeFlora, 1972; Zilva and Pannall, 1979; Stryer, 1981). In the latter case the cause of the pathological condition is thought to be the reduced NADPH availability in G6PDH deficiency, which is required to reduce oxidised glutathione. The reduced glutathione is thought to be essential for the maintenance of normal erythrocyte structure.

In some cases the G6PDH deficiency may be advantageous. For instance a lack of G6PDH appears to protect against falciparum malaria, presumably because the parasites require reduced glutathione for optimal growth (Luzzato, et al. 1969; Motulsky, 1964; WHO Tech. Ser. 366, 1967).

Increased G6PDH activity has been associated with hepatocarcinogenesis (Bannasch et al., 1984). These authors reported that the advance of carcinogenesis
**Fig. 1.1:** The oxidative branch of the pentose phosphate pathway.

\[ \text{β-D-Glucose 6-Phosphate} \rightarrow \text{6-Phospho-D-Gluconolactone} \]

\[ \text{NADPH} \rightarrow \text{6-Phospho-D-Gluconate} \]

\[ \text{CO}_2 \]

\[ \text{D-Ribulose 5-Phosphate} \]

Key:

1 = Glucose 6-Phosphate Dehydrogenase
2 = Gluconolactonase
3 = 6-Phosphogluconate Dehydrogenase
was characterised by a progressive shift away from glycogen metabolism towards glycolysis and the PPP.

These considerations highlight the physiological significance of the PPP and justify investigations in the mechanisms involved in the regulation of this pathway.

2. Regulation of the oxidative branch of the PPP

A number of metabolic processes utilize NADPH and one could postulate that the oxidation of glucose 6-phosphate (G6P) and its control is important for the modulation of the NADPH-consuming processes and vice versa. The initial substrate for the PPP, G6P, can also be utilized by other pathways, notably glycolysis and glycogen synthesis. Thus it would be expected that the regulatory enzymes of the PPP could be closely regulated in response to external influences.

The demand of the cells for NADPH is also continually changing and the pathway must be regulated in such a way that it adjusts quickly and efficiently to changing needs. This regulation is thought to occur at the level of G6PDH and 6PGDH, both in the long and short-term (Glock and McLean, 1955; Rudack et al., 1971a, 1971b, 1971c; Sapag-Hagar et al., 1973; Eggleston and Krebs, 1974; Levy and Christoff, 1983; Kelley and Kletzien, 1984; Stumpo and Kletzen, 1984, 1985).

a. Long term regulation

Several workers (Tepperman and Tepperman, 1958; 1964; Fitch et al., 1959; Fitch and Chaikoff, 1960; Muto and Gibson, 1970; Dao et al., 1982; Kelly and Kletzen, 1984) have demonstrated quantitative changes in the PPP dehydrogenases in response to hormonal and nutritional stimuli. In particular it has been shown
that starvation, alloxan-induced diabetes and fat-rich diets cause a marked reduction of both dehydrogenases in rat liver (Glock and McLean, 1955c; Yugari and Matsuda, 1967) and that carbohydrate refeeding of the animals leads to a characteristic increase in G6PDH and 6PGDH activity (Glock and McLean, 1955a, 1955c; Tepperman and Tepperman, 1958, 1963; Fitch et al., 1959, 1960; Niemeyer et al., 1962; Johnson and Sassoon, 1967; Novello et al., 1969). G6PDH is induced to a greater extent than 6PGDH under these conditions (Cohen et al., 1972). Fructose feeding results in greater degrees of induction compared to glucose feeding.

This increase in enzyme activity resulting from carbohydrate refeeding of previously starved rats has also been demonstrated for other lipogenic enzymes (Burton et al., 1969; Cohen et al., 1972; Craig et al., 1972). Cohen et al. (1972) also observed that sucrose induced NADPH generating enzymes to a greater extent than isocaloric amounts of starch. These authors claimed that in livers of rats fed a normal balanced diet G6PDH is the rate-limiting enzyme, whereas prolonged sucrose-feeding results in 6PGDH being the rate-limiting enzyme. However, it was G6PDH rather than 6PGDH, which was shown by these authors, to be most susceptible to nutritional stimuli.

The profound effect of sucrose feeding on lipogenic enzymes is consistent with the elevated serum lipid levels and increased hepatic content and synthesis of lipids which have been observed in sucrose feeding experiments (Portman et al., 1956; McDonald and Roberts, 1965; Allen and Leahy, 1966; Cohen and Teitelbaum, 1966).

Rudack et al. (1971a, 1971b) and Procsal et al. (1976) have demonstrated that both G6PDH and 6PGDH synthesis is increased by feeding carbohydrate rich diets. Furthermore, Rudack et al. (1971b) showed that
both the rate of synthesis and the rate of degradation increases upon high carbohydrate refeeding of fasted rats.

Johnson and Sassoon (1967) attempted to elucidate the dietary component responsible for triggering this increase in the PPP dehydrogenases. They failed in their objective but indicated that the site at which the induction of G6PDH is regulated is at the transcriptional level. It has been further suggested that there are two forms of molecular control in rat liver involved in the response of lipogenic enzymes to nutritional stimuli. These are changes in the concentration of the mRNA species (Nakanishi et al., 1976; Flick et al., 1975; Flick et al., 1977; Hutchison and Holten, 1978; Miksicek and Towle, 1982; Yoshimoto et al., 1983) and possibly changes in the translational efficiency of the mRNA species (Sun and Holten, 1978; Towle et al., 1980; Schwartz and Abraham, 1983).

Stumpo and Kletzien (1984) have measured the levels of functional mRNA encoding G6PDH in hepatocytes from fasted and fasted/carbohydrate-refed rats. Using a cell-free protein synthesis system in vitro, they found that hepatocytes from fasted/carbohydrate-refed rats contain twelve times as much of the mRNA as those from starved rats. The elevation in mRNA content found by these authors and Miksicek and Towle (1982) is sufficient to account for the increase in the activity of G6PDH observed in the induced hyperlipogenic state. In the light of these experiments Stumpo and Kletzien (1984) have concluded that there is no need to postulate possible translational control in the manner proposed by Sun and Holten (1978).

Hormones have been shown to be involved in the induction of the lipogenic enzymes due to fasting followed by refeeding carbohydrate-rich diets. Garcia and Holten (1975) demonstrated that cAMP (or glucagon) decreases the rate of enzyme synthesis in vivo in the case of G6PDH and has no effect on 6PGDH synthesis or degradation. Insulin
injection (Rudack et al., 1971b; Nepokroeff et al., 1974) has been shown to increase G6PDH activity. Rudack et al., (1971b) attributed this effect of insulin to the ability of this hormone to stimulate the appetite. However, recent work by Stumpo and Kletzien (1984, 1985) suggests that the insulin-effected increase in G6PDH is due to increases in the level of G6PDH mRNA. Furthermore, these authors have provided evidence which suggests that glucocorticoids amplify the insulin effect on G6PDH. The effects of insulin and glucocorticoids on the level of mRNA encoding G6PDH in cultured hepatocytes are additive, but the glucocorticoid-induced mRNA appears not to be translatable in the absence of insulin (Stumpo and Kletzien, 1984). These findings are in disagreement with those reported by Nakamura et al. (1982) who observed that in their hepatocyte culture system glucocorticoids do not affect the basal or insulin stimulated G6PDH. The discrepancy in results can probably be attributed to the different conditions of incubation employed by the two groups. The latter group included serum and glucocorticoids in their culture media whereas the former did not.

Kelley and Kletzien (1984) have reported that glucocorticoids by themselves do not increase G6PDH activity but amplify the effects of insulin. In the course of their studies, these authors also found that ethanol strongly potentiated the increase in G6PDH activity caused by glucocorticoids and insulin. This observation could account for the occurrence of alcohol-induced fatty liver, since the effect of ethanol would result in a greater availability of NADPH for lipogenesis in addition to providing a carbon source for acetyl-CoA synthesis.

Insulin appears to exert a regulatory effect at the pre-translational level (Yoshimoto et al., 1983; Stumpo and Kletzien, 1984). The findings of Stumpo and Kletzien (1984) suggest that glucocorticoids may regulate G6PDH activity and synthesis at the pre-translational level, while insulin may also exert control over translation, in such a
way that the glucocorticoid-induced mRNA is efficiently translated.

It is important to note that Stumpo and Kletzien (1984) could not account for all the mRNA encoding for G6PDH found in starved/carbohydrate-refed rats. Glucocorticoid and insulin together only raised the translatable G6PDH mRNA to less than 50% of the level observed in hepatocytes from carbohydrate-refed rats. It is possible, therefore, that other hormones and regulatory effectors are involved in the regulation of enzyme synthesis.

Miksicek and Towle (1982) have shown that triiodothyronine ($T_3$) administration to rats maintained on a diet of laboratory chow increases G6PDH activity, relative synthetic rate and functional mRNA activity. Spence and Pitot (1982) have reported similar results with cultured hepatocytes. These authors have also reported that glucose can only induce lipogenic enzymes in the presence of insulin, whereas insulin is not necessary for the induction of lipogenic enzymes by fructose or glycerol. Recently, Grigor and Gain (1983) have demonstrated that a parallel increase in serum insulin and prolactin levels accompanies the induction of lipogenic enzymes caused by starvation followed by refeeding a high carbohydrate diet.

6PGDH is also under genetic regulation, and a good correlation between the rate of appearance of 6PGDH mRNA and the increase in the rate of 6PGDH synthesis has been demonstrated to occur in primary cultures of hepatocytes, (Hutchison et al., 1984). However, it has been suggested that 6PGDH regulation may differ from that of G6PDH since its rate of synthesis is not affected by either glucagon or dibutyryl cyclic AMP under conditions where these compounds reduce the rate of synthesis of G6PDH (Procsal et al., 1976). The mRNA levels coding for 6PGDH are unaffected by glucagon or dibutyryl cyclic AMP (Hutchison and Holten, 1978).
A 4-fold increase in 6PGDH mRNA in the induced animals (48h starved/carbohydrate refed), when compared to control animals, has been reported by Hutchison et al. (1984). This observation is in agreement with those obtained by Hutchison and Holten (1978) and Miksicek and Towle (1982). Insulin was shown to stimulate large increase in the synthesis of 6PGDH and of translatable mRNA for the enzyme in cultured hepatocytes (Hutchison et al., 1984). However, it is not known yet if insulin regulates mRNA levels directly or indirectly.

Mariash et al. (1981) and Miksicek and Towle (1982) have demonstrated a synergism between T₃ and high-carbohydrate diet for the in vivo induction of 6PGDH in rats. A small stimulatory effect, in the presence of insulin, on 6PGDH induction by T₃ has been demonstrated by Hutchison et al. (1984), using cultured hepatocytes.

Acetate was also found to increase 6PGDH in the presence of insulin (Hutchison et al., 1984). These authors have also confirmed that cAMP does not alter the extent or kinetics of the induction of 6PGDH in cultured hepatocytes indicating that insulin does not exert its effects via changes in the second messenger.

In addition to carbohydrate, the lipid content of diets also affects the activity of lipogenic enzymes, which include G6PDH and 6PGDH. For instance Muto and Gibson (1970) have reported the selective lowering of the activities of lipogenic enzymes by administration of exogenous polyunsaturated fatty acids to rats adapted to the hyperlipogenic state. The same authors have also reported a severe deficiency in linoleic and arachidonic acid in rat liver which coincides with the adaptive rise in lipogenic enzymes when rats are maintained on a high-carbohydrate, low-fat diet.

Tepperman and Tepperman (1958, 1963) demonstrated a marked enhancement of lipid synthesis which parallels the
diet-induced increases of G6PDH activity. Taketa et al. (1970) showed that it was not necessary to induce G6PDH in order to increase NADPH output under conditions of adaptive hyperlipogenesis. In other words, the apparent induction of G6PDH by high carbohydrate diets appears to be secondary to enhanced lipogenesis - an observation favouring the "PULL" hypothesis which states that the primary event in the observed increased capacity of the PPP dehydrogenases is the removal of NADPH by processes such as lipogenesis. This statement could apply to any system involving NADPH oxidation and regeneration and could have consequences for the short term regulation.

An overview of the documentation on the long term regulation of the PPP dehydrogenases and other lipogenic enzymes shows that the mechanisms involved are varied and complex. Furthermore the understanding of the regulation of the PPP at the genetic level is still in its early stages and investigation is likely to continue in this field.

b. Short-term regulation

The mechanism for the short-term regulation of the PPP dehydrogenases remains largely unresolved. In particular, it is not known how the potent NADPH inhibition of the two PPP dehydrogenases is overcome in the cell. In vitro experiments with liver extracts in which attempts were made to duplicate physiological conditions have revealed that G6PDH and 6PGDH are extensively inhibited by NADPH (Sapag-Hagar et al., 1973; Eggleston and Krebs, 1974; Thompson et al., 1976; Holten et al., 1976). It has been argued (Thompson et al., 1976; Holten et al., 1976) that the residual activity of these dehydrogenases is sufficient for the NADPH requirements of the cell. However this conclusion has been based on the unlikely assumption that there are no other physiological inhibitors of these enzymes in vivo, apart from NADPH.
Eggleston and Krebs (1974) demonstrated that the NADPH inhibition of the dehydrogenases depends on the NADPH / NADP⁺ ratio, and that the inhibition of the dehydrogenases approaches 100% when this ratio is near 9:1. The in vivo ratio of the free nicotinamide dinucleotide phosphates is thought to be 99:1 (Veech et al., 1969; Lagunas et al., 1970; Greenbaum et al., 1971). For this reason Eggleston and Krebs (1974) emphasized the need for a de-inhibition mechanism to counteract the NADPH inhibition.

In the course of their search for this mechanism of de-inhibition, Eggleston and Krebs (1974) reported that oxidised glutathione de-inhibits G6PDH, in dialysed liver extracts. A cofactor was also involved in this process, and glutathione could not de-inhibit G6PDH in its absence. Rodriguez-Segade et al., (1978, 1979 and 1980) reported the isolation, purification and characteristics of this cofactor. It was, apparently, a peptide with a molecular weight of about 10,000 in rat liver (Rodriguez-Segade et al., 1979). Other workers have failed to confirm these observations (Gonzalez and Lagunas, 1977; Levy and Christoff, 1983). The latter group has presented evidence which suggests that the observations made by Eggleston and Krebs (1974) were artifacts of the methods used in the investigation. Another key difficulty is in the understanding of how GSSG could exert its effects on G6PDH in vivo where its concentration has been shown to be very low in the normal fed state (7.1 nM according to Veech et al. (1969)). Eggleston and Krebs (1974) reported that reduced glutathione (GSH), which is the abundant form of glutathione in the cell, could not replace GSSG in this mechanism. In these experiments 50μM-GSSG was required to overcome the NADPH inhibition by about 50%; 200μM totally de-inhibited the enzyme.

In the course of the present study no evidence for the participation of glutathione in the regulation of the PPP dehydrogenases was found. However, in view of the many reports of the involvement of thiol/disulphide ratios
in the regulation of general metabolism, and of G6PDH activity in particular (Hilf et al., 1975b, 1975a, 1978; Isaacs and Binkley, 1977; Francis and Ballard, 1980a, b; Puente et al., 1981), the possible involvement of glutathione cannot be lightly dismissed.

Significantly, Eggleston and Krebs (1974) confirmed that the inhibition of G6PDH by NADPH is competitively reversed by NADP+, and observed that the potent inhibition of rat liver G6PDH occurs despite the fact that the $K_i$ for NADPH is greater than the $K_m$ for NADP+. Sapag-Hagar et al. (1973) reported $K_m$ values for NADP+ of 6PGDH and G6PDH to be 9µM and 6µM, respectively. The $K_i$ values for NADPH were found to be 13µM for 6PGDH and 30µM for G6PDH by the same authors. These values were obtained with rat liver from animals which had been starved for two days and refed a high glucose diet for three days. These authors have also reported $K_m$ and $K_i$ values of 7µM and 5µM for NADP+ and NADPH, respectively, for rat liver 6PGDH obtained from 48h starved rats. The values for G6PDH are not given under these conditions. These figures would suggest that NADPH is a more effective inhibitor of 6PGDH in vivo. However, there is little evidence to suggest that this is so.

In the normal fed state the $K_m$ values of 6PGDH for 6PG and NADP+ were found to be 71 and 13µM by Procsal and Holten (1972). The $K_i$ for NADPH under these conditions was 20µM. The values for G6PDH, provided by Thompson et al. (1976) are 2, 29 and 13µM for NADP+, G6P and NADPH, respectively. These figures (except for those obtained for 6PGDH from starved rats) would suggest that both enzymes have a higher affinity for NADP+ than for NADPH but presumably the high in vivo free NADPH / NADP+ ratio more than offsets the effects of the affinity of the enzyme for the two nicotinamide dinucleotide phosphates.

A comparison of the $K_m$ value for G6P and the cellular concentrations of the substance under the three nutritional states (table 1.1b) implies that G6P concentration
Table 1.1a: The effect of the nutritional status on the NADPH : NADP⁺ ratio.

<table>
<thead>
<tr>
<th>Nutritional state</th>
<th>NADPH : NADP⁺ ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fed (control)</td>
<td>110</td>
</tr>
<tr>
<td>Fasted</td>
<td>175</td>
</tr>
<tr>
<td>Fasted/fat re-fed</td>
<td>203</td>
</tr>
<tr>
<td>Fasted/carbohydrate re-fed</td>
<td>45</td>
</tr>
<tr>
<td>Diabetic</td>
<td>186</td>
</tr>
</tbody>
</table>

The table shows the data obtained by Greenbaum et al., (1971) in their experiments with rat liver.

Table 1.1b: The cellular concentrations of G6P and 6PG under different nutritional conditions.

<table>
<thead>
<tr>
<th>Nutritional state</th>
<th>6PG (µM)</th>
<th>G6P (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fed (control)</td>
<td>14.2</td>
<td>217.0</td>
</tr>
<tr>
<td>Fasted</td>
<td>13.7</td>
<td>85.7</td>
</tr>
<tr>
<td>Fasted/carbohydrate re-fed</td>
<td>55.7</td>
<td>120.0</td>
</tr>
</tbody>
</table>

The data recorded was obtained from Greenbaum et al., (1971).
would not limit the activity of G6PDH, which catalyses the first committed step of the oxidative branch of the PPP. However, Thompson et al. (1976) have shown that the kinetic mechanism of the G6PDH catalysed reaction is such that the binding of the substrates to the enzyme occurs in an ordered, sequential manner, with NADP⁺ binding first. This means that the reaction can be limited by the availability of NADP⁺. The absolute concentration of free NADP⁺ is thought to be very low (in the order of 4μM), since the total NADPH (free and bound) is about 0.38mM (Williamson et al., 1971) and since the NADPH / NADP⁺ ratio is about 100. This calculation assumes that most of the NADPH is present in the free form. A consideration of the Km values for NADP⁺ for both G6PDH and 6PGDH in the fed state (2 and 13μM, respectively) and the expected cellular concentration of NADP⁺ in rat liver (4μM or less) would suggest that it is NADP⁺ rather than G6P availability that regulates these two dehydrogenases. It is conceivable that in the cell the requirement for NADP⁺ can be met by reactions involved in the re-oxidation of NADPH. The NADP⁺ so provided would act both as substrate and de-inhibitor of the NADPH-inhibited PPP dehydrogenases. These reactions would also remove the inhibitor (NADPH), which would further increase the capacity of the PPP dehydrogenases.

Another factor which is thought to have general significance in the regulation of the PPP dehydrogenases is the ATP concentration. ATP has been shown to inhibit the PPP dehydrogenases (Kauffman et al., 1979; Avigad, 1966). The Kᵢ for G6PDH and 6PGDH have been reported to be 1.85 and 0.11mM, respectively (Kauffman et al., 1979) Van Schaftingen et al. (1980a) reported that the ATP concentration of isolated hepatocytes is 3.42 ± 0.02mM. These values enable one to postulate a physiological role for ATP. However, addition of equimolar amounts of Mg²⁺ with ATP lowers the inhibition of G6PDH by 30% (Kauffman et al., 1979) and since ATP exists to a considerable extent chelated with Mg²⁺ in vivo (Alberty, 1968; Rose, 1968;
Veloso et al., 1973), ATP inhibition of G6PDH may not be significant in vivo. G6PDH appears to be inhibited regardless of the presence or absence of Mg\(^{2+}\). The low \(K_i\) value for this enzyme suggests that it may be inhibited by ATP in vivo.

The ATP inhibition is competitive with the cofactor NADP\(^+\) (Avigad, 1966; Kauffman et al., 1979). Since ATP shares structural similarities with NADP\(^+\), it is likely that ATP binds to the cofactor site.

Rat liver G6PDH has also been shown to be inhibited by long chain acyl-CoAs (Eger-Neufeldt et al., 1965; Taketa and Pogell, 1966). The \(K_i\) for stearyl-CoA has been reported to be 0.4\(\mu\)M. The \(K_i\) values for palmityl-CoA and lauryl-CoA were found to be 0.6 and 8.7\(\mu\)M respectively. These figures suggest that the extent of inhibition by long chain acyl-CoAs diminishes with the shortening of the chain-length of the fatty acid moiety. Eger-Neufeldt and co-workers showed that the affinity of G6PDH for G6P is reduced by stearyl-CoA such that the \(K_m\) increases from 23\(\mu\)M to 172\(\mu\)M. The maximal velocity is not affected by stearyl-CoA, indicating that the stearyl-CoA-induced inhibition of G6PDH is competitive with respect to G6P. At a fixed concentration of G6P, stearyl-CoA causes a shift in the \(K_m\) for NADP\(^+\) from 17\(\mu\)M in the absence of the inhibitor to 54\(\mu\)M in its presence. In this case the \(V_{max}\) is also reduced by 54\%. The long chain acyl-CoAs could act as negative feedback inhibitors in vivo. For instance, the decrease in G6PDH activity during starvation, fat feeding or acutely decompensated, diabetes may be in part due to the inhibition described here, since tissue long chain acyl-CoAs have been shown to increase markedly under these conditions (Bortz and Lynen, 1963; Garland and Tubbs, 1963; Wieland, 1964).

Mita and Yasumasu (1979) have reported that the
inhibition by palmityl-CoA of purified yeast G6PDH can be reversed by polyamines. The polyamines, putrescine, spermidine and spermine, are thought to be essential for the growth and proliferation of the mammalian cell (Heby et al., 1984). A reduction in the polyamine content of the cell has been associated with a reduced growth rate. The polyamine depletion apparently reduces the rate of DNA synthesis by affecting the nucleotide pools. The reversal of the palmityl-CoA induced inhibition of G6PDH by polyamines suggests that the latter would cause an elevation of G6PDH activity in the presence of high intracellular concentrations of long chain fatty acyl-CoA thioesters. This relationship between polyamines and G6PDH would appear to highlight the connection between the PPP, protein metabolism and nucleotide metabolism (see fig. 1.2), since polyamines have been found to exert a stimulating effect on the activities of DNA and RNA polymerases (Jänne et al., 1975). Since the PPP provides pentose for nucleotide synthesis and possibly NADPH for the reduction of thioredoxin, which in turn is used as a reductant in the ribonucleotide reductase reaction, the elevation of G6PDH activity by the polyamines would lead to an increase in intracellular levels of DNA and RNA and would be consistent with the proposed role of polyamines in cell growth.

The physiological significance of the inhibition of the PPP enzymes by long chain acyl-CoAs has been questioned because of the apparently indiscriminate inhibition of various unrelated enzymes by these compounds as a result of their detergent properties. However, an allosteric model for the inhibition of glucokinase by these compounds has been proposed by Tippett and Neet (1982a,b). Their results suggest that the physiological role of fatty acyl-CoAs has to be further investigated before any conclusions can be reached.
Fig. 1.2: The relationship between the PPP, RNA and DNA synthesis and polyamines.

Oxidative arm of the PPP

Fatty acid synthesis

L-Arginine

Agmatine

palmiformyl-CoA

Ornithine

Putrescine

Spermidine

Spermine

D-Ribulose-5-phosphate

D-Ribose-5-phosphate

5-Phospho-D-ribosyl-1-pyrophosphate

Ribonucleotide

Deoxyribonucleotide

Thioredoxin (SH₂)

Thioredoxin (S-S)

NADP⁺

NADPH
Long chain fatty acids have also been shown to inhibit rat liver G6PDH (Yugari and Matsuda, 1967). These authors showed that the fatty acids disaggregate the active enzyme into inactive sub-units and that NADP⁺ (and to a lesser extent, G6P) protects the enzyme against this inactivation. ATP was shown to facilitate the inhibition by fatty acids. However, this effect was reversed by ADP and AMP. The same observation was made for other nucleotide triphosphates and their corresponding di- and monophosphates.

Myristic (C₁₄) and lauric (C₁₂) acids were the most effective inhibitors, among saturated fatty acids of G6PDH. Overall, the unsaturated fatty acids (linoleic and oleic acids) were shown to have a much higher inhibitory activity than laurate or stearate. On the basis of their results, Yugari and Matsuda (1967) concluded that fatty acid synthesis was controlled in two ways by free fatty acids: one by inhibiting acetyl-CoA carboxylase and the other by restricting the supply of NADPH through the inhibition of G6PDH.

The short term regulation of the PPP dehydrogenases is still an area of intense research; it has, however, become apparent from a consideration of the available evidence that the two enzymes are primarily regulated by the NADPH / NADP⁺ ratio. Furthermore, it is possible that the reoxidation of NADPH, a known inhibitor of the two enzymes, with the consequent production of NADP⁺ (an activator of the dehydrogenases) may, at least in part, enable the normal functioning of the PPP (May, 1981; Elsayed et al., 1982; Kather et al., 1972a,b; Levy and Christoff, 1983; Yeh et al., 1984). This mechanism of control does not rule out regulation of the enzymes by de-inhibition in the manner proposed by Eggleston and Krebs (1974).
B. THE PRODUCTION AND UTILIZATION OF NADPH IN RAT LIVER

1. General considerations

Since the discovery of the nicotinamide dinucleotides (Warburg et al., 1935) the basic structural features of NADP$^+$ have been characterised and correlated with its major biochemical functions. The structure of NADP$^+$ is shown below:

![Structure of NADP$^+$]

Redox reactive site

Cleaved in adenylation

Fig. 1.3: The structure of NADP$^+$

The dihydroband formed in reduced nicotinamide dinucleotide phosphate (NADPH) causes an absorption at 340nm, a fact that has been made use of in biological studies. Biologically, NADPH is considered to be the major electron donor in energy metabolism.
donor in reductive biosyntheses, such as fatty acid synthesis.

In liver the total nicotinamide adenine dinucleotide phosphate (NADP$^+$ + NADPH) content has been reported to be $428 \text{ nmoles g}^{-1} \text{ wet weight}$, compared to $804 \text{ nmoles g}^{-1} \text{ liver}$ for nicotinamide adenine dinucleotide (NAD$^+$ + NADH) (Jacobson and Kaplan, 1957; Klingenberg, 1961; Bücher et al., 1964; Heldt et al., 1965; Williamson, 1969; Tischler et al., 1977).

A considerable amount of work still remains to be done on the regulation of the total cellular level as well as the subcellular distribution of the nicotinamide nucleotides. However, it has been shown that the biosynthesis of nicotinamide nucleotides occurs in different compartments. The synthesis of NAD$^+$ from nicotinamide mononucleotide (NMN) and adenosine tri-phosphate (ATP) by NAD$^+$ pyrophosphorylase occurs almost exclusively in the nucleus (Hogeboom and Schneider, 1952). Since the mitochondrial NAD$^+$ synthesizing activity is low, the nuclear synthesis is thought to be the main supplier of NAD$^+$ to the extramitochondrial spaces of the cell (Siebert and Humphrey, 1965). The mitochondrial pool is generally thought to be supplied by the mitochondrial synthesizing system (Lehninger, 1955; Purvis and Lowenstein, 1961; Grumicke et al., 1975).

The synthesis of NMN from nicotinamide (Imsande and Handler, 1961) as well as the conversion of NAD$^+$ to NADP$^+$ by NAD$^+$ kinase (Vignais, 1962) are cytosolic processes. The regulation of the latter process is not fully understood.

The NADP$^+$ system is 65% mitochondrial whereas the NAD$^+$ system is 20% mitochondrial in rat liver (Sies, 1982). The NADP$^+$ system is more reduced than the NAD$^+$ system and the difference in reduction depends on the availability of energy (Klingenberg and Slenczka, 1959).
In terms of total values, measured in terms of nmol g\(^{-1}\) liver wet weight, the ratio of NADPH / NADP\(^+\) is 65:47 in the extramitochondrial spaces (Sies, 1982). This ratio is substantially different from that obtained for free NADPH / NADP\(^+\), obtained by using redox indicator metabolite systems (Sies et al., 1977; Tischler et al., 1977). These authors calculated the redox potential (\(E_h\)) of the cytosolic NADP\(^+\) system in isolated hepatocytes by using the Nernst equation:

\[
E_h = E_m^1 - 2.3 \frac{RT}{nF} \log \frac{[\text{Reduced substrate}]}{[\text{oxidised substrate}]}
\]

where \(E_m^1\) is the mid-point potential at pH 7.0 at a defined ionic strength (usually \(I = 0.25\)) and temperature. \(E_h\) is measured in mV, and \(n\) is the number of electrons. Basing their calculation on the subcellular fractionation of the components of the NADP\(^+\) - isocitrate dehydrogenase indicator system, the redox potential was shown to be about -400mV. This is equivalent to a free NADPH / NADP\(^+\) ratio of about 100 (Sies, 1982) at pH 7.0. This value is in agreement with that obtained from data of total cellular contents of the components of the isocitrate dehydrogenase (NADP\(^+\)), malic enzyme and pentose phosphate dehydrogenase systems (Veech et al., 1969; Greenbaum et al., 1971). The enzymes used in the calculation of the redox state of the free nicotinamide-adenine dinucleotide phosphate in the cytoplasm of rat liver are assumed to satisfy the following conditions: (i) they are only (or almost entirely) active in one cell compartment (ii) they are active enough to establish a near equilibrium state (iii) their products and reactants are present in sufficiently high concentrations to allow for their accurate determination. In addition the equilibrium constant (\(K_{eq}\)) of the system must be known for the purified enzyme at the pH, temperature and ionic strength of the tissue. Veech et al. (1969) used the equilibrium equation shown below in their calculation:
\[
\frac{[\text{oxidised substrate}]}{[\text{reduced substrate}]} = \frac{[\text{NADPH}]}{[\text{NADP}^+]} = \text{Keq}
\]

Determination of the oxidised and reduced substrates would permit the calculation of the NADPH / NADP+ ratio if the Keq for the reaction is known.

Since the ratio of total contents of NADPH / NADP+ is 65:47 and that of the free nicotinamide adenine dinucleotide phosphates is 99:1, preferential binding of NADP+ in the manner demonstrated for NADH (Bücher, 1970; Bücher et al., 1972, Sies et al., 1972; Brauser et al., 1972; Bücher and Sies, 1980) has been postulated to explain the discrepancy between the two methods of determining the ratio.

Kauffman et al. (1979) have questioned the validity of assuming near-equilibrium conditions for the reactions catalysed by the NADP+-dependent dehydrogenases since they claim that these enzymes, in particular malic enzyme (\(K_i = 9 \mu\text{M}\)), isocitrate dehydrogenase (\(K_i = 181 \mu\text{M}\)) and 6PGDH (\(K_i = 114 \mu\text{M}\)), are likely to be inhibited by ATP in vivo. However, the authors concede that the degree of inhibition of these enzymes by ATP may be largely diminished in vivo since ATP exists largely as MgATP, and since Mg2+ diminishes the degree of inhibition, by ATP, of G6PDH, malic enzyme and NADP+-dependent isocitrate dehydrogenase.

The mitochondrial ratio of NADPH / NADP+ at pH 7.0 has been estimated to be about 100 (Sies et al., 1974; Bücher and Sies, 1976; Williamson, 1976; Sies et al., 1977) but the lack of a suitable redox indicator metabolite system in the mitochondria has given rise to uncertainties about the absolute accuracy of these values. However, this discourse is mainly concerned with the cytosolic NADP+ pool and its consequences for the availability of NADPH for reductive biosyntheses in the cytoplasm.
2. The Cytosolic NADPH-generating systems in rat liver

The enzymes known to be responsible for the production of NADPH in the rat liver cytosol are the PPP dehydrogenases (fig.1.1), malic enzyme (fig.1.6) and NADP⁺-linked isocitrate dehydrogenase (fig.1.4).

NADP⁺-linked isocitrate dehydrogenase, 6PGDH and malic enzyme are β-hydroxyacid decarboxylases which catalyse reversible reactions of the general type:

\[
R - \text{CH}_2\text{OH} + \text{NADP}^+ \rightleftharpoons R' - \text{C} = \text{O} + \text{CO}_2 + \text{NADPH} + \text{H}^+
\]

On the other hand, G6PDH oxidises G6P to 6-phospho-D-gluco lactone.

a. NADP⁺-linked isocitrate dehydrogenase

Isocitrate dehydrogenase (NADP⁺) was first purified by Adler et al. (1939), who established the stoichiometry of the reaction and the requirement for divalent ions and NADP⁺. The enzyme, which was found to be widely distributed in nature, was shown to catalyse a reversible reaction by Ochoa (1948):

\[
\begin{align*}
\text{COO}^- & \quad \text{NADPH H}^+ \\
\text{CH}_2 & \\
\text{CH} \quad \text{COO}^- & \quad \text{CH}_2 \quad \text{COO}^- \\
\text{CH} \quad \text{COO}^- & \quad \text{C} = \text{O} \\
\text{-OOC.CH.OH} & \\
\end{align*}
\]

Isocitrate \( \rightarrow \) Ketoglutarate

The distribution of the enzyme is tissue-specific ranging from 80% extramitochondrial in liver to 90% mitochondrial in heart (Pette, 1966; Fatania and Dalziel, 1978). This distribution is consistent with the function of NADP⁺-linked isocitrate dehydrogenase.
Fig. 1.4: NADP⁺-linked isocitrate dehydrogenase and the production of NADPH

The figure shows the series of reactions leading to the generation of NADPH by NADP⁺-dependent isocitrate dehydrogenase (3). Citrate is diverted from the citrate lyase (1) reaction by the reaction catalysed by aconitate hydratase (2). In the mitochondria the α-ketoglutarate is reduced (4) and the resulting isocitrate is reconverted to citrate (5). The diagram is essentially that of Weigl and Sies (1977).
In the liver, the enzyme contributes to the cytosolic NADPH pool, the latter being required for hepatic reductive reactions. The heart, on the other hand, is a tissue with a low demand for cytosolic NADPH.

The $K_m$ value of NADP$^+$-dependent isocitrate dehydrogenase for the metal-tribasic isocitrate complex, at pH 7.0, is $1.65\mu M$ (Colman, 1975) and that for NADP$^+$ is $2.5\mu M$ (Dalziel, 1980). Ahn and Kim (1984) have reported $K_m$ values of $54\mu M$ and $4.28\mu M$ for DL isocitrate and NADP$^+$, respectively. The concentration of Mg$^{2+}$ is important in the determination of the $K_m$ of the enzyme for isocitrate. For example, the $K_m$ for total threo-DL-isocitrate decreases from $90\mu M$ in the presence of $0.2mM-Mg^{2+}$ to $10\mu M$ with $5mM-Mg^{2+}$ (Dalziel, 1980). On the other hand, the $K_m$ for the Mg$^{2+}$-isocitrate complex is $3\mu M$ - independent of the concentration of Mg$^{2+}$. Mn$^{2+}$ has also been shown to increase the affinity of the enzyme for isocitrate. The concentration of the divalent ions does not affect the $K_m$ for NADP$^+$.

NADP$^+$-isocitrate dehydrogenase exhibits a random mechanism for the binding of its substrates, with catalysis more rapid than product release (Uhr et al., 1974). It has been reported that the enzyme binds isocitrate firmly in the absence of cofactor (Villafranca and Colman, 1972; Erlich and Colman, 1975; Reynolds et al., 1978).

In experiments with perfused rat liver, Brunengraber et al. (1973) calculated that extramitochondrial NADP$^+$-isocitrate dehydrogenase is capable of providing up to 54% of the total NADPH required for fatty acid synthesis. Under their experimental conditions, using a perfusate containing 25mM-glucose and no amino acids, the rate of fatty acid synthesis was $170\mu moles$ of acetyl groups incorporated into fatty acids per g dry wt. per hour.

NADP$^+$-isocitrate dehydrogenase is also thought to
play a significant role in the provision of NADPH for drug oxidation. On the basis of their results obtained with isolated hepatocytes, Weigl and Sies (1977) concluded that during drug oxidations dependent on cytochrome P-450, the activity of NADP⁺-isocitrate dehydrogenase is essential because of the inhibition of the PPP by NADPH. This conclusion was based on the observation that during drug metabolism, citrate and isocitrate levels and the citrate / α-ketoglutarate ratio decreased in a manner correlated to the overall decrease in the NADPH / NADP⁺ ratio observed under the same conditions. These authors postulated that the supply of NADPH by cytosolic NADP⁺-isocitrate dehydrogenase accompanies the NADPH supply by the PPP dehydrogenases. The decrease in the NADPH / NADP⁺ ratio caused by the oxidation of NADPH by the cyt. P-450 system results in the activation of the PPP dehydrogenases, which can then contribute to the NADPH pool.

Apart from the tricarboxylates, Weigl and Sies (1977) have presented evidence which suggests that aminotransferases may also be significant in the supply of NADPH to the cytosol during starvation. These authors have shown that the rate of drug oxidation is reduced by about 33% in the presence of aminotransferase inhibitors. This suggests that there is a requirement for transamination steps in the transfer of NADPH-reducing equivalents from the mitochondria to the cytosol under these conditions. This observation may be accounted for by the possible requirement for the aspartate aminotransferases in the NADPH transfer system. A scheme for the operation of this mechanism of NADPH transfer is shown in fig. 1.5.

It is of interest that of the four cytoplasmic NADPH generating enzymes, NADP⁺-isocitrate dehydrogenase activity was not shown to vary significantly with the dietary status (Fitch and Chaikoff, 1960).
Fig. 1.5: The role of aminotransferases in the transfer of reducing equivalents from mitochondria to the cytosol. The figure shows the system incorporating the cytosolic and mitochondrial aspartate aminotransferases in the supply of NADPH to the cytosol (Weigl and Sies, 1977). The requirement for the aminotransferase could result from a rate limitation of the malate/\(\alpha\)-ketoglutarate exchange system (fig. 1.4). If such a condition arises the transamination of \(\alpha\)-ketoglutarate would provide glutamate, which can then enter the mitochondria on the efficient aspartate/glutamate exchange system (La Noue et al., 1974). Transamination of mitochondrial glutamate with oxaloacetate would provide \(\alpha\)-ketoglutarate, which would in turn fuel the cytoplasmic NADP\(^+\)-dependent isocitrate dehydrogenase catalysed reaction.
The enzyme is also not significantly reduced by starvation for 48h, (Veech et al., 1969). The results reported by Taketa et al. (1970) indicate that NADP$^+$-linked isocitrate dehydrogenase is not increased by refeeding 48h starved rats with casein, glucose or casein plus glucose. In fact, the activity was shown to decrease by 12, 30 and 22% when rats were refeed casein, glucose and glucose plus casein, respectively.

Under the same conditions, G6PDH, 6PGDH and malic enzyme activities increased considerably. For instance, for the rats refeed the glucose-casein diet 6PGDH, G6PDH and malic enzyme activity increased to 143, 451 and 516% of the control values. These results suggest a different role for NADP$^+$-linked isocitrate dehydrogenase.

