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IN THE UNIVERSITY OF OXFORD

STUDIES ON GLYCERALDEHYDE 3 PHOSPHATE DEHYDROGENASE

G.D. Clark Walker

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<tr>
<td>ADP</td>
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<td>ADPR</td>
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<td>G3P</td>
<td>Glyceraldehyde-3-phosphate</td>
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<td>G3PD</td>
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<td>LDH</td>
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<td>NAD⁺</td>
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**Definitions**

**Absorptivity.**

This term is equivalent to 'extinction' or 'optical density' and is given by the expression

$$ \log_{10} \frac{I_o}{I} = \text{absorptivity} \,$$

where $I_o$ and $I$ represent the intensities of incident and transmitted light.

**Molar absorptivity.**

Is the absorptivity of a sample with a 1 cm light path having a concentration of 1 g. mole/litre.

i.e. molar absorptivity = \frac{\text{absorptivity}}{c \times l}

where $c$ is in g moles/L and $L$ is in cm.
ABSTRACT

The enzyme glyceraldehyde-3-phosphate dehydrogenase E.C.1.2.1.12 has been prepared from rabbit muscle by ammonium sulphate fractionation. After four refractionations the levels of the contaminating enzymes α GDH & TP1 were found to be 25 parts and 1 part per million and contamination by myokinase was 3 parts per thousand. The activity of the purified enzyme was found to be in the region of 14,000 - 17,000 moles NADH/min/10^5 g protein.

During refractionation of the purified enzyme in the presence of excess NAD+ the enzyme was found to crystallize in rectangular plates instead of the familiar rhomboidal plates. This observation was investigated in terms of the nucleotide content of the protein and the presence of a nucleotide differing from both NAD+ and NADH was detected. Different preparations of the enzyme from rabbit muscle were found to contain amounts of the unidentified nucleotide varying from 0.60 - 1.63 moles/10^5 g of protein. In conjunction with NAD+ the total nucleotide bound to the protein always amounted to 2.45 moles/10^5 g protein.

The nucleotide has been isolated by passage of the protein down a DEAE sephadex column at pH 9.5 - 10.0 and has subsequently been shown to contain adenine ribose and phosphate in the ratio of 1 : 2 : 2. The nucleotide was also shown to have the same electrophoretic mobility as NADH but no 340 μ absorption and
to have a molar absorptivity of $26.0 \times 10^3$ at the absorption maximum of 266 $\mu m$.

The nucleotide was compared with NADH-X prepared by the G3PD catalysed transformation of NADH and the nucleotide arising from the phosphate catalysed transformation of NADH. Although the initial products of both these transformations appear to be identical with the nucleotide isolated from the enzyme the compounds obtained, with absorption maxima at 264 $\mu m$ and molar absorptivities between $21.0 - 22.2 \times 10^3$, are thought to have undergone further modification during the isolation procedures.

The nucleotide isolated from the protein has not been unequivocally characterized but by analogy with the spectra of other transformed nicotinamides it is thought to be the 6- OH adduct formed by hydration of the 5:6 double bond of NADH.

The finding that the protein isolated from rabbit muscle contains an additional nucleotide other than NAD$^+$ resolves the discrepancy in the literature relating to the number of binding sites on the protein and the apparent liberation of a further binding site on charcoal treatment of the protein.

The increased absorption and unexpected shoulders in the absorption spectrum of the holo enzyme containing bound NAD$^+$ have been investigated by a difference spectrum technique and found to be due to the shielding of a tryptophan residue of the protein from interaction with the solvent. The shielding or burying of tryptophan has also been observed when
NADH and ADP are bound to the protein and in the former case the protein is thought to undergo a configurational change leading to the exposure of a previously 'buried' tyrosine residue. The extent of binding of NAD⁺ to the enzyme has been followed by observing the increase in the 360 mÅ absorption band resulting from the interaction between nucleotide and protein. The formation of the charge transfer complex was found to be markedly different from the expected titration curve based on the small dissociation constant for protein and NAD⁺. Protein concentration was shown to influence the titration curves and this has suggested that subunit effects may be responsible.

The extent of reduction of enzyme bound NAD⁺ by added G3P has been investigated and shown to be associated with the binding of G3P to the protein : NAD⁺ complex. Moreover the reduction of protein bound NAD⁺ was also found to be influenced by the protein concentration, proportionally less NADH being produced on increasing the protein concentration.

No evidence was found that the hydration product of NADH, was concerned in the non-stoichiometry of reduction of bound NAD⁺. Also, the observation was
made that NADH produced in situ by reduction of protein bound NAD$^+$ was not transformed into NADH - X in the slow NADH transformation reaction catalysed by the enzyme.

These facts have led to the conclusion that the NADH - X found on the protein after isolation from rabbit muscle is an artifact of the isolation procedure and is formed as a result of the breakdown of acyl enzyme which leads to the catalysed transformation of the bound NADH to NADH - X.

The titration of the protein with G3P in the presence of phosphate, sulphate or arsenate was found to yield identical curves for the reduction of NAD$^+$. It therefore appears that the overall equilibrium of the enzyme catalysed reaction is not established and that a further step must be included in the currently accepted reaction mechanism of the enzyme.

The number of sulphydryl groups on the protein has been determined using an amperometric technique with phenyl mercuric hydroxide. The value of $10^-2 - 10^-4$ SH residues/$10^5$ g of protein, gives a value of $12 - SH$ residues/117,000 g of protein. A value for the molecular weight in the region of 120,000 is supported by the finding that the holo enzyme binds 3 NAD$^+$ molecules/122,000 g of protein.

The disappearance in protein sulphydryl groups on acyl enzyme formation was studied with acetyl phosphate, acetaldehyde, and G3P as substrates of the enzyme.

Only 0.55 and 0.75 SH groups/$10^5$ g of protein
were found to disappear in the presence of the first two substrates and no difference in sulphydryl titre could be found between protein in the presence or absence of G3P. The results with acetylphosphate support earlier workers who found that acetyl phosphate caused a disappearance in titratable -SH groups of the protein and in addition the data with acetaldehyde confirm the view that the thiol ester so formed is a true catalytic intermediate. The fact that no thiol ester formation can be detected with the natural substrate, which is oxidized 50,000 times faster than acetaldehyde, was not surprising as a true catalytic intermediate must be expected to be broken down as rapidly as it is formed.
INTRODUCTION

A. GENERAL

The ease of preparation and interesting reaction which it catalyses have resulted in the enzyme glyceraldehyde 3-phosphate dehydrogenase (G3PD) E.C.1.2.1.12 being extensively studied both in regard to its physical and chemical properties and in relation to the reaction mechanism. It was first purified and crystallized from yeast by Warburg and Christian (1939) and later by Rafter and Krebs (1950). Dixon and Caputto (1945) and Cori et al. (1945, 1948) isolated the protein from rabbit muscle where it is located in the sarcoplasm and represents 16-23% of the soluble protein (Czok and Möhler, 1960). The enzyme, however, occurs in all cells with a glycolytic pathway (Entner and Doudoroff, 1952) and catalyses the first oxidative step in carbohydrate breakdown converting D-glyceraldehyde-3-phosphate (G3P) into 1,3-diphosphoglyceric acid (1,3 diPGA).

\[ \text{NAD}^+ + \text{G3P} + \text{Pi} \rightleftharpoons \text{NADH} + \text{H}^+ + 1,3\text{diPGA}. \]

NAD\(^+\) is involved in the oxidation, and inorganic phosphate is incorporated to form a 'high energy' bond which is transferred to ADP in the subsequent glycolytic reaction catalysed by phosphoglycerate kinase E.C.2.7.2.3. to form 3-phosphoglyceric acid (3PGA) and ATP.

\[ 1,3\text{diPGA} + \text{ADP} \rightleftharpoons 3\text{PGA} + \text{ATP}. \]

The enzyme is situated at a key point in the glycolytic pathway, where it is linked with \( \alpha \)-glycerolphosphate dehydrogenase (\( \alpha \text{GPDH}\)) E.C.1.1.1.8. and triosephosphate isomerase (TPI) E.C.5.3.1.1. in determining the subsequent metabolism of the triose phosphates produced from fructose 1:6 diphosphate

\[ \text{fructose 1:6 diphosphate} \]

\[ \text{dihydroxyacetone phosphate} \]

\[ \text{glycerolphosphate} \]

\[ \text{synthesis of triglycerides} \]

G3PD + \( \alpha \text{GPDH} \) together with lactic dehydrogenase (LdH) E.C.1.1.1.27. are
the three major dehydrogenases in muscle sarcoplasm (Czok and Bücher, 1960) and various authors have indicated that NADH bound to G3PD can be utilized by LDH in the reduction of pyruvate to lactate (Cori et al., 1950; Astrachan et al., 1957; Nygaard and Rutter, 1956). This result has been interpreted as evidence for the in vivo association of the two enzymes. G3PD has often been suggested as the target site in the Pasteur effect (Van Eyk, 1961) since Lipmann (1942) proposed that O2 acted by inhibiting this enzyme. Hatch and Turner (1960) and Balaza (1963) have made similar observations on the apparent oxygen inhibition of G3PD in pea seed and in brain mitochondria preparations. Wu and Böcker (1959) on the other hand indicate that orthophosphate by its requirement in the G3PD catalysed reaction may be the critical factor in respiratory control of glycolysis. Product inhibition of G3PD by 1,3diPGA has been suggested to account for the Pasteur effect by Velick and Furfine (1963) and on the basis of their kinetic studies they have indicated that a lowering of the steady state concentration of ATP would lead to a rise in 1,3diPGA and hence an inhibition of glycolysis.

Racker (1961) has drawn attention to the similarity between the substrate level phosphorylation catalysed by G3PD and oxidative phosphorylation of mitochondria. He has suggested that the enzyme can act as a model system for studying oxidative phosphorylation as it catalyses a 'tightly coupled' oxidation and phosphorylation. The comparison can be extended by the finding that the glycolytic reaction can be 'uncoupled' if the enzyme is treated with chymotrypsin (Krimsky and Racker, 1963). NAD+ bound to the protein digest fragment is still reduced, but the phosphorylation step of the reaction is lost.

Two recent reviews (Czok and Bücher, 1960; Velick and Furfine, 1963) provide detailed and interesting accounts of the available information on this enzyme; consequently only the apparently aberrant and debatable properties of the enzyme will be presented below.
Since the initial isolation of the crystalline enzyme from rabbit muscle over fifteen years ago, a number of unexplained and conflicting properties of this protein have been published. These features are listed under the following headings:

1. The number of NAD$^+$ binding sites per mole.
2. The non-stoichiometry of reduction of protein bound NAD$^+$ by G3P.
3. The nature of the visible absorption band exhibited by the G3PD-NAD$^+$ complex.
4. The G3PD catalysed transformation of NADH to NADH-X.
5. The requirement for NAD$^+$ in transferase and hydrolase activities of the enzyme.

In what follows, the findings summarized under the first two headings have been considered together due to the fact that the reduction of the coenzyme in situ was initially used to determine the number of binding sites.

The molecular weight of the enzyme has shown a considerable variation and values ranging from 117,000 to 150,000 have been obtained (Velick and Furfine, 1963). As the nucleotide content of the protein has also shown a large variation no unambiguous value for the molecular weight can be decided on, hence an arbitrary molecular weight of 100,000 has been chosen and all nucleotide values appearing in the literature have been expressed in terms of this figure.

1. A 2. The amount of NAD$^+$ bound to the protein and its reduction in situ.

Rapkine (1938) used charcoal to remove NAD$^+$ from crude enzyme preparations; his studies led him to suggest that NAD$^+$ protects the sulphhydryl groups of the protein. Taylor et al. (1948) found that NAD$^+$ was bound to the crystalline protein and estimated the amount bound by the increase in absorption at 340 nm on addition of G3P and arsenate, and also by enzymic reduction of the nucleotide in the supernatant after TCA precipitation of the protein. Their values of 1.2 moles NAD$^+/10^5$ g protein and 1.6 moles NAD$^+/10^5$ g protein, in retrospect, pointed to the non-stoichiometry of reduction of NAD$^+$ bound to the protein. These results were further
complicated by their finding of 2.0 moles of dinucleotide phosphorus and 1.95 moles of dinucleotide ribose/10^5 g of protein. These results by chemical assay indicate another discrepancy between total protein bound nucleotide and total enzymatically reducible NAD^+.

Cori et al. (1950) found that NADH was bound by the protein but was readily displaced by NAD^+. They also reported that enzyme bound NAD^+ was only 70% reduced upon addition of an equivalent amount of G3P. If the protein at this stage was precipitated with ammonium sulphate and then redissolved, a further reduction of NAD^+ could be obtained by addition of more G3P.

The binding of NAD^+ was studied by Velick et al. (1952) who found that the native enzyme contained 1.67 - 1.77 moles NAD^+/10^5 g protein as estimated by the increase in absorption at 340 m\(\mu\) on reduction with G3P. After charcoal treatment, which removes 95% of the bound nucleotides, the protein had the capacity to bind 2.5 moles NAD^+/10^5 g protein and they have concluded that charcoal treatment has altered the configuration of the protein and liberated a 'third' binding site. In addition, they found that P^{32} labelled NAD^+ would rapidly exchange with enzyme bound NAD^+. However, this result only indicated 2 binding sites per mole of enzyme assuming a molecular weight of 120,000.

Fox and Dundiker (1956) were the first to realize that NADH production on addition of substrate is not equivalent to bound NAD^+. In their paper they draw attention to the earlier findings of Taylor et al. (1948) and Cori et al. (1950), but did not realize that the discrepancies in these earlier papers could be due to more than one source of error. Their studies led them to the complete removal of bound coenzyme by charcoal treatment of the protein. They subsequently reconstituted the enzyme:coenzyme complex by addition of NAD^+ and observed the binding by following the appearance of the weak 360 m\(\mu\) absorption (discussed below). From this titration, they obtained a figure of 2.17 moles NAD^+/10^5 g of protein. NAD^+ bound to the 'native' enzyme was assayed by a different technique which consisted of heat denaturation of the protein followed by enzymic assay of the released NAD^+ in the supernatant. By this technique they were able to find results varying from 1.92 to 2.35 moles NAD^+/10^5 g of
protein in three different preparations of the crystalline enzyme.

These authors also studied the reduction of NAD\(^+\) in a reconstituted sample of protein which contained only 40\% of its total capacity of bound nucleotide. The reduction was carried out with excess glyceraldehyde in arsenate buffer to make the reaction irreversible, only 70\% of the NAD\(^+\) present was reduced and the authors have attributed this result in the light of the findings of Cori et al. (1950) as being due to a partial reduction and not to a lowered extinction coefficient of enzymically bound NADH. On the other hand Koeppe et al. (1956) think that the lowered 340 m\(\mu\) absorption on reduction of NAD\(^+\) results from the enzyme catalysed transformation of NADH to NADH-X (discussed below).

Stockell (1959) has used yeast G3PD for studying the number of binding sites and dissociation constants of NAD\(^+\) over a wide range of pH. By following the titration of apo enzyme with NAD\(^+\) at 405 m\(\mu\) she obtained a figure of 1.67 moles of NAD\(^+\) bound/10\(^5\) g protein, this figure requires correction however, as the apo enzyme used in this study initially contained approximately 0.25 moles NAD\(^+\)/10\(^5\) g of protein as estimated by addition of G3P.

In summary, the main problem underlying the number of binding sites per mole stems from the non-stoichiometry of reduction of NAD\(^+\) bound to the protein. The problem of assessing the results has been further complicated by the uncertainty regarding the state of purity of the enzyme preparations and by the presence of varying amounts of an unidentified nucleotide bound to the native protein. However, none of the workers who have studied the enzyme have commented on or realised the significance of the discrepancies in the analytical results which might have led them to infer the existence of such a nucleotide.

3. The nature of the weak G3PD:NAD\(^+\) absorption band.

Hacker and Krinsky (1952 a, b) first observed the production of a weak absorption band with an ill defined maximum at 360 m\(\mu\) and extending into the visible when NAD\(^+\) was added to apo protein. The absorption was stated to be abolished by iodoacetate, p-chloromercuribenzoate and acyl
phosphates, but from an inspection of their absorption spectra of iodoacetate treated enzyme it can be seen that the extinction is not even halved at 360 μμ. On the basis of this evidence they indicate that the absorption is due to a reaction between a protein bound -SH group and the oxidized form of the nicotinamide ring.

The absorption band due to the G3PD:NAD⁺ complex was also noted independently by Velick (1953), who first used the complex to titrate the apo enzyme with NAD⁺; using this procedure he found maximal absorption at 2.5 moles NAD⁺/10⁵ g protein. Velick (1954) also found that p-chloromercuribenzenesulphonate titration of the complex decreased the absorption, a minimum being reached at 50% of the initial absorption when 3 equivalents of the mercurial had been added. He also found by an ultra centrifugation technique that acetyl phosphate which abolished the 360 μμ band did not bring about the release of the bound NAD⁺.

Chance (1954), with the aid of a difference recording spectrophotometer, has used the absorption complex to study in vivo pyridine nucleotide levels in yeast; he indicates that iodoacetate and H₂O₂ abolish the band. Krinsky and Racker (1955) write the formula of the G3PD:NAD⁺ complex as an SH addition to the 4 position of the pyridine ring; protein bound glutathione is thought to provide the thiol in question. Support for their argument has come from Van Eys and Kaplan (1957), who have obtained spectral evidence for thiol addition compounds with NAD⁺. In particular, they report a complex formation between NAD⁺ and glutathione with a maximum absorption at 335 μμ. The addition in this case is thought to occur at the 4 position by analogy with the cyanide NAD⁺ complex.

Studies of the binding of NAD⁺ analogues to G3PD by Kaplan et al. (1957) have indicated that the 3-acetyl derivative of NAD⁺ can form a much 'tighter' complex having a definite absorption maximum at 350 μμ. The complex, in contrast to NAD⁺, is hardly affected by the addition of a tenfold excess of iodoacetate. The 3-formyl derivative on the other hand displaces bound NAD⁺ with a drop in 360 μμ absorption. This compound is a potent inhibitor of the enzyme and is thought to add by way of the aldehyde group to the essential sulphhydril of the protein.
Spectroscopic titrations by Stockell (1959) using the NAD$^+$ protein absorption band over a wide range of pHs have led her to the proposal that her dissociation pK values of 8.0 or 8.5 could be the result of the ionization of a sulphydryl group connected with the binding of the nucleotide.

An alternative explanation of the protein nucleotide absorption band has been made by Kosower (1956) who suggested that the broad featureless absorption has the characteristics of a charge transfer spectrum. More recently Cilento and Tedeschi (1961) have suggested on the basis of pyridinium-indole complexes that the G3PD:NAD$^+$ absorption arises from a tryptophan nicotinamide charge transfer complex. They conclude that such an interaction could account for the failure of iodoacetate to abolish the 3-acetyl pyridine nucleotide absorption reported by Kaplan et al. (1957). Further support for this idea comes from the work of Alvisatos et al. (1961) who record that an equimolar mixture of NAD$^+$ and 5-hydroxytryptamine produced a deep yellow colour with absorption between 300-400 nm. A charge transfer interaction has been proposed to explain this result.

At present the experimental support for either the sulphydryl covalent addition complex or the tryptophan charge transfer complex is equivocal; however, the theoretical calculations of Pullman and Pullman (1958), (1959), indicate that NAD$^+$ has a very low energy unfilled electron orbital and that tryptophan should be a moderately good $\pi$ electron donor. In the light of their calculations tryptophan could easily form a charge transfer complex with NAD$^+$.

4. The G3PD catalysed transformation of NADH to NADH-X.

Bafter et al. (1954) and Chaykin et al. (1956) have observed the enzymic transformation of NADH into a compound of unknown structure designated NADH-X. Both yeast and muscle enzyme catalyse the transformation, the former being much more efficient. The reaction occurs slowly with a dialysed sample of the yeast enzyme and is markedly stimulated by phosphate, pyrophosphate and citrate ions; the pH optimum of the reaction is around 4.5 - 5.0. The new compound is characterised by an increased absorption in
the 260-300 μm region with no 340 μm absorption; the greatest change relative to NADH is at 290 μm. Although the spectrum of the compound is similar to the acid modification of NADH, it is not identical. An enzyme has been discovered in yeast which catalyses the conversion of NADH-X back to NADH; ATP is utilised in this reaction on a stoichiometric basis (Weinhart et al. 1956).

\[
\text{NADH-X + ATP } \overset{\text{Mg}^{++}}{\longrightarrow} \text{NADH + ADP + Pi}
\]

Velick et al. (1953) and more recently Tucker and Grisolia (1962) and Ameluxen and Grisolia (1962) have also noted the transformation reaction; the latter authors indicate that between 4 - 10 moles of NADH-X are formed per mole of rabbit muscle enzyme. They also state that there is no evidence for inhibition of enzymic activity due to NADH-X formation and that iodoacetate fails to inhibit NADH-X formation. The questions as to the possible significance of NADH-X in metabolism or to its possible role in the reaction mechanism of the enzyme remain unanswered.

