STUDIES ON ACINETOBACTER α-OXOGLUTARATE DEHYDROGENASE

by

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GENERAL INTRODUCTION

The importance of α-oxoglutarate dehydrogenase as a key enzyme in intermediary metabolism

This thesis is primarily concerned with the enzyme α-oxoglutarate dehydrogenase (α-OGDH) from the bacterium Acinetobacter lwoffi. The reaction catalysed by this enzyme, namely:

α-oxoglutarate + NAD^+ + CoA ⟶ succinyl-CoA + CO_2 + NADH + H^+ . . . (1)

is one of the reactions involved in the tricarboxylic acid cycle (Fig. 1). This cycle, first postulated by Krebs & Johnson (1937), is fundamentally important in two ways. It provides a mechanism for breakdown of acetyl-CoA to CO_2 with the concomitant reduction of coenzymes (NAD^+, $NADP^+$ and FAD), whose re-oxidation yields most of the energy used by organisms growing in the presence of oxygen. Secondly, it provides intermediates which act as precursors for the synthesis of many compounds. α-Oxoglutarate is situated at a branch-point in this cycle, since it can be converted either by α-oxoglutarate dehydrogenase to succinyl-CoA (and thence to other tricarboxylic acid cycle intermediates) or by glutamate dehydrogenase to glutamate and thence to other amino acids (arginine, glutamine and proline). The regulation of α-OGDH must therefore play an important role in determining the metabolic fate of α-oxoglutarate.

Investigations carried out by Weitzman and his associates have revealed a novel form of control of the tricarboxylic acid cycle enzymes from A. lwoffi. In vitro studies suggest that several of these enzymes might be regulated by the adenylate system, i.e. by the levels, or relative levels, of AMP, ADP and ATP. This adenylate sensitivity has been demonstrated for pyruvate dehydrogenase (Jaskowska-Hodges, 1975; Jaskowska-Hodges & Weitzman, 1977), citrate synthase (Weitzman & Jones, 1968), isocitrate dehydrogenase (Parker & Weitzman, 1970; Self, Parker & Weitzman, 1973), α-oxoglutarate dehydrogenase

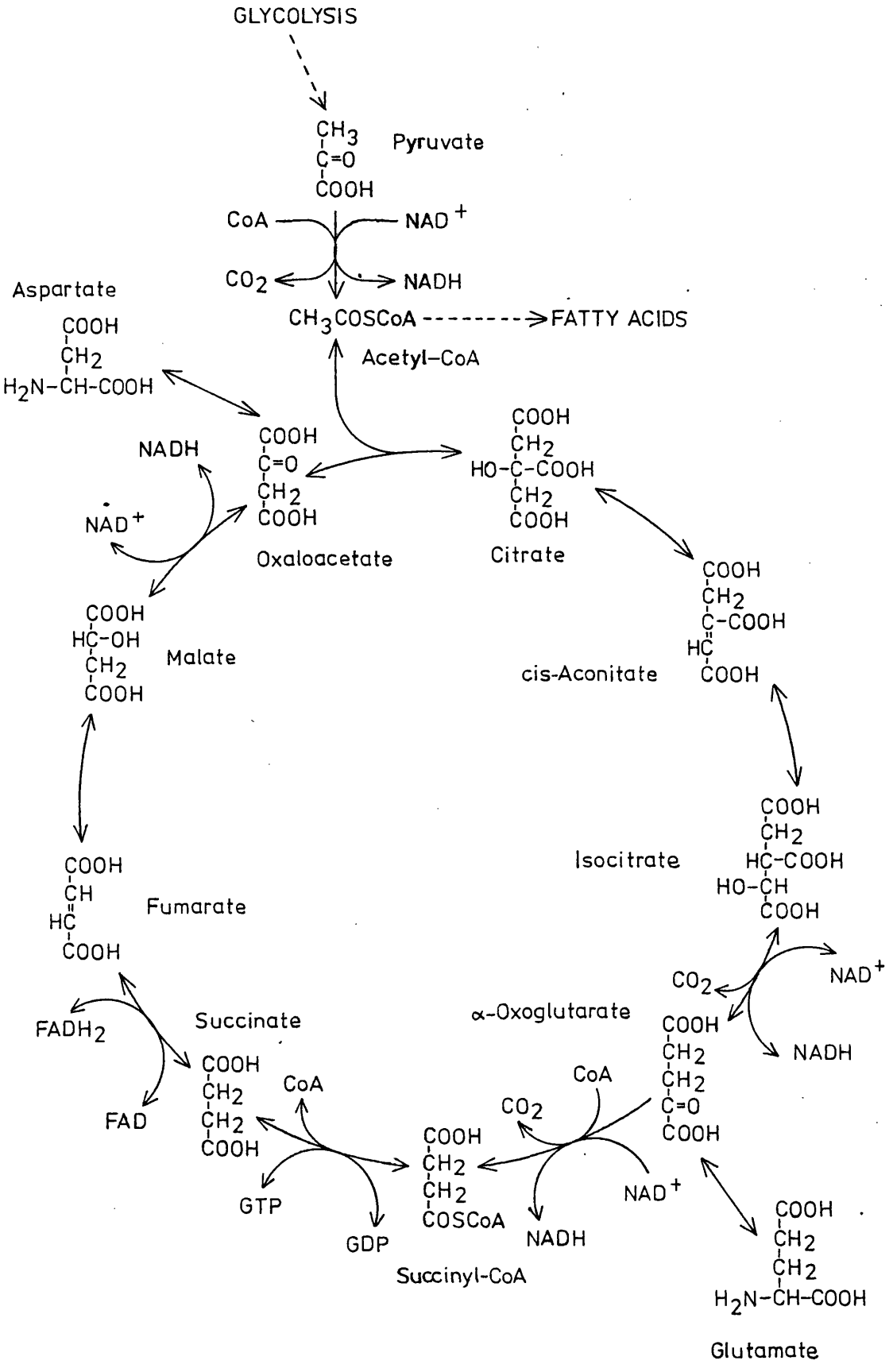


Fig. 1. TRICARBOXYLIC ACID CYCLE

(Weitzman, 1972; Parker & Weitzman, 1973) and succinate thiokinase (Weitzman & Jaskowska-Hodges, 1977). The regulation of α-OGDH in A. lwoffi thus constitutes one element of a multipoint control system and renders this particular α-OGDH complex worthy of further detailed study.

Previous work (Weitzman, 1972; Parker & Weitzman, 1972, 1973; Parker, 1973) has shown that the α-OGDH complex from A. lwoffi is stimulated by AMP (by a reduction in the K_m for α-oxoglutarate with little effect on V_{max}) and inhibited by NADH (both the K_m and V_{max} being affected). This latter control can be looked upon as being a type of feedback inhibition since NADH can be regarded as an end-product of the tricarboxylic acid cycle. On the other hand, high levels of AMP in the cell signify a low ATP level and hence under these conditions it would be advantageous to catalyse the oxidative processes of the tricarboxylic acid cycle at a higher rate.

The importance of α-OGDH as a multienzyme complex

Apart from its important regulatory properties, the α-OGDH complex of A. lwoffi is interesting for another reason - it is a multienzyme complex. The existence of such a complex presents a number of advantageous features:

(1) The enzyme components are concentrated rather than being dispersed throughout the cell.

(2) It is probable that an intermediate produced by one enzyme can meet the next enzyme more rapidly if the two enzymes are physically associated with each other than if they are structurally independent.

(3) An enzyme complex may be especially efficient if the metabolic intermediates are strongly bound to it and cannot readily escape before the series of reactions is completed.

Thus the short sequence of reactions catalysed by the α-OGDH complex would be expected to be more efficient than if three independent enzymes had been involved.

Reaction mechanism and structure of α-oxo-acid dehydrogenases

α-OGDH complexes have been successfully isolated and purified from a number of sources including E. coli (Koike et al., 1960), A. lwoffi (Parker & Weitzman, 1973), Saccharomyces cerevisiae (Hirabayashi & Harada, 1971), cauliflower (Poulsen & Wedding, 1970), pigeon breast muscle (Severin & Gomazkova, 1972), pig heart muscle (Hirashima et al., 1967) and bovine kidney (Ishikawa et al., 1966). In all cases the enzyme has been shown to consist of three types of enzyme subunits as follows:

E1 - α-oxoglutarate dehydrogenase (EC 1•2•4•2)

E2 - dihydrolipoamide transsuccinylase (EC 2•3•1•12)

E3 - dihydrolipoamide dehydrogenase (EC 1•6•4•3)

The overall reaction catalysed by α-OGDH (equation 1) is achieved by a co-ordinated sequence of steps as shown in Fig. 2. It can be seen that the E1 component catalyses two distinct reactions - oxidative decarboxylation of α-oxoglutarate and reductive transfer of the succinyl moiety from thiamine pyrophosphate (TPP) to lipoamide. E2 then catalyses the transfer of the acyl group to coenzyme A and finally the oxidation of the reduced lipoamide at the expense of NAD^+ is effected by the E3 subunit.

The multienzyme complex pyruvate dehydrogenase (PDH) resembles α-OGDH in many respects. Although it is not actually part of the tricarboxylic acid cycle, it catalyses the reaction by which acetyl-CoA is formed from pyruvate. Thus, being located at the major entry point to the cycle, it plays an important role in intermediary metabolism. Both the overall reaction and the individual steps catalysed by the component enzymes of the PDH complex are very similar to those catalysed by α-OGDH. The E3 components of the two complexes from E. coli have, by several criteria, been shown to be indistinguishable and are, in fact, functionally interchangeable (Pettit & Reed, 1967; Brown & Perham, 1972). This has been confirmed genetically by the demonstration that E. coli possesses a single dihydrolipoamide dehydrogenase gene (lpd) which specifies the E3 component of both α-OGDH and

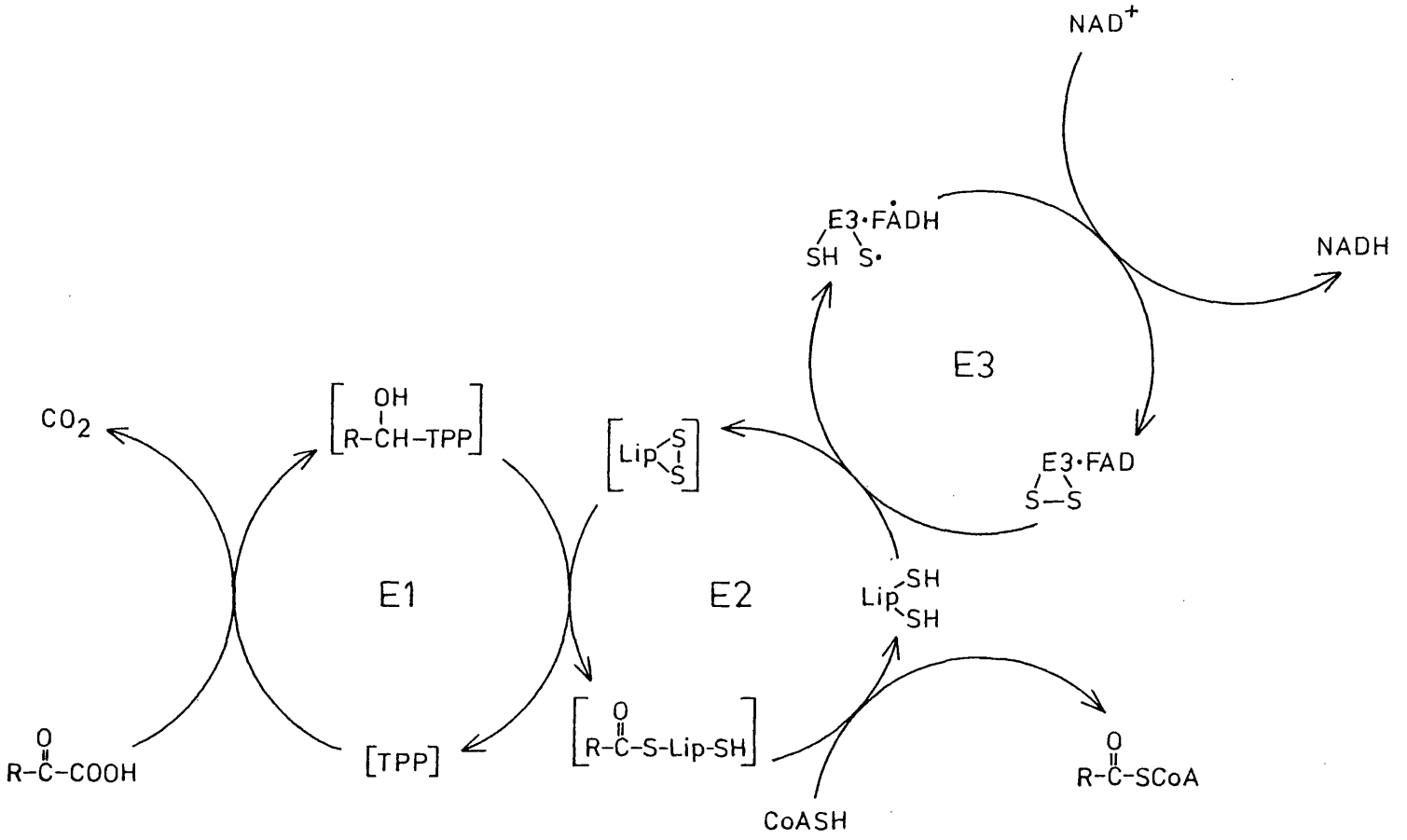


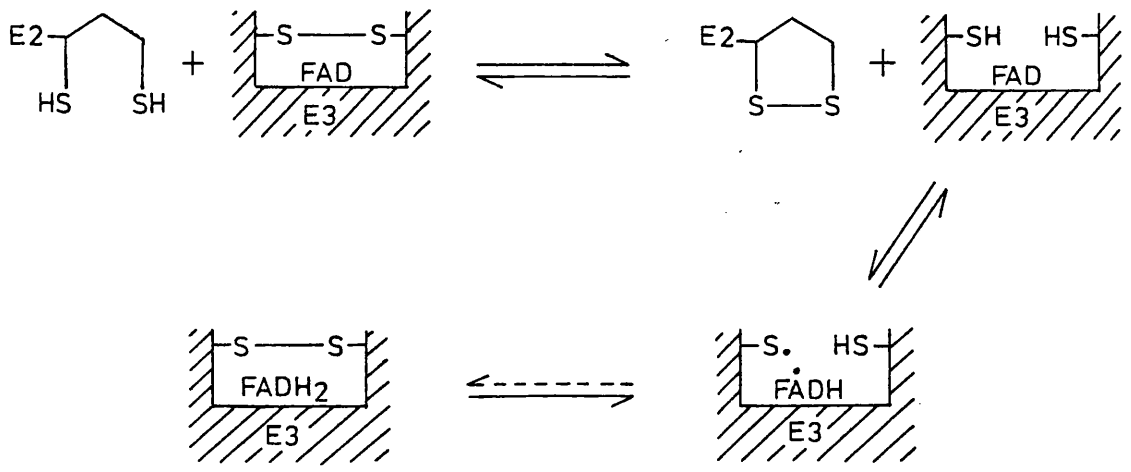
Fig. 2. Reaction sequence for α-OGDH and PDH

R equals $(CH_2)_2COOH$ for the α-OGDH complex and CH_3 for the PDH complex.

PDH complexes (Guest & Creaghan, 1972, 1973; Guest, 1974). Finally, it has been reported that the amino acid sequences of a peptide containing the disulphide bridge associated with the active site of the PDH dihydrolipoamide dehydrogenase component of both E. coli and pig heart exhibit a high degree of homology (Brown & Perham, 1974). It would therefore seem that the bacterial and mammalian dihydrolipoamide dehydrogenase components are derived by divergent evolution from a common ancestral protein.

Three cofactors are involved in the reaction mechanism of the α-OGDH complex, namely TPP, FAD and lipoic acid. TPP is known to be bound fairly strongly to the E1 component although some depletion can occur during enzyme purification (Bisswanger, 1974; Hayakawa et al., 1966; Moe & Hammes, 1974) and consequently TPP is generally added when assaying purified enzyme preparations for whole complex and E1 activities.

FAD, on the other hand, is attached to the E3 component, giving it its characteristic yellow colour. This cofactor plays an important role in the re-oxidation of enzyme-bound lipoamide during the catalytic sequence by alternate oxidation and reduction of a disulphide bridge at the E3 active site (Massey & Veegner, 1961; Massey et al., 1960; Brown & Perham, 1974) which is thought to proceed by a coupled di-radical (Massey, 1963; Searls et al., 1961; Mahler & Cordes, 1971):



Finally, lipoic acid is attached covalently to the E2 subunit which, in the case of E. coli PDH, involves a peptide bond between its carboxyl group and the ϵ-amino group of a lysine residue of the enzyme protein. Lipoic acid undergoes a cycle of transformations including reductive acylation, acyl transfer and finally electron transfer. Since lipoic acid must interact with acyl-TPP-E1, E2-CoA and E3-FAD, the spatial arrangement of the three enzyme components is very important. It has been suggested that the linkage of the lipoic acid to the ϵ-amino group of a lysine residue may provide a flexible arm of about 14Å in length enabling the lipoic acid to rotate between the catalytic sites of the three enzyme components (Nawa et al., 1960). The results of a recent kinetic analysis of the mammalian PDH complex are consistent with this proposed mechanism (Tsai et al., 1974). Such a mechanism would predict that the active sites of the three components must lie within a circle of about 28Å in diameter. However, it has been shown by a technique involving fluorescence energy transfer between the TPP and FAD binding sites that this distance is 45 ± 15Å, casting some doubt as to whether a 14Å flexible arm would be long enough (Moe et al., 1974). A possible explanation suggested by Koike et al. (1963) is that thiol-disulphide interchange and acyl transfer may take place between several lipo-lysyl moieties:

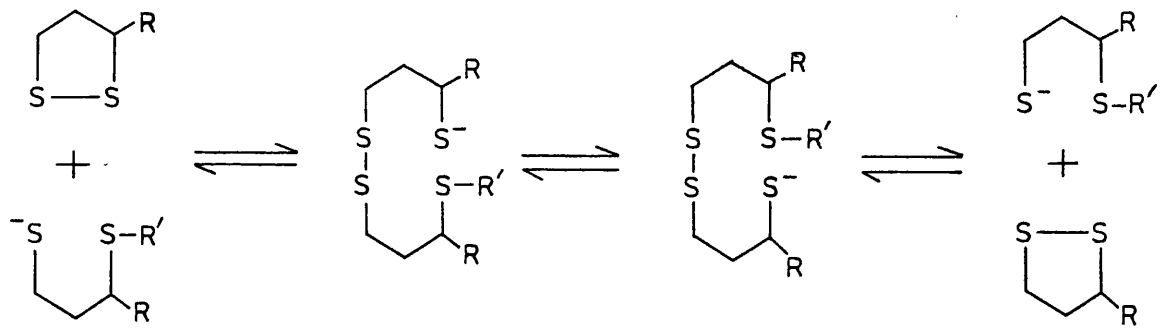


Table 1 summarizes the subunit stoichiometry of E. coli and mammalian α-OGDH and PDH complexes. In general the E2 components from E. coli form cubic cores (117-300Å in diameter) to which E1 and E3 dimers are non-covalently attached to the edges (two-fold positions) and faces (four-fold positions) respectively. Currently there exists some controversy as to the molecular weight and precise subunit stoichiometry of the E. coli PDH complex (Reed, 1974; Moe & Hammes, 1974; Speckhard & Frey, 1975; Vogel et al., 1972a,b; Perham & Danson, personal communication). This may possibly be due in part to the use of different E. coli strains (e.g. Crookes and K12).

It is evident from Table 1 that PDH complexes from mammalian sources (Hayakawa et al., 1969; Linn et al., 1972) are considerably more complex in structure than the E. coli enzyme. The mammalian complex is composed of a pentagonal dodecahedral based on icosahedral (5,3,2) symmetry with E3 dimers at the edges and E1 tetramers on the faces. The E1 polypeptide chains consist of two types (designated α and β) which form $\alpha_2\beta_2$ tetramers (Roche & Reed, 1972; Barrera et al., 1972). It seems likely that α subunits catalyse the decarboxylation of pyruvate to produce α-hydroxyethyl-TPP, whereas the β subunits catalyse the reductive acylation of the E2-bound lipoyl moieties by α-hydroxyethyl-TPP. In addition to the three main subunit types (E1, E2 and E3) mammalian PDH complexes also contain two regulatory enzymes, PDH kinase and PDH phosphatase (about five molecules of each), which are joined to the E2 core. PDH kinase phosphorylates the E1 α subunits thereby causing inhibition of whole complex activity (Barrera et al., 1972; see also Fig. 3). This is reversed by PDH phosphatase to regenerate active enzyme. It should be emphasised that this form of regulation is not found in either bacterial PDH complexes or in mammalian and bacterial α-OGDH complexes.

Genetic analysis of α-oxo-acid dehydrogenase loci

Table 1. Subunit stoichiometry of E. coli and mammalian α-OGDH and PDH complexes.

Complex	E1 subunits	E2 subunit	E3 subunits	References
α-OGDH (E. coli) MW: 2.4×10^6	MW: 190,000 6 dimers of identical chains (each 95,000)	MW: 1×10^6 24 identical chains (each 40,000). Half the chains contain bound lipoic acid.	MW: 112,000 6 dimers of identical chains (each 56,000)	Pettit et al. (1973)
PDH (E. coli) MW: 4.6×10^6	MW: 192,000 12 dimers of identical chains (each 96,000)	MW: 1.7×10^6 24 identical chains (each 65-70,000). All chains contain bound lipoic acid.	MW: 112,000 6 dimers of identical chains (each 56,000)	Reed (1969); Reed (1974) Koike et al. (1960) Eley et al. (1972) Reed et al. (1975)
α-OGDH (mammalian) MW: 2.7×10^6	MW: 216,000 6 dimers of identical chains (each 108,000)	MW: 1×10^6 24 identical chains (each 48,000). Only one third contain bound lipoic acid.	MW: 112,000 6 dimers of identical chains (each 56,000)	Hirashima et al. (1967) Tanaka et al. (1972) Ishikawa et al. (1966) Reed & Oliver (1968) Koike et al. (1974)
PDH* (bovine kidney) MW: 7×10^6	MW: 154,000 20 tetramers of non-identical subunits ($\alpha_2\beta_2$-MW: 41,000 & 36,000 respectively)	MW: 3.1×10^6 60 identical chains (each 52,000). All contain bound lipoic acid. Also binds two other subunits - PDH kinase & phosphatase (about 5 of each).	MW: 112,000 5 dimers of identical chains (each 56,000)	Hayakawa et al. (1969) Linn et al. (1972) Barrera et al. (1972) Reed (1974)

*Note: PDH from bovine heart has an E2 core which can bind 30 E1 tetramers, thus accounting for its somewhat higher molecular weight of 9×10^6.

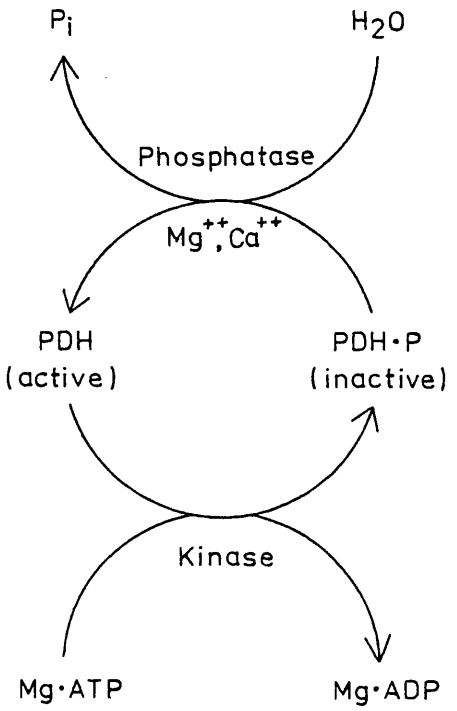


Fig. 3. The interconversion of active (unphosphorylated) and inactive (phosphorylated) forms of mammalian PDH complex.

Some preliminary results indicating how the synthesis of the individual components may be controlled has been obtained by genetic analysis. The structural genes for the α-OGDH and PDH complexes of E. coli K12 have been successfully mapped. It has been shown that there are two pairs of closely linked genes, ace E and ace F (coding for the E1 and E2 components of PDH) and suc A and suc B (coding for the E1 and E2 components of α-OGDH) (Henning & Hertz, 1964; Herbert & Guest, 1968). In both cases the E1 gene is transcribed before the E2 gene (Henning et al., 1966; Creaghan & Guest, 1972). As mentioned earlier the E3 components of both complexes are specified by a single gene, lpd, which is located adjacent to the distal gene (ace F) of the ace region. Thus the expression of the lpd gene may be governed by a secondary transcription promoter in the ace F - lpd region in order to produce E3 components for the α-OGDH complex (Guest, 1974).

Regulation of α-oxo-acid dehydrogenase complexes

As mentioned earlier the central positions of α-OGDH and PDH in metabolism make them important regulatory enzymes. In some systems these multienzyme complexes have been shown to be subject to product inhibition by succinyl-CoA and NADH in the case of α-OGDH (Garland, 1964; Erfle & Sauer, 1969) and by acetyl-CoA and NADH for PDH (Reed, 1969; Tsai et al., 1974). In both cases these inhibitions are competitive with respect to CoA and NAD^+. From these observations it would seem that the sites of action of acyl-CoA and NADH are the E2 and E3 subunits respectively. In contrast, however, succinyl-CoA inhibition of the α-OGDH complex of A. lwoffi is only observed at very high concentrations and it is therefore doubtful whether this effect is of physiological significance (Parker, 1973). On the other hand, this complex exhibits both NADH inhibition and AMP stimulation. It has been shown directly that the regulatory effects of AMP and NADH are exerted on the E1 and E3 subunits respectively. However, for technical reasons it was not possible to demonstrate directly whether NADH also

affects E1 activity although indirect kinetic evidence suggested that this is the case (Parker & Weitzman, 1973).

Aims of present work

When the present work was begun, a preliminary characterization of the α-OGDH complex of A. lwoffi had already been carried out. The present study was undertaken to obtain a more detailed understanding of the enzyme's structural, catalytic and regulatory properties with particular emphasis on the latter. Attempts have been made to establish more conclusively which subunits are involved in adenylate control and whether this regulation is allosteric in nature. As an alternative approach, dissociation of the complex into its component subunits has been investigated, in an attempt to further clarify the sites of regulation. Finally, the molecular weights of the whole complex and the individual subunits of the α-OGDH of A. lwoffi have been determined and compared with those from other systems to ascertain whether the additional regulatory properties of the A. lwoffi enzyme are associated with a more intricate enzyme structure.

CHAPTER I

ENZYME ASSAYS

I.1 INTRODUCTION

In order to carry out a detailed study of the catalytic, regulatory and chemical nature of the α-OGDH multienzyme complex from Acinetobacter lwoffi it is essential to have suitable assay procedures for the individual subunit activities as well as for the whole complex.

I.1·1 α-OGDH complex activity

At the onset of this work a suitable, sensitive assay was already available for the whole complex activity (Parker & Weitzman, 1973). All necessary substrates (α-oxoglutarate, thiamine pyrophosphate, CoA and NAD^+) were provided and the production of NADH was followed by monitoring the increase in absorbance at 340nm.

I.1·2 Dihydrolipoamide dehydrogenase (E3) activity

Two assay methods had already been described for dihydrolipoamide dehydrogenase (Parker & Weitzman, 1973). The first involves measuring the reaction in the backward direction, by following the decrease in absorbance at 340nm as oxidized lipoamide is reduced at the expense of NADH. In the second method the reaction is measured in the forward direction by following the formation of oxidized lipoamide polarographically. The former assay was routinely used in this work; it was chosen for its simplicity and the commercial availability of substrates.

I.1·3 α-Oxoglutarate dehydrogenase (E1) activity

The oxidative decarboxylation reaction catalysed by the E1 components of both PDH and α-OGDH may be used to reduce an artificial electron acceptor resulting in a spectral change. Both dichlorophenol-indophenol (DCPIP) (Dietrich & Henning, 1970; Parker & Weitzman, 1973) and ferricyanide (Massey,

1960; Das et al, 1961; Reed & Mukherjee, 1969) have been used in this way, the stoichiometry of the reactions being:

$$R\cdot CO\cdot COOH + DCPIP + H_2O \xrightarrow[TPP]{E1} R\cdot COOH + CO_2 + DCPIP\cdot H_2$$

$$R\cdot CO\cdot COOH + 2Fe(CN)_6^{3-} + H_2O \xrightarrow[TPP]{E1} R\cdot COOH + CO_2 + 2Fe(CN)_6^{4-} + 2H^+ .$$

Das et al. (1961) postulated a mechanism for the latter reaction (Fig. 4). The sensitivities of the ferricyanide and DCPIP assays vary according to the source of the α-OGDH. Thus, for example, the complex from A. lwoffi gives a relatively fast rate with DCPIP, but a slow rate with ferricyanide; however the latter is a good electron acceptor for the E. coli enzyme.

Although the DCPIP assay has been routinely employed throughout this work there are instances when this method is unsuitable, for the following reasons:

(1) Since DCPIP is non-enzymically reduced by a number of compounds, such as NADH, it is obviously impossible to study directly any effect of these substances on the E1 subunit. In particular it would be advantageous to have an alternative assay that could yield direct evidence as to whether this component is a site of NADH inhibition.

(2) An apparent rate is obtained with the DCPIP assay in the absence of E1, but in the presence of large amounts of protein, e.g. bovine serum albumin, casein or insulin. This effect is discussed more fully later (section I.3·2). However it should be emphasized that the interference is negligible at the protein concentrations normally used to determine E1 activity.

(3) The DCPIP E1 assay is between 50 and 100 times less sensitive than that of either the whole complex or the E3 component. Obviously this can be a disadvantage when studying dissociation and chemical modification of the complex on a small scale.

In view of the above disadvantages several possible alternative assays for the E1 component were investigated.

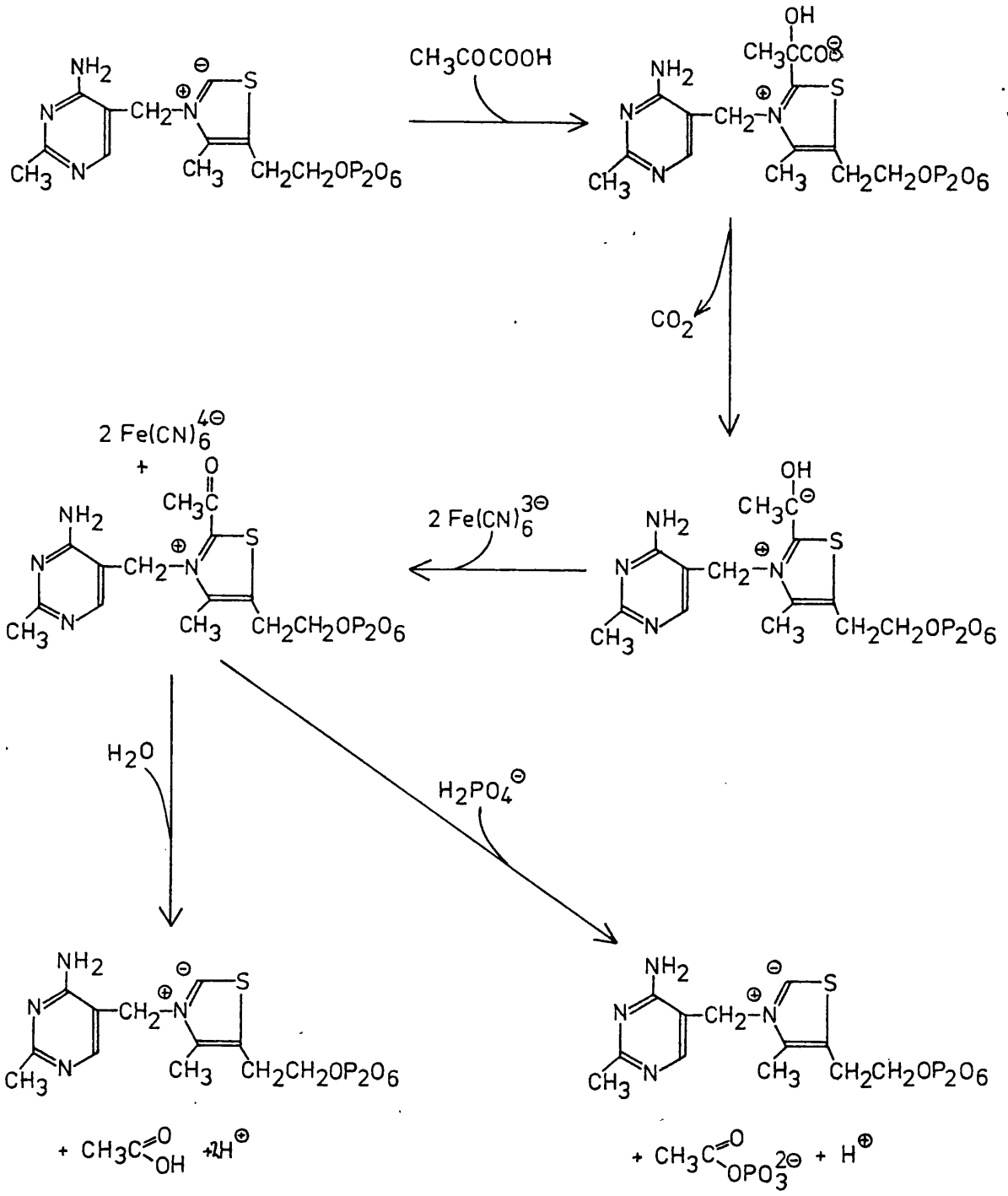
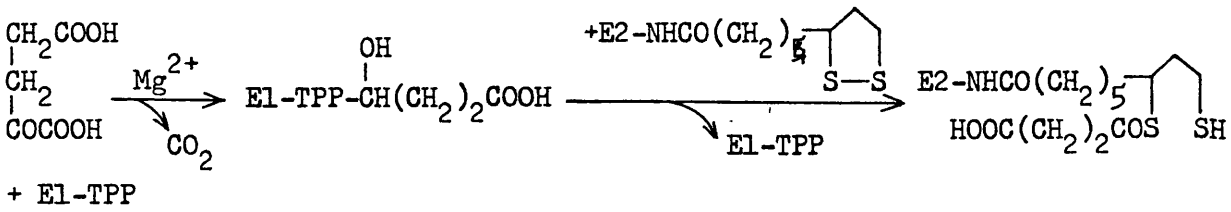


Fig. 4. The mechanism for the reduction of ferricyanide during the pyruvate dehydrogenase component (E1) assay; as proposed by Das et al. (1961).

(i) DTNB (5,5'-dithiobis[2-nitrobenzoic acid]) assay

Incubation of the α-OGDH complex with α-oxoglutarate, thiamine pyrophosphate and Mg^{2+} should result in decarboxylation of α-oxoglutarate followed by reductive acylation of the E2 bound lipoic acid moiety.



Assuming that exogenously added lipoamide can be acylated in a similar manner to enzyme bound lipoamide (see section I.3·2), it should be possible to monitor the extent of the E1 reaction, represented by the amount of acylated lipoamide formed, using DTNB. This reagent should react with the free sulphydryl group formed by acylation of lipoamide and result in concomitant release of the strongly absorbing 5-thio,2-nitrobenzoate ion (TNB^-) which can be measured at 412nm. Since DTNB inactivates the E1 component (section IV.3·1) these assays must be done in a discontinuous manner.

(ii) Hydroxylamine assay

In principle this is very similar to the DTNB assay described above. Acylated lipoamide produced by the E1 reaction (in the presence of α-oxoglutarate, TPP, lipoamide and Mg^{2+}) was determined as stable thioester by the hydroxylamine assay of Lipmann & Tuttle (1945).

(iii) Radiochemical assay

Both the E1 component and the whole complex activities of A. lwoffi have been successfully measured using a modification of the radiochemical assay previously described for the PDH complexes of E. coli (Schwartz & Reed, 1970) and bovine kidney (Roche & Reed, 1972).

This assay is based on the measurement of $[^{14}C]CO_2$ production from [1-^{14}C]α-oxoglutarate, CoA and TPP as shown by reactions (a-d) in Fig. 5.