The in vitro activity of NADP$^+$-isocitrate dehydrogenase (cytoplasmic) in rat liver has been reported to be between 22.4 to 32.5 μmoles min$^{-1}$ g$^{-1}$ (Veech et al., 1969; Taketa et al., 1970). Under conditions where the activity of NADP$^+$-isocitrate dehydrogenase was reported to be 32.5 μmoles min$^{-1}$ g$^{-1}$ (Taketa et al., 1970) the rates of G6PDH, 6PGDH and malic enzyme were reported to be 2.90, 6.94 and 3.71 μmoles min$^{-1}$ g$^{-1}$ liver, respectively. These values (see also Veech et al., 1969) imply that NADP$^+$-isocitrate dehydrogenase is the most active of the four cytoplasmic NADPH-generating enzymes.

In the mitochondria of isolated hepatocytes, the quantitative contribution of the flux through mitochondrial NADP$^+$ isocitrate dehydrogenase can contribute more than 80% of the NADPH required for urea synthesis, in model systems restricted to utilization of NH$_4$Cl and lactate (Petcu and Plaut, 1980). Hence both the mitochondrial and the cytoplasmic NADP$^+$-isocitrate dehydrogenase activities are important sources of reducing equivalents in rat liver.
b. Malic enzyme

NADP⁺-specific malic enzyme was discovered by Ochoa et al., (1952) who established the reaction stoichiometry and metal ion requirement for this enzyme. Like NADP⁺-dependent isocitrate dehydrogenase, malic enzyme is mainly extramitochondrial (95%) in the liver and 70% intramitochondrial in heart muscle. Unlike isocitrate dehydrogenase, which is a small dimer (90,000 Mₑ), malic enzyme is tetrameric with a molecular weight of about 200,000 (Dalziel, 1980).

Malic enzyme catalyses the reaction shown in fig.1.6. The $K_m$ values for malate and NADP⁺ have been reported to be 25μM and 1-3μM, respectively (Hsu and Lardy, 1967) for the purified enzyme from pigeon liver. Schimerlik and Cleland (1977) reported $K_m$ values of 4.1μM and 0.29mM for NADP⁺ and malate, respectively.

A simple ordered mechanism (co-enzyme binding first) has been demonstrated for malic enzyme in pigeon liver (Hsu et al., 1967), and NADPH release appears to be the rate-limiting step in the oxidative decarboxylation (Schimerlik and Cleland, 1977).

Malic enzyme is believed to play a significant role in the provision of NADPH for lipogenesis both in rat liver and adipose tissue (Young et al., 1964; Wise and Ball, 1964; Rognstad and Katz, 1979; Yoshimoto et al., 1983). Young et al. (1964) have presented evidence which implies that both liver and adipose tissue can transhydrogenate NADH to NADPH by coupling cytoplasmic malate dehydrogenase to malic enzyme.

Wise and Ball (1964) showed that white adipose tissue has a higher activity of malic enzyme than liver in rats. The distribution of the enzyme is tissue specific and probably relates to the function of the
Fig. 1.6: The generation of NADPH by malic enzyme.
Enzymes 1, 2 and 3 are malate dehydrogenase, pyruvate carboxylase and pyruvate dehydrogenase respectively.
enzyme in that particular tissue. Since the enzyme is 90 and 95% extramitochondrial in rat adipose and hepatic tissue, respectively, and is 26 and 31% cytoplasmic in rat heart and brain, respectively, one could postulate a role for malic enzyme in the provision of NADPH for lipogenesis and other NADPH-requiring processes. However, large variations in the levels of NADP^+-malic enzyme in livers of different species (ranging from no activity in beef liver to 69 U/g in humming bird liver) have been reported by Brdiczka and Pette (1971). These variations could be related to differences in lipogenic rates between various species or would suggest that malic enzyme is not an essential source of NADPH in some species.

The role of malic enzyme in the provision of NADPH for lipogenesis in hepatocytes has been investigated by Rognstad and Katz (1979). These authors used the malic enzyme inhibitor, 2,4 dihydroxybutyrate (K_i = 3.4 mM; Schimerlik and Cleland, 1977), to determine the effect of blocking this source of NADPH on lipogenesis. They found that while 10mM 2,4 dihydroxybutyrate greatly reduced the randomization of [14C] into lactate from substrates such as [6 - 14C]fructose or [6 - 14C] glucose, it did not inhibit lipogenesis significantly. This implied, to the authors, that the pyruvate cycle is operative in rat liver and that a compensatory increase in the activity of the PPP or some other NADPH-generating system was necessary. Rognstad and Katz (1979) showed that 2,4 dihydroxybutyrate increased the flux through the PPP by as much as 90% of the control values. This increase in the flux through the PPP, leads to the production of NADPH, by the pathway, which exceeds the amount required for lipogenesis under the conditions used in their experiments. These results suggest that the PPP dehydrogenases normally operate below their capacity in hepatocytes from starved/re-fed rats, and that when malic enzyme is inhibited, the PPP is capable of producing an increased amount of NADPH. A role for malic enzyme in the provision of NADPH for lipogenesis.
is indicated by these observations.

The production of NADPH by the PPP over that required for lipogenesis can be used for other NADPH-utilizing reactions such as microsomal NADPH reactions, glutathione reduction and amino acid syntheses.

Young et al. (1964) showed that malic enzyme is under dietary regulation. The activity of the enzyme in liver and adipose tissue was found to be decreased by starvation, and increased by carbohydrate refeeding of previously starved rats. Recently, Wilson and McMu"_"en (1981) have shown that hormones are involved in the induction of malic enzyme in cultured rat hepatocytes. T\textsubscript{3} was found to induce malic enzyme by 36%. This induction by T\textsubscript{3} increased to 164% in the presence of insulin and cortisol. The concentration of T\textsubscript{3} required for maximum induction was found to be 1.54 x 10^{-6} M. Thyroxine was found to be effective in the same range of concentration, although it had a negative effect at concentrations higher than 10^{-6} M. The potentiating effect of insulin and cortisol was found to be maximal at high concentrations - 10^{-5} and 10^{-4} M, respectively. Cortisol and insulin, individually, only caused a 19-24% induction of the enzyme. These results suggest that cortisol and insulin act synergistically in their potentiating effect on the T\textsubscript{3} induction.

In the work with chick liver hepatocytes, Goodridge and Adelman (1976) have reported that malic enzyme was increased 2-, 23-, and 77- fold by insulin, T\textsubscript{3}, and insulin plus T\textsubscript{3}, respectively. Glucagon suppresses the induction of malic enzyme. None of these hormones were shown to affect the rate of degradation of the enzyme. Goodridge and Adelman further demonstrated that T\textsubscript{3} induction of malic enzyme was blocked by the specific RNA polymerase II inhibitor, α-amanitin. This observation suggests that the induction of malic enzyme by T\textsubscript{3} requires
the continued synthesis of mRNA. These authors also reported that the suppressive effect of glucagon resulted from decreased synthesis of enzyme protein, and further postulated that the site of action of glucagon was at the level of translation or at the cytoplasmic mRNA processing stage.

Yoshimoto et al. (1983) have demonstrated reciprocal effects of epidermal growth factor (EGF) on G6PDH and malic enzyme in primary cultures of adult rat hepatocytes. EGF was found to inhibit both lipogenesis and the induction of malic enzyme. Furthermore, the induction of malic enzyme by insulin or by insulin plus \( T_3 \) was found to be inhibited by EGF or Br2 cAMP (dibutyryl cAMP). On the other hand, EGF or Br2 cAMP did not affect G6PDH induction. In addition the induction of malic enzyme by insulin or \( T_3 \) was lower at low cell densities. Since fatty acid synthesis has been shown to be higher at high cell densities, the increased induction of malic enzyme by insulin and \( T_3 \) at high cell densities is in agreement with its role as a lipogenic enzyme. G6PDH, on the other hand can be induced substantially at low cell densities, where the rate of DNA synthesis has been shown to be high, and this induction is not prevented by EGF. These observations are consistent with the dual role of G6PDH as a supplier of NADPH for lipogenesis (and possibly the thioredoxin reductase-catalysed reaction in DNA synthesis) and pentose for nucleic acid synthesis.

c. **Glucose-6-phosphate dehydrogenase (G6PDH)**

G6PDH is a cytoplasmic enzyme catalysing the oxidation of G6P (Cori and Lipman, 1952). The immediate product, 6-phosphogluconolactone, is rapidly hydrolysed by a lactonase to 6-phosphogluconate. These two reactions have a large free energy change which has been estimated to be about \(-37\)kJ mol\(^{-1}\) at 25°C, pH 7.0 (Bassham and Krause, 1969). These characteristics mean that the G6PDH catalysed reaction would be expected to be irreversible, favouring the oxidation of G6P in vivo. However, as discussed previously,
G6PDH is potently inhibited by NADPH.

Holten et al., (1976) reported that the enzyme is inhibited by over 98% in rat liver. If this figure is applied to the activity of G6PDH in rat liver, in vitro, reported by Veech et al. (1969), the activity of G6PDH in vivo would be expected to be in the region of 0.02 μmoles min⁻¹ g⁻¹ liver wet weight. This is comparable to the capacity of 6PGDH in the fed cell. Crawford and Blum (1983) using isolated hepatocytes incubated with \([1\text{-}^{14}\text{C}]\) glucose, have estimated the total flux through the oxidative arm of the PPP to be 7.4 nmol/20min mg⁻¹ of protein, corresponding to approximately 74 nmol min⁻¹ g⁻¹ liver. On the other hand Bacquer et al. (1976) using liver slices incubated with \([1\text{-}^{14}\text{C}]\) glucose estimated a flux through the PPP of about 46 nmol min⁻¹ g⁻¹. The flux through the PPP was shown to increase to 150 nmols min⁻¹ g⁻¹ in the presence of phenazine methosulphate.

The effect of this compound, which has also been demonstrated by McLean (1960), implies that the PPP is not maximally stimulated, even under conditions of dietary carbohydrate induced hyperlipogenesis.

Rognstad and Katz (1979) have shown that the PPP can provide 50-75% of the NADPH required for lipogenesis in rat liver. It is of interest that in the avian liver the activity of the PPP dehydrogenases does not change significantly with dietary manipulations (Goodridge, 1968), indicating that this pathway plays a minor role in the provision of NADPH for lipogenesis. Malic enzyme is believed to be the major source of NADPH in avian liver. Rognstad and Katz (1979), as a result of their work with isolated hepatocytes from fasted-refed rats incubated with \([1\text{-}^{14}\text{C}]\) galactose, have concluded that in rat liver, as in adipose tissue, the main sources of cytoplasmic NADPH are malic enzyme and the PPP dehydrogenases.

Bauer et al., (1983) have suggested that since the initial G6PDH catalysed reaction has a standard free energy at pH 7.0 of only -400 J mol⁻¹, and since the physiological NADPH / NADP⁺ ratio is unfavourable,
the G6PDH reaction will not favour the oxidation of G6P unless the cellular levels of 6-phosphogluconolactone are less than 10 nmol g\(^{-1}\). This necessitates the presence of 6-phosphogluconolactonase (6PGL), an enzyme which has been shown to be highly active in fatty acid-synthesizing organs such as liver and epididymal fat (Bauer et al., 1983). In rat liver the activity of 6PGL is 7 times that of G6PDH, when measured in vitro. Hence 6PGL forms an important link between the two dehydrogenases of the oxidative branch of the PPP and appears to be a pre-requisite for the normal function of the PPP dehydrogenases in generating NADPH. 6PGL does not appear to be rate-limiting.

Eggleston and Krebs (1974) have postulated that even though the G6PDH catalysed reaction is far removed from equilibrium, the steady state established by this system, as a result of the reversible inhibition of the reaction by NADPH in vivo, would behave like a near-equilibrium system inasmuch as withdrawal of NADPH decreases the inhibition and causes the withdrawn NADPH to be replaced. Hence the basic control of this first step of the oxidative arm of the PPP may still be considered to be a matter of near-equilibrium relations. In addition, Gumaa et al., (1971) have also concluded that control is not achieved by G6P limitation but rather by NADP\(^{+}\) limitation.

Multiple forms of rat liver G6PDH have been reported (Holten, 1972). These forms appear to be based on the same basic active unit which is a dimer of about 130,000. The higher molecular weight forms are formed by the aggregation of dimers. 90% of the purified enzyme exists in at least three different dimeric forms and the remaining 10% is made up by the higher molecular weight aggregates.

These multiple forms of G6PDH can be separated by electrophoresis on polyacrylamide gels; the bands that have been obtained have been found to correspond to
dimers, tetramers and hexamers of an inactive monomeric sub-unit of molecular weight 60,000-64,000. The dimer can be further resolved into three bands, designated I, II and III, by inclusion of NADP⁺ in the cathode buffer (Schmukler, 1970; Holten, 1972; Taketa and Watanabe, 1971). Band I corresponds to the fastest moving band, and band III the slowest. In most tissue homogenates the dimer form predominates and in rat liver only forms II and III are present and the latter predominates (Holten, 1972; Taketa and Watanabe, 1971; Watanabe et al., 1972; Chang et al., 1979). The patterns of the liver dimers can be manipulated to show all three forms. For instance, depletion of GSH in rat liver supernatants by treatment with diamide results in the conversion of slower moving components to faster ones (Watanabe et al., 1972). Regeneration of GSH has the reverse effect. Liver extracts from carbon tetrachloride-injured cells also exhibited an increased proportion of faster moving forms of the dimers of G6PDH, with a concomitant decrease in the GSH concentration. Overall, the increased intensity of the faster moving components observed in rat liver was found to be closely associated with an increased ratio of G6PDH activity to GSH levels.

Although the regulatory significance of the interconversions between these dimeric forms of G6PDH is not clear it is conceivable that a differential susceptibility to disaggregation of the various forms of the dimer to the inactive monomer could be a regulatory process. In this context Grigor (1984) have argued that the three dimeric forms could be significant in the turnover of G6PDH from the mammary gland. He has postulated that there are two disulphide bonds per dimer, which can be reduced by sulphydryl reagents. Following reduction, a portion of the peptide is exposed, altering the total charge of the protein. The author has argued that form I has two intact disulphide bonds, form II has one disulphide bond
reduced and form III has both disulphide bonds reduced. These three forms have the same specific activity and similar kinetics. However, the oxidised form is more rapidly inactivated by chymotrypsin or a mammary gland microsomal preparation. In the liver the predominant form of G6PDH is the fully reduced dimer, and it is possible that the high levels of GSH found in this organ maintain the relatively stable, fully reduced dimeric form in its reduced state. This could also stabilize the enzyme by protecting it against general proteolysis.

The role of thiol and disulphide groups have been further implicated in the regulation of G6PDH by Francis and Ballard (1980 a,b) who have shown that the inactivation of G6PDH by a rat liver microsomal membrane protein (Francis and Ballard, 1980 b) is partially reversed by dithiothreitol (DTT). Furthermore, pre-incubation of the microsomal protein with DTT, followed by the removal of DTT, resulted in the loss of the protein's capacity to inactivate G6PDH. A possible explanation for this being that the microsomal activity is dependent on the presence of intact disulphide bonds and that DTT reduces these bonds. NADPH was shown to protect G6PDH against inactivation, whereas the disulphide, L-cystine was shown to stimulate the inhibition of G6PDH by the protein. These results led Francis and Ballard (1980 b) to conclude that the inactivation of G6PDH proceeds by a disulphide-thiol exchange mechanism (fig. 1.6a), the rationale being that DTT reduces the disulphide links in the inactivating protein, thereby rendering it ineffective against G6PDH. Isaacs and Binkley (1977) have presented data which shows that G6PDH has a diurnal variation with activity highest during the period of maximal and lowest during the period of minimal mixed disulphide formation. All these observations suggest a role for thiols and disulphides in the regulation of G6PDH.
Fig. 1.6a: The proposed mechanism for the involvement of thiols and disulphides in the degradation of G6PDH (Francis and Ballard, 1980b).

1. The active enzyme (G6PDH) is oxidised to an inactive form by the disulphides;
2. (2) the reduced thiols in the microsomal protein are reoxidised by disulphides such as oxidised glutathione (GSSG);
3. (3) the inactive enzyme can now be denatured (Ballard et al., 1974; Francis and Ballard 1980 a,b).
6-Phosphogluconate dehydrogenase (6PGDH)

6PGDH is a dimeric, cytoplasmic enzyme (Mr 94,000-101,000) which catalyses the oxidation of 6PG to ribulose-5-phosphate (fig. 1.1) with the release of NADPH and carbon dioxide. It has been purified from rat liver (Procsal and Holten, 1972) and $K_m$ values of 71$\mu$M and 13$\mu$M for 6PG and NADP$^+$, respectively, have been reported. Rudack et al. (1971a) showed that the maximal capacity of normal rat liver to oxidise 6PG is 5.8 $\mu$moles min$^{-1}$ g$^{-1}$ liver. When the rats were induced to produce higher levels of 6PGDH (starvation followed by high carbohydrate re-feeding) the capacity was increased to 14.8 $\mu$moles min$^{-1}$ g$^{-1}$ liver. However, at the concentration of substrates and products expected in vivo, this capacity decreases to 0.02 and 0.06 $\mu$mole min$^{-1}$ g$^{-1}$ liver for the control and induced rats, respectively (Procsal and Holten, 1972). These calculations are based on an NADPH / NADP$^+$ ratio of 100, and a cellular concentration of 30$\mu$M 6PG and indicate that the enzyme is almost completely inhibited in vivo.

Unlike G6PDH, the $K_m$ for 6PG of 6PGDH (71$\mu$M) and the cellular concentration of 6PG (14.2-30$\mu$M) are related in such a way that 6PG could be rate-limiting for the 6PGDH catalized reaction in vivo. It is therefore conceivable that an increase in cellular concentrations of 6PG such as that occurring under lipogenic conditions (table 1.1b) could activate the enzyme.

The understanding of the precise role of 6PGDH in the provision of NADPH in the cytoplasm has been hindered by the lack of knowledge concerning the short-term regulation of the enzyme in vivo. However, as an integral part of the PPP, it is likely to be a significant source of NADPH.
3. The major cytosolic NADPH-consuming processes in rat liver.

a. Lipogenesis.

(i). General considerations: A number of biosynthetic pathways use NADPH in the rat liver cytosol. Notable among these is lipogenesis which has been extensively studied. Other abiotic reactions using NADPH, such as drug oxidation via the mixed function oxidase system, have been shown to be major processes utilizing NADPH (Smith and Wills, 1981). Rognstad and Katz (1979) using isolated hepatocytes from rats previously fasted for two days and refed a high-carbohydrate diet for one day, reported that fatty acid synthesis uses 805 μmol g⁻¹ dry weight h⁻¹ of NADPH. Under the same conditions the PPP was reported to produce 450 μmoles of NADPH g⁻¹ dry weight h⁻¹. Assuming that the dry weight is approximately 23% of the wet weight (Brunengraber et al., 1973), these figures correspond to 185 and 103 μmoles NADPH g⁻¹ h⁻¹ for fatty acid synthesis and the PPP, respectively. These figures suggest that lipogenesis is a major NADPH-consuming process.

Lipogenesis in the liver involves the conversion of acetyl-CoA to palmitate. The physiological substrate for fatty acid biosynthesis in rat liver is a matter of current investigation. Recently Geelen and Hindricks (1984) have suggested that lactate may be the substrate in rat liver. They have shown that glycogen, like glucose, is not a major substrate for fatty acid synthesis. In isolated hepatocytes incubated with hepatic glycogen pre-labelled with ¹⁴C from glucose, only 20% of the total carbon used in fatty acid synthesis is derived from glycogen. Experiments with D-[U-¹⁴C] glucose showed that only high concentrations of glucose (50mM) contributed appreciably (25%) to the carbon pool for fatty acid synthesis.
Nevertheless, the acetyl-CoA required for lipogenesis is mainly derived from the mitochondria and is exported to the cytoplasm as citrate (figs. 1.4, 1.5, 1.6). Acetyl-CoA is generated from citrate by the action of ATP-citrate lyase (fig. 1.8). Endemann et al., (1982) have proposed that the formation of acetoacetate in the mitochondria and its utilization in the cytosol may be a secondary pathway of acetyl group translocation operating concurrently with the predominant citrate cleavage pathway. Using isolated perfused rat livers and measuring the incorporation of ketone bodies into fatty acids and 3-β-hydroxy sterols, and comparing the values obtained with the values for total lipid synthesis measured by incorporation of tritium from tritiated water, Endemann et al. (1982) have reported that in fed rats ATP-citrate lyase contributes 31% to the carbon incorporated into fatty acid while acetoacetyl-CoA synthase provides only 4%. In the presence of oleate and hydroxycitrate, which inhibit the citrate cleavage pathway, the contribution of acetoacetyl-CoA synthetase increases to 8.6%. When livers from starved rats were used the flux through acetoacetyl-CoA synthetase increased to 26%. The specific activity of tracer amounts of β-hydroxy [3-14C] butyrate were measured in these experiments. These results suggest that other carbon sources are involved since the citrate cleavage pathway and the flux through the acetoacetyl-CoA synthetase together only account for 85% of the carbon incorporated into fatty acids. Cytoplasmic acetyl-CoA can also be furnished by acetyl-CoA synthetase which utilizes acetate and CoA (Veech and Guynn, 1974).

In rat liver the NADPH required for lipogenesis is derived mainly from the PPP dehydrogenases and malic enzyme (Rognstad and Katz, 1979). The role of NADP+-isocitrate dehydrogenase has been discussed. It has been suggested that at low rates of lipogenesis the PPP
dehydrogenases are able to supply all the NADPH required for fatty acid synthesis in both rat liver and adipose tissue (Katz et al., 1966; Saggerson and Greenbaum, 1970; Thompson et al., 1976; Holten et al., 1976). In cells actively synthesizing fatty acids, malic enzyme can contribute as much as 50% of the NADPH required for lipogenesis (Flatt and Ball, 1964; Wise and Ball, 1964; Kornacker and Ball, 1965; Ball, 1966; Rognstad and Katz, 1966; Rognstad and Katz, 1979).

The committed step of lipogenesis is the carboxylation of acetyl-CoA to form malonyl-CoA. The reaction is catalysed by the biotin containing acetyl-CoA carboxylase. The intermediates in fatty acid synthesis are then attached to an acyl-carrier protein (ACP) by the action of transacylase and fatty acid synthesis proceeds as in fig. 1.7. The overall stoichiometry of the reaction is:

\[
\begin{align*}
8 \text{CH}_3\text{C}^=\text{O} + \text{ACP} + 7 \text{HCO}_3^- + 7 \text{ATP} + \frac{1}{4} \text{NADPH}^+ \\
\to \text{CH}_3(\text{CH}_2)_4\text{C}^=\text{O}_{\text{ACP}} + 8 \text{CoA} + \frac{1}{4} \text{NADP}^+ + 7 \text{H}_2\text{O} + 7 \text{ADP}
\end{align*}
\]

\[+ 7 \text{Pi} + 7 \text{CO}_2\]

Rates of fatty acid synthesis are dependent on the nutritional status of the animal (Winegrad and Renold, 1958; Wieland et al., 1963; Wieland and Eger-Neufeildt, 1963; Butterworth et al., 1966; Brunengraber et al., 1973; Endemann et al., 1982). Fatty acid synthesis has been shown to be markedly reduced by fasting, fat feeding or in the diabetic state. Similar conditions have been shown to result in a diminution of the level of the PPP dehydrogenases.
Enzyme activity

1. Biotin carboxylase
   
   Reaction
   \[
   \text{BCCP} + \text{HCO}^- + \text{ATP} \xrightarrow{\text{BCCP} \sim \text{COO}^- + \text{ADP} + P_i} \]

2. Carboxyl transferase
   
   Reaction
   \[
   \text{BCCP} \sim \text{COO}^- + \text{CH}_3\text{C}^=\text{O} \xrightarrow{\text{BCCP} + \text{CH}_2\text{C}^=\text{O} \rightarrow \text{COO}^-} \]

3. Acetyl transacylase
   
   Reaction
   \[
   \text{CH}_3\text{C}^=\text{O} + \text{ACP-SH} \xrightarrow{\text{CH}_3\text{C}^=\text{O} + \text{CoA-SH}} \text{CoO}^- \xrightarrow{\text{CH}_2\text{C}^=\text{O} + \text{ACP-SH}} \text{CH}_3\text{C}^=\text{O} + \text{ACP-SH} \]

4. Malonyl transacylase
   
   Reaction
   \[
   \text{CH}_2\text{C}^=\text{O} + \text{ACP-SH} \xrightarrow{\text{CH}_2\text{C}^=\text{O} + \text{CoA-SH}} \text{CoO}^- \xrightarrow{\text{CH}_3\text{C}^=\text{O} + \text{ACP-SH}} \text{CH}_3\text{C}^=\text{O} + \text{ACP-SH} \]

5. \(\beta\)-Ketoacyl-ACP synthase (condensing enzyme)
   
   a. Reaction
   \[
   \text{CH}_3\text{C}^=\text{O} + \text{Enz-SH} \xrightarrow{\text{CH}_3\text{C}^=\text{O} + \text{ACP-SH}} \text{CH}_3\text{C}^=\text{O} + \text{ACP-SH} \]

   b. Reaction
   \[
   \text{CH}_3\text{C}^=\text{O} + \text{ACP-SH} \xrightarrow{\text{CH}_3\text{C}^=\text{O} + \text{ACP-SH}} \text{CH}_3\text{C}^=\text{O} + \text{ACP-SH} \]

   + \text{CO}_2 + \text{Enz-SH}
6. $\beta$-Ketoacyl-ACP reductase

$$\begin{align*}
\text{CH}_3\text{CH}_2\text{C}&=\text{O} \quad \text{S-ACP} \\
+\text{NADPH} &
\Downarrow
\text{OH} \\
\text{D-CH}_3\text{CHCH}_2\text{C}&=\text{O} \quad \text{S-ACP} \\
+ \text{NADP}^+ &
\end{align*}$$

7. $\beta$-Hydroxyacyl-ACP dehydratase

$$\begin{align*}
\text{CH}_3\text{CHOH CH}_2\text{C}&=\text{O} \quad \text{S-ACP} \\
\Downarrow
\text{trans-CH}_3\text{CH}=\text{CHC}&=\text{O} \quad \text{S-ACP} \\
+ \text{H}_2\text{O} &
\end{align*}$$

8. Enoyl-ACP reductase

$$\begin{align*}
\text{CH}_3\text{CH}=\text{CHC}&=\text{O} \quad \text{S-ACP} \\
\Downarrow
\text{CH}_3\text{CH}_2\text{CH}_2\text{C}&=\text{O} \quad \text{S-ACP} \\
+ \text{NADPH} &
\end{align*}$$

Recycle 5-8 five times

9. Thioesterase

$$\begin{align*}
\text{CH}_3\text{(CH}_2\text{)}_{14}\text{C}&=\text{O} \quad \text{S-ACP} \\
\Downarrow
\text{CH}_3\text{(CH}_2\text{)}_{14}\text{C}&=\text{OH} + \text{ACP-SH} \\
+ \text{H}_2\text{O} &
\end{align*}$$

Fig. 1.7: The reactions of lipogenesis (Wakil et al., 1983).

Reactions 1 and 2 are catalysed by acetyl-CoA carboxylase, and 3-9 by FAS.
Fig. 1.8: Utilization of acetoacetate in rat liver (Endemann et al. 1982). The numbers refer to the enzymes: 1, citrate synthetase; 2, ATP-citrate lyase; 3, acetoacetyl-CoA thiolase; 4, \( \beta \)-hydroxybutyrate dehydrogenase; 5, acetoacetyl-CoA synthetase; 6, hydroxymethylglutaryl (HMG)-CoA synthetase.
Boca and Flatt (1969) have postulated that the impairment of lipogenesis found in the fasted animal is primarily due to a decrease in the tissues ability to generate acetyl-CoA from glucose rather than the inhibition of or change in the level of the enzymes directly involved in lipogenesis. In contrast, Butterworth et al. (1966) have reported quantitative changes in the amount of fatty acid synthase in response to starvation.

Brunengraber et al. (1973) using tritiated water to measure lipogenesis in perfused rat liver have reported a lipogenic rate of about 0.65 μmoles of acetyl groups incorporated g⁻¹ min⁻¹ in fed rats, whilst Endemann et al. (1982) have reported values of 0.32 and 0.007 μmoles of acetyl groups incorporated g⁻¹ min⁻¹ for the fed and 48h starved rats, respectively. A higher rate for the 48h starved rats (0.02 μmoles acetyl groups incorporated g⁻¹ min⁻¹) has been reported by Brunengraber et al. (1973).

Lipogenesis is under both hormonal and nutritional regulation and lipogenic enzymes have been shown to be co-ordinately induced or inhibited by various factors. Spence and Pitot (1982) have demonstrated that in primary cultures of rat hepatocytes G6PDH, malic enzyme, ATP-citrate lyase, acetyl-CoA carboxylase, fatty acid synthase and stearoyl-CoA desaturase are co-ordinately induced by insulin or T₃. These hormones produce a 2-3 fold increase in the individual enzyme activities, whereas glucagon slightly decreases the enzyme activities. Fructose and glycerol, both in the absence and presence of insulin, were shown to increase the individual enzyme activities to the same extent as insulin or T₃. These increases in activity were shown to be the result of enhanced de novo synthesis of the enzymes. Hence a relationship between carbohydrate metabolism and lipogenesis, independently in part of hormonal effects, was demonstrated. Further involvement of hormones in
the regulation of lipogenesis has been indicated by the observation that vasopressin, adrenaline and angiotensin II inhibit lipogenesis (Hems, 1977; Ma and Hems, 1975).

Non-esterified fatty acids are thought to be acute regulators of lipogenesis since these compounds have been shown to inhibit lipogenic enzymes (Jeffcoat and James, 1977; Jeffcoat et al., 1979). In addition elevated levels of free fatty acids in vivo, as is the case in starved, fat-fed or diabetic rats, is associated with depressed lipogenesis (for a review see Volpe and Vagelos, 1976). Topping and Mayes (1982) have presented evidence which suggests that insulin can stimulate lipogenesis in perfused rat liver, in the presence of elevated concentrations of serum non-esterified fatty acids. The implication of this finding is that insulin could counteract the inhibitory effects of fatty acids in starved-refed, fed or insulin-treated diabetic rats.

ii. ATP-citrate lyase

ATP-citrate lyase, the enzyme catalysing the cleavage of citrate into acetyl-CoA and oxaloacetate (fig 1.8), is a key lipogenic enzyme since it provided most of the acetyl-CoA required for fatty acid synthesis (Endemann et al., 1982). The reaction catalysed by the enzyme is shown below:

\[
\text{Citrate} + \text{CoA} + \text{ATP} \rightleftharpoons \text{Acetyl-CoA} + \text{ADP} + \text{P}_i + \text{oxaloacetate}
\]

The \(K_m\) values of ATP-citrate lyase have been reported to be 0.07mM, 0.28mM and 1.6μM for citrate, ATP and CoA, respectively (Plowman and Cleland, 1967). The citrate required for this reaction is derived from the mitochondria where it is formed by the activity of citrate synthase. The substrates of this latter enzyme are mitochondrial oxaloacetate and acetyl-CoA, which are derived from pyruvate and generated by the action of pyruvate
carboxylase and pyruvate dehydrogenase.

The free acetyl-CoA / CoA ratio in rat liver cytoplasm has been found to be high in starved and fed rats, and in conditions of rapid fat synthesis. This suggests that the supply of acetyl-CoA is never limiting for fat synthesis under most in vivo conditions (Veech and Guynn, 1974). The activity of ATP-citrate lyase in both starved and fed rats is more than four times that of acetyl-CoA carboxylase and fatty acid synthase (Veech and Guynn, 1974). Recently, Spence and Pitot (1982) have reported that ATP-citrate lyase activity is not depressed by starvation.

ATP-citrate lyase is known to be regulated in response to hormones and metabolites (Spence et al., 1979; Spence and Pitot, 1982). Spence et al. (1979), using cordycepin and cycloheximide (protein synthesis inhibitors) have shown that the induction of ATP-citrate lyase in isolated hepatocytes by insulin, insulin plus glucose, glycerol and fructose is dependent on protein synthesis. These authors immunoprecipitated ATP-citrate lyase, and by following the incorporation of $[^{2}$H$]_{\text{leucine}}$ into this specific protein, showed that the changes in the activity of the enzyme correlate with changes observed in the rate of synthesis of the enzyme. Similar observations have been made by Pearce (1980) for rats fed a fructose-based diet.

Fat-feeding results in a diminution of the enzyme and Schwartz and Abraham (1981) have reported the conversion of ATP-citrate lyase into a catalytically inactive form by polyunsaturated fatty acids.

Glucagon treatment of hepatocytes and thyroidectomy diminish the measurable activity of ATP-citrate lyase (Spence et al., 1979). Although phosphorylation of the enzyme by a glucagon-stimulated cAMP-dependent protein kinase and by an insulin-stimulated cAMP-independent
mechanism has been demonstrated (Janski et al., 1979; Ranganathan et al., 1980; 1982; Alexander et al., 1982), the regulatory significance of this phosphorylation is not clear, since no changes in the regulatory or catalytic properties of the enzyme have been found. Hence the proposal that the enzyme may be subjected to hormonal influences which alter its susceptibility to degradation (Osterlund et al., 1980; Vogel and Bridger, 1981).

iii. Acetyl-CoA carboxylase.

Acetyl-CoA carboxylase catalyses the ATP- and bicarbonate-dependent carboxylation of acetyl-CoA to form malonyl-CoA. The carboxylation of acetyl-CoA requires the presence of biotin carboxyl carrier protein (BCCP), biotin carboxylase (BC) and carboxyl transferase (CT). The carboxylation proceeds in two stages:

1. \[ \text{BCCP} + \text{HCO}_3^- + \text{ATP} \xrightarrow{\text{BC, Mo}^{2+}} \text{BCCP} \rightleftharpoons \text{COO}^- + \text{ADP} + P_i \]

2. \[ \text{BCCP} \rightleftharpoons \text{COO}^- + \text{CH}_3\text{C}^\text{S-CoA} \xrightarrow{\text{CT}} \text{BCCP} + \text{CH}_2\text{C}^\text{S-CoA} \]

The \( K_m \) of acetyl-CoA carboxylase for acetyl-CoA is 80\( \mu \)M, that for the bicarbonate is 6mM and that for ATP is 0.2mM (Alberts and Vagelos, 1972; Soler-Argilaga et al., 1978; Song and Kim, 1981).

Rat liver acetyl-CoA carboxylase consists of two apparently identical sub-units of \( M_r 230,000 \) (Tanabe et al., 1975; Nakanishi et al., 1976; Song and Kim, 1981). The acetyl-CoA carboxylase protomer is the multifunctional 230,000 (\( M_r \)) sub-unit which contains the functions of BCCP, BC and CT.

Several studies have shown that the activity of acetyl-CoA carboxylase measured in crude extracts
correlates well with the rate of fatty acid synthesis, and that changes in the rate of fatty acid synthesis imposed by insulin and glucagon are due to alterations in the activity of acetyl-CoA carboxylase (Allred and Roering, 1973; Watkins et al., 1977; Geelen et al., 1978; Beynen et al., 1979; Witters et al., 1979). Hence, acetyl-CoA carboxylase provides a point of control and has been regarded as a rate-limiting enzyme of lipogenesis (Lane et al., 1974; Volpe and Vagelos, 1976; Giffhorn and Katz, 1984).

The hormonal regulation of hepatic acetyl-CoA carboxylase is thought to occur through changes in the cellular concentration of citrate (Watkins et al., 1977; Wakil et al., 1983), and/or by a phosphorylation-dephosphorylation mechanism (Carlson and Kim, 1973; Hardie and Cohen, 1978, 1979; Guy and Hardie, 1981), or both (Geelen and Beynen, 1981).

Citrate has been shown to be able to activate acetyl-CoA carboxylase by converting the inactive protomer to the active polymer (for reviews see Lane et al., 1974; Moss and Lane, 1971; Lane and Moss, 1971). In the cell the enzyme is believed to exist as a polymeric structure (Meredith and Lane, 1978; Buechler et al., 1984). The physiological significance of this citrate-(or tricarboxylic acid-) induced polymerisation of the enzyme has been questioned since citrate does not activate the enzyme in the presence of physiological concentrations of ATP, and the $K_a$ for citrate is 3-4mM, ten times the concentration of citrate thought to exist in the cell. However, Wakil et al., (1983) have explained the regulatory role of citrate in the regulation of fatty acid synthesis in terms of the inhibitory effect of glucagon on phosphofructokinase-1 and its effect of decreasing cytosolic citrate concentration. They have argued that the consequent decrease in citrate concentration results in a decrease in acetyl-CoA carboxylase activity, which results in a reduced
rate of fatty acid synthesis.

The polymerisation of acetyl-CoA carboxylase is enhanced by a high enzyme concentration, inorganic phosphate and pH in the range 6.5 - 7.0. Depolymerisation is induced by alkaline pH, HCO$_3^-$, fatty acyl-CoA and ATP-Mg$^{2+}$ (Lane et al., 1974). CoA has also been shown to cause the allosteric activation of acetyl-CoA carboxylase by inducing polymerisation (Yeh and Kim, 1980). CoA acts at a different site, on the enzyme, from citrate, but appears to compete with palmityl-CoA for the same site (Yeh and Kim, 1980).

Fatty acyl-CoAs, on the other hand, regulate fatty acid synthesis by inhibiting acetyl-CoA carboxylase as a result of its depolymerisation (Wakil et al., 1983). Both the binding of the acyl-CoA and depolymerisation of the carboxylase is competitive with citrate and is reversed by albumin (Goodridge, 1972, 1973). One acyl-CoA binds per carboxylase subunit with a dissociation constant of 5nM (Ogiwara et al., 1978 and Sreekrishma et al., 1980). The inhibition is reversed by phosphatidylcholine in the presence of citrate. Palmitoyl-, stearoyl- and arachidyl-CoA are the most effective inhibitors of the carboxylase (Nikawa et al., 1979). Since palmitoyl-dephospho-CoA is a poor inhibitor of acetyl-CoA carboxylase with a 40-fold higher $K_i$ than palmitoyl-CoA a specific binding site for the acyl-CoA dependent on the chain length of the fatty acid and the 3'-phosphate CoA is implied by these observations. Wakil et al. (1983) have stated that the regulation of the carboxylase by the long-chain acyl-CoAs is reciprocal to that by citrate and may reflect the physiological role of these metabolites in regulating fatty acid synthesis.

Physiological concentrations of guanine nucleotides (Witters et al., 1981) stimulate acetyl-CoA carboxylase activity in dialysed rat liver supernatant preparations.
but have no effect on the purified enzyme. On the other hand, CoA can activate even the purified enzyme and this activation is accompanied by both a change in the value of $K_m$ for acetyl-CoA from 0.2mM to about 4μM, and by polymerisation of the enzyme independent of citrate (Yeh and Kim, 1980; Yeh, 1981).

Malonyl-CoA has been reported to be a potent inhibitor of the enzyme exhibiting a $K_i$ of 10μM (Gregolin et al., 1966; Chang et al., 1967). This inhibition could, conceivably prevent unnecessary consumption of acetyl-CoA for lipogenesis.

Acetyl-CoA carboxylase is inactivated by phosphorylation (Hardie and Cohen, 1978). It has been suggested by Hardie and Cohen (1978) that glycogen and fatty acid synthesis may be regulated synchronously, since both glycogen synthetase and acetyl-CoA carboxylase are phosphorylated in the same manner. The phosphorylation may involve both cAMP-dependent and independent protein kinases (Guy and Hardie, 1981).