5. Transferase and hydrolase activities of the enzyme.

Meyerhof and Junowicz-Kocholaty (1942) observed that 1,3-diphosphoglyceric acid is dephosphorylated in the presence of arsenate in a catalytic reaction involving G3PD. Later, Racker and Krimsky (1952) demonstrated the arsenolysis of 1,3-diphosphoglyceric acid and acetyl phosphate by both yeast and muscle enzymes. A definite requirement for pyridine nucleotide was found, and the remaining arsenolytic activity of the apo protein was ascribed to a residual amount of firmly bound NAD+. NADH was found to be as effective as NAD+ in restoring the activity of the charcoal treated enzyme.

Iodoacetate treated enzyme retained 25 - 50% of the arsenolytic activity of the untreated enzyme; however, as the authors point out, this effect was only noted in the presence of added glutathione. The enzyme was also shown to catalyse a transacylase reaction involving the transfer of the acetyl group of acetyl phosphate to the sulphydryl of glutathione. Harting and Velick (1954 b) using acetyl phosphate have shown
that both yeast and muscle enzymes catalyse:

(i) phosphate exchange between acetylphosphate and radioactive phosphate;

(ii) arsenolysis of acetylphosphate;

and

(iii) acetylation of the thiol groups of coenzyme A and glutathione.

Removal of NAD$^+$ from the muscle enzyme reduced the rate of phosphate exchange to 1/20th of its former value; however, activity could be partly restored by re-addition of NAD$^+$. To account for these various reactions, the authors have envisaged that an acyl enzyme compound occurs as a common intermediate.

\[
\text{acetaldehyde} \xrightarrow{\text{NAD}^+} \text{acyl enzyme} \xrightarrow{\text{PO}_4} \text{acetylphosphate}
\]

\[
\text{H}_2\text{O} \text{ or AsO}_4 \quad \text{or}\quad \text{GSH or Co.A.}
\]

\[
\text{Acetate} \quad \xrightarrow{\text{Acyl thiol.}}
\]

Harting (1954) has indicated that the enzyme possesses an acyl phosphatase activity and correlates this with the oxidized form of the enzyme.

Caspar (1954) using 1,3diphasphoglycerate has found a phosphate exchange and an arsenolysis reaction catalysed by both muscle and yeast enzymes. p-Chloromercuribenzoate was shown to inhibit both reactions and the inhibition could be reversed with cysteine.

Krimsky and Racker (1955) have found that prior treatment of the enzyme with iodoacetate stimulates the phosphatase activity in contrast to its effect on the arsenolysis reaction. Carbonyl reagents such as hydroxylamine and phenyl hydrazine were found to inhibit the phosphatase activity of both iodoacetate treated and untreated enzyme without affecting the arsenolysis activity. Further evidence that the arsenolysis and phosphate exchange reactions proceed as depicted by Harting and Velick (1954) (see above) can be seen from Krimsky and Racker's report that 'acyl enzyme', which they isolate, is very rapidly hydrolysed in the presence of arsenate.
Good evidence that the phosphatase activity of the protein is different from its transaminase property comes from the studies of Rafter (1957), who found a sixfold increase in phosphatase activity of o-iodosobenzoate treated protein. Twenty-two reducing equivalents of o-iodosobenzoate were taken up by the protein and under these conditions the arsenolitic activity of the protein was abolished. In addition, the phosphatase activity was found to be very low with charcoal treated protein.

Concurrent with the preceding paper Rafter and Colowick (1957) made some interesting observations on the phosphatase activity. Using acetyl phosphate they found maximum phosphatase activity at pH 5.9; pyrophosphate and to a lesser extent orthophosphate were found to inhibit this activity competitively without affecting the arsenolysis reaction. At pH values above 7 and in the presence of NADH and enzyme, acetyl phosphate was found to promote the pyrophosphate stimulated transformation of NADH to NADH-X. The maximal effect of acetyl phosphate in this reaction was at pH 5.5; however, no disappearance of acetyl phosphate was found.

Kaplan et al. (1957) have studied pyridine nucleotide analogues in relation to the arsenolysis reaction. The 3-formyl derivative of NAD+ was found to inhibit arsenolysis of acetylphosphate; the 3-acetyl derivative on the other hand stimulated arsenolysis to 80% of the NAD+ value. It was also noted that the 3-acetyl derivative did not protect against iodoacetate inactivation of arsenolysis. Such a protection could have been implied for this analogue if the 'tightly complex' which they envisage to account for the increased 350 m\(\mu\) absorption of the 3-acetyl derivative is due to the reactive SH group. In addition, they state that the ability of the NAD+ analogues to promote the arsenolysis reaction, parallels their capacity to act as electron acceptors in the oxidation of triose phosphate.

Park and Koshland (1958) have studied the phosphatase activity with muscle enzyme. Destruction of protein bound NAD+ by Neurospora NADase abolished the phosphatase activity; both NAD+ and NADH could restore activity, the former being more effective. The phosphatase activity was found to be inhibited by disulphide reagents such as glutathione, cysteine, cyanide and bisulphite, and also by p-chloromercuribenzoate. The latter
compound is thought to displace NAD$^+$ in contrast to iodoacetate which had no effect on the phosphatase activity. Studies with $\text{H}_2\text{O}^{18}$ indicate that the enzyme catalyses a C-O cleavage of acetyl phosphate in contrast to the normal non enzymic cleavage at neutral pH which proceeds predominantly through P-O splitting. The phosphatase activity of the enzyme is explained by the above authors as arising from a modification of the normal site by at least the loss of one sulphydryl group rather than the creation of a new site.

Wolff and Black (1959) have found that thioacetoacetate can act as an acyl acceptor in the transferase reaction using 1,3-diphosphoglycerate. They note that after prolonged dialysis and charcoal treatment of the yeast enzyme a significant amount of thiol ester is still formed. Thiol ester enzyme is thought to be the intermediate in this transfer.

Another hydrolytic activity of the muscle (Park et al. 1961) and yeast (Taylor et al. 1963) enzymes has been described recently involving the cleavage of $p$-nitrophenylacetate. Acetyl enzyme has been found as the intermediate (Taylor et al. 1963), and a sulphydryl group is necessary for the reaction; moreover, NAD$^+$ inhibits the reaction and the observation is made that an enzyme with maximal $p$-nitrophenylacetate hydrolytic activity does not have an acetylphosphatase activity.

Recently, Glynn and Chappell (1964) have used the phosphate exchange activity of the enzyme coupled with phosphoglycerate kinase to produce terminal labelled ATP.

$$3\text{PGA} + \text{ATP} \rightleftharpoons 1,3\text{diph\text{PGA}} \rightleftharpoons i^{32} + \text{acyl enzyme}.$$ Furthermore, they state that NAD$^+$ which is present in the muscle enzyme is necessary for the reaction.

Finally, a modified preparation of the enzyme has been shown to have a diaphorase activity (Rafter and Colewick, 1957 b) and Keleti and Telagdi (1960) have demonstrated a very slow ATP - IMP transphosphorylation reaction in which NAD$^+$ is reduced accompanied by the loss of an equivalent amount of protein -SH groups.

These complicated results can be simplified by separation into two fundamental reactions. The first, which includes the phosphate exchange,
areneolysis and transferase activities, involves the reactive thiol of the enzyme and in all cases an acyl enzyme intermediate is thought to be produced. The second reaction of acetylphosphate hydrolysis is not associated with acyl enzyme formation, and occurs with modified enzyme in which the reactive sulphydryl group is either oxidized or blocked by a reagent. This reaction, which has been found to occur through C-O rather than P-O scission, is different from the normal hydrolytic cleavage (Di Sabato and Jencks, 1961) or the pyridine catalysed cleavage (Park and Koshland, 1958) of acetyl phosphate. Both types of enzyme catalysed reaction have been shown to be greatly stimulated by the addition of either NAD$^+$ or NADH (the exception being the hydrolysis of p-nitrophenylacetyl), and most probably the residual activity of the apo enzyme in these reactions can be accounted for by a small amount of firmly bound NAD$^+$. The multiple reactions which the enzyme catalyses have been interpreted as being due to two active sites (Taylor et al., 1963) one of which involves a sulphydryl group and the other an unknown group associated with the hydrolytic activity of the protein. However, the role of NAD$^+$ in these reactions remains unsolved except for the explanation that it maintains the protein in an appropriate configuration (Park and Koshland, 1958; Kacker, 1961).

C. THE PRESENT STUDY

Although the enzyme G3P has been studied extensively both in regard to its physical and chemical properties and in relation to the reaction mechanism, very few facts can be said to be well established.

The reaction mechanism has seen considerable change since the original proposal by Warburg and Christian (1939) that phosphate was added to G3P to form a phosphoaldehyde compound which was subsequently dehydrogenated to yield 1:3diphGA.

\[
R-CHO + H_2O_4^- \rightarrow R-CO-0^- + H^+ + O_2 \\
\text{NAD}^+ \rightarrow \text{1:3diphGA}
\]
Meyerhof and Cieser (1947) with kinetic studies were able to refute this pathway and favour the initial oxidation of G3P with reduction of NAD$^+$. Hacker and Krimsky (1952 a) by analogy with the glyoxalase reaction (Racker, 1951) proposed that an acyl enzyme is formed as an intermediate; glutathione was thought to be involved and the first step in the reaction was envisaged as an 'aldehydolysis' of the nicotinamide thiol addition compound with direct formation of NADH and thiol ester. The acyl glutathione so formed was thought to be transferred to another site on the enzyme for the subsequent phosphorolysis reaction.

Further evidence for this reaction mechanism came from Krimsky and Racker (1955) who found phosphoglyceryl protein with the acyl group test of Lipmann and Tuttle (1945). They re-write the thiol NAD$^+$ addition compound as occurring at the 4 position.

Segal and Boyer (1953) proposed that a thiohemiacetal enzyme was formed prior to the reduction of NAD$^+$ and support for their argument came from the studies of Holzer and Holzer (1953) who found that G3P exerted a protective action on iodosacetate inactivation of the enzyme. Koeppe, Boyer and Stulberg (1956), however, on reflection point out that no decision can be made between their thiohemiacetal reaction and the aldehydolysis reaction proposed by Racker and Krimsky.

The nature of the acyl enzyme intermediate was never established by Krimsky and Racker (1955) but was assumed to involve the thiol group of glutathione; this view, however, has never been substantiated and in fact Weaver and Lardy (1961) have found that phosphoglyceryl glutathione and apo enzyme are completely inert in the NADH oxidation reaction reported by Krimsky and Racker for their isolated acyl enzyme.
The best evidence in the literature relating to the nature of the acyl enzyme compound comes from the studies of Koeppe et al. (1956) who find 1.9 fewer \(-\text{SH}\) groups/105g of protein when acetyl phosphate is incubated with the NAD\(^+\) enzyme. The present situation regarding the nature of the acyl enzyme compound has been summarized by Jover (1959) who states that 'there is strong but not conclusive evidence that a thiol ester enzyme is a catalytic intermediate in the G3PD reaction'.

The phosphorolysis of acyl enzyme to yield acylphosphate has proved difficult to study due to the fact that phosphate does not appear to form an intermediary complex with the enzyme before entering into the phosphorylation reaction (Velick and Hayes, 1953). The cleavage of acyl phosphate by the enzyme, however, has been studied by Cohn (1956) who has shown that a C=O scission is involved and that the same bond is made in the reverse direction.

The work described above indicates that our knowledge of the intermediary stages in the overall catalytic reaction of the enzyme is far from satisfactory; the reaction mechanism, however, first postulated by Racker and Krimsky (1952 a) has been generally accepted.

The present study was undertaken in the hope of clarifying some of the anomalous and unexplained findings in the literature, with particular reference to headings 1 - 4 in section B described above. In addition the reaction mechanism of the enzyme has been investigated by studying the nature of the acyl enzyme formed from acetaldehyde and G3P as well as from acetyl phosphate, as it follows that a true catalytic intermediate should be identical from either side of the overall reaction.
CHAPTER I
PREPARATION AND ASSAY
OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

I.I. PREPARATION

The method of preparation of the rabbit muscle enzyme was based on the fractionation procedure of Cori et al. (1946) as modified by Racker and Krimsky (1952), who used EDTA in the initial extraction of the muscle in order to reduce inactivation of the enzyme by heavy metal ions. The routine procedure finally adopted is described below and is a typical preparation involving the muscle obtained from three rabbits.

I.I.(1) Isolation.

The animals were killed by stunning, bled, skinned, disembowelled, and the carcasses plunged into ice. All following operations were carried out at 0 - 2°C. Leg and back muscles were removed and finely minced in a meat grinder, and the mince (1460g) was extracted for 30 minutes with an equal volume (g/ml) of 50 mM EDTA adjusted to pH 8.5 with isopiestically distilled NH₃. The mixture was then centrifuged for 30 minutes in the International FS3 centrifuge at 2,100 r.p.m., (r.c.f. - 1,500). The residue was extracted and centrifuged as before; the total volume of combined extracts was 2,870 ml.

Dried (NH₄)₂SO₄ (300g/L of extract) was added from a vibrator (Racker and Schroeder, 1955) to the mechanically stirred extract over the course of 1½ hours; the solution was then allowed to stand for 30 minutes to allow complete equilibration of the protein mixture. The (NH₄)₂SO₄ concentration at this stage was equivalent to 52% saturation at 0°C. The suspension was then centrifuged for 30 minutes in the International FS3 at 2,100 r.p.m. and the precipitate discarded. The pH of the supernatant (3,100 ml) was checked and adjusted to 7.4 with isopiestic NH₃ and the concentration raised to 60% saturation by the further addition of 363g of (NH₄)₂SO₄ (117 g/L of solution). After addition of the (NH₄)₂SO₄ and equilibration for one hour the suspension was centrifuged for 1 hour at 2,500 r.p.m. (r.c.f. - 2,000), the precipitate was again discarded and 3,140 ml of supernatant were recovered. The (NH₄)₂SO₄ concentration was
further increased to 70% saturation by the addition of 41g (13g/L of solution) and the solution set aside at -1°C overnight in order to induce crystallisation. Crystallisation was found to be incomplete the following morning, so a further 20g of (NH₄)₂SO₄ were added and the suspension left for another 24 hours. This step is usually necessary in order to improve the yield of crystalline enzyme. At the end of this time the crystals were collected by centrifugation at 2,500 r.p.m. for one hour and then dissolved in the minimal volume of 5mM EDTA pH 7.5.

I.I.(2) Refractionation

Refractionation of the enzyme was accomplished by an empirical procedure. (NH₄)₂SO₄ was added slowly until a faint turbidity appeared, the solution was then centrifuged as soon as possible in the Spinco model L ultracentrifuge and (NH₄)₂SO₄ added to the supernatant to bring most of the protein out of solution. After 4 hrs the crystals were collected by centrifugation and redissolved in 5mM EDTA pH 7.5. This refractionation procedure was repeated three times, and a total of 4.5g of enzyme was obtained. The protein crystals at this stage were rhomboidal plates (Fig.1) and a solution of the protein was lemon yellow in colour. It was generally found in enzyme preparations from rabbit muscle that most of the myoglobin remained in solution at the first refractionation step and most of the contamination by other proteins as evidenced by the precrystallisation turbidity was removed by the second refractionation.

I.I.(3) Refractionation in the presence of added NAD⁺

In view of the conflicting reports as to the amount of bound NAD⁺ found on isolation of the native protein and also the known protective function of NAD⁺ in preventing sulphhydryl group oxidation (Rapkine, 1938), it was thought worth while to refractionate the protein in the presence of added NAD⁺. Accordingly, a solution of NAD⁺ brought to pH 7.5 with isopiestic NH₃ was added to a once-refractionated sample of enzyme to the extent of 10 moles/10⁵g of protein. The protein was then refractionated at pH 7.5 in the usual way and after four hours the crystals were examined under a microscope. The crystals appeared as elongated rectangular plates (Fig.2) instead of the familiar rhomboidal plates. This interesting observation was further investigated, and the results are described in detail in Chapter 2.
Figure 1. Rhomboidal plate crystals of rabbit muscle. The photograph is enlarged $\times 620$. 
Figure 2. Rectangular plate crystals of rabbit muscle G3PD prepared in the presence of excess NAD⁺. The photograph is enlarged X620.
I.I. Preparation from sheep muscle.

The high cost of enzyme preparations from rabbit muscle and the knowledge that the enzyme could be prepared from other mammalian muscles (Elodi and Szorenyi (1956) prompted the investigation of isolation of the enzyme from slaughterhouse material. Accordingly, sheep muscle was subjected to the same isolation procedure as rabbit muscle: the results, however, were not encouraging. At the second refractionation step, two fractions were collected at 65 - 70% and 70 - 75% (NH$_4$)$_2$SO$_4$ saturation. These fractions, together with the remaining 75% supernatant, showed very little difference with regard to either enzymic activity (see Table 2) or myoglobin contamination.

The low activity of these fractions and the fact that refractionation with (NH$_4$)$_2$SO$_4$ did not increase the activity or lower the myoglobin contamination did not commend sheep skeletal muscle as a source for preparation of purified enzyme. Attempts to obtain the enzyme from this source were therefore discontinued.

1.2. Measurement of enzyme activity

The method of estimation of the activity of G3PD by following NADH formation has been thoroughly studied by Ferdinand (1962), (1964). His method was based on the method of Koeppe et al. (1956) but with triethanolamine replacing pyrophosphate, as Biesenheres et al. (1953) had reported higher activities of the enzyme using this buffer.

(1) Method.

The enzymic activity was measured by following the optical density increase at 340 m$_u$ in the Optica CF4 recording spectrophotometer. The enzyme was diluted 10,000 fold to approximately 0.01 mg/ml in glassware which had been treated with dichlorodimethylsilane to form a non-wettable surface. The dilution was carried out in two stages, 0.1 ml of concentrated enzyme (100 mg/ml) was added to 10 ml of mechanically stirred 5 mM EDTA pH 7.5; 0.1 ml of this solution was diluted similarly and 0.1 ml of the second solution was pipetted into an 'adder-mixer' (Koeppe et al. (1956)) for addition to the cuvette. The cuvette contained:
2.0 ml. of a solution containing 0.2 mM EDTA, 50 mM \( \text{Na}_2\text{HPO}_4 \)
and 40 mM triethanolamine adjusted to pH 9.7 with
isopiestically distilled HCl.

0.2 ml. of 10 mM NAD\(^+\).

0.2 ml. of 7 mM G3P.

The final pH of the solution was 8.6, which was in the pH range of maximal
activity of the enzyme. The final concentrations of NAD\(^+\), G3P and Pi of
0.8 mM, 0.56 mM and 40 mM were well above the respective Km's of these
substrates of 0.013 mM, 0.090 mM and 0.290 mM reported by Velick and
Furhine (1963) at this pH.

The reaction was carried out at 25°C and was started by the addition
of enzyme in the 'adder-mixer'. By this procedure the addition of enzyme
and mixing of the solution in the cuvette took 3 seconds. The 'chart-drive'
was set on the fastest speed of 8 in/min. and the time taken for an optical
density increase of 0.2 was measured from the chart. The rate of increase
of 340 nm absorption under these conditions was found to be directly
proportional to the enzyme concentration.

(ii) Results.

With three times recrystallised enzyme the reaction gave a linear
increase in 340 nm absorption for at least 60 secs; with the first crystals
and once-refractionated enzyme, however, the rate of optical density change
was never quite linear and tended to decrease. This fact limited the
precision of the activity estimations with the initial refractionated enzyme;
with three times refractionated enzyme, however, the precision of the method
was found to be \( \pm 5\% \). The results have been expressed as the moles of NADH
produced/min./10^5 g of protein.

The activity of the preparation from rabbit muscle described under
I.I was followed at each fractionation step and the results are given in
Table 1. It can be seen that the activity did not increase beyond the third
refractionation.
The activities and yields of 6 different preparations from rabbit muscle are given in Table 2, these values being measured after the enzyme had been fractionated 3 times. The preparations showed a variation in activity which could in part be due to a variation in trace amounts of heavy metal ions, or to a variation in the contamination by triosephosphate isomerase and \( \alpha \) glycerophosphate dehydrogenase. These enzymes when present together could lower the rate of \( \text{NADH} \) formation by producing dihydroxyacetone phosphate which could then oxidize \( \text{NADH} \) to \( \text{NAD}^+ \).

\[
\begin{align*}
\text{G3P} & \rightleftharpoons \text{dihydroxyacetone phosphate} & \text{NADH} \\
\alpha \text{ glycerolphosphate} & \rightleftharpoons \text{NAD}^+
\end{align*}
\]

On the other hand, triosephosphate isomerase alone could affect the initial rate of \( \text{NAD}^+ \) reduction by considerably reducing the concentration of G3P. That this is a possibility can be inferred from the equilibrium concentrations of 96% dihydroxyacetone phosphate and 4% G3P (Oesper and Meyerhof (1950) and from the very large turnover number of triosephosphate isomerase of 550,000 moles of G3P/min. \( 10^5 \) g of protein at 25°C and pH 7.5, (Cock and Misher (1960). Accordingly, the occurrence of these two enzymes in preparations of G3P were tested for and the results are described in the next section.
The enzyme was found to be fairly stable if kept as a crystalline suspension in 80% saturated (NH₄)₂SO₄ at 2°C. A sample with an original activity of 15,000 was found to have dropped to 13,500 after 3 months and to 9,200 after 15 months.