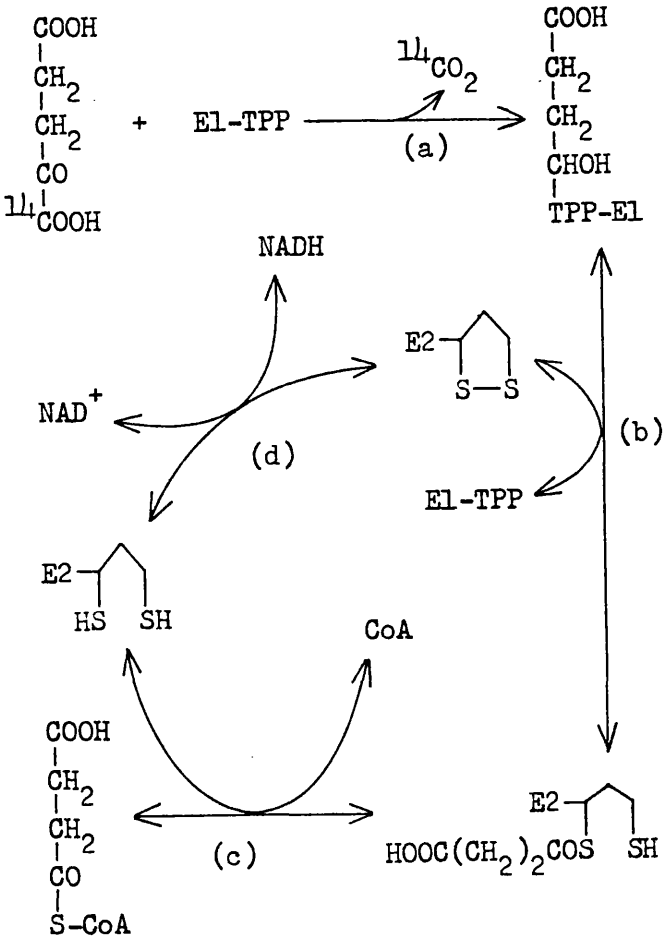


Fig. 5. The reaction sequence of the α-OGDH complex showing the release of $[^{14}C]CO_2$ from [1-^{14}C]α-oxoglutarate.

To assay the E1 component, however, CoA is omitted and consequently only reaction (a) in Fig. 5 is measured. In both cases the reaction is stopped at suitable time intervals by the addition of sulphuric acid and the released $[^{14}C]CO_2$ absorbed by potassium hydroxide. Aliquots of the alkali are then assayed for radioactivity.

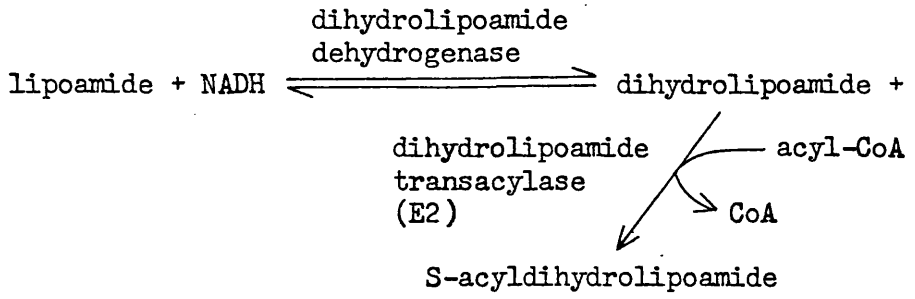
I.1.4 Dihydrolipoamide transsuccinylase (E2) activity

At the onset of this work the need for a new transsuccinylase assay was apparent since the assay used by other workers has many disadvantages. The basis of this method, which follows the back reaction, is the transsuccinylation of succinyl-CoA (produced by the action of succinic thiokinase on succinate, ATP and CoA) to dihydrolipoamide (Knight & Gunsalus, 1962). Succinyl dihydrolipoamide is then cleaved by hydroxylamine and reacted with ferric chloride to produce a strongly absorbing iron complex which may be measured spectrophotometrically at 500nm (Lipmann & Tuttle, 1945). An analogous procedure may be employed for the dihydrolipoamide transacetylase component of the PDH complex. In this case, however, acetyl-CoA is generated from acetyl phosphate, CoA and phosphotransacetylase. Before estimation of acetyl dihydrolipoamide, residual acetyl phosphate is destroyed by heating at low pH. Alternatively, the formation of acetyl dihydrolipoamide from acetyl phosphate may be followed continuously at 240nm (Knight & Gunsalus, 1962), but unfortunately this method is not suitable for the transsuccinylase component. Consequently only a discontinuous assay was available for the latter enzyme, requiring the preparation of both dihydrolipoamide and succinic thiokinase. A continuous assay for dihydrolipoamide transsuccinylase which does not require the preparation of these two components has been successfully developed. In addition, this method also enables the activity of dihydrolipoamide transacetylase to be assayed.

Principle of the dihydrolipoamide transacylase assay

This method is based on following the transfer of acyl groups from

CoA to dihydrolioamide by coupling to an equilibrium system of the dihydrolipoamide dehydrogenase reaction, and may be represented as follows:



An excess of exogenous dihydrolipoamide dehydrogenase is allowed to act on oxidized lipoamide and NADH and thereby produce an equilibrium mixture of lipoamide, dihydrolipoamide, NADH and NAD^+. After introducing E2 enzyme and the appropriate acyl-CoA, dihydrolipoamide is removed by acylation. The rapid establishment of an equilibrium by the dihydrolipoamide dehydrogenase allows this removal to be monitored continuously by following the oxidation of NADH at 340nm.

I.1.5 E2-E3 interaction test and lipoic acid test

An alternative way of detecting the presence of E2 component is by means of its lipoic acid content, rather than its enzymatic activity. A suitable method has been devised based on that of Erfle & Sauer (1968) and Perham (personal communication). The assay utilizes the back reaction of dihydrolipoamide dehydrogenase to reduce the oxidized lipoic acid moiety of the E2 component at the expense of NADH. One of the resulting sulphydryl groups may now react with DTNB with the concomitant release of the yellow 5-thio,2-nitrobenzoate ion (TNB^-). Oxidized lipoic acid is subsequently regenerated by intramolecular attack and loss of the TNB^- (Fig. 6). A cyclic series of reactions is thereby established which may be monitored by following the production of TNB^- at 412nm.

If the E3 component of the α-OGDH complex is utilized to reduce the E2-bound lipoic acid, then one might reasonably expect that a maximal rate of lipoic acid reduction will only occur if there is good spatial inter-

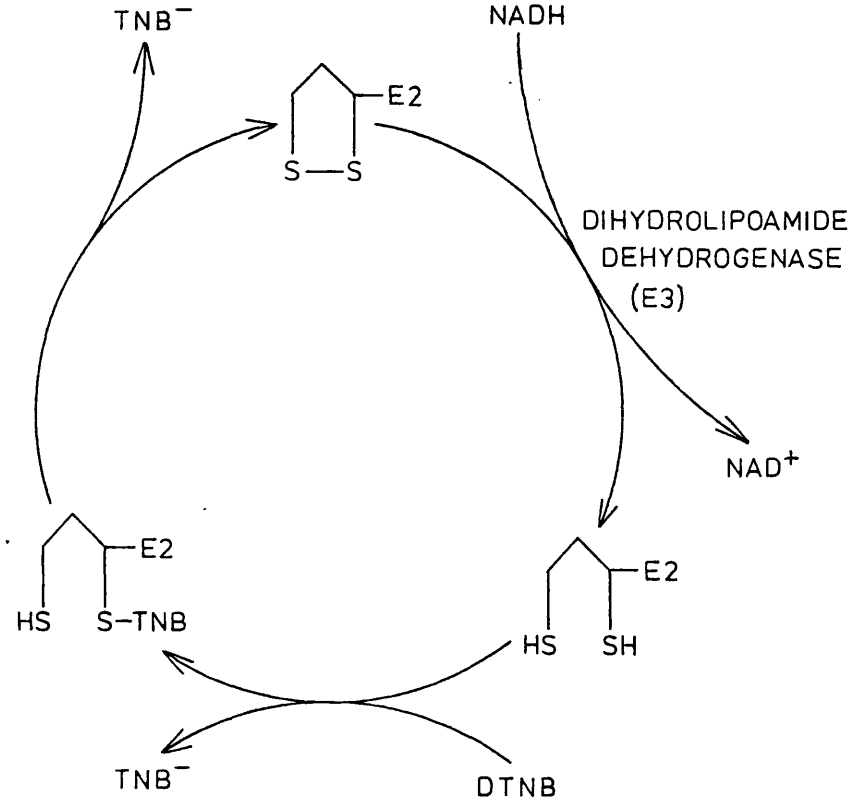


Fig. 6. Mechanism of the E2-E3 interaction test.

action between these two enzyme components. Consequently conditions which interfere with this spatial interaction (e.g. dissociation and separation of E3 from the whole complex) may be expected to reduce the reaction rate. Upon addition of a large excess of dihydrolipoamide dehydrogenase to such an E3-depleted assay a marked increase in rate is observed, presumably because the reduction of E2-bound lipoic acid by the E3 component is no longer rate limiting. Consequently the system may now be used as a means of detecting lipoic acid and hence the E2 component.

I.2 MATERIALS AND METHODS

I.2·1 Materials

[1-^{14}C]α-oxoglutarate (sodium salt) was purchased from New England Nuclear, Mass., USA (0·28mg ml^{-1}; 51·43mCi mmol^{-1}). Lipoamide (Sigma Chemical Co. Ltd., Surrey, UK) was prepared as a 10mM solution in absolute ethanol, acetyl-CoA and succinyl-CoA were prepared by acylation of CoA (Boehringer Mannheim) (with acetic and succinic anhydride respectively) (Simon & Shemin, 1953; Stadtman, 1957). All other chemicals were of analytical grade or the finest grade available and were obtained from Sigma Chemical Co. Ltd., Surrey, UK or BDH Chemicals Ltd, Poole, Dorset, UK.

Methods

All spectrophotometric assays were preformed in a Unicam SP 1800 spectrophotometer fitted with a thermostatically controlled cuvette holder, maintained at 25°C and linked to a Unicam AR25 external chart recorder.

I.2·2 Measurement of α-OGDH complex activity

Assay mixtures containing 0·1M Tris-HCl, pH 8·0, 10mM $MgCl_2$, 1mM EDTA, 5mM α-oxoglutarate, 0·5mM NAD^+, 0·1mM TPP, 0·1mM CoA and 2·5mM cysteine hydrochloride were preincubated for 10 min at 25°C in the spectrophotometer. The reaction was started by the addition of enzyme solution to give a total volume of 1·0ml and followed by measuring the formation of NADH at 340nm. Enzyme activities are expressed as units of μmoles of NADH formed min^{-1} ml^{-1}.

I.2·3 Measurement of PDH activity

PDH complex activity was measured in an identical manner to α-OGDH described above, except that 5mM pyruvate was included in the assay in the place of the α-oxoglutarate.

I.2·4 Measurement of E3 activity

This component was assayed in the backward direction by measuring the decrease in absorbance at 340nm as oxidized lipoamide is reduced at the

expense of NADH. Assay mixtures contained 0·5mM oxidized lipoamide, 0.2mM NADH, 10mM $MgCl_2$, 1mM EDTA, 0·1M Tris-HCl, pH 8·0 and the reaction was started by the addition of enzyme solution to give a total volume of 1·0ml. Enzyme activities are expressed as units of μmoles of NADH used min^{-1} ml^{-1}.

I.2·5 Measurement of E1 activity

(i) DCPIP assay

The absorbances of reaction mixtures containing 5mM α-oxoglutarate, 0·1mM TPP, 0·5mg phenazine methosulphate (stored in the dark), 0·05mM DCPIP, 0·5mM $MgCl_2$ in 0·1M phosphate buffer, pH 8·0, were monitored at 578nm to obtain the small endogenous rate present in the absence of enzyme. The reaction was then started by the addition of a small volume of enzyme solution to give a total volume of 1·0ml and the reduction of DCPIP was followed at 578nm. The rate due to the enzyme-catalyzed reaction was obtained by subtraction of the endogenous rate. Enzyme activities are expressed as the change in absorbance units min^{-1}.

(ii) DTNB assay

A solution of 0·2mM AMP, 1mM lipoamide, 5mM α-oxoglutarate, 0·1mM TPP, 10mM $MgCl_2$, 1mM EDTA, 0·1M Tris-HCl, pH 8·0, was preincubated at 37°C for 10min. The reaction was then started by the addition of 0·5mg of α-OGDH complex from A. lwoffi. Aliquots (0·1ml) were removed at intervals for up to 30min and transferred to cuvettes containing 0·9ml of 0·22mM DTNB, 10mM $MgCl_2$, 1mM EDTA, 0·1M Tris-HCl, pH 8·0, and the absorbance at 412nm was determined. Enzyme activities are expressed as change in absorbance units min^{-1}

(iii) Hydroxylamine assay

Solution A: 10mM α-oxoglutarate, 5mM lipoamide, 10mM TPP, 0·1M phosphate buffer, pH 7·5. Solution B: 2M hydroxylamine solution (prepared according to the method of Lipmann & Tuttle, 1945). Solution C: 1·67% (w/v) ferric chloride, 4% (w/v) trichloroacetic acid in 1M HCl.

To 4·0ml of solution A (preincubated at 37°C for 10min), 0·1mg of

α-OGDH complex from A. lwoffi was added and incubation at 37°C continued. Aliquots (1·0ml) were removed after 30sec, 10min, 20min and 30min, added to 1ml of solution B, mixed well and left at room temperature for 10min. 2·4ml of solution C were then added and the absorbance at 500nm determined after a 10min incubation at 25°C in the spectrophotometer. Control samples were processed as above but contained no enzyme. Enzyme activities are expressed as change in absorbance units . min^{-1}

(iv) Radiochemical assay

The reaction vessel consisted of a small glass bottle (approximately 25mm in diameter and 30mm tall) containing a small glass tube attached to one side (Fig. 7). The neck of the main vessel was sealed by a tightly fitting Suba-seal through which additions could be made by means of a long needled syringe.

El component assay

The reaction mixture contained 1mM [1-^{14}C]α-oxoglutarate (2 x 10^5counts min^{-1} μmol^{-1}), 0·2mM TPP, 10mM $MgCl_2$, 1mM EDTA, 50mM Tris-HCl, pH 8·0, in a final volume (after addition of 0·3ml of enzyme) of 1·0ml.

α-OGDH complex assay

In this case the reaction mixture contained 1mM [1-^{14}C]α-oxoglutarate (2 x 10^5counts min^{-1} μmol^{-1}), 0·2mM TPP, 0·5mM NAD^+, 0·1mM CoA, 10mM $MgCl_2$, 1mM EDTA, 50mM Tris-HCl, pH 8·0, in a final volume (after enzyme addition) of 1·0ml.

Assay procedure

All assays were performed in a 30°C constant temperature room. Buffers and other stable solutions were pre-equilibrated at this temperature for 3-4h. The reaction vessel, containing one of the above reaction mixtures (without enzyme) in its main chamber and 0·5ml of 10% (w/w) aqueous KOH in the side-tube, was closed with a Suba-seal and gently stirred with a bar magnet for 5min to ensure complete equilibration at the ambient temperature (30°C). The reaction was started by addition of 0·3ml of enzyme solution

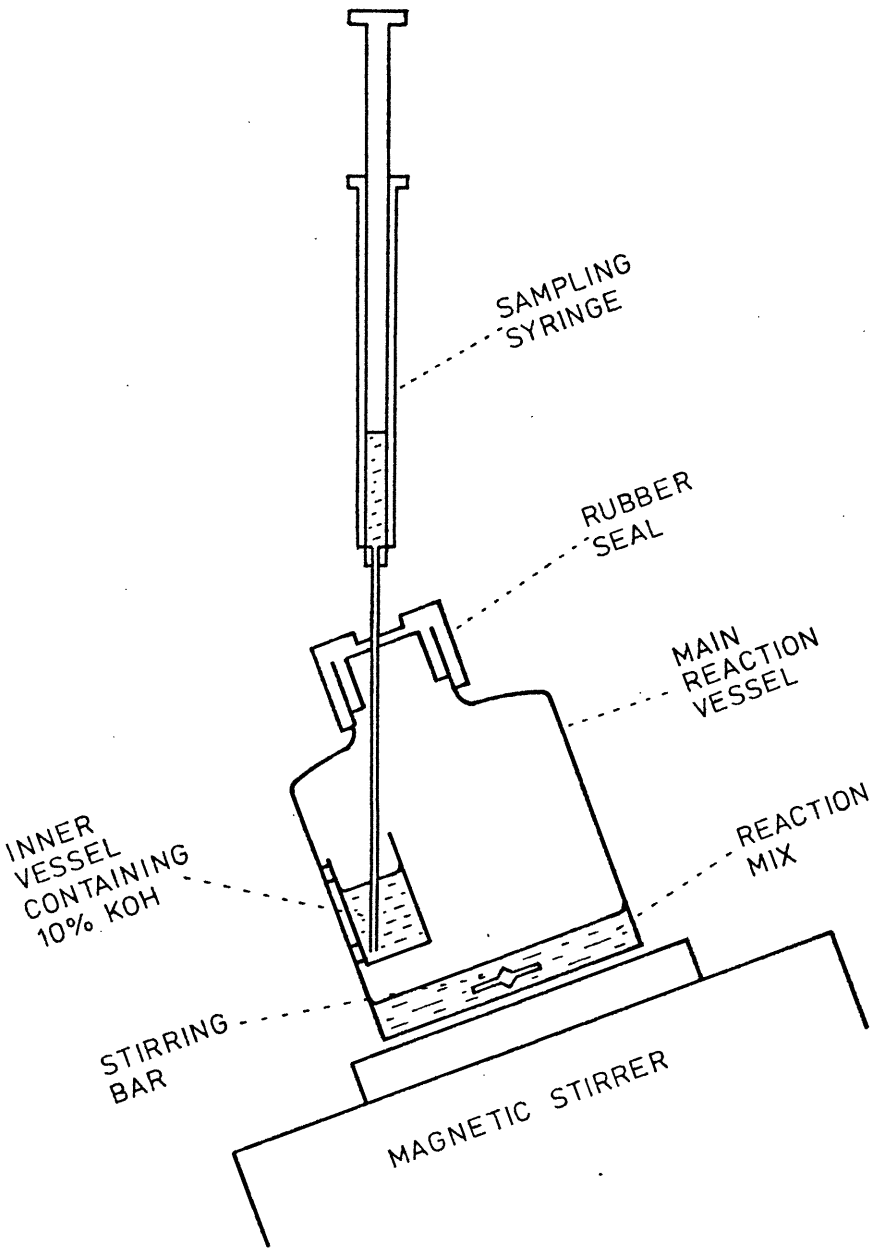


Fig. 7. The reaction vessel used for radiochemical assays

(stored on ice but briefly incubated at 30°C before addition) using an accurately calibrated glass syringe. After a suitable time, typically 5min, the reaction was stopped by the addition of 1·0ml of 0·5M H_2SO_4 (with a glass syringe) and gently stirred for 30min to ensure complete absorption of carbon dioxide by the KOH solution. Duplicate 0·1ml aliquots of the KOH solution were added, together with 0·9ml of water, to 10ml of aqueous scintillation fluid (5·5g of PPO and 0·1g of dimethyl POPOP dissolved in 667ml of toluene and 333ml of Fisons emulsifier mix no. 1) and counted for 20min in a Packard 3385 scintillation spectrometer. The optimum gain setting for ^{14}C in this scintillant was found to be 18% at discriminator (window) settings of 35-1000. No significant quenching by KOH was observed (over the range 0-500µl of 10% KOH per 10ml of scintillation fluid). The efficiency of counting, obtained using a standard [^{14}C]toluene solution (The Radiochemical Centre, Amersham, UK), was found to be 87·5%.

ENZYME ACTIVITIES ARE EXPRESSED AS nMol CO_2 PRODUCED .min^{-1}

Controls

(a) The specific radioactivity of the [1-^{14}C]α-oxoglutarate was checked (for non-enzymatic loss of [^{14}C]CO_2) during each series of assays, by counting 0·1ml of the [1-^{14}C]α-oxoglutarate (of known concentration), 0·1ml of 10% KOH solution and 0·8ml of water in 10ml of aqueous scintillation fluid. In no case was degradation of the [1-^{14}C]α-oxoglutarate found to occur.

(b) Assays were also carried out in the absence of enzyme to check for non-enzymatic [^{14}C]CO_2 production under the assay conditions. This was found to vary from 1-10% of the CO_2 produced enzymically depending on the reaction followed, and was corrected for accordingly.

(c) The time required for complete CO_2 absorption by the KOH solution was checked and 30min was found to be adequate.

I.2·6 Measurement of E2 activity

Approximately 5units of commercial dihydrolipoamide dehydrogenase was added to a cuvette containing 0·2µmol of NADH and 0·2µmol of lipoamide in

10mM $MgCl_2$, 1mM EDTA, 0·1M Tris-HCl, pH 8·0, to give a total volume of 0·96ml. The approach to equilibrium of this reaction was followed at 340nm and took approximately 10min. It was found, however, that commercial dihydrolipoamide dehydrogenase contains a low level of NADH oxidase activity which results in a slow, linear decrease in absorbance at 340nm even when the dihydrolipoamide dehydrogenase reaction has reached equilibrium. This rate was corrected for by adding 20µl of α-OGDH or PDH complex and recording the endogenous rate of NADH oxidation. 20µl of 7·5mM succinyl- or acetyl-CoA were then added and the rate recorded. Subtraction of the endogenous rate from this final rate gave a measure of the activity of the E2 component.

Checks were made to ensure that the dihydrolipoamide dehydrogenase was not rate-limiting in this coupled system by increasing the amount of this enzyme used. No change in rate was observed, indicating that the measured rate was in fact due to E2 activity. Enzyme activities are expressed as changes in absorbance units.min^{-1}

I.2·7 E2-E3 interaction test and lipoic acid test

The reaction mixture for the interaction test contained 0·5µmol of DTNB and 0·2µmol of NADH in 10mM $MgCl_2$, 1mM EDTA, 20mM Tris-HCl, pH 8·0 (MET-8 buffer). The reaction was started by the addition of enzyme solution to give a total volume of 1·0ml, and the formation of TNB^- was followed spectrophotometrically at 412nm.

The lipoic acid moiety, bound to the E2 component, may be detected by adding 30µg of commercial dihydrolipoamide dehydrogenase to the above assay mixture and following the increase in absorbance at 412nm.

I.3 RESULTS AND DISCUSSION

I.3·1 Measurement of α-OGDH complex and E3 component activities

The assays for both the whole complex and E3 activities were found to be simple and sensitive. In each case the rate was proportional to enzyme concentration as expected. The whole complex assay has been used to demonstrate stimulation of the complex by AMP and inhibition by NADH (Fig. 8). The K_m for α-oxoglutarate was found to be 2·5mM in the absence and 0·25mM in the presence of 0·2mM AMP. On the other hand, AMP has only a slight effect on the maximum velocity of the enzyme.

I.3·2 Measurement of E1 component activity

(i) DCPIP assay

In most cases the DCPIP assay was routinely used for measuring the E1 component activity. However, on a few occasions this was not possible for the reasons outlined in section I.1·3. One of the disadvantages described was that high concentrations of proteins (bovine serum albumin, casein and insulin) gave rise to an endogenous rate with this assay. Fig. 9 illustrates the effect of adding increasing amounts of bovine serum albumin to the DCPIP assay. Although there were occasions when assays in the presence of high protein concentrations were desirable (section III.2), normally only about 25μg of protein were used in this assay. Clearly in the latter case the endogenous rate due to protein (about 0·0001 absorbance units min^{-1}) is negligible, since the rate due to the E1 component would be about 0·03 absorbance units min^{-1}.

(ii) DTNB assay

A possible alternative way of assaying the E1 component may be to react the free -SH group of acylated dihydrolipoamide, produced from the lipoamide of the E2 component, with DTNB. Unfortunately the expected absorbance change at 412nm due to the production of TNB^- would only be in the order of 0·008 absorbance units for 0·5mg of enzyme complex (assuming that the

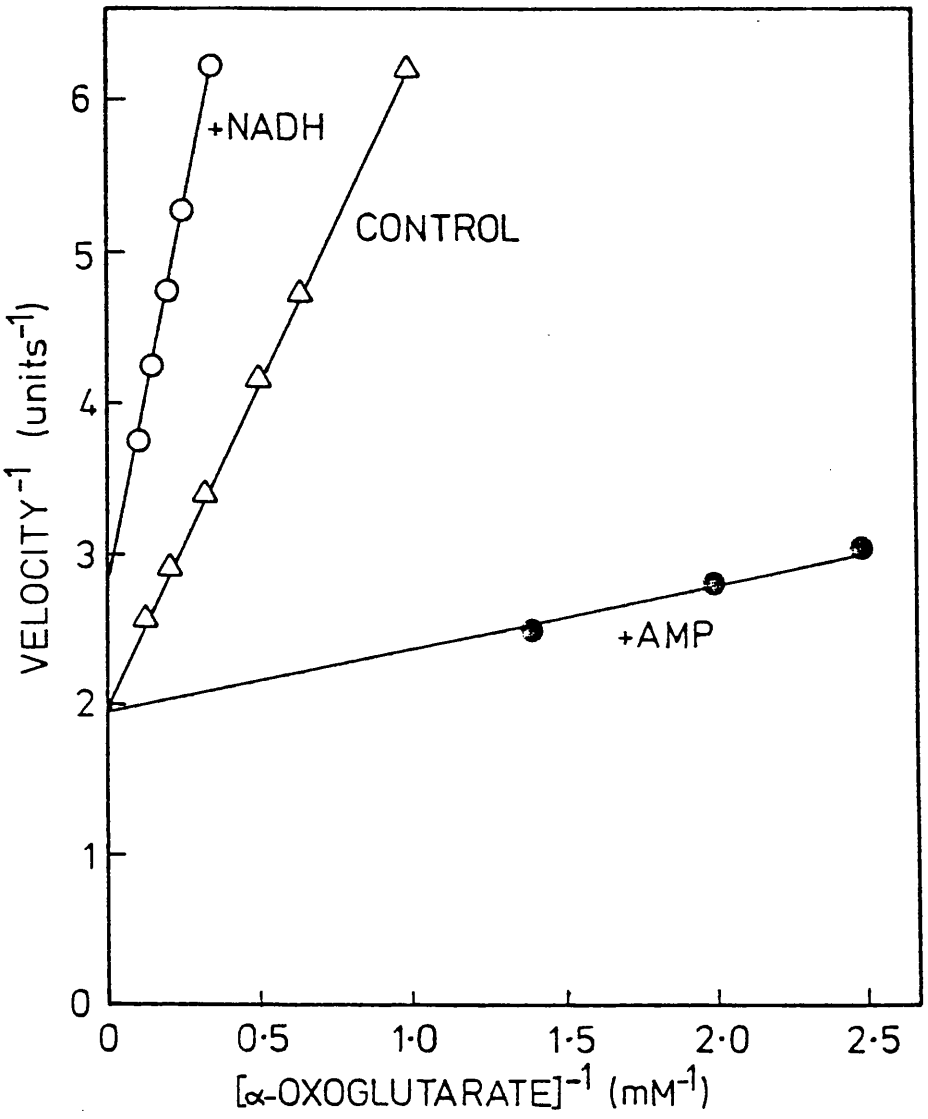


Fig. 8. Dependence of α-OGDH complex activity on α-oxoglutarate concentration.

Whole complex activity was measured on the native enzyme (△—△), or in the presence of 0·2mM AMP (●—●) or 0·2mM NADH (○—○). The assay procedure is described in section I.2·2. Each assay contained 20μg of α-OGDH.

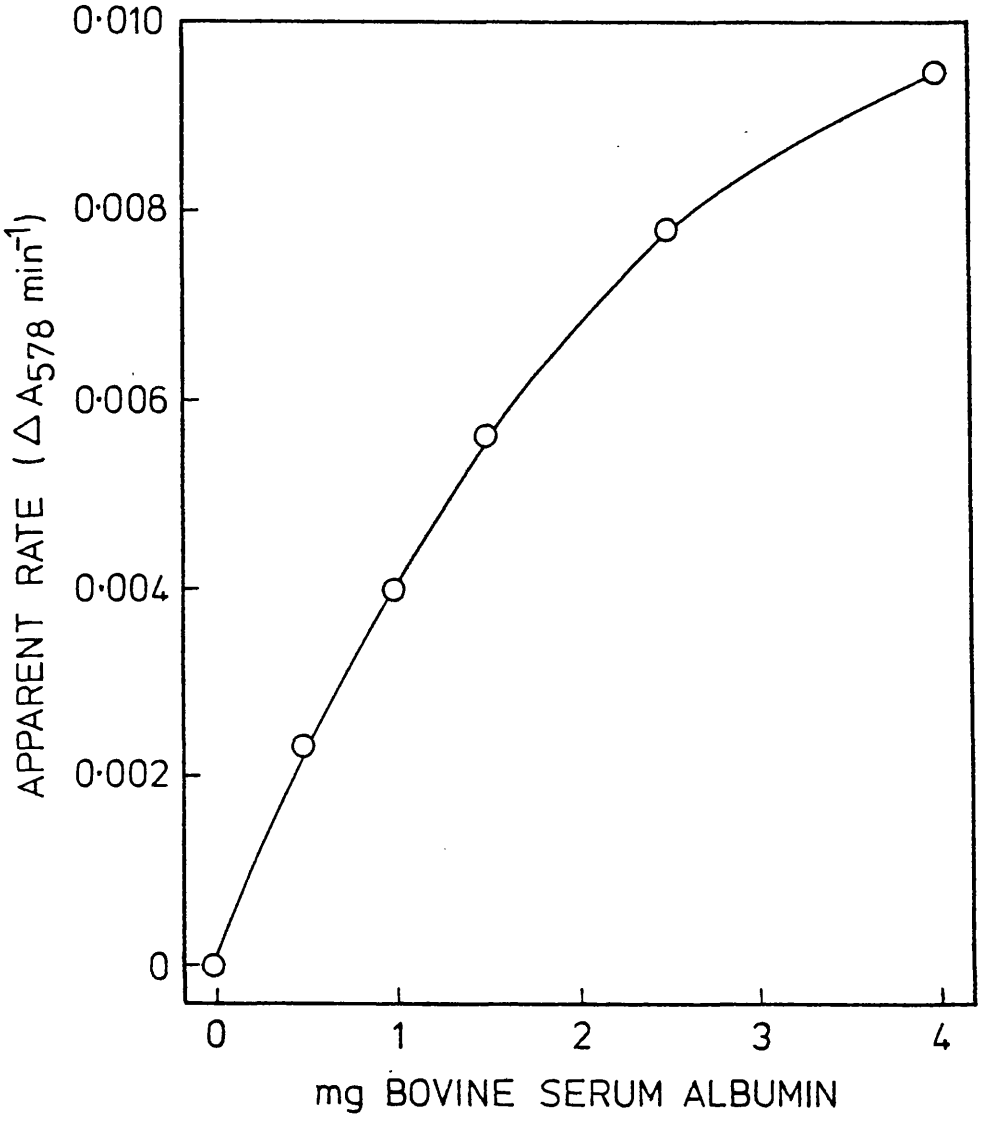


Fig. 9. Non-enzymic reduction of DCPIP by bovine serum albumin in the E1 assay.

The assay procedure is described in section I.2.5.

complex has a molecular weight of 2 x 10^6 and each molecule of the complex contains about 25 lipoamide residues). However, the sensitivity of the reaction would be considerably increased if the E1 component could utilize exogenous lipoamide and this is illustrated in Fig. 10. It can be seen that the absorbance increase due to TNB^- production, after a 30min reaction period, is only 0·075 absorbance units (cf. 0·008 in the absence of exogenous lipoamide) whereas the increase in lipoamide concentration is 160-fold. Consequently, it is obvious that the E1 component enzyme cannot utilize free lipoamide very efficiently. This would tend to support the view that the enzyme is essentially unable to acylate free lipoamide (Reed et al., 1958; Sanadi et al., 1958; Sanadi et al., 1959; Reed, 1960), despite evidence by other workers to the contrary (Gunsalus, 1954; Smith & Gunsalus, 1957).

In conclusion the DTNB assay, even in the presence of added lipoamide, appears to be considerably less sensitive than the DCPIP assay and was therefore not used.

(iii) Hydroxylamine assay

As with the DTNB assay described above, this assay did not prove to be a successful means of measuring E1 activity, probably due to the inability of the enzyme to acylate free lipoamide.

(iv) Radiochemical assay

The radiochemical assays for the whole complex and the E1 component activities have proved to be very useful, particularly for studies of the effect of NADH on these enzymes (since NADH interferes with the more routinely used DCPIP assay for the E1 component; see section I.1·3).

The reaction rates for whole complex and E1 component activities.

Fig. 11 illustrates the time course of $[^{14}C]CO_2$ production from [1-^{14}C] α-oxoglutarate by the α-OGDH complex and E1 component activities. In both cases the reaction was linear over the time period studied. Furthermore, a

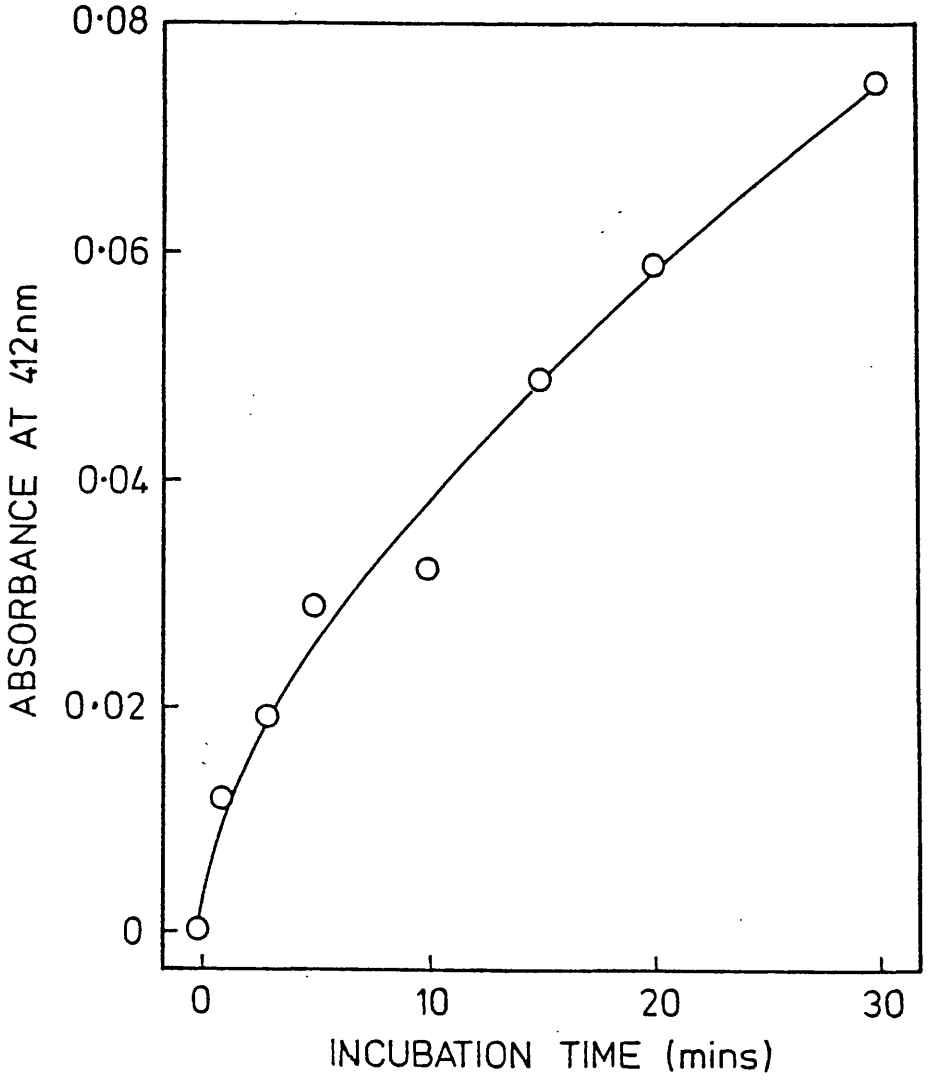


Fig. 10. Measurement of E1 activity using the DTNB assay.