Giffhorn and Katz (1984) have investigated the long-term regulation of acetyl-CoA carboxylase in primary hepatocyte cultures. These authors have demonstrated glucose- and insulin-dependent increases in the acetyl-CoA carboxylase activity, which they have attributed to increases in the amount of enzyme protein. This increase in the amount of enzyme was specifically effected by an increase in the rate of enzyme synthesis rather than by decreased enzyme degradation $\alpha$-amanitin, an inhibitor of RNA polymerase II (Faulstich, 1981), and cordycepin prevented the induction of acetyl-CoA carboxylase by glucose or glucose plus insulin. Buechler et al., (1984) have also demonstrated the short-term hormonal regulation of lipogenesis in isolated hepatocytes. In the presence of glucagon (10nM) the rate of lipogenesis from $\text{[}^{3}\text{H}_{2}\text{O]}$ decreased 10-fold from 0.154 μmol of acetyl units g$^{-1}$ min$^{-1}$. 
Insulin (85nM) on the other hand, increased the rate to $0.246 \text{ mmol g}^{-1} \text{ min}^{-1}$. These authors also attempted to relate the rate of fatty acid synthesis to the activity of acetyl-CoA carboxylase. They found that when the enzyme was prepared under conditions of maximal activity (i.e. preincubation with citrate) the rate elicited was comparable to the rate of fatty acid synthesis, but insulin and glucagon appeared to have no effect on enzyme activity. However, under certain conditions such as the homogenization of the cells in the absence of Triton X-100 and without preincubation, acetyl-CoA carboxylase appeared to be hormone-sensitive, the activity being suppressed by glucagon and increased by insulin. Under these conditions acetyl-CoA carboxylase activity was shown to be only 13%, 16% and 57% of the rate of lipogenesis when compared to the untreated, insulin - and glucagon - treated hepatocytes, respectively. Hence a role for acetyl-CoA carboxylase in the short-term regulation of lipogenesis is implied.

iv. Fatty acid synthase (FAS)

Fatty acid synthase catalyses the de novo synthesis of long-chain fatty acids from acetyl-CoA and malonyl-CoA (Simonis et al., 1967; Prescott and Vagelos, 1972; Volpe and Vagelos, 1973). The fatty acid synthase (FAS) isolated from animal tissues is a multifunctional enzyme consisting of two identical subunits ($M_r 250,000$). Unlike the yeast enzyme, animal FAS has no requirement for FMN as a cofactor and yields free palmitate and stearate as the end products (Wakil et al., 1983). The enzyme contains a total of seven enzymic activities. Dissociation of the enzyme into monomers results in the retention of six of the enzymic activities: acetyl transacylase, malonyl transacylase, $\beta$-hydroxyacyl dehydratase, enoyl reductase and thioesterase. The seventh activity, $\beta$-ketoacyl synthase (condensing enzyme), has an active center which requires the presence of two juxtapositioned thiols each derived from one subunit. This latter
activity can, therefore, only be detected in the native dimer (Wakil and Stoops, 1983). FAS therefore catalyses reactions 3-9 in fig.1.7.

The short-term regulation of FAS in animal tissues is not well understood. Nevertheless, several hypotheses have been advanced and reviews on the general regulation of FAS have appeared (Volpe and Vagelos, 1976; Stoops et al., 1977; Wakil et al., 1983). Phosphorylated sugars, especially fructose 1, 6-bisphosphate, have been shown to stimulate FAS. Plate et al. (1968) showed that the effect of fructose 1,6-bisphosphate is to reduce the $K_m$ of FAS for NADPH. However, this stimulation occurs at a non-physiological concentration (10mM) of the diphosphate.

Acetyl-CoA and malonyl-CoA competitively inhibit FAS (Katiyar and Porter, 1974; Srinivasan and Kumar, 1981). The liver enzyme is also inhibited by palmitoyl-CoA and free fatty acids (Hsu et al., 1969; Knoche et al., 1973) but evidence that this inhibition is of physiological significance is circumstantial. Indeed, Dorsey and Porter (1968) have shown that the inhibition by the thioester is dependent on the molar ratio of palmitoyl-CoA to protein, implying a detergent action rather than a site-specific action. The inhibition was found to be irreversible and similar to that produced by SDS (sodium dodecyl sulphate). In contrast, acetyl-CoA carboxylase exhibits a competitive, reversible, inhibition by long-chain acyl-CoAs (Ogiwara et al., 1978). The observations made with FAS do not support a role for long-chain acyl-CoA derivatives in the short-term regulation of this enzyme.

Stoops and Wakil (1982) have demonstrated a rapid, irreversible inactivation of chicken liver FAS by 1,3 dibromo-2-propanone which is used as a probe of the $\beta$-ketoacyl synthase site. Acetyl-CoA, but not malonyl-CoA, protects the enzyme against this inactivation. The
reason given for the different effects of these two substrates is that while acetyl-CoA can reside on both the cysteine-SH and the pantetheine-SH, malonyl-CoA can only reside on the latter, leaving the active cysteine-SH exposed for reaction with dibromopropanone with subsequent inactivation of the enzyme. It was shown in these experiments that dibromopropanone inhibits the enzyme by forming 3-bromo-2 keto derivatives of the cysteine residue. The effect of dibromopropanone was accompanied by the cross-linking of the two subunits.

In experiments with cold inactivated FAS, Stoops and Wakil (1982) showed that dibromopropanone inactivated FAS activity but failed to bring about the cross-linking of the two subunits presumably because of the dissociation of the dimer which occurs during the reversible cold inactivation of FAS (Fig.1.9). NADPH, but not NADH, was shown to enhance the restoration of the cold inactivated enzyme at room temperature. CoA, acetyl-, and malonyl-CoA had no effect on the rate of reactivation. As a result of these findings the authors suggested that the correct positioning of the phosphate group on NADPH may be critical in the reactivation process, and proposed a role for phosphates in the regulation of full FAS activity through a conformational change such as that shown in Fig.1.9.

In this respect the potent inhibition by NADP+ of goose uropygial gland FAS has been reported by Poulos and Kolattukudy (1981). They have shown that FAS activity is inhibited by increasing the NADP+ / NADPH ratio. The NADP+ induced inhibition of the overall activity of FAS, the ketoreductase and the enoyl reductase is competitive with respect to NADPH with $K_i$ values of 6μM, 40μM and 260μM, respectively. Furthermore the condensing activity of FAS was found to be severely inhibited, and this inhibition was abolished by modification of the NADPH
binding site of the enoyl reductase domain with pyridoxal phosphate. Under these conditions NADPH was unable to both induce the dimerization of the FAS subunits and prevent proteolysis of the modified enzyme. NADPH has been shown to fulfill both these functions in the presence of the unmodified enzyme. On the basis of these results, Poulose and Kalattukudy proposed that the enoyl reductase domain of FAS plays a key role in the interdomain interactions which regulate the activity of the enzyme via the $\text{NADP}^+ / \text{NADPH}$ ratio.

![Diagram of FAS inactivation](image)

**Fig 1.9:** The proposed mechanism for the cold inactivation of FAS (Stoops and Wakil, 1981)

The active dimer requires the juxtapositioning of the pantetheine-SH (Pant-SH) and the cysteine-SH (Cys-SH). Upon incubation at $0^\circ C$ a conformational change occurs resulting in two inactive forms which further dissociate into inactive monomers.
A role for CoA in the regulation of FAS has been postulated (Linn and Srere, 1980; Linn et al., 1980). These workers have shown that CoA depleting systems inhibit the activity of highly purified rat liver (FAS). Addition of CoA to these systems reverses the inhibition (Linn and Srere, 1980; Linn et al., 1980; Sedgwick and Smith, 1981). The rate of elongation of acyl moieties on the enzyme was reduced a 100-fold in the absence of CoA, which when present promoted the elongation process. Linn and Srere (1980) have suggested that CoA is required for the termination of the FAS reaction.

In common with other lipogenic enzymes, the synthesis of FAS is a well-regulated process, affected by diet and hormones. Morris et al., (1982) have measured directly the FAS mRNA levels in goose liver using cDNA probes isolated from cloned plasmids containing cDNA sequences prepared from FAS mRNA. These workers have correlated the rate of hepatic fatty acid synthesis in neonatal goslings to the increase in the amount of the mRNA for FAS.

It has been known for a long time now that fasting and the diabetic condition results in a reduction in the activity of FAS, while re-feeding with a high carbohydrate, low-fat diet and insulin administration increases the enzyme activity (Craig et al., 1972; Lakshmanan et al., 1972; Craig and Porter, 1973). Fructose has been reported to be a better inducer of the hepatic FAS than glucose (Bruckdorfer et al., 1972; Volpe and Vagelos, 1974).

Hormonal regulation of the dietary induction of liver FAS has been demonstrated (for review see Volpe and Vagelos, 1976). Glucagon and cAMP have been found to inhibit FAS induction in fasted-refed rats (Lakshmanan et al., 1972; Volpe and Marasa, 1975). These authors have suggested that the relative levels of insulin and glucagon in the blood regulate the activity of this enzyme. Some authors
have attributed the hormone-induced changes to alterations in the rate of enzyme synthesis operating at the translational level of protein synthesis (Lakshmanan et al., 1972, 1975; Pry and Porter, 1981). In diabetic, but not normal, rats, adrenalectomy can restore the depressed (FAS) levels (Volpe and Marasa, 1975). Spence and Pitot (1982) have demonstrated the effect of insulin and other hormones in rat hepatocytes (see 3.a.(i)). Joshi and Kasturi (1982) have reported that antimicrotubular agents (colchicine, colcemid, and vinblastine) inhibit the insulin induction of FAS and stearoyl-CoA desaturase in cultured liver explants. For instance 1μM colchicine inhibits the insulin induction of the two lipogenic enzymes by 90%. The colchicine effect was shown to be specific for the insulin-induced synthesis of the synthase and the Δ⁹ desaturase. Since colchicine has no effect on the induction of malic enzyme by T₃, colchicine appears to interfere with the induction of synthase by insulin. Lumicolchicine, an inactive isomer of colchicine, does not inhibit insulin induction of lipogenic enzymes.

The overall regulation of lipogenesis has been reviewed recently (Saggerson, 1980; Wakil et al., 1983). It is clear from these reviews that while the long-term regulation of fatty acid synthesis is becoming clearer, the short-term regulation of the enzyme is still not well-understood.

b. Glutathione reductase

Glutathione reductase catalyses the reduction of oxidised glutathione as shown below:

\[
\text{GSSG + NADPH} \quad \rightarrow \quad 2\text{GSH} + \text{NADP}^+
\]

The enzyme has been purified from rat liver (Mize and Langdon, 1962a; Carlberg and Mannervik, 1975). The native enzyme is a dimer with a molecular weight of
125,000. The enzyme contains two FAD molecules which are reducible by NADPH, GSH or dithioerythritol (Carlberg and Mannervik, 1975). GSSG reoxidises the reduced FAD. However, Mize and Langdon failed to demonstrate the presence of FAD in the enzyme molecule. The flavoenzyme purified by Carlberg and Mannervik can also utilize NADH at about 10% of the rate with NADPH, under optimal conditions. The pH dependence of the two activities was, however, different, optimal activity with NADH being obtained between pH 5.0 and 5.5 and that with NADPH being about pH 7.0. Next to GSSG, the mixed disulphide of CoA and GSH (CoA-SSG) was shown to be the best substrate, being able to elicit a glutathione reductase activity of 10% of that obtained with GSSG. Cystine and Cy-SSG were shown to be very poor substrates (0.4% \( \text{V}_{\text{max}} \)). The pH dependence of the CoA-SSG and Cy-SSG reductions exhibited lower pH optima (about pH 6) than the reduction of GSSG (pH 7). On the other hand Mize and Langdon reported that cystine was utilized at about 5% of the rate with glutathione.

The \( K_m \) of glutathione reductase for NADPH was found to be 7.9\( \mu M \) and that for GSSG was 56.7\( \mu M \) (Carlberg and Mannervik, 1975). These values are comparable with the figures reported by Mize and Langdon (1962a). NADP\(^+\) was shown to be a non-competitive inhibitor with respect to NADPH. The inhibition was 50% with 100\( \mu M \)-NADP\(^+\) when the concentrations of NADPH and GSSG were 10\( \mu M \) and 1.0mM, respectively. NADPH (340\( \mu M \)) was shown to fully inhibit glutathione reductase (Mize and Langdon, 1962a).

Mize and Langdon (1962b) proposed that one thiol residue on the enzyme protein participates in the catalytic event leading to the reduction of oxidised glutathione, as shown below:
The GSH produced by the action of glutathione reductase is involved in a wide range of enzymatic reactions. A major function of GSH is to serve as a reductant in oxidation-reduction processes, a function resulting in the regeneration of GSSG (Boyland and Chasseaud, 1969; Meister, 1982). The reduction of GSSG is consequently of fundamental importance in organs exhibiting high activities of most of the GSH-dependent biochemical reactions, such as mammalian liver. In phagocytosis, for example, the $H_2O_2$ levels are raised in order to destroy bacteria ingested during phagocytosis; this $H_2O_2$ has to be removed to prevent tissue damage. Under these conditions, reduced glutathione, the predominant form of glutathione (Owens et al., 1965), can be oxidised to GSSG with consequent removal of $H_2O_2$ as shown below (Reed, 1969):-

\[ H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O \]

The GSSG can be reduced to GSH by the action of glutathione reductase, this being accompanied by the removal of NADPH and the production of NADP$^+$ which would favour the stimulation of the PPP.
May (1981) has attempted to determine the role of glutathione in the short-term regulation of the PPP activity in rat adipocytes. He showed that incubation of adipocytes with \( \text{H}_2\text{O}_2 \) (60\( \mu \text{M} \)) in the presence of 1.1mM glucose over a 20min period resulted in a small reduction in GSH concentration and a more rapid recovery when compared to incubations performed in the absence of glucose. In addition, \( \text{H}_2\text{O}_2 \)-treated cells showed an increase in glucose C-1 oxidation when compared to cells incubated with glucose alone. The implication of these results is that the PPP activity was increased by \( \text{H}_2\text{O}_2 \) treatment and that the cycle was providing NADPH for rapid regeneration of GSH via glutathione reductase. Lowering of GSH to 35-60\% of control values by addition of agents such as t-butyl hydroperoxide, diamide or the sulfhydryl blocker N-ethyl-maleimide resulted in the stimulation of the PPP.

May (1981) did, however, demonstrate that the PPP could also be stimulated by reducing the \( \text{NADPH} / \text{NADP}^+ \) ratio without affecting the cellular GSH content (for example by addition of methylene blue). The conclusion drawn from this work was that the PPP contributes to the regeneration of GSH, and that glutathione metabolism, directly or indirectly, may play a significant role in the regulation of the PPP. Furthermore, the PPP is likely to be regulated by a mechanism independent of glutathione metabolism.

Elsayed et al., (1982) have related the activity of the PPP in lungs of mice to glutathione metabolism and dietary selenium levels. They have observed that when animals are exposed to air, selenium deficiency causes a reduction in glutathione peroxidase activity when compared to animals on selenium-supplemented diets. However, exposure to ozone results in an increase in glutathione reductase, G6PDH and 6PGDH activity, which is in proportion to the dietary selenium levels. Under these conditions glutathione peroxidase activity remained
constant. The stimulation of the PPP was found to be dependent on the presence of selenium in the diet.

An important aspect of this inter-relationship between the PPP and glutathione metabolism is that, coupled together the two systems form an important cellular defence mechanism by which the cell can withstand the damaging effects of oxidant stress from exogenous (inhaled O³, NO₂ or high levels of O₂) or endogenous (such as hydrogen peroxide or other peroxides formed in the membrane) sources. Since the PPP has been reported to contribute to the biosynthetic processes in cells under oxidant stress (Tierney et al., 1973), inhibition of the PPP would interfere with the capacity of the injured cells to regenerate new cell components.

It is important to note that the regulation of the PPP dehydrogenases by GSH/GSSG ratios depends on the oxidation of GSH to GSSG, which is in turn reduced to GSH by glutathione reductase. Since in the normal animal most of the glutathione is in the reduced form (Owens et al., 1965; Veech et al., 1969) it is difficult to ascertain the role of glutathione reductase in the regulation of the PPP in the absence of oxidant stress. This consideration emphasises the need for a regulatory mechanism independent of the glutathione reductase catalysed reaction.

Another cytoplasmic enzyme that could utilize NADPH indirectly is thioltransferase (Axelson et al., 1978). This enzyme catalyzes the thiol/disulphide interchange of glutathione and disulphides as shown below:

\[
\text{RSSR'} + 2\text{GSH} \underset{\text{NADPH}}{\rightarrow} \text{R'SH} + \text{RSH} + \text{GSSG}
\]

The regulatory significance of the consumption of NADPH by
these thiol-disulphide exchange reactions is still an area that is little understood. The elucidation of the precise effect that these reactions have on the cytoplasmic NADP⁺ pool and the NADP⁺ / NADPH ratio still remains to be determined.

c. Other NADPH-dependent disulphide reducing reactions

Tietze (1970b) has reported the presence of a non-specific NADPH-dependent disulphide reductase in the soluble fraction of rat liver homogenates. This enzyme promotes the reduction of the disulphide bonds of protein and non-protein substrates in the presence of NADPH, but not NADH. The activity could not be detected in the mitochondrial or microsomal fractions and was therefore assumed to be exclusively cytoplasmic. The enzyme is completely inhibited by incubation with 10⁻⁶M-arsenite, a treatment which, according to Tietze, had no effect on glutathione reductase, under similar experimental conditions. Mize and Langdon (1962a) have, however, reported that sodium arsenite (3x10⁻⁷M) inhibited their preparation of glutathione reductase provided the enzyme was not preincubated with EDTA (3.3x10⁻³M) or GSSG (5.6x10⁻⁸M). Unlike glutathione reductase, the non-specific reductase was destroyed by heating to 70° for 30 min (Tietze, 1970b).

The stoichiometry of this reaction was reported to be:

\[
R-S-S-R' + NADPH \rightarrow R'SH + RSH + NADP^+ 
\]

The enzyme shares some common characteristics with the enzyme system participating in the reduction of ribonucleotides in deoxyribonucleotide synthesis (Fig.1.10). These characteristics are: the specificity towards NADPH, the sensitivity to arsenite and the capacity to be resolved into heat-stable and heat-labile components (Reichard, 1968; Tietze, 1970b).
The enzyme system involved in the reduction of ribonucleotides includes thioredoxin reductase which is NADPH-dependent and ribonucleotide reductase which is thioredoxin specific but shows a broad specificity toward the disulphide substrates (Gonzales-Porque et al., 1970; Hall et al., 1971). This is similar to the enzyme system of Tietze (1970b). The reaction scheme for the thioredoxin system is shown below:

![Reaction Scheme](image)

**Fig.1.10:** The Thioredoxin system: oxidised thioredoxin is reduced by NADPH dependent thioredoxin reductase. Reduced thioredoxin then reduces ribonucleotide reductase which can then catalyse the reduction of ribonucleotides.

Luthman and Holmgren (1982a) have purified reduced thioredoxin and thioredoxin reductase from rat liver. These authors have failed to purify the oxidised form of thioredoxin. The molecular weight of thioredoxin was found to be 12 000 and the compound was shown to contain about 110 amino acids including 4 half cystines and an N-terminal valine. Thioredoxin reductase has a native molecular weight of 116 000. The enzyme is highly specific for NADPH \( (K_m = 6\mu M) \), contains FAD as the prosthetic group, is sensitive to inhibition by arsenite and exhibits a \( K_m \) of 2.5\( \mu M \) for rat liver thioredoxin.
Holmgren (1977) reported that thioredoxin will not form a stable complex with thioredoxin reductase when the two purified proteins are mixed in solution and the two proteins separate completely when a crude extract of liver is subjected to gel filtration. Holmgren (1977) has proposed that the non-specific NADPH-dependent disulphide reductase described by Tietze (1970b) is identical with the mammalian thioredoxin system. This is feasible since the dialyzed rat liver high speed supernatants would contain both the thioredoxin reductase and the ribonucleotide reductase.

d. The mixed function oxidase system

The mixed function oxidation (MFO) of abiotic compounds, by the liver, involving cytochrome P-450, is thought to be a quantitatively important use of hepatic cytoplasmic NADPH (Altman and Chayen, 1970; Chayen et al., 1973; Smith and Wills, 1981). In glycogen-rich livers from fed rats the supply of NADPH reducing equivalents for the monooxygenase system is thought to be met by the PPP, whereas in glycogen-depleted livers, the cytochrome P-450 linked monooxygenase system depends on the mitochondrial-derived production of reducing equivalents (Thurman and Scholz, 1969; Moldeus et al., 1974; Junge and Brand, 1975). The cytosolic and mitochondrial NADP⁺-linked isocitrate dehydrogenases and the tricarboxylate/dicarboxylate translocators provide a transfer system for NADPH reducing equivalents (Papa, 1969; Hoek and Ernster, 1974) from the mitochondria.

Weigl and Sies, (1977) have presented evidence which suggests that the supply of NADPH for the monooxygenase system by the PPP is accompanied by the supply of NADPH by extra-mitochondrial NADP⁺-linked isocitrate dehydrogenase even in the fed state.

Changes in nicotinamide and adenine dinucleotides
that accompany mixed function oxidation have been shown to have significant effects on intermediary metabolism. For example, steady-state concentrations of intermediates associated with glycolysis, the PPP and citric acid cycle have been found to change during mixed function oxidation (Sies and Kendal, 1970; Kauffman et al., 1977). Furthermore, the \( \text{NADPH} / \text{NADP}^+ \) ratio in rat liver is selectively decreased (by about 42%) during MFO, whereas the \( \text{NADH} / \text{NAD}^+ \) ratio remains unchanged (Sies et al., 1981). The oxidation of NADPH occurs in both the mitochondrial and cytosolic compartments of the hepatocyte (Weigl and Sies, 1977). Kauffman et al. (1979) have demonstrated that fructose infusion of starved rats increases the rate of p-nitro-anisole O-demethylation by 50%. Fructose has been shown to lower intracellular ATP levels (Woods et al., 1970; Mäenpaa et al., 1968) and Kauffman et al. (1979) have attributed the increase in MFO to the de-inhibition of NADPH-cytochrome c reductase resulting from the decreased ATP concentration.

Since cytochrome c reductase is the rate-limiting component of mixed function oxidation (Vermilion and Coon, 1978), the increased consumption of NADPH and the reduced levels of ATP (see A.1.b) could, in turn, relieve the PPP and other NADPH-generating enzymes.

Thurman and Scholz (1973) have postulated that the cyt. P-450-linked monooxygenase system may compete with lipogenesis for NADPH, since inhibition of lipogenesis occurs during the oxidation of aminopyrine. The authors found that in livers of phenobarbital-pretreated rats, the addition of aminopyrine to the perfusate inhibited the incorporation of \( ^3\text{H}_2\text{O} \) into total lipids by about 40%. It is possible that this apparent inhibition is an artifact resulting from isotopic dilution of the \( ^3\text{H} \) in NADPH, which would be expected to occur as a consequence of the increased turnover in the NADPH pool due to the enhanced mixed-function oxidation. The authors
eliminated this possibility by demonstrating a similar inhibition of incorporation of $^{14}$C glucose into lipids.

The aminopyrine effect was found to be induced by phenobarbital, this being a known inducer of MFO. Aminopyrine by itself only caused an 18% inhibition of lipogenesis. This suggested to the authors that it was the metabolism of aminopyrine, rather than the compound itself, that elicited the inhibition of lipogenesis. Aminophenylpyrazolone, a product of aminopyrine metabolism was shown to have no inhibitory effect on lipogenesis. Since aminophenylpyrazolone can be acetylated with acetyl-CoA, the lack of effect of this compound on lipogenesis, and the observation that the rate of acetylation of aminoantipyrine (another product of aminopyrine metabolism) was insignificant, implies that there was no diversion of acetyl-CoA from fatty acid synthesis to acetylation. Aminopyrine was shown not to affect the ATP/ADP ratio. This eliminates the involvement of an altered energetic state of the liver.

Under their experimental conditions Thurman and Scholz (1973) estimated that the NADPH requirement for the MFO was about $40\mu$mol NADPH g$^{-1}$ liver h$^{-1}$ and that for lipogenesis was $50\mu$mol NADPH g$^{-1}$ liver h$^{-1}$. The total requirement of NADPH under these conditions may surpass the capacity of the liver for NADPH generation and result in the inhibition of one or more of the NADPH utilizing pathways.

Smith and Wills (1981b) have reported that the cytochrome P-450 content of rat liver is increased by fat-feeding, whereas feeding a fat-free diet was shown to result in a decrease in both the cyt. P-450 content and the rate of aminopyrine N-demethylation, relative to the stock diet-fed rats. Fat-feeding depressed fatty acid synthesis, and feeding a fat-free diet had the reverse effect. The feeding period was 10-24 days. Sodium phenobarbitone, which was shown to increase both the cyt. P-450
content and the rate of aminopyrine N-demethylation, only had a slight inhibitory effect on the activity of fatty acid synthase (4.94 to 4.71 μmol NADPH oxidised min⁻¹ g⁻¹ of cytosolic protein).

G6PDH activity was shown to be twice as high in the centrilobular hepatocytes as in the periportal hepatocytes from stock-diet fed rats. Furthermore sodium phenobarbitone selectively increased G6PDH in the centrilobular hepatocytes. On the other hand, transferring rats from a stock diet to a fat-free diet caused a significant increase in the activity of G6PDH in both regions of the liver, the increase being more marked in the periportal hepatocytes. A diet containing herring oil, but not corn oil, significantly reduced the G6PDH activity in both regions. These observations, coupled with the finding that the ratio of the rate of drug oxidation to the rate of fatty acid synthesis was twice as high in the centrilobular hepatocytes as in the periportal cells suggest that more NADPH is available to the MFO in the former cells, and that these cells which have a higher cyt. P-450 content. (Smith and Wills, 1981a) are more important in the oxidative metabolism of foreign compounds than periportal cells. The effect of phenobarbitone, a known inducer of MFO, implies that more NADPH is channelled towards MFO in the centrilobular hepatocytes after phenobarbitone pre-treatment. This implies a competition for NADPH between the two pathways of NADPH consumption under consideration. Two groups support the view that the rate of supply of NADPH may be the rate limiting factor in controlling MFO and lipogenesis in whole liver cells (Thurman and Scholz, 1973; Thurman et al., 1977; Smith and Wills, 1981b), although it has been argued that NADPH cannot be limiting since the activities of the NADPH-generating enzymes are an order of magnitude higher than the activity of FAS. The latter argument is, however, based on in vitro observations which may not necessarily reflect in vivo
conditions (see 1.2).

Data on the absolute quantitative distribution of NADPH between the various pathways of NADPH utilization is not available. Research workers in this field have, in the main, assumed that lipogenesis is the major NADPH-utilizing biosynthetic process and have based their calculations on this. However, it is possible that there are other pathways of comparable rates, which use NADPH (for instance the reduction of disulphides) and significantly affect the cytosolic NADPH pool.

C. FRUCTOSE 2,6-BISPHOSPHATE

1. General considerations

Fructose 2,6-bisphosphate (F\textsubscript{2,6P}\textsubscript{2}) was first reported to be a positive effector of ATP-D-fructose-6-phosphate-1-phospho-transferase (PFK-1) a key regulatory enzyme of glycolysis by Van Schaftingen et al. (1980a). This role of fructose 2,6-bisphosphate has been confirmed by other groups of workers (Claus et al., 1981; Kitajima and Uyeda, 1983; Pilkis et al., 1981). PFK-1 is now believed to be regulated primarily by AMP, fructose 2,6-bisphosphate and fructose 6-phosphate (Van Schaftingen et al., 1980a,b,c). These authors have indicated that fructose 2,6-bisphosphate and AMP act synergistically in their stimulation of PFK-1. Under these conditions of stimulation the K\textsubscript{m} of PFK-1 for fructose 6-phosphate (F6P) is reduced from 6mM to 0.6 mM, the latter being closer to the physiological value. Fructose 2,6-bisphosphate, AMP and inorganic phosphate counteract the inhibition of PFK-1 by citrate and ATP. Conversely, fructose-1,6-bisphosphatase, a key regulatory enzyme of gluconeogenesis, is inhibited by fructose 2,6-bisphosphate and AMP (Pilkis et al., 1981). A review on the physical and regulatory properties of fructose 2,6-bisphosphate has appeared (Hers and Van Schaftingen, 1982).
A number of workers (Van Schaftingen et al., 1981a,b,c; Hue et al., 1981a; El-maghrabi et al., 1981) have shown that an enzyme which is distinct from PFK-1 catalyses the formation of fructose 2,6-bisphosphate from fructose 6-phosphate and ATP. This enzyme has been called PFK-2 and the stoichiometry of the reaction it catalyses is shown below:

\[
\text{F}_6\text{P} + \text{ATP} + \text{Mg}^2+ \rightarrow \text{ADP} + \text{Mg}^2+ + \text{F}_2\text{,6P}_2
\]

Furuya and Uyeda (1981) have shown that PFK-1 is almost 500 times as active as PFK-2. This is in agreement with the concept that PFK-1 catalyses a major step in the glycolytic pathway and that only a small amount of fructose 2,6-bisphosphate is required to stimulate PFK-1. The \(K_m\) values reported for ATP and F6P are 0.2-0.4 and 5 mM, respectively.

An enzyme catalysing the breakdown of F2,6 P2 has been isolated and is a specific fructose-2,6-bisphosphatase (Van Schaftingen et al., 1982a,b). PFK-2 and FBPase-2 (or F2,6BPase) activities appear to be present on the same protein.

The level of fructose 2,6-bisphosphate in rat liver is regulated by both hormonal and metabolic factors. Hue et al. (1981a) have reported that vasopressin, phenylephrine and A23187 cause an accumulation of fructose 2,6-bisphosphate in hepatocytes from fed rats. The effect was abolished by overnight starvation, \(\text{Ca}^{2+}\)-depletion and phosphorylase kinase deficiency. On the other hand glucose can cause the accumulation of fructose 2,6-bisphosphate regardless of the state of activation of phosphorylase or of the glycogen content of the cells. These findings indicate that it is the increased concentration of F6P, due to increased glycogeneolysis resulting from treatment with these hormones, which is responsible for the observed increase in F2,6P2. This hypothesis is reinforced by the correlation observed between hexose 6-
phosphate concentration and the level of fructose 2,6-bisphosphate (Hue et al., 1981a). Treatment of rats with glucagon or incubation of hepatocytes with this hormone was also shown to cause an inactivation of PFK-2. This inactivation was attributed to a stable modification of the enzyme, which was accompanied by changes in the kinetic properties of the enzyme such that the activity is reduced at both low and high concentrations of substrate. The implication of these results is that the effect of glucagon to decrease the level of fructose 2,6-bisphosphate is the result of cyclic AMP-dependent phosphorylation and inactivation of the enzyme responsible for its synthesis. Van Schaftingen et al. (1981b) have shown that treatment of PFK-2 with the catalytic sub-unit of cAMP-dependent protein kinase reduces the affinity of PFK-2 for fructose 6-phosphate, its $V_{\text{max}}$, and its sensitivity to the stimulatory effect of P$_i$. The susceptibility of the enzyme to inhibition by phospho-enol-pyruvate and citrate were increased. Since glucagon also increases the concentration of phospho-enol-pyruvate in rat liver (Exton and Park, 1968), these two mechanisms would act in concert to reduce fructose 2,6-bisphosphate levels. The rapid disappearance of fructose 2,6-bisphosphate observed in glucagon-treated cells is the result of an increased fructose 2,6-bisphosphatase activity.

Alloxan diabetes and starvation (72h) were shown to decrease the level of fructose 2,6-bisphosphate to 10% of the value found in livers of normal, fed rats (10nmol g$^{-1}$ liver) (Neely et al., 1981). The activity of PFK-2 was also shown to be reduced by diabetes. Insulin administration for 24h to diabetic rats restored the fructose 2,6-bisphosphate levels. Refeeding a high carbohydrate diet for 24h to the 72h-starved rats resulted in a fructose 2,6-bisphosphate level that was 2.5 fold higher than that found in livers of fed rats. The decrease in fructose 2,6-bisphosphate levels correlates well with the decreased rates of glycolysis and enhanced rates of gluconeogenesis that are observed in diabetes.
and starvation, hence supporting the role of this effector in the regulation of carbohydrate metabolism. The decreased levels of fructose 2,6-bisphosphate can be attributed to the increase in glucagon which occurs during starvation and diabetes. The elevated glucagon content (and decreased insulin levels) would inactivate PFK-2 and reduce fructose 2,6-bisphosphate concentration as discussed above. Furthermore, Neely et al. (1981) have demonstrated a decreased level of glucokinase in starvation and diabetes - a condition which would result in the reduction of F6P content. Insulin has been shown to antagonise the effects of glucagon (Pilkis et al., 1983).

Bartrons et al. (1983) have shown that PFK-2 and fructose-2,6-bisphosphatase (FBPase 2) are poor substrates for cyclic AMP-dependent protein kinase, in isolated hepatocytes, when compared to pyruvate kinase and phosphorylase. They also showed that PFK-2 and FBPase 2 were inactivated and activated, respectively, in parallel and that the time required to reach half-maximal modification was 5-10-fold longer than for the inactivation of pyruvate kinase. The half maximal effect of glucagon was usually obtained at 0.2-0.4nM, which is comparable to that reported for phosphorylase and pyruvate kinase, and implies that the fructose 2,6-bisphosphate system is equally sensitive to the cAMP dependent phosphorylating system. This suggests that fructose 2,6-bisphosphate concentration plays a significant role in the control of gluconeogenesis by glucagon. No evidence has been presented for a cyclic-AMP independent mechanism for phosphorylating the apparently single bifunctional protein, which is thought to contain PFK-2 and FBPase-2 activity.

These experimental observations imply that the physiological regulators of fructose 2,6-bisphosphate concentration in rat liver are glucagon and fructose 6-phosphate availability. Van Schaftingen et al. (1984) have reported that ethanol administration to fed rats
(intragastric) causes a 20-fold decrease in the concentration of fructose 2,6-bisphosphate, an activation of FBPase, an inactivation of PFK-2 but no change in cAMP. Isolated hepatocytes incubated in the presence of ethanol were found to exhibit a rapid increase in sn-glycerol 3-phosphate content and a continuous decrease in fructose 2,6-bisphosphate, with no change in the hexose 6-phosphate content. These authors found that at a given concentration of hexose 6-phosphate, there was an inverse relationship between the concentration of fructose 2,6-bisphosphate and that of sn-glycerol 3-phosphate. Sn-Glycerol 3-phosphate inhibits PFK-2 and counteracts the inhibition of FBPase by F6P. Sn-Glycerol 3-phosphate was also shown to accelerate the inactivation of PFK-2 by cAMP-dependent protein kinase.

Since the effect of ethanol is shared by other substrates of alcohol dehydrogenase (butan 1-ol and crotyl alcohol) and by acetaldehyde, and is inhibited by methylpyrazole, the authors argued that the decrease in the concentration of fructose 2,6-bisphosphate in the liver is mediated by the reduction of NAD\(^+\). Further, 1\(\mu\)M fructose 2,6-bisphosphate inhibited the inactivation of PFK-2 by cAMP-dependent protein kinase. Glycerol 3-phosphate (1mM) had the reverse effect. On the basis of these results, Van Schaftingen et al. (1984) have concluded that the changes in the activity of PFK-2 and FBPAse-2 are the result rather than the cause of the decrease in the concentration of fructose 2,6-bisphosphate. The cellular concentration of fructose 2,6-bisphosphate is also decreased by adenosine (Bartrons et al., 1984).

The highest concentration of fructose 2,6-bisphosphate has been found in liver (Kuwajima and Uyeda, 1982). Livers of genetically obese rats, starved for 24h were shown to contain more fructose 2,6-bisphosphate, glucose 6-phosphate, fructose 6-phosphate and glycogen and more pyruvate kinase and PFK-2 activities than livers of control lean rats (Hue et al., 1983). This was attributed to the change in
cAMP concentration, which was found to be decreased in livers of obese starved rats.

All these observations suggest that fructose 2,6-bisphosphate is a key regulator of carbohydrate metabolism.

2. Fructose 2,6-bisphosphate, the PPP and lipogenesis.

Since the PPP and glycolysis share a common substrate, G6P, and since the cofactor of Eggleston and Krebs (1974) has not been identified the initial aim of the project was to determine the effect of fructose 2,6-bisphosphate on the activity of the PPP dehydrogenases, with particular reference to the NADPH inhibition. Furthermore, the isomers of fructose 2,6-bisphosphat, fructose 1,6-bisphosphate (F1,6P₂) and glucose 1,6-bisphosphate (G1,6P₂) have been shown to inhibit 6PGDH (Dyson and D'Orazio, 1971; Beitner and Nordenberg, 1979) and F1,6P₂ has been reported to be an activator of the fatty acid synthase complex (Plate et al., 1968).

and (1982) have found that the stimulatory effect of 6PG on PFK-1 was additive to that of fructose 2,6-bisphosphate in the presence of physiological concentrations of ATP, AMP and ADP. Since 6PG is not an intermediate metabolite of glycolysis and since its stimulatory effect on hepatic pyruvate kinase is more pronounced during lipogenesis (Smith and Freedland, 1979; 1981), 6PG can be assumed to be a means of communication between two pathways central to lipogenesis.

Blackmore and Shuman (1982) have demonstrated a parallel increase in the conversion of altro heptulose 7-phosphate (sedoheptulose-7-phosphate) to altro heptulose 1,7-bisphosphate with increasing concentrations of F2,6P₂. The F2,6P₂ levels were increased by perfusion with glucose. An obvious criticism of this work is that F2,6P₂ does not have to act directly on the kinase catalysing this reaction in order to increase the product-
ion of altro heptulose 1,7-bisphosphate. Karadsheh et al. (1973) have shown that PFK-1 can catalyse this reaction, and the observed increase in the rate of the reaction may just be a reflection of the enhanced activity of PFK-1 due to the elevated levels of fructose 2,6-bisphosphate. Conversely, the effect of glucagon (which was shown to decrease the reaction rate) can be attributed to the inhibition of PFK-1 and the activation of FBPase-1, since Bonsignore et al. (1963) have shown that FBPase-1 can catalyse the reverse reaction. The observations reported by Blackmore and Shuman (1982) can be, therefore, attributed to the known effects of fructose 2,6-bisphosphate on these two enzymes. These results do, however, suggest that F2,6P2 may affect the flux through the PPP, albeit indirectly.

The aim of this project was to attempt to demonstrate a direct effect of F2,6P2 on the PPP and/or on lipogenesis in particular the fatty acid synthase complex. This approach was based on the observation that the PPP, lipogenesis and glycolysis are interrelated and share common regulators most of which have been mentioned. In addition the pathways show an interdependence since glycolysis via pyruvate provides acetyl-CoA for lipogenesis, and the PPP supplies the NADPH required for the reductive steps of the biosynthesis. Given these considerations it was reasonable to postulate that a potent regulator of glycolysis, such as F2,6P2 has been shown to be, would have a direct effect on either the PPP or one of the regulatory enzymes of lipogenesis.

However, in the course of this work no such observation was made. In fact, apart from PFK-1 and FBPase-1, the only other enzyme so far shown to be affected by F2,6P2 is bacterial and plant PP1-PFK (pyrophosphate: fructose-6-phosphate phosphotransferase).

Having failed in the initial objective a broader
approach to the regulation of the PPP and, in particular, the mechanisms involved in the activation of the PPP dehydrogenases, was adopted. The effect of glutathione and other metabolites was investigated and an attempt was made to isolate the cofactor reported by Eggleston and Krebs (1974). The observations made are reported in the "Results and Discussion" section.
II. MATERIALS AND METHODS

A. MATERIALS

1. Chemicals

Analytical grade chemicals and solutions prepared in glass distilled demineralized water were used throughout the course of the work. Unless otherwise stated, all enzymes and substrates were obtained from Sigma (London), as were all column materials except Bio-Gel P-3, which was purchased from Bio-Rad (Watford, Herts.). BDH Chemicals Ltd., Poole, Dorset supplied the solvents and acid solutions. All radioactive materials were supplied by the Radiochemical Centre (Amersham, Bucks.).

Sera: Normal sheep serum, fraction V (fully acid-free), from Sigma, was used for hepatocyte preparations. Normal sheep serum, pentobarbitone was the anaesthetic used and was obtained from Southern Vet. Services Ltd. (Lewes, East Sussex).

2. Specialised equipment

Plastic-coated silicate petri dishes (B.D. - non-sterile) were products of Becton and Dick, Cockeysville, Md. - USA. Polystyrene microtiter plates were obtained from Merck. Sharp and Dohme, Rahway, New Jersey. PTFE filter membranes were obtained from Millipore. Macerators and glassware were obtained from Haris (Macclesfield, Cheshire), and glassware from the local glass-blower in Manchester.