**TABLE 2**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Amount of muscle used in g</th>
<th>Yield of 3 times refractionated protein in g</th>
<th>Yield as g protein/Kg muscle</th>
<th>Activity moles NADH/min./10⁵g</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>700</td>
<td>0.7</td>
<td>1.0</td>
<td>14,000</td>
</tr>
<tr>
<td>2</td>
<td>550</td>
<td>0.6</td>
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<td>3</td>
<td>640</td>
<td>1.8</td>
<td>2.8</td>
<td>5,000</td>
</tr>
<tr>
<td>4</td>
<td>800</td>
<td>2.1</td>
<td>2.5</td>
<td>15,000</td>
</tr>
<tr>
<td>5</td>
<td>1200</td>
<td>1.8</td>
<td>1.5</td>
<td>17,000</td>
</tr>
<tr>
<td>6</td>
<td>1460</td>
<td>4.5</td>
<td>3.1</td>
<td>14,700</td>
</tr>
</tbody>
</table>

**sheep**

65 - 70% saturated (NH₄)₂SO₄

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Yield as g protein/Kg muscle</th>
<th>Activity moles NADH/min./10⁵g</th>
</tr>
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<tbody>
<tr>
<td>3.3</td>
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<td>1,600</td>
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</tbody>
</table>

70 - 75% saturated (NH₄)₂SO₄

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<th>Preparation</th>
<th>Yield as g protein/Kg muscle</th>
<th>Activity moles NADH/min./10⁵g</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td></td>
<td>2,500</td>
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</tbody>
</table>

75% supernatant

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Activity moles NADH/min./10⁵g</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>1,900</td>
</tr>
</tbody>
</table>

* The sheep muscle enzyme was refractionated twice.
I.3. **ESTIMATION OF CONTAMINANTS**

I.3.(1) **Myoglobin**

Absorption spectra of the rabbit muscle enzyme after 3 refractionations revealed no Soret band due to myoglobin contamination. However, the preparation from sheep muscle was red in colour and an estimation of the amount of myoglobin contamination was undertaken. The procedure involved measuring the optical density at 542 m\(\mu\) of a protein solution diluted 1:10 with \(10^{-2}\text{M KCH}\) in \(5 \times 10^{-3}\text{M EDTA pH 7.7}\). The \(\varepsilon_{1\text{em}}\) of 6.13 was used to calculate the amount of myoglobin (Boardman, 1961).

The sheep muscle enzyme after two refractionations contained 3.6% myoglobin in the 65 - 70% saturated \((\text{NH}_4)_2\text{SO}_4\) fraction and 3.8% in the 70 - 75% fraction.

I.3.(2) **Triosephosphate isomerase E.C.5.3.1.1.**

This enzyme has a turnover number of 550,000 moles G3P/min./10^5 g of protein at 25°C and pH 7.5 (Czok and Bücher (1960), and was assayed in the Optica at 25°C by the following procedure:

The cuvette contained:

- 2.0 ml. of 50 mM EDTA pH 7.5.
- 0.2 ml. 7 mM G3P.
- 0.3 ml. 1.5 mM NADH
- 0.01 ml. of a suspension of \(\alpha\) GDH.
- 0.1 ml. of G3P.D.

The rate of optical density decrease was measured at 340 m\(\mu\), and the estimation was carried out on once and thrice refractionated samples of preparation 4. The once refractionated sample contained 0.15° TPI and the thrice refractionated sample approximately 0.0001°.

I.3.(3) \(\alpha\) **Glycerophosphate dehydrogenase E.C.1.1.1.8.**

This enzyme has a turnover number of 10,300 moles NADH/min./10^5 g of protein calculated from the data given by Czok and Bücher (1960) and was assayed in the same system as for TPI, but with the addition of 0.01 ml. of TPI suspension replacing \(\alpha\) GDH. Once refractionated enzyme contained 0.085° and thrice refractionated enzyme contained approximately 0.0025° \(\alpha\) GDH.
1.3.(4) **Myokinase E.C.2.7.4.1.**

The presence of this enzyme as one of the largest possible contaminants of G3PD was suspected from the data issued by Boehringer and Sons on the contaminants tested for in their preparations.

The enzyme has a turnover number of 220,000 moles ADP/min./10^5 g of protein at 30°C (Noda and Kuby (1957)), and was assayed on the same samples of protein as used in the TPI and α-GDH estimations.

The assay at 25°C contained:

- 2.0 ml. of 50 mM triethanolamine/HCl pH 7.5
- 0.15 ml. of 8 mg/ml. phosphoenolpyruvate cyclohexylamine salt
- 0.025 ml. 100 mM ATP
- 0.1 ml. 10 mM ADP
- 0.25 ml. 100 mM MgCl₂
- 0.2 ml. 2 mM NADH
- 0.01 ml. pyruvate kinase 10 mg/ml. crystalline suspension
- 0.01 ml. lactate dehydrogenase 10 mg/ml. crystalline suspension
- 0.1 ml. G3PD.

The assay consists of following the drop in optical density of NADH by coupling to the myokinase catalysed reaction through the following reactions:

\[
\text{ATP} + \text{AMP} \rightleftharpoons 2 \text{ADP} \\
\text{ADP} + \text{phosphoenol pyruvate} \rightleftharpoons \text{pyruvate} + \text{ATP} \\
\text{pyruvate} + \text{NADH} \rightleftharpoons \text{lactate} + \text{NAD}^+.
\]

The once refractionated preparation contained 0.95% myokinase and the thrice refractionated enzyme contained 0.35%.

1.3.(5) **Alcohol dehydrogenase E.C.1.1.1.1.**

The presence of this enzyme in rabbit muscle was not suspected, but nevertheless, as the G3P used in the standard assay was prepared by the hydrolysis of the diethyl acetal (see Appendix), 4 moles of ethanol would be produced for every mole of D-G3P and could perhaps interfere in the assay of G3PD. Accordingly, alcohol dehydrogenase activity was tested for under the standard assay conditions by replacing the G3P solution for one containing the same concentration of ethanol. No production of NADH was found.
1.4. CONCLUSION

The method of preparation of G3PD is simple and the enzyme can be obtained in high yield from rabbit muscle. The turnover number of the enzyme was found to be in the region of 16,300 measured at 27°C and pH 8.6, which is the value reported by Koeppe et al. (1956) for their best preparation. This value is the highest in the literature and is well above the previously reported values of 6,700 at 27°C and pH 8.6, Cori et al. (1948), 4,100 at pH 8.7, Riesenbergs et al. (1953), and 9,700 at 28°C and pH 8.5, Astrachan et al. (1957).

The preparation after three refractions contains very little triosephosphate isomerase and α-glycerolphosphate dehydrogenase; the contamination by myokinase, however, is still significant. The usual 50% increase in activity during refractionation of the initial crystals without a large loss in refractionated protein can most readily be accounted for by the elimination of TII and α-CDH. The very small contamination of 3 times refractionated enzyme by these two proteins did not interfere in the titration studies described in Chapter 5, where substrate amounts of G3PD are used.
CHAPTER 2
A STUDY OF THE TWO CRYSTALLINE FORMS OF THE ENZYMES

It was noted in Chapter 1 that the enzyme appeared in a different crystalline form in the presence of added NAD\(^+\). This observation led to the investigation of the nucleotide content of the enzyme as it was thought that the two crystal shapes may help to clarify the findings of Velick et al. (1953) that the number of NAD\(^+\) binding sites were variable and depended on the conditions the protein had been exposed to. Different crystal forms of the rabbit muscle enzyme similar to those illustrated in Figures 1 and 2 have been reported by Elodi and Szorenyi (1956) but no attempt was made to relate the differences to the amount of bound nucleotide. Accordingly, chemical and enzymic analyses for NAD\(^+\) on the two crystalline forms were undertaken in conjunction with spectroscopic investigations.

2.1. ANALYSES FOR PROTEIN BOUND NUCLEOTIDE

The initial values for enzymically reducible NAD\(^+\), phosphate and ribose were obtained on solutions prepared from sedimented crystals; however, when the protein was crystallised in the presence of added NAD\(^+\), more consistent results were obtained if the crystals were washed by resuspension in 80% saturated ammonium sulphate. After 2 washings the mother liquor had been effectively removed and the nucleotide values remained constant.

Protein concentrations were determined by the biuret method (see Appendix) which had been standardised against dry weights of the dialysed protein.

2.1.(1) Chemical analysis for phosphate and ribose.

The procedure of Berenblum and Chain (1938) and the 'orcinol' procedure outlined by Ashwell (1957) were used to determine phosphate and ribose values on samples of the protein; these methods are described in the Appendix.

2.1.(2) Enzymic analysis for NAD\(^+\).

Due to the non-stoichiometry of reduction of enzyme bound NAD\(^+\) (see Introduction) it was necessary to develop some independent method for estimating NAD\(^+\) enzymically in the presence of a large amount of protein.
NAD⁺ is known to be stable to acid (Kaplan, 1960), and therefore pepsin digestion of the protein at acid pH seemed a possible method of destroying the protein under mild conditions which do not destroy the nucleotide. Preliminary investigations revealed that pepsin rapidly digested the acid denatured enzyme at pH 2.5 and 30°C. In addition, pepsin had no effect on NAD⁺ at this pH either in the presence or absence of added protein. The final procedure adopted was as follows:

0.2 ml. of G3PD, approximately 70 mg/ml.
0.4 ml. H₂O
0.4 ml. 0.2N HCl
1 mg pepsin

The resulting suspension was left at 30°C until the precipitate had been completely solubilized, this digestion usually took about 1 hr. The NAD⁺ content of a 0.4 ml. sample of the above digest was assayed either by reduction with ethanol and alcohol dehydrogenase (Colowick et al. (1951), or by G3P and G3PD as described in the Appendix.

2.I.(3) Presence of bound NADH.

The direct spectroscopic observation of NADH was complicated by the G3PD:NAD⁺ absorption band. However, the analytical figures cannot be accounted for by NADH as the absorption at 340 mJ decreases with decreasing amounts of enzyme bound NAD⁺ (see below).

2.I.(4) Results.

It can be seen from Table 3 that the amount of reducible NAD⁺ found on the preparations of 'native' protein varied from 0.6 - 1.85 moles/10⁵ g protein while the total amount of nucleotide represented by phosphate and ribose figures remained relatively constant (2.4 - 2.6 moles/10⁵ g protein). The protein recrystallized in the presence of added NAD⁺ on the other hand contained an average of 2.45 moles of nucleotide/10⁵ g of protein (Table 4) which could be accounted for as reducible NAD⁺.
<table>
<thead>
<tr>
<th>Preparation</th>
<th>Moles of NAD$^+$/10^5g protein (enzyme assay)</th>
<th>Moles of dinucleotide phosphate/10^5g protein</th>
<th>Moles of dinucleotide ribose/10^5g protein</th>
<th>Activity as moles NADH/min./10^5g protein</th>
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<td>1</td>
<td>0.82</td>
<td>2.60</td>
<td>2.68</td>
<td>14,000</td>
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<tr>
<td>2</td>
<td>0.62</td>
<td>-</td>
<td>-</td>
<td>13,900</td>
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<tr>
<td>4</td>
<td>1.38</td>
<td>-</td>
<td>-</td>
<td>15,000</td>
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<tr>
<td>5</td>
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<td>2.60</td>
<td>2.45</td>
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<td>2.50</td>
<td>2.45</td>
<td>14,700</td>
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<tr>
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<td>1.25</td>
<td>-</td>
<td>2.45</td>
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<tr>
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<td>1.63</td>
<td>-</td>
<td>2.33</td>
<td>11,000</td>
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<tr>
<td>Preparation</td>
<td>Moles of NAD$^+$/10$^5$g protein (enzyme assay)</td>
<td>Moles of dinucleotide phosphate/10$^5$g protein</td>
<td>Moles of dinucleotide ribose/10$^5$g protein</td>
<td>Activity as moles NADH/min./10$^5$g protein</td>
</tr>
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<tr>
<td>1</td>
<td>2.70</td>
<td>2.84</td>
<td>-</td>
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<td>2</td>
<td>2.88</td>
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<td>2.96</td>
<td>14,500</td>
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<td>2(a)</td>
<td>2.41</td>
<td>2.48</td>
<td>2.43</td>
<td>14,500</td>
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<td>4</td>
<td>2.44</td>
<td>-</td>
<td>2.41</td>
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<td>6</td>
<td>2.47</td>
<td>-</td>
<td>2.44</td>
<td>15,000</td>
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</table>

(a) These values and subsequent ones were obtained with washed crystals as described in the text.

The preparations were recrystallized in the presence of 10 moles NAD$^+$/10$^5$g of protein at pH 7.2 - 7.6 in 5 x 10$^{-3}$M EDTA. In all cases the crystals appeared as rectangular plates as illustrated in Figure 2.
2.2. SPECTROSCOPIC INVESTIGATIONS

A comparison was made between holo enzyme (preparation 4) containing 2.44 moles of reducible NAD$^+$/10$^5$g of protein and a preparation (Boehringer sample 1) which fortuitously contained only half as much reducible NAD$^+$ having 1.25 moles NAD$^+$ and 2.45 moles dinucleotide ribose/10$^5$g of protein. The spectra of these two proteins were compared with apo enzyme which was prepared (see appendix) by passing the protein through a charcoal column (Fox and Dandliker, 1956). Apo protein prepared by this procedure contained 0.15 moles of dinucleotide ribose/10$^5$g of protein.

The spectra were recorded both by hand on the Hilger U.V. spectrophotometer and automatically on the Optica CF4 and Beckman DK2 recording spectrophotometers. The proteins were diluted with 5 mM triethanolamine/HCl buffer pH 7.5, concentrations of approximately 20g/l were used to determine the spectra above 320 m$\mu$ and dilutions of 1 in 40 were used for wavelengths below this.

A difference spectrum was obtained on the Beckman DK2 spectrophotometer between the two nucleotide containing proteins which had both been adjusted to the same concentration of 1.62g/l. The spectra are illustrated in Figures 3 and 4 and the molar absorptivities and absorption maxima are presented in Table 5 together with the values in the literature.

It can be seen from the difference spectrum and also from the absorption spectra that the enzyme containing only 1.25 moles of reducible NAD$^+$ has an increased absorption in the 290 - 270 m$\mu$ region compared to the holo enzyme. Both these spectra contain shoulders which in the case of the holo enzyme would not be expected from a simple addition of the component absorption curves of NAD$^+$ and apo protein. Furthermore, it can be calculated that the 280 m$\mu$ absorption of 0.965, for a protein concentration of 1g/l, is greater than the sum of the absorptions of apo protein 0.819 for 1g/l and 0.100 for 0.0245 mM NAD$^+$ (calculated from a molar absorptivity for NAD$^+$ of 4.10 x 10$^{-3}$ at 280 m$\mu$, from the data of Siegel et al. (1959)).
Figure 3. Absorption spectra of G3PDH.
Figure 4. Difference spectrum between holo enzyme in the reference cuvette and Boehringer sample 1 in the test cuvette. Both proteins were adjusted to a concentration of 1.62 g/L.
### Table 5

**Spectral Data of G3PD**

<table>
<thead>
<tr>
<th>Protein sample</th>
<th>λ max.</th>
<th>Molar absorptivity x 10^5</th>
</tr>
</thead>
<tbody>
<tr>
<td>apo enzyme</td>
<td>280</td>
<td>0.819</td>
</tr>
<tr>
<td>holo enzyme</td>
<td>268 - 269</td>
<td>1.060</td>
</tr>
<tr>
<td>Boehringer sample 1</td>
<td>277</td>
<td>1.126</td>
</tr>
<tr>
<td>apo enzyme, Fox and Dandliker (1956)</td>
<td>280</td>
<td>0.829</td>
</tr>
<tr>
<td>enzyme containing 2.14 moles NAD+/10^5 g of protein, Fox and Dandliker (1956)</td>
<td>280*</td>
<td>1.002</td>
</tr>
<tr>
<td>enzyme of Kloppe et al. (1956)</td>
<td>276</td>
<td>1.23</td>
</tr>
<tr>
<td>enzyme containing 1.67 moles NAD+/10^5 g of protein, Velick et al. (1953)</td>
<td>276</td>
<td>1.06</td>
</tr>
</tbody>
</table>

* This value is not the absorption maximum.
The long absorption band for holo enzyme had an ill-defined absorption maximum at 360 m\(\mu\) and an absorptivity of 0.254 expressed at a concentration of 10 g/L; the sample containing only 1.25 moles of NAD\(^+\), however, had a 360 m\(\mu\) absorptivity of 0.146 expressed at a concentration of 10 g/L which is greater than the calculated value of 0.130 using the previously determined figure for the holo enzyme. In addition, the shape of the long absorption bands are not comparable below 340 m\(\mu\), the Boehringer sample having a much sharper increase in absorption in this region.

These spectral peculiarities have been further investigated and the results are presented in Chapter 4.

2.3. Discussion

The results presented above indicate that a difference exists between the two crystalline forms of the enzyme which can be related to the presence of an unidentified nucleotide bound to the 'native' enzyme. The nucleotide differs from NAD\(^+\) by having an increased absorption in the 260 m\(\mu\) region and in addition contains no appreciable 340 m\(\mu\) band. The two crystalline forms of the enzyme showed no difference in activity under the standard assay conditions.

A doubt concerning the nature of the crystalline forms of the enzyme existed due to the report by Elodi and Szorenyi (1956) who indicated that the crystal shape was dependent on pH, rectangular plates being produced in the pH region 5.7 - 7.0 while above pH 7.0 the familiar rhomboidal plates were found. However, attempts to repeat their crystallization with 'native' enzyme at pH 6.5 yielded rhomboidal plates and moreover holo enzyme was found to crystallize as rectangular plates as high as pH 8.5. In view of these results it is tempting to suggest that the presence of the unidentified nucleotide has altered the protein configuration as reflected in the changed crystal shape. In this regard the work of Mirhead and Perutz (1963) has shown that the change in crystal shape which occurs on oxygenation of haemoglobin, (Haurowitz (1938), is related to a real structural change in the configuration of the haemoglobin subunits.
The earlier confusion in the literature relating to the amount of nucleotide bound to the protein is thought to have been resolved.

Although data is presented in the paper of Taylor et al. (1948) pointing to the discrepancy between the amount of reducible NAD$^+$ and the total nucleotide content of the enzyme, the significance of their findings was not realized by the above authors. The results also help to clarify the findings of Velick et al. (1953) on the apparent liberation of a third binding site upon charcoal treatment of the enzyme. The fact that their enzyme preparation contains some of the unidentified nucleotide can be inferred from the absorption maximum at 276 m$\mu$ (see Table 5) for their preparation which is almost identical to the sample of Boehringer enzyme containing 1.25 moles of reducible NAD$^+$/10$^5$g of protein.

Although Fox and Dondliker (1956) realized that reduction of protein bound NAD$^+$ by G3P gave erroneous results they did not measure total nucleotide ribose or phosphate to establish an absolute value for protein bound nucleotide. The absorption maximum of 276 m$\mu$ for their enzyme preparations and their liberated reducible NAD$^+$ analyses of 1.92, 2.2 and 2.35 moles of NAD$^+$/10$^5$g of protein testify to the presence of a variable amount of an unidentified compound bound to the protein.

During the course of this work a report has appeared (Pfeiderer and Stock, 1962) which describes the presence of a nucleotide bound to rabbit muscle G3P in the ratio of 2 moles of NAD$^+$/1 mole of nucleotide. They have isolated this compound and present spectroscopic evidence that it is similar to the NADH-X described by Chaykin et al. (1956). However, the results presented above indicate that the amount of unidentified nucleotide bound to the protein varies considerably and does not occur in a fixed ratio to NAD$^+$.

The isolation and partial characterization of the anomalous nucleotide is described in the next chapter.
CHAPTER 3

ISOLATION AND IDENTIFICATION OF THE ENZYME BOUND NUCLEOTIDE

The realisation that the enzyme isolated from rabbit muscle contained a nucleotide which differed from NAD$^+$ and NADH by an increased 260 - 300 m$\mu$ absorption was followed by investigating various methods for isolating the compound in a pure state. The isolation and characterisation of the nucleotide were undertaken as a preliminary to investigating the possible involvement of the nucleotide in the reaction mechanism of the enzyme.