The dependence of the extent of reaction (represented by absorbance at 412nm) on the assay incubation time is shown. Experimental details are given in section I.2·5. 0·5mg of αOGDH complex from A. lwoffi was used per assay.

very similar rate was obtained for the whole complex activity with this radiochemical assay to that obtained with the spectrophotometric assay (Fig. 11). However, the reaction rate for the E1 component was only 18% that of the whole complex. A possible explanation for this is that acylation of free TPP is considerably slower than enzyme bound TPP. Since acylated TPP is presumably the end-product of this E1-catalysed reaction, once acylation of enzyme-bound TPP has taken place free TPP must be utilized for the reaction to continue. It is not unreasonable to suppose that this latter process may be rate-limiting. Furthermore, if this is indeed the case, addition of phenazine methosulphate and DCPIP may be expected to regenerate enzyme-bound TPP (as shown in Fig. 4) with a concomitant increase in reaction rate. Fig. 12 shows that this does occur but only to a limited extent. However, this is not really surprising since DCPIP appears to provide a rather inefficient regenerating system for TPP as suggested by the slow rate obtained in the DCPIP E1 assay, compared with the whole complex assay.

Determination of the K_m for α-oxoglutarate.

The K_m values for α-oxoglutarate for both the whole complex activity and the E1 component have been determined using the radiochemical assay (Fig. 13). It can be seen that the K_m values are essentially the same for the two enzyme activities (whole complex: 2·0mM [1 determination]; E1: 2·5-2·8mM [3 determinations]). Furthermore, the value obtained for the whole complex activity using this method compares favourably with that obtained by the spectrophotometric method (2·5mM; Fig. 8).

I.3·3 Measurement of E2 component activity

The linear dependence of enzymic rate on enzyme concentration measured by the E2 assay has been demonstrated for the transacylase component of both α-OGDH and PDH complexes of A. lwoffi and also for the PDH complex of E. coli (Fig. 14). Moreover, unlike the hydroxylamine assay (section I.1·4), this method also permits an examination of the dependence of transacylase

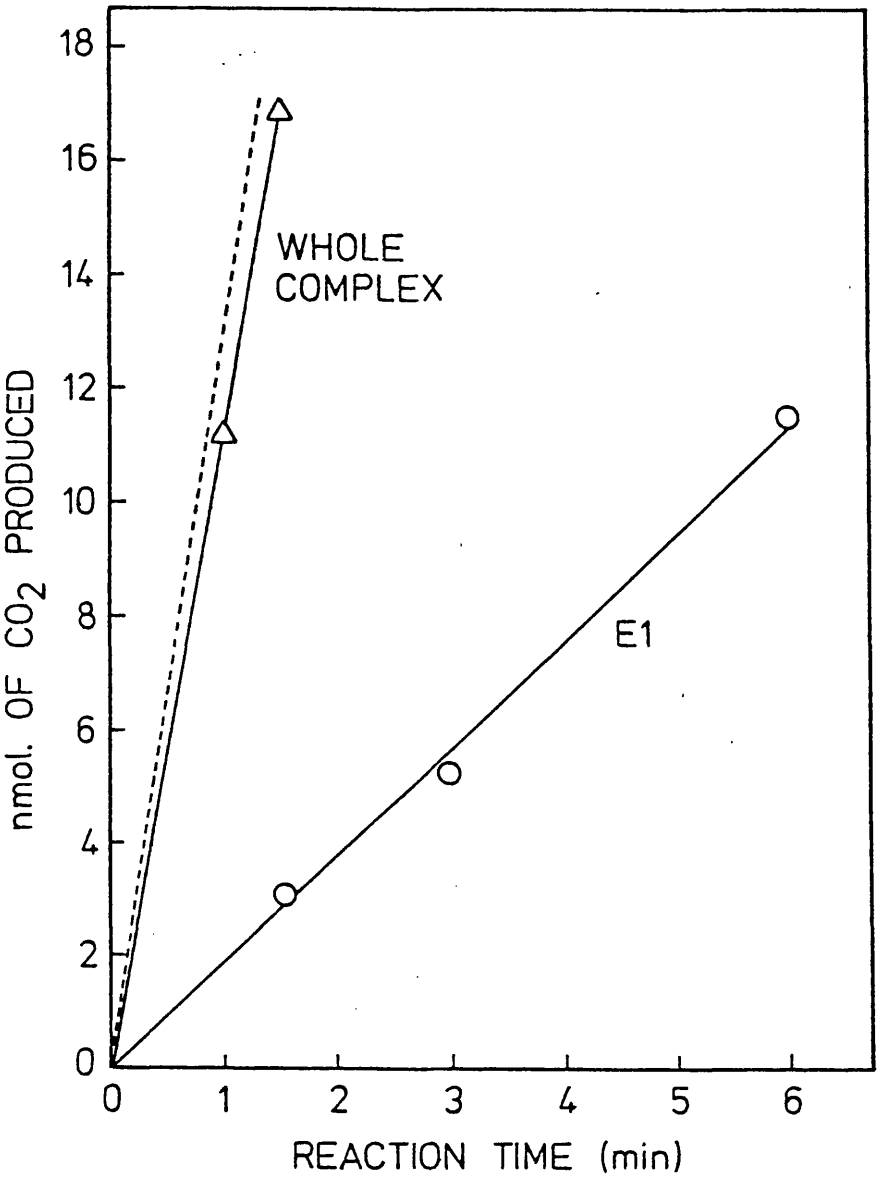


Fig. 11. Measurement of E1 and whole complex activities using radiochemical assays.

The kinetics for both whole complex (△—△) and E1 (○—○) activities are shown. The experimental procedure is given in section I.2·5, using 3μg of A. lwoffi α-OGDH complex per assay and 1mM [1-^{14}C]α-oxoglutarate (200,000cpm μmol^{-1}). (------) compares whole complex activity obtained using the spectrophotometric assay (section I.2·2).

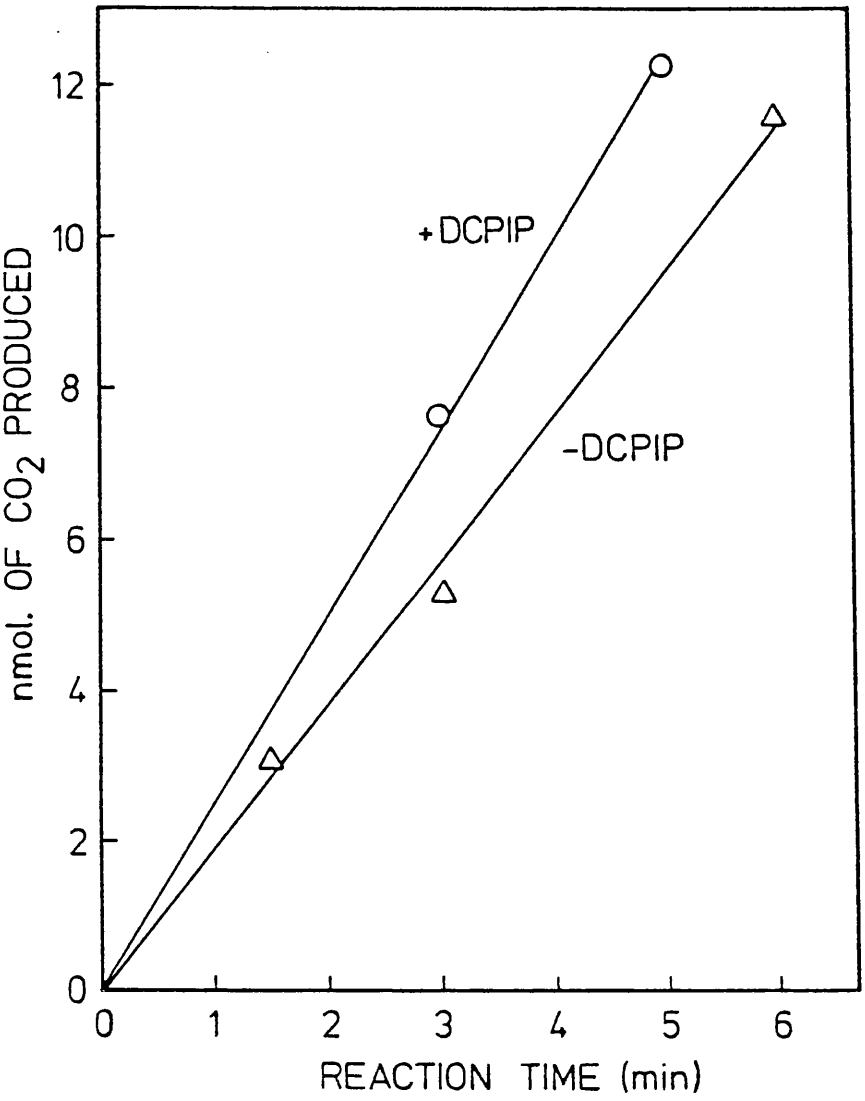


Fig. 12. The effect of DCPIP on the E1 radiochemical assay.

The kinetics of E1 activity in the absence (△—△) and presence (○—○) of 0·05mM DCPIP and 0·5mg of phenazine methosulphate are shown. The assay procedure is given in section I.2·5. All data were corrected for controls lacking enzyme. 3µg purified A. woodii dCOGDH were used per assay.

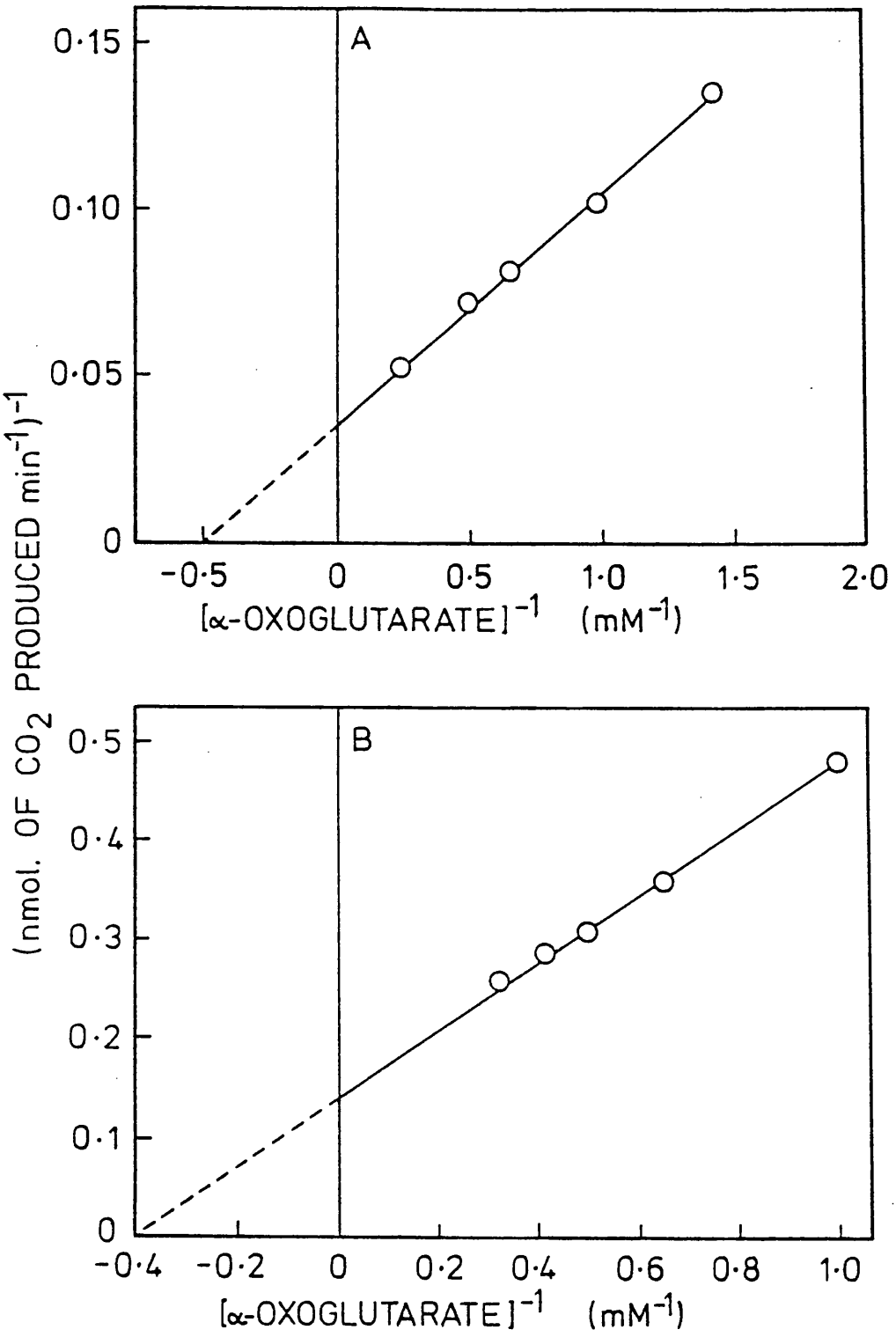


Fig. 13. Determination of the K_m for α-oxoglutarate using the E1 and whole complex radiochemical assays.

The dependence of whole complex activity (A) and E1 activity (B) on α-oxoglutarate concentration was determined as described in section I.2·5, using 3µg purified *A. vinelandii* αOGDH per assay.

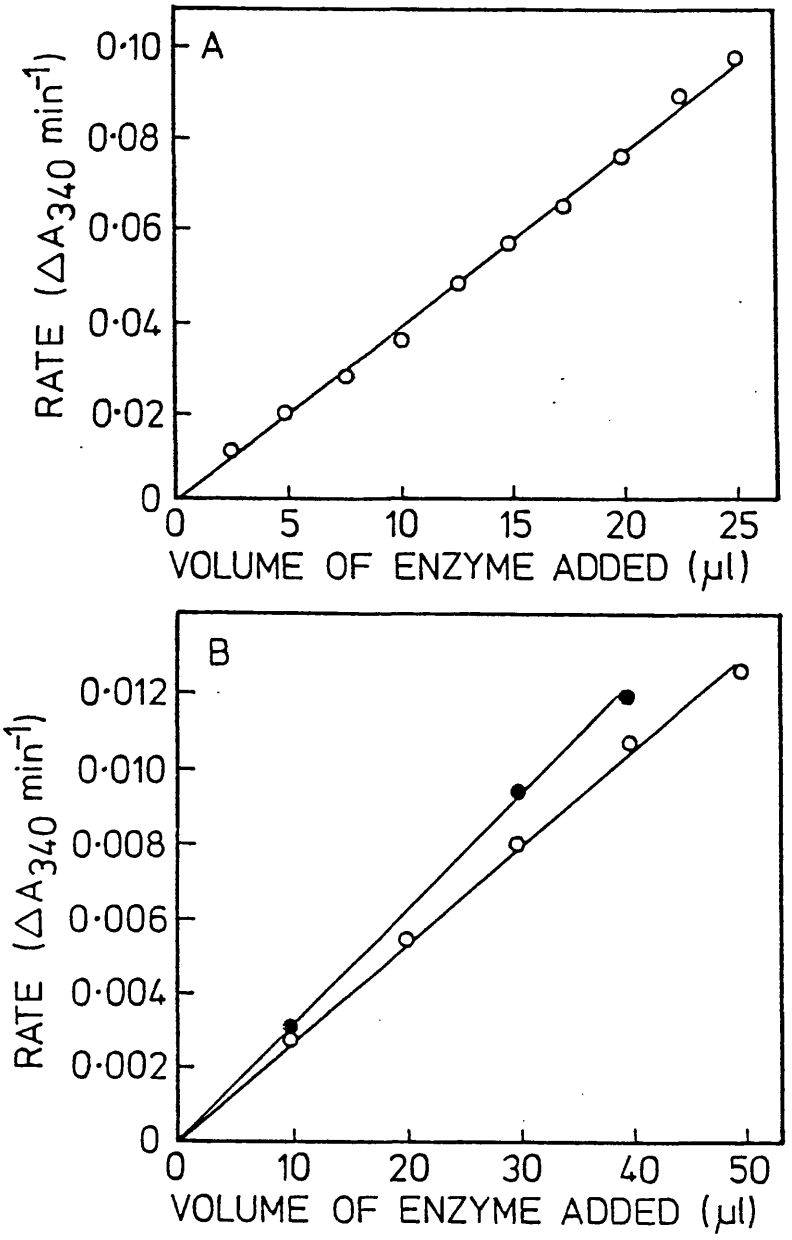


Fig. 14. Dependence of the observed rate on the concentration of dihydrolipoamide transacylase (E2).

A. The transsuccinylase activity of purified α-OGDH from *A. lwoffi* (protein content: 9·5mg ml^{-1}).

B. The transacetylase activity of PDH from *A. lwoffi* (o—o; protein content: 12·5mg ml^{-1}) and *E. coli* (●—●; protein content: 75mg ml^{-1}).

The purification of these enzymes is described in section II.2 and assay procedures are given in section I.2·6.

activity on acyl-CoA concentration (Fig. 15). The apparent K_m for succinyl-CoA of the dihydrolipoamide transsuccinylase component from A. lwoffi was found to be 60μM (from the double reciprocal transformation of Fig. 15.

The sensitivity of this assay is not as great as that of the whole complex or E3 assays, but is comparable with that of the DCPIP assay for the E1 component of A. lwoffi. For the E. coli enzyme, however, the sensitivity is much lower. A further disadvantage arises from the equilibrium nature of this method, in that the removal of one molecule of dihydrolipoamide by the transacylase reaction does not result in the oxidation of exactly one molecule of NADH by the dihydrolipoamide dehydrogenase reaction. However, these disadvantages are offset to some extent by the following advantages this method possesses over the hydroxylamine assay:

(i) It is a continuous assay.

(ii) No preparation of dihydrolipoamide and succinic thiokinase is required; the coupling enzyme (dihydrolipoamide dehydrogenase) is readily available commercially.

(iii) The assay permits examination of the dependence of E2 activity on acyl-CoA concentration.

It was mentioned earlier (section I.2·6) that commercial dihydrolipoamide dehydrogenase was found to contain a low level of NADH oxidase activity. This resulted in a slow decrease in absorbance at 340nm even when the dihydrolipoamide dehydrogenase reaction had reached equilibrium. Attempts were made to remove this NADH oxidase by gel filtration on Sephadex G200. Unfortunately this proved to be unsuccessful; the NADH oxidase and dihydrolipoamide dehydrogenase co-eluted. Consequently correction for removal of NADH by NADH oxidase had to be made to the transacylase assays as described in section I.2·6.

Very recently, a new assay has been described for the dihydrolipoamide transacetylase activity of bovine kidney (Butterworth et al., 1975). This

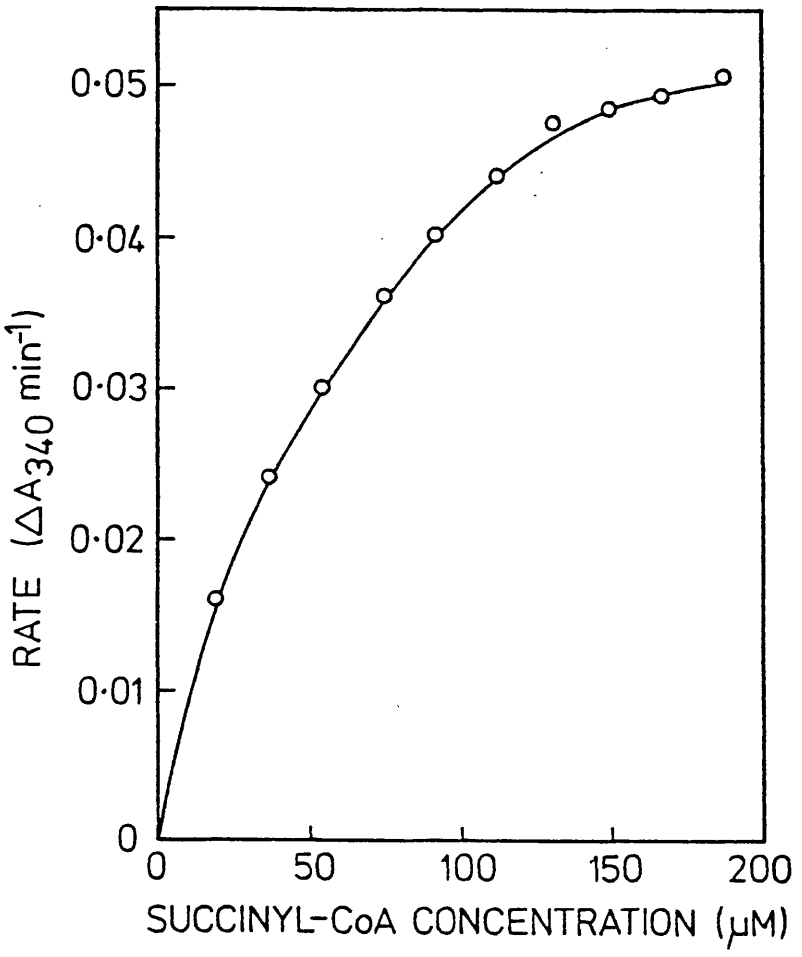


Fig. 15. Dependence of A. lwoffi dihydrolipoamide transsuccinylase activity on succinyl-CoA concentration.

Assays were performed as described in section I.2.6 using 120μg of purified A. lwoffi α-OGDH complex.

method also measures the reaction in the backward direction. Radioactively labelled S-acetyl dihydrolipoamide is produced by the transfer of the acetyl group of [1-^{14}C]acetyl-CoA to dihydrolipoamide and extracted into benzene. Unreacted [1-^{14}C]acetyl-CoA remains in the aqueous phase. This assay has the disadvantage of being discontinuous but has the advantage over the hydroxylamine assay of being simpler and more sensitive.

I.3.4 E2-E3 interaction test and lipoic acid test

The reaction rate of the E2-E3 interaction test was found to be proportional to the enzyme concentration as shown in Fig. 16. Both this test and the lipoic acid test (section I.1.5) proved to be extremely useful in the dissociation and chemical modification studies (chapters III and IV respectively).

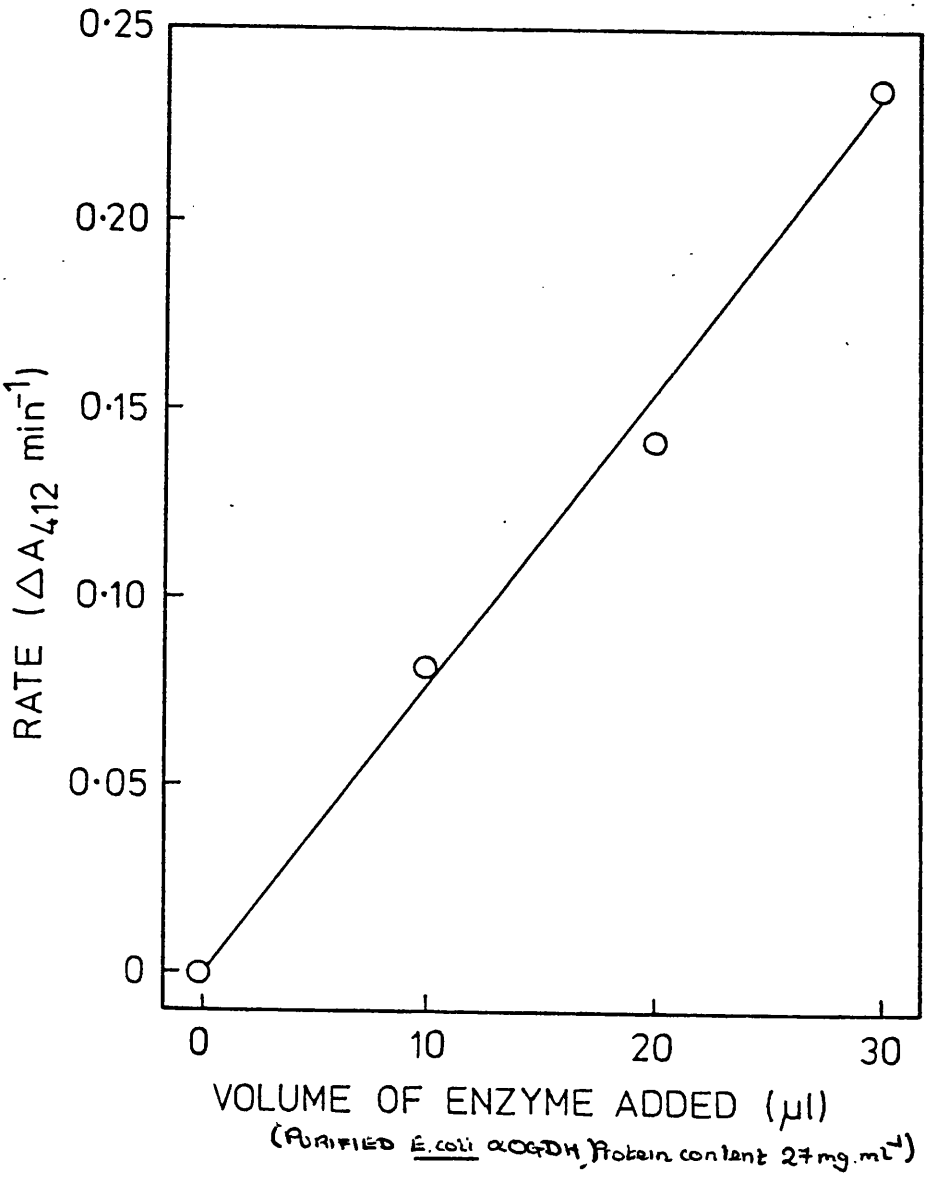


Fig. 16. Dependence of the observed rate on the concentration of α-OGDH using the E2-E3 interaction test.

Experimental details are described in section I.2·7.

CHAPTER II

ENZYME PURIFICATION AND MOLECULAR PROPERTIES

II.1 INTRODUCTION

In order to carry out a detailed kinetic study of an enzyme it is necessary to obtain at least a partial purification so that interfering reactions are eliminated. On the other hand, determination of the structural properties (e.g. number of subunits, number of polypeptide chains, etc.) requires a much more extensive purification, preferably to homogeneity.

The α-OGDH complex from A. lwoffi has already been purified (Parker & Weitzman, 1973) to a specific activity of 2lunits mg^{-1} protein. This purification procedure has been adopted with several minor modifications in the present work and yields essentially pure enzyme as indicated by electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulphate and analytical ultracentrifugation. Procedures are also described for the partial purification of the α-OGDH complex from E. coli and the PDH complexes from both A. lwoffi and E. coli for use in E2 assays (section I.2·6) and dissociation studies (section III.2).

In order to understand fully how a particular enzyme functions it is helpful to have a knowledge of its physical properties. In the case of the α-OGDH complex from A. lwoffi information about its molecular size and the size and number of the component polypeptide chains, for example, would enable comparison with the complexes from E. coli and mammalian cells which lack AMP regulation, to be made.

This section also describes the determination of the polypeptide chain molecular weights of the three different enzyme components which constitute the α-OGDH complex of A. lwoffi. These data were obtained by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate using the

method of Weber & Osborn (1969). Finally, the molecular weight of the whole complex was obtained using a novel theory derived by Rowe (1975) involving the determination of the sedimentation coefficient of the complex by analytical ultracentrifugation but without requiring the diffusion coefficient or any other properties of the enzyme to be measured.

II.2 MATERIALS AND METHODS

II.2·1 Materials

Protamine sulphate was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.; DEAE-cellulose (Whatman DE-11) from H. Reeve Angel and Co. Ltd., London EC4, U.K. and Sepharose 4B from Pharmacia (G.B.) Ltd., London W5, U.K. All other chemicals were obtained from either Sigma (London) Chemical Co. Ltd., Kingston, Surrey, U.K. or BDH Chemicals Ltd., Poole, Dorset, U.K.

II.2·2 Determination of protein concentration

Protein concentration was routinely determined by the method of Lowry et al. (1951) using bovine serum albumin (Cohn fraction V; Sigma) as a standard. However, when a large number of column fractions had to be assayed for protein, this was more conveniently determined from the ratio of absorbances at 280 and 260nm (Layne, 1957). These two methods gave very similar values with purified α-OGDH from A. lwoffi.

II.2·3 Growth of bacteria

(i) A. lwoffi 4B

Cultures of A. lwoffi were maintained on nutrient agar slopes. A "starter culture" was prepared when required by inoculating a 500ml Erlenmeyer flask containing 200ml of 2·5% (w/v) nutrient broth (Oxoid) with cells from an agar slope. They were grown for approximately 8h at 37°C with vigorous shaking. This culture was then used to inoculate 20-30 two-litre Erlenmeyer flasks, each containing 1 litre of 2·5% (w/v) nutrient broth and grown at 37°C overnight on a rotary shaker (300rev. min^{-1}).

(ii) E. coli (Crookes strain)

Cultures of E. coli were maintained and grown as described above, except that 1·3% (w/v) nutrient broth was used and the growth temperature was 30°C.

(iii) E. coli K12 (strain CA244)

Glycerol-grown cells of E. coli K12 were obtained as a frozen paste from

the Microbiological Research Establishment, Porton Down, Wilts., U.K.

II.2·4 Purification of A. lwoffi α-OGDH complex

The purification procedure was essentially that described by Parker & Weitzman (1973) with some modifications as follows:

(a) Preparation of cell-free extract

A late log-phase culture of A. lwoffi (20-30 litres) was harvested using a Sharples continuous flow centrifuge and the cells washed with MET-8 buffer at 4°C; then stored at -5°C until required. α-OGDH complex activity remained stable for several weeks in frozen cells.

The frozen pellet of cells was slowly thawed in cold MET-8 buffer and the cell density adjusted to give an absorbance at 680nm of about 100. This slurry was passed twice through a pre-cooled French pressure cell at 12000 lb in^{-2} and then centrifuged (20000xg, 2h, 4°C) to remove unbroken cells and cell debris.

All subsequent steps were performed at 4°C.

(b) Removal of nucleic acid

The crude extract was adjusted to a protein concentration of 20mg ml^{-1} with MET-8 buffer and stirred continuously while a 2% (w/v) aqueous solution of protamine sulphate was added dropwise, to a final ratio of 0·9mg protamine sulphate per 10mg protein. The mixture was allowed to stand on ice for 20 min before centrifugation (25000xg, 30min, 4°C) and the pellet, consisting mainly of protamine-bound nucleic acid, was discarded.

(c) Isoelectric precipitation

The pH of the supernatant from the protamine sulphate step was adjusted to 5·3 by the dropwise addition of 7% aqueous acetic acid with continuous stirring. The suspension was centrifuged immediately (25000xg, 20min, 4°C) and the pellet discarded. The pH of the supernatant, which contained all of the α-OGDH complex activity, was then adjusted to 4·8 with acetic acid. The precipitated α-OGDH complex was collected by centrifugation (25000xg, 30min,

4°C) and resuspended in MET-8 buffer in 20% (v/v) glycerol.

(d) Ion-exchange chromatography

DEAE-cellulose (Whatman DE-11) was precycled according to the Whatman Technical Bulletin IE2. This was then used to pack a 45cm x 2·5cm column (Pharmacia) and washed with 5-6 column volumes (1-1·2 litres) of MET-8 buffer in 20% (v/v) glycerol. The sample (from (c)) was applied to the top of the column, allowed to run in and washed with approximately 2 column volumes of MET-8 buffer in 20% (v/v) glycerol. A 600ml linear salt gradient (0·15-0·40M KCl) was then applied; 100 x 5ml fractions were collected and assayed for protein and complex activity. The α-OGDH complex eluted at a KCl concentration of about 0·23M. Fractions containing high complex activity ($\leqslant$5 units mg^{-1} protein) were combined.

(e) Ammonium sulphate fractionation

Finely ground ammonium sulphate was slowly added, with continuous stirring, to the combined enzyme fractions to 50% saturation (31·3g of ammonium sulphate per 100ml of solution). After standing for 30min the precipitated protein, containing all of the α-OGDH complex, was collected by centrifugation (25000xg, 30min, 4°C) and resuspended in a small volume (typically 2ml) of MET-8 buffer in 20% (v/v) glycerol.

(f) Gel filtration

A 45cm x 2·5cm column containing Sepharose 4B (checked for even packing with Dextran Blue) was equilibrated with 6 column volumes of MET-8 buffer. The concentrated enzyme solution was loaded onto the top of the column and eluted at a flow rate of 15ml h^{-1}. Fractions (2·0ml) were collected and assayed for enzyme activity and protein and those containing a high enzyme specific activity ($\geqslant$10 units mg^{-1} protein) were combined.

(g) Ammonium sulphate fractionation

The enzyme was concentrated by precipitation with ammonium sulphate (31·3g per 100ml Of enzyme solution) as described in section (e). The

resulting protein pellet was resuspended in 1·0-1·5ml of MET-8 buffer in 20% (w/v) glycerol and dialysed overnight against 2 litres of the same buffer. The purified α-OGDH complex was stored at 4°C and showed a gradual loss of activity over a number of weeks.

II.2·5 Purification of E. coli α-OGDH complex

A late log phase culture of E. coli (20 litres) (section II.2·3) was harvested with a Sharples continuous flow centrifuge and the pellet washed with 20mM phosphate buffer, pH 7·0. The cells were then resuspended in 0·1M phosphate buffer, pH 7·0, and sonicated (MSE 100 watt ultrasonic disintegrator) at 0°C at an amplitude of 7μm for 2 x 1·5 min periods with a 1 min interval for cooling. Unbroken cells and cell debris were removed by centrifugation (25,000xg, 30 min, 4°C).

Purification of the α-OGDH complex was then carried out as described by Reed & Mukherjee (1969).

II.2·6 Purification of A. lwoffi PDH complex

Attempts to purify the PDH complex from A. lwoffi have been reported to be unsuccessful (Jaskowska-Hodges, 1975), mainly due to the instability of this enzyme. However, I have succeeded in preparing a partially purified enzyme which was considerably more concentrated than a crude cell extract.

All stages in the purification were performed at 4°C.

(a) Preparation of a cell-free extract

A late log-phase culture of A. lwoffi (10 litres), grown as described in section II.2·3, was harvested using a Sharples continuous flow centrifuge, washed and resuspended in MET-8 buffer. The cells were broken by passage through a pre-cooled French pressure cell at 12000lb in^{-2}. This procedure was found to produce a 5-fold greater yield of PDH activity than sonication. Cell debris and unbroken cells were removed by centrifugation (25000xg, 30 min, 4°C).

(b) Removal of nucleic acid

Since the enzyme was found to be precipitated by protamine sulphate, nucleic acids were removed by treatment with streptomycin sulphate, as follows. The protein concentration of the extract was adjusted to 20-25mg ml^{-1} by addition of MET-8 buffer. A 25% (w/v) aqueous solution of streptomycin sulphate was added dropwise, with continuous stirring, to a final concentration of 0·035mg of streptomycin sulphate per mg of protein. After allowing the mixture to stand on ice for 15 min, the nucleic acid precipitate was removed by centrifugation (25000x*g*, 30 min, 4°C).

(c) Isoelectric precipitation

The pH of the supernatant (from (b)) was lowered to 5·8 by the dropwise addition of 7% acetic acid, and the resulting precipitate removed by centrifugation (25000x*g*, 20 min, 4°C). The supernatant (containing the PDH activity) was adjusted to pH 5·2 with acetic acid and the precipitate collected by centrifugation (25000x*g*, 30 min, 4°C). The pellet, which contained most of the PDH activity, was resuspended in about 5ml of MET-8 buffer.

(d) High speed centrifugation

The resuspended enzyme was centrifuged at 72000x*g* for 30 min (at 4°C) in a MSE 50 ultracentrifuge, and the pellet was resuspended in 2ml of MET-8 buffer.

(e) Gel filtration

The concentrated enzyme solution was loaded onto a 40 x 2·5cm column containing Sepharose 4B (pre-equilibrated with MET-8 buffer) and eluted with the same buffer. Fractions (2ml) were collected and assayed for enzyme activity and protein concentration. Fractions containing high PDH specific activities ($\geqslant$0·2 units mg^{-1} protein) were combined.

(f) Ammonium sulphate fractionation

Finely-ground ammonium sulphate was added slowly with continuous stirring, to the combined fractions to 75% saturation (51·6g per 100ml of

enzyme solution). After standing for 20min on ice the pellet, containing all of the PDH activity, was obtained by centrifugation (25000xg, 30min, 4°C) and resuspended in 5ml of MET-8 buffer.