The perfusion system consisted of a metal chamber with a glass door. Inside the chamber, a thin layer of distilled water was maintained at 25°C, and on the left-hand side of the chamber there was an air-tight box maintained at a temperature of 25°C by a temperature control unit. The air supply was provided by a pump and filter. The perfusion medium was circulated by a Water-Marshal Ltd. pump. The perfusion medium was sterilized by a Water-Marshal Ltd. filter sterilizer (Water-Marshal Ltd., Westbury, Wiltshire).
A. MATERIALS

1. Chemicals

Analar grade chemicals and solutions prepared in glass distilled de-ionised water were used throughout the course of this work. Unless otherwise stated, all enzymes and substrates were obtained from Sigma (London), as were all column materials except Bio-Gel P-2, which was purchased from Bio-Rad (Watford, Herts.). BDH Chemicals Ltd. (Poole, Dorset) supplied the solvents and acid solutions. All radioactive materials were supplied by the Radiochemical Centre (Amersham, Bucks.).

Bovine serum albumin, fraction V (fatty acid-free), from Sigma, was used for hepatocyte preparations. Sagatal (sodium pentobarbitone) was the anaesthetic used and was obtained from Southern Vet. Services Ltd. (Lewis, East Sussex).

2. Specified equipment

Plastic-backed silica gel t.l.c. - ready-foils (F 1500, 20x20 cm, acid resistant) were products of Schleicher and Schull (GmbH D-3 354 Dassel, W. Germany). Ultrafiltration membranes were bought from Amicon Ltd. (Stonehouse, Glos.). Sterile liver cannuli were obtained from Armour Pharmaceutical Co. Ltd. (Eastbourne, Sussex) and nylon mesh (Nybolt, No.10, 132 micron) was from J. Staniar and Co. (Manchester, U.K.).

The perfusion cabinet consisted of a metal cabinet with a glass door. Inside the cabinet, a fan heater connected to a thermostat maintained the temperature at 37°C. In addition the cabinet contained a glass lung made to specification (Miller and Smith, 1973). The perfusion medium was circulated with a Watson-Marlow H.R. flow inducer (Watson-Marlow Ltd. Bucks.) and Swinnex-13
filter units (Buc. France) were used to filter the circulating perfusate.

3. Animals

The rats (Wistar strain) were bred in the laboratory, animal house and were maintained on a standard commercial diet, Diet PMD obtained from Labsure (High Wycombe, Bucks; Table 2.1). The rats were subjected to a 12h light/12h dark regime in an animal house maintained at 22 ± 2°C. The light period commenced at 6 a.m. and ended at 6 p.m.

Table 2.1: Composition of Diet PMD

<table>
<thead>
<tr>
<th>Constituent</th>
<th>% of feed</th>
</tr>
</thead>
<tbody>
<tr>
<td>crude oil</td>
<td>2.7</td>
</tr>
<tr>
<td>crude protein</td>
<td>19.7</td>
</tr>
<tr>
<td>crude fibre</td>
<td>5.3</td>
</tr>
<tr>
<td>calcium</td>
<td>0.6</td>
</tr>
<tr>
<td>phosphorous</td>
<td>0.7</td>
</tr>
<tr>
<td>NaCl</td>
<td>1.0</td>
</tr>
<tr>
<td>carbohydrate</td>
<td>53.48</td>
</tr>
</tbody>
</table>

*Values obtained from the suppliers (Labsure, High Wycombe, Bucks.). The total metabolisable energy was reported to be 2568 kcal/kg.
B. METHODS

1. Enzyme Assays

a. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)

G6PDH activity was determined by a method of assay similar to that used by Eggleston and Krebs (1974) and a molar absorption coefficient of $6.22 \times 10^3 \text{ Mol}^{-1} \text{ cm}^{-1}$ was assumed in the calculations. The activity of the enzyme was measured by following the reduction of NADP$^+$ at 340nm. The assay mixture (total vol. 1ml) was composed of 0.1M -bis-tris propane (BTP) pH 7.4, 100μM-NADP$^+$, 1mM-MgCl$_2$ (where present), 100μM-G6P, and 50μl of a dialysed crude high speed supernatant of homogenized rat liver or a partially purified preparation (cf. II. B.4a.) of the enzyme. The reaction was allowed to proceed at 25°C. In some experiments NADPH was added to a concentration of up to 200μM.

Crystalline baker's yeast G6PDH (Sigma, type VII) activity was determined in the same manner.

b. 6-Phosphogluconate dehydrogenase (EC. 1.1.1.44)

6PGDH was assayed in the manner described by Procsal and Holten (1972), with minor modifications. The assay mixture was as detailed for G6PDH except that G6P was replaced by 100μM-6PG. The liver enzyme was obtained from dialysed high speed supernatants. In some experiments the enzyme was partially purified (cf. II. B.4b.). The crystalline yeast enzyme (Sigma) was assayed in the same manner.

c. Fatty acid synthase

Fatty acid synthase was assayed both (i) spectrophotometrically (Linn, 1981) and (ii) radiochemically (Butterworth et al., 1966). In the spectrophotometric method the
oxidation of NADPH was followed at 340nm at 25°C in a reaction mixture (total vol. 1ml) consisting of: 0.09M-KP$_4$, pH 6.8, 5mM-EDTA, 30μM-acetyl-CoA, 200μM-NADPH and 100μl of the partially purified (cf. II. B.4c.) or crude preparations of the enzyme. Malonyl-CoA (10μM) was used to start the reaction. In the radiochemical method the assay mixture (total vol. 1ml) comprised of 440,000 d.p.m. [1 - $^{14}$C] acetyl-CoA, 30μM-acetyl-CoA, 200μM-DTT, 200μM-NADPH, EDTA, 0.1M-KP$_4$, pH 6.8 and 5-80μM-malonyl-CoA. The reaction was allowed to proceed for 10min before it was stopped by the addition of HClO$_4$ to a final concentration of 1.8% (w/v). The synthesized fatty acids were extracted (cf. II. B.3b.) and the incorporation of [1 - $^{14}$C] acetyl-CoA determined by assaying for the radioactivity in toluene scintillant (5g PPO/L toluene; 10ml) using a Beckman LS 7500 liquid scintillation spectrometer.

d. Phosphofructokinase-1 (EC 2.7.1.11)

PFK-1 activity was determined in desalted high speed (100,000g) supernatant preparations derived from liver homogenates by the method of Van Schaftingen et al. (1980a,b.). The oxidation of NADH in the presence of ATP and F6P was followed at 340nm. The auxiliary enzymes phosphoglucone isomerase, triose phosphate isomerase, glycerophosphate dehydrogenase and aldolase were used as auxiliary enzymes in this assay system. The assay mixture (total vol. 1ml) contained 50mM-Hepes, pH 7.1, 100mM-KCl, 5mM-MgCl$_2$, 100μM-NADH, 1mM-NH$_4$Cl, 50μl of the desalted auxiliary enzymes, 5mM-KP$_4$, pH 7.1, 100μM-AMP, 50μl PFK-1 and 20-50μl 100mM/350mM-F6P/G6P. The reaction was initiated with 1.5mM-ATP-Mg$^{2+}$ (pH 7.0). In experiments with F2,6P$_2$, 10μl of the compound was added to give the specified concentration.

e. The NADPH-dependent disulphide reductase

The reductase responsible for the oxidation of NADPH as described in III.c. was assayed by following the decrease in the absorbance at 340nm. The incubation
mixture (total vol. 1ml) contained: 100mM-BTP/HCl, pH 7.5, 100μM-NADPH, an appropriate volume of the di­sulphide-containing preparation and enzyme. The sources of the enzyme and the substrate are indicated in indi­vidual legends. The assay was routinely performed at 25°C. Deviations from this standard assay system are detailed in the appropriate sections.

f. Glutathione reductase (EC 1.6.4.2)

The method of assay used was essentially that of Mize and Langdon (1962) except that BTP/HCl was used as the buffer. The oxidation of NADPH (100μM) in the presence of 100μM-GSSG and 100mM-BTP/HCl at pH 7.0 and 25°C, in a total volume of 1ml, was followed at 340nm.

2. Metabolite Assays

a. F2,6P2

When F2,6P2 was prepared in the laboratory, its presence in preparations was determined by its capacity to stimulate PFK-1 (Van Schaftingen et al. 1980c; Hers and Van Schaftingen, 1982). The assay was performed as in II. B. 1d. above. A standard curve, based on the PFK-1 assay, was prepared by plotting y/Vmax against F2,6P2 concentration at sub-optimal concentrations of the effector. The latter was synthesized as in II. B.5.

b. Phosphates

(i) The method of Fiske and SubbaRow (1925)

The labile phosphate-content of the samples under study was determined by this method as detailed by Leloir and Cardini (1957). The intensity of the blue colour resulting from the formation of the phosphomolybdate complex was measured at 660nm. The absorbance at 660nm
was used to determine the phosphate content from a standard curve prepared with inorganic phosphate (Leloir and Cardini, 1957).

(ii) Hydrolysis of the phosphate bonds with alkaline phosphatase

Test samples were hydrolysed by incubation with alkaline phosphatase (6 units ml\(^{-1}\)) from porcine intestinal mucosa, at pH 8.3. The incubation mixture contained 0.1M-BTP/HCl, pH 8.3, the appropriate volume of the test sample, alkaline phosphatase and, where included, 0.25mM-ZnCl\(_2\) and 2.5mM-MgCl\(_2\). The incubation period was 17h at 4°C and the reaction was terminated by heating to 80°C for 15min. Parallel control incubations, which did not contain alkaline phosphatase, were included and heat-treated in the same manner.

c. Carbohydrates

The phenol-sulphuric acid method (Dubois et al., 1956) was used for the determination of the carbohydrate content of test samples. The experiments were performed in the manner described by Ashwell (1966) and the reaction mixture was composed of 2ml of the test sample and 0.05ml of the phenol reagent. To this mixture 5ml of conc. H\(_2\)SO\(_4\) was added to initiate the reaction. The colour was allowed to develop for 30min after which the absorbance was measured at 485nm.

d. Proteins and amino acids

(i) The Folin-Lowry method

Protein was assayed by the method of Lowry et al. (1951). In the present study 50ml of 2% (w/v) Na\(_2\)CO\(_3\) in 0.1M-NaOH (A) was mixed with 1ml 0.5% CuSO\(_4\) in 1% sodium potassium tartarate (B) to give solution C. The Folin and Ciocalteu's phenol reagent was a commercial preparation.
containing tungstate, molybdate, phosphoric and hydrochloric acids.

The samples (0.1ml) were treated with 0.25ml of 10% trichloroacetic acid. The precipitate was removed by centrifugation and resuspended in 1ml 0.1M-NaOH. Solution C (5ml) was added to these samples and allowed to stand for 10min. After this preincubation period 0.5ml of a \( \frac{1}{4} \) dilution of the Folin and Ciocalteu's phenol reagent was added. The colour was allowed to develop for 30min, after which absorbance readings were taken at 750nm. The concentration of the protein was determined from a standard curve prepared using a standard BSA (500μg ml\(^{-1}\)) solution.

(ii) Peptide hydrolysis with peptidase and carboxypeptidase-A

Peptidase treatment

A peptidase (1 unit ml\(^{-1}\)) from porcine intestinal mucosa with general proteolytic and aminopeptidase activity was incubated with samples at pH 7.1 and 37°C. The reaction was stopped by heating to 90°C for 15min and the supernatant, collected after centrifugation, was assayed for substrate content by determining the activity of the NADPH-dependent disulphide reductase as in II. B.1.e.

Carboxypeptidase-A (EC 3.4.12.2.) treatment

Carboxypeptidase-A (142.8 units ml\(^{-1}\)) from bovine pancreas was incubated with samples at pH 7.5 and 37°C. The reaction was stopped at time intervals in the same manner as in the peptidase experiments. The substrate content was determined as above.

(iii) Amino acid analysis with an amino acid autoanalyser

A Joel JLC-6AH analyser was used to determine the...
amino acid content of the peptide substrate (III. C.). The samples were hydrolysed with HCl (pH 1.2) at 100°C for 24h and then stored at 4°C. Control samples were also heated (in the absence of HCl) to 100°C for 24h and stored in the same manner.

Both the control and test samples were run automatically, each being allowed to develop for 5h. The temperature conditions were 90°C for the reaction bath and 55°C for the two columns. A B.D.H. amino acid standard sample (Sepramar calibration standard A) was diluted to give a sample concentration of 0.1M, and this was used as the reference standard.

When calculating the molar concentrations of the amino acids, glycine was used as the reference amino acid. The control samples were found not to have free amino acids after analysis.

(iv) t.l.c. analysis of the dansylated products of chemical and carboxypeptidase hydrolysis products

The N-terminal amino acid of the peptide described in III.C. was determined by reacting the peptide with dansyl-chloride (Gray, 1967). In this procedure, 1ml of the Sephadex G-25 product (Fig. 3.18) was transferred to a small test tube and allowed to dry in vacuo. The peptide preparation was then redissolved in 20μl of NaHCO₃ (0.2N, pH 8.5). To this was added an equal volume of the dansyl-chloride solution (2.5 mg ml⁻¹ acetone) to give a final concentration of dansyl-chloride of 5mM. The tubes were sealed and the reaction allowed to proceed for 2h at 37°C. At the end of this period the solution, which was colourless, was dried in vacuo and redissolved in 50μl 6.7N-HCl. The tube was quickly sealed in a flame. The sealed tube with its contents was then heated in an oven for 24h at 105°C. After the acid hydrolysis was complete, the HCl was removed in vacuo over NaOH pellets and the products were redissolved in 50% v/v aqueous pyridine.
Standard dansyl amino acids were prepared by dissolving individual amino acids in 0.5M-NaHCO₃ to give a final concentration of 10mM. To 0.1ml of each of these solutions an equal volume of dansyl-chloride was added and the solutions were incubated for 2h at 37°C. After the incubation period the solutions were further diluted with 2ml water and stored in sealed tubes.

The standards and the test samples were chromatographed by t.l.c. on plastic backed silica gel t.l.c.-ready-foils (F 1500, 20x20cm). The samples were spotted on the t.l.c. plates so that they were 1.5cm apart and 1cm away from the edge of the plate. The plastic backed foils were placed in beakers (for support) and placed in a solvent-vapour-saturated chromatographic tank. The solvent used for the run was ethylacetate: pyridine: acetic acid (80:20:2).

At the end of the run, the spots of fluorescent material were visualized under u.v. light (254nm), while the plates were still damp. R_f values were determined, and when an R_f value coincided with a standard, the standard and test samples were mixed and co-chromatographed. The identification was considered positive if the amino acids co-chromatographed.

In experiments with carboxypeptidase, the amino acids released at different time intervals were reacted with dansyl-chloride in the manner described for the standards. These were chromatographed against the standards as described above. A zero time control was included in the experiments.

e. Preparation of the cofactor described by Eggleston and Krebs (1974)

The cofactor (Eggleston and Krebs, 1974) was prepared from a high speed supernatant of a 33% w/v rat liver homogenate. In this case the test system for the
cofactor activity was composed of 100μM-GSSG, 33.3μM-ZnCl₂, 100μM-G6P, 100μM-NADP⁺ and 200μM-NADPH, in a final volume of 1ml. GSSG was added to dc-inhibit G6PDH, and ZnCl₂ to inhibit GSSG-reductase.

Alternatively, the high speed supernatants were treated with an equal volume of 10% perchloric acid and the precipitated protein removed by centrifugation. The supernatants were then treated with 0.25 vol. K₂CO₃ and the precipitate removed by centrifugation. Portions of this preparation were then added to the incubation mixture of G6PDH as described above.

f. Determination of the disulphide content of peptides

The assay system used for the determination of disulphides was essentially that described by Zahler and Cleland (1968). The disulphides were reduced with DTT. The resulting monothiols were then reacted with 5,5′ dithiobis (2-nitrobenzoic acid) (DTNB), and the absorbance of the resulting yellow complex was measured at 412nm. A molar absorption coefficient of 1.36x10⁶ Mol⁻¹ cm⁻¹ was used to calculate the concentration of monothiols.

The experiment was performed by mixing 0.2ml of the peptide preparation with 0.1ml of 0.05M-Tris/HCl, pH 9.0 and 0.1ml of 3mM-DTT. The reduction was allowed to proceed for 25min, after which 0.2ml of 1M-Tris/HCl, pH 8.1, 1.5ml of 5mM-NaAsO₂ and water, to give a final volume of 2.9ml, was added. This mixture was incubated for 10min. At the end of the incubation period, DTNB (3mM in 0.05M-sodium acetate, pH 5.0, 0.1ml) was added to the mixture to start the reaction. The increase in absorbance at 412nm was followed until no further change was observed.

3. Preparation and use of hepatocytes

a. Preparation of hepatocytes
Hepatocytes were prepared by the method of Berry and Friend (1969), modified as described by others (Cornell, et al., 1973; Krebs, et al., 1974; Daneshmand, 1983). Rats were anaesthetized with Sagatal (60 mg ml$^{-1}$), 0.1 ml/100g body weight, approximately. The anaesthetic was injected intraperitoneally. The peritoneal cavity was opened by lateral incisions so that the portal vein and the inferior vena cava were exposed. After removing the surrounding tissue, as far as possible, two pieces of thread were loosely tied around the portal vein, and one around the inferior vena cava just anterior to the right renal vein. A sterile luer cannula was inserted carefully into the portal vein such that the blood flowed freely out of the exposed end of the cannula. The two ligatures were tied firmly and the liver perfused with Krebs-Ringer bicarbonate (KRB) buffer without Ca$^{2+}$ (Krebs and Henseleit, 1932 (Table 2.2) which had been previously incubated at 37°C and gassed with 95% O$_2$ and 5% CO$_2$. The inferior vena cava was then severed below the right kidney to prevent swelling of the liver and to allow the free flow of the perfusate through the liver. The rib cage was opened to allow access to the thoracic cavity. The inferior vena cava was cannulated via the right atrium of the heart, the cannula being held in place by a tight ligature. The ligature around the inferior vena cava in the peritoneal cavity was then tied. The perfusion was continued at a flow rate of about 20 ml min$^{-1}$. The liver was transferred to a perfusion cabinet (37°C) and the perfusion continued until the liver appeared pale and the perfusate was free of blood. The liver was then perfused with collagenase (clostridiopeptidase A, EC 3.4.24.3) from Clostridium histolyticum (30 mg/100 ml KRB with Ca$^{2+}$) until the liver was smooth and the leakage of medium from the liver was substantial. Depending on the batch of collagenase, this took between 15 and 25 min. The liver was freed from the rest of the rat and transferred to a plastic beaker (250 ml capacity) containing 50 ml of KRB (without Ca$^{2+}$) which was gassed.
Table 2.2: Composition of Krebs-Ringer bicarbonate

<table>
<thead>
<tr>
<th>Solutions required</th>
<th>Krebs-Henseleit original Ringer HCO$_3^-$ (parts by vol.)</th>
<th>Final concn mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.90% NaCl (0.154 M)</td>
<td>100</td>
<td>118</td>
</tr>
<tr>
<td>1.15% KCl (0.154 M)</td>
<td>4</td>
<td>3.5</td>
</tr>
<tr>
<td>1.22% CaCl$_2$ (0.11 M)</td>
<td>3</td>
<td>2.5</td>
</tr>
<tr>
<td>2.11% KH$_2$PO$_4$ (0.154 M)</td>
<td>1</td>
<td>1.2</td>
</tr>
<tr>
<td>3.82% MgSO$_4$.7H$_2$O (0.154 M)</td>
<td>1</td>
<td>1.2</td>
</tr>
<tr>
<td>1.3% NaHCO$_3$ (0.154 M)</td>
<td>21</td>
<td>24.8</td>
</tr>
</tbody>
</table>

The solutions indicated, except CaCl$_2$, were mixed together and gassed with CO$_2$ for 1h and stored at 4°C. CaCl$_2$ was added at the start of each experiment. All buffer solutions were gassed with O$_2$ : CO$_2$ (95 : 5 v/v) before the start of each experiment.
with 95% \( \text{O}_2 \) and 5% \( \text{CO}_2 \). The tissue was minced and passed through a funnel with a nylon mesh so that the cells filtered into centrifuge tubes (MSE 50ml capacity). The cells were sedimented by centrifugation at 50g for 50s in an MSE minor bench centrifuge. The supernatant was discarded and the cells resuspended in 20ml of KRB (with \( \text{Ca}^{2+} \)) containing 1.5% (w/v) fatty acid-free bovine serum albumin. The number of cells per ml was determined by examining a hepatocyte suspension in 0.25% trypan blue (in 0.9% \( \text{NaCl} \)) under a microscope using a haematocrit chamber. The hepatocytes were diluted 1:1 (v/v) with the trypan blue preparation. The cellular concentrations were adjusted as required. The cells were preincubated for 20min in KRB (with \( \text{Ca}^{2+} \)) containing 1.5% BSA and gassed continuously with 95% \( \text{O}_2 \) and 5% \( \text{CO}_2 \) to restore the ATP / ADP ratio to normal (Claus et al., 1975).

Siliconized glassware was used throughout the preparation, and the method used for siliconization involved rinsing the glassware with a solution of dimethyl chlorosilane (BDH Chemicals Ltd., Poole, England) in 1,1,1-trichloroethane (2% w/v) and air-drying overnight. The glassware was rinsed with distilled water to remove \( \text{HCl} \) formed in the treatment. The glassware was left to air-dry.

Structural viability of the hepatocytes was determined by counting the cells which excluded trypan blue and expressing this as a percentage of the total number of cells. The cell preparations used in the experiments recorded in Table 3.1 had viability values of 90 and 95%, indicating a substantial integrity of the plasma membrane.

It has been demonstrated by other workers in this laboratory that under these conditions of extraction, the hepatocytes prepared by this method are metabolically viable as indicated by their ATP levels and their capacity to form glucose from \( \text{L-}^{14}\text{C} \) lactate (Daneshmand,
b. Lipogenesis from \[^{1-1^4C}\text{acetate}\]

Hepatocytes were prepared as in 3a above and pre-incubated for 20 min before the addition of substrates and effectors. Each incubation mixture contained 10^6 cells ml\(^{-1}\), 1.5 mM acetate, \[^{1-1^4C}\text{acetate}\] (4.4x10^5 d.p.m./assay), 25 mM - and 10^-5 M - glucose and glucagon, respectively, in a final volume of 1 ml. The incubations were carried out in siliconized conical flasks in a shaking water bath (200 cycles min\(^{-1}\)) at 37°C, for 30 min. The rate of fatty acid synthesis was linear for up to 30 min, and the cells were gassed continuously with 95% O\(_2\) and 5% CO\(_2\) throughout the incubation period. The reaction was stopped by adding 1 ml of 6% w/v perchloric acid.

The lipids formed during this time period were estimated by an adaptation of the method of Folch et al. (1957), as described by Daneshmand (1983).

The perchlorate extracts were treated with methanol: chloroform (2:1 v/v) (6 ml) and the samples ultrasonicated for 15 s (MSE ultrasonic disintegrator, maximum amplitude). After 16 h the samples were transferred to plastic, centrifuge tubes and 7.6 ml of methanol:chloroform:water (2:1:0.8) was added to each tube. This was followed by further sonication for 15 s. Chloroform (4 ml) and water (4 ml) were added and the two phases were separated by centrifugation in a bench centrifuge (MSE minor) at 2,000 g for 10 min. The chloroform layer was retained and treated with 25 ml water. The chloroform layer was again retained and filtered into scintillation vials through phase separating paper (Whatman IPS) to remove precipitated protein and water. The chloroform extract was evaporated to dryness, under a stream of nitrogen. The radioactivity was then assayed in 10 ml of a toluene scintillant solution.
(5g PPO/L toluene) using a Beckman LS 7500 (microprocessor controlled).

c. **Determination of the NADPH-oxidising activity in hepatocytes**

Hepatocytes, prepared as in 3a, were harvested by centrifugation and then resuspended in 0.25M-sucrose, 1mM-EDTA 20mM-BTP/HCl, pH 8.3. The cells were disrupted by sonication at maximum amplitude for 15s and then centrifuged at 10,000g for 10min. The supernatant was heated to 60°C for 1min, centrifuged and assayed for the NADPH oxidising activity as in II.B.1.e.

4. **Purification procedures**

a. **Glucose-6-phosphate dehydrogenase**

(i) **Purification by affinity chromatography**

Blue Sepharose CL-6B, which consists of the dye Cibacron Blue F3G-A and Sepharose CL-6B, was the chromatographic material used. The Blue Sepharose CL-6B was equilibrated with 20mM-BTP/HCl, pH 7.0, 5mM-EDTA and 10% glycerol, and packed into a column (20ml capacity).

Livers from rats treated as in II.B.3.a. were homogenized in 3 vol. of 20mM-BTP/HCl, pH 7.5, containing 5mM-EDTA, 10% glycerol (v/v) and 0.25M-sucrose. The homogenate was then centrifuged at 100,000g for 1h and the resultant supernatant treated with 3M-acetic acid such that the final pH was 5.0. The supernatant was stirred for 30min at 4°C. The precipitate formed was then removed by centrifugation at 100,000g for 30min and the pH of the supernatant was then adjusted to pH 7.0 with KOH. The preparation was clarified by centrifugation at 10,000g for 10min. The supernatant was applied to the Blue Sepharose CL-6B column, which was then washed with the equilibrating buffer (containing 0.2M-KCl). The
enzyme was then eluted with 1.4mM-NADP⁺ and 0.3M KCl in 20mM-BTP/HCl/1mM-EDTA, pH 7.0, 10% glycerol and 0.25M-sucrose. Fractions (4.5ml) were collected and assayed for protein content and G6PDH activity. The purification procedure was carried out at 4°C. The specific activity of the enzyme obtained in this way was 0.63µmol min⁻¹ mg⁻¹ protein, representing a 105-fold purification and a recovery of 24% compared to the high speed supernatant fraction.

(ii) Purification of rat liver G6PDH by the method of Matsuda and Yugari (1967)

This method of purification of G6PDH was used in the initial part of this work. The procedure followed was essentially that of Matsuda and Yugari (1967), with minor modifications. Livers were obtained and homogenized and centrifuged as above. The high speed (100,000g) supernatant was then treated with ethanol, chilled to 0°C, so that the final concentration of ethanol was 25% (v/v). This mixture was stirred for 30min at 4°C. The precipitate was removed by centrifugation at 10,000g for 10min. The supernatant was then treated with 0.03 vol. of freshly prepared zinc acetate (0.1M) and stirred for 30min. The precipitate was collected by centrifuging at 10,000g for 20min. The precipitate was then resuspended in one-fifth of the original volume of 20mM-BTP/HCl, pH 7.5, containing 5mM-EDTA. This preparation was further treated with acetic acid (3M) so that the final pH of the solution was 5.0. The precipitate was then removed by centrifugation and the supernatant applied to a DEAE-cellulose column (2.5x12cm) previously equilibrated with 20mM-BTP/HCl and 5mM-EDTA, pH 7.5. The enzyme was eluted with a linear gradient of NaCl (0-1M) in 20mM-BTP/HCl, pH 7.5 and 5mM-EDTA, in a total volume of 300ml. The specific activity of this enzyme was about 0.24µmols min⁻¹ mg⁻¹ protein representing a 40-fold purification and a 10% recovery. It was found that G6PDH purified in this manner was very unstable and this instability accounts for the low
recovery and degree of purification. It was for this reason that the method in (i) was adopted.

b. 6-Phosphogluconate dehydrogenase

The partial purification of 6PGDH was achieved by following the procedure reported by Procsal and Holten (1972), with minor differences. The livers from fed female rats were weighed and homogenized in four volumes of 0.15M-KCl and 0.16mM-KHCO\(_3\) at pH 7.0. The homogenate was centrifuged at 100,000g for 1h and the supernatant dialyzed for 16h, during which time the buffer was changed four times. The supernatant was treated with an equal volume of saturated (NH\(_4\))\(_2\)SO\(_4\) (4.1M or 767 g/l of salt). The supernatant was stirred for 30min at 4°C. The precipitate was removed by centrifugation and discarded. The supernatant was treated with 2/3 vol. of saturated (NH\(_4\))\(_2\)SO\(_4\), at pH 7.0. The mixture was allowed to stand for a further 30min, with continual stirring at 4°C. The 50-70% (NH\(_4\))\(_2\)SO\(_4\) precipitate was retained and dissolved in 1mM-EDTA (pH 7.0) and dialysed against the homogenising buffer at pH 7.0. At this stage the specific activity was 0.4μmol min\(^{-1}\) mg\(^{-1}\) protein, representing an 18-fold purification and a 40% recovery. Although activity was gradually lost over a period of time (2-3 weeks), this enzyme preparation was more stable than that of G6PDH. In some experiments, the enzyme was further purified by chromatography on DEAE-cellulose. In this case, the dialyzed 50-70% (NH\(_4\))\(_2\)SO\(_4\) fraction was applied to a DEAE-cellulose column (4.2x12cm), which was previously equilibrated with 5mM-potassium phosphate - 1mM-EDTA, pH 7.0. The column was eluted with a linear gradient from 5 to 40mM-KP\(_i\), pH 7.0 and 1mM-EDTA in a total volume of 300ml. The fractions (3.5ml) containing 6PGDH activity were pooled and dialyzed before use. The specific activity of the enzyme was only 0.5μmol min\(^{-1}\) mg\(^{-1}\) protein, representing a purification factor of 24.5 and a recovery of 28.3%. In the purification procedure, the enzyme activity was determined at pH 9.0, and not
pH 7.0, as the former represents the pH optimum of the enzyme. Other conditions of assay were: 200μM-6PG, 100μM NADP⁺ and 100mM-BTP/HCl, pH 9.0.

c. Partial purification of rat liver FAS

The method used for the partial purification of FAS was that of Linn (1981), the only deviation from the described procedure being that DEAE-cellulose (DE52) (Whatman) was replaced by DEAE-cellulose from Sigma and that rabbit serum was omitted from the buffers. The procedure was followed up to the DEAE-cellulose step and the product at this stage had a specific activity of 5.5nmol min⁻¹ mg⁻¹ protein in fresh extracts. This activity rapidly declined within days (Table 3.3 in 'Results and Discussion').

In some experiments, the crude high speed supernatant (100,000g) was the enzyme source. This preparation was obtained by homogenizing livers, from 48h starved/3 days refed female rats, in 5 vol. of KPᵢ buffer, pH 7.2, containing 1mM-MgCl₂, 100μM-EDTA, 10% glycerol and 1mM-DTT and centrifuging at 100,000g. The resultant supernatant was then dialyzed against the homogenization buffer for 16h (3 buffer changes) before assay. The enzyme activity was determined as in II.B.1.c (spectrophotometrically).

d. The NADPH-dependent reductase and the peptide substrate from rat liver

The reductase (cf. III.C.) was purified from high speed supernatants derived from rat liver homogenates (1:2, w/v). The homogenising buffer was 20mM-BTP/HCl, pH 8.3 containing 1mM-EDTA and 0.25M-sucrose and the centrifugation (100,000g) lasted for 1h at 4°C. These preparations contained both the enzyme and the substrate. In order to separate the enzyme from the substrate, without losing the latter, the supernatant was either (i) heated to 60°C for 1min in a water bath, cooled, centrifuged and the precipitate discarded or (ii) ultrafiltered.
through Amicon ultrafiltration membranes (M cut-off 10,000), which were stored in ethanol (50% v/v) and extensively washed with distilled water before use. The product of either of these methods was then assayed for NADPH oxidising activity (II.B.1.e.). At this stage in the purification procedure the enzyme was still associated with the peptide substrate.

In some experiments the initial preparative steps outlined above were performed at pH 10.0. When this was the case, 20mM-glycine/NaOH was the homogenisation buffer and it contained 0.25M-sucrose. Solid BTP to a final concentration of 20mM and an appropriate volume of HCl (6N) were added to adjust the pH to 7.5 before assaying for the NADPH-oxidising activity.

These preparations were then used for the purification of (i) the enzyme-substrate complex (cf. III.C.2a), (ii) the enzyme (cf. III.C.2b) and (iii) the peptide substrate (cf. III.C.2c).

(i) Purification of the enzyme-substrate complex

Two procedures were followed for the attempted purification of the enzyme-substrate complex.

In scheme I, the heat treated extracts were treated with an equal volume of chilled ethanol (-20°C) and stirred for 30min at 4°C. The precipitated protein was removed by centrifugation at 100,000g for 30min. Both the supernatant and the precipitate, resuspended in the original volume of buffer (20mM-BTP/HCl, 1mM-EDTA, pH 8.3), were tested for NADPH-oxidising activity (II.B.1.e). The activity was found to be associated with the supernatant.

The ethanolic supernatant was then ultrafiltered through Amicon membranes (PM 10, M cut-off 10,000) at 4°C under a nitrogen pressure of 3.8 Kg/cm². A standard
Amicon stirred cell (diameter 4.3 mm) was used. The ultrafiltrate was assayed for NADPH-oxidising activity and protein content.

In earlier experiments the ultrafiltration step was followed by barium acetate precipitation (Cardini and Leloir, 1957). This, however, was omitted in later experiments because of the large losses in activity incurred by the procedure.

Regardless of whether barium acetate was used or not, the enzyme-substrate complex was further purified by precipitating it out with 3 vol. of chilled ethanol and stirring the solution at 4°C for 30 min. The precipitate was collected by centrifugation at 100,000g for 30 min and resuspended in 0.2 vol. of the original volume of buffer (20mM-BTP/HCl, 1mM-EDTA, pH 8.3). This sample was then applied to a small (5ml capacity) DEAE-Sephadex A-50 column previously equilibrated with 20mM-BTP/HCl and 1mM-EDTA, pH 8.3. The complex was eluted with a discontinuous gradient of KCl (0-1M) in 20mM-BTP/HCl and 1mM-EDTA, pH 8.3.

In scheme II, PEG-6000 fractionation followed the heat treatment. PEG-6000 was added as a solid to the heat extracts to a final concentration of 10g/100ml. The preparation was stirred for 30 min at 4°C and the precipitate removed by centrifugation at 100,000g for 30 min. The supernatant was diluted with an equal volume of buffer (20mM-BTP/HCl, 1mM-EDTA, pH 8.3) and applied to a Sephadex A-50 column (Fig. 3.11), equilibrated as above. The complex was eluted with a continuous gradient of KCl (0-1M) in buffer, as above, in a total volume of 300ml.

The eluates containing the complex were pooled, diluted 2-fold with the buffer and reapplied to a smaller Sephadex A-50 column and eluted with a discontinuous
gradient of KCl as above.

(ii) Partial purification of the enzyme moiety

The enzyme-substrate complex was resolved by treating the heat-treated extracts with 30g/100ml of PEG-6000, stirring for 30min at 4°C, and centrifuging at 100,000g for 30min. The precipitate contained the enzyme and the supernatant the substrate.

The enzyme-containing substrate was resuspended in 20mM-BTP/HCl, 1mM-EDTA, pH 8.3 and made 10% (w/v) with PEG-6000. The resultant precipitate was removed by centrifugation and discarded. The supernatant was then treated with PEG-6000 so that the final concentration of the polymer was 30% (w/v). The precipitate was collected by centrifugation and resuspended in the original volume of buffer.

The enzyme preparation was then applied to a DEAE-cellulose column previously equilibrated with 20mM-BTP/HCl, 1mM-EDTA, pH 7.5. The enzyme was eluted with a continuous gradient of NaCl (0-1M) in the equilibrating buffer (Fig. 3.14).

(iii) The purification of the peptide substrate

The 30% PEG-6000 supernatant (see (ii) above) was the substrate source. The supernatant was diluted with an equal volume of buffer (20mM-BTP/HCl, 1mM-EDTA, pH 8.3) and applied to a Dowex Cl\(^-\) 1 (x8-200) column (Fig. 3.16). PEG-6000 was washed off the column with 300ml of buffer. The substrate was then eluted with a continuous gradient of NaCl (0-1M), in buffer, in a total volume of 350ml.

The substrate-containing fractions were pooled, diluted with an equal volume of buffer and applied to a Dowex 50 H\(^+\) 50 (x8-400) column. The method was essentially that of Cowgill (1957). After the
application of the substrate the column was washed with 0.01M-NH$_4$OH (60ml, and pH 8.5). The substrate was then eluted with a linear concentration and pH gradient (0.01M, pH 8.5 - 0.1M, pH 13.0) of NH$_4$OH in a total volume of 300ml (Fig. 3.17). The substrate-containing fractions were again pooled, adjusted to pH 8.3 with solid BTP (20mM) and 6N-HCl and divided into three portions.

These portions were applied individually to a Sephadex G-25 column (previously equilibrated with 20mM-BTP/HCl, 1mM-EDTA, pH 8.3 and 100mM NaCl), a Bio-Gel P-2 column (equilibrated with 20mM-BTP/HCl, 1mM-EDTA, pH 8.3) and a small Dowex Cl$^-$ 1 (x8-200) column. The first two columns were eluted with the equilibration buffers (Fig. 3.18 and 3.19), and the Dowex Cl$^-$ 1 (x8-200) column with a discontinuous gradient of NaCl (0-1M) in buffer (Fig. 3.20).

e. Fractionation of the liver homogenates

Rat liver homogenates obtained as in (II.B.4d) were fractionated by differential centrifugation. The method was essentially that of de Duve et al. (1955), with minor modifications. The nuclear fraction was precipitated at 650g, the lysosomal and mitochondrial fraction at 22,500g and the microsomal at 100,000g. Each particulate fraction was resuspended in the original volume of the homogenising buffer. The supernatant obtained after centrifugation at 100,000g for 1h was taken to be the soluble fraction. All the centrifugation steps were carried out at 4°C for 15min, except that performed at 100,000g.

All the fractions were sonicated at max. amplitude for 30s, heated to 60°C for 1min as described previously, and the supernatants were assayed for NADPH-oxidising activity as in II.B.1.e.
5. Synthesis of fructose 2,6-bisphosphate

The method used was that of Van Schaftingen and Hers (1981a), which was a modification of the method of Pontis and Fischer (1963) for the synthesis of fructose-2-phosphates.

The initial step of this method was to convert the sodium salt of F1,6P₂ to the free acid by ion exchange chromatography on Dowex 50 H⁺ (x8-200). This was then converted to the pyridinium salt by neutralizing the solution with pyridine to give a final concentration of 0.2M-F1,6P₂. At the same time, 24g of dicyclohexyl-carbodi-imide (DCC) was dissolved in 10ml aqueous pyridine.

The cyclisation reaction was performed by incubating 6ml of the pyridine salt of F1,6P₂ (0.2M) with 0.5ml triethylamine (a strong base which prevents further reaction of the cyclic phosphodiester formed with DCC), 20ml pyridine and the DCC/pyridine solution (10ml). The reaction was allowed to proceed for 24h at 20°C. The crystalline material formed during the incubation was filtered off, after stopping the reaction with 40ml water. The product was extracted four times with a total volume of 200ml ether. The aqueous phase was retained.

The aqueous solution (40ml) was mixed with 0.2 vol. of 2.5M-NaOH, in order to hydrolyse the cyclic diester formed. The reaction was allowed to proceed for 30min at 37°C.

At the end of the incubation period, glycine (pKₐ 9.9) was added to a final concentration of 20mM and the pH adjusted to 9.4 with 2M-HCl. MnCl₂ was also added to a final concentration of 0.5mM. F₁,6BPase (0.2 unit ml⁻¹) was then added to remove the untreated F₁,6P₂.