3.1. ISOLATION OF THE UNIDENTIFIED NUCLEOTIDE

3.1.1. Charcoal Adsorption of the Nucleotides.

Preliminary experiments for recovering the nucleotide fraction from charcoal columns after preparation of apo enzyme revealed a poor return of the adsorbed nucleotide. The recoveries from charcoal by elution with 10$\%$ V/V aqueous pyridine were variable but as little as 20$\%$ of the known nucleotide was obtained by this procedure. The low recoveries are thought to be due to adsorbed and denatured protein preventing desorption of the nucleotide. This was suggested by the fact that recovery of apo protein from charcoal was never 100$\%$.

In view of the poor yields obtained by this method it was decided to investigate enzymic digestion of the protein followed by DEAE sephadex fractionation of the liberated nucleotides.

3.1.2. Enzymic Digestion of the Protein.

Preliminary experiments revealed that 'nagase', a crystalline proteinase from B. subtilis (Matsubara et al., 1958), was more effective in digesting the protein at neutral pH than papain, trypsin or chymotrypsin. Accordingly 120 mg of G3PD (Boehringer Sample 1) containing 1.25 moles NAD$^+$ and 2.45 moles of dinucleotide ribose/10$^5$g in 4 ml. of 50 mM Tris HCl pH 8.0 was incubated with 3 mg of 'nagase' at 25$^\circ$C for 3 hours. At the end of this time a sample of the protein when acidified showed no precipitation. The solution was subsequently applied to a G25 DEAE sephadex column 7 cm x 1 cm equilibrated with 0.01 M glycylglycine buffer pH 7.5. The column
effluent was monitored at 253 m\textmu m with an LKB Unicord and Fraction collector. It was seen that a large faint yellow band passed rapidly through the column, 100 ng of biuret reacting material being collected in 6 ml. Subsequent analysis of this peak revealed that it had a maximum absorption at 275 m\textmu m, an absorption of 0.845 for 1g/L and contained 1.64 moles NAD$^+$ and 2.32 moles of dinucleotide ribose/10$^5$g protein. The enzymic activity of this digested protein was determined and found to be 1100 moles NADH/min./10$^5$g protein.

The column was washed thoroughly with 0.01 M glycylglycine at pH 7.5 to remove the trailing protein and then a convex gradient to 0.5 M NaBr was established. Two peaks were collected from the column, peak I had a maximum absorption at 275 m\textmu m and a shoulder at 288 m\textmu m (see Figure 5) which strongly suggested the presence of tryptophan. Moreover, the ribose content of this fraction could only account for 4% of the 260 m\textmu m optical density as NAD$^+$. The second peak had an absorption maximum at 266 m\textmu m (see Figure 5) and the ribose content gave a dinucleotide molar absorptivity of 25.9 x 10$^3$. A sample of this peak was assayed for NAD$^+$ with ADH; no NAD$^+$ was found however.

The analytical results show that although some nucleotide could be obtained free from NAD$^+$ by this procedure the digested protein still contained 47% of the initial unidentified nucleotide. Moreover, it is not certain whether 'nagase' had hydrolysed all the protein leaving a 'core' with residual activity or whether some intact protein remained after the 3 hrs incubation. The fact that a tryptophan containing peptide was released from the hydrolysed protein along with the anomalous nucleotide served to focus attention on the possibility that tryptophan was in a part of the enzyme which was accessible to the proteinase. This, together with the fact that NAD$^+$ still remained bound to the unhydrolysed part of the enzyme, has been taken as suggestive evidence that the release of the tryptophan peptide, together with the nucleotide, may be the result of configurational changes in the protein associated with the bound nucleotide which would make this part of the protein more susceptible to digestion.
Figure 5. Absorption spectra obtained from the elution peaks after DEAE sephadex fractionation of the 'nagase' digest of G3PD. The spectrum obtained from peak 1 has been plotted on an arbitrary scale, while that from peak 2 has been plotted on a molar absorptivity scale based on the ribose assay.
It was decided to try and improve the yield of liberated nucleotide at this stage by mild urea denaturation of the protein in place of the enzymic digestion.

3.I.(3) Urea denaturation of the protein.

Boyer and Schulz (1959) and Elodi and Jecsai (1960) have studied the effect of urea on G3PD. Both groups indicate that 1.5 - 2.0 M urea produced no marked physical changes in the protein but all enzymic activity is destroyed. It was thought in the light of the enzymic digestion that under these circumstances NAD$^+$ may still be bound whereas the extra nucleotide would be liberated. Therefore, 2 M urea was chosen to denature the protein. Enzyme (Boehringer Sample 1) 225 mg in 3 ml. of 5 mM EDTA pH 7.5 was made to 2 M by the addition of 8 M urea. After 2 minutes the protein was applied to a 1 x 7 cm column of DEAE sephadex at 20°C, the column gradually ceasing to flow and the protein had to be forced through with compressed air. The column was then washed with 30 ml. of H$_2$O under compression to remove as much of the denatured protein as possible. A linear gradient to 0.3 M triethylamine bicarbonate pH 8.5 was then applied to the column and a main peak followed by a very small peak were collected. Analysis of these peaks revealed that the first had a maximum absorption at 266 m$\mu$ and a molar absorptivity of $25.8 \times 10^3$ based on the dimucleotide ribose content. Analysis by ADH and ethanol revealed no NAD$^+$ in this peak. The total yield of nucleotide was 0.7 $\mu$ moles which represents 26% of the initial unidentified nucleotide. The second peak revealed no ribose and no absorption above 255 m$\mu$; it was found later to elute from the column at the same position as EDTA.

Subsequently the solution of the first peak was taken to dryness on the rotary evaporator to remove the triethylamine bicarbonate buffer and then redissolved in distilled water. The pH of this solution was found to be 1.5 and the absorption spectrum revealed that the maximum had shifted to 255 m$\mu$. On neutralisation with dilute NH$_3$ the absorption dropped and shifted to the red but did not resemble the original nucleotide spectrum at neutral pH. The low pH on redissolving the evaporated nucleotide
is unexplained and complicated by the fact that during evaporation of the triethylamine bicarbonate buffer the pH rises to around 10.

The experiment was repeated as before with a smaller sample of enzyme; this time, however, the protein denaturation was greater and on gradient elution of the column and an NAD\(^+\) peak was found to precede and tail into the unidentified nucleotide peak. This method of isolation was discontinued.


It was known from the physical studies of Slodi et al. (1960) that at pH's above 9.0 unfolding and irreversible disorganization of G3PD occurs. Hence, it was thought that mild alkali treatment of G3PD would facilitate a rapid separation of the nucleotides from the protein.

Enzyme (Boehringer Sample 1) 157 mg in 5 ml. representing 1.88 \(\mu\)moles of the unknown nucleotide was taken to pH 9.5 with iso piestic \(\text{NH}_3\) and applied to a 1 x 7 cm column of DEAE sephadex at pH 9.5 in .01 M triethyamine bicarbonate. The protein passed rapidly through the column and emerged a bright yellow colour, 114 mg in 10.3 ml. of eluate was collected, the pH being 9.8.

The protein peak contained 2.16 moles \(\text{NAD}\(^+\)\) ribose/\(10^5\)g giving a total of 2.46 \(\mu\)moles of nucleotide or 64\% of the total nucleotides. Linear gradient elution of the column to 0.3 M triethylamine bicarbonate at pH 8.5 revealed only one peak having an absorption maximum at 266 m\(\mu\) and a molar absorptivity of 26.0 x \(10^3\) based on the ribose assay.

Concentration of the peak, which amounted to 90 ml. was carried out on the rotary evaporator. The volume was reduced to approximately 5 ml., rediluted to 15 ml. and the pH lowered by bubbling in \(\text{CO}_2\). This was repeated twice to remove as much triethylamine bicarbonate as possible. The final volume of the sample was 4.5 ml., the pH 7.7 and 1.1 \(\mu\)moles of nucleotide were recovered. This figure represents 60\% of the unknown nucleotide and in all 93\% of the total nucleotide was accounted for.

A subsequent experiment showed that lowering of the pH to 9.0 reduced the yield of unknown nucleotide to 5\% of the amount present in the protein sample.
3.2. IDENTIFICATION OF THE ISOLATED NUCLEOTIDE

The spectrum of the isolated nucleotide is clearly different from both NAD$^+$ and NADH, having an increased absorption, a red shifted absorption maximum at 266 m$\mu$ and no 340 m$\mu$ band. Perhaps the most striking feature of the spectrum however is the large absorption in the 280 - 290 m$\mu$ region. The dinucleotide ribose figures indicate that the compound has a molar absorptivity at 266 m$\mu$ of 25.9, 25.8 and 26.0 x 10$^3$ and a phosphate analysis on the third sample gave a molar absorptivity of 25.6 x 10$^3$.

Adenine analysis on the third sample (see Appendix) gave 80% of the expected value, however, as the adenine estimation requires a large amount of material and is not very reproducible the analysis was not repeated. Paper electrophoresis in 0.1 M EDTA pH 7.5 for 3½ hrs at 150/cm revealed that the compound moved to the same position as NADH but did not fluoresce under U.V. light. No contaminants were revealed by this procedure.

Finally, a difference spectra between the nucleotide and NAD$^+$ was obtained by adjusting the concentrations of the two nucleotides by their ribose contents. The spectrum is given in Figure 6. The interesting feature is that it differs considerably from the one obtained with the two nucleotides bound to the enzyme (see figure 4). The two spectra should have been comparable; however, an explanation became apparent during the spectroscopic investigations described in the next chapter.

The fact that the isolated nucleotide contains adenine, ribose and phosphate in the proportions 1:2:2 and that it has the same charge as NADH at neutral pH indicated that it could be a modification product of NADH and in all probability identical with the GpFD catalysed modification of NADH termed NADH-X (Chaykin et al., 1956). Accordingly, a comparison was made between this nucleotide and two other modified NADH's, one from the enzyme catalysed transformation of NADH and one from the modification of NADH which occurs in high molarity phosphate buffer (Stock et al., 1961).
Figure 6. Difference spectrum between NAD⁺ in the reference cuvette and NADH-X isolated from the enzyme in the test cuvette. Both nucleotides were adjusted to 18.2 µM by dilution with 5 mM triethanolamine/HCl pH 7.5.
3.3. COMPARISON OF THE ISOLATED NUCLEOSIDE WITH NADH MODIFICATION PRODUCTS

3.3.1. Phosphate modified NADH

A suitable aliquot of weighed NADH was added to 3 M potassium phosphate buffer pH 6.75 and the changes in the absorption spectrum was followed in the DK2 spectrophotometer at 25°C (Figure 7). The change in absorption is seen to be complex; this is apparent from the diffuse isobestic point beginning at 304 mμ and extending to 307 mμ during the course of the reaction. In addition, the absorption at 264 mμ reached a maximum after 3 hrs and thereafter began to fall. This secondary change in the absorption spectrum of phosphate modified NADH complicated the comparison with the spectrum of the nucleotide isolated from the protein. However, from the ratio of the decrease in optical density at 340 mμ over the increase at 260 mμ, an initial change in molar absorptivity of $11.2 \times 10^3$ can be calculated from the molar absorptivity of $6.22 \times 10^3$ for NADH at 340 mμ. This figure has been obtained for the change in optical density over the first 30 minutes of the reaction and it has been assumed that the secondary reaction leading to a decrease in 260 mμ absorption is negligible. From the initial change in molar absorptivity of $11.2 \times 10^3$ and from the molar absorptivity of NADH of $14.4 \times 10^3$ at 259 mμ (Siegel et al., 1959), the initial phosphate modification compound of NADH has a molar absorptivity of $25.6 \times 10^3$ which is near the figure obtained for the nucleotide isolated from the enzyme.

In view of this result the isolation of the phosphate modified NADH was undertaken.

NADH (100 mg) was dissolved in 5 ml. of 3 M potassium phosphate buffer pH 6.75 and after 3 hrs 5 ml. of 2 M BaBr₂ was added followed by 85 ml. of water. The heavy flocculent precipitate was filtered and the supernatant diluted with 5 volumes of chilled ethanol and stood at -15°C for 48 hrs. During this time the solution turned opalescent and finally a light precipitate appeared which was collected in the centrifuge. After washing with ethanol, ether and drying over P₂O₅ in vacuo 66 mg of material was obtained. The compound was solubilized by dropwise addition of 1 M Na₂SO₄ and removal of BaSO₄ by centrifugation.
Figure 7. The effect of 3M potassium phosphate pH 6.75 on the absorption spectrum of NADH. The temperature was 25°C.
The spectrum differed from the initial NADH modified spectrum by having a molar absorptivity at 264 μ of 21.0 x 10^3, although the 290 μ absorptions were almost the same (see Figure 8). Analysis for adenine (see Appendix) by hydrolysis of the nucleotide in 0.5 N H₂SO₄ followed by Ag⁺ precipitation of the liberated adenine gave 80% of the calculated amount of adenine based on the dinucleotide ribose value. Curiously, the silver purine precipitate was seen to darken in the case of the modified NADH but not in the NADH controls; this has suggested that a reducing group is present in the former. Electrophoresis of the compound, as before, revealed that it moved slightly ahead of NADH and did not fluoresce.

As the compound obtained by this procedure was clearly different from the initial phosphate transformation product, the method of isolation was modified in order to try and minimise any secondary reactions. NADH (60 mg) was dissolved in 5 ml of 3 M potassium phosphate pH 6.75, left for 1 hr at 20°C and finally diluted to 1 L with water previously chilled to 2°C. At this molarity of phosphate (0.015 M) the nucleotide was retained by a DEAE sephadex column equilibrated to pH 8.0 with 0.01 M Tris. Linear gradient elution with 0.3 M triethylamine bicarbonate was started as soon as the dilute solution had been run on to the column (5 hrs.) Only one peak was obtained with an initial shoulder which subsequently proved to be modified NADH moving slightly ahead of the main NADH peak.

Analysis of this fraction revealed that it had a molar absorptivity of 21.6 x 10^3 based on ribose and a lower 290 μ absorption than either the modified NADH prepared above or the nucleotide isolated from the enzyme, (see Figure 8 and Table 6). The absorption spectrum at 340 μ revealed there was no significant contamination by NADH. The experiment was repeated in 2.5 M K phosphate at pH 7.5; after 1 hr the solution was diluted to 1 L with chilled water at 2°C, and the pH raised to 8.5 with NaOH prior to application to the column. Subsequent examination of the compound which appeared in the same elution position as before revealed a molar absorptivity of 21.0 x 10^3 but with a much higher λ max/290 ratio than any of the other nucleotides, see Figure 8 and Table 6.

Although the initial spectral changes of NADH in 3 M phosphate
Figure 8. Absorption spectra of NADH transformation products.
### TABLE 6

**SPECTRAL DATA ON THE MODIFIED NADH NUCLEOTIDES**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Absorption maxima.</th>
<th>$\varepsilon \times 10^{-3}$ based on the dimucleotide ribose assay.</th>
<th>$\varepsilon$ at the $\lambda_{max}$ at 290 m(\mu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleotide isolated from the enzyme</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>266</td>
<td>25.9</td>
<td>2.10</td>
</tr>
<tr>
<td>2</td>
<td>266</td>
<td>25.8</td>
<td>2.04</td>
</tr>
<tr>
<td>3</td>
<td>266</td>
<td>26.0</td>
<td>1.94</td>
</tr>
<tr>
<td>Ba$^{++}$ precipitated phosphate modified NADH</td>
<td>264</td>
<td>21.0</td>
<td>1.76</td>
</tr>
<tr>
<td>Phosphate modified NADH at pH 6.75</td>
<td>264</td>
<td>21.6</td>
<td>2.26</td>
</tr>
<tr>
<td>Phosphate modified NADH at pH 7.5</td>
<td>264</td>
<td>21.0</td>
<td>2.66</td>
</tr>
<tr>
<td>G3PD catalysed modification of NADH</td>
<td>264</td>
<td>22.2</td>
<td>2.60</td>
</tr>
</tbody>
</table>
encouraged attempts to isolate a compound with similar spectral properties to the nucleotide isolated from the enzyme this has not succeeded and it is thought that the secondary modification of NADH may still be occurring under the isolation conditions used in the present study.

3.2.2) G3PD modified NADH.

The preparation of NADH-X from G3PD has been described by Chaykin et al. (1956). In their preparation the transformation was carried out at pH 6.0 in the presence of 0.15 M pyrophosphate followed by Ba⁺⁺ precipitation. However, in experiments to be described in the next chapter, it was observed that apo enzyme catalysed the transformation of NADH in 0.05 M Tris buffer pH 8.6 in the complete absence of phosphate. An isosbestic point was observed at 303 mμ for this reaction and the ratio of the decrease at 340 mμ over the increase at 260 mμ gave a change in molar absorptivity of 10.3 x 10³ in the absence of phosphate and 10.3 x 10³ in the presence of 50 mμ pyrophosphate at pH 8.6. Accordingly, the conditions of Chaykin et al. (1956) were modified to minimise the acid modification reaction of NADH which these authors state interferes with NADH-X production at lower pH's.

Apo enzyme (180 mg) in 3.2 ml. of water was added to 50 mg of NADH (68 μ moles) in 1.8 ml. and the solution made to 10 ml. with 0.1 M sodium phosphate at pH 7.0. After 1 hr the optical density at 340 mμ had ceased to drop and the remaining NADH was converted to NAD⁺ by the addition of 0.05 ml. acetaldehyde and ADH. The initial drop in optical density at 340 mμ corresponded to 15.3 μ moles of NADH transformed or 3.4 moles of NADH per NAD⁺ binding site on the protein.

The resulting mixture of modified NADH and NAD⁺ was diluted to 30 ml. and applied to a Diı Advisors sephadex column 15 cm x 1.5 cm and eluted with a linear gradient to 0.3 M LiCl. Two peaks were obtained which tailed into each other. The first was identified as NAD⁺ by ADH assay and the second yielded a component with a maximum absorption at 264 mμ, a molar absorptivity of 22.2 x 10³ and a λmax/290 mμ ratio of 2.60 (see Figure 8 and Table 6).
The compound obtained by the above enzyme catalysed modification of NADH resembled the phosphate modified NADH in spectral properties rather than the nucleotide isolated from the native enzyme. No further attempts to prepare the nucleotide from this source were undertaken.

3.4. DISCUSSION

The modified nucleotide isolated from the enzyme is thought to be identical to the nucleotide initially produced by the enzyme catalysed transformation of NADH and the initial compound produced from NADH in 3 M phosphate. However, in neither case has it proved possible to isolate a nucleotide with identical spectral properties to those of the nucleotide from native enzyme. Part of the difference may be in the fact that small variations in pH and ionic strength could affect the absorption spectra of a chromophore which may be highly polarised in the excited state. In this regard direct comparison with spectra in the literature could be misleading.

Chaykin et al. (1956) have reported that the molar absorptivity for NADH-X at 265 μm is 28.7 x 10^3. This value has been calculated, however, from the observed optical density increase at 260 μm and is based on the molar absorptivity of 17.5 x 10^3 for NADH which is much higher than the currently accepted value of 14.4 x 10^3. Also the 265 μm/290 μm ratio on their isolated sample of NADH-X was 2.52, but this product was stated to contain 2% NAD⁺ and 15% NADH. Unfortunately, no molar absorptivity value of the compound was reported after isolation.

The extensive studies of Stock et al. (1961) on the acid and polyanion degradation of NADH have led them to the conclusion that the modified NADH in 3 M phosphate has an identical spectrum to that of NADH-X of Chaykin et al. The reported molar absorptivity of 28 x 10^3 was calculated from the increase in absorption at 260 μm and is again based on a high molar absorptivity for NADH.

During the course of this work the above authors, Pfleiderer and Stock (1962), have published a report on the isolation of NADH-X from rabbit muscle G3PD; no molar absorptivity figures are given for their isolated compound however.
The exact structure of NADH-X is unknown but studies on the nature of the chromophore responsible for the increased 260 m\(\mu\) absorption have been undertaken by Anderson and Berkelhammer (1958). These authors have found that the first compound produced when weak acid acts on \(N\) substituted dihydro-3-acetyl pyridine compounds is the water adduct to the 5:6 double bond.

![Chemical structure](image)

1-Benzyl-2-hydroxy-5-acetyl-1,2,3,4-tetrahydropyridine has been isolated and found to have an absorption maximum at 295 m\(\mu\). A similar absorption maximum of 295 m\(\mu\) has been found by Marti et al. (1956) for the compound \(N\)-methyl-1,4,5,6-tetrahydronicotinamide.

Meinhart and Hines (1957) have found the incorporation of deuterium into NADH-X when the enzyme catalysed reaction is carried out in \(D_2O\) and Krebs (1963) has indicated that NADH-X may be the 6 - OH derivative of NADH formed by the addition of water to the 5:6 double bond.

Stock et al. (1961) indicate from studies on model compounds that the chromophore accounting for the 260 m\(\mu\) absorption is an unsaturated carbonyl of the type \(-N-C=O^{\ddagger}\) and by analogy with the work of Anderson and Berkelhammer indicate that the initial acid and polyanion transformation product of NADH is the 6 - OH compound. The transformation in 3 M phosphate is thought to occur through a phosphate intermediate.

![Chemical structure](image)
However, the above authors suggest that the NADH-X of Chaykin et al. may have the amino group of adenine added at the 6 position.