(g) High speed centrifugation

The PDH complex was collected by centrifugation at 50000rev. min^{-1} (200000xg) for 2h at 4°C in an MSE 50 ultracentrifuge and the pellet resuspended in a small volume (about 0·2ml) of MET-8 buffer.

II.2·7 Purification of E. coli PDH complex

A partially purified, concentrated preparation of PDH complex from E. coli was prepared as follows:

(a) 12g of a frozen paste of E. coli K12 (strain CA244) cells were slowly thawed in about 2vol. of MET-8 buffer. The suspension was passed through a pre-cooled French pressure cell at 12000lb in^{-2} and then centrifuged (25000xg, 30min, 4°C) to remove cell debris.

(b) Nucleic acid was removed from the cell-free extract by precipitation with protamine sulphate, followed by isoelectric precipitation (as described in section II.2·4). PDH from E. coli was precipitated between pH 5·2 and 4·8.

(c) The isoelectric precipitate was resuspended in 2ml of MET-8 buffer and subjected to high speed centrifugation (200000xg, 2h, 4°C). Finally, the pellet was resuspended in a small volume of MET-8 buffer to give a total volume of 0·2ml.

II.2·8 Polyacrylamide gel electrophoresis

Gel electrophoresis at pH 8·9 was performed by the method of Davis (1964) with 7% acrylamide / 0·18% (w/w) bis-acrylamide gels. The gels were prepared in 10 x 0·5cm glass tubes and pre-run at 2mA per gel for 1-2h. Up to 20µl of sample (containing approximately 100µg of protein and 0·2µg of the marker dye, bromophenol blue) in buffer containing 10% (w/v) sucrose, were layered onto the tops of the gels and electophoresis

performed at 0·5mA per gel (20-40 volts) until the dye entered the gel. The current was then increased to 2mA per gel (about 150 volts) and maintained until the marker dye approached the bottom of the tube. After electrophoresis the gels were either treated with a protein stain, or sliced and assayed for E3 activity or lipoic acid content.

(a) Protein stain

Gels were stained with Coomassie Brilliant Blue according to the method of Chrambach et al. (1967), as follows. Gels were first fixed for 30min in 12·5% (w/v) trichloroacetic acid. They were then stained for 60min in 1% (w/v) Coomassie Brilliant Blue in 12·5% (w/v) trichloroacetic acid. Finally the gels were destained and kept in 10% (w/v) trichloroacetic acid.

(b) Localisation of E3 activity or lipoic acid

The gels were cut into 1-2mm slices with a razor blade and the position of each slice in the gel recorded. Each slice was transferred to a separate test-tube, covered with 0·2ml of buffer containing 10mM $MgCl_2$, 1mM EDTA, 0·1M Tris-HCl, pH 8·0, and left at 4°C overnight. The buffer and slice were then assayed for E3 activity or lipoic acid content as described in sections I.2·4 and I.2·7. In the case of the E3 assay, absorbance readings at 340nm were taken at 15 or 30 sec intervals and the solution was mixed in between.

II.2·9 Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate

By incorporating sodium dodecyl sulphate into the buffers used for polyacrylamide gel electrophoresis protein complexes are dissociated and denatured (Shapiro et al. (1967)). As a result it has been observed that the mobilities of these denatured proteins are usually proportional to the log of their molecular weights. 10% polyacrylamide gels containing 0·1% sodium dodecyl sulphate were used throughout, prepared according to the method of Weber & Osborn (1969). Bovine serum albumin (Sigma; Cohn fraction

V, molecular weight 68000), catalase (EC 1•11•1•6; MW 62500), pyruvate kinase (EC 2•7•1•40; MW 57000), fumarase (EC 4•2•1•2; MW 49000), aldolase (EC 4•1•2•13; MW 40000) and glyceraldehyde-3-phosphate dehydrogenase (EC 1•2•1•12; MW 36000) were used as standards. Electrophoresis was carried out at 0•5mA per gel until the bromophenol blue marker dye entered the gels and the current then increased to 2mA per gel. The gels were stained for 2h in a 0•2% (w/v) solution of Coomassie Brilliant Blue in 50% methanol / 7% acetic acid. Destaining was performed by transverse electrophoresis (1 amp, 100 volts) for 45min. The destaining solution was composed of acetic acid / methanol / water (7:6:88 parts by volume). Gels were stored in 7% acetic acid.

The mobility of a protein (R_f value) was calculated as:

$$R_f = \frac{\text{distance of protein migration}}{\text{distance of marker dye migration}} \times \frac{\text{length of gel before staining}}{\text{length of gel after destaining}}$$

II.2•10 Analytical ultracentrifugation

The sedimentation coefficient of the α-OGDH complex from A. lwoffi was determined by sedimentation velocity in an MSE Centriscan 75 ultracentrifuge. Purified α-OGDH was extensively dialysed against 0•1M Tris-HCl, pH 8•0, in 8% (v/v) glycerol and then diluted to give a range of concentrations from 0•5 to 10mg ml^{-1}. Samples were centrifuged at 40000rev. min^{-1} at 5°C, the position of the boundary being observed at suitable intervals by Schlieren optics. Sedimentation coefficients were computed on a Hewlett-Packard 9100B calculator by linear regression analysis of ln x against time from the relationship

$$s = \frac{dx/dt}{xw^2} = \frac{d \ln x}{w^2 dt}$$

(where s = sedimentation coefficient; x = distance of boundary from centre of rotor and w = angular velocity (rad. sec^{-1})).

These values were corrected to $S_{20,w}$ values (i.e. water at 20°C as the

solvent) from the relationship:

$$s_{20,w} = s_{5,solv} \cdot \frac{\eta_{5,solv}}{\eta_{20,w}} \cdot \frac{1-\bar{\nu}\rho_{20,w}}{1-\bar{\nu}\rho_{5,solv}} \qquad \text{(Bowen,1970a)}$$

(where $s_{5,solv}$ = sedimentation coefficient in 0·1M Tris-HCl in 8% (v/v) glycerol at 5°C; η = viscosity; $\bar{\nu}$ = partial specific volume and ρ = density of solvent.)

$\eta_{5,solv}$ and $\eta_{20,w}$ were determined using a capillary viscometer (Bowen, 1970b). $\rho_{5,solv}$ and $\rho_{20,w}$ were determined by accurately weighing 5cm^3 of buffer and water at 5°C and 20°C. A value of 0·73 was used for $\bar{\nu}$.

The protein concentration of each sample, determined both by the method of Lowry et al. (1951) and from the ratio of absorbances at 280 and 260nm (Layne, 1957), was corrected for 10% dimerization (calculated from the observed Schlieren peaks) and for radial dilution (Bowen, 1970c).

II.3 RESULTS AND DISCUSSION

II.3·1 Purification of α-OGDH from A. lwoffi

Table 2 illustrates a typical purification table for the α-OGDH complex from A. lwoffi. The specific activity of the purified enzyme (20 units mg^{-1} protein) was comparable to that reported by Parker & Weitzman (1973), although the preparation was on a slightly larger scale and produced a somewhat higher percentage yield. Nevertheless, the enzyme preparation was on a comparatively small scale and frequent repetition was required. Since the enzyme was found to be stable for only a few weeks [possibly because of progressive proteolytic degradation during storage as has been found for the PDH complex from E. coli (Perham & Thomas, 1971)],larger yields would have been wasted since they could not be successfully stored. However, for certain types of work, e.g. dissociation studies, much larger amounts of complex would be desirable and possible ways of achieving this were investigated as follows.

As the laboratory was not equipped with fermentors for the growth of large amounts of bacteria, frozen slurries of acetate-grown A. lwoffi cells were obtained from the Microbiological Research Establishment, Porton Down, Wilts, U.K. However, although these cells have proved to be of use for the isolation of other tricarboxylic acid cycle enzymes they were not suitable for the preparation of α-OGDH. This was mainly due to the extreme sensitivity shown by the complex to treatment with protamine sulphate, making the removal of nucleic acid by this means impossible and the subsequent isoelectric precipitation both difficult and inefficient. As an alternative 40 and 60 litre batches of A. lwoffi cells were grown up as described in section II.2·3 and the α-OGDH complex purified in the usual manner. To overcome overloading problems during chromatography multiple columns were used but unfortunately enzyme purified in this way was neither as pure nor as stable as that prepared in the usual small scale manner.

Table 2. Purification of the α-OGDH complex from A. lwoffi

α-OGDH complex was prepared from 20 litres of a late log-phase culture of A. lwoffi cells as described in section II.2.4.

Purification step	Volume (ml)	Total Activity (units)	Total Protein (mg)	Specific Activity (units mg^{-1})	Yield (%)
Crude extract	200	1163	4010	0.29	100
Protamine sulphate precipitation	150	1140	2550	0.447	98
Isoelectric precipitation	30	872	300	2.9	75
DEAE-cellulose chromatography	60	540	61.5	8.9	46.4
Ammonium sulphate precipitation	5	500	55	9.69	43
Sepharose gel filtration	50	350	19.4	18	30
Dialysis	17	337	17	20	29

II.3·2 Purification of α-OGDH from E. coli

A purification table for the α-OGDH complex from E. coli is shown in Table 3. It can be seen that the same degree of purification was not obtained in this case (specific activity: 8·3 units mg^{-1}) compared to the A. lwoffi enzyme (20 units mg^{-1}). Nevertheless, the degree of purity was acceptable for the work for which this enzyme was intended (see section III.2).

II.3·3 Purification of PDH from A.lwoffi and E. coli

Typical purifications of PDH from A. lwoffi and E. coli are illustrated in Tables 4 and 5 respectively. In both cases the final specific activity was somewhat low but nevertheless was considerably greater than in a crude cell extract and was adequate for the proposed work.

II.3·4 Polyacrylamide gel electrophoresis

The purified α-OGDH complex from A. lwoffi was examined by electrophoresis at pH 8·9 on 7% polyacrylamide gels (section II.2·8) as a test for homogeneity. A typical gel, stained with Coomassie Brilliant Blue to show protein bands, is depicted in Fig. 17. The minor bands are unlikely to be impurities or products arising from proteolytic breakdown since there is no evidence for these in gels run in the presence of sodium dodecyl sulphate (see section II.3·5) or from the analytical ultracentrifugation data (section II.3·6). Consequently it seems likely that these bands represent dissociation of the complex into subunits. In an attempt to overcome dissociation of the complex during electrophoresis, several of the electrophoretic parameters were varied, e.g. 4%, 5% and 7% polyacrylamide gels at pH 8·9 and 7·0 (Costen & Looms, 1969) run at 4°C or room temperature, but without success.

It should be pointed out that this distribution of protein bands is different to that obtained by Parker & Weitzman (1973). They reported a major band (R_f = 0·07 relative to bromophenol blue) and two faster-moving

Table 3. Purification of the α-OGDH complex from E. coli (Crookes)

α-OGDH complex was prepared from 20 litres of a late log-phase culture of E. coli (Crookes) cells as described in section II.2.5.

Purification step	Volume (ml)	Total Activity (units)	Total Protein (mg)	Specific Activity (units mg^{-1})	Yield (%)
Crude extract	92	1120	3882	0.28	100
Protamine sulphate precipitation	25	112	112.5	0.99	10
High-speed centrifugation	4	105	30	3.5	9.4
Isoelectric precipitation	0.6	102	12.3	8.26	9.0

Table 4. Purification of the PDH complex from A. lwoffi

PDH complex was prepared from 10 litres of a late log-phase culture of A. lwoffi cells as described in section II.2.6.

Purification step	Volume (ml)	Total Activity (units)	Total Protein (mg)	Specific Activity (units mg^{-1})	Yield (%)
Crude extract	45	105	1899	0.055	100
Streptomycin sulphate precipitation	40	100	1667	0.06	95
Isoelectric precipitation	5.2	44	330	0.13	42
High-speed centrifugation	1.2	22.5	90	0.25	21
Sepharose gel filtration	59	15.2	56	0.27	14
Ammonium sulphate precipitation	5.5	10	38.5	0.26	9.5
High-speed centrifugation	0.5	8.8	23	0.38	8.4

Table 5. Purification of the PDH complex from E. coli (K12)

A partially purified, concentrated preparation of PDH complex was prepared from 12g of a frozen paste of E. coli K12 (strain CA244) cells as described in section II.2.7.

Purification step	Volume (ml)	Total Activity (units)	Total Protein (mg)	Specific Activity (units mg^{-1})	Yield (%)
Crude extract	31	51	542	0.09	100
Protamine sulphate precipitation	32.5	46	377	0.12	90
Isoelectric precipitation	2.5	28	41	0.69	55
High-speed centrifugation	0.2	27	17.6	1.52	53

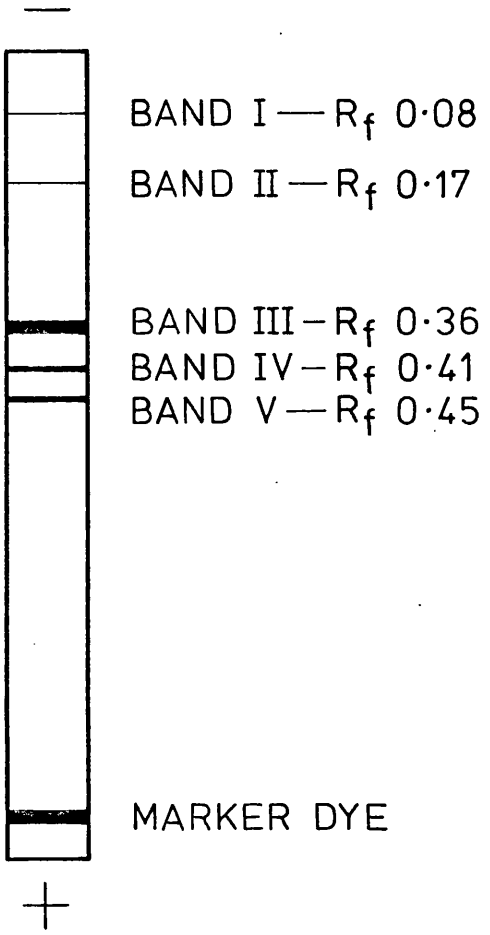


Fig. 17. Diagrammatic representation of a 7% polyacrylamide gel after electrophoresis of purified A. lwoffi α-OGDH complex.

The gel was stained for protein with Coomassie Brilliant Blue. All experimental details are described in section II.2·8.

minor bands (R_f = 0·6 and 0·7), but the reason for the discrepancy between these results is not known.

With a view to understanding the precise identity of the various protein bands shown in Fig. 17, parallel unstained gels were sliced immediately after electrophoresis, eluted with buffer overnight and assayed for whole complex activity, E3 activity and lipoic acid content. Low levels of whole complex activity were detected in the top few slices of the gel up to an R_f value of 0·08, corresponding to band I in Fig. 17. E3 activity was also observed in the first few slices (R_f = 0 to 0·1) as well as in a slice of R_f = 0·45 coincident with band V. Finally, lipoic acid could be detected in the first few slices at the top of the gel and again at high R_f values (0·65 to 0·80).

In conclusion it would appear that band I in Fig. 17 represents whole enzyme complex and the other bands are dissociated subunits. In particular band V contains the E3 component, either solely or in combination with another subunit.

II.3·5 Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate

Electrophoresis of purified α-OGDH complex from A. lwoffi on polyacrylamide gels in the presence of sodium dodecyl sulphate resulted in only three major bands (Fig. 18). This is consistent with the complex being composed of multiple copies of three individual polypeptide chains as shown by other workers using α-OGDH from other organisms (Perham & Thomas, 1971; Reed, 1974; Koike et al., 1974; see also "General Introduction").

It has been observed that proteins which have been dissociated and denatured by sodium dodecyl sulphate have mobilities which are proportional to the logarithm of their molecular weights when subjected to electrophoresis on polyacrylamide gels in the presence of this detergent (Weber & Osborn, 1969). Consequently six different protein standards of known polypeptide

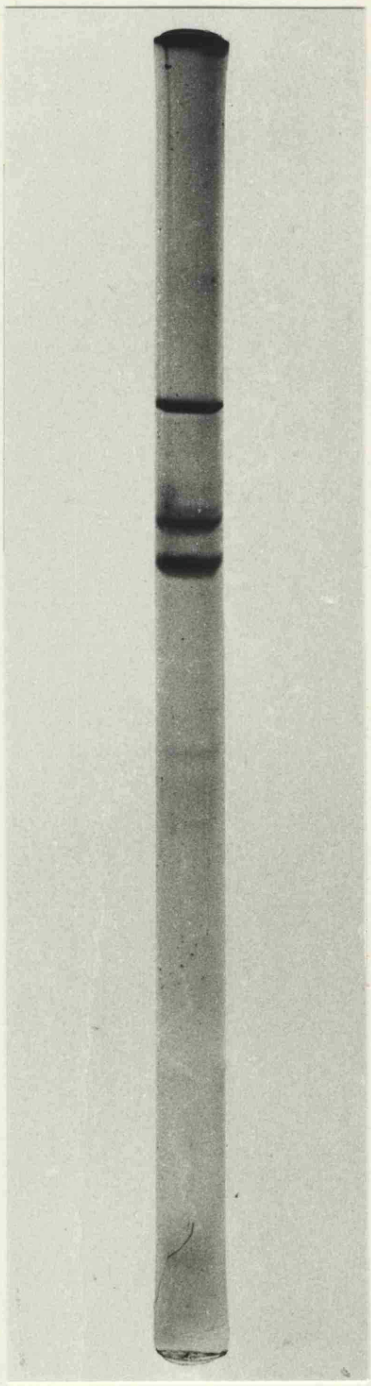


Fig. 18. Polyacrylamide gel electrophoresis of A. lwoffi α-OGDH complex in the presence of sodium dodecyl sulphate.

The gel was stained for protein with Coomassie Brilliant Blue. All experimental details are described in section II.2·9.

chain molecular weight were pretreated as described by Weber & Osborn (1969) and run on different gels in the presence of sodium dodecyl sulphate. A plot of their relative mobilities against the logarithm of their molecular weights was found to be linear (Fig. 19) as expected. Molecular weights could then be assigned to the three protein bands of the α-OGDH complex of A. lwoffi run on parallel gels to the standard proteins. The results of three separate determinations are shown in Table 6. Comparison of these chain molecular weights with those reported for the same enzyme from other sources (Table 7) does not yield conclusive evidence as to the identity of the three bands. It is interesting to note that the polypeptide chain molecular weights for the α-OGDH complexes from E. coli and pig heart, two widely different sources, are very similar yet those from A. lwoffi appear to be somewhat different. However, perhaps it is not so surprising when one considers that the latter complex exhibits quite a different kind of regulatory sensitivity to the other two enzymes and this may be reflected in structural differences. Furthermore, this control is exerted on the E1 component, whose polypeptide chain molecular weight seems to differ the most.

II.3.6 Determination of the molecular weight of the α-OGDH complex of A. lwoffi

(a) Determination of sedimentation coefficient

Analytical ultracentrifugation of purified α-OGDH complex from A. lwoffi, using schlieren optics to detect the protein boundary, resulted in a single, virtually symmetrical peak (Fig. 20) indicating that the complex was essentially pure. A small shoulder was observed at the base of the leading edge of the peak, believed to be due to dimerisation of the complex and comprising 10% of the total peak area. A small amount of dimerisation has also been observed during analytical ultracentrifugation of the PDH complex from E. coli (Danson & Rowe, personal communication).

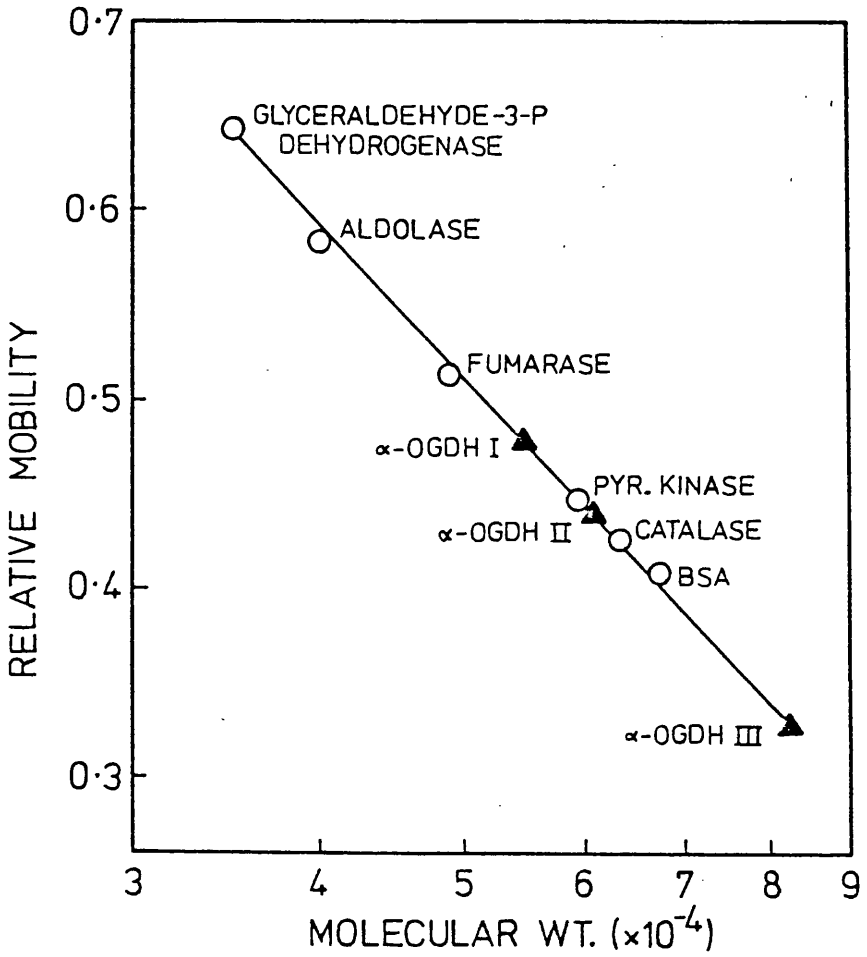


Fig. 19. Dependence of log molecular weight on the relative mobility after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. SDS gel electrophoresis was performed using standard proteins of known molecular weight and A. lwoffi α-OGDH complex, as described in section II.2·9.

Table 6. Chain molecular weights of the A. lwoffi α-OGDH complex subunits.

The polypeptide chain molecular weights of A. lwoffi α-OGDH complex were determined by gel electrophoresis in the presence of sodium dodecyl sulphate. The results of three independent determinations are given. Experimental details are described in section II.2.9. Band numbers correspond to those given in Fig. 18.

	Molecular weights of protein bands		
	Band 1	Band 2	Band 3
Determination 1	55,000	60,000	82,000
Determination 2	54,000	60,000	80,000
Determination 3	56,000	60,230	80,080
Mean mol. wt.	55,000	60,080	80,900

Table 7. Summary of polypeptide chain molecular weights for α-OGDH complexes from various sources.

Subunit	Source of α-OGDH complex	Chain molecular weight	References
E1	*E. coli*	95,000	Perham & Thomas (1971)
	E. coli	94-95,000	Reed (1974); Pettit *et al.* (1973)
	Pig heart	97-113,000	Koike *et al.* (1974)
E2	*E. coli*	42-47,000	Reed (1974); Pettit *et al.* (1973)
	E. coli	51,000	Perham & Thomas (1971)
	Pig heart	41-48,000	Tanaka *et al.* (1974)
E3	Mammalian and bacterial cells	55-56,000	Reed (1974); Koike et al. (1963); Barrera *et al.* (1972); Vogel & Henning (1973); Brown & Perham (1974)

Note: The E3 subunits from various sources (including mammalian and bacterial cells) have been shown to be very similar in many respects and chain molecular weights are in good agreement. However, Perham & Thomas (1971) have reported a slightly higher value of 59,000 for the *E. coli* E3 subunit.

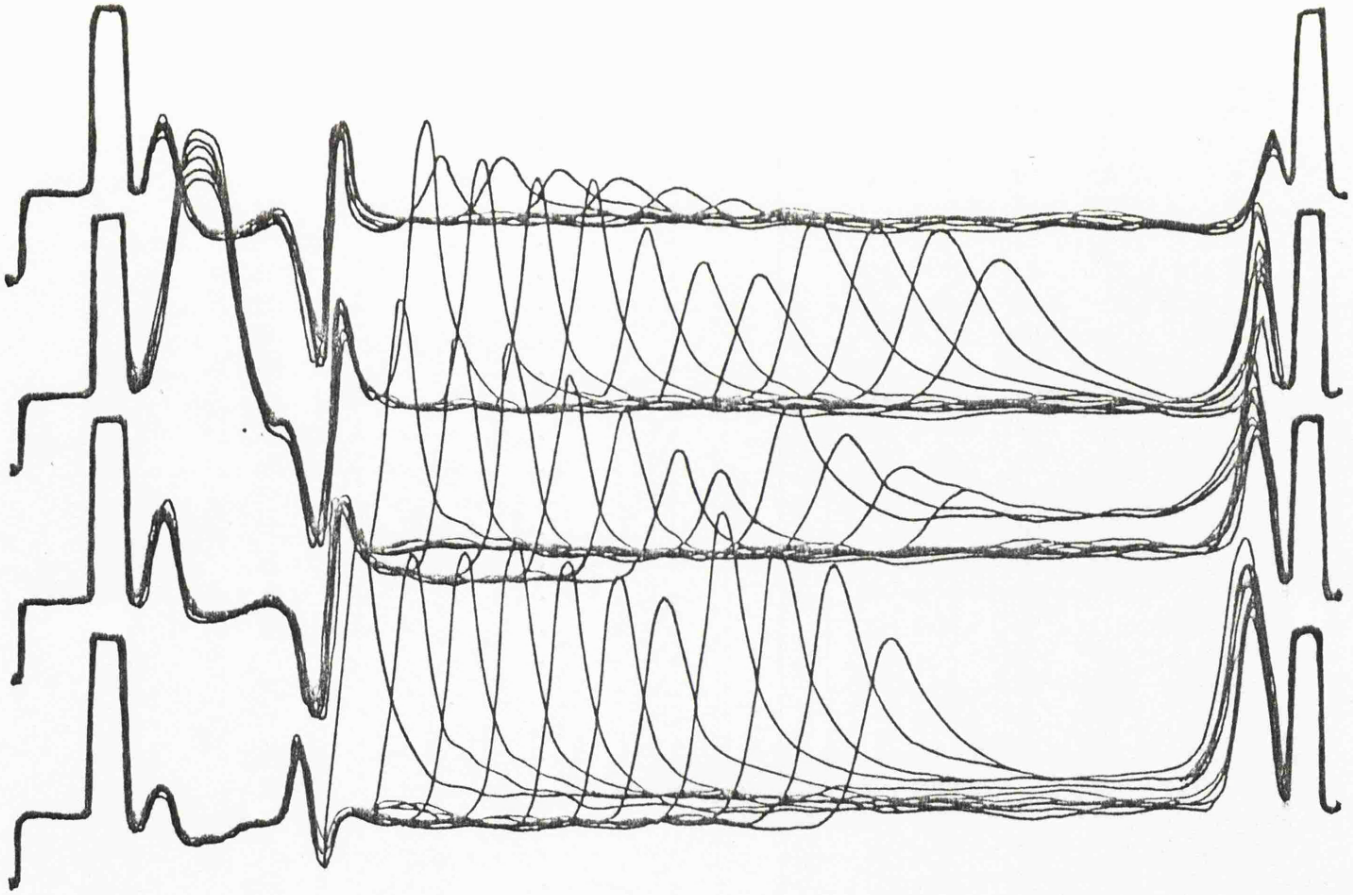


Fig. 20. Sedimentation analysis of purified α-OGDH complex from A. lwoffi.
Samples of α-OGDH (2·56, 5·13, 6·83 and 10·25mg ml^{-1} in 0·1M Tris-HCl, pH 8·0 containing 8% glycerol) were sedimented at 40,000rpm at 5°C in an MSE Centriscan 75 ultracentrifuge using Schlieren optics to observe the boundary position at suitable time intervals. Experimental details are given in section II.2·10.

Sedimentation values for α-OGDH were determined at a number of different protein concentrations and corrected for solvent and temperature (to give $s_{20,w}$ values) as described in section II.2.10. The $s_{20,w}$ values were then extrapolated to zero protein concentration (Fig. 21) giving a $s^{o}_{20,w}$ of 29.86S.

At very low protein concentrations the sedimentation values deviated from normal, such that a decrease in $s_{20,w}$ value was observed with decrease in protein concentration within this range. Parker & Weitzman (1973) have reported a similar observation using the same enzyme, but failed to observe the more typical concentration dependence at high protein concentration (Schachman, 1959) since their data were restricted to low enzyme concentrations (Fig. 21). It is probable that at very low concentrations dissociation of the complex occurs resulting in a reduction in the apparent $s_{20,w}$ value as observed by Danson & Rowe (personal communication) using the PDH complex of E. coli.

(b) Molecular weight determination

The molecular weight of the α-OGDH complex was calculated using an equation derived by Dr. A.J. Rowe (personal communication):

$$M = N\left(\frac{6\pi\eta s^{o}_{20,w}}{1-\bar{\nu}\rho}\right)^{3/2}\left(\frac{3K_s}{16\pi}\right)^{1/2} \quad \ldots\ldots (1)$$

where M = molecular weight; $\bar{\nu}$ = partial specific volume = 0.73 (assumed); ρ = density of water at 20°C = 0.998203g cm^{-3}; η = coefficient of viscosity of water at 20°C = 0.01005g cm^{-1} s^{-1}; N = Avogadro's number = 6.023×10^{23}; and where:

$$K_s = K'_s - \bar{\nu} \quad \ldots\ldots (2)$$

$s^{o}_{20,w}$ and K'_s (a constant) were derived from the relationship: $s = \frac{s^{o}}{1+K_s c}$ (Bowen, 1970d) and found to be 29.86×10^{-13}s and 17.70cm^3 g^{-1} respectively. By substituting these values in equation (1) the molecular

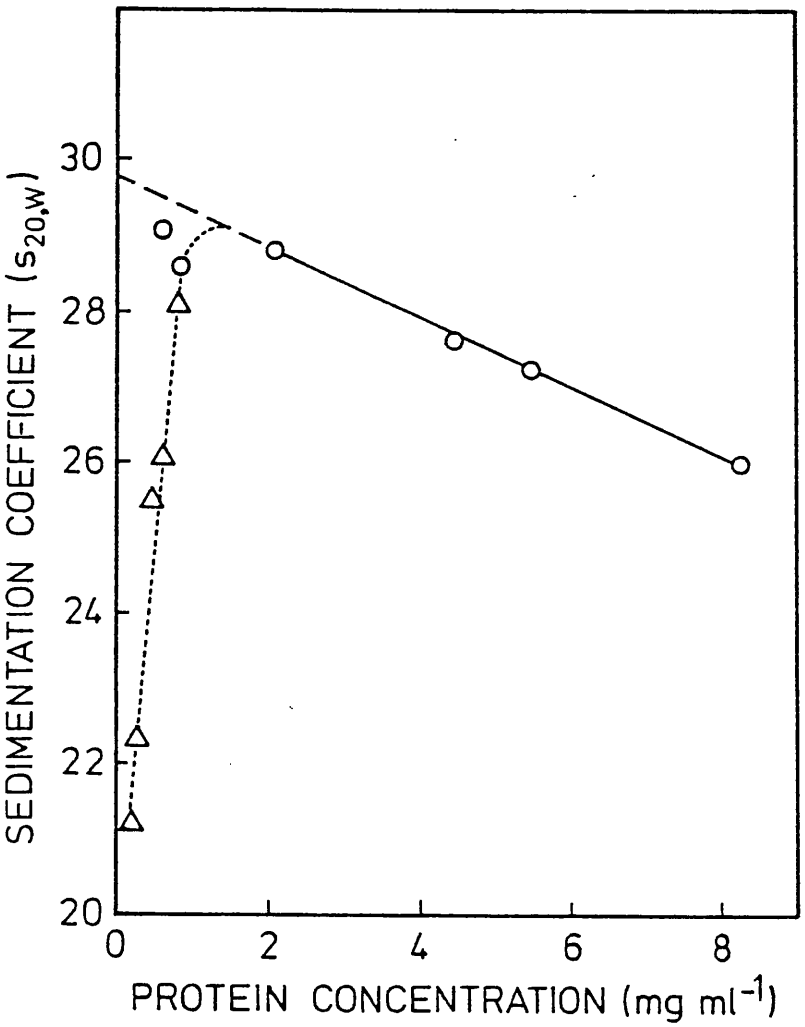


Fig. 21. Dependence of $S_{20,w}$ on protein concentration. Sedimentation coefficients were computed from the sedimentation data given in Fig. 20 and corrected to $S_{20,w}$ values as described in section II.2·10; (o—o). (Δ---Δ); $S_{20,w}$ values for the same enzyme obtained by Parker & Weitzman (1973).

weight of the complex was calculated to be $1 \cdot 82 \times 10^6$. Table 8 shows a comparison of the molecular weight and sedimentation coefficient with those from other sources. It would appear that the α-OGDH complex from A. lwoffi is slightly smaller than the complexes from other organisms. This difference is consistent with measurements of the diameters of complexes made by electron microscopy. The A. lwoffi enzyme has been shown to be a polyhedron of 200 to 220Å diameter (Parker & Weitzman, 1973) compared with 260Å for the pig heart complex (Tanaka et al., 1972).

Table 8. Comparison of the molecular weights and sedimentation coefficients of α-OGDH complexes isolated from various sources.

Enzyme source	$S^{o}_{20,W}$	Molecular weight	Reference
E. coli	40	2.4×10^6	Koike *et al.* (1960)
Pig heart	35.7	2.7×10^6	Hirashima *et al.* (1967)
Pig heart	31.5	2.7×10^6	Tanaka *et al.* (1972)
Pigeon breast muscle	31	-	Severin & Gomazkova (1972)
A. lwoffi	29.9	1.82×10^6	Section II.3.6

CHAPTER III

DISSOCIATION STUDIES

III.1 INTRODUCTION

It was described in the General Introduction how α-OGDH and PDH complexes from various sources are composed of three types of subunits (E1, E2 and E3) each with its own catalytic function, the E2 subunit forming a core to which E1 and E3 subunits are non-covalently attached. The E1 subunit of the α-OGDH complex of A. lwoffi is of particular interest since regulation of this component by AMP and possibly NADH has been demonstrated (Parker & Weitzman, 1972, 1973). Clearly a detailed study of the regulatory properties of the complex would be facilitated if the individual subunits could be isolated in enzymically active forms. Thus one of my main aims was to resolve the complex into its three different components, enabling the following questions to be investigated:

(a) Are the regulatory sites for AMP and NADH really located on E1 and does this subunit modulate its activity in response to these effectors when it is separated from the other components of the complex?

(b) Can the E1 subunit from the A. lwoffi complex be reassociated with E2·E3 from another organism (in particular one that does not show nucleotide control e.g. E. coli or Pseudomonas sp.) and thereby reconstitute a nucleotide-regulated α-OGDH complex? Such hybridization would suggest a close similarity between the subunit structures of the α-OGDH complexes from different organisms.

(c) How does the E1 component of A. lwoffi differ from those of E. coli or Pseudomonas sp.? Is its sensitivity to effectors associated with a relatively more complex structure?

Both α-OGDH and PDH complexes from E. coli and mammalian cells have

been successfully resolved into their component subunits and Table 9 summarizes the methods that have been employed. α-OGDH and PDH complexes from E. coli are readily dissociated to E1•E2 and E3 in the presence of urea and into E1 and E2•E3 at high pH values. E3 may be dissociated from the mammalian complexes by urea, but the use of high pH values results in a loss of E1 activity (Glemzha et al., 1966; Hayakawa & Koike, 1967). Alternative procedures for the resolution of E1 from E2 were therefore employed including the use of potassium iodide and guanidine hydrochloride (see Table 9).

A novel procedure has recently been described for the dissociation of pig heart α-OGDH (Imai & Tomika, 1974; Tomika et al., 1974). It was shown by sucrose density gradient analysis that the addition of basic polypeptides, such as poly-L-lysine, to the complex caused dissociation into its component subunits. Unfortunately, however, the authors did not actually separate the dissociated subunits.