After 3h at 30°C, the reaction was stopped by a 5-fold
dilution of the solution with water. The solution was then passed down a column (30x0.6cm) of Dowex AG-1 Cl- form. The phosphoric esters were eluted with a NaCl gradient (100-400mM) in 250ml. F2,6P2 was determined by its capacity to stimulate PFK-1 under the conditions defined in II.B.1.d. Fractions containing F2,6P2 were pooled, diluted 3-fold and rechromatographed.

\[
\sum \chi = \text{sum of } \chi
\]

\[
\bar{\chi} = \text{observed values}
\]

\[
N = \text{number of observations}
\]

\[
SD = \text{standard deviation}
\]

\[
(t) = \frac{\bar{\chi}_1 - \bar{\chi}_2}{\sqrt{\frac{(SD_1)^2}{n_1} + \frac{(SD_2)^2}{n_2}}}
\]

where \(\bar{\chi}_1\) = mean or arithmetic average \(\frac{\sum \chi_1}{N}\)

(iii) E.E.M. = \(\frac{SD}{\sqrt{N}}\)

where E.E.M. = standard error mean
6. Statistical analysis

The t-test was used for statistical analysis and the calculations of the standard deviation and 't' were based on the following formulae:—

(i) \[ SD = \sqrt{\frac{\sum(x^2) - \left(\frac{\sum x}{N}\right)^2}{N-1}} \]

where \( \sum \) = "sum of"

\( x = \) observed values

\( N = \) number of observations

\( SD = \) standard deviation

(ii) \[ t = \frac{\overline{x}_1 - \overline{x}_2}{\sqrt{\frac{(SD_1)^2}{N_1} + \frac{(SD_2)^2}{N_2}}} \]

where \( \overline{x} = \) mean or arithmetic average \( \frac{\sum x}{N} \)

(iii) S.E.M. = \[ \frac{SD}{\sqrt{N}} \]

where S.E.M. = standard error mean
The recent discovery of F2,6P2, a potent stimulator of glycolysis and inhibitor of gluconeogenesis in rat liver, has resulted in a search for possible regulatory enzymes which respond to the metabolite, other than F6P-1 and F3,6 PPase. A number of regulatory enzymes which could conceivably be regulated by F2,6P2 are considered in this thesis. It is well known that high rates of hepatic glycolysis are associated with increased lipogenesis and that some of the enzymes which regulate lipogenesis have been investigated. In addition, the regulation of the supply of NADPH for lipogenesis by the PPP has been investigated and a cytosolic NADPH consuming-reaction characterized.

A. F2,6P2 AND LIPOGENESIS

A positive correlation between rates of lipogenesis and glycolysis was long ago observed (Trach and Guynn, 1954). It seemed appropriate, therefore, to examine the rate of lipogenesis under conditions of raised and reduced levels of F2,6P2, a known positive effector of glycolysis (Van Schaftingen et al., 1980a,b and c; Van Schaftingen et al., 1981a,b,c). These authors have shown that incubation of rat hepatocytes with glucose raises the intracellular levels of F2,6P2 and glucagon has the reverse effect.

The rate of lipogenesis from $^{14}$C acetate was followed in the presence and absence of glucose and glucagon as shown in Table 3.1. It was observed that incubation of hepatocytes with glucose increased lipogenesis and that glucagon had the reverse effect. These results support the theory that the regulation of glycolysis and lipogenesis may be co-ordinated.

However, in the absence of a demonstrable direct affect
The recent discovery of F2,6P2, a potent stimulator of glycolysis and inhibitor of gluconeogenesis in rat liver, has resulted in a search for possible regulatory enzymes which respond to the metabolite, other than PFK-1 and F1,6BPase. A number of regulatory enzymes which could conceivably be regulated by F2,6P2 are considered in this thesis. It is well known that high rates of hepatic glycolysis are associated with increased lipogenesis and thus some of the enzymes which regulate lipogenesis have been investigated. In addition, the regulation of the supply of NADPH for lipogenesis by the PPP has been investigated and a cytosolic NADPH consuming-reaction characterized.

A. F2,6P2 AND LIPOGENESIS

A positive correlation between rates of lipogenesis and glycolysis has long been established (Veech and Guynn, 1974). It seemed appropriate, therefore, to examine the rate of lipogenesis under conditions of raised and reduced levels of F2,6P2, a known positive effector of glycolysis (Van Schaftingen et al., 1980a,b and c; Van Schaftingen et al., 1981a,b). These authors have shown that incubation of rat hepatocytes with glucose raises the intracellular levels of F2,6P2 and glucagon has the reverse effect.

The rate of lipogenesis from 14C acetate was followed in the presence and absence of glucose and glucagon as shown in Table 3.1. It was observed that incubation of hepatocytes with glucose increased lipogenesis and that glucagon had the reverse effect. These results support the theory that the regulation of glycolysis and lipogenesis may be co-ordinated.

However, in the absence of a demonstrable direct effect
Table 3.1: The effect of incubating hepatocytes with glucose and glucagon on lipogenesis from [1-^{14}C]acetate

<table>
<thead>
<tr>
<th>Effector</th>
<th>Incorporation of [1-^{14}C]acetate mmol min^{-1} per 10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.131 ± 0.098</td>
</tr>
<tr>
<td>glucagon (10^{-9} M)</td>
<td>0.137 ± 0.062</td>
</tr>
<tr>
<td>glucose (25mM)</td>
<td>1.147 ± 0.208</td>
</tr>
<tr>
<td>glucose (25mM) + glucagon (10^{-9} M)</td>
<td>0.128 ± 0.041</td>
</tr>
</tbody>
</table>

Hepatocytes isolated from 24h starved female rats were incubated with the indicated effectors for 30min after preincubation of the hepatocytes for 20min. Each incubation mixture (1ml) contained 10^6 cells and effectors as indicated in II.B.3b and the amount of radioactive label incorporated into fatty acids was determined as in II.B.2e.
of F2,6P2 on the regulatory enzymes of lipogenesis, namely acetyl-CoA carboxylase and fatty acid synthase, the postulation remains theory. With this in mind, an attempt was made to demonstrate a direct effect of F2,6P2 on FAS. Acetyl-CoA carboxylase has been studied by other workers (Davies et al., unpublished results).

1. Fatty acid synthase

Plate et al. (1968) in their work with pigeon liver fatty acid synthase have shown that this enzyme is sensitive to inhibition by malonyl-CoA, one of the substrates of FAS. They showed that the inhibition is of the mixed type with respect to acetyl-CoA and is competitive with respect to NADPH. The Km for NADPH is 19-fold greater at an inhibitory concentration (37.5μM) of malonyl-CoA than at the Vmax concentration (10μM) of the substrate.

These authors further showed that this inhibition by malonyl-CoA could be reversed by fructose 1,6-bisphosphate (F1,6P2), and that this reversal is reflected in a progressive decrease in the Km for NADPH with F1,6P2 concentration. F1,6P2 was shown not to markedly affect the Km values for either acetyl- or malonyl-CoA. In the work with human liver FAS, Roncari (1975) reported that 10mM-F1,6P2 activated FAS by decreasing its Km for NADPH. The range of concentration (10-40mM) of F1,6P2 required to reverse the inhibition of FAS activity by malonyl-CoA exceeds the expected liver content of F1,6P2, which has been estimated to be about 32 ± 4 nmol g⁻¹ liver wet weight (Van Schaftingen et al., 1980a).

In the present study the effect of F1,6P2 has been demonstrated, but in agreement with Plate et al. (1968) the concentration of F1,6P2 required to stimulate FAS is outside the physiological range. Since F2,6P2 has been shown to be a 1000-fold more effective than F1,6P2 in the stimulation of PFK-1 (Hers and Van Schaftingen, 1982), it seemed
appropriate to investigate the effect of \( F_2,6P_2 \) on FAS activity. The effect of \( F_1,6P_2 \) and malonyl-CoA on the rat liver enzyme were also investigated.

a. \( F_2,6P_2 \) and FAS activity (spectrophotometric assay)

The effect of \( F_2,6P_2 \) on FAS activity in a dialysed 100,000g supernatant of a rat liver homogenate was determined (Table 3.2). At subsaturating concentrations of malonyl-CoA and saturating concentrations of other substrates, \( F_2,6P_2 \) was found to cause an apparent stimulation of the enzyme activity, but it did not overcome inhibition of the enzyme by high concentrations of malonyl-CoA. The results shown in Table 3.2 confirm the reported inhibition of FAS by high concentrations of malonyl-CoA. In agreement with the results of Plate et al. (1968) \( F_1,6P_2 \) was shown to stimulate FAS activity at high and low concentrations of malonyl-CoA.

The apparent stimulation by \( F_2,6P_2 \) was also demonstrated with a partially purified FAS preparation and there was an apparent synergism between AMP and \( F_2,6P_2 \) (Table 3.3). AMP is known to act synergistically with \( F_2,6P_2 \) in the stimulation of PFK-1 activity at the concentration of effectors similar to those used in the present study (Hers and Van Schaftingen, 1982).

However, with these relatively crude preparations a high background rate of NADPH oxidation was observed before the addition of malonyl-CoA to start the reaction (Table 3.3). Since the FAS activity was determined by following the rate of oxidation of NADPH at 340nm, the high background rates cast some doubt on the validity of the results obtained by this method of assay since there was an apparent inhibition of the background rate of NADPH oxidation by \( F_2,6P_2 \) and AMP (Table 3.3). For this reason, the enzyme activity was measured by following the incorporation of \( [1-^{14}C] \) acetyl-CoA into fatty acids.
Table 3.2: The effect of F1,6P₂, F2,6P₂ and varying concentrations of malonyl-CoA on FAS activity

<table>
<thead>
<tr>
<th>Malonyl-CoA (µM)</th>
<th>No effectors</th>
<th>10mM-F1,6P₂</th>
<th>40mM-F1,6P₂</th>
<th>3µM-F2,6P₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.77</td>
<td>1.33</td>
<td>1.66</td>
<td>1.10</td>
</tr>
<tr>
<td>10</td>
<td>1.60</td>
<td>1.33</td>
<td>1.60</td>
<td>1.40</td>
</tr>
<tr>
<td>50</td>
<td>0.67</td>
<td>0.67</td>
<td>1.33</td>
<td>0.90</td>
</tr>
<tr>
<td>80</td>
<td>0.70</td>
<td>0.63</td>
<td>1.33</td>
<td>0.67</td>
</tr>
</tbody>
</table>

FAS activity was spectrophotometrically assayed (II B.1.c(i)). The enzyme source was a high speed supernatant of a rat liver homogenate. The supernatant was extensively dialysed against 0.1M-KPi, pH 7.5 before FAS activity was determined at pH 6.8. The results are expressed as the mean of triplicate readings. The reaction was initiated with the appropriate concentration of malonyl-CoA.
Table 3.3: The effect of F2,6P2 and AMP on FAS activity at subsaturating concentrations of malonyl-CoA

<table>
<thead>
<tr>
<th>Effectors/concn.</th>
<th>Total activity (nmol NADPH oxidised min(^{-1}) mg(^{-1}))</th>
<th>Background rate (nmol NADPH (o) min(^{-1}) mg(^{-1}))</th>
<th>Net FAS activity (nmol min(^{-1}) mg(^{-1}))</th>
<th>% of correc. (V_0) rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.550</td>
<td>0.473</td>
<td>0.077</td>
<td>100</td>
</tr>
<tr>
<td>F2,6P2 (1.5(\mu)M)</td>
<td>0.625</td>
<td>0.520</td>
<td>0.105</td>
<td>129</td>
</tr>
<tr>
<td>AMP (100(\mu)M)</td>
<td>0.580</td>
<td>0.510</td>
<td>0.070</td>
<td>87</td>
</tr>
<tr>
<td>F2,6P2 + AMP (1.5(\mu)M) (100(\mu)M)</td>
<td>0.659</td>
<td>0.360</td>
<td>0.299</td>
<td>375</td>
</tr>
</tbody>
</table>

A partially purified FAS (PEG fractionation followed by DEAE-cellulose fractionation (IIB.4.c)) was used in this experiment. The results are the mean of triplicate readings. The assay conditions were as in Table 3.2. The malonyl-CoA concentration used was 5 \(\mu\)M.
b. F$_2$,$\Delta$P$_2$ and FAS activity (radioactive assay)

The malonyl-CoA dependent incorporation of $[1^{-14}C]$ acetyl-CoA into fatty acids catalysed by a dialysed high-speed supernatant fraction from rat liver was followed and taken as a measure of the activity of FAS. This alternative method of assay overcame the inaccuracy introduced by high NADPH-dependent background rates associated with the spectrophotometric assay (Table 3.3).

Table 3.4 shows the results obtained with the radiochemical method. Two concentrations of F$_2$,$\Delta$P$_2$ were used: 10$\mu$M which approximates the in vivo concentration of F$_2$,$\Delta$P$_2$ in fed rats (Neely, et al. 1981), and 0.5$\mu$M, a concentration which might be expected to reflect the levels of F$_2$,$\Delta$P$_2$ under conditions of starvation.

At the higher concentration of F$_2$,$\Delta$P$_2$ an apparent inhibition of FAS activity by F$_2$,$\Delta$P$_2$ was observed at all concentrations of malonyl-CoA examined (Experiment I, Table 3.4). AMP (100$\mu$M) partially overcame this inhibition at lower concentrations of malonyl-CoA. Activation of the enzyme by AMP was not observed. At the lower concentration of F$_2$,$\Delta$P$_2$, an apparent stimulation of FAS activity was observed.

Since F$_2$,$\Delta$P$_2$ levels have been reported to increase 2.5-fold in rat liver during conditions of induced hyperlipogenesis (Neely, 1981), the results obtained in the present study suggest that F$_2$,$\Delta$P$_2$ at physiological concentrations is not a positive effector of FAS activity as one might expect. The observed inhibition (Table 3.4) does not correlate with the role for F$_2$,$\Delta$P$_2$ as a positive effector of glycolysis, the latter having been associated with high lipogenic rates (Veech and Guynn, 1974). These results are inconclusive and require further investigation. However, due to reasons of time and finance, this was not possible. It is quite possible that a stimulation can
Table 3.4: The effect of F2,6P2, F1,6P2 and malonyl-CoA on FAS activity

<table>
<thead>
<tr>
<th>Effectors</th>
<th>nmoles [1-14C]acetyl units incorporated min⁻¹ per mg⁻¹</th>
<th>5μM-malonyl-CoA</th>
<th>10μM-malonyl-CoA</th>
<th>50μM-malonyl-CoA</th>
<th>80μM-malonyl-CoA</th>
</tr>
</thead>
<tbody>
<tr>
<td>EXPT. I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NONE</td>
<td>0.46 ± 0.18</td>
<td>0.93 ± 0.31</td>
<td>0.31 ± 0.10</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>F2,6P2 (10μM)</td>
<td>0.20 ± 0.04*</td>
<td>0.42 ± 0.06**</td>
<td>0.15 ± 0.03*</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>AMP (100μM)</td>
<td>0.48 ± 0.22</td>
<td>0.74 ± 0.38</td>
<td>0.16 ± 0.14</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>AMP (100μM) + F2,6P2</td>
<td>0.44 ± 0.23</td>
<td>0.52 ± 0.16*</td>
<td>0.10 ± 0.04*</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>EXPT. II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NONE</td>
<td>0.46 ± 0.20</td>
<td>0.90 ± 0.20</td>
<td>0.47 ± 0.16</td>
<td>0.40 ± 0.20</td>
<td></td>
</tr>
<tr>
<td>F2,6P2 (0.5μM)</td>
<td>0.50 ± 0.23</td>
<td>1.20 ± 0.06*</td>
<td>1.43 ± 0.30***</td>
<td>0.93 ± 0.20**</td>
<td></td>
</tr>
<tr>
<td>F1,6P2 (10mM)</td>
<td>0.48 ± 0.16</td>
<td>1.16 ± 0.23</td>
<td>1.46 ± 0.73**</td>
<td>1.86 ± 0.36***</td>
<td></td>
</tr>
</tbody>
</table>

N.D. NOT DETERMINED
* p < 0.05
** p < 0.025
*** p < 0.005
Table 3.4:

The activity of FAS, in the presence of the indicated effectors, was determined. The incubation mixture was composed of 100mM-KPi pH 6.8, 200μM-NADPH, 30μM-DTT, 30μM-acetyl-CoA and the indicated concentration of malonyl-CoA in a final volume of 1ml. The enzyme source was an extensively dialysed crude high speed supernatant of a rat liver homogenate (II.B.4.C). The reaction was allowed to proceed for 10min after which the reaction was stopped with 10% perchloric acid. The fatty acids were extracted and monitored as in II.B.3.b. The results are expressed as the mean ± S.D. (n=3 for each experiment). The symbols *, **, *** represent the level of significance and refer to the difference between the test incubation and its appropriate control.
only be observed when the assay conditions are suitable and since only a limited number of experiments could be performed it may be that these conditions were not realised. By analogy, the other enzymes which are susceptible to F2,6P2 i.e. PFK-1 and F1,6BPase, the conditions under which the effector exerts an effect on enzyme activity are very critical.

Table 3.4 also shows the inhibition of FAS activity by high concentrations of malonyl-CoA and the reversal of this inhibition by F1,6P2. This is in agreement with the results obtained by Plate et al. (1968) with pigeon liver FAS and with the results obtained by the spectrophotometric assay in the present study (Table 3.2) for the rat liver enzyme. F2,6P2 (10μM) enhanced the malonyl-CoA-induced inhibition of the enzyme (Table 3.4). AMP, on its own, did not significantly affect the activity of FAS under the conditions of assay.

Otto et al. (1983) have reported very similar malonyl-CoA concentrations, 6.4 and 7.6 nmol g⁻¹ cells for starved and carbohydrate refed rats, respectively, whereas Veech and Guynn (1974) have reported a value of 25 nmol g⁻¹ liver in freeze clamped livers from fed rats. It is not likely, therefore, that the malonyl-CoA content of the liver cells exerts an important inhibitory effect on FAS in vivo, and consequently, the enhanced inhibition by F2,6P2 is probably of no physiological significance.

Unfortunately, the apparent stimulation of FAS activity by F2,6P2 and AMP observed with the spectrophotometric assay (Table 3.2) could not be shown when the radiochemical method was used, indicating that the result found previously was an artifact resulting from a change in the background rate. The significance of the effect of F2,6P2 observed at the lower concentration of the effector is uncertain as the experiment was only performed once.
2. Acetyl-CoA carboxylase

F₂₆P₂, at physiological concentrations, has been shown not to affect either the activity or the degree of polymerization, in the presence or absence of citrate, of acetyl-CoA carboxylase (Davies et al., unpublished results). This enzyme has, therefore, not been assayed in the present study.

B. THE PENTOSE PHOSPHATE PATHWAY

1. Fructose 2,6-bisphosphate and the PPP dehydrogenases

Fructose 2,6-bisphosphate has been shown to be a positive physiological effector of glycolysis (Hers and Van Schaftingen, 1982; Pilkis et al., 1983b). It was of interest, therefore, to determine the effect of this compound on the PPP dehydrogenases, the rate-limiting enzymes of the PPP, another pathway of glucose oxidation. The idea that the two pathways may be co-ordinately regulated has been supported by the observations of Sommercorn and Freedland (1982), who have demonstrated the stimulatory action of 6-phosphogluconate on PFK-1. Furthermore, this stimulation has been shown to be additive to that caused by F₂₆P₂ in the presence of physiological concentrations of AMP, ADP and ATP.

However, while indirect evidence that implicates F₂₆P₂ in the regulation of the flux through the PPP exists (Blackmore and Shuman, 1982), a direct effect of this compound on the PPP dehydrogenases has not been demonstrated.

In the course of this work much time was fruitlessly spent in altering conditions in the hope of demonstrating an effect by F₂₆P₂ on the PPP dehydrogenases. F₂₆P₂ was shown not to inhibit or stimulate the PPP dehydrogenases. The compound did not reverse the NADPH-induced
inhibition of G6PDH or 6PGDH. Addition of AMP, which has been reported to enhance the effect of F2,6P2 on PFK-1 (Van Schaftingen, 1981a), did not enable F2,6P2 to stimulate or inhibit the dehydrogenases. Various substrates and effectors which are related to the PPP, either because they are substrates for lipogenesis or known effectors of the enzymes of carbohydrate metabolism, were combined with F2,6P2 in an attempt to ascertain the role of the bisphosphate in the regulation of the PPP. These included ADP, ATP, Mg2+, lactate, citrate, Sn glycerol-3-phosphate, acetyl-, malonyl- and oleoyl-CoA. The results obtained were negative. F2,6P2 had no effect on the oleoyl-CoA-induced inhibition of the dehydrogenases.

The lack of positive results with F2,6P2 shifted the emphasis of the project from F2,6P2 to the general regulation of the PPP dehydrogenases, in particular to the possible mechanisms that may result in the reversal of the inhibition of the dehydrogenases by NADPH.

2. The regulatory role of GSSG in the de-inhibition of the PPP dehydrogenases

A physiological role for GSSG as a de-inhibitor of the PPP dehydrogenases has been postulated by Eggleston and Krebs (1974). These authors confirmed the inhibition of G6PDH by NADPH and reported that the inhibition was greater at low NADP+ concentrations and that the activity depended on the NADPH / NADP+ ratio. This inhibition was also reported to be competitive with respect to NADP+ (K1 7µM). The Km of the enzyme for the NADP+ under the same conditions of assay was reported to be 3µM.

Eggleston and Krebs (1974) reported that GSSG (10-100µM), in the presence of an unidentified small molecular weight cofactor, could reverse the NADPH-induced inhibition of G6PDH activity. This mechanism of de-inhibition was reported to be independent of the glutathione reductase catalysed reaction.
In the present study it was shown, using 100,000g supernatants from rat liver, that glutathione exerts a small inhibitory effect on 6PGDH (Fig. 3.1) and also on G6PDH activity (results not shown). The latter effect confirming the observation of Krebs and Eggleston (1974). However, under the experimental conditions used in this study, which were similar to those reported by Eggleston and Krebs, a high glutathione reductase activity comparable to that of 6PGDH (Fig. 3.2) was observed. It is likely, therefore, that the slight inhibition observed is due to glutathione reductase activity.

In order to determine the effect of GSSG and the cofactor on the NADPH-inhibited dehydrogenases, a 100,000g supernatant, which was not de-salted was used and Zn$^{2+}$ and GSSG were added. The de-inhibition of G6PDH by GSSG and the low M$_r$ cofactor reported by other workers (Eggleston and Krebs, 1974; Rodriguez-Segade et al., 1978, 1979) could not be repeated in the present study. It was observed in the course of this work that addition of Zn$^{2+}$ to the high speed supernatant caused a precipitation of the protein during the assay, which in turn resulted in a change of absorbance at 340nm which resembled the time course for an enzyme reaction. Furthermore, the rate of precipitation resulted in an apparent de-inhibition (results not shown) of both dehydrogenases.

Since then Levy and Christoff (1983) have also reported this Zn$^{2+}$ mediated phenomenon which results in a spurious rate of change in absorbance. They have inhibited GSSG reductase by using mercuric acetate instead of ZnCl$_2$, and under their conditions no de-inhibition of the dehydrogenases was found, in agreement with the results presented here and those of Gonzalez and Lagunas (1977).

Eggleston and Krebs reported that the cofactor for
The activity of 6PGDH was assayed in the presence of different concns. of glutathione (oxidised). The source of the enzyme was a dialysed high speed supernatant (100,000g) from a rat liver homogenate. The points are mean ± S.D (n=3). 6PGDH activity was determined as in II.B.1.b.
The relationship between 6PGDH activity and glutathione reductase activity

GSSG-reductase and 6PGDH activity were assayed as in II.B.1.a and II.B.1.b, respectively. The enzyme source was as in Fig. 3.1. The points represent the mean of three readings. Glutathione reductase activity is indicated by open circles (O) and 6PGDH activity by closed circles (•).
GSSG in the de-inhibition mechanism was present in perchloric acid extracts of high speed supernatant liver preparations. Addition of such extracts to the incubation mixture in the presence of GSSG did not result in a de-inhibition. On the contrary such extracts inhibited both dehydrogenases of the PPP (Fig. 3.3a).

The conclusions drawn from this work were essentially that GSSG, with or without the cofactor preparation, does not directly affect the activity of the PPP dehydrogenases. Furthermore, the inhibitory effect of GSSG in the absence of the cofactor preparation could be attributed to the activity of glutathione reductase, since even under conditions where Eggleston and Krebs (1974) claimed there was no GSSG reductase activity (such as when the GSSG concentration was less than 200\( \mu \text{M} \), it was found in the present study that glutathione reductase was active. In fact maximal rates were obtained with 200\( \mu \text{M} \)-GSSG.

A further consideration is that some of the activation of the enzyme reported by these authors could be the result of a shift in the NADPH / NADP\(^+\) ratio due to NADPH oxidation (III.C). Since the NADPH-oxidising activity reported in III.C would be expected to be present in the Amicon filtrates. The preincubation period of 15min at 38\( ^\circ \)C would result in the oxidation of virtually all the NADPH (see their Table 4). The inhibitor, NADPH, having been removed, addition of G6P to the reaction mixture would give an enhanced rate of G6P oxidation.

The pitfalls of the actual methods of determination of G6PDH activity in crude extracts have been extensively discussed by Levy and Christoff (1983) and will not be further discussed here.
Concentration dependence of the perchlorate extract-induced inhibition of G6PDH

The activity of G6PDH was determined in the presence and absence of HCIO₄ extracts, as detailed in II.B.1.a, obtained from fed (●) and 48h starved (○) rats. The enzyme source was a dialysed high speed supernatant obtained from fed rats.
Comparison of the inhibitor in the perchlorate extract with oleoyl-CoA

The effect of the inhibitor in the perchlorate extract described above on the pH profile of rat liver G6PDH was determined and compared to that found with oleoyl-CoA, since fatty-acyl CoA derivatives have been reported to be effective inhibitors of G6PDH (Eger-Neufeldt et al., 1965; Taketa and Pogell, 1966).

Both inhibitors were found to be most effective at alkaline pH values (Fig. 3.3b), although the profiles did not exactly parallel each other; the inhibitor in the HClO₄⁻ extract was more effective than oleoyl-CoA at the lower pH values.

The concentration of long chain acyl-CoA derivatives in rat liver has been reported to be about 0.03 μmol g⁻¹ fresh weight, and this increases to 0.1 μmol g⁻¹ liver in starved or fat-fed rats (Fritz et al., 1974). Furthermore it has been stated that the most abundant forms of long chain acyl-CoAs in vivo are palmitoyl-CoA and oleoyl-CoA (Tippett and Neet, 1982a,b). This implies that the final concentration of oleoyl-CoA in the HClO₄⁻ extract would be expected to be less than 0.25 μM. In the present study the Kᵢ for oleoyl-CoA was found to be about 26 μM (Fig. 3.4a). This suggests that oleoyl-CoA is unlikely to be the inhibitor in the perchlorate extract. However, the possibility remains that palmitoyl-CoA (Kᵢ value 0.6 μM as determined by Eger-Neufeldt et al., 1965) may be responsible for the inhibition. It is also possible that the inhibition was due to fatty acids, which are also reported to inhibit G6PDH (Yugari and Matsuda, 1967). That some of the inhibition was due to fatty acid or to fatty acyl-CoA was supported by the observation that addition of bovine serum albumin (fatty acid free) to a final concentration of 0.75% reversed the inhibition partially.
Enzyme activity was measured at the appropriate pH in BTP/HCl buffer with no effectors (O), 50µM oleoyl-CoA (A) or HClO₄⁻ extract (●). G6PDH was partially purified (DEAE-cellulose eluate) as described in II.B.4.a(ii) and assayed as in II.B.1.a. The concentration of NADP⁺ and G6P in the assay mixture was 100µM and 1.5mM, respectively.
To determine the degree of inhibition induced by oleoyl-CoA, desalted, purified yeast G6PDH (Sigma product) was used. It was assayed in the same manner as rat liver G6PDH (II.B.1.a) at pH 7.5. The reaction cuvette contained 2 ng of enzyme protein per ml of reaction mixture.

Fig. 3.4a: Oleoyl-CoA-induced inhibition of G6PDH activity
However, when the degree of inhibition by extracts from livers of fed and starved (48h) rats were compared it was found that the degree of inhibition obtained with equivalent amounts of tissue was greater with extracts from fed rats than from starved ones (Fig.3.3a). This is contrary to what one would expect if the inhibition was due to fatty acyl-CoA or to free fatty acids, since these compounds are increased by starvation (Bortz and Lynen, 1963; Garland and Tubbs, 1963; Wieland, 1964). This observation suggests that the inhibitor is not a fatty acyl-CoA or a fatty acid, and further work is required in order to identify the inhibitor.

b. The effect of oleoyl-CoA on the kinetic parameters of G6PDH from rat liver

Inhibition of liver G6PDH by acyl-CoA has been reported previously, but most studies of the effect of the inhibition on the kinetic properties have been on the purified yeast enzyme. A physiological concentration of acyl-CoA (50μM) was selected in order to examine the effect of acyl-CoAs on the kinetic parameters of partially purified (DEAE-cellulose eluate) rat liver G6PDH preparation. Oleoyl-CoA was used and this concentration of the inhibitor appeared to exert a potent inhibition (Table 3.6).

A plot of [S]/v against[S] indicated that the $K_m$ for NADP$^+$ was increased from 22μM to about 60μM and that the $V_{max}$ was lowered to 6% of the original value (Fig.3.4b). Similarly, the $K_m$ for G6P was increased from 42μM to 280μM (results not shown), and the $V_{max}$ reduced to 29% of the control value. The inhibition is therefore of the mixed type (Dixon and Webb, 1964) with respect to both NADP$^+$ and G6P. In contrast, Eger-Neufeldt (1965) reported that the acyl-CoA-induced inhibition only affected the $V_{max}$ of G6PDH from yeast when NADP$^+$ was varied.
Table 3.6: The oleoyl-CoA induced inhibition of rat liver G6PDH

<table>
<thead>
<tr>
<th>Concentration of NADP⁺ (μM)</th>
<th>Concentration of G6P (mM)</th>
<th>Control rate (nmol min⁻¹ mg⁻¹ protein)</th>
<th>Inhibited rate (nmol min⁻¹ mg⁻¹ protein)</th>
<th>% ( V_1/V_0 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>1.50</td>
<td>66</td>
<td>5</td>
<td>7.7</td>
</tr>
<tr>
<td>40</td>
<td>1.50</td>
<td>72</td>
<td>6</td>
<td>8.3</td>
</tr>
<tr>
<td>60</td>
<td>1.50</td>
<td>172</td>
<td>11</td>
<td>6.6</td>
</tr>
<tr>
<td>80</td>
<td>1.50</td>
<td>249</td>
<td>10</td>
<td>3.9</td>
</tr>
<tr>
<td>100</td>
<td>1.50</td>
<td>222</td>
<td>15</td>
<td>6.7</td>
</tr>
<tr>
<td>200</td>
<td>1.50</td>
<td>223</td>
<td>15</td>
<td>6.7</td>
</tr>
<tr>
<td>100</td>
<td>0.02</td>
<td>60</td>
<td>7</td>
<td>11.0</td>
</tr>
<tr>
<td>100</td>
<td>0.04</td>
<td>90</td>
<td>7</td>
<td>7.3</td>
</tr>
<tr>
<td>100</td>
<td>0.08</td>
<td>120</td>
<td>7</td>
<td>5.5</td>
</tr>
<tr>
<td>100</td>
<td>0.16</td>
<td>120</td>
<td>25</td>
<td>20.5</td>
</tr>
<tr>
<td>100</td>
<td>0.50</td>
<td>126</td>
<td>18</td>
<td>14.3</td>
</tr>
<tr>
<td>100</td>
<td>1.00</td>
<td>159</td>
<td>60</td>
<td>37.7</td>
</tr>
<tr>
<td>100</td>
<td>1.50</td>
<td>180</td>
<td>55</td>
<td>30.6</td>
</tr>
</tbody>
</table>

The concentration of NADP⁺ and G6P were varied as shown in the table, and the activity of G6PDH, in the presence and absence of oleoyl-CoA (50μM), determined as in II.B.1.a. The enzyme source was the DEAE-cellulose eluate (II.B.4.a(ii)). The assay was performed at pH 7.5 and 25°C with a 2min pre-incubation period.
Fig. 3.4b: Determination of the kinetic parameters of G6PDH in the presence of oleoyl-CoA at subsaturating concentrations of NADP^+.

The data used is the same as that recorded in Table 3.6.
Pre-incubation with NADP⁺ (Table 3.6) appeared to protect the enzyme from complete inhibition by oleoyl-CoA. It is known that NADP⁺ protects G6PDH against inactivation; for instance NADP⁺ has been shown to antagonise the fatty acid-induced disaggregation of crystalline rat liver G6PDH (Yugari and Matsuda, 1967) and to protect against protein-induced inactivation of the enzyme (Bonsignore and DeFlora, 1972).

The physiological significance of the acyl-CoA-induced inhibition of various enzymes has been questioned because of the lack of specificity with respect to the enzymes inhibited. It has been postulated that the inhibition of numerous unrelated enzymes by the acyl-CoAs can be attributed to their detergent properties (Tippett and Neet, 1982a,b). However, recent work by these authors with glucokinase suggests that the inhibition by acyl-CoAs of this enzyme is an allosteric, site-specific process. On the basis of their results, they have proposed a physiological role for palmitoyl- and oleoyl-CoA in vivo. Further work is necessary to determine whether the inhibition of G6PDH described in the present study is physiologically relevant.

4. ATP and the PPP dehydrogenases

ATP has been reported to be an inhibitor of rat liver G6PDH and 6PGDH (Kauffman et al., 1979). However conflicting observations have been reported for G6PDH (Bonsignore and DeFlora, 1972) and it has been argued that the effect of ATP may depend on the degree of purity of the enzyme. Avigad (1966), using a crude rat liver preparation as the enzyme source, claimed an inhibition of G6PDH by ATP. Similarly, Kauffman et al. (1979) using partially purified preparations, were able to demonstrate an effect of ATP on both G6PDH and 6PGDH. On the other hand, Yugari and Matsuda (1967) using extensively purified rat liver G6PDH failed to demonstrate any inhibitory effect by ATP on G6PDH, although ATP was shown to
facilitate the fatty acid-induced inhibition of crystalline rat liver G6PDH.

Avigad (1966) also reported that the ATP effect was more pronounced at neutral pH values than at pH values greater than 8.0, and further postulated that the effect may be dependent on the concentration of NADP⁺. This inhibition was found to be substantially reduced in the presence of Mg²⁺, an observation which has since been confirmed by others.

In the present study the effect of ATP-Mg on both 6PGDH and G6PDH from rat liver and yeast has been determined.

a. G6PDH:

Table 3.7 shows the effect of ATP-Mg on purified G6PDH from yeast and a partially purified rat liver enzyme preparation. The yeast enzyme was found to be more susceptible to inhibition than the liver enzyme. The physiological concentration of ATP in the liver cell has been reported to be \(3.42 \mu\text{mol g}^{-1}\) liver wet weight (Van Schaftingen et al., 1980a). At a similar concentration of ATP-Mg the rat liver enzyme was less than 33% inhibited. In contrast the yeast enzyme was almost 50% inhibited.

The physiological relevance of this ATP-induced inhibition of rat liver G6PDH is difficult to ascertain in view of the known potent inhibition of G6PDH at the calculated in vivo NADPH / NADP⁺ ratio, which has been discussed previously. Fig. 3.6a shows almost complete inhibition of G6PDH at NADPH / NADP⁺ ratios which are much lower than those found in the cell. With this consideration in mind, the effect of ATP-Mg on an NADPH-inhibited rat liver G6PDH preparation was determined (Table 3.8). Further inhibition by ATP-Mg
Table 3.7: The effect of ATP-Mg on G6PDH from yeast and rat liver

<table>
<thead>
<tr>
<th>ATP-Mg (mM)</th>
<th>Yeast G6PDH % V&lt;sub&gt;max&lt;/sub&gt;</th>
<th>Rat liver G6PDH % V&lt;sub&gt;max&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.03</td>
<td>100</td>
<td>N.D.</td>
</tr>
<tr>
<td>0.15</td>
<td>100</td>
<td>N.D.</td>
</tr>
<tr>
<td>0.3</td>
<td>96</td>
<td>N.D.</td>
</tr>
<tr>
<td>0.6</td>
<td>97</td>
<td>N.D.</td>
</tr>
<tr>
<td>1.2</td>
<td>85</td>
<td>N.D.</td>
</tr>
<tr>
<td>1.5</td>
<td>64</td>
<td>82</td>
</tr>
<tr>
<td>3.0</td>
<td>52</td>
<td>77</td>
</tr>
<tr>
<td>4.5</td>
<td>N.D.</td>
<td>67</td>
</tr>
<tr>
<td>6.0</td>
<td>N.D.</td>
<td>65</td>
</tr>
<tr>
<td>7.5</td>
<td>N.D.</td>
<td>56</td>
</tr>
</tbody>
</table>

N.D. Not determined

The activity of yeast and rat liver G6PDH was assayed at pH 7.4 and 25°C in the presence of saturating concentrations of NADP<sup>+</sup> (140μM) and G6P (1.33mM). The specific activity of the rat liver enzyme was 0.63μmol min<sup>-1</sup> mg<sup>-1</sup> protein, being a Blue Sepharose CL-6B eluate (II.B.4.a.1). The final concentration of crystalline yeast G6PDH protein (Sigma product) was 9 ng ml<sup>-1</sup> per assay.
Table 3.8: The effect of ATP-Mg on the NADPH-inhibited G6PDH from rat liver

<table>
<thead>
<tr>
<th>ATP-Mg concn. (mM)</th>
<th>% ( V_{\text{max}} ) determined at pH 7.1</th>
<th>% ( V_{\text{max}} ) determined at pH 8.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1.5</td>
<td>80</td>
<td>115</td>
</tr>
<tr>
<td>2.5</td>
<td>92</td>
<td>115</td>
</tr>
<tr>
<td>3.5</td>
<td>92</td>
<td>116</td>
</tr>
<tr>
<td>4.5</td>
<td>80</td>
<td>131</td>
</tr>
<tr>
<td>5.0</td>
<td>80</td>
<td>108</td>
</tr>
</tbody>
</table>

The assay was performed as in Table 3.7 except that 50\( \mu \text{M} \)-NADP\(^+\) and 100\( \mu \text{M} \)-NADPH were used, and the reaction was started with 100\( \mu \text{M} \)-G6P. The rat liver enzyme preparation was as in Table 3.7.
was found at pH 7.1, but not pH 8.0.

The data shown above indicates that ATP-Mg may exert a small inhibitory effect in vivo, but the NADPH / NADP⁺ ratio appears to be much more important. Substantial inhibition of the liver enzyme has only been demonstrated with free ATP but since ATP exists in the cell as the Mg²⁺ complex, and since ATP-Mg exhibits a reduced inhibitory effect, ATP would not be expected to exert a large inhibitory effect on G6PDH in vivo. The possibility of the existence of isoenzymes of G6PDH with different kinetic properties warrants further investigation as it was observed that some fractions eluted from Blue Sepharose columns with different concentrations of NADP⁺ and KCl did not appear to be sensitive to inhibition by ATP-Mg. That the multiple molecular forms of G6PDH may respond differently to regulatory effectors is supported by the work of Martins et al. (1985) who have shown the presence of three different molecular forms of G6PDH which respond to varying degrees when animals are treated with insulin. All three dimer species are increased by insulin treatment but it was found that electrophoretic forms I, II and III, which correspond to the dimeric forms of G6PDH, increased 5, 3 and 2-fold respectively. These authors also showed that starvation (72h) only decreased the amounts of enzyme corresponding to bands II and III. Although these observations are related to the long-term regulation of G6PDH it would be interesting to know if multiple molecular forms are important in the short-term regulation.

b. 6PGDH:

6PGDH from rat liver has been reported to be more sensitive to ATP inhibition than G6PDH (Kauffman et al., 1979), the $K_i$ for the latter enzyme being 16-fold higher than that for 6PGDH. Again these figures apply to free ATP and not ATP-Mg. In the present study, an inhibition of the rat liver enzyme by ATP-Mg could not be demonstrated.
in the presence (Fig. 3.5) or absence (Table 3.9) of NADPH. Some inhibition was obtained with crystalline yeast (Sigma) 6PGDH (Table 3.9). This inhibition of yeast 6PGDH by ATP-Mg could only be demonstrated on concentrations of 6PG that were less than 500\(\mu M\).