An alternative suggestion for the nature of NADH-X has been given by Kaplan (1960) and Burton and Kaplan (1963) who state that the primary acid modification product of NADH could be the ring open aldehyde form V whereas the enzymically produced compound could be the ring open enol form VI.

\[
\begin{align*}
    & \text{V} \\
    & \text{VI}
\end{align*}
\]

Both formulations of Burton and Kaplan may be misleading in this respect as compound II (which is thought to represent the structure of NADH-X) by analogy with the pyran ring of carbohydrates could be considered to contain a masked aldehyde group and as such would enter into reactions with carbonyl reagents which would make it indistinguishable from either V or VI.

An explanation for some of the secondary spectral changes which occur with the phosphate modified NADH and enzyme catalysed modification of NADH may be connected with a phosphate catalysed unmasking of the aldehyde group.
Phosphate ion was present in the enzyme catalysed NADH modification whereas no polyanion was present with NADH-X isolated from the native enzyme.

Once the aldehyde group of NADH-X is unmasked it may undergo a cyclization with the amide group leading to ring closure and possible further re-arrangement to yield a variety of products.
CHAPTER 4

SPECTROSCOPIC INVESTIGATIONS OF G3PD NUCLEOTIDE INTERACTIONS

It had been found previously that the absorption spectrum of G3PD containing bound NAD$^+$ showed some unexpected shoulders at 283 and 277 nm and had a molar absorptivity at 280 nm that could not be explained by a simple addition of the molar absorptivities of the apo enzyme and NAD$^+$. Furthermore, it was found that the difference spectrum between isolated NADH-X and NAD$^+$ was not the same when the nucleotides were bound to the protein. These findings were subsequently investigated and the results are described in the present chapter. It was also decided to determine the number of NAD$^+$ binding sites on the protein by following the appearance of the G3PD: NAD$^+$ absorption band at 360 nm. Stockell (1959) had previously used the appearance of this absorption band to determine the number of NAD$^+$ binding sites on yeast G3PD.

4.1. INTERACTION BETWEEN G3PD AND NAD$^+$ OR NADH STUDIED BY DIFFERENCE SPECTRA

The availability of a Beckman DK-2 spectrophotometer with a -0.3 to +0.7 optical density scale enabled difference spectra to be determined (Wetlauffer, 1962), using the 'tandem cell' technique of Herskovits and Laaskowski (1962). The procedure consisted of obtaining difference spectra between nucleotide and protein in the reference cells on one hand and nucleotide combined with protein in the test cells. This technique was chosen in order to study the small absorption differences resulting from the binding of NAD$^+$ to G3PD.

1. Method.

The difference spectra were obtained by using four matched 1 cm. cuvettes into which were placed 2.5 ml. of buffer together with apo protein and nucleotide according to the following diagram:
<table>
<thead>
<tr>
<th>test cells</th>
<th>reference cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 ml. apo enzyme</td>
<td>0.1 ml. apo enzyme</td>
</tr>
<tr>
<td>0.05 ml. nucleotide</td>
<td>0.05 ml. H₂O</td>
</tr>
<tr>
<td>0.1 ml. H₂O</td>
<td>0.1 ml. H₂O</td>
</tr>
<tr>
<td>0.05 ml. H₂O</td>
<td>0.05 ml. nucleotide</td>
</tr>
</tbody>
</table>

A reference line was drawn with the protein solutions before addition of the NAD⁺ or NADH.

The final protein concentration, given in the legend to the figures, was in the region of 1-3 g/L, and an amount of nucleotide was added to the cuvettes equivalent to the number of NAD⁺ binding sites on the protein using the figure of 2.45 moles NAD⁺/10⁵ g. of protein. Additions to all four cuvettes were made with the same pipettes in order to minimize volume errors. The supporting buffer was 50 mM triethanolamine/HCl/NH₃ at pH 7.5 or 8.7 which in some cases contained 50 mM sodium sulphate or phosphate as indicated. If the reference cells were reversed the intensity of the difference spectra were only altered by 2-4% at wavelengths below 270 μm. This could possibly be due to a small difference in the light scattering between the two solutions. However in all the spectra recorded the positions of the reference cells were kept constant with the protein solution placed first in the light path.

ii. Results.

The spectra are presented in Figures 9 and 10. In the case of NAD⁺ the spectra clearly show an increase in absorption over the viewed range from 360 to 245 μm. The shoulders noted previously in the absorption spectrum now appear as peaks located at 278 and 269 μm. Moreover, the spectra reveal that a minimum occurs at 287 μm and that a small decrease in intensity over the whole region is noted when the pH is changed from 7.5 to 8.7.
Figure 9. Difference spectra showing the effect of NAD$^+$ on the absorption of G3PD. The spectra were obtained using the 'Tandem cell' technique as described in the text, a base line was recorded for each preparation before the addition of nucleotide. The final protein concentration was 2.62 g/L in each case.
Difference spectrum showing the effect of the binding of NADH to G3PD. The supporting medium was 50mM triethanolamine pH 8.7 (no phosphate present) and the final protein concentration 2.69 g/L. The change in the difference spectrum due to NADH-X formation was followed with time.
The presence of phosphate at pH 7.5 was found to increase the absorption in the 295–275 μm region. However, this effect is probably due to an increase in ionic strength of the medium as the replacement of phosphate by an equal molarity of sulphate gave rise to the same enhancement.

The spectra obtained with NADH replacing NAD⁺ show some pronounced differences. A hypochromic effect can be seen in the 340 μm region which represents an average drop in optical density of 15%, the maximum drop being at 333 μm. The 287 μm 'trough' noted in the NAD⁺ spectra extends well below the reference line with NADH although the two peaks at 277 μm and 268 μm can still be seen.

The difference spectra with NADH and enzyme were found to alter with time, a drop in 340 μm absorption being accompanied by an increase in the 290–260 μm region. The isosbestic point for this reaction was at 303 μm. This reaction undoubtedly represents the enzyme catalysed transformation of NADH to NADH-X; moreover, it was found to occur at a pH as high as 8.7 and in the complete absence of phosphate, although the rate was much faster in the presence of phosphate and at the lower pH.

iii. Discussion.

The interpretation of U.V. spectral changes of proteins which accompany alterations in their secondary and tertiary structures was considerably clarified by the work of Yanari and Bovey (1960). These authors indicate that the change in polarizability of the environment surrounding the aromatic side chains of a protein is responsible for the spectral effects. In their view the blue shift and lower absorption accompanying urea denaturation of proteins is due to the exposure of previously hydrophobic bonded aromatic residues of the protein to an aqueous environment of lower polarizability. In this regard an environment of higher polarizability would stabilize the excited state of a τi → τi* transition provided that the dipole moment of the excited state is greater than the ground state. Such a stabilisation by increased solvation of the excited state would result in a red shift in the spectrum.
In studying changes accompanying protein denaturation the solvent perturbation spectrum technique of Herskovitz and Laskowski (1962) has been used by Donovan (1964). His studies on aldolase have indicated that 20% aqueous ethylene glycol can cause much the same perturbation of tryptophan and tyrosine chromophores as occurs when these groups are 'buried' in the native protein. His published difference spectra of tyrosine and tryptophan in 20% ethylene glycol show that tyrosine has two peaks at 265 m\(\mu\) and 277 m\(\mu\) and tryptophan has peaks at 290 and 283 m\(\mu\) and a pronounced minima at 287 m\(\mu\).

In view of these findings the spectral perturbation accompanying the binding of NAD\(^+\) to G3PD can be interpreted as a shielding of a tryptophan residue or residues from an aqueous environment by the NAD\(^+\). Tryptophan is thought to be the only residue involved in this case as the pronounced 'trough' at 287 m\(\mu\) is similar to the 'trough' observed with tryptophan in 20% ethylene glycol, whereas tyrosine shows a maximum red shift in this region.

Recently a report has appeared (Shifrin, 1964) which provides good evidence that the G3PD:NAD\(^+\) absorption band at 360 m\(\mu\) is the result of a 'charge transfer' interaction between the pyridine ring of NAD\(^+\) and a tryptophan residue on the protein. The model compound N-(p-indolylethyl)-3-carboxamide pyridinium chloride has been found to have a long weak absorption band extending into the visible. This absorption is stated to arise from the interaction of the two ring systems which overlap but are not necessarily parallel in this compound.

However, the enhancement of tryptophan absorption when NAD\(^+\) is bound to the protein, is opposite to what might have been expected from the studies of Shifrin (1964). The charge transfer production with his model tryptophan:nicotinamide compound is accompanied by a lower absorption in the 260-270 m\(\mu\) region which has been interpreted as resulting from an interaction between the oscillators of the nicotinamide ring and the benzene ring of tryptophan. The conclusion is reached therefore that the shielding of the protein tryptophan residue or residues from an aqueous environment by NAD\(^+\) is sufficiently large to overcome the hypochromic effect which may
be inferred as a result of the G3PD: \( \text{NAD}^+ \) charge transfer interaction.

The effect of phosphate and sulphate is to produce a further increase in protein absorption in the 290-270 nm region in the presence of \( \text{NAD}^+ \). This finding could be interpreted from the knowledge that increases in ionic strengths are known to produce long wavelength shifts by stabilizing the excited states of \( \pi \rightarrow \pi^* \) transitions. However, the effect is more likely to be due to a change in protein configuration which could lead to the shielding of a tyrosine residue from the solvent, this latter explanation is favoured by the observations of changes in protein absorption in the presence of \( \text{NADH} \) discussed below.

The \( \text{NADH} \)-protein difference spectrum is more complicated than that of \( \text{NAD}^+ \). A hypochromic effect on the \( \text{NADH} \) absorption is noted in the 340 nm region. A further hypochromic effect is found in the 287 nm region which suggests that in the presence of \( \text{NADH} \) a tyrosine residue or residues are now in a less polarizable or more aqueous environment.

Tryptophan is thought to be shielded again but the effect is not as great as might have been expected by the removal of the hypochromatic contribution of the charge transfer interaction.

The interpretation of the spectral perturbation of G3PD by \( \text{NAD}^+ \) and \( \text{NADH} \) outlined above is supported in part by the fluorimetric studies of Velick (1958), (1961). The protein was found to quench the fluorescence of \( \text{NADH} \); this is reflected in the difference spectra by a hypochromic effect at 340 nm. Moreover, Velick found that \( \text{NADH} \) quenched protein fluorescence 40% whereas \( \text{NAD}^+ \) only quenched the fluorescence 27%. These results led Velick to suggest that the nucleotide may be situated near a tryptophan 'stack'. Tryptophan was thought to be the only residue quenched by either nucleotide, however, an exposure of a tyrosine residue or residues to the solvent could also result in a quenching effect.

**4.2. OTHER NUCLEOTIDE-ENZYME INTERACTIONS**

The difference spectrum between \( \text{NAD}^+ \) and \( \text{NADH-X} \) (figure 6) has been shown to change in the presence of protein (figure 4), similarly \( \text{NAD}^+ \) and \( \text{NADH} \) when bound to the protein produced different perturbations of the
protein absorption in the 290-260 μm region. Consequently it was decided to study the effect of adenosine diphosphate ribose (ADPR) and nicotinamide mononucleotide (NMN) on the absorption of the protein in order to determine if a particular residue in the nicotinamide adenine dinucleotide could be associated with the perturbations.

4.2.1 ADPR enzyme.

Addition of ADPR to apo enzyme produced only a very small difference spectral change which could not be resolved from possible instrumentation error. Accordingly it was decided to try to overcome the large enzyme nucleotide dissociation constant by attempting to crystallize the protein in the presence of excess ADPR.

ADPR (10 moles/binding site) was added to a preparation of apo enzyme at pH 7.5 and ammonium sulphate added gradually to this solution until a slight cloudiness appeared; the solution was then set aside at 2°C. After 1 month a slight silkiness appeared on swirling the solution and after a further 6 weeks the crystals were examined with a phase contrast microscope. The crystals appeared as tiny needles, (Figure 11) and were collected by centrifugation, washed three times with 50% saturated ammonium sulphate solution and finally dissolved in a minimal volume of water.

The absorption spectrum (Figure 12) of the resulting solution showed a maximum at 268.5 μm, had an absorptivity of 0.990 for Ig/L and clearly resembled the spectrum obtained on addition of NAD⁺ to the apo enzyme. The protein contained 2.49 moles of dinucleotide ribose and 2.35 moles of dinucleotide phosphate/10⁵ g. of protein and only 0.18 moles of reducible NAD⁺ by the pepsin digest technique. Moreover, the absorption spectrum of a concentrated solution of the enzyme showed a small shoulder in the 330 μm region; this shoulder was far larger than could be accounted for by the residual amount of NAD⁺.

A subsequent attempt over a period of six weeks to prepare crystalline ADPR enzyme with ADPR at 4 moles/binding site yielded a preparation which contained only 1.58 moles of dinucleotide ribose, revealed no 330 μm shoulder and had an absorptivity of 0.915 for Ig/L. The activity
Figure 11. Crystals of G3PD prepared in the presence of excess ADP. The crystals appear as rods. The photograph is enlarged $17\times 0$. 
Figure 12. Absorption spectrum of G3PD containing bound ADPR.
of this latter preparation was determined and found to be 8100 moles NADH/min./10^5 g. of protein. The difference between these two preparations in regard to nucleotide content is probably due to the lower initial ratio of ADPR and to the shorter length of time allowed for crystallization.

4.2.2 NAD enzyme.

An attempt to prepare crystalline enzyme containing bound NMN was unsuccessful. The apo protein was allowed to stand in the presence of 4 moles NMN per binding site for 6 weeks at 2°C. After this time a stickiness of the solution was observed but the crystals were too small to be seen under the phase contrast microscope at a magnification of 1500. The preparation contained 0.53 moles of mono nucleotide ribose (0.26 moles of dinucleotide ribose)/10^5 g. and had an absorptivity of 0.853 for 1L/L. These figures are scarcely different from those obtained for the best preparations of apo enzyme.

The fact that this enzyme preparation showed a stickiness on swirling indicates that the apo enzyme can be crystallized although the crystals are extremely small. The activity of this preparation after 6 weeks was 11,000 moles NADH/min./10^5 g. of protein. Hitherto the crystallization of apo protein has not been reported and it is significant that the activity of this sample was not markedly reduced by the long crystallization procedure.

4.2.3 Discussion.

Astrachan (1954) has investigated the binding of NAD^+ scission products to the enzyme and has found that only ADPR and desamino NAD^+ retain the ability to bind to the protein. In view of his results it was not surprising to find that only ADPR enzyme could be prepared. The formation of G3PD:ADPR in the presence of ammonium sulphate can be explained by invoking a lower solubility for this complex than for apo enzyme. Hence if sufficient time is allowed for crystallization, all the binding sites on the protein should eventually be occupied by nucleotide.

In this regard the observation by Velick (1961) that NADH exists in the folded configuration (the planes of the adenine and nicotinamide
rings are thought to lie one above the other) when bound to the enzyme may be relevant to the large dissociation constant for ADPR and G3PD. It can be envisaged that ADPR must assume a folded configuration before binding to the enzyme occurs.

The absorption spectrum below 290 m\(\mu\) obtained when ADPR is bound to the protein is similar to the absorption spectrum of the enzyme with bound NAD\(^+\). The position of the 'shoulders' in the spectrum indicate that ADPR serves the same function as NAD\(^+\) in shielding tryptophan from the solvent. This effect could be achieved with ADPR when it is considered that it is probably bound to the NAD\(^+\) binding site in the folded configuration.

The observation of a small 330 m\(\mu\) shoulder in the absorption spectrum of G3PD:ADPR could not be repeated in a subsequent preparation. Consequently an explanation for this preliminary result is not attempted.

4.3. TITRATION OF G3PD WITH NAD\(^+\) STUDIED IN THE NEAR ULTRA VIOLET

Stockell (1959) has determined the number of NAD\(^+\) binding sites for yeast G3PD by following the appearance of the G3PD:NAD\(^+\) absorption band at 405 m\(\mu\). The high sensitivity of her double beam spectrophotometer enabled her to use this wave length rather than the maximum at 360 m\(\mu\), as high concentrations of NAD\(^+\) were found to contribute to the absorption at the lower wave length. Similarly, it was hoped by repeating her technique with the muscle enzyme that a value for the number of NAD\(^+\) binding sites could be determined for rabbit muscle G3PD.

However, the maximum absorption of the complex at 360 m\(\mu\) was chosen to follow the titrations due to the limited sensitivity of the Hilger U.V. spectrophotometer used in the present study.

1. Method.

Apo protein from two different samples of G3PD was prepared as described in the appendix. The samples of protein were; Preparations 2 and 6, initial activities 13,900 and 14,700 moles NADH/min./10\(^5\) g protein. Preparation 2 was used 3 months after isolation and Preparation 6 was used 3 weeks after isolation. The titrations were carried out by the addition
of 20 μl aliquots of NAD$^+$ from a micrometer syringe to apo protein in 2.5 ml of 50 mM triethanolamine/HCl/Na$_3$ buffer at pH 7.5 or 8.6 or 50 mM EDTA pH 7.5 as indicated in the legend to figure (13). Preparation 2 was used at concentrations of 7.2 and 13.0 g/L while preparation 6 was used at 3.67 and 6.71 g/L. The NAD$^+$ was diluted with supporting buffer prior to the titration.

ii. Results.

The titration curves measured by the absorptivity increase at 360 nm are given in figure (13). Each curve is the mean of duplicate titrations, which agreed with one another within the experimental error. Unfortunately in these initial studies different supporting buffers were used for the two protein preparations. The reason for the pronounced difference in the results obtained with 6.71 g/L (preparation 6) and 7.2 g/L (preparation 2) has not been investigated. However, it may be seen with each protein preparation, that the amount of charge transfer complex formed with excess NAD$^+$ is not proportional to the protein concentration. In view of these preliminary results the attempt to estimate the number of NAD$^+$ binding sites of the protein by this method was discontinued.

iii. Discussion.

Although the titration curves of NAD$^+$ binding to G3PD were not reproducible with the two protein preparations, it appears in each case that the extent of formation of the charge transfer complex between protein and NAD$^+$ is less at the higher protein concentration. Moreover, the curves show no well defined end point. From the small dissociation constant for NAD$^+$ and rabbit muscle enzyme, in the region of 0.1 - 1.0 μM (Velick and Furfine 1963), at the concentrations of NAD$^+$ and enzyme employed in the titrations a well defined end point in the titration curves would have been expected. It therefore appears that the NAD$^+$ binding to the protein under these conditions is anomalous and influenced by the protein concentration.
Figure 13. The effect of protein concentration on the formation of the G3PD: NAD$^+$ charge transfer complex, 50 mM triethanolamine/HCl/NH$_3$ pH 7.5 was used as the supporting medium for the two lowest protein concentrations and 50 mM EDTA pH 7.5 for the higher concentrations.
Recently Derenyi et al. (1963) and Parham and Harris (1963) have produced evidence that the protein is composed of three or four identical subunits depending on the chosen molecular weight. Schachman (1963) has discussed the effect of protein concentration on the subunit aggregation of proteins and concludes that many proteins are composed of homo polymer subunits which undergo increased aggregation with increased protein concentration. Hence an interaction between the identical NAD\(^+\) binding sites dependent on the protein subunit aggregation may be the cause of the anomalous titration curves.

The first studies of the binding of NAD\(^+\) by G3PD were carried out by Velick et al. (1953). These workers used an ultracentrifugation technique and protein concentrations of 1-3 g/L. Their results showed that the binding of successive amounts of NAD\(^+\) increased the affinity of the enzyme for NAD\(^+\). However, subsequent work by Velick (1958) with a fluorometric technique and much smaller protein concentrations produced no evidence for interaction between binding sites. Similarly Stockell (1959) using the appearance of the charge transfer complex found no evidence for binding site interaction with yeast G3PD at protein concentrations in the region of 0.6 - 1.8 g/L, but states that a protein concentration of 2.0 g/L led to an increase in the NAD\(^+\) dissociation constant of about 80%. It therefore seems that binding site interaction occurs for yeast G3PD at protein concentrations above 2.0 g/L. The present preliminary results suggest that with muscle enzyme binding site interaction may occur at a lower protein concentration.
CHAPTER 5

INVESTIGATION OF THE EFFECT OF G3P ON ENZYME BOUND NAD^+

Various workers have found that the addition of excess G3P to G3PD containing bound NAD^+ results in a lower absorption at 340 μm than would be expected for the total reduction of NAD^+. Cori et al (1950) produced evidence that NAD^+ could be recovered after G3P addition to the enzyme, on the other hand Koeppe et al (1956) have attributed the lower than calculated 340 μm absorption to a production of NADH-X from NADH. Recently Velick and Furfine (1963) have discussed the non stoichiometry and indicate that the effect can in part be accounted for by a hypochromic displacement of the bound NADH.

Preliminary experiments with purified enzyme confirmed the observations in the literature and further investigations relating to the non stoichiometry of reduction of protein bound NAD^+ are described in the present chapter.