It was clear, therefore, at the onset of my work that a number of successful procedures were already available by which α-OGDH and PDH complexes from other sources could be resolved. Consequently, these procedures were applied to the α-OGDH complex of A. lwoffi in the hope that one would be suitable for this complex. As a control α-OGDH from E. coli was also studied.

Table 9A. Methods used for the dissociation and resolution of the component subunits of PDH complexes

Enzyme source	Method	Separation achieved	References
E. coli	(i) Calcium phosphate-cellulose chromatography in the presence of 4M urea.	E3 from E1·E2 or from E2.	Koike *et al.* (1963); Reed & Willms (1966); Koike & Reed (1961)
	(ii) Calcium phosphate-cellulose chromatography at pH 9·5.	E1 from E2·E3 or from E2.	
Pig heart	(i) Calcium phosphate-cellulose chromatography in the presence of 4M urea.	E3 from E1·E2 or from E2.	Hayakawa & Koike (1967); Hayakawa *et al.* (1969)
	(ii) Ammonium sulphate fractionation in the presence of 0·3M potassium iodide.	E1 from E2.	
Bovine kidney and heart	(i) Incubation with 10mM dithiothreitol at high salt concentration followed by Sepharose gel filtration.	E2 from E1 and E3.	Linn *et al.* (1972)
	(ii) Ammonium sulphate fractionation of E1 and E3 in the presence of 10mM dithiothreitol.	E1 from E3.	

Table 9B. Methods used for the dissociation and resolution of the component subunits of α-OGDH complexes.

Enzyme source	Method	Separation achieved	References
E. coli	Calcium phosphate-cellulose chromatography: (i) Elution of E1 at pH 10. (ii) Elution of E2 with 1% ammonium sulphate in 0·1M phosphate buffer followed by 4M urea. (iii) Elution of E3 with 4% ammonium sulphate and 2·5M urea.	E1 from E2·E3 E2 from E3	Mukherjee *et al.* (1965)
E. coli	(i) Incubation at pH 9 in high salt concentration followed by gel filtration on Sepharose 6B. (ii) Gel filtration on Sepharose 4B in the presence of 6M urea and 5% ammonium sulphate.	E1 from E2·E3 E2 from E3	Pettit *et al.* (1973)
Pig heart	Calcium phosphate-cellulose chromatography in the presence of 2·5M urea.	E3 from E1·E2	Massey (1960)
Pig heart	(i) Calcium phosphate-cellulose chromatography in the presence of 2·5M urea and 1% ammonium sulphate. (ii) Gel filtration in the presence of 0·7M guanidine-HCl and 2mM dithiothreitol (Tanaka *et al.* also include 0·5% Triton X100).	E3 from E1·E2 E1 from E2	Koike *et al.* (1971) Tanaka *et al.* (1972)
Pigeon breast muscle	(i) Alternating abrupt temperature changes and ammonium sulphate precipitations to make the complex labile, followed by calcium phosphate-cellulose chromatography in the presence of 3M urea. (ii) Calcium phosphate-cellulose chromatography at pH 7·5, followed by precipitation of E1 with ammonium sulphate and further purification by gel filtration on Sephadex G200.	E3 from E1·E2 E1 from E2·E3	Severin & Gomazkova (1972)

III.2 MATERIALS AND METHODS

III.2·1 Preparation of calcium phosphate suspended on cellulose

Calcium phosphate suspended on cellulose was prepared by the method of Reed & Willms (1966) as follows:

(1) To 31g of cellulose powder (Whatman CF11) suspended in 200ml of distilled water were added 50ml of a solution containing 2M $CaCl_2$, 1·34M KH_2PO_4 and 0·66M HCl.

(2) The suspension was stirred rapidly for 2min, 50ml of 8M NH_4OH added, and stirring continued as the mixture thickened.

(3) After allowing to stand overnight at 5°C the supernatant fluid was decanted off. The calcium phosphate-cellulose was washed by decantation with several 3 litre volumes of distilled water until the supernatant fluid gave a negative result with Nessler's Reagent. "Fines" were removed during the washing procedure.

(4) Finally, the calcium phosphate-cellulose was collected by centrifugation (5000xg) and resuspended in 20mM phosphate buffer, pH 6·0. The suspension was stored at 5°C for at least a week before use.

III.2·2 Separation of E. coli α-OGDH complex subunits by the method of Mukherjee et al. (1965)

(1) A column (1·5 x 1·0cm) of calcium phosphate suspended on cellulose was equilibrated with 50mM ethanolamine-phosphate buffer, pH 10·0, (50mM ethanolamine solution brought to pH 10·0 with KH_2PO_4) containing 1M NaCl.

(2) Purified E. coli α-OGDH complex (0·2ml, 22mg ml^{-1}) was applied to the column and left to bind for 30min.

(3) The column was washed with 10ml of 50mM ethanolamine-phosphate buffer, pH 10·0, containing 1M NaCl and 6 x 0·5ml fractions were collected. The E1 component should be eluted at this stage.

(4) The column was then eluted with 0·1M potassium phosphate buffer, pH 7·5, containing 1% $(NH_4)_2SO_4$ and 0·5ml fractions (which should contain

E2 activity) were collected.

(5) To remove final traces of E2 activity the column was eluted with 0·1M potassium phosphate buffer, pH 7·5, containing 4M urea and 1% $(NH_4)_2SO_4$ and 6 x 0·5ml fractions were collected.

(6) Finally the column was eluted with 0·1M potassium phosphate buffer, pH 7·5, containing 2·5M urea and 6 x 0·5ml fractions were again collected (which should contain E3 activity).

All fractions were assayed for E1, E2, E3 and whole complex activities.

III.2·3 Separation of E. coli α-OGDH complex subunits by gel filtration in the presence of 4M urea

(1) Purified α-OGDH complex (36·8mg) in 2·0ml of buffer containing 4M urea and 5% $(NH_4)_2SO_4$ was left on ice for 90min to dissociate.

(2) The mixture was then applied to a column (23·6 x 1·6cm) of Sepharose 4B, pre-equilibrated with 0·1M phosphate buffer containing 4M urea and 5% $(NH_4)_2SO_4$.

(3) The column was eluted with the same buffer and 60 x 1·0ml fractions were collected. Fractions were assayed for protein, lipoic acid content and E3 activity.

III.2·4 Separation of E. coli α-OGDH complex subunits by gel filtration at pH 9·0 in the presence of 1M NaCl

(1) E. coli α-OGDH complex (1·0ml; 18·4mg ml^{-1}) was brought to pH 9·0 by the addition of 0·1ml of 1M glycine buffer, pH 9·5, (or 0·1M 2-amino, 2-methyl, 1,3-propanediol buffer, pH 9·5) and NaCl was added to a final concentration of 1M.

(2) After standing on ice for 30min the mixture was applied to a column (23·5 x 1·6cm) of Sepharose 4B pre-equilibrated with 0·1M glycine buffer, pH 9·0, (or 0·1M 2-amino, 2-methyl, 1,3-propanediol buffer, pH 9·0) containing 1M NaCl.

(3) The column was eluted with the same buffer and 60 x 1·0ml fractions

were collected. These were assayed for protein, E2-E3 interaction (with and without commercial dihydrolipoamide dehydrogenase) and E1, E3 and whole complex activities.

III.2.5 Separation of A. lwoffi α-OGDH complex subunits by gel filtration in the presence of 6M urea

(1) Purified A. lwoffi α-OGDH complex (2.2mg) in 0.2ml of buffer containing 6M urea and 5% $(NH_4)_2SO_4$ was left on ice for 1h to dissociate.

(2) The mixture was then applied to a column (23.5 x 1.6cm) of Sepharose 4B pre-equilibrated with 0.1M phosphate buffer, pH 7.5, containing 6M urea and 5% $(NH_4)_2SO_4$.

(3) The column was eluted with the same buffer and 70 x 1.0ml fractions were collected and assayed for protein, E2-E3 interaction (with and without commercial dihydrolipoamide dehydrogenase) and E3 activity.

III.2.6 Separation of A. lwoffi α-OGDH complex subunits by gel filtration at pH 9.0 in the presence of 1M NaCl

(1) A. lwoffi α-OGDH complex (0.2ml; 10.8mg ml^{-1}) was brought to pH 9.0 by the addition of 1M glycine buffer, pH 9.5, and NaCl was added to a final concentration of 1M. It was left on ice for 1h to dissociate.

(2) The enzyme was then applied to a column (23.5 x 1.6cm) of Sepharose 4B, pre-equilibrated with 0.1M glycine buffer, pH 9.0, containing 1M NaCl.

(3) The column was eluted with this buffer and 60 x 1.0ml fractions collected and assayed for protein, E2-E3 interaction (with and without commercial dihydrolipoamide dehydrogenase), E3 activity and whole complex activity.

III.3 RESULTS AND DISCUSSION

Before attempting to dissociate the α-OGDH complex of *A. lwoffi* into its component subunits using the methods described for other systems (see section III.1), it was necessary to investigate the effects of the various dissociating conditions on the component activities, since it was already known that the *A. lwoffi* complex is unstable under some of the conditions employed, e.g. high pH values (greater than 9·0) and high salt concentrations.

III.3·1 Effects of dissociating conditions on enzyme activities

(a) *Urea.* Fig. 22 summarizes the effects of urea on both the whole enzyme complex and the individual component activities. The E1 subunit was found to be extremely sensitive to urea; even at the lowest concentration of urea investigated (2M) apparently complete loss of activity was observed. The E2 component was also inactivated by urea but to a lesser extent. Progressively more inactivation was seen as the urea concentration was increased from 2 to 5M. Finally, the E3 subunit was stable when incubated with 2-3M urea and only at higher concentrations (4-5M) was inactivation observed.

The inactivation of whole complex activity by urea was found to be irreversible by both dilution and removal of urea by gel filtration. Under the standard assay conditions described for the whole complex (section I.2·2) the enzyme is diluted up to 100-fold in the assay cuvette. Consequently, on assaying complex that has been incubated with urea, the urea concentration may be reduced 100-fold by omitting further urea from the assay mixture. Under these conditions no rate was observed, even after 30min. It would therefore seem likely that the effect of urea is irreversible. Secondly, complex that had been incubated for 3h in the presence of 4M urea was passed through a column (12 x 1·5cm) of Sephadex G25 which had been pre-equilibrated with MET-8 buffer. Fractions (1·0ml) were collected and assayed for whole complex activity, but none could be detected. In a control experiment using

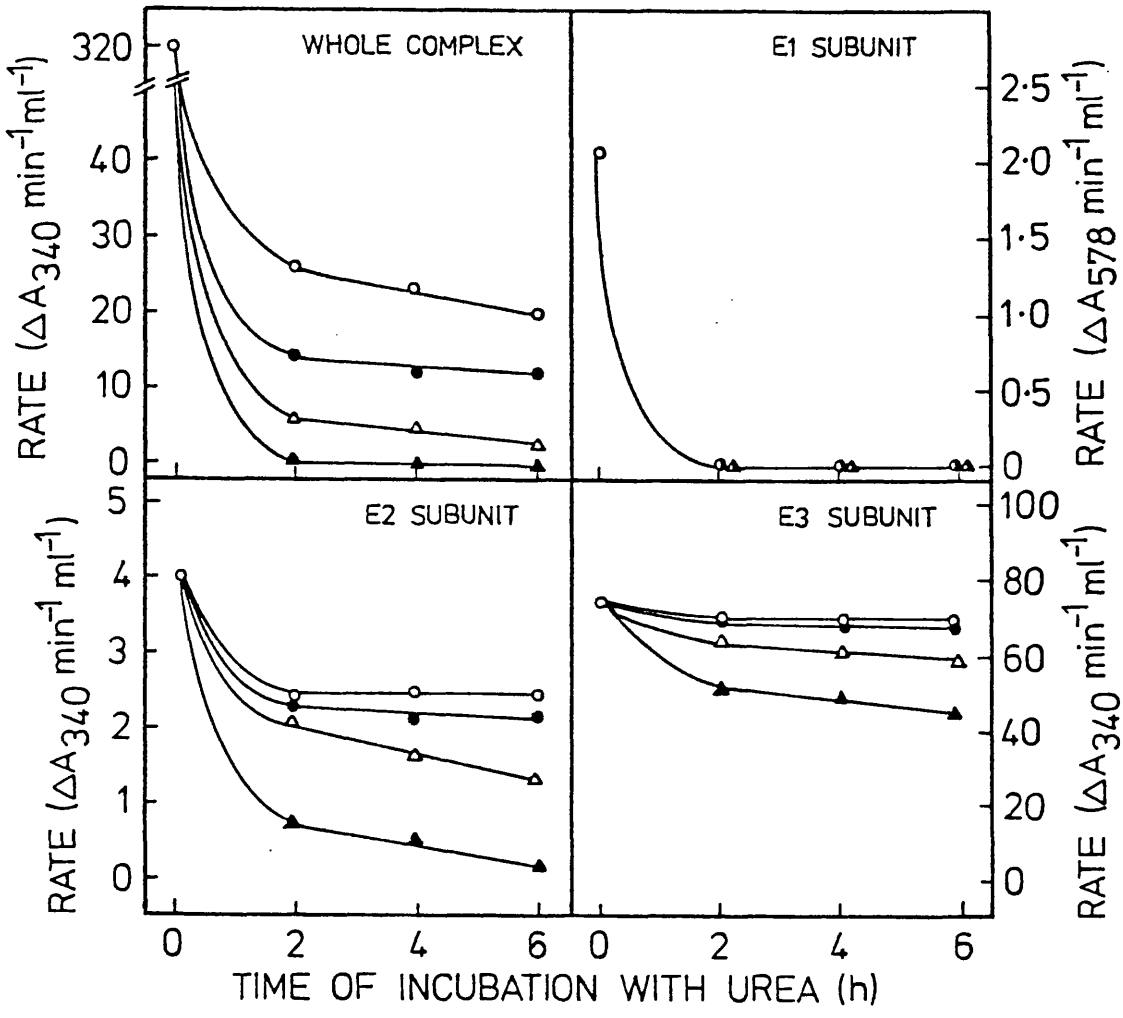


Fig. 22. The effect of urea on A. lwoffi α-OGDH complex and its component enzyme activities.

Samples of purified α-OGDH complex were diluted to 2·5mg ml^{-1} with MET-8 buffer and made 2, 3, 4 or 5M with respect to urea. At 2h intervals aliquots were assayed for complex and component activities. (o——o), 2M urea; (•——•), 3M urea; (Δ——Δ), 4M urea; (▲——▲), 5M urea.

enzyme that had not been treated with urea a peak containing all of the original activity was obtained.

The extreme sensitivity of E1 to urea was unfortunate since this subunit is of particular interest due to its regulatory properties. Protection against loss of E1 activity was investigated using a variety of substrates and effectors, but could not be achieved. However, marginal protection was obtained in the presence of 0·2mM AMP at a urea concentration of 2M, 25% of the E1 activity remaining after 2h of incubation. As the urea concentration was increased this protection was no longer observed.

(b) Potassium iodide, sodium bromide, guanidine hydrochloride and high pH values. The effects of 0·3M KI, 0·3M NaBr, 0·6M guanidine hydrochloride and high pH values on A. lwoffi α-OGDH complex are summarized in Table 10. Since KI was found to interfere with the E1 assay its effect on this component enzyme could not be studied. Under all the conditions investigated, inactivation of the E1 component was observed with partial protection in the presence of 0·2mM AMP. The E2 subunit was partially inactivated in the presence of NaBr, KI or at pH 9·5, whilst loss of E3 activity was seen on incubation with guanidine hydrochloride.

III.3·2 Preliminary attempts at dissociation and separation of enzyme subunits

(a) α-OGDH complex from A. lwoffi

Attempts were made to dissociate and separate the subunits of the A. lwoffi α-OGDH complex by incubation with 2·5M urea followed by Sepharose gel filtration. Successful dissociation of the whole complex into E1·E2 and E3 should result in elution of the larger molecular weight species first, i.e. E1·E2 followed by E3. However, it can be seen from Fig. 23A that the E3 activity eluted with the main protein peak. Unfortunately, E1 and E2 activities could not be detected, presumably due to inactivation and/or dilution during gel filtration. Fractions containing E3 activity were re-run on a second column together with lactate dehydrogenase (M.W. 140000)

Table 10. Stability of A. lwoffi α-OGDH complex and component enzyme activities to various treatments.

Enzyme preparation	Treatment	Incubation time (h)	Activity (absorbance units min^{-1} ml^{-1}) Complex	E1	E2	E3
1	None	-	372	1·02	5·1	150
1	0·3M KI	2	284	-	2·5	150
1	0·3M KI	4	89·2	-	-	150
1	0·3M NaBr	2	202	0·14	3·76	148
1	0·3M NaBr	4	156	-	2·8	148
2	None	-	319	9·2	2·64	146
2	0·6M GuHCl	2	196	0	2·48	78
3	pH 8·0	2	336	8·1	12·3	138
3	pH 9·5	2	0	0	6·6	140

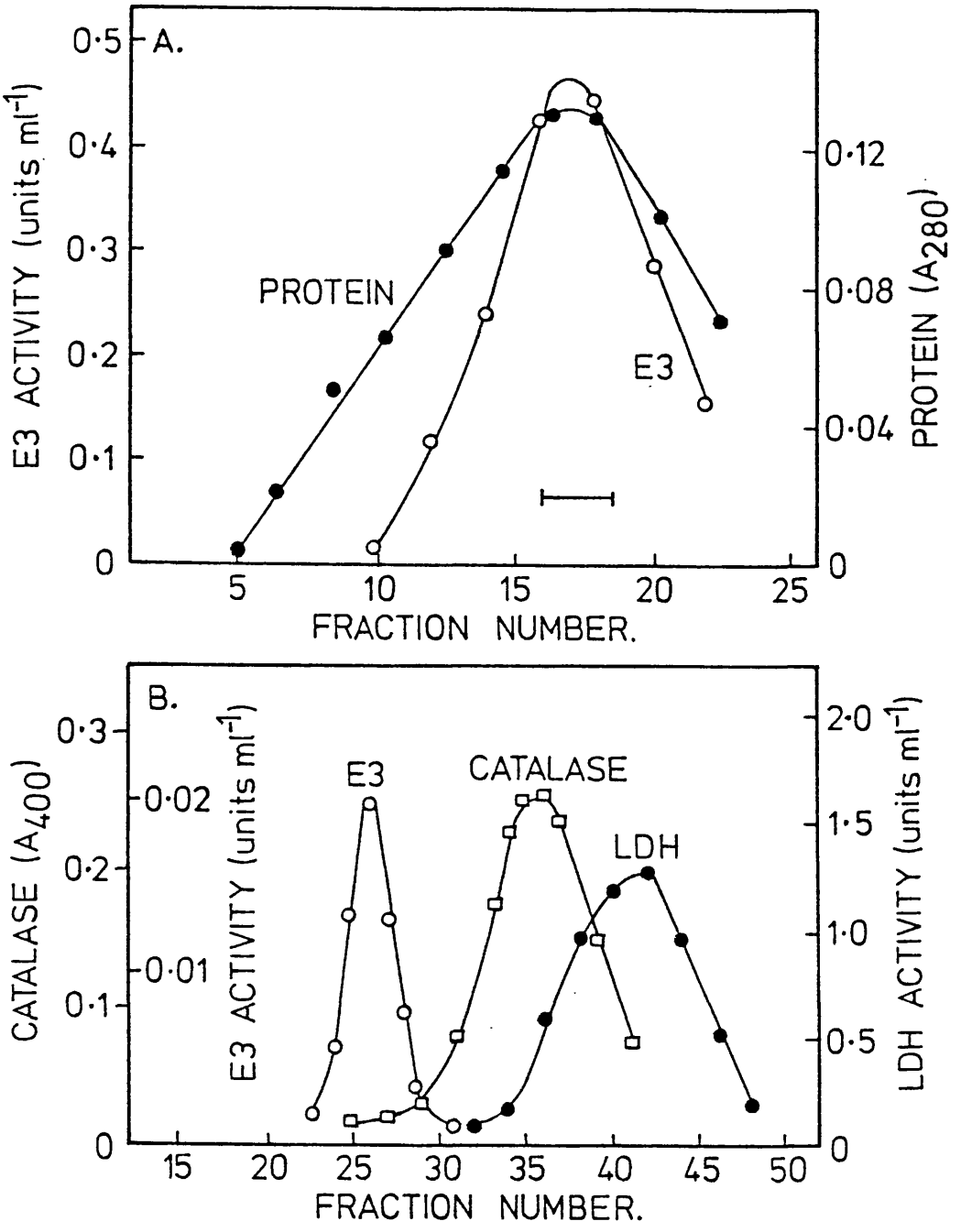


Fig. 23. Gel filtration of A. lwoffi α-OGDH complex in the presence of 2·5M urea.

1·25mg of purified α-OGDH complex in MET-8 buffer containing 2·5M urea was applied to a Sepharose 4B column (30x2·5cm) and eluted with the same buffer (Fig. 23A). Fractions (0·5ml) were assayed for E3 activity (o——o) and protein (●——●).

Fractions containing high levels of E3 activity were pooled, mixed with catalase and lactate dehydrogenase, and re-chromatographed on a Sephadex G200 column (32x2·5cm) equilibrated with MET-8 buffer. Fractions (2ml) were assayed for the three enzyme activities (Fig. 23B).

and catalase (M.W. 248000) using a Sephadex G200 column. From Fig. 23B it can be seen that the E3 activity was eluted first, in the void volume, suggesting that it was in the form of a large molecular weight species, presumably undissociated but inactivated complex.

Further attempts were performed in the presence of higher urea concentrations (ranging from 2 to 4M) and using either Sepharose gel filtration or calcium phosphate-cellulose chromatography for the separation of the component enzymes. It has been found that the E3 components of E. coli and mammalian complexes are more strongly bound to calcium phosphate-cellulose than the other two component enzymes (Massey, 1960; Koike et al., 1963) such that after dissociation with urea, E1·E2 can be eluted with 50mM phosphate buffer, pH 7·5, containing 1% $(NH_4)_2SO_4$ and E3 then washed off with 0·1M phosphate buffer containing 4% $(NH_4)_2SO_4$. This method was therefore applied to the α-OGDH complex of A. lwoffi in the presence of 0·2mM AMP (to protect E1 against inactivation) using either 0·3M NaBr or 2-4M urea for dissociation. Unfortunately, these experiments were not very successful since E1 and E2 activities often proved to be difficult to detect after chromatography. Furthermore, in many cases complete dissociation did not occur so that whole complex activity was eluted at high salt concentration. Nevertheless, a limited degree of success was obtained as shown by the presence of E1 and E2 activities in fractions eluted at low salt concentrations, in the absence of E3 activity.

Experiments involving Sepharose gel filtration in conjunction with 4M urea resulted in a similar pattern to that obtained with 2·5M urea in Fig. 23A.

(b) α-OGDH complex from E. coli (Crookes)

Due to the very limited success achieved with the α-OGDH complex from A. lwoffi, control experiments were done using the complex from E. coli. Preliminary studies were performed to investigate the effect of 4M urea and 1% $(NH_4)_2SO_4$ on the three component enzyme activities. The E3 component was

found to be relatively stable (as with the A. lwoffi complex), whereas large loss of E1 activity occurred (greater than 50% in 45min). The rates obtained for the E2 assay in the E. coli system were very poor and hence this component could not be followed during these studies.

Perhaps it was the sensitivity of the E. coli α-OGDH E1 component to urea that led other workers (Mukherjee et al., 1965; Pettit et al.,1973) to use high pH values to dissociate the E1 component, followed by urea to dissociate the remaining E2•E3 subcomplex. In addition, Severin & Gomazkova (1972) reported that the degree of inactivation of pigeon breast muscle α-OGDH complex by urea depends not only on the time of contact between the enzyme and urea, but also on the protein concentration. Since the present experiments were performed at a relatively low protein concentration compared with those used in other systems, exogenous protein was added to the enzyme complex to see if this would protect it against urea inactivation. Bovine serum albumin was added to α-OGDH solutions to give a final concentration of 50mg ml^{-1} prior to incubation with urea or at pH 9.5. Unfortunately, bovine serum albumin was found to interfere with the DCPIP E1 assay such that an apparent rate was obtained in the absence of α-OGDH (see section I.3.2). Therefore, E1 assays performed in the presence of bovine serum albumin had to be corrected for this non-enzymic rate. Table 11 shows the effect of exogenously added bovine serum albumin on the inactivation of α-OGDH complex by urea and high pH values. Bovine serum albumin partially protected both the A. lwoffi and the E. coli complexes against loss of E1 activity. In conclusion, therefore, inactivation of α-OGDH by urea and high pH values is probably increased at low protein concentrations.

The α-OGDH complex from E. coli was subjected to gel filtration on a Sepharose 4B column (12.0 x 1.0cm) in the presence of 4M urea and 5% $(NH_4)_2SO_4$. On the basis of other published work E3 would be expected to be

Table 11. Protection by bovine serum albumin against loss of E1 activity.

All samples were incubated at 4°C for 2h prior to being assayed.

Experimental conditions	Activity (absorbance units min^{-1} ml^{-1})			
	Whole complex	E1	E2	E3
A. lwoffi α-OGDH				
0·17mg of α-OGDH in 0·5ml of MET-8 buffer.	336	8·1	12·3	138
0·17mg of α-OGDH in 0·5ml of MET-8 buffer containing 4M urea.	0	1·0	0	120
0·17mg of α-OGDH in 0·5ml of MET-8 containing 4M urea and 25mg BSA.	0	3·0	0	129
0·17mg of α-OGDH in 0·5ml of 50mM 2-methyl, 2-amino 1,3-propanediol buffer, pH 9·5.	0	0	6·6	141
0·17mg of α-OGDH in 0·5ml of buffer, pH 9·5, containing 25mg of BSA.	60	2·0	6·0	140
E. coli α-OGDH				
0·34mg of α-OGDH in 0·5ml of 20mM phosphate buffer, pH 7·0.	610	8·7	-	22·8
0·34mg of α-OGDH in 0·5ml of buffer, pH 7·0, containing 4M urea.	98	0	-	6·0
0·34mg of α-OGDH in 0·5ml of buffer, pH 7·0, containing 4M urea and 25mg of BSA.	75	8·0	-	12·0
0·34mg of α-OGDH in 0·5ml of buffer, pH 9·5.	420	2·3	-	10·2
0·34mg of α-OGDH in 0·5ml of buffer, pH 9·5, containing 25mg of BSA.	462	4·4	-	15·6

dissociated from E1•E2 in the presence of urea, the latter species being eluted first due to its larger molecular weight. It can be seen from Fig. 24 that two protein peaks were obtained, E3 activity being coincident with both of them. On the other hand E1 activity was detected solely in Peak 1. Presumably, incomplete dissociation had occurred with the E1•E2 subcomplex eluting with the undissociated complex due to the relatively small difference in their molecular weights (2•1 and 2•8 x 10^6 respectively). Thus Peak 2 should consist solely of E3 component.

This experiment was repeated after a prolonged preincubation of the complex with urea to obtain a more complete dissociation and resulted in the disappearance of E3 activity from the first peak, but the E3 activity remained in Peak 2.

Finally, dissociation and separation of E. coli α-OGDH complex activities by calcium phosphate-cellulose chromatography was found to be partially successful. Table 12 shows the results obtained using the dissociation procedure of Mukherjee et al. (1965). Although no E1 activity could be detected, the presence of E2 and E3 activities in the appropriate fractions indicates that at least partial resolution had occurred.

III.3•3 Further attempts to dissociate and separate α-OGDH subunits

With the availability of the E2-E3 interaction and lipoic acid tests, further information could be obtained from dissociation studies. The position of the E2 component could be detected by its lipoic acid content and comparison of the rate for the E2-E3 interaction assay in the presence and absence of added dihydrolipoamide dehydrogenase could confirm whether the E3 component was present in fractions containing lipoic acid (i.e. the E2 component).

(a) α-OGDH complex from E. coli

Dissociation and separation of the E. coli α-OGDH complex subunits was investigated by Sepharose gel filtration in the presence of 4M urea and 5%

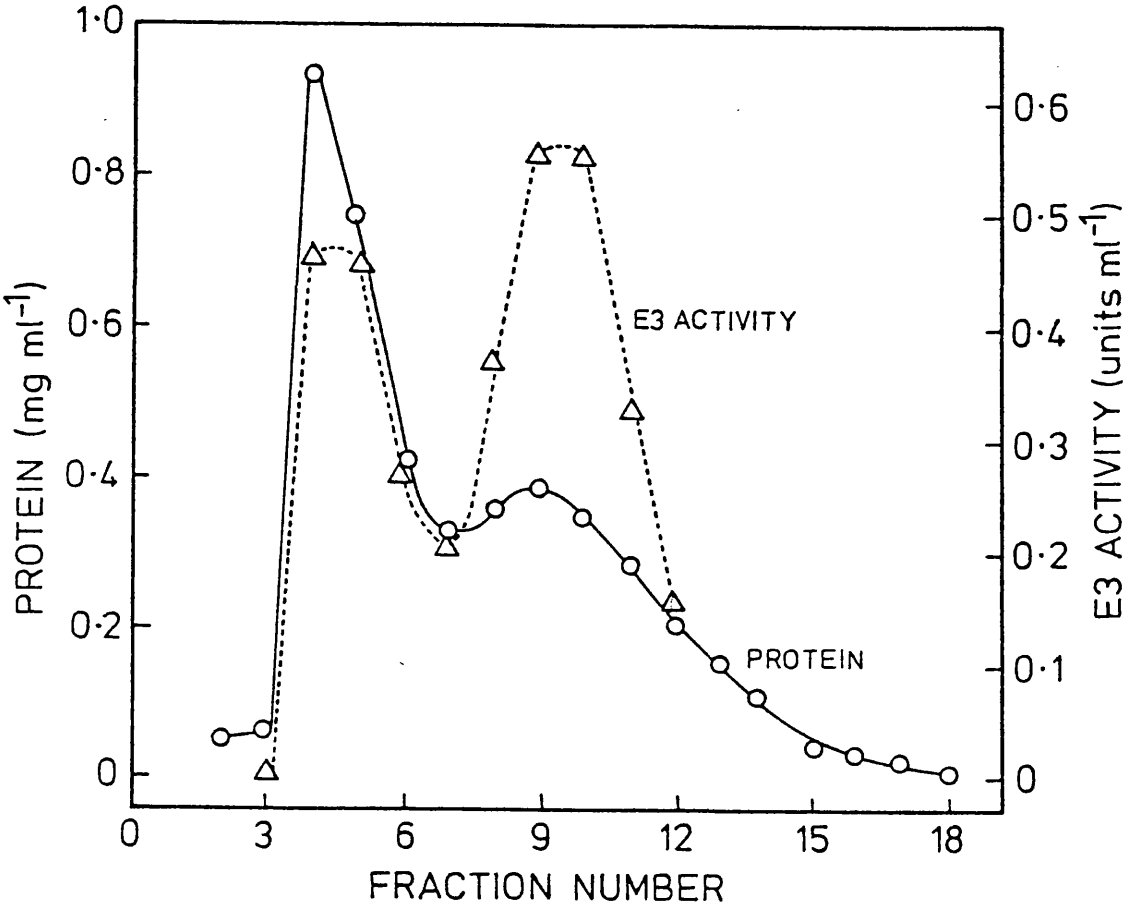


Fig. 24. Resolution of the E. coli α-OGDH complex by gel filtration in the presence of 4M urea and 5% ammonium sulphate.

0·2ml of purified complex (30mg ml^{-1}) was made 4M with respect to urea and applied to a Sepharose 4B column (12 x 1cm) equilibrated with 0·1M phosphate buffer, pH 7·5, containing 4M urea and 5% ammonium sulphate. Fractions (1ml) were collected and assayed for protein (o—o) and E3 activity (Δ···Δ).

Table 12. Resolution of E. coli α-OGDH complex by the method of Mukherjee et al. (1965).

Detailed experimental conditions are described in section III.2.2. (+), activity detectable; (-), activity not detectable.

Elution buffer	Expected elution	Activity detected			
		Complex	E1	E2	E3
50mM ethanolamine-phosphate buffer, pH 10, containing 1M NaCl	E1	-	-	-	-
0.1M potassium phosphate buffer, pH 7.5, containing 1% ammonium sulphate	E2	-	-	+	-
0.1M potassium phosphate buffer, pH 7.5, containing 1% ammonium sulphate and 4M urea	E2	-	-	-	-
0.1M potassium phosphate buffer, pH 7.5, containing 4% ammonium sulphate and 2.5M urea	E3	-	-	-	+

$(NH_4)_2SO_4$ (to dissociate the E3 component from the complex) or at pH 9·0 in the presence of 1M NaCl (to dissociate E1 from the complex).

(i) Dissociation by 4M urea and 5% $(NH_4)_2SO_4$

The separation obtained on a column (23·5 x 1·6cm) of Sepharose 4B after dissociation with 4M urea and 5% $(NH_4)_2SO_4$ is shown in Fig. 25. It can be seen that almost complete resolution of E3 from E2 (detected by its lipoic acid content) occurred, with the E3 peak being eluted in virtually the same position as Torula yeast dihydrolipoamide dehydrogenase. E1 activity was not detectable but should still be associated with the E2 peak. An anomalous protein peak was observed in Fraction 14. This was believed to be due to a slight precipitate that had formed in the enzyme solution on storage.

(ii) Dissociation at high pH values in the presence of 1M NaCl

Secondly, a column (23·5 x 1·6cm) of Sepharose 4B was run at pH 9·0 in the presence of 1M NaCl and the results are shown in Fig. 26. It would seem that partial dissociation of E3 from the complex occurred resulting in the appearance of E3 activity in Peak 2 (eluting in the same position as Torula dihydrolipoamide dehydrogenase). The remaining E3 activity co-eluted with E2 (detected by its lipoic acid content), presumably as an E2·E3 subcomplex. This is consistent with the observed stimulation of the E2-E3 interaction test by added dihydrolipoamide dehydrogenase in Peak 1 but not in Peak 2, suggesting that the ratio of E3 to E2 is greater in Peak 2 than in Peak 1. Since whole complex activity was detected in Peak 2 but not in Peak 1, this would suggest that the E1 component is eluted in Peak 2. This was confirmed directly by the E1 assay in subsequent experiments. The relatively low level of whole complex activity is presumably due to the presence of only small amounts of E2 in Peak 2 and possibly also inactivation of E1 due to the high pH.