An overview of the results obtained in this section suggests that since ATP \textit{in vivo} exists to a considerable extent chelated with Mg\(^{2+}\) (Alberty, 1968; Rose, 1968; Veloso et al., 1975), it is unlikely to be of general regulatory significance as far as rat liver G6PDH and 6PGDH are concerned.

5. Phosphorylated hexose metabolites and the PPP dehydrogenases

The effect of hexose phosphates and inorganic phosphate on G6PDH and 6PGDH was investigated. It was found that G6PDH activity was not affected by any of these compounds which included F1P (up to 100\(\mu M\)), F6P (up to 5\(\mu M\)), F1,6P\(_2\) (up to 5\(\mu M\)), F2,6P\(_2\) (up to 12\(\mu M\)) and G1,6P\(_2\) (up to 100\(\mu M\)), at pH 7.0.

It was, however, shown that G1,6P\(_2\) does inhibit 6PGDH, confirming the observations made by Beitner and Nordenberg (1979). Table 3.11 shows the results obtained for the rat liver enzyme. The liver enzyme preparation in the present study was somewhat less sensitive to inhibition than that used by Beitner and Nordenberg (1979); these authors recorded a 77\% inhibition at 100\(\mu M\) G1,6P\(_2\), whereas the enzyme in the present study was only 43\% inhibited. The only difference between their experimental conditions and those used here was that they used Tris-HCl buffer, whereas bis-tris propane-HCl was used in the present study. Beitner and Nordenberg (1979) reported a cellular concentration of G1,6P\(_2\) of 6.3 \(\pm\) 0.3\(\mu M\) in rat liver. This concentration of G1,6P\(_2\) would be expected to cause an inhibition of less than 18\% (Table 3.11). The physiological range of G1,6P\(_2\) concentration was reported to be 9–30\(\mu M\) in
Fig. 3.5: The effect of ATP-Mg on NADPH-inhibited 6PGDH

ATP-Mg (1.5mM) was added to the incubation mixture of 6PGDH (II.B.1.b) in the presence of NADP⁺ (50μM) and the indicated concentration of NADPH, where used. The assay was performed at pH 7.0 and 25°C, and the graph shows the effect of ATP-Mg (•) on the NADPH-inhibited enzyme (○). The enzyme source was the 50-70% (NH₄)₂SO₄ fraction (II.B.4.b.).
Table 3.9: The effect of ATP-Mg on crystalline yeast and partially purified rat liver 6PGDH

<table>
<thead>
<tr>
<th>ATP-Mg (mM)</th>
<th>% V/V₀ (yeast)</th>
<th>% V/V₀ (liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.5</td>
<td>87</td>
<td>105</td>
</tr>
<tr>
<td>1.5</td>
<td>80</td>
<td>95</td>
</tr>
<tr>
<td>2.5</td>
<td>N.D.</td>
<td>116</td>
</tr>
<tr>
<td>5.0</td>
<td>67</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D. Not determined

Yeast (Sigma product) and rat liver 6PGDH (DEAE-cellulose eluate) activity was determined as in II.B.1b in the presence of the indicated concentration of ATP-Mg; ADP and AMP were absent from the incubation medium.
rat liver by the same authors. Beitner and Nordenberg (1979) have proposed that the diminished levels of G1,6P2 in the dystrophic muscle may be responsible for the diversion of glucose from the PPP to glycolysis.

In the present study it was found that an NADPH-inhibited 6PGDH was insensitive to further inhibition by G1,6P2 (100μM) (Table 3.11). Since 6PGDH is known to be inhibited by NADPH in vivo, the inhibitory effect of G1,6P2 on 6PGDH activity would appear to be of limited relevance, in the absence of mechanisms to enhance this inhibitory effect.

Since G1,6P2 was shown to inhibit 6PGDH in vitro, and since F1,6P2 has been reported to be an inhibitor of sheep liver 6PGDH (Dyson and D'orazio, 1971), the effect of F1,6P2 and F2,6P2 on 6PGDH from rat liver was determined. However, it was found under the experimental conditions used in this study that F1,6P2 had no effect at concentrations up to 5mM. The K_i reported for the sheep liver enzyme by Dyson and D'orazio (1971) was 70.8μM, a physiologically relevant concentration (Van Schaftingen et al., 1980a). The reasons for the lack of effect of F1,6P2 on the rat liver enzyme (Table 3.12) are not clear, but Procsal and Holten (1972) also failed to demonstrate an inhibitory effect of this compound on 6PGDH. Similarly, F2,6P2 failed to inhibit rat liver 6PGDH (Table 3.12).

It was thought that F2,6P2 could have a role in the regulation of 6PGDH because of its structural similarity to G1,6P2, but as the results obtained indicate, F2,6P2 does not appear to affect 6PGDH activity in vitro. Furthermore, F2,6P2 did not enhance or reverse enzyme inhibition by various metabolites as discussed previously.
Table 3.11: The effect of glucose 1,6-bisphosphate on 6PGDH activity

<table>
<thead>
<tr>
<th>G1,6P2 (μM)</th>
<th>6PGDH activity (nmol min⁻¹ mg⁻¹ protein)</th>
<th>% v/v₀</th>
<th>% v/v₀*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9.5</td>
<td>100</td>
<td>39</td>
</tr>
<tr>
<td>12.5</td>
<td>7.7</td>
<td>81</td>
<td>39</td>
</tr>
<tr>
<td>25</td>
<td>7.0</td>
<td>74</td>
<td>39</td>
</tr>
<tr>
<td>50</td>
<td>6.0</td>
<td>63</td>
<td>39</td>
</tr>
<tr>
<td>100</td>
<td>5.4</td>
<td>57</td>
<td>39</td>
</tr>
</tbody>
</table>

* in the presence of NADPH

6PGDH activity was measured at pH 7.6 (BTP/HCl, 0.2M) in the presence of 100 μM-NADP⁺, 100μM-6PG, 1mM-Mg Ac and 50μl of 6PGDH. The enzyme was partially purified as in II.B.4.b, and was assayed at 25°C. v₀ is the velocity in the control cuvette i.e. in the absence of G1,6P₂. V is the velocity at a given concentration of G1,6P₂. The results are the mean of three experiments. In experiments with NADPH, 50μM-NADP⁺ and 100μM-NADPH was used.
Table 3.12: F1,6P2, F2,6P2, and 6PGDH activity

<table>
<thead>
<tr>
<th>F2,6P2 (µM)</th>
<th>% V/Vo</th>
<th>F1,6P2 (µM)</th>
<th>% V/Vo</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>113</td>
<td>25</td>
<td>90</td>
</tr>
<tr>
<td>2</td>
<td>106</td>
<td>50</td>
<td>89</td>
</tr>
<tr>
<td>3</td>
<td>106</td>
<td>100</td>
<td>89</td>
</tr>
<tr>
<td>6</td>
<td>107</td>
<td>200</td>
<td>95</td>
</tr>
<tr>
<td>12</td>
<td>116</td>
<td>250</td>
<td>93</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>500</td>
<td>89</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>1000</td>
<td>100</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>5000</td>
<td>110</td>
</tr>
</tbody>
</table>

6PGDH activity (II.B.4b) was determined as in II.B.1b in the presence of 100µM-NADP* and 100µM-6PG at pH 7.0. F1,6P2 and F2,6P2 were varied as indicated.
6. Concluding remarks

Although a wide variety of compounds have been tested for the ability to reverse the well-established NADPH-induced inhibition of G6PDH and 6PGDH (Fig. 3.6) in the present study, no de-inhibition has been demonstrated.

In physiological terms it is possible that the inhibition of the PPP dehydrogenases is essential in the resting cell in order to prevent the over-production of NADPH. It is conceivable that in the normal resting fed cell NADP⁺-dependent malic enzyme and isocitrate dehydrogenase could supply all the necessary NADPH, assuming that these two enzymes are able to function in vivo. The effect of NADPH / NADP⁺ ratios on the activity of these two enzymes is not well documented although inhibition of both enzymes by NADPH has been reported, with $K_i$ values of 30 and 10μM for malic enzyme and isocitrate dehydrogenase, respectively (Fabregat et al., 1985). Nevertheless, the activity of cytosolic NADP⁺-isocitrate dehydrogenase is one order of magnitude higher than the PPP dehydrogenases so that one would expect this enzyme to produce more NADPH even if both enzymes were inhibited to the same extent in vivo. Hence the enzyme could contribute sufficient NADPH to the liver cytosol in concert with malic enzyme and the PPP dehydrogenases to meet the demands of the resting cell, even though the flux through the latter would be low. On the other hand increased demand for NADPH, such as is the case during enhanced lipogenesis or mixed function oxidation, would cause an alteration in the NADPH / NADP⁺ ratio which would favour the activation of the PPP dehydrogenases. This view is supported by recent work by Fabregat et al. (1985) who have shown that inhibition or activation of NADPH-consuming pathways correlate with the activity of the PPP, and that NADPH / NADP⁺ ratios are inversely proportional to the activity of the PPP. Hence, an active PPP, in conjunction with the other NADPH-producing enzymes would meet the extra requirement for NADPH and
Fig. 3.6: The NADPH-induced inhibition of 6PGDH and G6PDH

The effect of NADPH on G6PDH (a) and 6PGDH (b) was determined. Partially purified G6PDH (specific activity 0.24 μmoles min⁻¹ mg⁻¹ protein) and 6PGDH (0.4 μmol min⁻¹ mg⁻¹ protein) were assayed in the presence of varying concentrations of NADPH as shown. G6PDH activity was determined at pH 7.4 in the presence of 70μM-NADP⁺ and 100μM-G6P. 6PGDH was assayed at pH 7.0 in the presence of 100μM-6PG and 50μM-NADP⁺. BTP/HCl was the buffer and the experiments were performed at 25°C. The results are the mean of three readings.
restore the \( \text{NADPH} / \text{NADP}^+ \) ratio. The latter effect would, in turn, reduce the flux through the PPP dehydrogenases and possibly the other NADPH producing enzymes.

C. ISOLATION AND CHARACTERIZATION OF A CYTOSOLIC NADPH-CONSUMING REACTION

In the course of trying to find a de-inhibitor for the PPP dehydrogenases, a major NADPH-utilizing reaction has been characterized. The procedure used to obtain liver cytosolic extracts containing the reactive components of this reaction was essentially that of Dunaway and Segal (1976). These authors purified what they called 'a peptide stabilizing factor of rat liver phosphofructokinase'.

This stabilizing factor, reported to have a molecular weight of 3800 (Dunaway and Segal, 1976), shares some common properties both with \( \text{F}_2\text{,6P}_2 \) and the cofactor reported (Eggleston and Krebs, 1974) to act in conjunction with GSSG in reversing the NADPH-induced inhibition of the PPP dehydrogenases.

The peptide stabilizing factor of PFK-1 was reported to contain glutamate, glycine and a half-cystine (1 : 0.96 : 0.85). However, Dunaway and Segal (1976) dismissed the possibility that this peptide was identical to GSSG or GSH. Like the cofactor reported by Eggleston and Krebs (1974), the peptide stabilizing factor was shown to be acid labile, sensitive to storage at \(-20^\circ\text{C}\) and heat labile. On the other hand, Van Schaftingen and Hers (1983) have claimed that the so-called peptide is in fact \( \text{F}_2\text{,6P}_2 \) since the peptide stabilizing factor of PFK-1 co-migrates with \( \text{F}_2\text{,6P}_2 \) on gel filtration. In addition, like \( \text{F}_2\text{,6P}_2 \), the peptide (i) stimulates PFK-1 by increasing the affinity of the enzyme for \( \text{F}_6\text{P} \), (ii) is an inhibitor of \( \text{F}_1\text{,6BPase} \) and (iii) is acid labile. However, more recent work by Kruep
and Dunaway (1984a,b) provides further support for the existence of two peptide stabilizing factors, neither of which is $F_26P_2$.

The common properties shared by $F_26P_2$, the PFK-1 stabilizing peptide and the cofactor reported by Eggleston and Krebs (1974) justified an investigation into the effect of the PFK-1 stabilizing factor on the PPP dehydrogenases.

However, in the present study, it was demonstrated that addition of heat treated liver extracts, which were devoid of $G_6PDH$ and $6PGDH$ activity, to the reaction mixtures of either of these enzymes results in an apparent concentration-dependent inhibition of their activities (Fig.3.7a). Upon further investigation it became evident that (a) constituent(s) of the heat extracts remove(s) the NADPH produced by the action of the dehydrogenases. The basis of this conclusion was the observation that addition of exogenous NADPH to the heat extract in the absence of added enzyme and substrate resulted in a rate of NADPH oxidation faster than the activity of $G_6PDH$ (Fig.3.7b). Furthermore, it was observed that the inhibition only occurred for a limited time period and that this time period was dependent upon the volume of the heat extract added to the incubation mixture (Fig.3.7c). This latter fact suggested that the substance(s) responsible for the inhibition was being removed by some other reaction or that an opposing reaction was occurring concurrent to the $G_6PDH$-catalysed reaction, and that the former reaction was running out of substrate, when the apparent inhibition ceased. Since NADPH production was being followed at 340nm, it seemed likely that the apparent inhibition could be due to a reaction utilizing the NADPH produced by the $G_6PDH$-catalysed reaction, and hence causing a zero net change in the absorbance. This theory was shown to be the case by the results shown in Figs.3.7b, and 3.8a.
Fig. 3.7a, b, c and d.
Fig. 3.7a: Apparent inhibition of the PPP dehydrogenases by the heat extract

Rat liver G6PDH (0) and 6PGDH (•) were assayed as in II.B.1a and b, respectively, in the presence of the indicated volumes of the heat extract (II.B.4.d), at pH 7.5 in the presence of 100μM-6PG or G6P and 100μM-NADP⁺. Both G6PDH and 6PGDH were partially purified by PEG-6000 precipitation (3.5 - 20%) followed by DEAE-cellulose chromatography.

Fig. 3.7b: NADPH oxidation by the heat extract

The activity of G6PDH (0) assayed as described above and the oxidation of NADPH (100μM) (•) by a constituent of the heat extract (50μl) were followed at 340nm.

Fig. 3.7c: The relationship between the volume of the heat extract added and the"activity"of rat liver G6PDH

Rat liver G6PDH activity was determined in the absence (△) of or in the presence of either 50μl (0) or 100μl (•) of the heat extract. The conditions of assay were the same as in Fig. 3.7a.

Fig. 3.7d: Estimation of the molecular weight of the apparent inhibitor of G6PDH

The heat extract (1ml) was applied to a Bio-Gel P-2 column (Void volume 50.5ml) which was standardised and equilibrated with 20mM-BTP/HCl, pH 8.3 containing 1mM-EDTA and eluted with the same buffer. The volume of fractions collected was 3ml.
Fig. 3.8a: The enzyme-substrate velocity curve

The curve shows the effect of varying the volume of the combined enzyme-substrate complex present in the heat extract, and assaying for NADPH-oxidising activity (II.B.1.e). The heat extract used contained 12mg ml\(^{-1}\) of protein.
Table 3.13: Effect of the substrate moiety on G6PDH activity

<table>
<thead>
<tr>
<th>Additions</th>
<th>G6PDH activity (nmol min$^{-1}$ mg$^{-1}$ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt. I</td>
</tr>
<tr>
<td>Control incubation</td>
<td>3.9 ± 0.0</td>
</tr>
<tr>
<td>Control incubation + substrate (100μl)</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>Control incubation + NADPH (100μM)</td>
<td>N.D.</td>
</tr>
<tr>
<td>Control incubation + NADPH (100μM) + substrate (100μl)</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D. Not determined

The G6PDH sources were DEAE-cellulose preparations (II.B.4a(ii)) and the substrate was the Dowex 50 H$^+$ eluate (cf. C.2.c.). The assay conditions were as in II.B.1a except that 100μM NADPH and 100μM NADP$^+$ were present. The final concentration of the substrate was estimated to be about 116μM NADPH equivalents. The results are expressed as the mean ± S.D. (n=3). Experiments I and II were done at different times with different preparations of enzyme.
The NADPH-oxidising activity, as determined by the apparent inhibition of G6PDH activity by a constituent of the heat extracts, was eluted from both Sephadex G-25 and Bio-Gel P-2. On Sephadex G-25 two peaks of "inhibitory activity" were obtained. The first peak appeared 16ml after the void volume (36ml) and the second at 36ml after the void volume. On the Bio-Gel P-2 column (void volume 50.5ml) the "inhibitory activity", which was measured by the degree of inhibition of G6PDH, eluted as a single peak 8ml after the void volume. This corresponded to a molecular weight of about 680 (Fig.3.7d).

Since the component of the heat extract was initially identified by its capacity to negate the G6PDH (or 6PGDH) activity, the effect of the components of this constituent (C.3) on G6PDH activity was determined. The fraction containing the enzyme (cf. C.3b) had no effect, whereas the substrate moiety (cf. C.3c) exhibited a slightly inhibitory effect (Table 3.13). Francis and Ballard (1980a,b) have reported the existence of a protein inhibitor of G6PDH, as discussed previously. However, it is unlikely that this is identical to the substrate found in the present study, since the degree of inhibition induced by the protein reported by Francis and Ballard is much greater.

1. Preparation of crude liver extracts containing the NADPH-oxidising activity

Three month old female rats were either anaesthetized or killed by cervical dislocation. The livers were removed, weighed and homogenised in 20mM-bis-tris propane (BTP/HCl) pH 8 containing 1mM-EDTA (pH 7.0) and 0.25M-sucrose (1 : 2, w/v). The homogenate was centrifuged at 100,000g for 90min. The resulting supernatant was heated in a water bath to 60°C for 1min and cooled on ice. The denatured protein was removed by further centrifugation at 100,000g for 30min. The supernatant was used as
the source of the component under study and is referred to as the "heat extract". The NADPH-consuming component of the extract was determined by following the oxidation of NADPH in BTP/HCl buffer, pH 7.5, in the presence of the heat extract.

a. The stabilizing effect of EDTA

When EDTA was excluded from the homogenisation buffer but added to the reaction mixture, the NADPH-oxidising activity was not affected. However, when different concentrations of EDTA were added to portions of this extract, and stored for 24h at -20°C (Table 3.14), it was found that concentrations of EDTA up to 3mM conferred some stability upon the NADPH-oxidising activity (Table 3.14). Higher concentrations of EDTA caused an inhibition of the activity. Since 1mM-EDTA was as effective as 3mM-EDTA in stabilizing the activity 1mM-EDTA was routinely included in the homogenising buffer.

b. Selection of pH of extraction

The NADPH-oxidizing activity was found to be abolished by pH values less than 3 and greater than 13 after 15min incubation at 25°C (results not shown). To determine the effect of the pH on the stability and abundance of the NADPH-oxidising activity, the extraction was performed at pH 8.3 and pH 10.0.

It was found that the pH 10 preparation did not lose activity after storage at -20°C for 24h. In the same time period and under the same conditions, the pH 8.3 extract lost 50% of the NADPH-oxidising activity. However at pH 10 protein denaturation was less than at pH 8.3. The pH 10 heat treated extract contained 20.9 ± 0.1 mg protein ml⁻¹ whereas the pH 8.3 extract had 12.6 ± 0.06 (n = 6) mg protein ml⁻¹. The total
Table 3.14: Stabilization of the NADPH-oxidising activity by EDTA after 24h storage at -20°C

<table>
<thead>
<tr>
<th>EDTA (mM)</th>
<th>Initial activity (nmol min⁻¹ mg⁻¹)</th>
<th>Activity after 24h (nmol min⁻¹ mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>27.2</td>
<td>9.8</td>
</tr>
<tr>
<td>1</td>
<td>27.2</td>
<td>15.7</td>
</tr>
<tr>
<td>3</td>
<td>27.2</td>
<td>15.2</td>
</tr>
<tr>
<td>4</td>
<td>27.2</td>
<td>11.5</td>
</tr>
<tr>
<td>5</td>
<td>27.2</td>
<td>8.8</td>
</tr>
</tbody>
</table>

The data represent the mean of triplicate readings determined at each of the five EDTA concentrations indicated. The heat treated extract was obtained as in II.B.4.d, with the important difference that EDTA was omitted from the extraction buffer. NADPH-oxidising activity was determined as in II.B.1.e. Portions of the extract (10ml) were treated with the various concentrations of EDTA and assayed for NADPH-oxidising activity before and after storage at -20°C for 24h.
activity as assayed at pH 6 and 7 (Fig. 3.8b) was slightly higher in the pH 10, than in the pH 8.3 extract and this difference was pronounced when the assay was performed at pH values greater than 7.5 (Fig. 3.8b). Since the latter extract contained less protein and since it was easier to adjust the pH of the extract to the pH of the assay, pH 8.3 was chosen for routine extraction purposes.

c. Selection of temperature of extraction

Fig. 3.9 shows the effect of temperature on the NADPH-oxidising activity in the crude heat extract. The rapid decline in activity at temperatures higher than 70°C clearly indicated that lower temperatures should be used for extraction. The loss of activity is presumably due to the denaturation of the enzyme moiety of this complex (cf. III C.2), since the purified substrate was later found to be stable under these conditions. Since the yield of the NADPH-oxidising activity did not apparently change between 60° and 70°, the former temperature was selected for routine extraction purposes.

d. Comparison of the effect of the heating process and ultrafiltration on the NADPH-oxidising activity.

To determine that the reaction was not an artifact resulting from the heating process, the heating step was omitted and ultrafiltration of high speed supernatants through Amicon filters with a nominal molecular weight cut-off of 10,000 was performed at 4°C.

The ultrafiltrate was tested for 6PGDH, G6PDH and NADPH-oxidising activity. 6PGDH (Mr 102,000) and G6PDH (Mr 110,000) activity were excluded from the ultrafiltrate, indicating that there was no leakage through the filters of higher molecular-weight substances. However, the NADPH-oxidising activity was found to be present.
The heat treated extracts obtained at pH 10 (●) and pH 8.3 (○) were assayed for NADPH-oxidising activity at the pH values indicated as in II.B.1.e. The extracts were adjusted to pH 7.5 with 6N-HCl, and in the case of the pH 10 extract, solid BTP was added to a final concentration of 20mM to maintain the pH at 7.5. The pH 10 extract was prepared in glycine/NaOH buffer (II.B.4.d).
Fig. 3.9: Effect of temperature on the NADPH-oxidising activity

The heat treated extract was further incubated at the indicated temperature and at pH 8.3 for 1 min, cooled on ice and assayed for NADPH-oxidising activity as in II.B.1.e. The points represent the mean ± S.D (n=3).
This experiment excluded the possibility that the reaction was an artifact of the heating process. Ultrafiltration could not, however, be used routinely as the filters were blocked by the protein in the high speed supernatant. The volume of extract that could be obtained by this method was, therefore, restricted to a maximum of about 10ml. Hence the heating process was used for routine purposes.

e. Intracellular localization of the NADPH-oxidising activity.

The rat liver homogenate was prepared as described above (C.I) and fractionated by differential centrifugation (II.B.4.e) to give nuclear, microsomal, mitochondrial and soluble fractions. The particulate fractions were re-suspended in BTP/HCl pH 8.3 and sonicated for 30s (2x15s) at maximum amplitude at 4°C and assayed for NADPH-oxidising activity after heating each extract to 60°C for 1min, cooling on ice, centrifuging and discarding the precipitate formed. There was no detectable activity in any of these fractions (results not shown) and it was therefore concluded that the reaction was associated exclusively with the cytoplasmic compartment.

As a result of the observations made in this section of the work the NADPH-oxidising activity was routinely obtained from the soluble fraction of the rat liver homogenate. The extractions were performed at pH 8.3, in the presence of 1mM-EDTA, 0.25M-sucrose, and the high speed supernatants were heated to 60°C for 1min. Dialysis of the heat-treated extracts was found to result in a loss of the activity, presumably as the result of the dissociation of the enzyme-substrate complex.

2. Purification

In the early stages of the work the NADPH-oxidising activity was thought to be present on a single molecular
unit, as it could be isolated and partially purified in an intact state by two different purification schemes that would be expected to separate an enzyme from its substrate (Tables 3.16 and 3.17). However, at a later stage in the study a polyethylene glycol (PEG-6000) fractionation procedure was found to resolve the NADPH-oxidising activity into two fractions which were inactive separately but when re-combined were active in carrying out the oxidation of NADPH. Hence it was concluded that the heat-extract contained the enzyme-substrate complex and that the inactive fractions were the enzyme and the substrate moieties of the complex.

a. Purification of the enzyme-substrate complex

The results shown in Tables 3.16 and 3.17 are for the schemes for the attempted purification of the enzyme-substrate complex.

In scheme I the heat-treated extract was obtained as in C.1 and the activity and protein content determined. An equal volume of chilled ethanol was added to the extract and the resulting precipitate, which did not contain NADPH-oxidising activity, was discarded. The supernatant was then passed through an Amicon ultrafiltration filter with a nominal molecular weight cut-off of 10,000. At this step 44% of the initial activity was lost, compared to 20% activity lost between the heat step and the ethanol-precipitation stage. The larger loss at the ultrafiltration step could be attributed to the loss of some of the enzyme assuming that the $M_r$ of the enzyme is near to 10,000.

The ultrafiltrate was then treated with three volumes of chilled ethanol and the precipitated complex was collected by centrifugation and resuspended in bis-tris propane (20mM), EDTA (1mM), pH 8.3. This sample was
<table>
<thead>
<tr>
<th>Purification step</th>
<th>Fraction volume (ml)</th>
<th>Protein (mg ml(^{-1}))</th>
<th>Total protein (mg)</th>
<th>Enzyme activity (nmol min(^{-1}) ml(^{-1}))</th>
<th>Specific activity (nmol min(^{-1}) mg(^{-1}) protein)</th>
<th>Total activity (nmol)</th>
<th>Yield (%)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat for 1 min at 60°C</td>
<td>93</td>
<td>13.80</td>
<td>1283.4</td>
<td>360</td>
<td>26.1</td>
<td>33480</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>1:1 (v/v) chilled ethanol precipitation</td>
<td>186</td>
<td>2.96</td>
<td>550.6</td>
<td>144</td>
<td>48.6</td>
<td>26784</td>
<td>80</td>
<td>1.86</td>
</tr>
<tr>
<td>10,000 Mr cut-off ultrafiltration</td>
<td>186</td>
<td>2.40</td>
<td>446.4</td>
<td>65</td>
<td>27.1</td>
<td>12090</td>
<td>36</td>
<td>1.04</td>
</tr>
<tr>
<td>Ethanol (3v) precipitation followed by DEAE-Sephadex A-50</td>
<td>9</td>
<td>0.015</td>
<td>0.135</td>
<td>45</td>
<td>3000</td>
<td>405</td>
<td>1.2</td>
<td>115</td>
</tr>
</tbody>
</table>

The data represents the purification and yield of the NADPH-oxidising activity. The heat treated extract was assumed to contain 100% activity for calculation purposes as the activity could not be measured in the high speed supernatant and the assays were performed as in II.B.1e.
applied to a DEAE-Sephadex A-50 column (5ml capacity) and eluted in a stepwise manner with KCl (Fig. 3.10). The peak activity was obtained at a KCl concentration of 0.16M. The total activity obtained was only 1.2% of the initial activity but the protein content was very low after this step, hence the high specific activity observed. In retrospect the large loss in activity observed after ion-exchange chromatography was probably the result of enzyme denaturation or substrate loss. Nevertheless enough enzyme and substrate co-purified through the procedure to yield an active component.

In scheme II (Table 3.17) a polyethylene glycol precipitation followed the heat step. The heat extract obtained in the same manner as in scheme I, was treated with 10g/100ml of solid polyethylene glycol 6000. The precipitate was removed by centrifugation, and the supernatant, which contained the NADPH-oxidising activity was applied to a DEAE-Sephadex A-50 column (Fig. 3.11). It was found that only 10% of the activity was lost, and this loss could be attributed to the volume of the supernatant lost between transfers from tube to tube in the course of the experiment.

The sample applied to the DEAE-Sephadex A-50 column was eluted with a continuous gradient (0-1M) of KCl in a total volume of 300ml (Fig. 3.11). The enzyme-substrate complex was eluted between 0.48 and 0.60M KCl. The eluates containing activity were pooled, diluted with an equal volume of BTP/HCl, pH 8.3 and re-applied to a smaller Sephadex A-50 column and eluted with KCl (Fig. 3.12). In scheme II 96% of the NADPH-oxidising activity was lost at the first ion-exchange stage relative to the preceding stage, and a further 75% (compared to the first ion-exchange stage) was lost at the second ion-exchange step. These large losses are probably due to the loss of the enzyme or dissociation of the enzyme-substrate complex on the ion-exchangers, as found for scheme I.
The ethanol concentrated extract (5ml) (II.B.4.d.(i)) was passed down a small DEAE-Sephadex A-50 column (5ml capacity) equilibrated at pH 8.3 with BTP/HCl. Fractions (3ml) were collected and assayed for activity as in section II.B.1.e. The volume of eluate used was 350μl in a final volume of 1ml.
The figure shows the elution pattern of the NADPH-oxidising activity on DEAE-Sephadex A-50. The sample (90ml), which was the 10% PEG supernatant, was applied, after a two-fold dilution with BTP at pH 8.3, to the column. The column (2.5x18cm) had been previously equilibrated with BTP/HCl pH 8.3. The excess polyethylene glycol was washed off with BTP (until the flow through was clear) and a continuous gradient of potassium chloride (0-1M) applied in a total volume of 300ml. The flow rate was about 12ml h\(^{-1}\) and 3.3ml fractions were collected and assayed for NADPH-oxidising activity (II.B.1.e). The volume of eluate used in the assay was 300μl in a final volume of 1ml.
Fig. 3.11: Ion exchange chromatography on DEAE-Sephadex of the enzyme-substrate (continuous gradient)
**Fig. 3.11b**: Protein content and the activity profile of the NADPH-oxidising activity

The conditions of assay are as those described in the legend to Table 3.11. The sample material is the same as in Fig. 3.11 and the protein content (●) was estimated as detailed in II.B.2.d(i).
Fig. 3.12: Ion exchange chromatography of the enzyme-substrate complex (Scheme II)

A portion of the pooled DEAE-Sephadex A-50 column eluates (5ml), diluted with an equal volume of BTP/HCl pH 8.3, was applied to a smaller (5ml capacity) column. Fractions (3.3ml) were collected and assayed for protein content (▲) as in section II.B.2.d.(i) and NADPH-oxidising activity (●) as in II.B.1.e. The volume of eluate used in the assays was 250μl in a final volume of 1ml.
Table 3.17: Purification Scheme II

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Fraction volume (ml)</th>
<th>Protein (mg·ml⁻¹)</th>
<th>Total protein (mg)</th>
<th>Enzyme activity (nmol min⁻¹ ml⁻¹)</th>
<th>Specific activity (nmol min⁻¹ mg⁻¹ protein)</th>
<th>Total activity (nmol min⁻¹)</th>
<th>Yield (%)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat for 1 min at 60°C</td>
<td>100</td>
<td>12.4</td>
<td>1240</td>
<td>315.2</td>
<td>25.4</td>
<td>31520</td>
<td>100</td>
<td>1.00</td>
</tr>
<tr>
<td>10% PEG-6000 supernatant</td>
<td>90</td>
<td>7.8</td>
<td>702</td>
<td>315.2</td>
<td>40.4</td>
<td>28368</td>
<td>90</td>
<td>1.59</td>
</tr>
<tr>
<td>DEAE-Sephadex A-50 (continuous gradient 0-1M)</td>
<td>17</td>
<td>0.19</td>
<td>3.23</td>
<td>68.6</td>
<td>361</td>
<td>1166</td>
<td>3.70</td>
<td>14.21</td>
</tr>
<tr>
<td>DEAE-Sephadex A-50 (step-wise)</td>
<td>10</td>
<td>0.01</td>
<td>0.1</td>
<td>29.46</td>
<td>2950</td>
<td>295</td>
<td>0.93</td>
<td>116.14</td>
</tr>
</tbody>
</table>

The Table represents the yield and purification of the NADPH-oxidising activity. Experimental details of the procedure are as detailed in II.B.4d (1).
Both purification methods resulted in low yields with a purification factor of just over 100. The purification factor is expressed relative to the heat extract (which on average contained 20% of the protein content of the high speed supernatant) and is therefore an underestimate. Nevertheless, an attempt was made to improve the degree of purification and to minimise the apparent inactivation and loss of NADPH-oxidising activity. Since the minimum loss of activity occurred at the polyethylene glycol fractionation stage (Table 3.17), an attempt was made to further fractionate the sample at this point in the purification procedure. It was during this phase of the work that it was shown that polyethylene glycol treatment could resolve the complex into an enzyme and a substrate (Table 3.18).

The 10-30% (w/v) PEG-6000 precipitate was thought to contain the enzyme since the activity was lost when this fraction was subjected to temperatures greater than 70°C or to pH values less than 3 or greater than 11 (data not shown). These treatments did not affect the activity of the 30% PEG-6000 supernatant when added to an untreated 10-30% PEG fraction. The former was, therefore, thought to be the substrate-containing fraction.

Fig. 3.13a shows the substrate saturation curve of the enzyme. Since the molar concentration of the substrate was unknown, the amount of NADPH that could be oxidised by a given volume of the supernatant containing the substrate was determined. Hence the concentration of the substrate was expressed as "NADPH-equivalents". On this basis it was determined that if a 1 : 1 NADPH : substrate stoichiometry was assumed, the heat treated extract contained 1.07 ± 0.21 (n=6) nmoles of NADPH equivalents per ml of extract. The $K_m$ of the enzyme for the substrate was found to be about 80μM NADPH equivalents (Fig.3.13b). The $V_{max}$ under these conditions was found to be 133 nmoles min$^{-1}$ mg$^{-1}$ protein.
Table 3.18: Dissociation of the enzyme-substrate complex with PEG-6000

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Rate of NADPH oxidation (nmol min⁻¹ ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat extract</td>
<td>424.2</td>
</tr>
<tr>
<td>10% PEG supernatant</td>
<td>420.0</td>
</tr>
<tr>
<td>10-30% PEG precipitate</td>
<td>7.2</td>
</tr>
<tr>
<td>30% PEG supernatant</td>
<td>0.0</td>
</tr>
<tr>
<td>30% PEG supernatant + 10-30% PEG precipitate (equal volumes)</td>
<td>376.4</td>
</tr>
</tbody>
</table>

Polyethylene glycol-6000 (PEG) was added as a solid. The activity of each fraction was assayed as in II.B.1.e. The precipitate was resuspended in BTP/HCl pH 8.3 containing 1mM-EDTA to the original volume of the heat extract. This fractionation has been used routinely in the various purification procedures employed in the course of this work.
Fig. 3.13a: The substrate saturation curve of the NADPH-oxidising enzyme

The heat treated extract was treated with PEG-6000 as in Table 3.18. The 10 - 30% precipitate re-suspended in 20mM-BTP/HCl pH 8.3 containing 1mM-EDTA was used as the enzyme source. The 30% supernatant was the substrate source and the assay conditions were as in Table 3.18. The points are the mean ± S.D. (n=3).
Fig. 3.13b: Determination of $K_m$ of the enzyme for the substrate
For data refer to Fig. 3.13a
Having determined that the NADPH-oxidising activity was composed of an enzyme and a substrate, which are apparently very tightly bound together, an attempt was made to partially purify the enzyme and to identify the substrate.

b. Partial purification of the enzyme moiety

A partial purification of the NADPH-oxidising enzyme was attempted and the procedure adopted is recorded in detail in II.B.4.d (ii). The scheme involved four steps as shown in Table 3.19.

The heat treated extract was obtained as in III. C.1 and treated with 30g/100ml of PEG 6000. The precipitate was collected by centrifugation at 10,000g. This treatment separated the enzyme from the substrate. The precipitate, which was the enzyme-containing fraction was re-suspended in the original volume of 20mM-BTP/HCl, 1mM-EDTA, pH 8.3. The preparation was then treated with 10% (w/v) PEG 6000 and the resultant precipitate was discarded. The supernatant, was assayed for activity and then treated with solid PEG 6000 such that the solution was made 30% with PEG 6000. The 10-30% PEG 6000 precipitate was collected and suspended in the original volume of buffer. This fraction was assayed for enzyme activity. DEAE-cellulose chromatography was attempted and it was at this point that the greatest loss of activity occurred. Fig. 3.14 shows the elution profile of the enzyme upon DEAE-cellulose chromatography. Although a major peak of the enzyme activity was found to elute at 0.15M-NaCl, the possible existence of iso-enzymes is indicated by minor peaks at 0.19M and 0.21M NaCl. It would be of interest to determine whether the existence of these isoenzymes is related to their specificity for the cofactor or the substrate, or to their intracellular localization.

The overall purification was poor being only 12-fold
Fig. 3.14: DEAE-cellulose chromatography of the enzyme

The 10 - 30% PEG 6000 precipitate (see text) was resuspended in 80ml of 20mM-BTP/HCl pH 8.3 and 1mM-EDTA, pH 7.0. The sample was washed onto a DEAE-cellulose column, previously equilibrated with 20mM-BTP HCl pH 7.5, with an equal volume of buffer. The enzyme was eluted with a continuous linear gradient of NaCl (0-1M) in 20mM-BTP/HCl pH 7.5 and 1mM-EDTA. A total volume of 300ml was used for the elution. The activity of the enzyme was determined as in II.B.1.e. The protein content was determined as in II.B.2.d.(ii). The graphs represent the activity (○) and the specific activity (▲) profiles.
Fig. 3.14: DEAE-cellulose chromatography of the enzyme
Table 3.19: Partial purification of the reductase

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Fraction volume (ml)</th>
<th>Protein (mg ml(^{-1}))</th>
<th>Total protein (mg)</th>
<th>Enzyme activity (nmol min(^{-1}) ml(^{-1}))</th>
<th>Specific activity (nmol min(^{-1}) mg(^{-1}))</th>
<th>Total activity (nmol min(^{-1}))</th>
<th>Recovery (%)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat extract</td>
<td>100</td>
<td>11.11</td>
<td>1111</td>
<td>420.0</td>
<td>37.8</td>
<td>42000</td>
<td>100</td>
<td>1.00</td>
</tr>
<tr>
<td>10% PEG supernatant</td>
<td>100</td>
<td>4.84</td>
<td>484</td>
<td>390.0</td>
<td>80.6</td>
<td>39000</td>
<td>92.8</td>
<td>2.13</td>
</tr>
<tr>
<td>10-30% PEG precipitate resuspended</td>
<td>100</td>
<td>1.26</td>
<td>100.8</td>
<td>376.0</td>
<td>298</td>
<td>37600</td>
<td>89.5</td>
<td>7.88</td>
</tr>
<tr>
<td>DEAE-cellulose (fractions 7-10 pooled)</td>
<td>15.6</td>
<td>0.10</td>
<td>1.56</td>
<td>45.71</td>
<td>457.1</td>
<td>713</td>
<td>1.69</td>
<td>12.09</td>
</tr>
</tbody>
</table>

The polyethylene glycol fractionation was performed as in Table 3.18 and the DEAE-cellulose elution as in Fig. 3.14. The enzyme activity was assayed as in "Materials and Methods" section II.B.1.e.
and the percentage recovery was low (Table 3.19). It is, however, noteworthy that the purification factor, as stated previously, is an under-estimate since it is compared to the heat treated extract, as it was not possible to detect a significant rate of NADPH oxidation in high speed supernatant preparations, even in the presence of added substrate.

The lack of activity in the high speed supernatants could be due to a number of reasons. One is that the reaction is inhibited by a macromolecule which is removed by the heating process or by ultrafiltration. Another possibility is that there are two redox reactions of comparable magnitude working in opposition in the high speed supernatant so that there is little change in the concentration of NADPH.