5.1. TITRATION OF THE ENZYME WITH G3P

Initial studies with once refractionated enzyme showed that the virtually instantaneous increase in 340 μm absorption upon addition of excess G3P to substrate amounts of the protein was followed by a slow drop in optical density. This secondary reaction was traced to the contamination of the enzyme by triosephosphate isomerase and α-glycerolphosphate dehydrogenase (see Chapter I). When these two enzymes had been removed by 4 refractions of the G3PD no secondary drop in 340 μm absorption was observed.

Holo enzyme prepared by addition of NAD^+ to apo enzyme was used for the titration studies and samples were prepared having 2.45 moles of NAD^+/10^5g. of protein.

5.1.1 Effect of phosphate, sulphate and arsenate on the reduction of NAD^+

The study of the effect of adding G3P to enzyme containing bound NAD^+ in the presence of phosphate, sulphate and arsenate was undertaken, as phosphate is a participant in the overall reaction and arsenate renders the reaction irreversible (Warburg and Christian, 1939).
The titrations were carried out in 1 cm. cuvettes containing 2.0 ml. of 50 mM triethanolamine/HCl/KH₂PO₄ buffer pH 8.9 with either 50 mM sodium sulphate, phosphate or arsenate. Protein was added to the cuvette to give an initial concentration of 3.06 g/L prior to the addition of 20 μl aliquots of 1.385 mM DL G3P from a micrometer syringe. The titrations were followed at 340 μm and 410 μm in the Hilger U.V. spectrophotometer.

The titration curves at 340 μm and 410 μm for the protein in sulphate and phosphate are given in figure (1) together with the 340 μm absorption found with the protein in the presence of arsenate after a single addition of G3P. The final absorptions and titration curves are seen to be the same with the three different anions.

5.1. (2) Titration of the enzyme with DL G3P.

DL G3P was used in place of the DL mixture in order to find if the L isomer was involved in inhibiting the reduction of enzyme bound NAD⁺.

The titration was carried out as before with the same sample of protein and in the presence of phosphate, the DL G3P concentration was 1.295 mM. The absorption curve obtained (figure 1) was similar to the ones for the DL mixture although the absorptivity change towards the end of the titration was not as great.

5.1. (3) Effect of the protein concentration on the titration with DL G3P.

The preliminary experiments on NAD⁺ binding, described previously, suggested that the protein concentration may affect the extent of binding. It was reasoned that the extent of reduction of bound NAD⁺ by substrate may also vary in an anomalous manner with the protein concentration. Accordingly the reduction of protein bound NAD⁺ was studied with protein concentrations in the same region of 1 - 10 g/L as used for the NAD⁺ binding. The titrations were carried out in 1 cm. cuvettes in 2.0 ml of 50 mM triethanolamine/HCl/KH₂PO₄, 50 mM sodium phosphate pH 8.9 to which was added 0.5 ml of protein to give protein concentrations of 1.18, 2.27, 4.64 and 6.96 g/L. These solutions were expressed as molarity of the D isomer.
Figure 14

The effect of D and DL G3P on the reduction of enzyme-bound NADH in the presence of 5 mM phosphate, sulphate or arsenate. The titrations were carried out in 50 mM triethanolamine/HC1/NaOH in pH 8.9. The curves obtained with phosphate and sulphate are identical and the arrow indicates the absorptivity of the solution in the presence of arsenate.
titrated with 0.691, 0.925, 1.65 and 2.77 mM DL G3P (expressed here as molarities of the D isomer) which was diluted with the supporting buffer immediately before the titrations. The activity of the enzyme used in these studies was 10,000 moles NADH/min/10^5 g protein.

Throughout the titration, the measured absorptivity at 340 μm was corrected for dilution by titrant, and also for the contribution of the charge transfer complex. The latter correction was taken to be twice the absorption at 410 μm measured concurrently as it was found that the absorption of the charge transfer complex at 410 μm is approximately half that at 340 μm (figure 3).

The results are presented in figure 15. The reciprocal of the corrected absorptivity at 340 μm, which may be taken as a measure of the NADH concentration, is plotted against the reciprocal of the free G3P concentration. This value is calculated from the initial G3P concentration and the amount of NADH formed, assuming that the molar absorptivity of the latter is the same as for free NADH. The plots are not linear. The intercepts on the ordinate, although clearly uncertain, correspond to 74%, 69%, 64% and 59% reduction of the NADH for the protein concentrations of 1.18, 2.27, 4.64 and 6.96 g/L respectively. These values of reduced NADH at infinite G3P concentration have been calculated from the NADH content of the enzyme (2.45 moles/10^5 g protein) and the molar absorptivity of free NADH, 6.22 x 10^3. The absorption maximum of the product was at 340 μm and the stability of the absorptivity showed there was no slow formation of NADH-X.

The results presented in the three sections above are discussed at the end of the chapter.
Figure 15. Double reciprocal plots showing the effect of protein concentration on the reduction of protein bound NAD$^+$ with DL G3P. The protein concentrations, reading from the top of the graph are: 1.18, 2.27, 4.64 and 0.96 g/L. The titrations were carried out in the presence of 50 mM triethanolamine/HCl/NH$_3$ containing 50 mM sodium phosphate pH 8.9.
Concurrent with the previous experiments difference spectra were produced to compare the absorption of the NADH formed in situ by reduction of protein bound NAD$^+$ to that of NADH added to the apo enzyme. By correlating the changes occurring at 340 μm with those in the 290-260 μm region compounds with increased 280-290 μm absorption over that of NAD$^+$ should be immediately revealed as the transformation of NAD$^+$ to NADH has an isosbestic point at 282 μm.

Two sets of difference spectra were produced in the presence and absence of phosphate using the Beckman DK2 spectrophotometer. Additions to 2.5 ml. of 50 mM tris/HCl pH 6.7 were made according to the following diagram.

<table>
<thead>
<tr>
<th>test cuvette</th>
<th>reference cuvette</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 ml. protein</td>
<td>0.2 ml. protein</td>
</tr>
<tr>
<td>0.2 ml. NADH 1.015 mM</td>
<td>0.2 ml. NAD$^+$ 1.03 mM</td>
</tr>
<tr>
<td>0.1 ml. G3P 6.85 mM</td>
<td>0.1 ml. G3P 6.85 mM</td>
</tr>
</tbody>
</table>

The final protein concentration was 2.75 g./L and the amount of nucleotide added to the cuvettes was equivalent to 2.5 moles/10$^5$g. of protein.

The spectra were recorded with a minimum of delay due to the production of NADH-X in the cuvette containing NADH added to apo enzyme. Hence spectra with added G3P were recorded 3 minutes after the spectra obtained upon adding the nucleotides to the apo enzyme.

The difference spectrum between enzyme bound NAD$^+$ and NADH before addition of G3P (figure 16) yields only 72% of the calculated NADH absorption at 340 μm. This is due to the hypochromic effect of the protein on NADH absorption accounting for a 15% drop and the remaining 13% drop to the 'charge transfer' interaction. The isosbestic point is at 292 μm and the absorption difference of 0.100 at the normal isosbestic point for NAD$^+$/NADH at 282 μm reflects the change in the protein absorption.
Figure 16. Difference spectrum between NAD$^+$ and NADH bound to G3PD in 50mM tris/HCl and 50mM sodium phosphate pH 8.7 before and after the addition of 0.685 μmoles of G3P.
already found indirectly (Chapter 4), between protein in the presence of NAD$^+$ and NADH.

Upon addition of G3P to both cuvettes the resulting difference spectra show there is still a 15-18% difference (based on the calculated unbound NADH absorption) between the two solutions. A control experiment showed that G3P had no effect on the absorption of apo enzyme. This result indicates that the 340 m$m$ non stoichiometry cannot be accounted for by the hypochromic effect of the protein on the in situ produced HAD$^+$. In addition the spectra of the two protein solutions after treatment with G3P are almost identical between 305 and 285 m$m$ and it is felt that this result provides compelling evidence against the immediate production of NADH-X (or compounds with a similar absorption) upon reduction of protein bound NAD$^+$. The production of 20% NADH-X would have caused a 0.200 difference in absorptivity at 283 m$m$. This calculation is based on the molar absorptivity difference between NAD$^+$ and NADH-X of $15.1 \times 10^3$ at 283 m$m$ determined from the absorption spectrum of NADH-X (Chapter 3).

The difference spectra recorded 15 and 30 minutes after G3P addition, which show a steadily increasing 280-290 m$m$ absorption indicate that NADH-X is being produced in the cuvette with NADH added to apo enzyme. These spectra by showing an increased 280-290 m$m$ absorption also exclude the possibility of a slow NADH-X formation from NADH produced in situ, and furthermore exclude the possibility that G3P inhibits this formation.

5.3. THE PRODUCTS OF THE REACTION OF G3P ON THE NAD$^+$ ENZYME

The previous studies pointed to the fact that incomplete reduction of enzyme bound NAD$^+$ occurs on addition of excess G3P. Nevertheless, it was felt that the non stoichiometry of reduction may still be due in part to a hypochromic effect of the protein on NADH produced in situ as is found when NADH is added to apo enzyme.
5.3. (1) Recovery of acid stable NAD\(^+\).

A sample of protein (13.2 mg.) containing 2.46 moles of \(\text{NAD}^+ /10^5\) g. in 2.5 ml. of 50 mM triethanolamine/HCl/NH\(_3\) pH 8.7 had an absorptivity at 340 m\(\mu\) of 0.124 and 0.066 at 410 m\(\mu\). After addition of 0.4 ml. of 1.9 mM G3P the absorptivity at 340 m\(\mu\) was 0.470 and 0.043 at 410 m\(\mu\). This solution was then taken to pH 1.7 by the addition of 0.3 ml. of HCl and digested with 1 mg. of pepsin. After 30 min. 0.4 ml. samples were withdrawn and analysed for NAD\(^+\) by G3PD as described in the appendix. The concentration of NAD\(^+\) after addition of G3P was then calculated to be 0.053 mM or 47.5\% of the initial amount present on the protein.

The amount of NADH produced was calculated by subtracting the absorptivity contribution due to the remaining 'charge transfer' interaction at 340 m\(\mu\) and the absorptivity of the G3P solution determined independently. These values of 0.078, determined by subtracting the doubled remaining absorption at 410 m\(\mu\) after volume corrections and 0.040 for the G3P solution, gave an absorptivity change of 0.352 which corresponds to a concentration of 0.0565 mM NADH or 50.5\% of the initial NAD\(^+\) present. These figures indicate that 96\% of the initial NAD\(^+\) can be accounted for as NAD\(^+\) or NADH.

5.3. (2) Effect of the protein on the absorption of NADH produced in situ.

A sample of protein (3.97 mg.) containing 2.78 moles of \(\text{NAD}^+ /10^5\) g. protein (addition of NAD\(^+\) to apo enzyme) in 2.65 ml. of 50 mM triethanolamine/HCl/NH\(_3\) buffer pH 8.7 was treated with 0.1 ml. of 0.96 mM G3P. The absorptivity of the solution at 340 m\(\mu\) was 0.214. The solution was then plunged into a boiling water bath for 60 seconds, cooled and centrifuged. The absorptivity of the resulting solution was 0.201. This experiment has been repeated a number of times on various preparations and a 7-10\% drop in absorptivity at 340 m\(\mu\) was always observed on heat denaturation and removal of the protein. Furthermore, the supernatant solution had no 410 m\(\mu\) absorption and in this regard the corresponding drop in 340 m\(\mu\) absorption upon heat denaturation of the protein can be attributed to the destruction of the G3PD:NAD\(^+\) charge transfer complex.
5.4. DISCUSSION

The non-stoichiometry of reduction of NAD\(^+\) when excess G3P is added to enzyme containing bound NAD\(^+\) is thought to be resolved in favor of the incomplete reduction of the protein bound NAD\(^+\) rather than the formation of NADH-X or like compounds or to the hypochromic effect of the protein on NADH absorption. The experimental findings in support of this conclusion may be summarized as follows.

1. Evidence supporting the presence of NAD\(^+\) after addition of excess G3P.
   i. The absorption due to the 'charge transfer' complex between G3PD and NAD\(^+\) is never entirely abolished as judged by the 410 m\(\mu\) absorption.
   ii. Acid stable NAD\(^+\) can be recovered after denaturation of the protein treated with excess G3P.
   iii. Difference spectra between the protein with added NADH (which show a hypochromic absorption) and NADH produced in situ with excess G3P still show a 15 - 18\% difference in 340 m\(\mu\) absorption under the particular conditions of the experiment.
   iv. The increase in absorptivity at 340 m\(\mu\) upon titration of the enzyme with G3P varies with protein concentration. It appears that relatively smaller amounts of NADH are produced with excess G3P as the protein concentration is increased.

2. Factors against the hypochromic absorption of NADH.
   i. Heat denaturation of the enzyme containing NADH produced in situ leads to a decrease rather than an increase in 340 m\(\mu\) absorption. The decrease however can be attributed to the destruction of the remaining 'charge transfer' complex.
ii. Recovery of NADH\(^+\) together with the 340 m\(\mu\) estimation of NADH can account for 98% of the nucleotide originally present.

iii. There is no slow NADH\(-\)X formation from NADH produced by the addition of G3P, whereas NADH added to apo enzyme has a hypochromic absorption and is transformed slowly into NADH\(-\)X.

3. Miscellaneous findings.

i. Difference spectra and recoveries of NAD\(^+\) and NADH show that there is no rapid formation of NADH\(-\)X or compounds with a similar absorption spectrum upon addition of G3P to the enzyme.

ii. The effect is not due to an inhibition of the enzyme with the L isomer of G3P.

iii. The effect is not associated with the establishment of the overall equilibrium of the enzyme catalysed reaction as titrations of the enzyme with G3P in the presence of phosphate, sulphate or arsenate yield the same 340 m\(\mu\) absorption curves.

The fact that the 'charge transfer' absorption measured at 410 m\(\mu\) does not entirely disappear on addition of excess G3P to the enzyme is by itself strong evidence for the incomplete reduction of enzyme bound NAD\(^+\).

Also it has been shown that the presence of phosphate has no effect on the G3P titration curve and in addition, although it has not been shown directly, it may be reasoned that the absence of NADH\(-\)X formation from NADH produced in situ supports the view that the acyl enzyme is stable even in the presence of phosphate. These results could perhaps be best explained by the establishment of the
foll"wing equilibrium.

The experimental evidence outlined above is thought to favour the view that the acyl enzyme NADH complex is largely dissociated into free NADH and acyl enzyme. This view would in part support the recently proposed reaction mechanism of the enzyme (Krimsky and Racker 1963) whereby the NADH produced in the initial step must be either oxidized or replaced by NADH for the phosphorylation reaction to proceed. However, an alternative possibility is that an allosteric factor (Monod et al. 1963) acting at a distant site, may cause the protein to assume a configuration leading to the catalysis of the phosphorylation reaction.

The finding that at infinite G3P concentration the percentage reduction of NADH varied from 75% to 59% at the lowest and highest protein concentrations is presumably related to the observation described in the previous chapter that the formation of charge transfer complex decreased at the higher protein concentration. Although no explanation can be offered for the variation in percentage reduction of NADH with protein concentration a reason for the incomplete reduction at infinite G3P concentration with the lowest protein concentration may be that a proportion of the catalytic sites of this particular protein sample are inactive. The preparation, which had been isolated six weeks previously, had an activity of
10,000 moles of NADH/min/10^5 g of protein which is lower than the average activity immediately following isolation. A result which supports the conclusion that the enzyme is partially inactive is the finding that this sample contained 0.6 disulphide groups/10^5 g of protein (Chapter 6).

Harris (1964) has suggested that an intra chain disulphide bridge could form between the cysteine at the active site and the one situated four residues away. In this formulation the finding of 0.6 disulphide groups/10^5 g of protein would mean that 0.6 sulphydryl groups at the active site or 24% of the sites would be inactive. Throughout the experiments there has been no evidence to suggest that NADH-X or compounds with a similar absorption spectrum are produced when O3P is added to enzyme containing bound NAD^+. The NADH so produced is not transformed into NADH-X in the slow reaction catalysed by the enzyme. This has been associated with the fact that no evidence can be found for the hypochromic absorption of NADH produced in situ. These two results may be correlated with the 'induced fit' theory of Koshland (1963) which invokes a functional role for the flexibility of protein structure whereby the substrate induces the protein to form the final structure at the active site.

In the case of O3P the formation of acyl enzyme in the presence of O3P is thought to alter the nature of the nucleotide binding site so that the hypochromic absorption of NADH and the formation of NADH-X no longer occur. These findings provide compelling evidence against the participation of NADH-X in the mechanism of action of the enzyme.

The enzyme catalysed formation of NADH-X therefore, can be associated with the binding of NADH to the protein in the absence of the acyl enzyme intermediate. Configurational charges occurring at the nucleotide binding site in the absence of acyl enzyme may result in a close interaction between the tryptophan (associated in
the charge transfer complex with the nicotinamide ring of \( \text{NAD}^+ \) and the reduced nicotinamide ring of \( \text{NADH} \). An increase in electron density or increased polarization of the reduced nicotinamide ring by the tryptophan acting in much the same way as with the oxidised nicotinamide ring, would facilitate an enamine transition leading to protonation of the pyridine ring at the 5 position with subsequent \( \text{NADH}^+ \) formation.

![Chemical structure](image)

The study of the effect of \( \text{G3PD} \) on the reduction of bound \( \text{NAD}^+ \) at protein concentrations between 1 - 10 g/L is thought to be relevant to the \textit{in vivo} state of the enzyme. Gzok and Bucher (1960) have found that the protein concentration of rabbit muscle sarcoplasm, which contains \( \text{G3PD} \) together with the other proteins of the glycolytic pathway is probably higher than 200 g/L. This would make the \textit{in vivo} concentration of \( \text{G3PD} \) in the region of 40 g/L as this protein is stated to comprise about 20% of the soluble or myogen fraction of the muscle.
CHAPTER 6

SOME ASPECTS OF THE SULPHUR CHEMISTRY OF G3PD

A sulphydryl group on the enzyme has been implicated in the reaction mechanism of G3PD since Racker and Krimsky (1952 a) first proposed that a thiol ester intermediate is formed between the protein and the substrate. Koepe et al. (1956) found a disappearance in titratable protein sulphydryl groups in the presence of acetyl phosphate and Ferdinand (1962) confirmed this observation and produced evidence for the concomitant appearance of thiol ester. It was decided to extend these studies to the natural substrate and also to investigate the nature of the acyl enzyme produced with aldehyde instead of acyl phosphate. As a preliminary, the total number of sulphydryl and disulphide groups on the protein were determined.

6.1. ESTIMATION OF THE NON-METHIONINE SULPHUR RESIDUES OF G3PD

6.1.(1) Estimation of the Sulphhydryl Groups of G3PD.

1. Method.

The amperometric titration technique of Allison and Cecil (1958) using phenylmercuric hydroxide was adapted for the estimation of the total sulphydryl groups of G3PD. The titration was carried out at room temperature in the cell of the PO4 Polarograph (Radiometer Copenhagen) using a dropping mercury electrode which was connected to the reference calomel electrode by a 1M NH₄NO₃ bridge in 2% agar. Deoxygenation of the supporting media (2.5 ml.) containing 8M urea in 50 mM Na₂B₄O₇ pH 9.25 and 50 mM Na₂SO₃ or Na₂S₂O₃ was accomplished by bubbling N₂ through the solution for 10 minutes. Sodium sulphite or thiosulphate were included in the supporting solution to prevent 'overbinding' of the mercurial by the protein (Cecil and Snow, 1962) and also to detect the presence of disulphide groups. Sulphite but not thiosulphate reacts with disulphide groups

$$RSSR + 3O_3^- \rightarrow RS^- + RSSO_3^-$$

producing a thiol which can contribute to the sulphydryl titre. (Cecil, 1963).
Protein (10-15 mg. in 1 ml.) was added to the solution and deoxygenation continued for a further 5 minutes. To minimize frothing in the presence of protein during N$_2$ gasping a ring of silicone 'MS antifoam A' was applied around the top of the cell prior to the addition of the protein. The titration was carried out at a potential of -0.6 V by the addition of 20μl. aliquots of 10.18 mM phenylmercuric hydroxide from a micro burette, the full scale deflection on the recorder was 1μa.

ii. Results.

The results are given in Table 7. A direct comparison between the titrations in sulphite and thiosulphate with preparation 5 suggested the presence of a small amount of disulphide. Accordingly, a method for the direct determination of the disulphide content of the enzyme was developed and is described below.

6.1.(2) Estimation of the Disulphide Groups of G3PD.