In conclusion, therefore, it would appear that E1 has been successfully

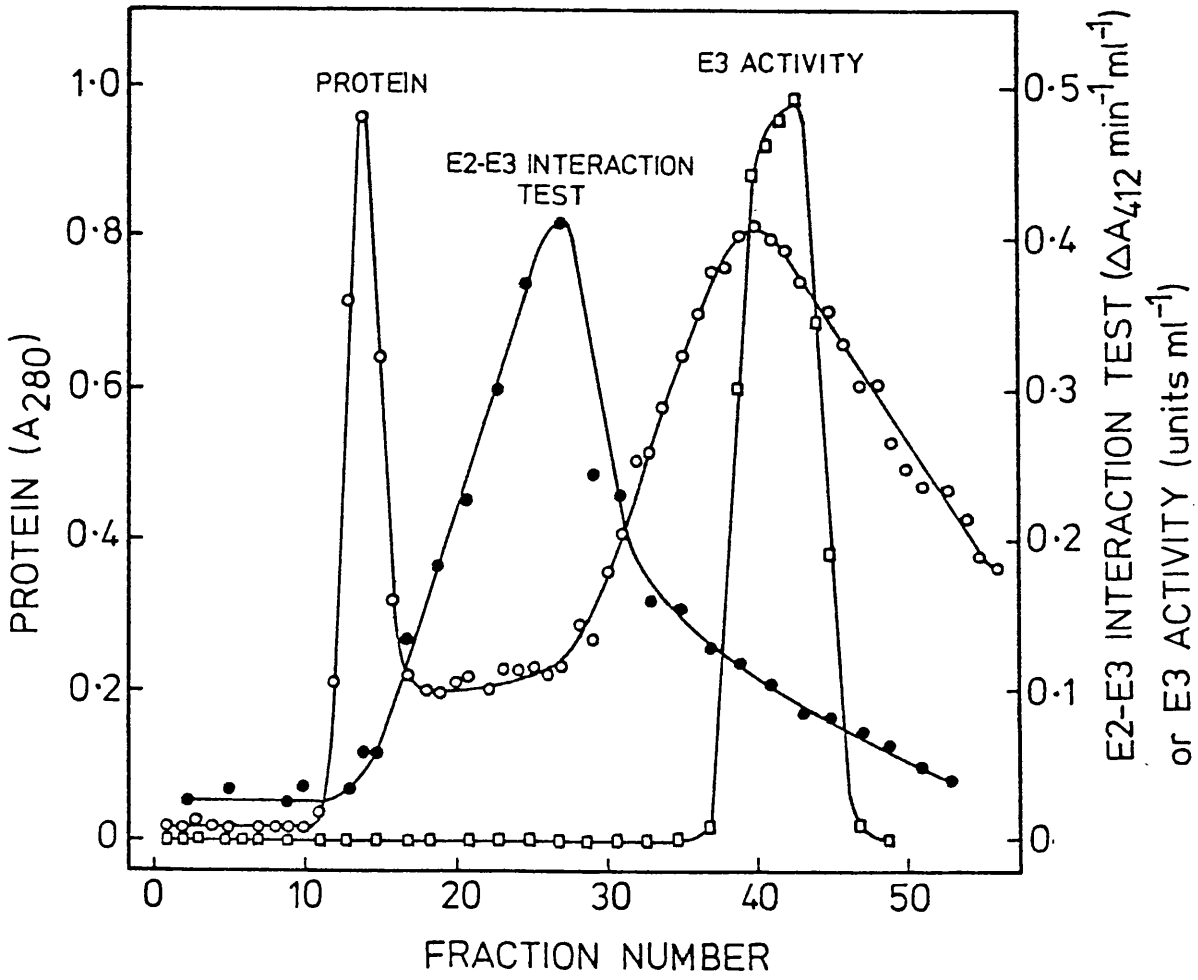


Fig. 25. Separation of the E. coli α-OGDH complex subunits by gel filtration in the presence of 4M urea and 5% ammonium sulphate.

All experimental details are given in section III.2.3. (o——o), protein; (•——•), E2-E3 interaction test; (□——□), E3 activity.

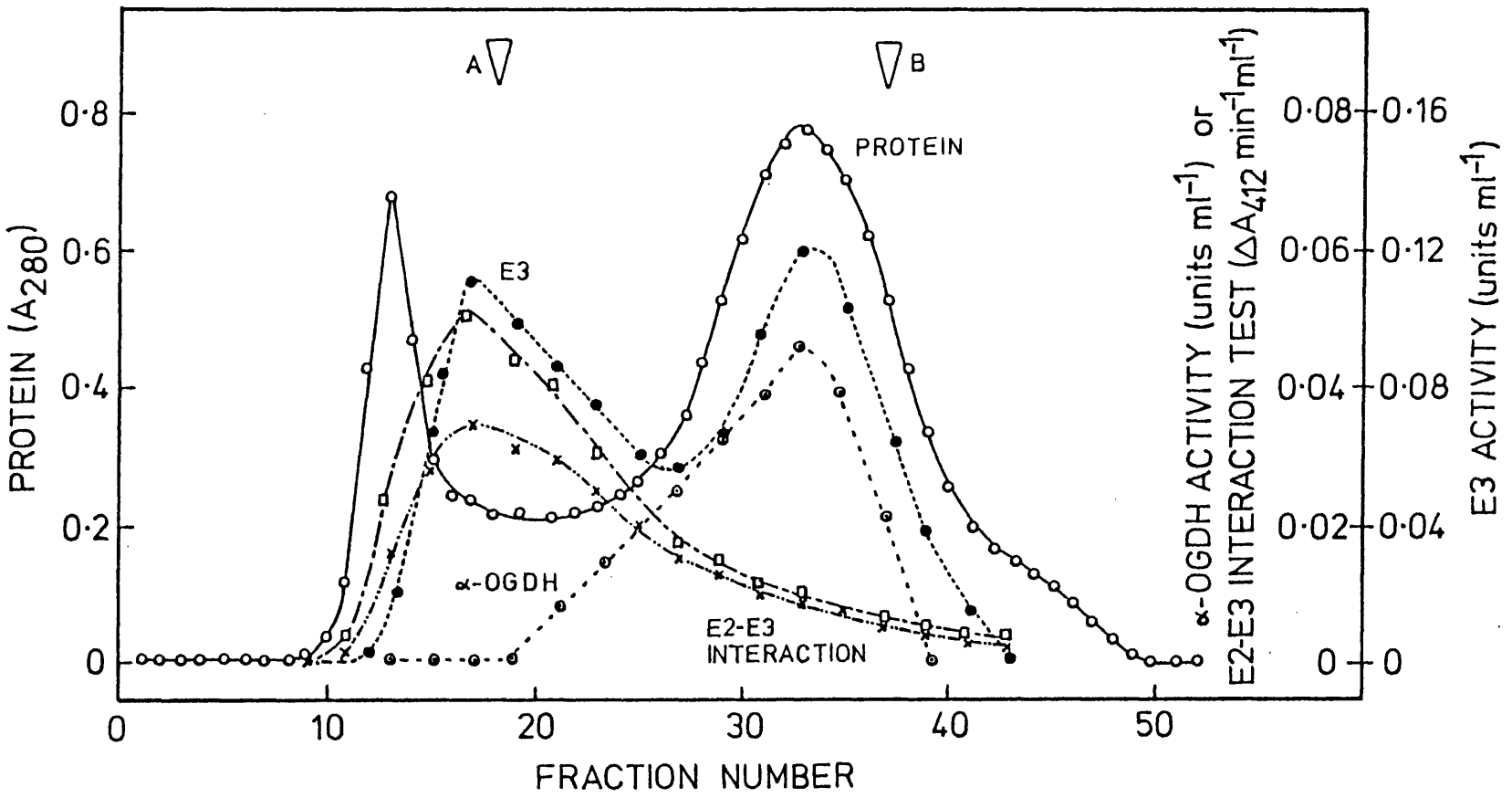


Fig. 26. Separation of E. coli α-OGDH complex subunits by gel filtration at pH 9 in the presence of 1M NaCl. All experimental details are given in section III.2·4. (o——o), protein; (⊙···⊙), whole complex activity; (•·····•), E3 activity; (×—··—×), E2-E3 interaction test (in the absence of added dihydrolipoamide dehydrogenase); (□·—·□), lipoic acid test (with added dihydrolipoamide dehydrogenase).

A, E. coli α-OGDH complex. B, Torula yeast dihydrolipoamide dehydrogenase.

dissociated and separated from this complex and, in addition, E3 has been partially dissociated.

(b) α-OGDH complex from A. lwoffi

(i) Dissociation in the presence of 6M urea and 5% $(NH_4)_2SO_4$

Fig. 27 shows the separation of A. lwoffi α-OGDH subunits obtained by Sepharose 4B gel filtration after dissociation in the presence of 6M urea and 5% $(NH_4)_2SO_4$. The E3 subunit was successfully dissociated, its activity peak coinciding with that of Torula dihydrolipoamide dehydrogenase. However the E2 component (as detected by its lipoic acid content) was eluted considerably later than with the E. coli system (see Fig. 25), its apparent molecular weight being only slightly larger than that of the E3 component which is known to exist as dimers of molecular weight 112000 (Pettit et al., 1973). Consequently, it would appear that the E1•E2 subcomplex of A. lwoffi α-OGDH has undergone a greater degree of dissociation under these conditions than its E. coli counterpart.

(ii) Dissociation at high pH values in the presence of 1M NaCl

Dissociation of the A. lwoffi α-OGDH complex at high pH values in the presence of 1M NaCl followed by gel filtration on Sepharose 4B produced a very similar result (Fig. 28) to that obtained with the E. coli complex (Fig. 26). However, in this case no whole complex activity was detected in the second (lower molecular weight) peak, possibly due to the absence of the E2 component which did not trail into the second peak as before. It was therefore not possible to infer whether dissociation of the E1 subunit occurred.

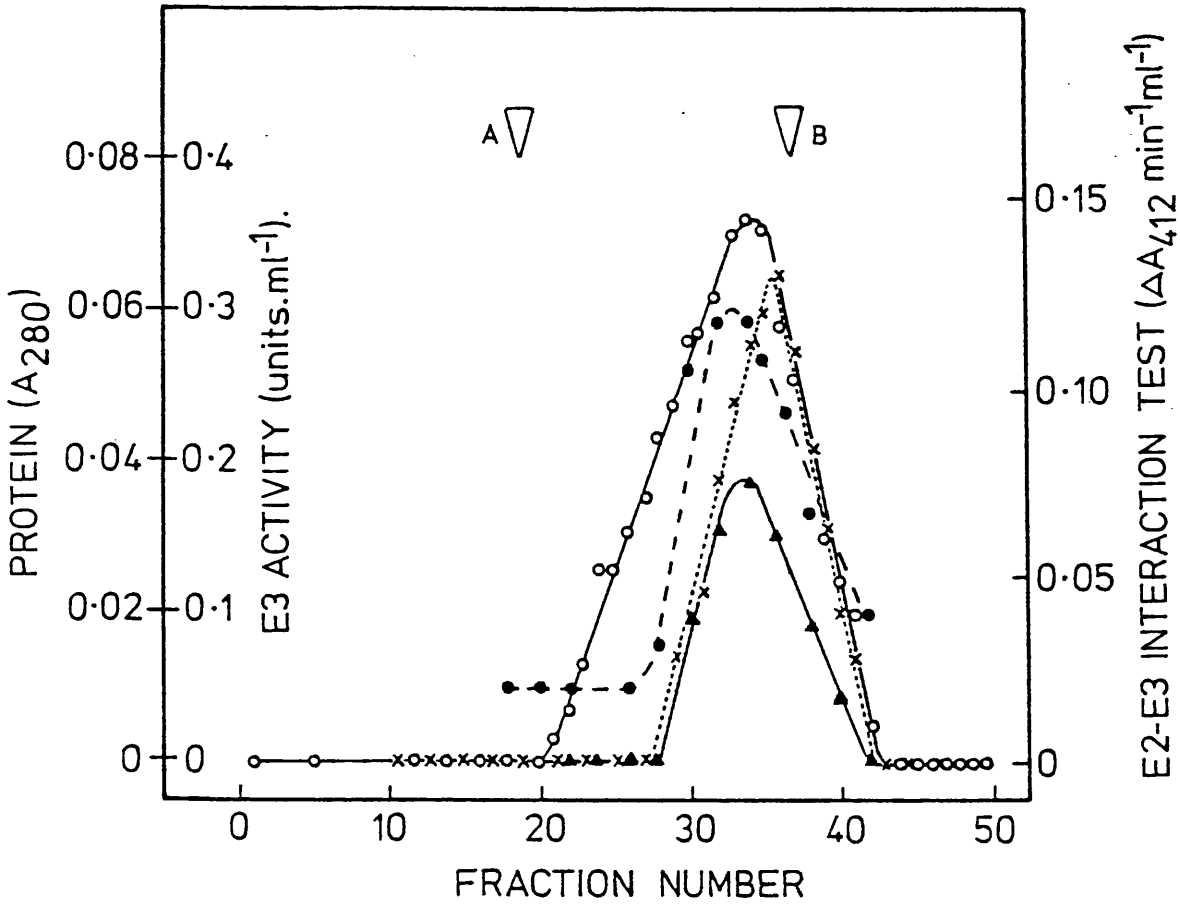


Fig. 27. Separation of A. lwoffi α-OGDH complex subunits by gel filtration in the presence of 6M urea and 5% $(NH_4)_2SO_4$. Experimental details are given in section III.2.5. (o——o), protein; (×·····×), E3 activity; (▲——▲), E2-E3 interaction test (without added dihydrolipoamide dehydrogenase); (●- - ●), lipoic acid test (with added dihydrolipoamide dehydrogenase). A. A. lwoffi α-OGDH complex. B. Torula yeast dihydrolipoamide dehydrogenase.

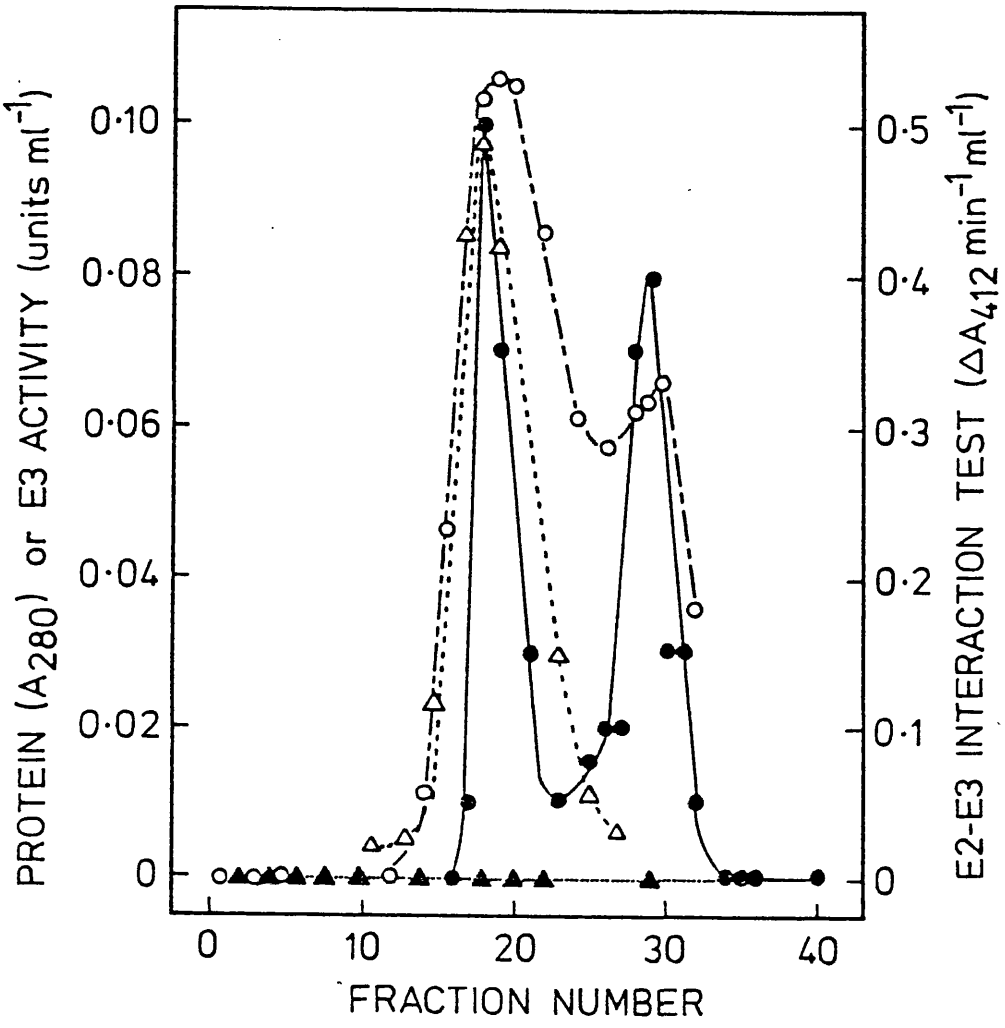


Fig. 28. Separation of A. lwoffi α-OGDH complex subunits by gel filtration at pH 9 in the presence of 1M NaCl.

Experimental details are given in section III.2·6.

(●—●), protein; (▲---▲), whole complex activity; (o—·o), E3 activity; (△···△), lipoic acid and E2-E3 interaction tests (with and without added dihydrolipoamide dehydrogenase).

CHAPTER IV

CHEMICAL MODIFICATION STUDIES

IV.1 INTRODUCTION

A knowledge of the chemical structure of an enzyme, particularly the functional groups involved at the catalytic and regulatory sites is essential for a complete understanding of the mechanism of catalysis and its regulation. Chemical modification of proteins is a well established biochemical method that has been widely used to gain information about the chemical nature of amino acid residues involved in enzyme function. It should, however, be emphasised that care must be taken in interpreting such results since changes in enzyme activity need not necessarily be due directly to modification of the active site, but may also result indirectly from a change in enzyme conformation induced by modification elsewhere.

In addition, in the case of a multienzyme complex, e.g. α-OGDH, it may be possible to modify chemically, and thereby inactivate, a single component activity. Complexes from different sources with different inactivated subunits could then be mixed, subjected to dissociating and reassociating conditions and finally assayed for whole complex activity (produced by hybridisation of active subunits from the different sources to form an active complex - see Fig. 29). It can be seen that there are four different possible combinations in which reassociation can occur. Consequently the maximum expected whole complex activity will only be 25% of the original if complete dissociation and reassociation occurs.

Experiments along these lines provide an alternative approach to the subunit separation studies described in Chapter III. Furthermore, this method should be more sensitive, requiring only relatively small amounts of enzyme.

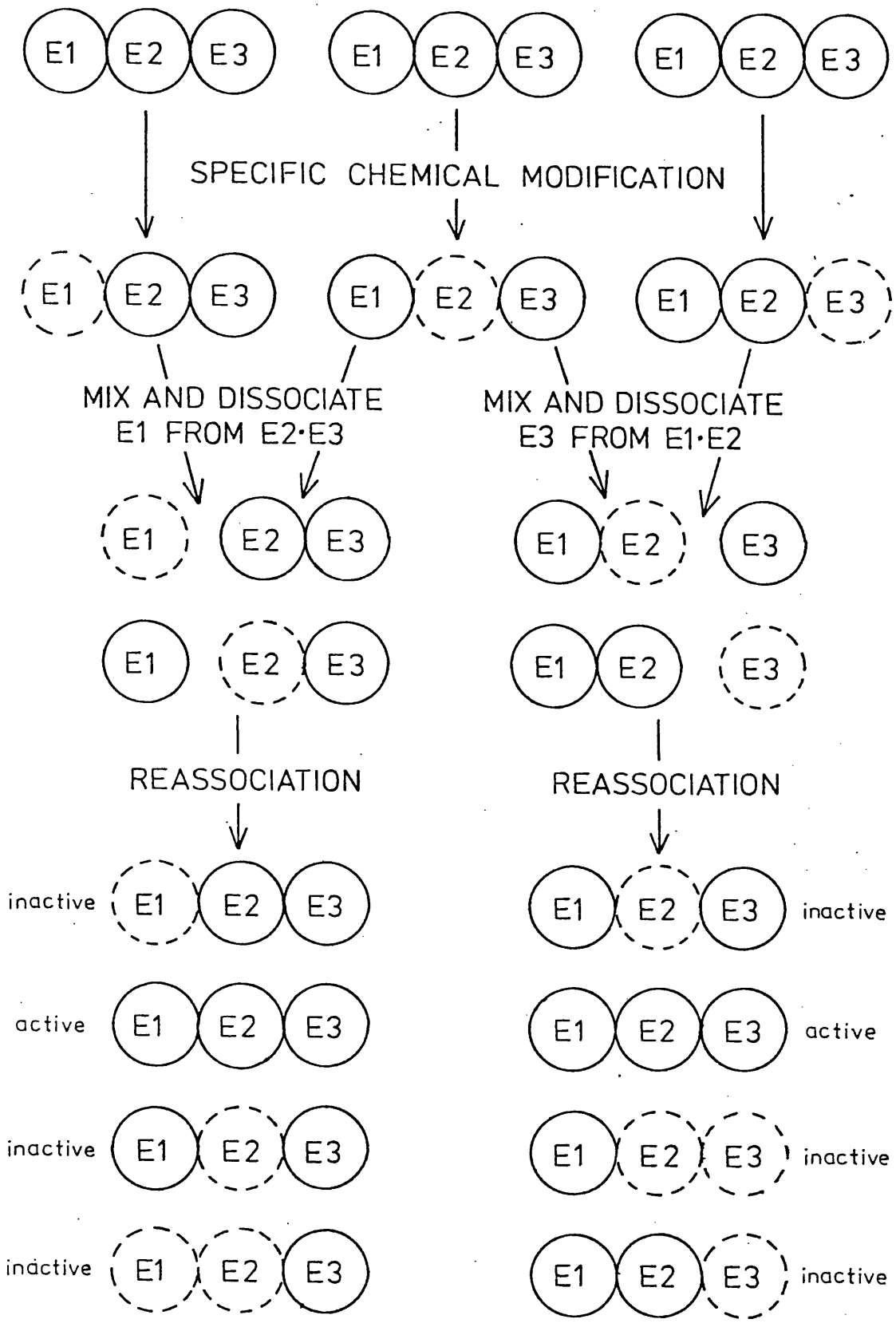


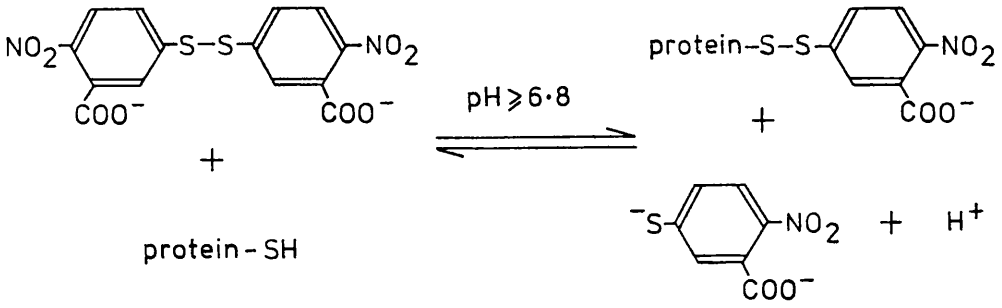
Fig. 29. A schematic representation of the use of specific chemical modification in dissociation and reassociation studies of the α-OGDH complex.

For further details see text (section IV.1).

With a view to applying these methods to the α-OGDH complex the following chemical modification reagents and techniques were investigated:

(i) 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB)

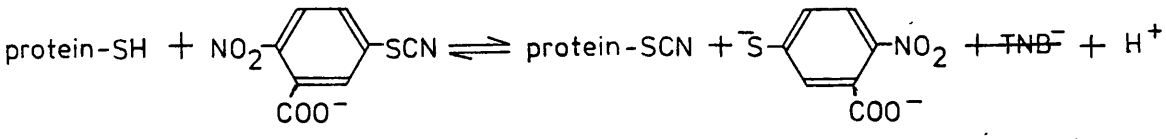
DTNB specifically reacts with free sulphydryl groups of proteins:



A mixed disulphide is formed and one molecule of free thionitrobenzoate anion (TNB^-) is released for each sulphydryl group that reacts in the protein. TNB^- has a strong yellow colour with a maximum absorbance at 412nm (A_{412nm} = 1·36 x $10^4 M^{-1}$ cm^{-1} at pH 8·0). The reaction of DTNB with sulphydryl groups can be readily reversed by adding an excess of thiol groups which competitively displace the TNB^- group (Means & Feeney, 1971b).

(ii) Nitrothiocyanobenzoic acid (NTCB)

This reagent reacts with proteins in an analogous way to DTNB, with the S-cyano derivative being formed instead of the mixed disulphide derivative (Degani & Patchornik, 1974):



NTCB should be preferable to DTNB since the cyano group is smaller and does not carry a negative charge. Consequently it is less likely to impose steric hindrance within the protein.

(iii) Iodoacetamide

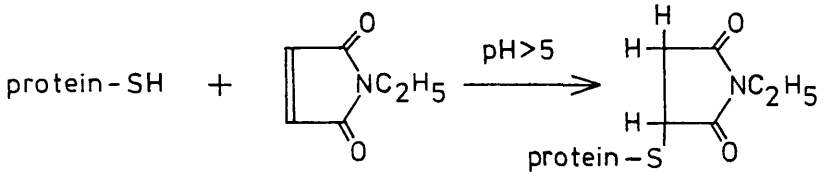
Iodoacetamide can react with sulphydryl, imidazole, thioester and amino groups depending on the pH of the reaction (Means & Feeney, 1971c). Of these the sulphydryl group is the most reactive and reactivity increases with increase in pH values since the reactive species is the ionic form of this group:

$$\text{protein-S}^- + \text{ICH}_2\text{CONH}_2 \xrightarrow{\text{pH}>7} \text{protein-S-CH}_2\text{CONH}_2 + \text{I}^-$$

However, to avoid unnecessary reaction with amino groups pH values should not be too high; the optimum for modification of sulphydryl groups is usually around pH 7-8.

(iv) N-ethylmaleimide (NEM)

NEM has been widely used as a sulphydryl-specific reagent both in the determination of the number of sulphydryl groups in a protein and also in establishing the effect of modification on enzyme activity.

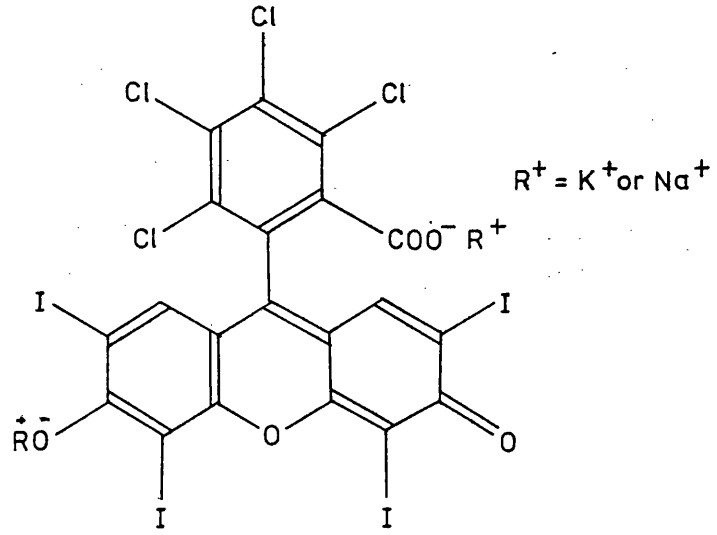


It also reacts with amino groups though at a slower rate than the sulphydryl reaction.

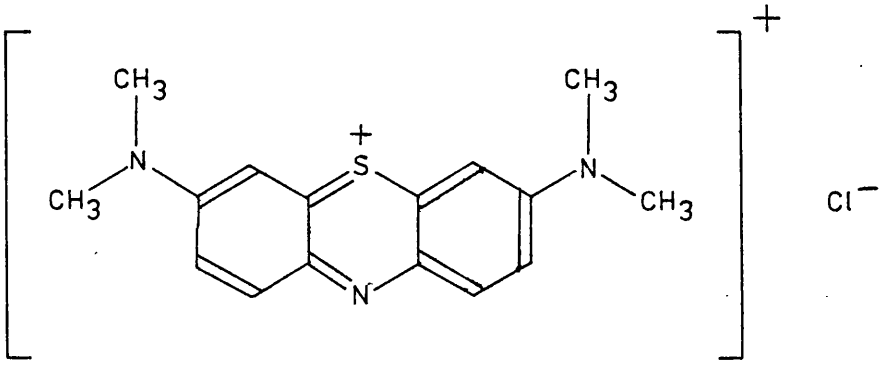
(v) Photooxidation

Photooxidation has been used as a method for the selective modification of amino acid side chains, both of free amino acids (Weil et al., 1951; Weil, 1965) and also those of polypeptides and proteins (Weil & Buchert, 1951; Martinez-Carrion et al., 1967). It has been shown that only five of the amino acids are photooxidised in the presence of a sensitizing dye (Weil et al., 1951; Weil, 1965), namely tyrosine, histidine, tryptophan, methionine and cysteine.

A wide range of dyes has been utilized as photosensitizers and these vary greatly in their efficiency. The two most widely used dyes for proteins are rose bengal and methylene blue, their structures being shown in Fig. 30.



ROSE BENGAL



METHYLENE BLUE

Fig. 30. Structures of rose bengal & methylene blue.

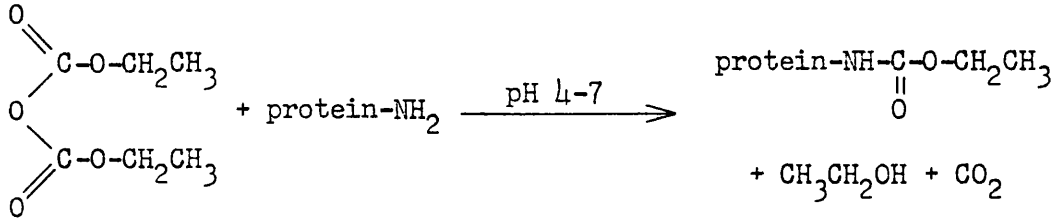
Although the latter has been the more popular of the two, it has recently been suggested that rose bengal may be somewhat more selective for histidine (Westhead, 1965; Bellin & Yankus, 1968). This could be due to the anionic properties of this particular dye which favours the formation of short-lived dye-imidazole complexes.

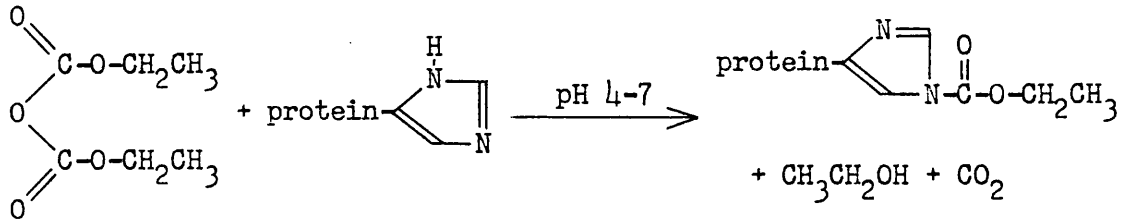
Some of the breakdown products following photooxidation are not well characterised, although it is clear that polypeptide bonds are not broken.

(vi) Diethylpyrocarbonate (DEPC)

DEPC is both an ester and an anhydride, but because of resonance involving the ester group it is less reactive than many anhydrides. It is slowly hydrolysed by water [half life at 25°C, pH 7·0 = 25min (Melchior & Fahrney, 1970)] to give two equivalents of ethanol and carbon dioxide.

At low pH values (around pH 4·0) DEPC reacts principally with the accessible amino and imidazole groups of proteins and is consequently a useful reagent for the chemical modification of histidine and lysine residues:



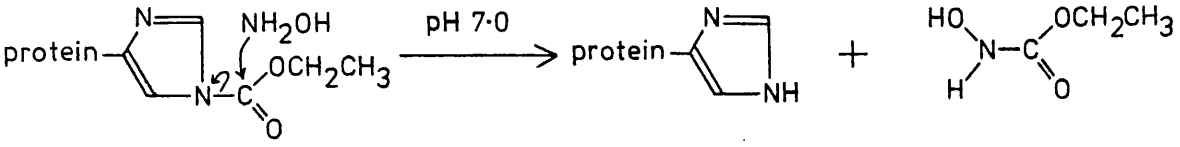


However, under certain conditions, DEPC may lose its specificity e.g. near neutral pH values (Rosén & Fedorcsák, 1966; Melchior & Fahrney, 1970).

The reaction of DEPC with the imidazole groups of proteins is accompanied by an increase in absorbance at 230 to 240nm, which provides a convenient means of measuring the extent of reaction (Melchior & Fahrney,

1970; Mühlrad et al., 1969).

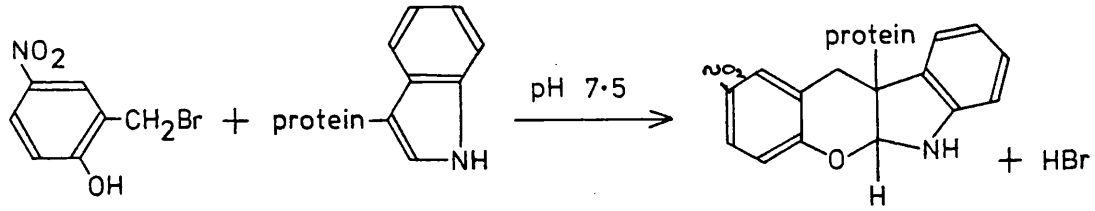
Finally, the modification of histidine residues can be readily reversed at neutral pH values by hydroxylamine:



However, reversal of modified lysine and arginine groups cannot be obtained in this way (Melchior & Fahrney, 1970).

(vii) 2-hydroxy-5-nitrobenzyl bromide (HNBBr)

At neutral or acid pH values HNBBr reacts very rapidly with tryptophan residues and also to a lesser extent (at about one-fifth the rate) with cysteine (Koshland et al., 1964). At alkaline pH values tyrosine is also modified by HNBBr (Horton & Koshland, 1965).



The rapid reaction of HNBBr is probably due to resonance stabilization of the carbonium ion. In aqueous solution it has a half-life of less than one minute. Only tryptophan (and cysteine to a lesser extent) can compete with water for the highly reactive carbonium ion.

IV.2 MATERIALS AND METHODS

IV.2·1 Materials

Rose bengal, DTNB and HNBBr were purchased from Sigma (London) Chemical Co. Ltd., Kingston, Surrey, U.K. Methylene blue and DEPC were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. NTCB was prepared according to the method of Degani & Patchornik (1971).

IV.2·2 Chemical modification by DTNB

(i) Time course of enzyme inactivation by DTNB

A solution of *A. lwoffi* α-OGDH complex (0·1-0·5mg ml^{-1}) in 10mM $MgCl_2$, 1mM EDTA, 0·1M Tris-HCl, pH 8·0, was made 0·2-1·0mM with respect to DTNB and incubated at 20°C. At suitable time intervals samples were removed and assayed for whole enzyme complex, E1, E2 and E3 enzyme activities. In the case of the E2 subunit the samples were made 2·5mM with respect to cysteine hydrochloride before being assayed for activity, in order to remove any remaining DTNB which would otherwise react with the dihydro lipoamide in the E2 assay.

(ii) Determination of exposed enzyme sulphydryl groups using DTNB

A cuvette containing 2mg of α-OGDH complex in a buffer composed of 10mM $MgCl_2$, 1mM EDTA, 0·1M Tris-HCl, pH 8·0, in a final total volume of 1·0ml (after addition of DTNB) was zeroed against an identical cuvette, but lacking enzyme, in a double-beam spectrophotometer set at a wavelength of 412nm. DTNB was added to both the assay and reference cuvette to give a final concentration of 2mM, and the increase in absorbance at 412nm was monitored continuously.

IV.2·3 Chemical modification by NTCB

A solution containing 0·5mg ml^{-1} of α-OGDH complex in MET-8 buffer was incubated at 20°C with 1mM NTCB. Samples were removed at suitable time intervals and assayed for whole complex activity.

IV.2·4 Chemical modification by iodoacetamide

Twenty microlitres of 10mM iodoacetamide in MET-8 buffer was added to 20µg of purified A. lwoffi α-OGDH complex in 200µl of the same buffer and incubated at 20°C. At suitable intervals 10µl samples were withdrawn, added to cuvettes containing 2·5mM cysteine hydrochloride in 1·0ml of 10mM $MgCl_2$, 1mM EDTA, 0·1M Tris-HCl, pH 8·0, and assayed for whole complex activity by the addition of the appropriate substrates.

Reactions were also carried out in the presence of either 2·5mM α-oxoglutarate plus 0·5mM TPP or 1mM NADH (added prior to the iodoacetamide).

IV.2·5 Chemical modification by N'ethylmaleimide (NEM)

(i) Time course of enzyme inactivation by NEM

Forty microlitres of 1mM aqueous NEM solution were added to 40µg of purified A. lwoffi α-OGDH complex in 400µl of MET-8 buffer. The mixture was incubated at 20°C and samples were removed at suitable time intervals for assay of whole complex activity.

Reactions were also carried out in the presence of either 40µl of 10mM NADH or 25µl of 10mM AMP plus 10µl of 10mM TPP and 20µl of 0·5M α-oxoglutarate (added prior to addition of NEM).

(ii) Effect of NEM on subunit activities under different incubation conditions

Four tubes were set up, each containing 500µg of purified α-OGDH complex in 500µl of MET-8 buffer together with the following additions:

Tube 1 : 100µl of MET-8 buffer

Tube 2 : 50µl of MET-8 buffer

Tube 3 : 50µl Of 10mM NADH

Tube 4 : 20µl of 0·5M α-oxoglutarate, 20µl of 10mM TPP and 10µl of MET-8 buffer

After incubation at 20°C for 2min, 50µl of NEM solution (1mM) were added to tubes 2,3 and 4, and incubation continued. At suitable time intervals 10µl aliquots were removed and assayed for whole complex activity,

100µl for E1 activity, 100µl for E2 activity, 50µl for E3 activity and 10µl for the E2-E3 interaction test (both in the presence and absence of Torula yeast dihydrolipoamide dehydrogenase).