The massive loss of enzyme activity upon DEAE-cellulose chromatography is consistent with the results obtained in the purification of the enzyme-substrate complex (Tables 3.16 and 3.17). Clearly, the enzyme is in some way inactivated on the DEAE-containing material. Since there was no enzyme activity in the flow through or in the buffer used to wash off excess protein and unbound material, one can only assume that the inactivation is either due to the interaction between the enzyme and the column material or, more likely, there is a cofactor involved in the reaction which is removed by the chromatographic procedure. It is also possible that some of the substrate is lost at this stage either as a result of disintegration or by remaining attached to the column.

A comparison of Fig. 3.11 with 3.14 shows that the enzyme-substrate complex requires a higher concentration of salt to elute than the enzyme. This implies that the substrate moiety is highly charged in comparison to the enzyme moiety. This theory is consistent with the higher salt concentrations required to elute the substrate off
the ion exchangers used in C.2.c.

In the course of this work it has not been possible in the time available to improve the degree of purification or indeed to characterize the enzyme in detail, and this work will have to await another project. The relatively crude enzyme preparation did not permit accurate substrate specificity studies.

c. Purification of the substrate

In order to attempt an identification of the reaction, it was imperative to purify and characterise the substrate. Of the various methods for the purification of the substrate tried, the best results were obtained with the scheme depicted in Fig. 3.15.

In this scheme, the 30% PEG-6000 supernatant was obtained by treating the heat extract with 30g/100ml PEG-6000. The protein precipitate was removed by centrifugation and discarded. The supernatant was applied to a Dowex Cl\(^-\) 1 (x8-200) column with an equal volume of 20mM-BTP/HCl, pH 8.3 containing 1mM EDTA, pH 7.0. The PEG-6000 was washed off with buffer (300ml). It was important to remove all the PEG-6000 since it was found that the presence of the polymer interfered with protein and carbohydrate estimations. The substrate was eluted with a linear concentration gradient (0-1M) of NaCl, in a total volume of 350ml. The substrate was found to elute between 0.21 and 0.42M - NaCl (Fig. 3.16). The fractions were assayed for carbohydrate and amino acid content (II.B. 2c and II.B. 2d(iii), respectively). The carbohydrate content did not correlate with the activity profile, but the presence of certain amino acids in the acid hydrolysates of the substrate correlated with the activity profile (Table 3.24). The distribution of activity in fractions off the Dowex Cl\(^-\) 1 (x8 - 200) column suggested heterogeneity of the substrate (Fig. 3.16).
1:2 (w:v) Rat Liver Homogenate

100,000g Supernatant

Heat 60°C 1min

Supernatant + 30g/100ml PEG-6000

30% PEG 6000 Supernatant

Dowex Cl⁻ (Elution with NaCl)

Dowex H⁺ (Elution with Ammonia)

Small Dowex Cl⁻ Column

Bio-Gel P-2

Sephadex G-25

Fig. 3.15: Purification of the substrate

The scheme depicts the various methods employed in the purification of the substrate. For experimental detail see section II.B.4.d.(iii).
Sodium chloride gradient (0–1 M)

The figure shows the elution profile of the substrate elution off Dowex Cl⁻ (X8-200)

obtained as in Table 3.13, was applied to the column (20 cm) and the excess NaCl was washed off with 20 mM

NaOH, pH 8.5 (300 ml). A continuous concentration gradient (0–1 M) of NaCl in 20 mM-Tris/HC1, pH 8.5

(150 ml) was applied. Fractions (1.6 ml) were collected and assayed for substrate content. The volume of

the fractions used in the assay, performed as in II.B.1.e., was 100 pl. Fractions 21–40 were pooled for further purification. The enzyme source was

10–50% PEG-6000 precipitate (Table 3.13).

Fig. 3.16: Substrate elution off Dowex Cl⁻-1 (X8-200)
Fig. 3.16: Substrate elution off Dowex Cl⁻ (x8-200)

The figure shows the elution profile of the substrate on Dowex Cl⁻ (x8-200). A 30% PEG supernatant obtained as in Table 3.18, was applied to the column (2x26cm) and the excess PEG was washed off with 20mM-BTP/HCl, pH 8.3 (300ml). A continuous concentration gradient (0-1M) of NaCl in 20mM-BTP/HCl, pH 8.3 (350ml) was applied. Fractions (4.8ml) were collected and assayed for substrate content. The volume of the fractions used in the assay, performed as in II.B.1.e, was 100μl. Fractions 21 - 32 were pooled for further purification. The enzyme source was the 10 - 30% PEG-6000 precipitate (Table 3.19).
The fractions containing the highest substrate content were pooled and the minor peak eluting at 0.28M-NaCl was discarded. The substrate-containing fractions were diluted with an equal volume of buffer as above and applied to a Dowex-50 H\(^+\) 50 (x8-400) column. The column was then washed with 0.01M-NH\(_4\)OH (60ml) and eluted with a linear concentration and pH gradient (0.01M, pH 8.5 to 0.1M, pH 13.0) of NH\(_4\)OH in a total volume of 300ml. The substrate eluted between pH 8.8 and pH 9.1; i.e. between 0.06M and 0.16M-NH\(_4\)OH. The most active fractions eluted as a single peak (Fig. 3.17). The substrate obtained at this stage of the purification was stable to storage at -20\(^\circ\)C. The fact that the substrate binds to both a cation and an anion exchanger suggests that the substrate has an amphoteric nature.

The substrate-containing fractions from the Dowex 50 H\(^+\) column were pooled and the pH adjusted to 8.3 by adding solid bis-tris propane to a final concentration of 20mM and adjusting the pH with 6N-HCl. This product was divided into three portions, which were applied separately to Sephadex G-25, Bio-Gel P-2 and a small Dowex Cl\(^-\) 1 (x8-200) column.

On Sephadex G-25, the substrate elution peak was found to be 16.4ml from the void volume (Fig. 3.18). This indicated a molecular weight of less than 5,000 but greater than 709, since the compound eluted before NADH. In the absence of NaCl, which was included in the elution buffer for the substrate, the standard cystine appeared to be retarded on the Sephadex G-25 column. This was probably due to adsorption of the compound on to the gel material, since it has been reported by the manufacturers (Pharmacia Fine Chemicals) that some small molecules behave anomalously on such columns. They have also reported that the use of buffers with an ionic strength greater than 0.02 overcomes the interaction between charged solutes and the matrix, hence the inclusion of NaCl (100mM) in the eluting buffer.
The pooled Dowex Cl\textsuperscript{−} eluates (57ml) were applied to a Dowex 50 H\textsuperscript{+} 50 (x8-400) column (1.5 x 19cm) which had been previously washed in 6N-hydrochloric acid and de-ionised water. The column was then washed with 60ml of 0.01M-NH\textsubscript{4}OH (pH 8.5) and a continuous gradient (total vol. 300ml) of NH\textsubscript{4}OH (0.01M pH 8.5 to 1M pH 13.0) was applied and 4.1ml fractions collected. The pH of the eluate fractions was adjusted to pH 7.5 with 6N-HCl immediately before assay. Portions of the eluates (100µl) were assayed for activity as in Fig. 3.16.
Fig. 3.17  Elution of the substrate off a Dowex 50 H⁺ column
Fig. 3.18: Sephadex G-25 chromatography of the substrate

A portion of the Dowex 50 H\textsuperscript{+} pooled sample (3ml) was applied to a Sephadex G-25 column (void volume 47ml) equilibrated with BTP/HCl (1mM-EDTA) pH 8.3, containing 100mM-NaCl. The substrate was eluted with the same buffer and 4.1ml fractions were collected and assayed for substrate content (II.B.1.e). A fixed volume of 300\textmu l of the eluates and 50\textmu l of the PEG precipitated enzyme were used.
Fig. 3.18: Sephadex G-25 chromatography of the substrate
of the substrate.

On Bio-Gel P-2 the substrate elution peak was found 30.4 ml after the void volume, indicating a molecular weight of between 440-529 (Fig. 3.19). It is clear that there is a large discrepancy between the results obtained with the Bio-Gel P-2 and the Sephadex G-25 columns. It is possible that the substrate is being retarded as a result of some interaction with the Bio-Gel P-2 material.

The portion of the substrate-containing pooled fractions from the Dowex-50 H⁺ column did not bind to the Dowex Cl⁻ 1 (x8 - 200) column, suggesting a change in the charge on the substrate molecule at the Dowex-50 H⁺ chromatography stage. The results obtained are shown in Fig. 3.20. The diagram also indicates the separation of the phosphate groups from the main substrate, implying a decomposition of the substrate molecule. This supposition is underlined by the fact that the phosphate-content peak is at 0.5 M NaCl, which is in the region normally associated with the substrate-elution peak on Dowex Cl⁻ 1 (x8 - 200) (Fig. 3.16 and Fig. 3.23). In this case, however, most of the substrate was found in the flow through fraction of this column.

Table 3.20 shows the recovery of the substrate at various stages of the purification. The recovery of the substrate was measured as a function of the activity obtained with a fixed amount of enzyme with known specific activity. The largest loss in activity was found to be between the PEG-6000 and the Dowex Cl⁻ 1 (x8 - 200) stages, the loss being about 58%. Some of the loss could be attributed to the fact that only fractions 21-32 were pooled (Fig. 3.16). Another possibility is that the 30% PEG-6000 fraction contains more than one substrate for the enzyme and that the ion exchange step is selective, resulting in an apparent decrease in the substrate content of the ion exchange product. Alternatively, the ion
Fig. 3.19: Bio-Gel P-2 chromatography of the substrate

The experiment was performed as in Fig. 3.18, with the difference that only 1 ml of the sample was applied to the Bio-Gel P-2 column (void volume 49 ml) and that NaCl was not present in the buffer. Fractions (3.8 ml) were collected and 300 μl portions assayed for substrate content as in Fig. 3.19.
Fig. 3.19: Bio-Gel P-2 chromatography of the substrate
Fig. 3.20: Ion-exchange chromatography of the substrate on Dowex Cl\(^{-1}\) (x8-200)

The Fig. shows the elution of the substrate (●) and the phosphate estimation (▲), on Dowex Cl\(^{-1}\) (x8-200). The sample (5ml) was applied in 20mM-BTP/HCl pH 8.3 and eluted with an ascending stepwise gradient of sodium chloride (0.0-1.0M). Fractions (5ml) were collected and assayed for NADPH oxidation (II.B.1.e.) and phosphate content (II.B.2.b.(i)). The enzyme source was the same as in Fig. 3.16.
### Table 3.20: Recovery and purification of the substrate

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity (nmol min(^{-1}))</th>
<th>% recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% PEG-6000 supernatant</td>
<td>19,290</td>
<td>100</td>
</tr>
<tr>
<td>Dowex Cl(^-) ion exchange</td>
<td>8,144</td>
<td>42</td>
</tr>
<tr>
<td>Dowex 50 H(^+) ion exchange</td>
<td>5,600</td>
<td>29</td>
</tr>
<tr>
<td>BIO-Gel P-2 (1ml)</td>
<td>196</td>
<td>16*</td>
</tr>
<tr>
<td>Sephadex G-25 (3ml)</td>
<td>588</td>
<td>16*</td>
</tr>
<tr>
<td>Dowex Cl(^-) ion exchange (5ml)</td>
<td>300</td>
<td>5*</td>
</tr>
</tbody>
</table>

* volume used relative to the previous step accounted for.

The Table shows the purification procedures and recovery of the peptide substrate. Details of the methods used are recorded in II.B.4 d(iii).
exchange step may be a poor method of purification for the substance under study. It was also found that 31% of the activity was lost between the anionic and cationic ion-exchange chromatographic steps.

The recovery after Sephadex G-25 chromatography was about the same as that obtained with Bio-Gel P-2 chromatography, representing a loss of about 13% relative to the previous step.

As NaCl and KCl were routinely used in the purification procedures it was important to determine the effect of these salts on the rate of the reaction (Fig.3.21 and 3.22). Both were found to be inhibitors of the reaction. However, KCl was a more potent inhibitor of the reaction than NaCl, being effective at concentrations as low as 0.05M. On the other hand, NaCl was shown to be an activator of the reaction at low concentrations (less than 0.15M). For this reason, in later experiments NaCl rather than KCl was used for elution purposes. Since the final concentration of NaCl in the reaction mixture never exceeded 0.2M for the various purification steps, the salt would not be expected to significantly interfere with the reaction.

In conclusion, it is clear from this section on purification that the enzyme and substrate form a stable complex, which is not the result of covalent bonding as the two can be separated with PEG-6000. The latter, being non-polar, can be readily separated from the substrate by ion exchange chromatography, since the polymer will not bind and can, therefore, be washed off the column prior to elution of the substrate.

It has been suggested that PEG removes water from the hydration envelope of protein molecules and causes a corresponding alteration in the dielectric constant of the medium, which in turn alters the steric relationship of the hydrophilic groups of the protein molecule (Chun et al.,
Fig. 3.21: The effect of NaCl on the rate of the reaction.

The substrate and enzyme were obtained by PEG-6000 fractionation as in Table 3.18 and 3.19. The enzyme source was the 10 – 30% fraction and the substrate source was the 30% PEG-6000. NADPH oxidation was followed as in II.B.1.e in the presence of increasing concentrations of NaCl as shown. The results are the mean of three readings.
**Fig. 3.22:** The effect of KCl on the rate of the reaction

This experiment was performed on the enzyme substrate-complex in the heat treated extract, since this was the stage at which the salt was used. The assay conditions were as in II.B.1.e and the initial rates were determined in the presence of increasing concentrations of KCl.
Since the substrate appears to be charged, it can be expected to be hydrophilic. If it is associated with the enzyme by virtue of its charge, then it is conceivable that as the PEG-6000 concentration increases, the two molecules will increasingly dissociate, and that the enzyme moiety will precipitate out as a result of the removal of the water from its hydration envelope.

Although no attempt has been made to characterize the enzyme in any detail, some conclusions about the nature of the enzyme moiety can be drawn from this section. One is that it appears to be a small molecule, as evidenced by the observation that the enzyme-substrate complex passed through Amicon ultrafilters ($M_r$ cut off 10,000), which excluded 6PGDH and G6PDH, and that a relatively high concentration of PEG-6000 was required to precipitate it out. The other observation is that the enzyme appears to be stable and survives the heating to 60° (which destroyed G6PDH and 6PGDH activity) and the ethanol fractionation treatment. Small molecular weight enzymes have been reported and include thioltransferase ($M_r$ 11,000) (Mannervik and Axelsson, 1975).

3. The components of the reaction

Since a number of reactions utilize NADPH it was important to exclude the possibility that the oxidation of NADPH was due to a well-known reaction such as the reduction of glutathione by glutathione reductase. An attempt was therefore made to characterize the substrates of the reaction.

a. The fate of NADPH

It was readily shown that NADPH was being oxidised to NADP⁺ (Table 3.21). The experiment was performed with partially purified preparations of both enzyme and substrate. It was determined that 97% of the NADPH added
Table 3.21: Production of NADP⁺ by the NADPH-dependent reductase

<table>
<thead>
<tr>
<th>Reactants</th>
<th>NADPH oxidized (nmoles ml⁻¹)</th>
<th>NADP⁺ reduced (nmoles ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Enzyme + substrate + NADPH</td>
<td>580</td>
<td>-</td>
</tr>
<tr>
<td>2. G6P + G6PDH + (1)</td>
<td>-</td>
<td>563</td>
</tr>
<tr>
<td>3. G6P + G6PDH + enzyme + substrate N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>but no NADPH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. NADPH + G6P + G6PDH</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D. No detectable change in absorbance

The partially purified enzyme (10-30% PEG 6000 fraction) was reacted with NADPH and the substrate (the charcoal-treated 30% PEG fraction) (1) until no further change in O.D. was observed at 340nm. NADPH was added in excess to ensure that the substrate was completely reduced. G6P (in excess) and crystalline yeast G6PDH were then added (2) and the reduction of NADP⁺ followed at 340nm. At the end of the reaction more G6P was added to ensure that all the NADP⁺ was reduced. The amount of NADPH oxidized and NADP⁺ reduced was calculated from the change in O.D. Control experiments (3) and (4) were also performed.
was converted to NADP⁺, as determined by the reaction of the latter with G6P and crystalline yeast G6PDH. Table 3.21 also shows that the NADP⁺ used in the G6PDH-catalysed reaction was the product of the NADPH-oxidising activity, since there was no oxidation of G6P in the absence of added NADPH. Conversely, addition of NADPH to the incubation mixture of G6PDH in the absence of the NADPH-oxidising activity did not result in G6P oxidation. Hence it was concluded that the reaction under discussion involves the transfer of reducing equivalents from NADPH to a second substrate.

b. Attempted characterization of the second substrate

The reaction was followed at various wavelengths to examine the possibility of an identification of the components of the reaction by a characteristic change in the absorption spectrum, but the only change in absorbance observed was at 340 nm (Table 3.22). There was no change in the absorbance when the reaction was followed at 290, 415, 520, 550, 556, and 640 nm. These wavelengths are associated with the oxidation and reduction of quinones and the cytochromes. The molar absorption coefficients of quinones, cytochromes and NADP⁺ in their reduced and oxidised forms are comparable at their wavelengths of maximum absorption (Dixon and Webb, 1964).

Another reaction that could be expected to consume NADPH is that catalysed by NADPH oxidase:

\[ \text{NADPH} + 20_2 \rightarrow 20_2^- + \text{NADP}^+ \]

To exclude the possibility that this reaction was involved, the incubation mixture was gassed with oxygen-free nitrogen. The reaction was allowed to proceed in sealed cuvettes. It was observed that the rate of the reaction was not diminished as a result of this treatment, and it was concluded that NADPH oxidase was not the enzyme involved in
Table 3.22: Absorbance changes induced by the reaction at different wavelengths

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>Reaction rate (nmols min(^{-1}) mg(^{-1}))</th>
<th>Substance likely to induce change in absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>290</td>
<td>0</td>
<td>quinones</td>
</tr>
<tr>
<td>340</td>
<td>18.5</td>
<td>NAD(H), NADP(H)</td>
</tr>
<tr>
<td>415</td>
<td>0</td>
<td>(\alpha)-oxidised cyt. a(_3)</td>
</tr>
<tr>
<td>520</td>
<td>0</td>
<td>(\beta)-reduced cyt.c.</td>
</tr>
<tr>
<td>550</td>
<td>0</td>
<td>(\alpha)-reduced cyt.c.</td>
</tr>
<tr>
<td>556</td>
<td>0</td>
<td>(\alpha)-reduced cyt.b(_5) (NADH)</td>
</tr>
<tr>
<td>450</td>
<td>0</td>
<td>reduction of cyt.P(_{450})</td>
</tr>
</tbody>
</table>

The partially purified substrate (Dowex 50H\(^+\) eluate) was incubated with 100\(\mu\)M-NADPH, 100mM-BTP/HCl pH 7.5 at 25°C and partially purified enzyme (30% PEG-6000 fraction) in a total volume of 1ml. The reaction was followed at the wavelengths indicated and the rates recorded.
the oxidation of NADPH observed in the present study.

The substrate was also assayed for carbohydrate content. Where carbohydrate was detected, the carbohydrate profile did not correlate with the NADPH-oxidation profile (Fig. 3.23), and in the more purified substrate preparations no carbohydrate was detectable. It was therefore assumed that the substrate did not contain carbohydrate.

Preliminary experiments had indicated that the substrate was a low molecular weight substance and that it was amphoteric (C.2.c). The possibility that the substrate was a peptide was therefore explored. The substrate was found to react positively with ninhydrin. The presence of peptide bonds in the substrate was investigated.

(i) Detection of peptide bonds in the substrate

Since it was suspected that the substrate was a peptide, the effects of a peptidase and a carboxypeptidase on the substrate was determined (Table 3.23).

Peptidase treatment destroyed the substrate completely within 3h, as determined by the lack of oxidation of NADPH. Since the degradation of the substrate was being determined by the loss of activity of the enzyme towards the substrate, it was important to ensure that the observed loss of activity was not due to the degradation of the enzyme by the peptidase. To demonstrate this, both the control (substrate incubated in the absence of the peptidase) and the test (substrate incubated with the peptidase) substrate incubation mixtures were heated to 90°C for 45min just before they were assayed for NADPH-oxidising activity. It was found that the heat treatment destroyed the peptidase activity without affecting the substrate. This was demonstrated by adding a portion of the heated peptidase-treated test sample to the reaction mixture containing the active enzyme and substrate. There was no reduction in
A barium acetate and ethanol treated 30% PEG-6000 supernatant (II.B.4.d.) was washed onto a Dowex Cl⁻1 (x8-200) column (5ml capacity). The column was washed with 50ml of 20mM-BTP/HCl, pH 8.3 containing 1mM-EDTA. The substrate was eluted with a discontinuous gradient of NaCl (0-1.4M). The samples were assayed for substrate content as in Fig. 3.16(a). The carbohydrate content was also determined (II.B.2.c.)(A). The points are the mean of 3 readings. The enzyme source was the 10 - 30% PEG-6000 fraction.
Table 3.23 : Detection of peptide bonds in the purified substrate

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>Activity after 3h incubation (nmol min⁻¹)</th>
<th>Activity after 18h incubation (nmol min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unhydrolysed control substrate (incubated in the absence of peptide hydrolysing enzymes)</td>
<td>15.27 ± 0.72</td>
<td>6.39 ± 0.69</td>
</tr>
<tr>
<td>Carboxypeptidase hydrolysed substrate</td>
<td>8.77 ± 0.84</td>
<td>N.D.</td>
</tr>
<tr>
<td>Peptidase hydrolysed substrate</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D. = not detectable

The Table shows the effects of peptide hydrolysing enzymes on the substrate. The experiment was performed in triplicate and the results are mean ± S.D. Three portions of the substrate were individually incubated with 142.8 units ml⁻¹ of carboxypeptidase, 1 unit ml⁻¹ of peptidase or with an appropriate volume of deionised water (II.B.2d(ii)). At the end of the incubation period the reaction mixtures were all heated to 90° for 45 min, to destroy peptidase activity, cooled and the reaction of the substrate with the enzyme preparation followed as in II.B.1e. In addition portions of the supernatants containing the enzyme-hydrolysed substrate were added to an active preparation to ensure that there was no peptide hydrolysing activity associated with these supernatants. It was found that the heat treatment had destroyed the carboxypeptidase and the peptidase activity and the enzyme under investigation was not being hydrolysed. The reduction in the reaction rates could therefore be attributed to the destruction of the substrate rather than the enzyme which showed no loss of activity during the course of the experiment. Experiments I and II are independent experiments and are not directly comparable.
the rate of NADPH oxidation, implying that there was nothing in the peptidase-treated substrate preparation that could account for the loss of NADPH-oxidising activity in the test incubation other than the destruction of the substrate itself.

If the enzyme was being hydrolysed by the peptidase activity in the 'test' supernatants, one would expect to observe a reduction in the reaction rate if a portion of this preparation (added to the same final concentration as in the 'test' incubations) was added to the control incubations. This was not found to be the case and the results obtained were therefore taken to be a valid indication of the presence of peptide bonds in the substrate.

To eliminate the possibility that the loss of activity with the peptidase-treated samples was due to the cleaving of peptide bonds, rather than destruction by heating to 90°, the control incubations were also heated to 90° and treated in a manner identical to that of the test incubations. The substrate was found to be stable under these conditions and no significant loss of activity was registered.

The effect of carboxypeptidase on the substrate was also investigated. It was found that the destruction of the substrate by this enzyme was slower than that caused by the peptidase. After 3h incubation the carboxypeptidase destroyed only 50% of the substrate, which was measured as a function of the activity with a fixed concentration of enzyme. Since the activity of the carboxypeptidase, as stated by the supplier was higher per ml of incubation mixture than the peptidase, it was concluded that the carboxypeptidase reaction was hindered in some way. Both enzymes were incubated with substrate under their optimum conditions (II.B.2 d (ii)).
A number of factors could account for the slower rate of hydrolysis of the substrate by carboxypeptidase. The carboxypeptidase used in these experiments (Carboxypeptidase A) requires a free carboxyl group and does not hydrolyse amides or esters of simple alcohols. The specificity of the enzyme is determined primarily by the amino acid bearing the free carboxyl group and only to a small extent by the amino acid adjacent to this. The enzyme acts best on peptides with a C-terminal aromatic amino acid, the configuration of which must be L-. As Table 3.24 shows, aromatic amino acids were not detected in the substrate, and this could account for the reduced rate of peptide hydrolysis observed with carboxypeptidase. Furthermore, it is possible that the substrate has a modified C-terminal. In addition the enzyme has been reported to act only slowly on dipeptides unless the amino acid group is acylated, although in tripeptides and higher peptides the length of the peptide chain has little effect. The length of the side chain is not critical (Dixon and Webb, 1964).

In contrast, the peptidase does not require the presence of aromatic amino acids in the peptide chain and acts with maximum efficiency on compounds containing an N-terminal amino acid with an aliphatic side chain, the efficiency increasing with the size of the side chain.

The peptidase does, however, require a free α-amino group and blocking of this group prevents the action of the enzyme. The enzyme lacks esterase activity and does not therefore hydrolyse the corresponding amino acid esters. Amides of most amino acids are hydrolysed, although those of leucine, norvaline and 2-aminocaproic acid are attacked more rapidly than others. The configuration of the amino acids on both sides of the bond which is split is important, particularly that on the N-terminal side of the bond. The presence of leucine on either side of the bond being hydrolysed has a particularly favourable effect on the rate

Another possibility that could account for the slow rate of carboxypeptidase inactivation of the substrate is that the enzyme may have to digest a substantial proportion of the substrate before a modified substrate is produced which has no affinity for the NADPH-dependent reductase.

Another explanation for the slower rate of hydrolysis obtained with carboxypeptidase is that the latter enzyme preparation is contaminated with a residual activity of peptidase which is responsible for the hydrolysis and that carboxypeptidase itself is inactive.

(ii) Amino acid content of the substrate

Amino acid analysis, following acid hydrolysis of a partially purified substrate preparation (Fig. 3.23) revealed that the substrate contained at least four different amino acids which coincided with the presence of the substrate (Table 3.24). These included methionine, cystine, valine and possibly leucine. The amino acid composition of the substrate was also determined by t.l.c. after carboxypeptidase treatment (Table 3.25). In this method of analysis, the substrate in the purest form obtained (Fig. 3.18), was treated with carboxypeptidase for various time intervals. After each time interval dansyl chloride derivatives of the released amino acids were prepared (II.B.2 d (iv) and the sample chromatographed on t.l.c. as described in the legend to Table 3.25. The amino acids appeared in the sequence valine, cystine, "a" and "b", the latter two not being readily identifiable by t.l.c. as they did not coincide with any of the standards used which were dansylated derivatives of the amino acids detected with the autoanalytical method.

In a separate experiment the N-terminal amino acid, determined by dansyl amino acid formation followed by acid hydrolysis and t.l.c. analysis, was found to be valine. Hence it appeared from these observations that
Table 3.24: Amino acid content of the Dowex Cl⁻¹ (x8 - 200) eluates

<table>
<thead>
<tr>
<th>NaCl (M)</th>
<th>Amino acid content of the fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>methionine*, leucine*, isoleucine</td>
</tr>
<tr>
<td>0.2⁺</td>
<td>methionine*, leucine*, isoleucine</td>
</tr>
<tr>
<td>0.4⁺</td>
<td>methionine, cystine, valine</td>
</tr>
<tr>
<td>0.6⁺</td>
<td>methionine, cystine, valine</td>
</tr>
<tr>
<td>0.8⁺</td>
<td>methionine, cystine, leucine</td>
</tr>
<tr>
<td>1.0⁺</td>
<td>methionine</td>
</tr>
<tr>
<td>1.2⁺</td>
<td>methionine</td>
</tr>
<tr>
<td>1.4</td>
<td>methionine</td>
</tr>
</tbody>
</table>

* Identification inconclusive but nearest to the expected peak of the indicated amino acid.

The amino acid content of the samples, which are the same as those recorded in Fig. 3.23, was determined with a Joel JLC-6AH analyser as in II.B.2d(iii). The unhydrolysed samples did not yield free amino acids, hence the recorded amino acids were taken to be the products of acid hydrolysis. The active samples are indicated in Fig. 3.23 and correspond to the NaCl concns. marked (+) in the table.
Table 3.25: t.l.c. analysis of the products of carboxypeptidase treatment of the purified substrate

<table>
<thead>
<tr>
<th>Time of incubation with carboxypeptidase (min)</th>
<th>Amino acids detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>10</td>
<td>valine</td>
</tr>
<tr>
<td>45</td>
<td>valine, cystine</td>
</tr>
<tr>
<td>60</td>
<td>valine, cystine</td>
</tr>
<tr>
<td>180</td>
<td>valine, cystine</td>
</tr>
<tr>
<td>360</td>
<td>valine, cystine, 'a'</td>
</tr>
<tr>
<td>1080</td>
<td>valine, cystine, 'a', 'b'</td>
</tr>
</tbody>
</table>

The substrate obtained at the Sephadex G-25 stage (Fig. 3.15) was hydrolysed with carboxypeptidase (II.B.2d(ii)) for the indicated time. The carboxypeptidase was removed by heating to 90° for 45 min followed by centrifugation. The supernatants were reacted with dansyl chloride (II.B.2d(iv)) and the dansylated derivatives of the free amino acids formed were separated by t.l.c. (II.B.2d(iv)). The dansyl derivatives were located under u.v. light. 'b' is a faster moving molecule ($R_f \approx 0.6$) than 'a' ($R_f \approx 0.5$).
valine was present in the peptide at both the N- and C-terminal ends.

The results in Table 3.23 and 3.25 indicate that after 3h, when some of the valine and cystine were shown to be removed by carboxypeptidase treatment, only about 50% of the original amount of the substrate was destroyed. The appearance of the amino acids referred to as "a" and "b" correlate with the total inactivation of the substrate.

It was noted in these time course experiments that the stationary spot at the base line due to the dansylated, unhydrolysed peptide diminished with prolonged incubation with carboxypeptidase, and eventually disappeared after 18h.

The conclusion drawn from this section of the work was that the substrate was a cystine-containing peptide and that valine was at both terminals of the peptide.

(iii) Correlation of the reduction of the disulphide bonds in the substrate molecule with NADPH oxidation

On the basis of the results obtained in the preceding section a working hypothesis, which assumed that the cystine associated with the substrate was being reduced by NADPH during the reaction, was adopted. The reaction proposed is depicted below:-

\[
R-S-S-R' + NADPH \rightarrow RSH + R'SH + NADP^+
\]

To test this hypothesis, the purified substrate was subjected to both chemical and enzymatic disulphide reduction procedures. The resulting thiol groups were assayed by the method of Zahler and Cleland (1968), which uses 5, 5'-dithiobis (2-nitro-benzoic acid) (DTNB). In the present study dithiothreitol (DTT) was used as the reducing agent as follows:-
DTT + RSSR\(^1\) \rightarrow DTT-SSR\(^1\) + RSH

DTT-SSR\(^1\) \rightarrow DTT + R\(^1\)SH (oxidised)

DTT was added in excess. In order to distinguish between the monothiols formed and the dithiols of DTT, arsenite, which forms a tight complex with the dithiols, but not the monothiols, was added after reduction. The formation of the dithiothreitol-arsenite complex is shown below:

\[
\begin{align*}
\text{CH}_2\text{SH} & \quad \text{CH}_2\text{S} \\
\text{CH.OH} & \quad \text{CH.OH} \\
\text{CH.OH} + \text{H AsO}_2 & \quad \text{CH.OH} \quad \text{AsOH} \\
\text{CH}_2\text{SH} & \quad \text{CH}_2\text{S}
\end{align*}
\]

The free monothiols were then reacted with DTNB and the formation of the yellow complex followed at \(412\)nm until there was no further change in the absorbance.

The free thiol content of the enzyme preparation, as determined by its reaction with DTNB was negligible under the conditions of assay. Fig.3.24 shows the results obtained when the purified substrate eluted from a Sephadex G-25 column was chemically reduced with DTT. There is a clear correlation between the NADPH oxidation rate and the thiol content of the substrate, after chemical reduction. There was no detectable free thiol in the substrate preparation before chemical or enzymatic reduction.

Free thiol production was also found to occur when the enzymatically-catalysed reduction of the substrate occurred (Fig.3.25). The rate of free thiol production catalysed by the enzyme was proportional to the substrate concentration and was correlated to the rate of NADPH oxidation.
Sephadex G-25 fractions

Fig. 3.24: Detection of disulphide bonds in the substrate

The Sephadex G-25 column chromatography fractions (Fig. 3.19) which reacted with the enzyme were assayed individually for disulphide content (▲) and NADPH oxidation (●). The disulphides were chemically reduced with DTT (Zahler and Cleland, 1967). The free monothiols were assayed by following the absorbance at 412 nm after addition of DTNB (II.B.2.f.).
A time course experiment (results not shown) was also performed. It was found that NADPH oxidation was essential for the release of the thiol groups. Table 3.26 shows that thiol production was dependent on the presence of the enzyme, the substrate and NADPH. The rate of thiol production, measured at 417nm, was proportional to that of NADPH oxidation, measured at 340nm. It was checked that the following reagents and combination of reactants did not react with DTT: NADPH, enzyme (the thiol content where detected), substrate (reduction), enzyme and substrate of NADPH, NADPH and substrate.

Since the analysis of the substrate had indicated the presence of a cysteine residue, and since the rate of thiol production was proportional to the substrate concentration (Fig. 3.25), it was concluded that the substrate was a disulphide-containing peptide. It is also clear from the records in this section that there is a direct proportion between NADPH oxidation and thiol production.

Fig. 3.25: Enzymatically catalysed thiol production and NADPH oxidation expressed as functions of substrate concentration.

The total NADPH (▲) oxidised and thiol groups produced (●) were determined after enzymatic reduction of the substrate II.B.1.e and II.B.2.f.). The thiol group content of the partially purified enzyme was determined (2.20 nmol SH/ml) and subtracted from the values obtained for the substrate. A portion (0.8ml from a total reaction volume of 1ml) of the substrate was assayed for the disulphide after NADPH oxidation had ceased (33mins.).
A time course experiment (results not shown) was also performed. It was found that NADPH oxidation was essential for the release of the thiol groups. Table 3.26 shows that thiol production was dependent on the presence of the enzyme, the substrate and NADPH. The rate of thiol production, measured at 412nm was proportional to that of NADPH oxidation, measured at 340nm. It was checked that the following reactants and combination of reactants did not react with DTNB: NADPH; enzyme (the thiol content where detected was subtracted); substrate (before reduction); enzyme and substrate or NADPH; NADPH and substrate.

Since amino acid analysis of the substrate had indicated the presence of a cystine residue, and since the rate of thiol production was proportional to the substrate concentration (Fig. 3.25) it was concluded that the substrate was a cystine-containing peptide. It is also clear from the results recorded in this section that there is a direct correlation between NADPH oxidation and thiol production.

(iv) Phosphate content of the substrate

Inorganic phosphate, as estimated by the method of Fiske and SubbaRow (II.B.2.b.(i)), was associated with the substrate at all stages of the purification and was therefore assumed to be an integral part of the substrate molecule. Figs. 3.20, 3.26, 3.27, 3.27b show that the $P_i$ content and the NADPH oxidation profiles coincide and indicate that the substrate may be a phosphorylated peptide. It was checked that the disulphide bonds of cystine in oxidised glutathione did not react in the method used for phosphate determination.

These results justified a further investigation of the role of phosphate in the substrate molecule, and the effect of alkaline phosphatase on the substrate was
Table 3.26: The NADPH-dependence of the reaction releasing thiols from the purified substrate

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>mmols thiol produced min⁻¹ mg⁻¹ protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>E + DTNB</td>
<td>0</td>
</tr>
<tr>
<td>E + DTNB + NADPH</td>
<td>0</td>
</tr>
<tr>
<td>E + DTNB + NADPH + S</td>
<td>64.1</td>
</tr>
<tr>
<td>S + DTNB</td>
<td>0</td>
</tr>
<tr>
<td>S + DTNB + NADPH</td>
<td>0</td>
</tr>
<tr>
<td>S + DTNB + NADPH + E</td>
<td>59.5</td>
</tr>
</tbody>
</table>

The enzyme (E), which was the 10-30% PEG 6000 resuspended precipitate was initially incubated with DTNB. The change in absorbance was followed at 412nm and the zero rate recorded. NADPH was then added and the absorbance change recorded again. Finally the substrate (S) was added and the rate recorded. The experiment was repeated but with the substrate replacing the enzyme in the incubation medium. The enzyme was used to start the reaction in the latter case. The assay was performed at pH 7.0 (100mM-BTP/HC1 and 1mM-EDTA, pH 7.0) in the presence of 100µM NADPH, 200µl of the charcoal treated substrate (Table 3.28) and 100µl of the enzyme in a final volume of 1ml.
Fig. 3.26: Phosphate content profile of the enzyme substrate complex

A purified enzyme-substrate complex containing sample (heat → PEG → DEAE-Sephadex) was applied to a small DEAE-Sephadex column and eluted with KCl (discontinuous gradient). The eluates were assayed for NADPH-oxidising activity (●) (II.B.1.e.) and phosphate content (▲) (II.B.2.b.(i)). The results are the mean of 3 readings. The data is the same as in Fig. 3.12.
Fig. 3.27: Phosphate content of substrate-containing fractions obtained from a Dowex Cl\(^{-1}\) (x8-200) column.

The 30% PEG-6000 supernatant was treated with activated charcoal for 20 min. The charcoal was removed by centrifugation and the supernatant (5 ml) was applied to a small Dowex Cl\(^{-1}\) (x8-200) and eluted with a discontinuous gradient of NaCl as indicated. The phosphate (•) and substrate content was determined (II.B.2.b.(i) and II.B.1.e.). The enzyme source was the 10 - 30% PEG-6000 fraction.
Fig. 3.27b: Phosphate-content of the substrate-containing fractions

A 30% PEG-6000 supernatant was passed down a Dowex Cl\(^-\) \(x8-200\) column and eluted as in Fig. 3.16. The samples were assayed for the substrate (●) as in Fig. 3.16, phosphate (▲) as in II.B.2.b(i) and total NADPH oxidation (○), the latter being estimated from the total absorbance change at 340nm. The volume of the fractions was 4.1ml.
tested. It was found that the activity of the enzyme towards the substrate was substantially reduced, but not abolished, by alkaline phosphatase treatment of the substrate (Table 3.27). The optical density changes at 340 nm indicated that the substrate had been destroyed in some way and control experiments ruled out the possibility that the observed reduction in the rate of reaction was due to the inhibition of the enzyme or destruction of NADPH by alkaline phosphatase. The experiment with alkaline phosphatase was repeated at least 3 times with different preparations and in each case it was observed that there was always a residual activity left after the alkaline phosphatase treatment. This implied that the presence of the phosphate in the molecule was not essential for the reaction of the substrate with the enzyme and NADPH but that the phosphate was required for maximum activity.

(v) The nature of the phosphate group in the substrate

Barium acetate precipitation:

The substrate was precipitated by barium acetate (0.2 M) in the presence of 80% ethanol. This suggested that if the compound was phosphorylated, it was likely to be a monophosphate (Cardini and Leloir, 1957). However, the substrate itself was also found to be precipitated by 80% ethanol in the absence of barium acetate. This experiment did not, therefore, provide conclusive evidence for the existence of a phosphate group in the substrate molecule.