For this study the very reactive N-2:4-dinitroanilino maleimide (Clark-Walker and Robinson, 1961) was chosen to block the sulphydryl groups of the denatured protein.

\[
\begin{align*}
RSH + &\begin{array}{c}
\text{N} - \text{N} \\
\text{NO}_2
\end{array}
\rightarrow \begin{array}{c}
\text{N} - \text{N} \\
\text{NO}_2
\end{array}
\rightarrow RS
\end{align*}
\]

The unreacted maleimide was destroyed rapidly by the addition of excess sulphite to yield a variety of products, (Robinson and Clark-Walker, unpublished experiments). The sulphite at the same time also cleaved any disulphide present and the thiol so produced was titrated with phenylmercuric hydroxide in the described manner.
### TABLE 7

The sulphydryl and disulphide content of GDH.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Activity (moles NADH/min/10^5 g protein)</th>
<th>Number of titratable sulphydryl groups/10^5 g protein with ( \text{SO}_3^- )</th>
<th>Number of directly determined disulphide groups/10^5 g of protein</th>
<th>Total non methionine sulphur residues/10^5 g protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>4. 15 months after isolation containing 2.41 moles NAD(^+)/10^5 g protein</td>
<td>9.200</td>
<td>-</td>
<td>9.3</td>
<td>-</td>
</tr>
<tr>
<td>5. 2 months after isolation containing 2.43 moles NAD(^+)/10^5 g protein</td>
<td>12.500</td>
<td>9.2</td>
<td>9.7</td>
<td>0.6</td>
</tr>
<tr>
<td>6. as above with NAD(^+) removed by charcoal</td>
<td>-</td>
<td>-</td>
<td>9.6</td>
<td>9.7</td>
</tr>
<tr>
<td>6. 3 months after isolation containing 2.46 moles NAD(^+)/10^5 g protein</td>
<td>10.000</td>
<td>9.0</td>
<td>9.7</td>
<td>0.6</td>
</tr>
<tr>
<td>Boehringer sample 1 containing 1.25 moles NAD(^+) and 2.15 moles nucleotide/10^5 g protein</td>
<td>11.600</td>
<td>-</td>
<td>9.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* These values were determined in the presence of 28.5 mM perchlorate at pH 4.3 as described under heading 6.2.
1. **Method.**

A solution (2.0 ml.) containing 9M urea in 0.1M sodium acetate pH 4.6 was deoxygenated by N₂ gassing for 10 minutes. Protein (15-20 mg. in 1.0 ml.) was added together with 0.2 ml. of 10 mM N-2,4-dinitroanilinomaleimide in dioxan and left to react at room temperature for 5 minutes during which time deoxygenation was continued. At the end of this time 0.25 ml. of 1M Na₂SO₃ was added and the solution left for a further 5 minutes. The pH of the solution was then raised to 9.5 with 0.1 ml. of isopiestic NH₃ and titrated amperometrically with 10.13 mM phenylmercuric hydroxide at -0.5 V and 1 µA full-scale deflection. A potential of -0.5 V was chosen to avoid the complex reduction wave of the destroyed maleimide which begins around -0.52 V under these conditions. A control was carried out by replacing the sulphite with 0.2M sodium thiosulphate. The titration with phenylmercuric hydroxide in this case was carried out at -0.55 V in order to avoid the anodic wave of the thiosulphate. No uptake of phenylmercury could be detected in the controls.

11. **Results.**

The results are given in Table 7 together with the sulphydryl titres. The amount of disulphide present in preparation 5 was 0.6 groups/10⁵ g protein determined by this direct method as compared to the value of 0.55 groups/10⁵ g protein determined from the difference in titres between the protein in sulphite and thiosulphate. A control carried out by leaving the denatured protein in the cell 20 minutes prior to the addition of maleimide revealed no increase in the number of disulphide groups on the protein. This experiment indicates that the measured disulphide does not appear as a result of a rapid oxidation occurring in the denatured protein.

6.1.(3) **Discussion.**

Values in the literature for the number of sulphhydryl groups of rabbit muscle G3PD have been determined by a variety of methods. Velick and Furfine (1963) found 9.1 cysteinyl residues/10⁵ g protein by amino acid analysis, whereas Koepe et al. using the spectrophotometric technique with
p-chloromercuribenzosuote found 11.7 SH groups/10^5 g protein after 40 minutes reaction in 1 M sodium acetate pH 4.6. Benesch et al. (1955) have found 9.15 SH groups/10^5 g protein using amperometric titration with silver ion at pH 7.4 in Tris buffer but Boyer and Snails (1959) find that urea denaturation is necessary for the rapid reaction of the total sulphhydryl groups of G3PD. They found 10.8 groups/10^5 g of protein in 4.8 M urea. Recently Harris (1964) has found four unique cysteines in amino acid sequence studies of rabbit muscle G3PD and this finding together with those of Perham and Harris (1963) and Devenyi et al. (1963) which indicate that the protein is composed of homopolymer subunits gives a definite limit of 10.2 - 11.6 SH groups/10^5 g of protein. These limits are the values expected from a protein composed of 3 subunits each with 4 cysteines assuming the minimum determined molecular weight of 118,000 (Taylor, 1950) or a protein made up of 4 subunits with a maximum molecular weight of 138,000, (Fox and Bandliker, 1956).

G3PD of molecular weight 138,000 formed from 3 subunits would have 8.7 SH groups/10^5 g protein while G3PD of molecular weight 118,000 made up of 4 subunits would have 13.5 SH groups/10^5 g of protein. The results obtained in the present study of 10.2 - 10.4 SH groups/10^5 g of protein (after correcting for the small amount of disulphide) favour the view that the protein is composed of three subunits with a combined molecular weight of 117,000. A value for the molecular weight in the region of 120,000 is supported by the finding that the protein contains an average of 2.45 moles of NAD*/10^5 g of protein. This value gives a molecular weight of 122,000 for 3 moles of bound NAD*.

The experimental results presented above show that there is no detectable difference between the sulphhydryl titres of enzyme containing NADH-X and holo enzyme, moreover the removal of NAD* from the protein does not change the amount of titratable sulphhydryl groups.

Although there have been no reports in the literature relating to the presence of disulphide groups in isolated G3PD, Tucker and Grisolia (1962) and Amelunxen and Grisolia (1962) have been able to detect the disappearance
of sulphydryl groups of the protein in the presence of NADH. Ferdinand (1962), working with a partially inactive preparation of G3PD found suggestive evidence for the presence of a disulphide group from the difference between the sulphydryl titres in sulphite and thiosulphate.

The experiments described in the present work indicate that a small amount of disulphide is present in some preparations of G3PD; however, the three samples used were at least two months old and showed less than the average activity of preparations immediately after isolation. Recently Harris (1964), on the basis of his amino acid sequence studies, has suggested that an intra-chain disulphide bond could form between the cysteines at the active site and a cysteine group situated four amino acid residues away.

The disulphide determinations presented above lend support to this view and as discussed previously could account for the incomplete reduction of enzyme bound NADH at infinite G3P concentrations. It is thought that freshly isolated and fully active G3PD contains no disulphide groups (Ferdinand, 1962; Cecil, 1963).

6.2. ESTIMATION OF THE SULPHHYDRYL GROUPS OF G3PD IN THE PRESENCE OF SUBSTRATES

An investigation of the amount of sulphydryl groups in G3PD was made after reaction of the holo enzyme with acetaldehyde and G3P. These substrates were chosen in order to establish that the disappearance of sulphydryl groups noted by Koeppe et al (1956) and Ferdinand (1962) in the presence of acetyl phosphate were due to the formation of a catalytic intermediate. It follows that the formation of a true catalytic intermediate should be identical from either direction of the overall reaction. The possibility exists however that more than one true catalytic intermediate may occur in an enzyme catalysed reaction. Such a possibility could come about in this particular case by acyl transfer from a sulphydryl group to another acyl acceptor group on the protein.

As a preliminary, the sulphydryl titre of G3PD was studied in the presence of acetyl phosphate in order to confirm the findings of Koeppe et al. (1956) and Ferdinand (1962).
6.2.1 The Effect of Acetyl Phosphate on the Sulphydryl Group Titrations of G3PD.

The protein used in these studies was preparation 6 which had been refractionationated four times. The protein was treated with charcoal to remove NADH-X and reconstituted by the addition of NAD⁺ to the extent of 2.45 moles/10⁵ g protein. Acetyl phosphate was purchased as the dilithium salt and the concentration was measured by hydroxamic acid formation as described in the Appendix.

1. Method.

The treatment of enzyme with acetyl phosphate was carried out in 1 cm. cuvettes under the conditions described in the legend to Table 8. The absorption of the solution was measured at 340 and 410 mµ before and after the addition of acetyl phosphate. The acetyl phosphate was diluted 1:1 with the triethanolamine buffer immediately before the addition to the cuvette. The final protein concentration was 4.73 g/L and the acetyl phosphate concentration was equivalent to 41.5 moles/mole of NAD⁺ binding sites. A second series of experiments were carried out with a final protein concentration of 6.21 g/L and 37 moles of acetyl phosphate/NAD⁺ binding site. Controls were prepared in the same manner but with buffer replacing acetyl phosphate.

The sulphydryl group titrations of the protein after 3 minutes and 45 minutes in the presence of acetyl phosphate were carried out on 1 ml. samples which were added to 2.5 ml. of 9 M urea in 0.1 M sodium acetate and 0.04 M perchlorate pH 3.5. The final pH of the solution was 4.3 and deoxygenation of the solution with protein present was continued for 5 minutes. The titration with 5.05 mM phenylmercuric hydroxide was carried out at -0.5 V and 0.7 µA full-scale deflection on the recorder.

2. Results.

The results of the titrations in the presence and absence of acetyl phosphate together with the absorptivity changes are given in Table 8. The average values of 9.07 and 8.42 SH groups/10⁵ g of protein clearly show that 0.55 SH groups/10⁵ g of protein disappear in the presence of acetyl phosphate. In addition the spectroscopic measurements indicate that there is a small but reproducible drop in the 'charge transfer' complex of the protein in the presence of acetyl phosphate.
**TABLE 6**

*The Sulphydryl Groups of G3PD in the Presence of Acetyl Phosphate*

The reaction cuvette contained:

- 2.0 ml. 50 mM triethanolamine/\(\text{HCl/KOH}\) and 50 mM \(\text{Na}_2\text{SO}_4\) pH 8.9.
- 0.2 ml. H₂O.
- 0.25 ml. G3PD (51 g/L) or 0.3 ml. G3PD (58 g/L) preparation 6.
- + 0.25 ml. 50-25 mM acetyl phosphate in triethylamine buffer.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protein concentration g/L</th>
<th>Absorption 340 μm 410 μm</th>
<th>Titrateable -SH groups/10⁵g protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.45 ml. protein</td>
<td>5.21</td>
<td>0.108 0.057</td>
<td>after 3 min. 8.4</td>
</tr>
<tr>
<td>+ 0.25 ml. acetyl phosphate</td>
<td>4.73</td>
<td>0.083 0.043</td>
<td>after 45 min. 8.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.21</td>
<td>0.107 0.056</td>
<td>after 3 min. 8.3</td>
</tr>
<tr>
<td></td>
<td>4.73</td>
<td>0.085 0.041</td>
<td>after 45 min. 8.3</td>
</tr>
<tr>
<td>2.45 ml. protein</td>
<td>5.21</td>
<td>0.105 0.056</td>
<td>after 3 min. 9.05</td>
</tr>
<tr>
<td>+ 0.25 ml. buffer</td>
<td>4.73</td>
<td>0.097 0.052</td>
<td>after 45 min. 9.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.21</td>
<td>0.108 0.057</td>
<td>after 3 min. 9.0</td>
</tr>
<tr>
<td></td>
<td>4.73</td>
<td>0.100 0.053</td>
<td>after 45 min. 9.1</td>
</tr>
<tr>
<td>2.5 ml. protein</td>
<td>6.97</td>
<td>0.164 0.083</td>
<td>after 3 min. 8.45</td>
</tr>
<tr>
<td>0.3 ml. acetyl phosphate</td>
<td>6.21</td>
<td>0.129 0.061</td>
<td>after 45 min. 8.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.97</td>
<td>0.165 0.082</td>
<td>after 3 min. 8.45</td>
</tr>
<tr>
<td></td>
<td>6.21</td>
<td>0.127 0.062</td>
<td>after 45 min. 8.45</td>
</tr>
<tr>
<td>2.5 ml. protein</td>
<td>6.97</td>
<td>0.165 0.083</td>
<td>after 3 min. 9.05</td>
</tr>
<tr>
<td>0.3 ml. buffer</td>
<td>6.21</td>
<td>0.147 0.074</td>
<td>after 45 min. 9.1</td>
</tr>
</tbody>
</table>
6.2.(2) The Effect of Acetaldehyde on the Sulphydryl Group Titre of G3PD.

The protein solution used for these experiments was the same as for acetyl phosphate. Acetaldehyde was redistilled and the concentration measured with liver alcohol dehydrogenase and NADH as described by Racker (1957).

1. Method.

The reduction of protein bound NAD$^+$ by acetaldehyde was carried out in 1 cm. cuvettes as described in the legend to Table 9. The reduction of NAD$^+$ was followed at 340 mμ, the temperature being 24°C. The final protein concentration was 4.73 g/L and the acetaldehyde concentration was equivalent to 145 moles/mole of NAD$^+$ binding sites. After 80 and 120 minutes 1 ml. samples of the solution were titrated in the same manner as before.

11. Results.

The time course of the reduction of NAD$^+$ by acetaldehyde is given in Figure 30 and the titrations of the protein sulphydryl groups are given in Table 9.

The average of 0.3 SH groups/10$^5$g of protein found after reduction of protein bound NAD$^+$ with acetaldehyde is equivalent to the disappearance of 0.75 SH groups/10$^5$g of protein. However, the amount of NAD$^+$ reduced after 80 minutes is equivalent to 1.4 moles/10$^5$g of protein. This figure has been calculated by subtracting the doubled remaining absorption at 410 mμ (see Chapter 2) from the final absorption at 340 mμ in order to allow for the contribution of the remaining 'charge transfer' complex at 340 mμ.

6.2.(3) The Effect of G3P on the Sulphydryl Group Titre of G3PD.

The protein solution used for these experiments was preparation 6 which had been reconstituted with NAD$^+$ to the extent of 2.53 moles of NAD$^+/10^5$g of protein.

1. Method.

The reduction of protein bound NAD$^+$ was carried out in 1 cm. cuvettes as described in the legend to Table 10.
Figure 17. The reduction of enzyme bound NAD⁺ by acetaldehyde.
The cuvette contained: 2.0 ml 50 mM triethanolamine/HCl/NH₃ and 50 mM Na₂SO₄ pH 8.9, 0.45 ml protein (28.4 mg/ml) and 0.25 ml 0.15M acetaldehyde, the temperature was 23°C.
TABLE 9

The Sulphhydryl Groups of G3PD in the Presence of Acetaldehyde

The reaction cuvette contained:

- 2.0 ml. 50 mM triethanolamine/HCl/NH₃ and 50 mM Na₂SO₄ pH 8.9
- 0.2 ml. H₂O
- 0.25 ml. G3PD (51 g/L) preparation 6.
- 0.25 ml. 0.15 M acetaldehyde.

<table>
<thead>
<tr>
<th>Absorption at 340 μμ</th>
<th>Absorption at 410 μμ</th>
<th>Titratable SH groups/10^5 g protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>initial after 80 min.</td>
<td>initial after 80 min.</td>
<td>after 80 min. after 120 min.</td>
</tr>
<tr>
<td>0.104</td>
<td>0.444</td>
<td>0.052 0.012</td>
</tr>
<tr>
<td>0.103</td>
<td>0.446</td>
<td>0.051 0.012</td>
</tr>
</tbody>
</table>
The absorption of the solution was measured at 340 and 410 µm and the G3P solution was diluted 1:1 with triethanolamine buffer prior to the addition to the cuvette. The final protein concentration was 6.21 g/L and 1 ml samples of the reacted solutions together with controls were titrated spectrophotometrically with phenylmercuric hydroxide as previously described.

11. Results.

The absorptivity changes and sulphydryl titres are given in Table 10. From these results it can be seen that there is no significant detectable difference between the sulphydryl titres in the presence of G3P or in the controls.

6.2.4 Discussion.

The estimation of the disappearance in titratable sulphydryl groups of G3PD in the presence of substrates was carried out at pH 4.3 as thiol esters are known to have a maximum stability between pH 3.0 - 5.0, (Stadtman, 1952; Noda et al., 1953; Jasnicke and Lynen, 1960). Phosphate was excluded from the medium in order to avoid the possibility that it may influence the concentration of acyl enzyme.

The disappearance in titratable sulphydryl groups with both acetyl phosphate and acetaldehyde gives strong support to the theory that the acyl enzyme so formed is a true catalytic intermediate and that it occurs by thiol ester formation. These findings are in qualitative agreement with those of Koepp et al. (1956) and Ferdinand (1962); however only 0.55 thiol groups/10^5 g of protein were found to disappear in the presence of acetyl phosphate in contrast to 1.9 fewer SH groups/10^5 g protein found by Koepp et al. using p-chloromercuribenzoate and 2.1 fewer SH groups/10^5 g protein found by Ferdinand using amperometric titration with HgCl2. The formation of acyl enzyme from acetyl phosphate appears to be rapid; however the reduction of protein bound MAD⁺ by acetaldehyde occurs slowly and even though 1.4 moles of MAD⁺/10^5 g of protein are reduced after 80 minutes only 0.75 SH groups disappear. This figure may mean that
TABLE 10

The Sulphydryl Groups of G3PD in the Presence of G3P.

The reaction cuvette contained:

- 2.0 ml. 50 mM triethanolamine/HCl/NH₃ and 50 mM Na₂SO₄ pH 8.9.
- 0.2 ml. H₂O.
- 0.3 ml. G3PD (58.0 g/L) preparation 6.
- 0.3 ml. 2.77 mM DL G3P in triethanolamine buffer.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Absorption 340 mμ</th>
<th>Absorption 410 mμ</th>
<th>Titrateable -SH groups/10⁵g protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 ml. protein</td>
<td>0.167</td>
<td>0.086</td>
<td>9.05</td>
</tr>
<tr>
<td>0.3 ml. buffer</td>
<td>0.150</td>
<td>0.078</td>
<td>9.1</td>
</tr>
<tr>
<td></td>
<td>0.168</td>
<td>0.087</td>
<td>9.1</td>
</tr>
<tr>
<td></td>
<td>0.152</td>
<td>0.079</td>
<td>9.15</td>
</tr>
<tr>
<td>2.5 ml. protein + 0.3 ml. G3P</td>
<td>0.163</td>
<td>0.085</td>
<td>9.1</td>
</tr>
<tr>
<td></td>
<td>0.524</td>
<td>0.047</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>0.168</td>
<td>0.085</td>
<td>9.05</td>
</tr>
<tr>
<td></td>
<td>0.540</td>
<td>0.049</td>
<td>9.1</td>
</tr>
<tr>
<td></td>
<td>0.166</td>
<td>0.085</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>0.543</td>
<td>0.050</td>
<td>8.95</td>
</tr>
</tbody>
</table>
acetyl enzyme is partially destroyed during the course of the titration procedure. The rapid breakdown of the phosphoglycerol-1 enzyme intermediate during the course of the titration procedure almost certainly accounts for the failure to detect any change in the sulphydryl titre of the protein after treatment with 63P. The breakdown of the thiol ester intermediate when the protein is denatured may be the result of protein amino groups causing the 'aminolysis' of the thiol ester even at pH 4.3. Such an 'aminolysis' has been reported for amino acid thiol esters by Stadtman (1952).

A small 10-15% drop in the amount of 'charge transfer' complex is found in the presence of acetyl phosphate. This finding is in contrast to those of Racker and Krimsky (1952 a) and Krimsky and Racker (1955) who state that treatment of the protein with acetyl phosphate and 1:3 diphosphoglycerate abolishes the 360 m\(\mu\) absorption of the protein. In the light of the tryptophan-nicotinamide charge transfer complex the above experimental findings with acetyl phosphate can be interpreted as a weakening of the tryptophan-nicotinamide interaction which probably accompanies configurational changes at the active site associated with the formation of acyl enzyme. This conclusion supports the discussion in the previous chapter concerning the 'flexibility' of G3PD. Configurational changes of enzymes associated with substrate binding have recently been discussed by Koshland (1963) and Jencks (1963).
GENERAL DISCUSSION

In the course of preparing G3PD in the presence of added NAD⁺ a different crystalline shape of the protein was found. This observation lead to the investigation of the nucleotide content of the two crystalline enzymes and as a result a compound having a similar analysis to the NADH-X of Chaykin et al. (1956) was isolated from the enzyme crystallized as rhomboidal plates. In subsequent experiments G3PD was found to catalyse the conversion of NADH to NADH-X and contrary to other workers (Rafter et al. 1954, Chaykin et al. 1956) the reaction was found to occur in the complete absence of phosphate or other polyanions. In addition it was found that NADH produced by the in situ reduction of enzyme bound NAD⁺ with G3P was not transformed into NADH-X. No evidence was found to suggest that NADH-X has a functional role in the reaction mechanism of the enzyme and the most likely explanation for its occurrence on the enzyme is that it is formed from NADH during the isolation procedure. The variable amounts of NADH-X found on different preparations of the enzyme are thought to reflect the amount of acyl enzyme and NADH existing at the moment of death of the animal. The NADH-X is thought to be formed during the isolation procedure by the breakdown of the acyl enzyme intermediate which would then result in the enzyme catalysed transformation of the bound NADH.