IV.2·6 Photooxidation

(i) pH dependence

Purified α-OGDH complex from A. lwoffi (100µl of a solution diluted to give a suitable assay rate) in 10mM phosphate buffer, pH 7·0, was added to a tube on ice containing 100µl of 30µM aqueous rose bengal or methylene blue and 800µl of 50mM buffer (either Tris-HCl, phosphate or 2-methyl, 2-amino-1,3 propanediol) at various pH values ranging from 7·0 to 9·5. After shaking, a sample (100µl) was immediately removed ("zero time sample") and added to a tube containing 400µl of 100mM Tris-HCl, pH 7·9, at 0°C. It was protected from light with aluminium foil and kept on ice until its activity was assayed. The rest of the enzyme-dye mixture was immediately immersed in a 20°C waterbath and illuminated by an Erro 150watt spotlight (Fig. 31). Further 100µl samples were withdrawn at suitable intervals during the illumination period and added to 400µl of Tris-HCl buffer, on ice, as above. After all of the samples had been collected they were assayed for whole complex activity.

Control samples were incubated in the dark to ensure that neither the pH of the reaction mixture nor the presence of the photosensitizing dye were inactivating the enzyme. Any inactivation rates obtained in these "dark controls" (which never exceeded 10% of the corresponding light reaction rate) were subtracted to obtain the true inactivation rate due to photo-oxidation.

(ii) Effect of photooxidation on enzyme subunit activities

α-OGDH complex from A. lwoffi (200µg) was added to a tube, on ice, containing 3µM rose bengal or methylene blue solution in a total volume of 500µl of 50mM 2-methyl, 2-amino 1,3-propanediol buffer, pH 9·0. After

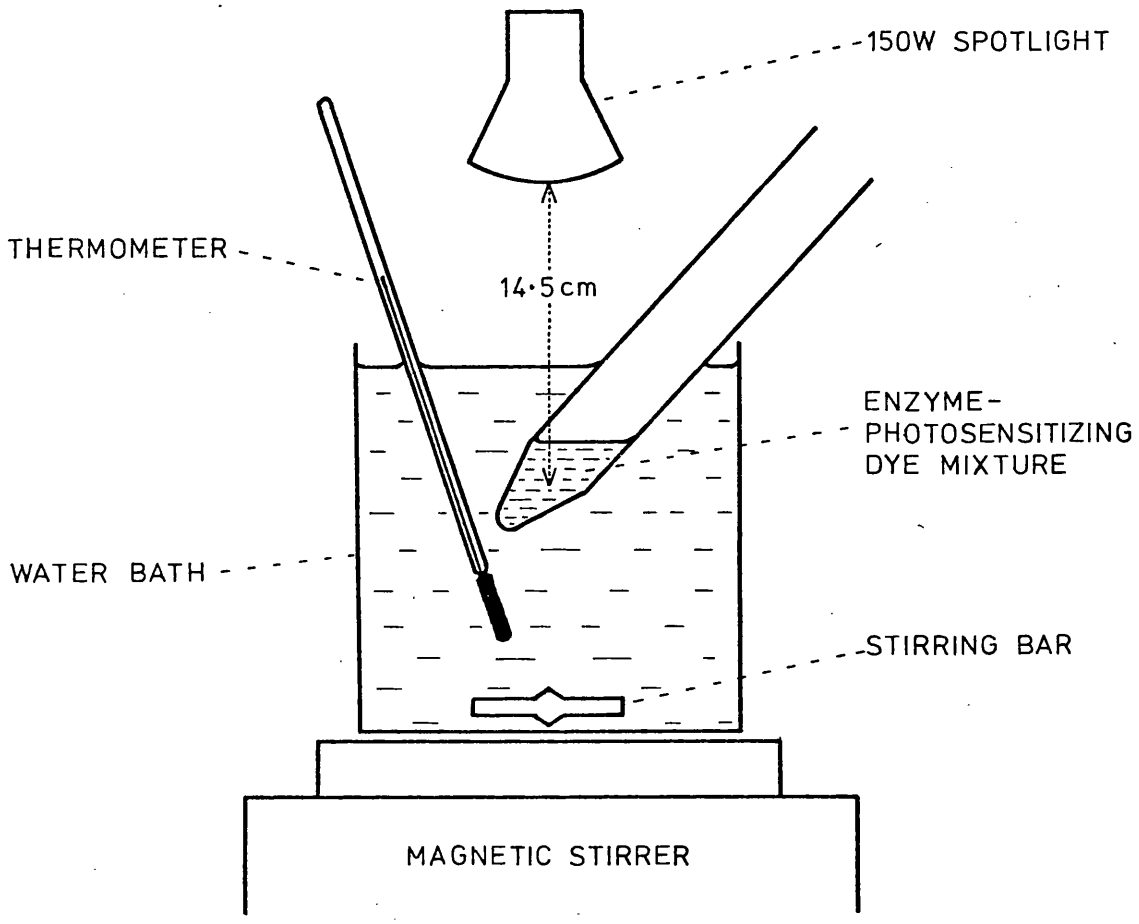


Fig. 31. Apparatus used for photooxidation.

Experimental details are described in section IV.2·6.

shaking, samples were removed (20µl for whole complex and E3 assays, 50µl for E1 and E2 assays) and added to four different cuvettes containing the appropriate assay buffers for the three subunit and whole complex assays. These cuvettes were protected from light with aluminium foil and stored on ice until assayed. The remaining enzyme-dye mixture was immediately immersed in a 20°C waterbath and illuminated by a 150watt spotlight for 2min. Further samples were then taken and added to cuvettes containing the same assay buffers as before. They were finally assayed for the appropriate subunit or whole complex activity, the reactions being started by the addition of substrates.

IV.2·7 Chemical modification by DEPC

The concentration of DEPC was determined from the increase in absorbance at 240nm in the presence of excess imidazole (0·3mM), due to the formation of N-ethoxyformylimidazole [A_{240} = 3·2 x 10^3 l mol^{-1} cm^{-1} (Ovádi *et al.*, 1967)].

(i) Time course of enzyme inactivation by DEPC

Twenty microlitres of 100µM DEPC solution in absolute ethanol was added to 0·2mg of purified *A. lwoffi* α-OGDH complex in 1·0ml of 0·1M phosphate buffer, pH 6·0. The mixture was incubated at 20°C and samples were withdrawn at suitable intervals and assayed for whole complex activity.

Controls were carried out using 20µl of pure ethanol instead of ethanolic DEPC solution, but were found to result in no loss of enzyme activity.

(ii) Reactivation of enzyme by hydroxylamine

α-OGDH complex was incubated for 5 or 45min in the presence of DEPC as described above. Samples (200µl) were withdrawn, added to tubes containing 50µl of 0·5M imidazole (at pH 7·0) and adjusted to pH 7·0. Twenty microlitres of 3·3M aqueous hydroxylamine solution, pH 7·0, were then added and incubated at 20°C. At suitable intervals aliquots were removed and assayed

for whole complex activity.

IV.2·8 Chemical modification by HNBBr

(i) Effect of HNBBr on whole complex activity

A 100mM solution of HNBBr in water-free acetone (dried with anhydrous sodium sulphate) was prepared directly before use. Twenty microlitres of this solution were quickly added to 0·2mg of purified A. lwoffi α-OGDH complex in 200µl of citrate-phosphate buffer (40mM citric acid, 120mM Na_2HPO_4, pH 5·7); the pH after addition of the HNBBr was found to be 5·6. After incubation at 20°C for 1min and 5min, 10µl aliquots were assayed for whole complex activity.

Control incubations were carried out using 20µl of water-free acetone instead of HNBBr solution.

(ii) Effect of HNBBr on subunit activities

Two hundred microlitres of 100mM HNBBr in water-free acetone were added to 1·0ml of citrate-phosphate buffer, pH 5·7, containing 0·45mg of α-OGDH complex. After 4min incubation at 20°C the solution was assayed for whole complex, E1, E2 and E3 subunit activities.

IV.3 RESULTS AND DISCUSSION

IV.3·1 Chemical modification by DTNB

(i) Time course of inactivation by DTNB

In order to establish whether enzyme modification by DTNB affects the catalytic properties of the complex, time courses of loss of enzyme activity were followed during incubation with DTNB. Under the conditions used, namely a 500-fold excess of DTNB to enzyme, pseudo-first order kinetics should be observed (Freedman & Radda, 1968). It can be seen from Fig. 32 that a biphasic time course was obtained, composed of two pseudo-first order reactions. The first phase of inactivation is about 7½ times faster than the latter phase. A similar pattern is seen with an even larger excess of DTNB (i.e. 2000-fold excess over protein).

From this result it would appear likely that DTNB reacts with two different classes of sulphydryl groups, one giving rise to a much faster inactivation of the enzyme than the other. This is indeed found to be the case in Section IV.3·1(iii).

(ii) Reactivation of enzyme by dithiothreitol (DTT)

The reaction of DTNB with sulphydryl groups can be readily reversed as the TNB group is competitively displaced by an excess of thiol groups (Means & Feeney, 1971b). Fig. 33 shows that incubation of DTNB-inactivated α-OGDH complex with a 250-fold excess of DTT resulted in complete reactivation. In fact the enzyme activity after reactivation by DTT was usually slightly higher than the original activity before inactivation. This apparent activation is not observed when native enzyme is treated with DTT, however, and hence its significance is not yet clear.

(iii) Effect of DTNB on individual subunit activities

As outlined in the introduction to this chapter, one of the main aims of the chemical modification studies was to find a reagent which could specifically inactivate one of the enzyme components. In the case of DTNB,

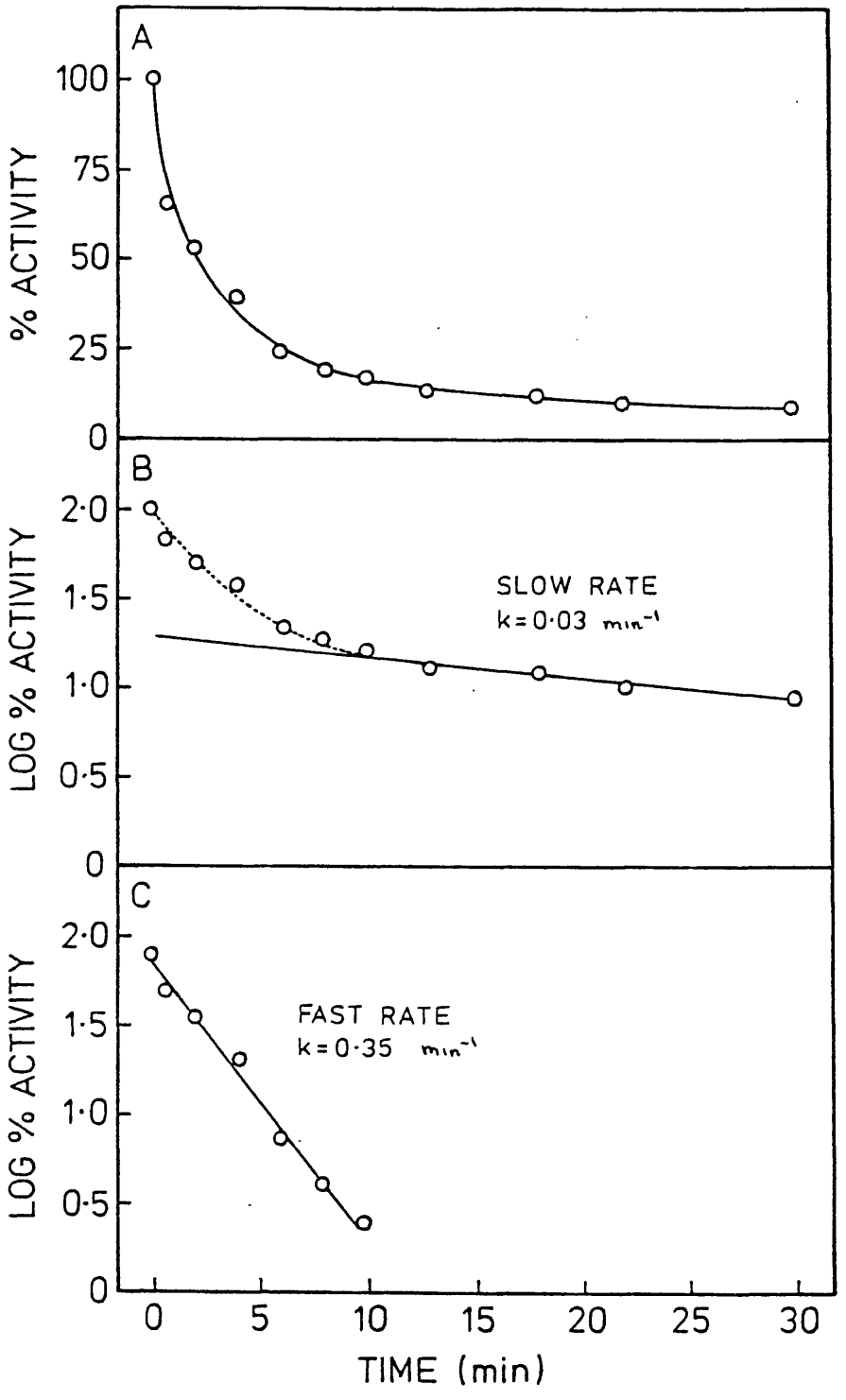


Fig. 32. Kinetics of the DTNB inactivation of A. lwoffi α-OGDH complex.

The experimental procedure is described in section IV.2·2. The semi-log plot for determination of the fast rate constant was obtained after subtraction of the slow rate.

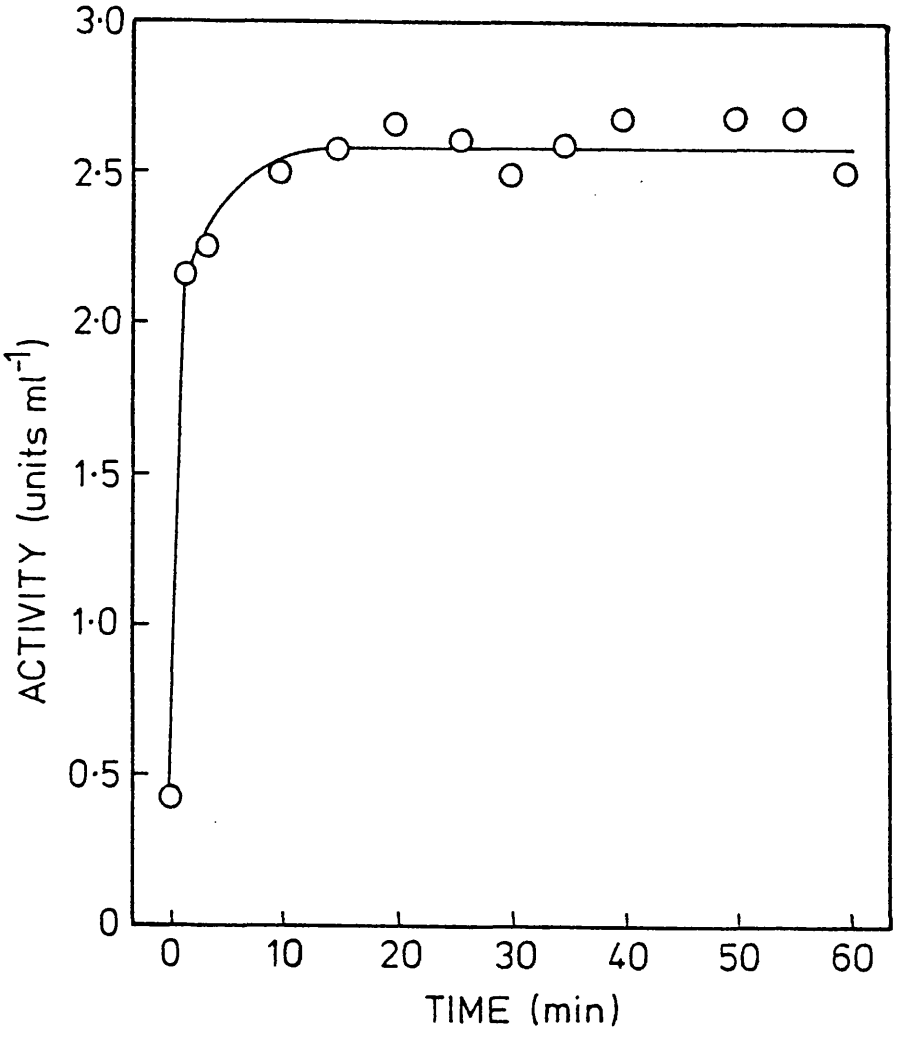


Fig. 33. Reactivation of A. lwoffi α-OGDH complex by DTT after DTNB inactivation.

0·1mg of α-OGDH complex in 10mM $MgCl_2$, 1mM EDTA, 0·1M Tris-HCl, pH 8·0, was incubated with 0·2mM DTNB at 20°C for 1h. The mixture was then made 50mM with respect to DTT and the incubation continued. Aliquots were removed at intervals and assayed for whole complex activity. Initial activity before DTNB inactivation = 2·0units ml^{-1}.

however, both the E1 and E2 components showed marked inactivation on incubation with this reagent although the E3 component lost no activity and in fact showed a slight activation (Table 13). Furthermore, it can be seen from Fig. 34 that the E1 component is inactivated at a much faster rate than is E2. This result is consistent with the biphasic inactivation kinetics observed for the whole enzyme complex (Fig. 32). It should be pointed out that only about 70% of the original E1 activity is lost. This could mean that only 70% of the E1 component subunits present in the whole complex are susceptible to DTNB attack; or else all the E1 components are inhibited by 70%.

Inactivation of E1 does not seem to be due to a reduction in the affinity of α-oxoglutarate for its binding site, the K_m of the whole complex for this substrate being virtually the same before (K_m = 2·5mM) and after (K_m = 2·0mM) DTNB inactivation.

The activation of E3 by DTNB appears to be real and is not simply due to an ~~increase~~ decrease in absorbance at 340nm as a result of TNB^- formation (which was found to be less than 1% of that due to NAD^+ ~~reduction~~ oxidation by E3). To see if this activation is a general property of dihydrolipoamide dehydrogenases, the effect of DTNB on Torula yeast enzyme was studied. It can be seen from Fig. 35A that this dihydrolipoamide dehydrogenase was not activated by DTNB but instead exhibited a very slow loss of activity. The possibility therefore existed that activation of the E3 component of the A. lwoffi enzyme is a consequence of its being part of a multienzyme complex and is not characteristic of the free enzyme. This was investigated by dissociating the E3 component from the α-OGDH complex of A. lwoffi with urea (see section III.3·3) prior to treatment with DTNB. This resulted in a virtually identical activation to that observed with undissociated complex (Fig. 36) and hence it would appear that this activation is a property of the E3 component of the A. lwoffi complex.

Table 13. The subunit specificity of DTNB inactivation of A. lwoffi α-OGDH complex. 1·25mg of α-OGDH complex in 0·2ml of MET-8 buffer were made 0·3mM with respect to DTNB and incubated at 20°C for 1h. Whole complex and individual subunit activities were then assayed and the results compared with those for enzyme incubated in the absence of DTNB.

	Activity (absorbance units min^{-1} ml^{-1})		Activity
	Without DTNB	With DTNB	(% of control)
Whole complex	468	28	6
E1 subunit	5·08	1·0	20
E2 subunit	3·4	0·92	27
E3 subunit	560	800	140

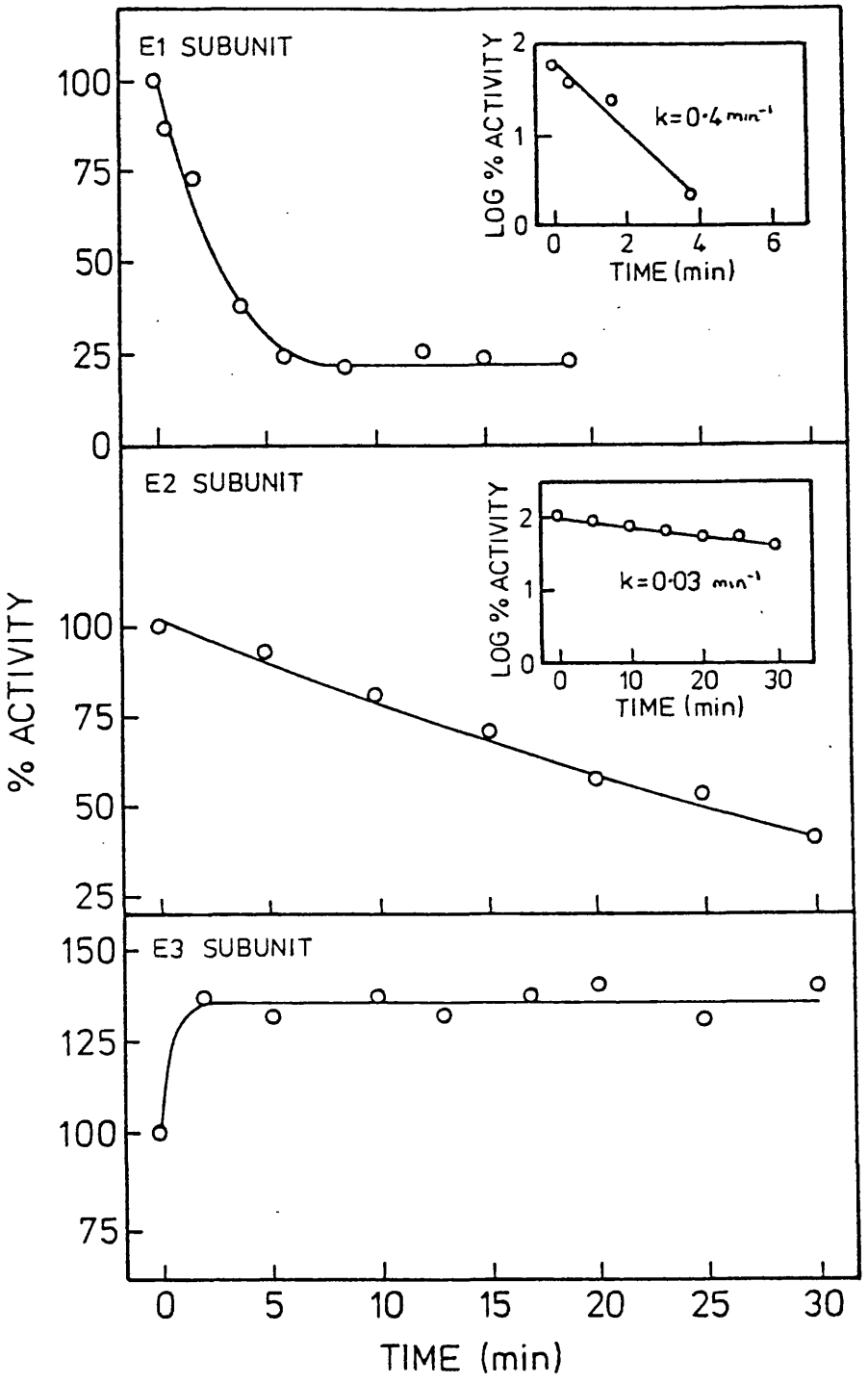


Fig. 34. Time dependence of the effect of DTNB on the subunit activities of A. lwoffi α-OGDH.

Experimental details are given in section IV.2·2.

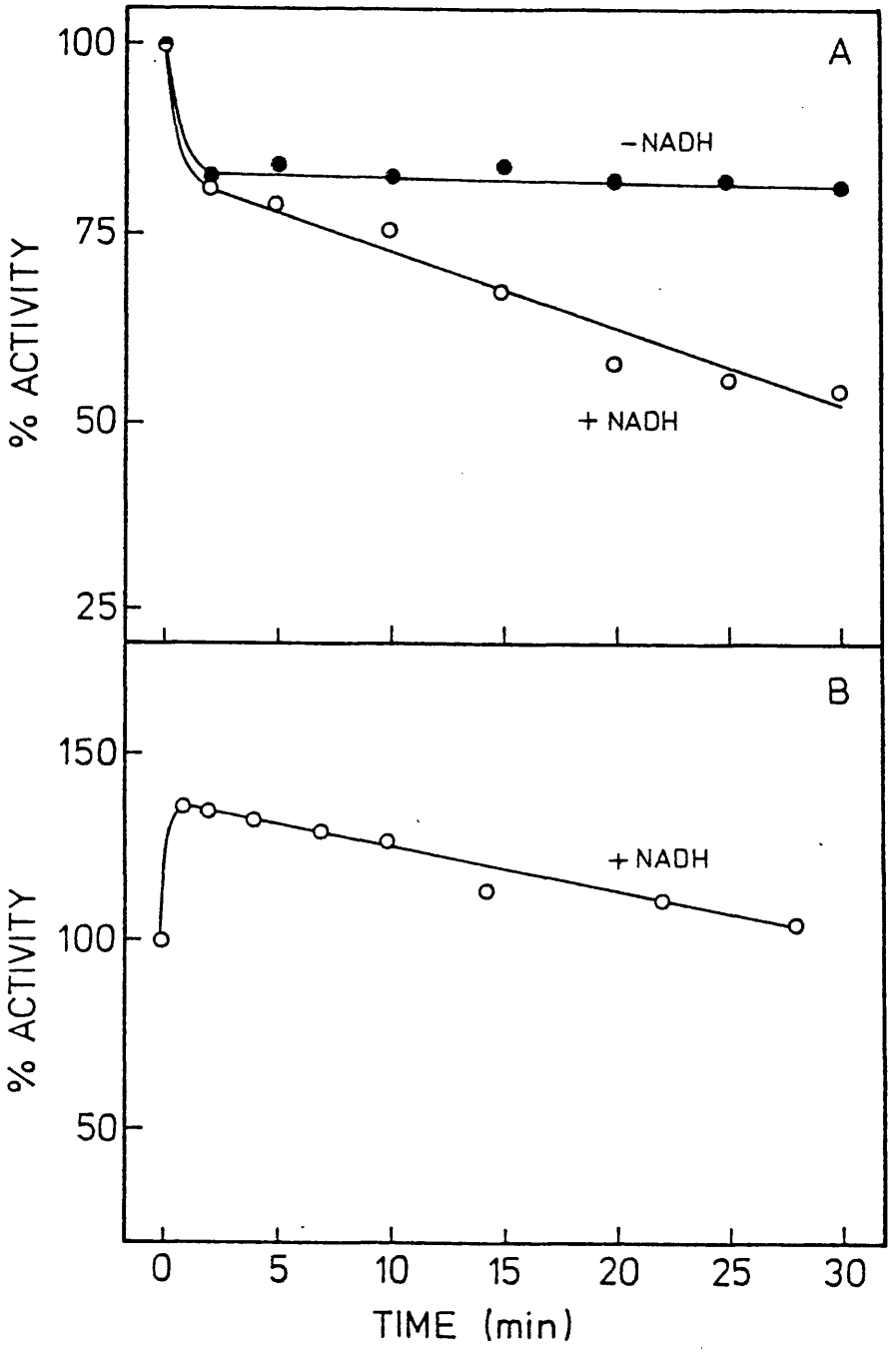


Fig. 35. The effect of NADH on DTNB inactivation of Torula yeast dihydrolipoamide dehydrogenase & E3 component of A. lwoffi α-OGDH. A. 0·25mg of Torula dihydrolipoamide dehydrogenase in 0·5ml MET-8 buffer was incubated with 0·2mM DTNB in the presence and absence of 0·4mM NADH. At intervals samples were assayed for activity. B. 0·1mg of A. lwoffi α-OGDH complex in 1ml of MET-8 buffer plus 0·4mM NADH was incubated at 20°C with 0·2mM DTNB. At intervals samples were removed and assayed for E3 activity.

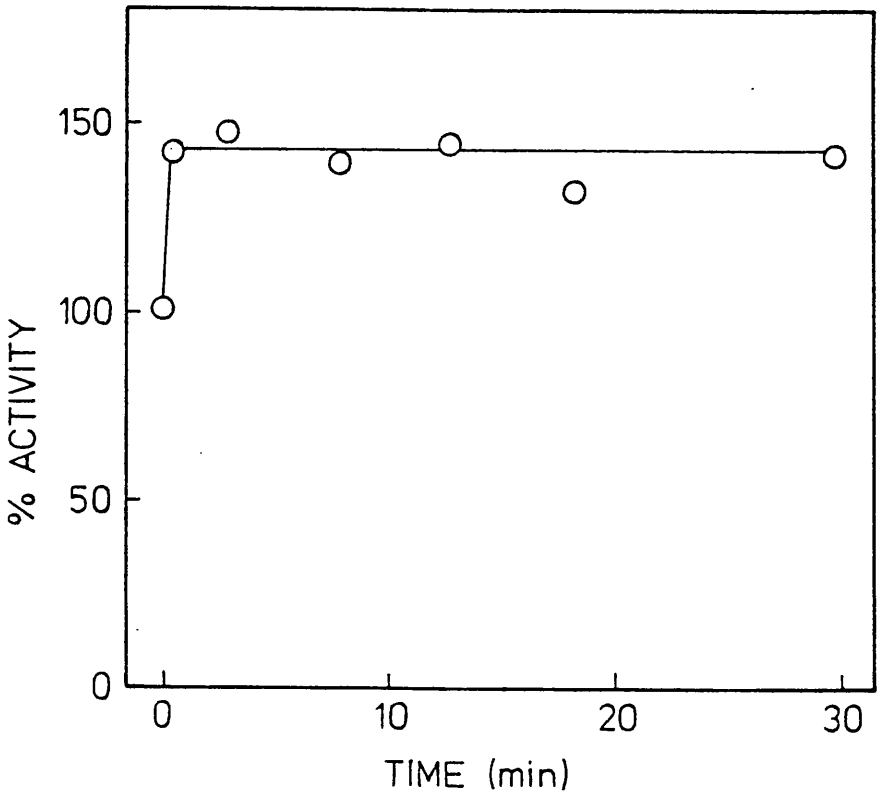


Fig. 36. The effect of DTNB on the E3 component after dissociation from the A. lwoffi α-OGDH complex.

Dissociation and separation of E3 from the complex was carried out in the presence of 6M urea as described in section III.2·5. 0·02ml of 10mM DTNB was added to 0·1mg of enzyme in 1ml of MET-8 buffer and the mixture was incubated at 20°C. At intervals samples were removed and assayed for E3 activity.

It has been shown that a disulphide bridge is located at the active site of E3 and is involved in the reaction mechanism (Massey et al., 1960; Massey & Veeger, 1961; Brown & Perham, 1974). This disulphide bridge is alternately reduced and oxidised during the reaction. Incubation of the enzyme with NADH would be expected to result in the reduction of this disulphide bond to produce sulphydryl groups susceptible to attack by DTNB. Consequently, it would be reasonable to expect a greater inactivation of E3 by DTNB in the presence of NADH than in its absence. Fig. 35 shows that this is indeed the case for both the E3 component of A. lwoffi α-OGDH and for the Torula yeast enzyme, although initial activation by DTNB is again seen in the former case.

(iv) Effect of modification by DTNB on the regulation of the complex

Chemical modification may be used to study the regulatory properties of an enzyme as well as the catalytic properties. Consequently, an investigation was carried out to see if incubation of A. lwoffi α-OGDH complex with DTNB resulted in any change in its regulatory properties. The kinetics of DTNB inactivation of whole enzyme activity were followed (as described in section IV.2.2) and after a suitable incubation period (typically 5min) the regulatory effects of AMP and NADH were studied. It can be seen from Table 14 that there was no loss in either the AMP stimulation or the NADH inhibition characteristic of the untreated complex. In conclusion, therefore, although the catalytic activity of the complex is affected by DTNB, the regulatory properties would appear to be unaffected.

(v) Protection afforded by substrates and effectors

If a substrate is able to protect an enzyme against loss of activity by a chemical modifier, it is likely that the modification occurs at or near the substrate binding site. Consequently, some information about the functional groups involved in substrate binding may be obtained. Modification of A. lwoffi α-OGDH complex by DTNB in the presence of either 5mM α-oxo-

Table 14. The effect of DTNB on the regulatory properties of α-OGDH complex.

0·1mg of α-OGDH complex was incubated for 50min at 20°C in the absence or presence of 0·2mM DTNB. The sensitivity of the complex to 0·2mM NADH and 0·2mM AMP was then determined, in the former case at an α-oxoglutarate concentration of 5mM and in the latter case of 1mM.

	NADH inhibition	AMP stimulation
Native complex	53%	400%
DTNB-treated complex	51%	400%
DTNB-treated complex reactivated by DTT	-	390%

glutarate or 0·2mM TPP had no appreciable effect on the DTNB inactivation. It should perhaps be mentioned that incubation of complex with α-oxoglutarate would result in the E2-bound lipoic acid being acylated by the E1 reaction and thus exposing a free sulphydryl group to DTNB attack. However, during the subsequent assay for activity the lipoic acid bound acyl group would be transferred to CoA and then intramolecular displacement could occur to release the TNB^- group and consequently regenerate the lipoic acid.

On the other hand, modification of whole complex by DTNB in the presence of 0·6 or 1·0mM NAD^+ resulted in a significant decrease in the loss of enzyme activity as shown in Fig. 37. Very similar results, however, were obtained for DTNB inactivation in the presence of either 0·4mM AMP or 0·2mM NADH (which are not substrates). It would therefore seem likely that NAD^+ exerts some indirect effect on the active site, rather than directly protecting the substrate binding site. This is further supported by the observation that the kinetics of inactivation in the presence of NAD^+, NADH or AMP were still biphasic, but only the initial fast loss of activity, which has been associated with E1 inactivation, was affected. Since NAD^+ is not a substrate for the E1 component it seems probable that these "protective agents" (NAD^+, NADH and AMP), which are all nucleotides, have a common indirect effect on E1 activity; possibly by interaction with the nucleotide regulatory sites which are thought to be located on this component.

It may at first seem surprising that the rate of whole complex inactivation by DTNB was not slightly faster in the presence of NADH than in the presence of either NAD^+ or AMP, since in the former case inactivation of E3 should occur by reduction and subsequent modification of the disulphide bond present at the active site. However, this was not observed since E3 inactivation in the presence of NADH occurred at a rather slow rate and even after 30min the activity of E3, which is stimulated by DTNB, had not dropped

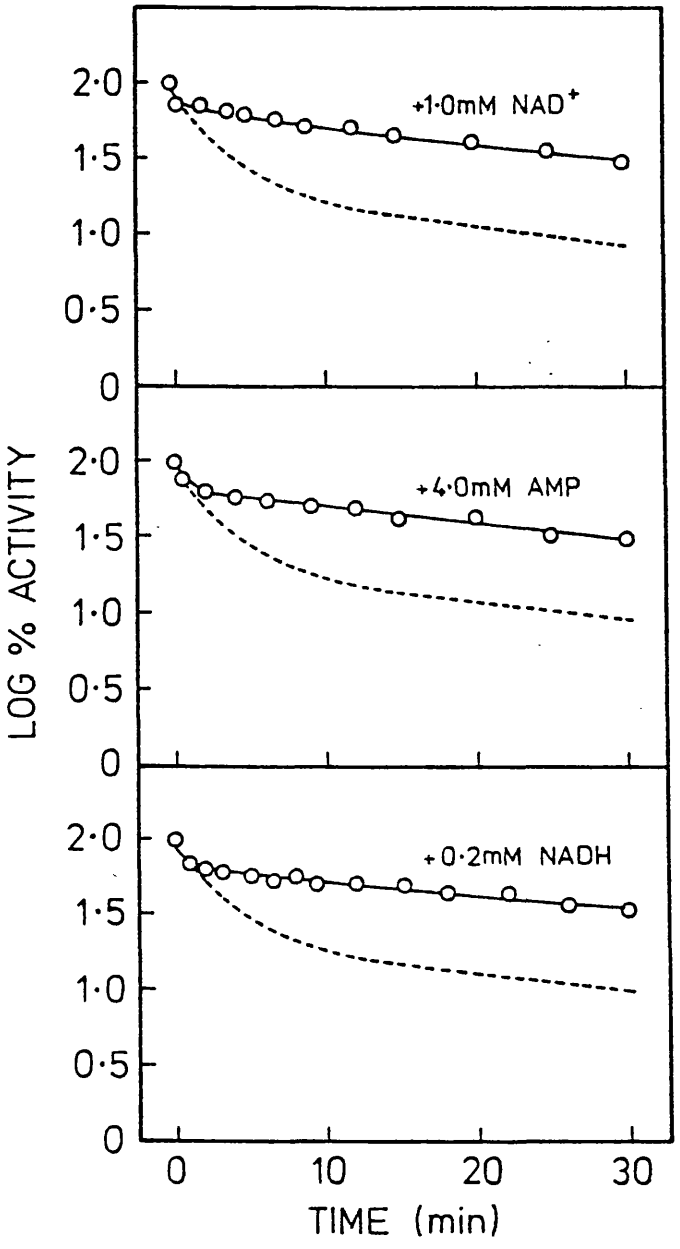


Fig. 37. The effects of NAD^+, AMP and NADH on the DTNB inactivation of A. lwoffi α-OGDH complex.