Charcoal treatment of the substrate:

Activated, acid washed, charcoal is known to selectively adsorb purines and pyrimidines and their derivatives. Inorganic phosphate, sugar phosphates, salts and proteins are not adsorbed significantly. In
Table 3.2: The effect of alkaline phosphatase treatment on the substrate

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rate of NADPH oxidation (µmol min⁻¹ ml⁻¹)</th>
<th>Total NADPH oxidised (µmol ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>0.48 ± 0.08</td>
<td>1.50</td>
</tr>
<tr>
<td>alkaline phosphatase treated substrate</td>
<td>0.09 ± 0.009</td>
<td>0.48</td>
</tr>
</tbody>
</table>

The table shows the effect of incubation of the substrate with alkaline phosphatase for 17h at 4°C. The results are mean ± S.D (n=5). Alkaline phosphatase (6 units ml⁻¹) was added to a portion of the substrate; a control incubation of the substrate was also performed in the absence of alkaline phosphatase. After the incubation period both the alkaline phosphatase-treated and untreated substrate were heated to 80°C for 15min. The precipitated alkaline phosphatase was removed by centrifugation and the resultant supernatant (designated the "test supernatant") was tested for the presence of the substrate by incubation with the enzyme and NADPH (II.B.1.e.). The destruction of the alkaline phosphatase by the heating process was confirmed by the observation that the addition of the "test supernatant" to the reaction mixture of G6PDH (the removal of G6P and NADP⁺ by alkaline phosphatase is assessed in this experiment) had no effect on the activity of G6PDH. If alkaline phosphatase was still active addition of the phosphatase would have destroyed the G6P. The fact that some inhibitory contaminant was not present in the "test supernatant" was shown by adding the same amount of test supernatant to the control substrate incubation; the latter was found to exhibit the same rate of NADPH.
Table 3.27 continued:

oxidation as that observed in the absence of the "test supernatant". Thus the reduction in activity following alkaline phosphatase treatment of the substrate is due to the destruction of the substrate, rather than the removal of NADPH or destruction of the enzyme in some way by alkaline phosphatase.
addition, activated charcoal adsorbs most of the nucleotide co-enzymes (Crane and Lipmann, 1953, Cardini and Leloir, 1957).

The substrate in the present study was stirred with about 50mg ml\(^{-1}\) of activated charcoal for 20min. The mixture was then centrifuged at 10,000g for a further 20min. The results obtained are shown in Table 3.28. The table shows that 78% of the substrate (measured as a function of its reactivity with the enzyme) was not adsorbed on charcoal. When the charcoal was washed with ammonia (5N) a further 19.2% of the substrate was recovered. It is possible that the substrate preparation at this stage is heterogeneous (see Fig.3.16) and that the substrate is contaminated with a nucleotide derivative such as GSS-CoA which can be expected to contribute to the rate of NADPH oxidation, since the enzyme preparation was shown to contain an active glutathione reductase activity.

As a result of this experiment, which was reproducible, it can be said that the substrate responsible for the main reaction is not a purine - or a pyrimidine-based phosphorylated compound. The results are consistent with the peptide nature of the substrate.

Phosphate has been shown to be covalently attached to amino acid residues in three forms: as acyl phosphate attached to the carboxyl function of aspartic or glutamic acid; as a phosphoamidate with either the imidazole nitrogens of histidine or with the epsilon amino group of lysine; and as an O-monoester with the hydroxyl group of serine, threonine or tyrosine (Buss and Stull, 1983; Taborsky, 1974; Weller, 1979). None of these amino acids were found in the hydrolysate of the substrate, and it is unlikely from the amino acid peaks observed on the amino acid autoanalyser chart that the unidentified amino acids in the substrate could be any of these. However,
Table 3.28: The effect of charcoal treatment of the substrate on the rate of NADPH oxidation

<table>
<thead>
<tr>
<th>Treatment of substrate</th>
<th>Rate of NADPH oxidation (nmol min⁻¹ mg⁻¹ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO TREATMENT</td>
<td>182.8</td>
</tr>
<tr>
<td>After charcoal treatment</td>
<td>142.8</td>
</tr>
</tbody>
</table>

The 30% PEG supernatant (Table 3.18) was stirred with 50mg ml⁻¹ of charcoal for 20min. The charcoal was removed by centrifugation at 10,000g. The supernatant was tested for its reactivity with the enzyme (II.B.1e). The results are the mean of three readings. The experiment was repeated 3 times with similar results.
if the reaction with alkaline phosphatase (Table 3.27) is genuine, the substrate is likely to be a monoester of phosphoric acid.

(vi) **Comparison of the substrate with GSSG and cystine**

Amino acid analysis of the peptide substrate under study showed that glycine and glutamic acid were absent from the peptide. Thus the possibility that the substrate was the tripeptide glutathione was unlikely. Furthermore, phosphate groups were always associated with the substrate. The possibility that the peptide may be the mixed disulphide of GSH and CoA was rendered unlikely by the observation that the peptide did not absorb light at 260 nm, was not significantly adsorbed on activated charcoal and was devoid of glutamic acid and glycine after acid hydrolysis. Since the ratio of cystine : glutamic : glycine in glutathione is 1 : 1 : 1, it is unlikely that the lack of detection of these amino acids was due to insufficient amounts of these amino acids being present, as the cystine peak was large and distinct and was only observed after acid hydrolysis.

Both GSSG and cystine were found mainly in the flow through fraction of the column. A small proportion of the glutathione applied to the column was retarded and was eluted as a peak emerging at 0.4 M-NaCl (Fig. 3.28). The peptide substrate on the other hand was totally retarded and emerged as a single peak at 0.6 M-NaCl. This is further evidence that the substrate under investigation is neither glutathione nor cystine. Furthermore, cystine was not a substrate for the reductase under study.

The cellular concentration of the substrate was estimated to be about 4.84 ± 1.50 (n=9) μmol g⁻¹ liver, assuming a stoichiometry of one disulphide bond cleaved per NADPH oxidised. In the absence of knowledge regarding the specificity of the enzyme under study, this figure
The elution profile of glutathione (○) and cystine (△) after ion exchange chromatography on a Dowex Cl\(^-\) column was determined. The experiment was performed at pH 8.3 20mM-BTP/HCl 1mM-EDTA pH 7.0) and eluted in a stepwise manner with NaCl in 20mM-BTP/HCl pH 8.3. The elution profile of the substrate under study was also determined (○). The assay conditions for glutathione and the disulphide reductase were as in II.B.1.e and f. The disulphide content of the cystine containing fractions was determined as in II.B.2.g.

Fig. 3.28: Comparison of GSSG, cystine and the substrate.

The elution profile of glutathione (○) and cystine (△) after ion exchange chromatography on a Dowex Cl\(^-\) column was determined. The experiment was performed at pH 8.3 20mM-BTP/HCl 1mM-EDTA pH 7.0) and eluted in a stepwise manner with NaCl in 20mM-BTP/HCl pH 8.3. The elution profile of the substrate under study was also determined (○). The assay conditions for glutathione and the disulphide reductase were as in II.B.1.e and f. The disulphide content of the cystine containing fractions was determined as in II.B.2.g.
can only be considered as a rough estimate of the substrate (or substrates) being reduced. The cellular concentration of GSSG has been reported to be about 18 mmol g\(^{-1}\) liver in perfused rat liver (Sies et al., 1983). Lower values have been reported by Veech et al. (1969) and Owen et al. (1965).

(vii) Comparison of the substrate to thioredoxin and glutaredoxin

The reduction of ribonucleotides to the corresponding deoxyribonucleotides, needed for DNA synthesis is catalyzed by ribonucleotide diphosphate reductase, which has been shown to depend on two different hydrogen donor systems (Thelander and Reichard, 1979; Luthman and Holmgren, 1982a,b). One of these is the NADPH-dependent thioredoxin system (Thelander and Reichard, 1979) and the other is a glutathione-dependent system where the protein glutaredoxin couples the oxidation of GSH to the reduction of ribonucleotides (Luthman and Holmgren, 1982b). Fig.3.29 shows the proposed scheme for the latter system. The thioredoxin system has already been discussed in the Introduction.

Since both these systems use NADPH and involve the reduction of disulphide bonds, it is not unlikely that glutaredoxin or thioredoxin or a phosphorylated derivative of these, could be the substrate under investigation in the present study. However, an analysis of the amino acid composition of the substrate in the present study shows that this is unlikely. While both thioredoxin and glutaredoxin have been shown to contain high levels of glutamic acid (Luthman and Holmgren, 1982a,b) relative to cystine, glutamic acid could not be detected in the present study at any stage of the purification of the substrate. Thioredoxin has also been shown to consist of high levels of lysine, aspartic acid and alanine relative to cystine, this was not found to be the case.
Fig. 3.29: The glutaredoxin system

In this system, the ultimate source of electrons is NADPH, but they are passed to glutathione (catalyzed by glutathione reductase). Reduced glutathione then reduces glutaredoxin, which in turn acts as the reducing substrate in the ribonucleotide reduction (Luthman and Holmgren, 1982).
for the substrate in the present study. Whereas the ratio of valine : cystine has been reported to be 2.75 and 2.22 for thioredoxin and glutaredoxin, respectively, it was found in this study that the ratio of valine to cystine was in favour of cystine (1: 0.4) in the various analytical experiments performed on the substrate.

Thioredoxin and the substrate in the present study do, however, share at least one property in common and that is that they both appear to have valine as the only N-terminal amino acid. The cellular concentration of thioredoxin and glutaredoxin (both have an $M_r$ of 12,000) has been reported to be 45 mg/kg wet weight and 14.0 mg/kg, respectively. These concentrations are three orders of magnitude lower than the estimated cellular concentration of the peptide under study. Furthermore, the molecular weight of the substrate under study would not appear to be greater than 5000 as determined from gel- and ultrafiltration experiments. Furthermore both thioredoxin and glutaredoxin are reported (Luthman and Holmgren, 1982a,b) to be eluted in the void volume when chromatographed on Sephadex G-50 (separation range 1,500-30,000), whereas the peptide under study separated from the macromolecules on Sephadex G-25 (1,000-5,000). Furthermore, DTNB cannot be substituted for the substrate under study, whereas Luthman and Holmgren (1982a) have reported that DTNB can act as a substrate instead of thioredoxin. In the reaction under study, the disulphide bonds in DTNB are only broken down after the reduced peptide substrate is formed.

There are sufficient differences between the substrate under investigation and both glutaredoxin and thioredoxin to eliminate the possibility that one of the latter may be identical to the former.
(viii) General properties of the substrate

The preceding discussion indicates that the substrate is a peptide, containing disulphide bonds and, possibly, phosphate groups. Furthermore, the substrate appears to be highly charged as evidenced by the high concentrations of NaCl required to elute the substrate off the anion-exchanger. For instance the concentration of NaCl required to elute F2,6P2 from Dowex Cl-1 (x8 - 200) was found to be between 0.175M and 0.250M (results not shown). The concentration range required for the substrate under study was 0.21 to 0.42M-NaCl. Leucine, valine, methionine and cystine were found not to bind to the ion exchanger under the same conditions.

Treatment of the 30% PEG supernatant fraction with HCl (pH 1) for 20min at 4°C resulted in a 43% reduction in the substrate content, as determined by the rate of NADPH oxidation. The substrate was heat stable in that it was not destroyed by heating to 90°C for 45min at pH 8.3.

4. Characteristics of the reaction and comparison with known NADPH-dependent disulphide reducing reactions

The evidence from the previous sections suggests that a NADPH-dependent reductase exists in rat liver cytosol with a specificity towards a cystine-containing peptide substrate, which can also be shown to occur in the same cellular compartment. The properties of the reaction between the partially purified enzyme and substrate were further investigated.

a. Kinetics of the reaction

A kinetic analysis shows that the enzyme exhibits simple Michaelis-Menten kinetics with respect to its natural substrate (Fig. 3.13a) and to NADPH (Fig. 3.30a). The estimated value of the $K_m$ of the enzyme for NADPH is 5μM (Fig. 3.30b) and that for the peptide substrate is
The NADPH-oxidising activity at varying NADPH concentrations was assayed as in II.B.1.e. The points are mean ± S.D (n=4). A partially purified enzyme (II.B.4.d.(ii).) and purified substrate (II.B.4.d.(iii).) were used in the assays.
Fig. 3.30b: Determination of Km of the enzyme for NADPH and NADH

The graph is based on the data in Fig. 3.30a.
82.5\text{\mu M} (Fig. 3.13b), assuming a 1 : 1 relationship between moles of peptide reduced and moles of NADPH oxidised. Although NADH was oxidised by crude heat extracts (5\% V_{\text{max}} \text{ of NADPH}) it was not oxidised by partially purified preparations of enzyme and substrate (results not shown).

b. **Effect of pH and temperature on the reaction**

The enzyme was found to be active over a broad pH range. The pH optimum of the reaction was, however, shown to be 6 (Fig. 3.31), and the acetate buffer was found to be somewhat inhibitory compared to BTP/HCl (Fig. 3.31). The temperature optimum was found to be 37°C (Fig. 3.32).

c. **Apparent irreversibility of the reaction**

An interesting feature of the reaction is that it appears to be irreversible. Addition of excess NADP^+ at concentrations up to 1\text{mM}, failed to reverse the reaction catalysed by the 10-30\% PEG-6000 fraction. The purified substrate was used. The reaction was allowed to proceed to completion in the direction of NADPH oxidation (II.B.1.e) after which NADP^+ was added in excess but, as stated, no reversibility could be demonstrated.

The rationale behind the experiment was that if the reaction, shown below, was reversible, one would expect that once the forward reaction had come to a stop, the equilibrium of the reaction could be displaced such that the addition of excess NADP^+ would favour the backward reaction. This was not found to be the case under the conditions used in the present study.

\[
\text{RSSR}^+ + \text{NADPH} \rightarrow \text{R}^+\text{SH} + \text{RSH} + \text{NADP}^+ 
\]
Two buffers were used to determine the effect of pH on the NADPH-oxidising activity. Sodium acetate/acetic acid buffer (0.2M) was used in the pH range 3-6 and TRIS/HCl (0.2M) in the pH range 6-9. The enzyme and substrate sources were the 10-30% and 30% PAG-fractions, respectively.
Fig. 3.31: **The effect of pH on the NADPH-oxidising activity**

Two buffers were used to determine the effect of pH on the NADPH-oxidising activity. Sodium acetate/acetic acid buffer (0.2M) was used in the pH range 3-6 and BTP/HCl (0.2M) in the pH range 6-9. The enzyme and substrate sources were the 10 - 30% and 30% PEG-fractions, respectively.
The effect of temperature on the reaction was determined and the rates assayed as in II.B.1.e. The incubation mixture was maintained at the appropriate temperature for 10 min. The temperature of the reaction chamber of the spectrophotometer was brought as near as practically possible to the required temperature. The results are the mean ± S.D. (n=4). The enzyme source was the 10 - 30% PEG fraction and the substrate source was the 30% PEG fraction.
There are several possible explanations for the irreversibility of the reaction. Firstly, the enzyme may indeed catalyse an irreversible reaction under normal physiological conditions, and that in vivo the reverse reaction may be catalysed by a different enzyme possibly with different cofactor requirements. In this context, an excess of NAD$^+$ did not reverse the reaction.

A second possibility is that the reaction is rendered irreversible by further metabolism or instability of the peptide product, such that it cannot be utilized in the reverse reaction despite the presence of an excess of NADP$^+$.

d. The stoichiometry of the reaction

The stoichiometry of the reaction was determined by following the rate of NADPH oxidation at 340nm and relating this to the reduction of disulphide at 412nm in the presence of DTNB. The initial rate of NADPH oxidation was found to be $10.4 \pm 2.3$ (n=6) mmol min$^{-1}$ mg$^{-1}$ protein. Under the same conditions of assay, with the same enzyme and substrate (Sephadex G-25 eluate) preparations and concentrations, the rate of thiol production was $17.2 \pm 2.7$ (n=6) mmol min$^{-1}$ mg$^{-1}$ protein. The ratio of NADPH oxidation to thiol production was therefore determined to be $1:1.7$ or, approximately, $1:2$. This indicates that 1 NADPH is oxidised for every two thiols produced. Fig. 3.25 shows a linear relationship between the total NADPH consumed, thiol groups produced and the volume of substrate reacted with the enzyme. In this case however the average thiol yield per mole NADPH oxidised was $3:1$, implying that 3 thiol groups were being produced per mole of NADPH. The reason for the discrepancy between the initial rate data and the redox titration data is not clear. It is possible that some NADPH is regenerated or that there is an autocatalytic breakdown of the substrate as a result of the initial
enzymatic cleavage of the disulphide.

e. **Comparison of the reductase under study with the thioredoxin - and glutathione-reductase catalysed reactions**

The relatively crude enzyme preparations used in the study did reduce glutathione but not cystine (up to 1mM). Mize and Langdon (1962) and Carlberg and Mannervik (1975) have shown that glutathione reductase can catalyse the reduction of cystine. Other differences between the reaction described here and that catalysed by glutathione reductase are that the pH optimum for the reaction in the present study was found to be 6.0, compared to 7.0 for the glutathione catalysed reaction (Carlberg and Mannervik, 1975); 64% of the activity was lost upon incubation of the enzyme under investigation at 70°C for 30 min, whereas Tietze (1970b) showed that glutathione reductase was stable under these conditions; NADP⁺ (200µM) which has been reported to inhibit glutathione reductase with a Kᵢ of 100µM (Carlberg and Mannervik, 1975) only caused a 20% inhibition of the reaction under study. MgCl₂ and CaCl₂ which have been reported to stimulate GSSG reductase (Mize and Langdon, 1962) had no effect on the rate of the reaction under study. These observations suggest that glutathione reductase is not the enzyme responsible for the reduction of the disulphide in the present study.

The involvement of thioredoxin reductase is thought to be unlikely for the following reasons: The Kₘ for the substrate in the present study is 33 times that of thioredoxin reductase for thioredoxin (Luthman and Holmgren, 1982a); The enzyme under consideration, unlike thioredoxin reductase, was not sensitive to inhibition by arsenite (10⁻¹¹M); This concentration of arsenite has been reported to inhibit thioredoxin reductase by 96% (Luthman and Holmgren, 1982a); Finally, these authors also reported that thioredoxin reductase catalysed the NADPH-dependent reduction of DTNB and that thioredoxin
inhibited this reaction. In this study, the enzyme under investigation did not reduce DTNB and the presence of DTNB did not inhibit the reduction of the substrate (Table 3.26).

Although an attempt has not been made to further characterize the reductase under study, experiments involving ultrafiltration indicated that the enzyme is small in size compared to thioredoxin reductase ($M_r 116,000$) and glutathione reductase ($M_r 125,000$), since it passed through ultrafiltration membranes with a $M_r$ cut off of 10,000.

These observations together with the considerations detailed in C.3. have led to the conclusion that the reaction reported here is separate and distinct from the glutathione and thioredoxin reductase catalysed reactions. The possibility that either of these enzymes is reducing a different substrate and thus exhibiting different properties has not been eliminated.

In this work the average rate of NADPH oxidation catalysed by the peptide reductase in the heat extract has been estimated to be $1.98 \pm 0.68$ (n=6) $\mu$mol min$^{-1}$ g$^{-1}$ liver during the light period and $0.64 \pm 0.12$ (n=3) $\mu$mol min$^{-1}$ g$^{-1}$ liver during the dark periods. The reaction was found to be associated with the parenchymal cells. When hepatocytes were isolated between 9 and 11 a.m., the rate of NADPH oxidation was found to be $2.29 \pm 0.26$ $\mu$mol min$^{-1}$ g$^{-1}$ cells. In these experiments with hepatocytes it was essential to transfer the cells from the Krebs-Hensleit buffer to BTP/HCl (pH 8.3, 1mM EDTA, 0.25M sucrose) before sonication of the cells, in order to obtain significant reaction rates.
5. **Physiological significance of the NADPH-oxidising system**

In an effort to determine the physiological significance of the NADPH-oxidising/disulphide reducing reaction described in the preceding chapters, a comparative study was carried out. In this study the initial rates of the reaction were determined in fed and starved rats; male and female rats; in anaesthetized and non-anaesthetized rats; and finally, the rates were also measured at different times of the day.

a. **Comparison of initial rates of NADPH oxidation by liver extracts from males and females**

When animals of similar weight and age (3 months old) were compared, it was found that there was no significant difference between the initial rate of the reaction in liver extracts from males \( (2.24 \pm 0.49 \mu\text{mol min}^{-1} \text{g}^{-1} \text{liver}, n=6) \) and females \( (2.42 \pm 0.57 \mu\text{mol min}^{-1} \text{g}^{-1} \text{liver}, n=6) \). In this experiment the rats were killed at 2:45 p.m.

b. **The effect of anaesthetics on the rate of the reaction in liver extracts**

In this set of experiments, a comparison was made between the rates of NADPH oxidation found in liver extracts of rats killed with a blow to the head, and those anaesthetized with Sagatal or with ether. These rats were killed at 2:45 p.m. It was found that there was no significant difference between the first group and the Sagatal-treated rats \( (P > 0.100) \). However, a significant difference \( (P > 0.01 < 0.025) \) was found between the ether-treated animals \( (4.20 \pm 1.52 \mu\text{mol min}^{-1} \text{g}^{-1} \text{liver}, n=6) \) and the Sagatal-anaesthetized rats \( (2.46 \pm 0.48 \mu\text{mol min}^{-1} \text{g}^{-1} \text{liver}, n=6) \). Since ether appeared to increase the rate of NADPH oxidation, and since this effect could be the result of an increase in an NADPH-dependent enzyme or reaction not related to the reaction under study, the
comparisons reported below were performed on Sagatal-anaesthetized rats.

c. Demonstration of a diurnal variation of the NADPH-oxidising activity and the effect of starvation

A diurnal variation was observed in the rates of NADPH oxidation catalysed by heat-treated extracts of rat liver isolated at various times during the day (Table 3.29a). It is clear from the table that the initial rate of the reaction is higher during daylight hours than at night. This difference was found to be statistically significant \( P < 0.005 \).

The rats used in these experiments were subject to a 12h light/12h dark regime, with the dark period starting at 6.00 p.m. Rats are known to be nocturnal feeders (Edwards et al., 1972) so that the decreased activity observed at 9:00 p.m. may be related to the feeding period of these animals and the higher rates found during the day may be coincident with the period of fasting.

To test this hypothesis, animals were starved for 48h, killed at 10.45 a.m., the livers extracted and assayed for NADPH oxidising activity (Table 3.30). This period of starvation resulted in a 1.8 fold increase in the activity \( P < 0.01 \), confirming the view that the diurnal changes observed could be related to feeding and fasting.

If the results in Tables 3.29a and 3.30 are considered together, it is apparent that there is a 5-fold difference between the activity observed when the animal is feeding (9:00 p.m.) and the activity found in the starved rats. This large increase in activity during starvation may be related to the high \( \text{NADPH} / \text{NADP}^+ \) ratio found in the livers of starved animals (Greenbaum et al., 1971).
Table 3.29a: Diurnal variation of the NADPH-oxidising activity

<table>
<thead>
<tr>
<th>Time of day</th>
<th>Total activity (μmol min⁻¹ g⁻¹)</th>
<th>Numbers of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.45 a.m.</td>
<td>2.91 ± 0.52</td>
<td>6</td>
</tr>
<tr>
<td>2.45 p.m.</td>
<td>2.70 ± 0.20</td>
<td>6</td>
</tr>
<tr>
<td>9.00 p.m.</td>
<td>1.09 ± 0.28</td>
<td>6</td>
</tr>
</tbody>
</table>

Litter mates of 3 month old female rats were used in the experiment. The heat extract was obtained as in II.B.4d. and assayed for NADPH-oxidising activity as in II.B.1e. The results are expressed as mean ± S.D. and represent the total activity per gramme liver. The experiment was repeated three times.

Table 3.29b: Statistical analysis of the data in Table 3.29a

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Statistical significance (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.45 a.m. and 2.45 p.m.</td>
<td>&gt; 0.100</td>
</tr>
<tr>
<td>10.45 a.m. and 9.00 p.m.</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>2.45 p.m. and 9.00 p.m.</td>
<td>&lt; 0.005</td>
</tr>
</tbody>
</table>
Table 3.30: The effect of starvation (48h) on the NADPH oxidising activity

<table>
<thead>
<tr>
<th>Treatment of the animals</th>
<th>Total specific activity (μmol min⁻¹ g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>48h starved</td>
<td>5.21 ± 0.43</td>
</tr>
<tr>
<td>fed</td>
<td>2.89 ± 0.19</td>
</tr>
</tbody>
</table>

Female rats were used in this experiment. The liver weights were within 1.06g of each other. There were 3 animals in each group and the experiment was performed twice. The results were found to be statistically significant (P < 0.005) and the results are expressed as mean ± S.D. (n=3).
The activity measured in the heat extracts reflects both the activity of the enzyme and the level of substrate. In order to determine whether the changes observed were the result of differences in enzyme activity or in substrate content of the liver, the total amount of NADPH oxidised in the fed animals was determined in each case (Table 3.31). This was taken to reflect the substrate content of the liver. Although a slight decrease in the substrate content was observed at 9.00 p.m. (Table 3.31) this was not found to be statistically significant. It would therefore appear that the observed decrease in the reaction at 9.00 p.m. was the result of either an inhibition or a decrease in the quantity of the enzyme rather than a change in the substrate content of the liver.

In interpreting these results it has been assumed that the reductase under investigation is saturated with the substrate both in vivo and in vitro. This assumption has been based on the comparison of the $K_m$ of the enzyme for the substrate and the substrate content of the heat extract. Furthermore, addition of excess purified substrate to the heat extract reaction mixture did not increase the rate of reaction significantly (results not shown).

d. Interpretation of the results obtained in physiological terms

Fig. 3.34 summarizes the conclusions drawn from the observations made in this study. Firstly, the continual oxidation of NADPH with consequent production of NADP$^+$ could conceivably stimulate the PPP dehydrogenases according to the 'Pull' hypothesis. This would result in an increased production of NADPH, which can be utilized by pathways such as lipogenesis and cholesterogenesis. However, it is important to realise that the reaction under study could also compete with cholesterogenesis...
Table 3.31: Diurnal variation of the substrate content of rat liver

<table>
<thead>
<tr>
<th>Time of day</th>
<th>Total NADPH oxidised μmol g⁻¹ liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.45 a.m.</td>
<td>4.16 ± 1.20</td>
</tr>
<tr>
<td>2.45 p.m.</td>
<td>4.08 ± 0.79</td>
</tr>
<tr>
<td>9.00 p.m.</td>
<td>3.48 ± 0.64</td>
</tr>
</tbody>
</table>

Litter mates of female rats were used in the experiments. The total amount of NADPH oxidised was determined for each liver by following the change in O.D. at 340nm (II.B.1e). The results are expressed as the mean ± S.D. (n=6). The differences between the different times were not statistically significant (P > 0.100).
Lipogenesis (stimulation of PPP) leads to increased NADPH-oxidising activity during lipogenesis and decreased during lipogenesis. Increased NADPH-oxidising activity is also observed in the absence of lipogenesis.

Fig. 3.34: Possible relationship between lipogenesis and the NADPH-dependent reductase.
and lipogenesis for NADPH in the normal fed cell and this may account for the lower NADPH-oxidising activity observed during feeding periods when lipogenesis is maximal (Kimura et al., 1970; Edwards et al., 1972; Hems et al., 1975). Under these conditions the NADPH-oxidising activity was found to be reduced by 50-60% of the activity found during periods when animals would be expected to be fasting. This would suggest that there may be competition for NADPH between the reaction under study and lipogenesis and cholesterogenesis.

It is clear that the reaction in the present study has a diurnal variation pattern which is the reverse of the exhibited by lipogenesis and cholesterogenesis.

The enzyme responsible for the oxidation of NADPH in fatty acid synthesis is FAS. However, although rates of fatty acid synthesis have been shown to vary diurnally, being highest during feeding hours, a comparable rhythm in assayable FAS activity has not been demonstrated (Bruckdorfer et al., 1974). This contrasts with the reductase under study, where the enzyme activity appears to be inhibited or reduced in some way. In contrast to FAS, however, hydroxymethylglutaryl-CoA (HMG-CoA) reductase, another NADPH-dependent enzyme, responsible for the regulation of cholesterogenesis, exhibits a circadian (diurnal) rhythm, as does cholesterol biosynthesis itself (Edwards et al., 1972; Zubay, 1983). The peak of activity of this enzyme has been shown to be around midnight.

The inverse relationship between the times of maximal flux through cholesterogenesis and lipogenesis, which are probably related to food intake, and pathways of NADPH utilization, such as the reaction under study, would limit the competition for the NADPH pool of the liver and overcome the problems which would be expected to arise as a result of excessive oxidation of NADPH and consequent
disturbance of the NADPH / NADP⁺ ratio. The possibility that NADPH content of the liver cell may, under conditions of enhanced NADPH oxidation, become a limiting factor in lipogenesis has been indicated by the work of Thurman and Scholz (1973), who found that enhanced mixed-function oxidation inhibits lipogenesis.

The total concentration of NADPH in the rat liver extramitochondrial compartment has been reported to be 65 nmol g⁻¹ liver wet weight (Sies, 1982). The Kₘ for NADPH of FAS is about 50μM (Volpe and Keshimoto, 1972). In contrast the Kₘ values for NADPH of all the disulphide reductases considered in this study are less than 10μM: thioredoxin reductase, 2.5μM; glutathione reductase, 7.9μM; the reductase under study, 5μM. This data suggests that if FAS competed with these enzymes for NADPH, it would be the least effective. Furthermore it has been reported that the maximum rate of fatty acid synthesis in liver is 0.12 μmol palmitate min⁻¹ g⁻¹ liver, equivalent to 1.68 μmol NADPH min⁻¹ g⁻¹ liver, (Holten et al., 1976). By comparison, the reductase in the present study is capable of oxidising NADPH at a maximal rate of about 2 μmol min⁻¹ g⁻¹ liver. These considerations would explain why it would be favourable to inhibit the disulphide reductase during periods of active lipogenesis.
IV. GENERAL DISCUSSION AND CONCLUSION

The original aim of the thesis was to establish a link between the regulation of glycolysis and of lipogenesis by examining the effect of \( \text{F}2,6\text{P}_2 \) on some lipogenic enzymes. From a consideration of the literature one would expect conditions in which there are high rates of lipogenesis to be associated with high \( \text{F}2,6\text{P}_2 \) levels. It is known, for example, that glucose, at high concentrations, increases \( \text{F}2,6\text{P}_2 \) (Van Schaftingen et al., 1980a,b,c and 1981a,b,c) and can also stimulate fatty acid synthesis independently of its role as a carbon source (Salmo et al., 1974). On the other hand, glucagon decreases \( \text{F}2,6\text{P}_2 \) and is a potent inhibitor of lipogenesis (Barrows et al., 1981; Wakhil et al., 1983)

Hence the possibility exists that \( \text{F}2,6\text{P}_2 \) may have a role in the co-ordinated regulation of lipogenesis and glycolysis. However, in the course of this work no physiologically relevant activation or inhibition of some of these enzymes was demonstrated. In fact, there are no reports in the literature which claim any regulatory role for \( \text{F}2,6\text{P}_2 \) other than in the control of the \( \text{F}6\text{P}/\text{F}1,\text{P} \) substrate cycle. The enzymes investigated in this study were \( \text{F}6\text{P} \), \( \text{6P}1\text{CDP} \) and \( \text{6P}1\text{F6P} \).

In the course of the investigation of the possible effects of \( \text{F}2,6\text{P}_2 \) on the pyruvate dehydrogenase, it was found that there was an NADH-sensing reaction, which was catalysed by a cytoplasmic liver enzyme and involved a cystine-containing protein substrate which was also found associated with the same cellular compartment.

A comparison of the substrate with other peptides, which are known to be reduced by NADH-dependent reductases revealed properties which distinguished it from oxidised glutathione, cysteine, thioredoxin and glutaredoxin. These properties include a high cysteine
The original aim of the thesis was to establish a link between the regulation of glycolysis and of lipogenesis by examining the effect of F2,6P2 on some lipogenic enzymes. From a consideration of the literature one would expect conditions in which there are high rates of lipogenesis to be associated with high F2,6P2 levels. It is known, for example, that glucose, at high concentrations, increases F2,6P2 (Van Schaftingen et al., 1980a,b,c and 1981a,b,c) and can also stimulate fatty acid synthesis independently of its role as a carbon source (Salmon et al., 1974). On the other hand, glucagon lowers F2,6P2 and is a potent inhibitor of lipogenesis (Bartrons et al., 1983; Wakil et al., 1983).

Hence the possibility exists that F2,6P2 may have a role in the co-ordinated regulation of lipogenesis and glycolysis. However, in the course of this work no physiologically relevant activation or inhibition of some of the key lipogenic enzymes by this metabolite was demonstrated. In fact, there are no reports in the literature which claim any regulatory role for F2,6P2 other than in the control of the F6P/F1,6P2 substrate cycle. The enzymes investigated in this study were FAS, 6PGDH and G6PDH.

In the course of the investigation of the possible effects of F2,6P2 on the PPP dehydrogenases, it was found that there was an NADPH-consuming reaction, which was catalysed by a cytoplasmic liver enzyme and involved a cystine-containing peptide substrate, which was also found associated with the same cellular compartment.

A comparison of the substrate with other peptides which are known to be reduced by NADPH-dependent reductases revealed properties which distinguished it from oxidised glutathione, cystine, thioredoxin and glutaredoxin. These properties include a high cystine
content relative to other amino acids in the substrate molecule, the acidic nature of the peptide as determined by its behaviour on ion-exchange chromatography, its phosphate content, and, finally, the apparently high cellular content of the oxidised form.

It is not readily apparent why the peptide under study exists in the oxidised form in the cell since it is readily converted to the reduced form in vitro by the enzyme isolated in the present study. Various possibilities could account for this phenomenon. It is possible, for instance, that the substrate, the reductase and the NADPH are compartmentalized in such a way as to prevent the reduction of the substrate. A second possibility is that NADPH-dependent reduction of the substrate is extensively inhibited in vivo by a high molecular weight component which is removed either by heating or by ultra-filtration. Alternatively, it is possible that the peptide exists in the reduced form in vivo but becomes oxidised during the extraction procedure. It would be instructive to explore the latter possibility.

The physiological role of the peptide substrate and the reductase described in this thesis remains a problem. It is known that there is a high level of disulphide residues associated with rat liver cytosol preparations of which oxidised glutathione content represents only a small fraction (Isaacs and Binkley, 1977). The remainder is assumed to be mixed disulphides of glutathione with proteins and disulphide residues within proteins. This thesis does show, however, that a significant proportion of the disulphide residues may in fact be associated with an acidic peptide. At this point it is worth noting that the existence of protein-GSH mixed disulphides in the cell, to the extent reported in the literature has been questioned (Meister and Anderson, 1983). It has been suggested that they may be artifacts of the isolation procedures.
The diurnal variation and the effects of feeding and fasting reported in this thesis, of the NADPH-oxidising activity in crude extracts indicates a possible physiological role for the NADPH-dependent disulphide reductase. Although the specificity of the enzyme under study with respect to disulphides is unknown and the possibility of other substrates being present in the crude extracts remains, it is tempting to speculate that this diurnal variation may be related to the regulation of other hepatic phenomenon known to exhibit such variations. It is well known that lipogenesis, cholesterogenesis and G6PDH activity undergo diurnal changes. Furthermore, these changes are inversely related to the change found for the reductase activity in the present study. The latter activity is also inversely related to changes in the total \(-SS- / SH\) ratio in rat liver extracts (Isaacs and Binkley, 1977).

It is possible that regulation of these cellular processes could be related to changes in the \(\text{NADPH} / \text{NADP}^+\) and the \(-SS- / -SH\) ratios in the hepatic cytoplasm. For example it has been reported that G6PDH is inactivated by a disulphide-dependent microsomal factor which may have a role in the degradation of the enzyme (Francis and Ballard, 1980a,b). It would be of interest to investigate the possible effects of the peptide under study on various key regulatory enzymes, the activities of which may be regulated by covalent modification with such a peptide.

It is generally recognised that G6PDH is a key rate-limiting enzyme in the PPP; yet a consideration of its kinetic properties suggests that the enzyme should be extensively inhibited in the presence of the \(\text{NADPH} / \text{NADP}^+\) ratios reported to occur in the cytoplasm. One aim of this thesis was to attempt to demonstrate the possible de-inhibition of the enzyme under certain physiological conditions. Eggleston and Krebs (1974) reported that GSSG and a low molecular weight cofactor
relieved the NADPH inhibition of G6PDH in crude liver extracts. In agreement with Levy and Christoff (1983) it was found in this study that the effect was the result of an artefact caused by the addition of Zn$^{2+}$ to the assay mixture to inhibit GSSG reductase. It has also become apparent that the apparent inhibition by GSSG of G6PDH reported by Eggleston and Krebs (1974) can be attributed to GSSG reductase activity. These observations highlight the problems of using relatively crude extracts to examine the effect of physiologically important regulators on enzyme activities. On the other hand, the use of purified preparations can also give misleading results. It is, nevertheless, important to show that effects observed with crude preparations can also be shown with a re-combined system with purified components.

In addition to the de-inhibition of G6PDH by metabolites an alternative method of de-inhibition - the so called 'PULL' hypothesis should be considered. This hypothesis states that the removal of NADPH (and the consequent regeneration of NADP*, the most effective known positive effector of G6PDH) is a pre-requisite for the activation of the PPP dehydrogenases. It is clear that the NADPH-dependent reduction of the disulphide substrate described in this thesis could have such a role. The diurnal variation in the reductase activity which is inversely correlated to that of lipogenesis and cholesterogenesis may be significant in this respect. It is possible that there is competition between FAS and the disulphide reductase for the cytoplasmic NADPH. A situation such as this would explain the apparent inhibition of the disulphide reductase activity during periods associated with high lipogenic rates.

Evidence for the 'PULL' hypothesis has accumulated in recent years. Yeh et al. (1984) have presented evidence correlating the regulation of erythrocyte G6PDH activity and NADPH-dependent pyrroline-5-carboxylate
reductase. Treatment of the normal erythrocyte with pyrroline 5-carboxylate causes a six-fold increase in the flux through the PPP. Under the same conditions 5-phosphoribosyl 1-pyrophosphate formation increased 2.5-fold, and the incorporation of hypoxanthine into inosine monophosphate increased 2.6-fold. In contrast, pyrroline 5-carboxylate had no effect on G6PDH-deficient erythrocytes. Hence, Yeh et al. (1984) have proposed that pyrroline 5-carboxylate is converted to proline by the reductase with concomitant production of NADP+ which, in turn, augments G6PDH activity with an increased production of ribose 5-phosphate.

Similarly, May (1981) has suggested that under conditions where GSH is oxidised, increased glutathione reductase activity, in adipose tissue, parallels increased flux through the PPP dehydrogenases. Elsayed et al. (1982) have also shown that oxidant stress (such as exposure to ozone) increases glutathione reductase, G6PDH and 6PGDH activities in the presence of selenium in mouse lung.

Further evidence for the regulation of the PPP dehydrogenases by alterations in the NADPH / NADP+ ratio, in rat liver, has come from the work of Fabregat et al. (1985) who have shown that inhibition of fatty acid synthesis with kynurenate results in an inhibition of the PPP and an increase in the NADPH / NADP+ ratio. This implies that the activity of the PPP is dependent on an active fatty acid synthesizing (NADPH utilizing) system. Conversely, the authors have shown that incubation of hepatocytes with tert - butyl - hydroperoxide or paraquat compounds, which are metabolized via NADPH-consuming pathways, activate the PPP and decrease the NADPH / NADP+ ratio.

These observations strongly support the view that the PPP dehydrogenases are regulated by factors which alter the NADPH / NADP+ ratio, and provides further evidence
for the 'PULL' hypothesis. This does not, however, exclude other mechanisms of regulation such as that proposed by Eggleston and Krebs (1974). Furthermore it is possible that G6PDH and 6PGDH are not as sensitive to NADPH inhibition in vivo as they are in vitro. Enzyme concentrations are generally much higher in vivo than under in vitro assay conditions, a fact that could significantly affect the regulatory behaviour of an enzyme. Thus in vitro assay conditions may not reveal the true regulatory properties of the enzyme in vivo.

In the light of the recent evidence presented by Martins et al. (1985), which suggests that individual multiple molecular forms of G6PDH may respond differently to hormonal factors, it would be instructive to determine the response of the different multiple molecular forms of G6PDH to NADPH / NADP⁺ ratios.

In conclusion, the available evidence suggests that the activity of the PPP dehydrogenases is primarily determined by changes in the NADPH / NADP⁺ ratio. These changes can be effected by a variety of conditions and in particular by NADPH utilizing reactions.
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