The binding of nucleotides to the apo enzyme has been studied by the aid of difference spectra. By this technique the anomalous shoulders noted in the absorption spectra of the enzyme containing NAD⁺ have been resolved in terms of a shielding of a tryptophan residue of the protein from contact with the solvent. Both NADH and ADPR were found to produce similar spectra to that of NAD⁺, however in the former case a further change occurs in the protein structure leading to the exposure of a previously 'buried' tyrosine residue. These results are particularly interesting in view of the recent work of Shifrin (1964) who has presented convincing evidence that the visible absorption band resulting from the
binding of \( \text{NAD}^+ \) to the enzyme is due to a 'charge transfer' interaction between tryptophan and the nicotinamide ring of \( \text{NAD}^+ \).

Studies of the binding of \( \text{NAD}^+ \) to \( \text{GDP} \) by following the increase in the charge transfer complex have shown that the extent of formation of the complex is proportionally smaller at higher protein concentrations. In this respect charge transfer complex formation resulting from interaction between the two ring systems is probably a very sensitive indicator of the alignment of the nucleotide and protein. It can be thought, by analogy with the oxygenation of haemoglobin, that an alteration in protein structure accompanying the binding of \( \text{NAD}^+ \) to one of the subunits may alter the alignment of subsequent molecules of \( \text{NAD}^+ \) at the remaining binding sites. Increases in protein concentration by causing increases in subunit interaction may enhance this effect. Similarly the extent of reduction of enzyme bound \( \text{NAD}^+ \) by \( \text{GDP} \) seems to vary with protein concentration and this effect may also be related to subunit interaction affecting the alignment of the \( \text{NAD}^+ \) on the protein.

The titration curves with \( \text{GDP} \) were found to be independent of the presence or absence of phosphate and this observation has been taken as evidence that the overall equilibrium of the enzyme catalysed reaction is not established and that a further step or steps in the catalysis must occur. Recently Velick and Furfine (1963) and Krinsky and Racker (1963) have found that \( \text{NADH} \) inhibits the enzyme catalysed oxidation of \( \text{GDP} \) and the latter workers postulate that the phosphorylation of the acyl enzyme intermediate takes place in the presence of \( \text{NAD}^+ \) rather than \( \text{FADH} \). Krinsky and Racker suggest that the \( \text{NADH} \) produced by the addition of \( \text{GDP} \) is oxidized in situ by coupling to another dehydrogenase system and Jegal and Boyer (1953) have suggested the other alternative that \( \text{NADH} \) is replaced by \( \text{NAD}^+ \). However, Boyer, in a latter publication (Koeppe et al. 1956) rewrote the reaction mechanism of the enzyme excluding the \( \text{NAD}^+ \).
requirement in the phosphorylation.

The recent formulation of Krimsky and Racker has received support from Hilvers and Weenen (1962) and Hilvers et al. (1964) who find that the rate of oxidation of NADH by 1:3diPGA catalysed by the NAD* free enzyme is stimulated by the addition of NAD*.

The involvement of NAD* in the phosphorylation step may be 'allosteric', as has been suggested by Park and Koshland (1958) and Racker (1961) or it may be involved directly. This latter hypothesis cannot be ignored as Barltrop et al. (1963) have found evidence that the adenine ring of NAD* may be associated with a phosphorylation reaction. An involvement of NAD* in the phosphorylation of the natural substrate may also help to explain the requirement for NAD* in the hydrolase and transferase activities of the enzyme and the anomalous result recorded by Loewus et al. (1956) that a small amount of deuterium is incorporated from the medium into the nicotinamide ring of NAD* during oxidation of G3P.

The formation of acyl enzyme from both acetylphosphate and acetaldehyde oxidation has been studied and evidence was found that a sulphydryl group of the protein was concerned. It thus seems that the thiol ester formation between acetylphosphate and protein noted previously by Koeppen et al. (1956) and Ferdinand (1962) is a true catalytic intermediate in the reaction mechanism of the enzyme. The failure to detect the presence of a thiol ester intermediate with the natural substrate, which is oxidized 50,000 times faster than acetaldehyde (Hartling and Velick, 1954) is not surprising in this regard as Jenks (1963) has pointed out that true covalent catalysis requires the acceleration of both the formation and breakdown of intermediates.

Evidence has been found from two sets of analytical data that the molecular weight of the enzyme is in the region of 120,000. Thus the enzyme crystallised in the presence of excess NAD* was found to contain 3 molecules per 122,000 g. of protein and sulphydryl analyses have shown there are 12 sulphydryl groups per 117,000 g. of protein. These results support the
120,000 molecular weight value determined by Taylor and Lowry (1956)
rather than the higher values found by Fox and Dandliker (1956) and Elias
et al. (1960).

Finally mention must be made of the 'flexibility' of the enzyme. The different crystalline shapes of the enzyme are thought to reflect real differences in the configuration of the protein associated with the binding of the different nucleotides. This view is supported by the difference spectra between NAD$^+$ and NADH-X in the presence and absence of protein as a decrease in absorption in the 260 μ of protein bound to the protein in place of NAD$^+$. Differences also exist between the absorption spectra of the protein in the presence of NAD$^+$ and NADH and these differences have been associated with the shielding and exposure of tryptophan and tyrosine residues of the protein which must result in the latter case from an alteration in protein structure. Similarly the titration studies involving the formation of the binary complex between NAD$^+$ and protein and the ternary complex between G3P and G3PD:NAD$^+$ have shown a dependence on the protein concentration. This dependence on protein concentration has been taken to reflect configurational changes associated with complex formation which are then transmitted to the rest of the 'active molecule' through subunit interaction.

* Schachman (1963) has defined the molecular weight of a protein which undergoes association, dissociation equilibria as the smallest unit which possesses the characteristic biological activity.
APPENDIX

I. MATERIALS

Acetyl phosphate

Dilithium salt, W.W. 152

Adenosine diphosphate ribose

Monosodium salt, M.W. 599 corrected for 1 H₂O
Fahrl Laboratories, Milwaukee 5, Wisconsin, U.S.A.

Adenosine monophosphate

Disodium salt, M.W. 391.
C.F. Boehringer, & Sons, Mannheim, Germany.

Adenosine triphosphate

Disodium salt, M.W. 623 corrected for 4 H₂O
Sigma Chemical Co., St. Louis, Missouri, U.S.A.

Ager

Davis Standard Agar, Davis galatine Ltd., Warwick, England
Suspended and washed ten times by decantation with distilled H₂O.

Alcohol dehydrogenase

Crystalline suspension of the yeast enzyme in ammonium sulphate
C.F. Boehringer & Sons, Mannheim, Germany.

Ammonia

Metal free ammonia was prepared by isoplastic distillation.
Distilled water is placed in a beaker over 0.330 ammonia in a
desiccator for 10 days during which time the system reaches equilibrium.
The concentration of the ammonia solution prepared in this manner is
approximately 3%.

Ammonium sulphate

Ammonium sulphate especially low in heavy metals was obtained from
British Drug Houses, Poole, England.

Apo enzyme

Apo enzyme was prepared by passage of the protein through a charcoal
column according to the procedure of Fox and Dandliker (1956). A
column of activated charcoal was prepared 3 cm x 0.8 cm. Protein
solution (5mls) containing 200-400 mg of protein was forced through
the column under a pressure of 1.5 Kg/cm² and the emerging colourless
solution was collected in a small graduated cylinder immersed in an
ice bath. The yield of apo enzyme was generally 30%.
Charcoal

Novite A charcoal was activated according to the procedure of Le Page and Mueller (1949). The charcoal (100 g) was suspended in 1L of 10% v/v HCl and boiled for 30 min. The suspension was filtered, washed and the 'fines' removed by several decantations. The charcoal was left in distilled water until used.

Chymotrypsin

Crystalline and salt free.
Armour Pharmaceutical Co. Ltd., Eastbourne.

Dichlorodimethylsilane (CH₃)₂SiCl₂
B.D.H. laboratory reagent.

M-2:4-Dinitroanilinomaleimide. M. 278.
Prepared by the method of Clark-Walker & Robinson (1961) and recrystallised twice from acetone-benzene.

Dowex Resin

Dowex 50 cation exchange resin was obtained from V. A. Howe Ltd. The resin was reconstituted by treatment with H/10 HCl and washed with water until free from acid.

Diethylaminoethyl Sephadex

A-50 anion exchange resin, medium particle size, containing 3.2 m equivalents of cationic groups/g dry weight.
Pharmacia, Uppsala, Sweden.
The resin was washed before use according to the procedure of Mandeles (1960).

Ethylene diamine tetra acetic acid

Disodium salt M.W. 372.
The B.D.H. laboratory reagent was recrystallised from water.

D-Glyceraldehyde-3-phosphate

The dimethylacetal cyclohexylamine salt was a gift from Dr. C. E. Ballou, the free D-3GP was prepared from the acetal according to the procedure of Ballou and Fischer (1955).

DL-Glyceraldehyde-3-phosphate

Obtained as the anhydrous barium salt of DL-glyceraldehyde-3-phosphate diethyacetal M.W. 380 from the Sigma Chemical Co., St. Louis, Missouri, U.S.A.
The free aldehyde was prepared according to the method of Harker et al. (1959). The diethylacetal (80 mg) was suspended in 3 ml. H₂O and mixed thoroughly with 1g of Dowex 50 H⁺. The tube was then placed in a boiling water bath for 3 minutes and agitated continuously by shaking, cooled rapidly in ice and the resin removed by filtration with a sintered glass filter. The filtrate was made to a final volume of 10 ml. and the concentration of the resulting solution (approximately 6-7 mM in DO₃F) was determined enzymatically.
Glyceraldehyde-3-phosphate dehydrogenase

The rabbit muscle enzyme was purchased from Boehringer & Sons, Mannheim, Germany in 1g amounts as crystalline suspensions in ammonium sulphate.

α-Glycerolphosphate dehydrogenase

Crystalline suspension of the rabbit muscle enzyme in ammonium sulphate. Boehringer & Sons, Mannheim, Germany.

Hydrochloric acid

Metal free hydrochloric acid was prepared by isopiestic distillation as described for ammonia.

The HCl so obtained from conc. HCl was approximately 9M.

Lactic dehydrogenase

Crystalline suspension of the rabbit muscle enzyme in ammonium sulphate.

Boehringer & Sons, Mannheim, Germany.

'Nagase'

Crystalline proteinase enzyme from B. subtilis (Matsubara et al. 1956) obtained from the Teikoku Chemical Industry Co. Ltd., Osaka, Japan.

Nicotinamide-adenine dinucleotide NAD⁺

Obtained from the Sigma Chemical Co., St. Louis, Missouri, U.S.A.

M.W. 735.4 corrected for 4 H₂O.

Nicotinamide-adenine dinucleotide (reduced) NADH

Disodium salt, M.W. 763 corrected for 3 H₂O.

Sigma Chemical Co., St. Louis, Missouri, U.S.A.

Nicotinamide mononucleotide

From the Pabst Laboratories, Milwaukee, Wisconsin, U.S.A.

M.W. 335.

Orcinol

Was B.D.H. laboratory reagent and was recrystallised from benzene.

Papain

Twice crystallized suspension in 0.05M sodium acetate.

British Drug Houses, Poole, England.

Pepsin

Porcine mucosa crystalline enzyme.

Phenyl mercuric hydroxide

Prepared from B.D.H. micro analytical phenyl mercuric acetate. M.W. 336.75 by addition of NaOH and gentle heating. The resulting solution was acidified to pH 9.5 with HNO₃ and could be stored without decomposition for 6 months. The concentration was estimated by potentiometric titration with KBr according to the procedure of Allison and Cecil (1958).

Phosphoenol pyruvate

Cyclohexylamine salt.
C. F. Boehringer & Sons, Mannheim, Germany.

Pyruvate kinase

Crystalline suspension of the rabbit muscle enzyme in ammonium sulphate.
C. F. Boehringer & Sons, Mannheim, Germany.

Silicone A: Antifoam A.

A silicone grease to prevent surface denaturation of proteins in the polarographic cell.
Hopkin and Williams Ltd., Chadwell Heath, Essex.

Triethanolamine

B.D.H. Laboratory Reagent was distilled under a pressure of 0.02 mm Hg, the fraction distilling at 124-126°C being collected. The oily liquid was stored at -18°C.

Triethylamine bicarbonate

This buffer was prepared by the procedure of Smith et al. (1962). Redistilled triethylamine (140 ml.) was added dropwise to 500 ml. H₂O cooled in an ice bath. Carbon dioxide was bubbled through the solution until the pH dropped to 7.5 (6-10 hrs), the solution was then made to 1M in triethylamine bicarbonate by dilution to 1L.

Triosephosphate isomerase

Crystalline suspension of the rabbit muscle enzyme in ammonium sulphate.
C. F. Boehringer & Sons, Mannheim, Germany.

Trypsin

Crystallized, salt free.

All other reagents were AR grade.
2 METHODS OF ESTIMATION

1. Acetyl phosphate.

The procedure outlined below for the estimation of acetyl phosphate has been adapted from the original method of Lipmann & Tuttle (1945) by Stadtman (1957). A 1.0 ml. solution of acetyl phosphate containing 0.5 - 3.0 μ moles is treated with an equal volume of hydroxylamine reagent. The hydroxylamine reagent is made from equal volumes of 4M NH₂OH.HCl and 3.5M NaOH, the final pH is 7.0. After allowing the acetyl-phosphate solution to react for 10 minutes 2 ml. of 0.1M FeCl₃ in standardized 1 N HCl is added and the absorption read at 500 μ within 10 min. against a control prepared similarly.

Under the conditions described above, which are slightly different from those of Stadtman, fresh acetyl phosphate gave an absorptivity of 0.190 for 1 ml. Butylthiolacetate under the same conditions gave an absorptivity of 0.195 for 1 ml. The butylthiolacetate was prepared by the method of Wessell and Reid (1937) and was standardized at 231 μ from the molar absorptivity of 4.3 × 10⁴ given by Noda et al. (1953) for the absorption of the compound in ethanol.

2. Adenine.

1. Silver precipitation of adenine.

This procedure for adenine analysis in nucleotides is adapted from Schmidt (1955). The sample containing 0.5 - 2.0 μ moles of adenine was heated in a boiling water bath with 2.5 ml. 0.5 N H₂SO₄ for 1 hr, cooled and 0.5 ml. 1 N Ag NO₃ added. The precipitation of the silver adenine complex was allowed to continue for 2 hrs then centrifuged and washed three times with H₂O. The adenine was redissolved by heating in a boiling water bath with 2.5 ml. of N HCl for 10 min and the precipitate of AgCl was removed by centrifugation. The supernatant and one washing of the AgCl precipitate were made to 50 ml. with H₂O and the absorption measured at 262.5 μ. The concentration of adenine was calculated from the molar absorptivity of 13.15 × 10⁴ (Burton 1959). Recovery of adenine from NADH by this method was 90% based on the 340 μ absorption of NADH.
ii. Bromine adduct of adenine.

The procedure outlined below is taken from Davis and Morris (1963). A 1.0 ml. sample containing 0.2 - 1.0 μ moles of adenine was treated with 0.5 ml. 0.04M KBr, 0.2 ml. 18 N H₂SO₄ and 0.3 ml. 1 N KMnO₄, left for 5 min. at room temperature and decolourised with 1-2 drops of 10% H₂O₂. The solution was made to a final volume of 3 ml. and the absorptivity measured at 330 mμ within 30 min. A standard curve was drawn with NAD⁺ as the authors state that nucleotides give only 90% of the absorption of free adenine. The amounts of 0.1325, 0.265, 0.53 and 1.06 μ moles of NAD⁺ were found to give absorptivities of 0.081, 0.205, 0.460 and 1.045 respectively.

3. D-Glyceraldehyde-3-phosphate.

G3PD was used to estimate the amount of D-G3P in the presence of arsenate as the reaction becomes irreversible under these conditions (Warburg and Christian 1939). A 0.5 ml. sample of G3P containing 0.2 - 0.6 μ moles was added to 0.2 ml. of 10 mM NAD⁺ and 2.0 ml. of a buffer solution comprised of 0.2M triethanolamine, 12 μM Na₂ HAsO₄ and 0.2 mM EDTA adjusted to pH 9.0 with isoplastic HCl. The absorptivity increase at 340 mμ was measured after the addition of 0.1 ml of G3PD (0.5 g/L). The molar absorptivity of 6.22 x 10³ for NADH was used to calculate the concentration of the D-G3P.

4. Nicotinamide adenine dinucleotide NAD⁺.

i. With G3PD.

The conditions for estimating NAD⁺ with G3PD were the same as for G3P except that 0.2 ml. of 7 mM G3P replaced the NAD⁺ solution.

ii. With yeast ADH.

A sample of NAD⁺ (0.4 ml.) containing 0.2 - 0.6 μ moles of NAD⁺ was added to a cuvette containing 2.0 ml. of 50 mM sodium phosphate buffer at pH 9.5 and 0.3 ml. 5M ethanol. The absorptivity increase at 340 mμ was
measured after the addition of 0.1 ml. of ADH prepared by diluting the ammonium sulphate suspension of the commercial crystalline enzyme 1 to 40 in 0.1M sodium phosphate pH 7.5.

5. Nicotinamide adenine dinucleotide (reduced) NADH.

The procedure for estimating NADH has been adapted from Ciotti and Kaplan (1957).

A solution of NADH (0.3 ml) containing 0.2 - 0.6 μ moles was added to 2.5 ml. of 0.1M sodium phosphate buffer pH 7.5 containing 5 mM acetaldehyde. The absorptivity decrease at 340 μm was measured after the addition of 0.1 ml of yeast ADH diluted as for the NAD⁺ assay.

6. Phosphate.

The procedure for estimating phosphate as inorganic phosphate in the presence of a large amount of organic matter is based on the procedure of Berenblum and Chain (1938). A sample of protein (0.1 - 0.25 ml) containing 2 - 10 μg of phosphorus is heated on an ashing rack with 1 ml. of perchloric-sulphuric acid mixture prepared by mixing equal quantities of conc H₂SO₄ and 65% v/v HClO₄. After 1 hour the mixture is allowed to cool and 1 ml. of 5% w/v ammonium molybdate is added and the solution brought to the boil again for 5 minutes to decompose any pyrophosphate formed in the ashing procedure. After the solution has cooled to room temperature a further 4 ml. of ammonium molybdate is added and the solution transferred to a separating funnel with 4 x 5 ml. washings with H₂O. The molybdate complex of phosphate is extracted into 7 ml. of isobutanol by shaking for 15 seconds, the aqueous layer is then discarded and the organic layer washed with 10 ml. of 0.1N H₂SO₄. The organic layer is then transferred to a 10 ml. volumetric flask and made to volume with isobutanol after the addition of 3 drops of 40% w/v SnCl₂ in conc HCl. The blue colour which was found to be quite stable was measured at 735 μm against an isobutanol blank. A standard curve was prepared from KH₂PO₄, 10 μg of phosphorus was found to give an absorptivity of 0.525.
7. Protein.

The Biuret method for estimating protein was taken from the procedure outlined by Layne (1957).

Biuret Reagent: 1.5g of CuSO₄ + 6.0g sodium potassium tartrate · 4 H₂O dissolved in 500 ml. H₂O. To this was added 300 ml. of 10% w/v NaOH and the solution made to 1L with H₂O. The biuret reagent was stored in a plastic bottle at 2°C and renewed every 3 months. Estimation procedure. A 1.0 ml. solution containing 0.5 - 3.0 mg of protein was added to 4 ml. of biuret reagent and left at room temperature for 1 hour. The colour developed was measured at 550 μ against a biuret control. The procedure was standardized against dialysed dry weights of G3PD, as this protein was found to give 14% higher values than standardized dry weights of porcine insulin and bovine serum albumin. The absorptivity variation of 0.062 - 0.066 for 1 mg. of protein was found to be due to variations in the different batches of biuret reagent. It was therefore found necessary to standardize each fresh preparation of biuret reagent against dry weights of the protein. In addition the biuret procedure did not obey Beer's Law above absorptivities of 0.300 and hence protein solutions were appropriately diluted.

8. Ribose.

The orcinol method for the estimation of ribose was taken from the procedure outlined by Ashwell (1957). A 1.0 ml. solution containing 0.01 - 0.08 μ moles of ribose was added to 0.5 ml. of 7% w/v orcinol in 95% ethanol and 2.0 ml. of 0.05% w/v FeCl₃ in conc HCl. After heating in a boiling water bath for 30 minutes the stoppered tubes were cooled in ice and the colour intensity measured at 670 μ. A standard curve was prepared from ribose and each fresh batch of FeCl₃ reagent was restandardized. The variation between the standard curves obtained yielded absorptivity values between 0.780 and 0.800 for 0.1 μ mole of ribose.
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