0·1mg of purified α-OGDH complex in 1ml of MET-8 buffer containing the appropriate nucleotide (1·0mM NAD^+, 4mM AMP or 0·2mM NADH) was made 0·2mM with respect to DTNB and the mixture incubated at 20°C. Samples were taken at suitable intervals and assayed for whole complex activity. The broken lines indicate DTNB inactivation observed in the absence of nucleotides (see Fig. 32).

below that of untreated enzyme.

Similarly, DTNB inactivation in the presence of NADH should result in reduction and subsequent modification of the lipoyl disulphide bond of the E2 component. However, this does not give rise to any significant loss of activity since intramolecular displacement of the lipoate-bound TNB^- group during the enzyme assay should result in regeneration of active enzyme.

(vi) Determination of the number of exposed sulphydryl groups using DTNB

By reacting a known amount of enzyme protein with a large excess of DTNB (more than 40-fold for first-order kinetics) the number of exposed thiol groups can be determined from the increase in absorbance at 412nm (Ellman, 1959; Freedman & Radda, 1968). Furthermore, by monitoring the increase in absorbance as a function of time the rate of modification can be calculated.

Analysis of *A. lwoffi* α-OGDH complex in this manner revealed that remarkably few sulphydryl groups are susceptible to modification even after a reaction time of 90min, and some variation between results of different determinations was observed. A total of 13 ± 3 sulphydryl groups (four determinations) were found to be modified assuming an enzyme molecular weight of 2×10^6. The kinetics of modification were found to be biphasic (c.f. kinetics of whole complex inactivation) with 10 ± 2 residues reacting rapidly ($k \simeq 0{\cdot}8min^{-1}$) and 3 ± 1 residues reacting more slowly ($k \simeq 0{\cdot}03min^{-1}$). Variations in results may reflect slight differences in enzyme conformation, which may be particularly important when comparing results from different enzyme preparations.

IV.3·2 Chemical modification by NTCB

It can be seen from Fig. 38 that modification of *A. lwoffi* α-OGDH complex by NTCB resulted in a rapid loss of about 90% of the original activity over a period of about 9min. No further inactivation was seen. The subunit specificity of NTCB revealed a similar pattern to that obtained

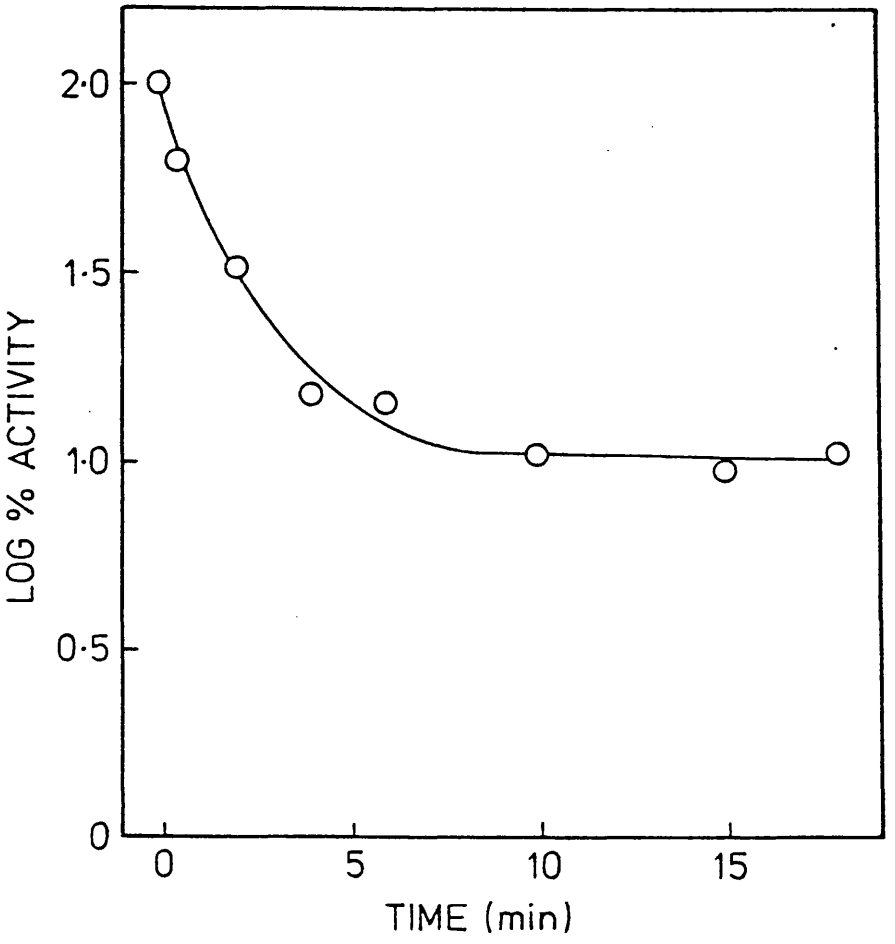


Fig. 38. Inactivation of A. lwoffi α-OGDH complex by NTCB.

Experimental details are given in section IV.2·3.

for DTNB. Table 15 shows that the loss of whole complex activity is due to partial inactivation of the E1 and E2 subunits; the E3 subunit being unaffected. In this case no stimulation of the E3 subunit was observed (c.f. the effect of DTNB on E3; section IV.3·1). It is possible that the cyano group, which becomes bound to the enzyme upon reaction with NTCB, is much less bulky and uncharged compared with the TNB^- group formed upon reaction with DTNB and is hence less likely to cause any ~~steric hindrance~~ conformational change.

NTCB inactivation of the E3 subunit was studied in the presence of NADH in the same way as with DTNB. In this case, however, no detectable inactivation (due to modification of the sulphydryl groups in the E3 active site) was found, even after 2h of treatment (Fig. 39A). This is perhaps not too surprising since it has been noted that modification by NTCB is slower than by DTNB (Degani & Patchornik, 1974). However, when this experiment was performed on Torula yeast dihydrolipoamide dehydrogenase, the rate of inactivation by NTCB was increased in the presence of NADH (Fig. 39B).

IV.3·3 Modification of the lipoyl sulphydryl groups

In section IV.3·1 the effect of DTNB on the lipoyl sulphydryl groups of the E2 subunit was described. However, several problems were encountered when using this reagent. In particular, in the presence of either α-oxo-glutarate or NADH, DTNB was unable to produce permanent modification of the lipoyl sulphydryl groups due to intramolecular displacement of the lipoyl-bound TNB^- group to regenerate unmodified lipoic acid. It is clear, therefore, that DTNB is not an efficient reagent for modifying the sulphydryl groups of the E2-bound lipoic acid. Consequently two other sulphydryl-specific reagents were examined:

(i) Iodoacetamide

Although iodoacetamide caused inactivation of whole complex activity, no increase was seen in the presence of either 1·0mM NADH or 25mM α-oxo-glutarate + 0·5mM TPP + 1·0mM AMP.

Table 15. The subunit specificity of inactivation by NTCB. 200µg of A. lwoffi α-OGDH complex in 0·4ml of MET-8 buffer were incubated with 1·0mM NTCB for 20min at 20°C. After incubation the enzyme was assayed for whole complex and subunit activities, and the results compared with those for untreated complex.

	Activity (absorbance units min^{-1} ml^{-1})	
	Before NTCB treatment	After NTCB treatment
Whole complex	398	17
E1 subunit	3·47	2·27
E2 subunit	6·2	2·0
E3 subunit	334	336

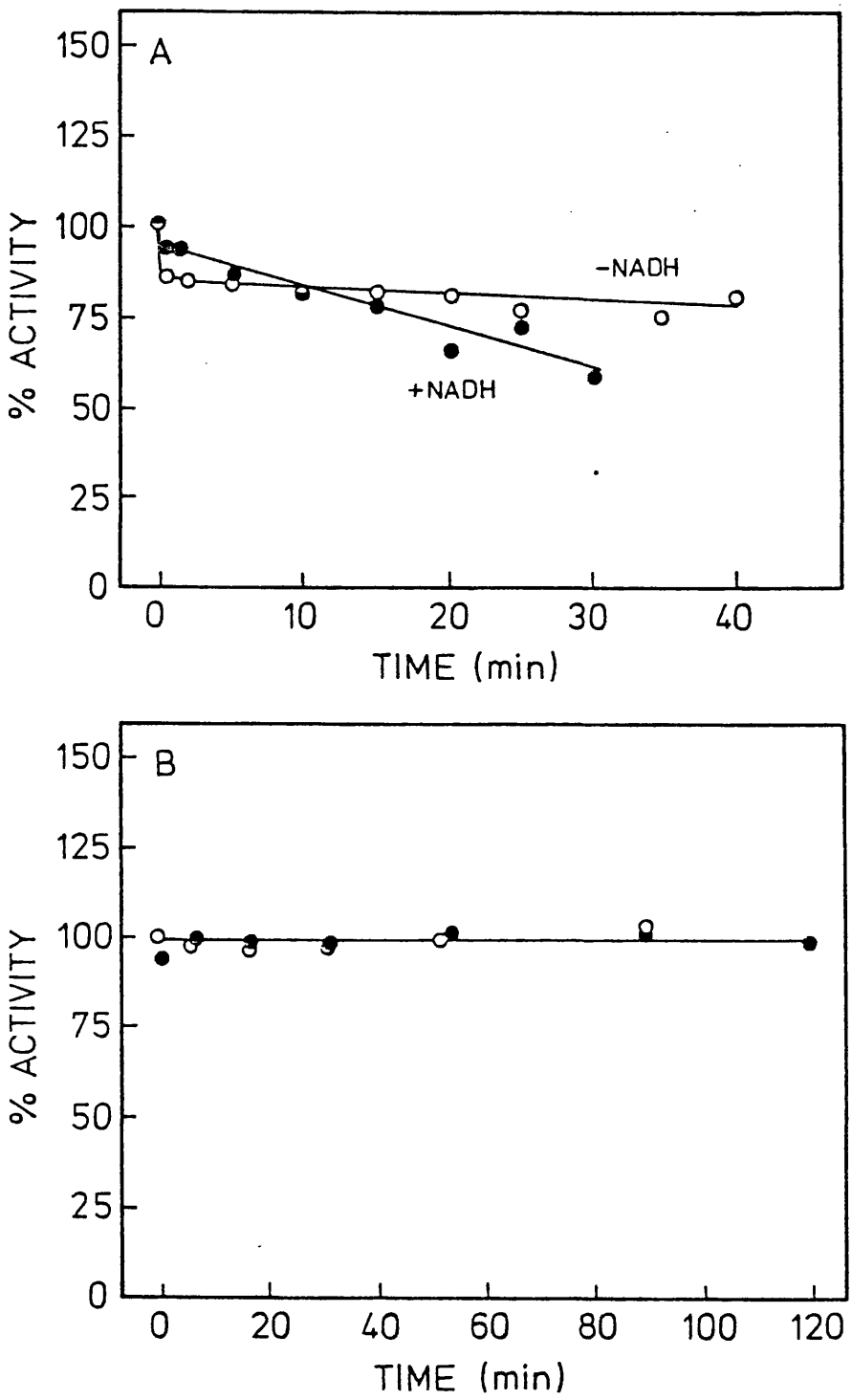


Fig. 39. The effect of NADH on NTCB inactivation of Torula yeast dihydrolipoamide dehydrogenase & E3 component of A. lwoffi α-OGDH. A. 0·25mg of Torula dihydrolipoamide dehydrogenase in 0·5ml MET-8 buffer was incubated at 20°C with 0·2mM NTCB in the presence or absence of 0·4mM NADH, and samples were assayed for activity. B. 0·1mg of A. lwoffi α-OGDH complex in 0·5ml MET-8 buffer was incubated with 0·4mM NTCB in the presence or absence of 0·4mM NADH. At intervals samples were assayed for E3 activity.

(ii) NEM

In the presence of 1·0mM NADH or 20mM α-oxoglutarate + 0·4mM TPP + 0·4mM AMP, both of which cause reduction of the disulphide bond of the E2-bound lipoic acid, a faster and more marked inhibition of whole complex activity was achieved on incubation with NEM (Fig. 40). This suggests that irreversible modification of the lipoyl sulphydryl groups has been achieved.

The effect of NEM modification on the individual subunit activities of the α-OGDH complex, both in the presence and absence of effectors and substrates was subsequently investigated (Table 16). It can be seen that NEM has its most pronounced effect on the E1 component, with a 75% inactivation after 3min. No increase in inactivation of either the E1 or the E3 subunit was observed in the presence of α-oxoglutarate, TPP and AMP. Similar results were obtained in the presence of NADH (although this could not be applied to the E1 component since NADH interferes with the assay). In contrast a complete loss of rate was observed for the lipoic acid and the E2-E3 interaction tests in the presence of either NADH or α-oxoglutarate + TPP + AMP; although only about a 45% loss of rate was observed in the presence of NEM alone. This suggests that in the presence of these substrates and effectors the E2-bound lipoic acid can be reduced and subsequently modified by NEM. No loss of E2 activity was seen under these conditions since a large excess of exogenous lipoamide was provided in the assay.

IV.3·4 Photooxidation

By studying the effect of pH on the photooxidation of a protein, some indication of which amino acids have been oxidised may be obtained (Westhead, 1965; Martinez-Carrion et al., 1967; Weil, 1965; Sluyterman, 1962; Weil et al., 1951), although the pattern becomes rather complex when more than one type of amino acid is affected. This approach was applied to the α-OGDH complex of A. lwoffi using rose bengal and methylene blue as the photo-

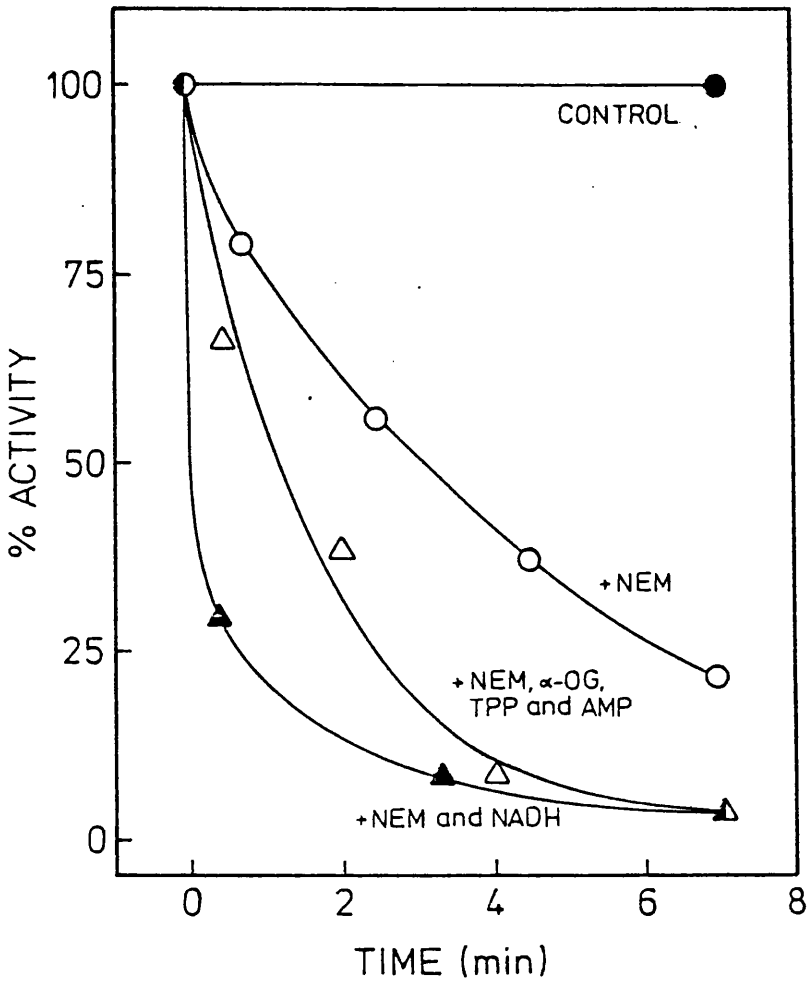


Fig. 40. Inactivation of A. lwoffi α-OGDH complex by NEM.

Experimental details are given in section IV.2.5.

Table 16. Effect of NEM on the individual subunit activities of α-OGDH complex.

The subunit specificity of α-OGDH modification by NEM was investigated, both in the presence and absence of substrates and effectors. Experimental details are given in section IV.2·5.

	Incubation time (min)	Activity (absorbance units min^{-1} ml^{-1}) Control	+ NEM	+ NEM + NADH	+ NEM + AMP + TPP + α-oxoglutarate
Whole complex assay	0	360	365	330	657
	0·5	-	313	120	510
	1·0	-	307	47	375
E1 subunit assay	0	0·65	-	-	-
	3·0	-	0·163	-	0·297
E2 subunit assay	0	0·34	-	-	-
	2·5	-	0·35	0·36	-
E3 subunit assay	0	6·6	-	-	-
	2·0	-	5·2	5·0	6·8
E2-E3 interaction test	0	11·6	-	-	-
	3·25	-	6·3	0	0
Lipoic acid test	0	11·6	-	-	-
	3·25	-	6·3	0	0

sensitizing dyes and some typical results are shown in Fig. 41. In both cases photooxidation was markedly enhanced above pH values of around 8·0, suggesting the oxidation of cysteine residues and consequently further supporting the evidence obtained from the DTNB studies (section IV.3·1) that cysteine residues are important in the catalytic mechanism of the enzyme.

(i) Subunit specificity of photooxidation

It can be seen from Table 17 that photooxidation (using rose bengal or methylene blue as the photosensitizing dye) of α-OGDH results in a marked loss of E1 and E2 subunit activities, with little or no effect on the E3 component. This is in reasonable agreement with the results obtained by chemical modification using DTNB and NTCB.

(ii) Effect of photooxidation on the kinetics and regulatory properties of α-OGDH

Photooxidation had no effect on the K_m of α-OGDH for α-oxoglutarate although the V_{max} was greatly reduced (from 0·15 to 0·026 units ml^{-1} min^{-1}). The regulatory properties of the complex were essentially the same after photooxidation.

Photooxidation with methylene blue was carried out in the presence of a number of substrates and effectors (i.e. AMP, NADH, cysteine, CoA, NAD^+, α-oxoglutarate, TPP and lipoamide) to examine any protection afforded. Although NADH and lipoamide showed some protection (Table 18), little useful information may be gained since lipoamide is thought to rotate through the catalytic sites of all three subunits and NADH may well act on more than one subunit (Parker & Weitzman, 1973). Furthermore, NADH can itself be photo-oxidised and may therefore give an apparent protection.

IV.3·5 Chemical modification by DEPC

The effect of chemical modification by DEPC, a histidine-specific reagent, on the α-OGDH complex of A. lwoffi was studied. Unfortunately

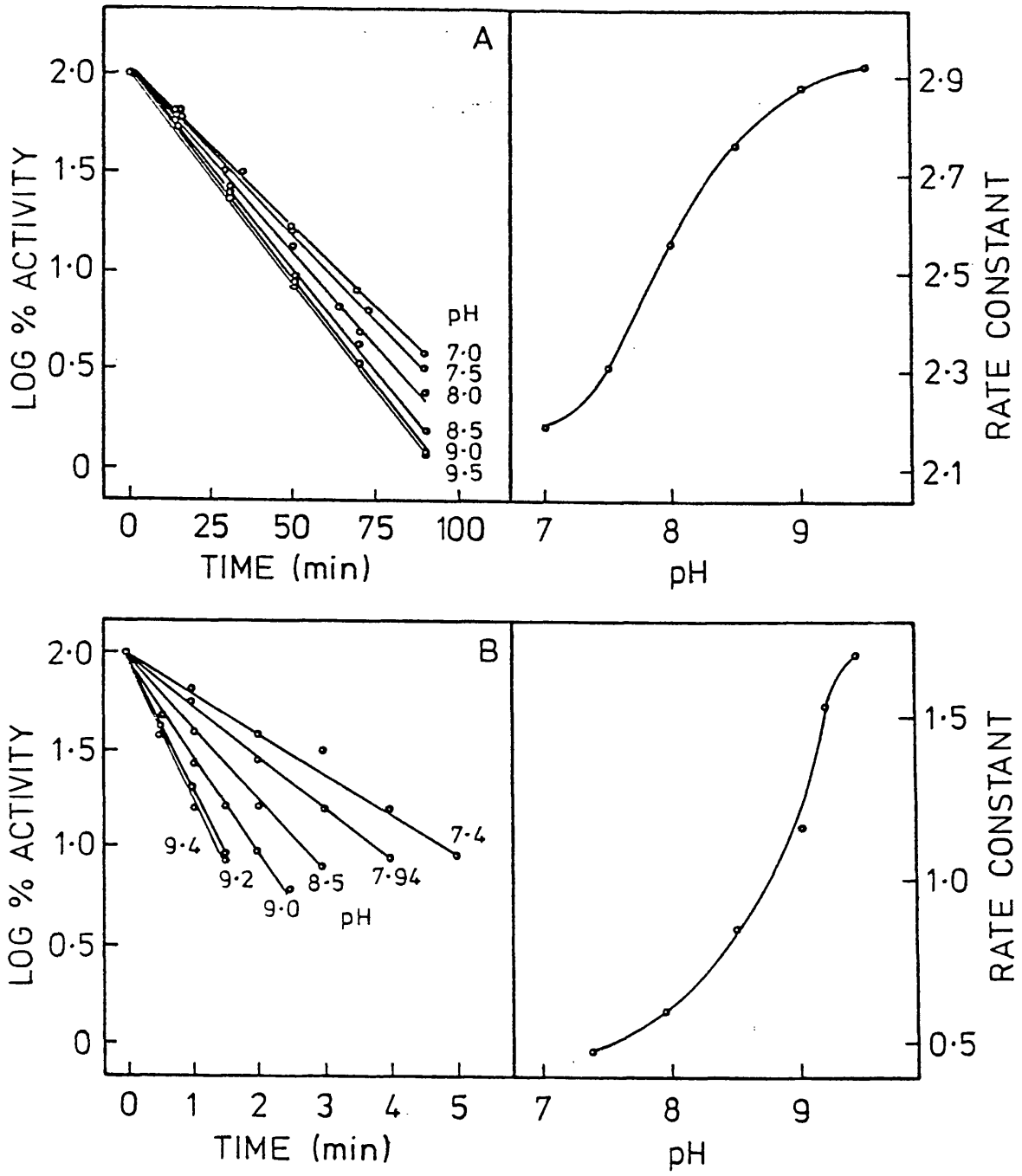


Fig. 41. Photooxidation of A. lwoffi α-OGDH complex at different pH values.

Purified α-OGDH complex was photooxidised in the presence of rose bengal (A) or methylene blue (B) in 2-methyl, 2-amino-1,3propane-diol buffer, as described in section IV.2.6. The loss of complex activity as a function of photooxidation time at various pH values is shown on the left from which the dependence of rate constant on pH may be derived (graphs on the right).

Table 17. Effect of photooxidation on the individual subunit activities.

Experimental details were as described in section IV.2•6. All activities are expressed in absorbance units min^{-1} ml^{-1}.

	Rose bengal as photosensitizing dye		Methylene blue as photosensitizing dye	
	Activity before photooxidation	Activity after photooxidation	Activity before photooxidation	Activity after photooxidation
Whole complex	349	61•9	252	27•5
E1 subunit	5•3	0•66	4•08	0•58
E2 subunit	12•9	7•5	13•0	6•0
E3 subunit	275	262•5	238	212

Table 18. Photooxidation of A. lwoffi α-OGDH complex in the presence of substrates and effectors.

Experimental details were described in section IV.2·6. The photosensitizing dye was rose bengal and photooxidation was carried out at pH 9·5 in 50mM 2-methyl, 2-amino 1,3-propanediol buffer.

Additions	Rate constant of inactivation by photooxidation (min^{-1})
None	2·0
0·2mM AMP	2·0
0·2mM CoA	1·9
5·0mM α-oxoglutarate	2·0
2·5mM cysteine	1·96
0·2mM NADH	1·1
0·5mM NAD^+	4·1
0·2mM TPP	2·0
0·5mM lipoamide	1·4

there was some variation in results obtained from different enzyme preparations possibly due to minor differences in protein conformation. Obviously this presents some difficulties in interpreting the effects of DEPC on the complex. In the forthcoming sections the results most frequently obtained will be presented, although the deviations from these will also be mentioned.

In the presence of a 2000-fold excess of DEPC to enzyme (i.e. under conditions of pseudo-first-order kinetics) the inactivation of the complex was essentially instantaneous at 20°C. However, if either the temperature was decreased (to 0°C) or the DEPC excess was decreased (to 20-fold) the kinetics of inactivation were normally found to be biphasic (Fig. 42), the initial rate ($k = 0.37\ min^{-1}$) being 15 times greater than the final rate ($k = 0.025\ min^{-1}$). Occasionally, however, some preparations did not show this typical biphasic pattern, but instead exhibited a linear mode of inactivation ($k = 0.025\ min^{-1}$, similar to the slower inactivation rate for the biphasic response).

(i) Subunit specificity of chemical modification by DEPC

Table 19 shows the typical effect of DEPC modification on the activities of the individual subunits of the α-OGDH complex. Inactivation can be seen to be specific to the E2 component which showed a loss of 85% of its activity during a 44min incubation with DEPC. E3 on the other hand showed only a very small loss of activity over this period (about 10%) and E1 activity was slightly stimulated (shown not to be due to an indirect effect of DEPC on the E1 assay). Occasionally with some preparations, however, a partial loss of E1 activity (of up to 30%) was observed. This could not be protected against to any appreciable extent by inclusion of AMP, TPP or α-oxoglutarate.

When the kinetics of E2 inactivation were followed a biphasic response was observed (Fig. 43) similar to that obtained for the whole complex (Fig. 42).

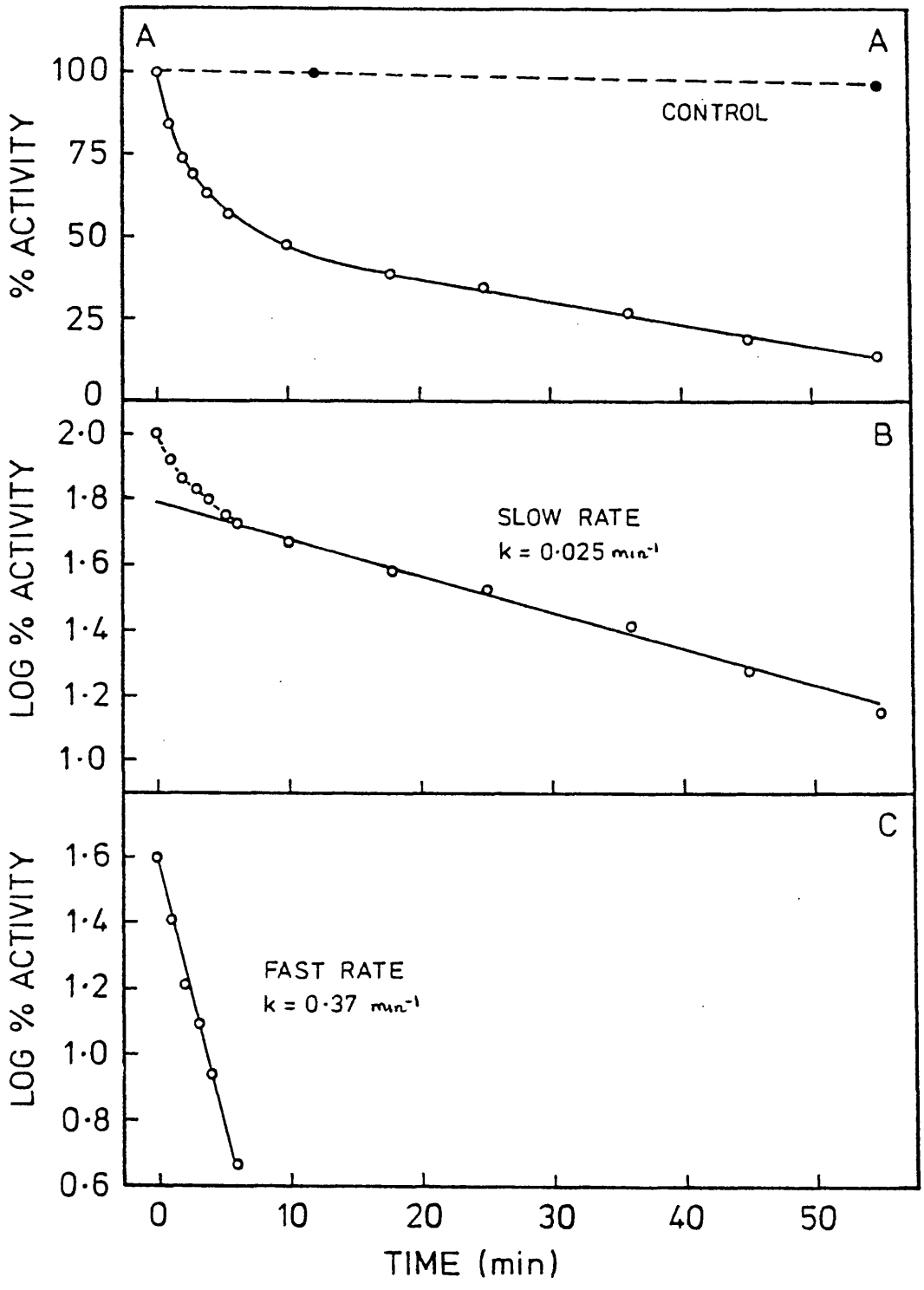


Fig. 42. Inactivation of A. lwoffi α-OGDH complex by DEPC.

Experimental details are given in section IV.2.7.

A. Time dependence of inactivation (o——o); "acetone" control (•- - -•).

B. Semilog plot for the determination of the slow rate constant.

C. Semilog plot for the determination of the fast rate constant (after subtraction of the slow rate).

Table 19. Subunit specificity of α-OGDH inactivation by DEPC.

0·5mg of A. lwoffi α-OGDH complex in 0·5ml of 0·1M phosphate buffer, pH 6·0, was made 10μM with respect to DEPC and then incubated at 20°C. At intervals samples were removed and assayed for whole complex and individual subunit activities.

	Incubation time (min)	Activity (absorbance units min^{-1} ml^{-1})
Whole complex	0	420
	11	310
	22	230
	42	192
E1 subunit	0	5·5
	14	6·3
	29	6·0
	47	7·0
E2 subunit	0	7·8
	5	3·4
	27	2·7
	44	1·2
E3 subunit	0	240
	9·5	230
	25	210
	41	215

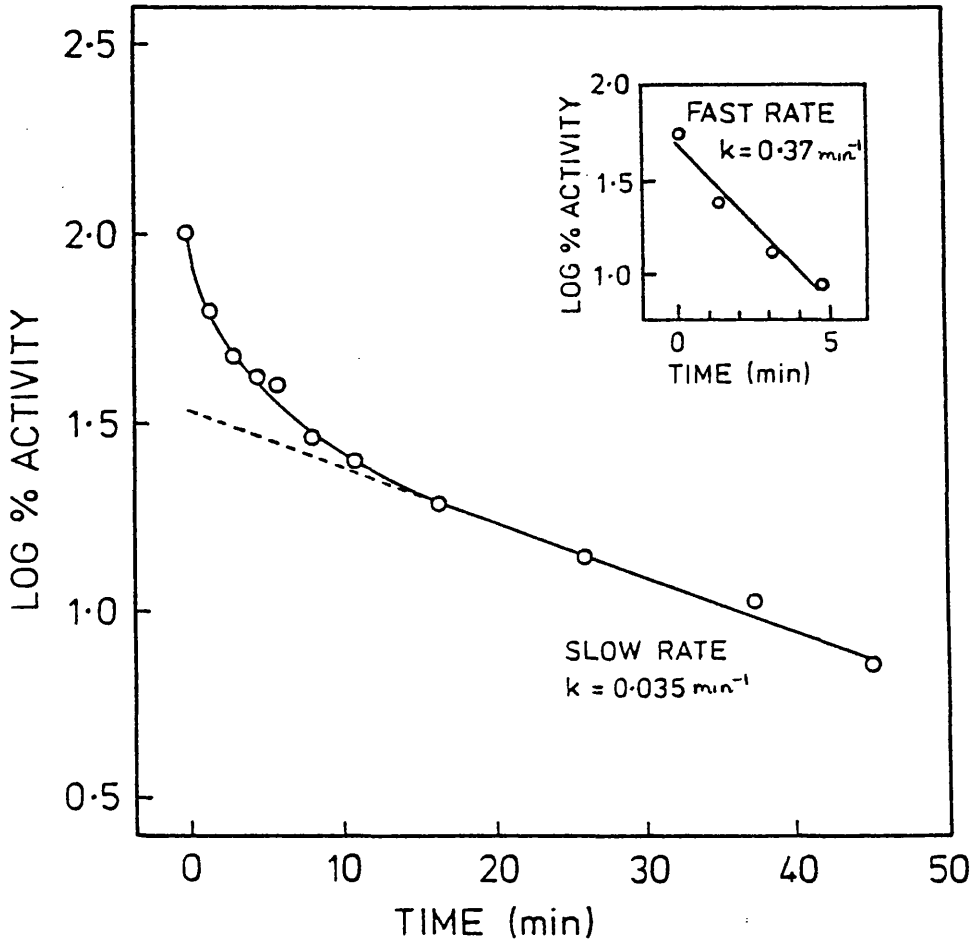


Fig. 43. Inactivation of the E2 component of α-OGDH by DEPC. A. lwoffi α-OGDH complex (1mg in 1ml of 0·1M phosphate buffer, pH 6·0) was made 10μM with respect to DEPC and then incubated at 20°C. At suitable intervals aliquots were removed and assayed for E2 activity.

(ii) Termination of DEPC inactivation by imidazole

Addition of a large excess of imidazole (1000 times that of the DEPC concentration) was found to prevent completely the DEPC inactivation of α-OGDH, by reacting with the DEPC and thereby removing it. In the absence of DEPC imidazole normally had no effect on the whole complex activity. Addition of imidazole may therefore be conveniently used to stop DEPC inactivation of the complex when studying time courses, and thereby prevent further inactivation during the subsequent enzyme assays. In this way the biphasic nature of inactivation of whole complex and E2 activities by DEPC was confirmed.

(iii) Reactivation of DEPC-inactivated complex by hydroxylamine

A. lwoffi α-OGDH complex was inactivated by DEPC for 5 or 45min and then tested for reversibility by hydroxylamine. It can be seen from Fig. 44 that only the second slow phase of inactivation of the complex was reversed by hydroxylamine. Furthermore, reactivation of this slow phase would appear to be complete since the reactivated 45min sample achieved the same activity as the 5min sample. The slight inactivation of the 5min sample and the reactivated 45min sample observed in Fig. 44B is due to the action of hydroxylamine on whole complex activity.

From these results it can be concluded that the second slow phase of complex inactivation by DEPC is due to modification of one or more histidine residues, whereas the initial faster rate of inactivation is likely to reflect modification of some other residues, possibly arginine or lysine.

(iv) Effect of DEPC modification on the kinetic and regulatory properties of α-OGDH

(a) Effect on the K_m for α-oxoglutarate. An investigation into the effect of DEPC modification of α-OGDH on the K_m for α-oxoglutarate was carried out to see if the modification interfered with α-oxoglutarate binding. Fig. 45 shows that DEPC inactivation caused a decrease in the V_{max}

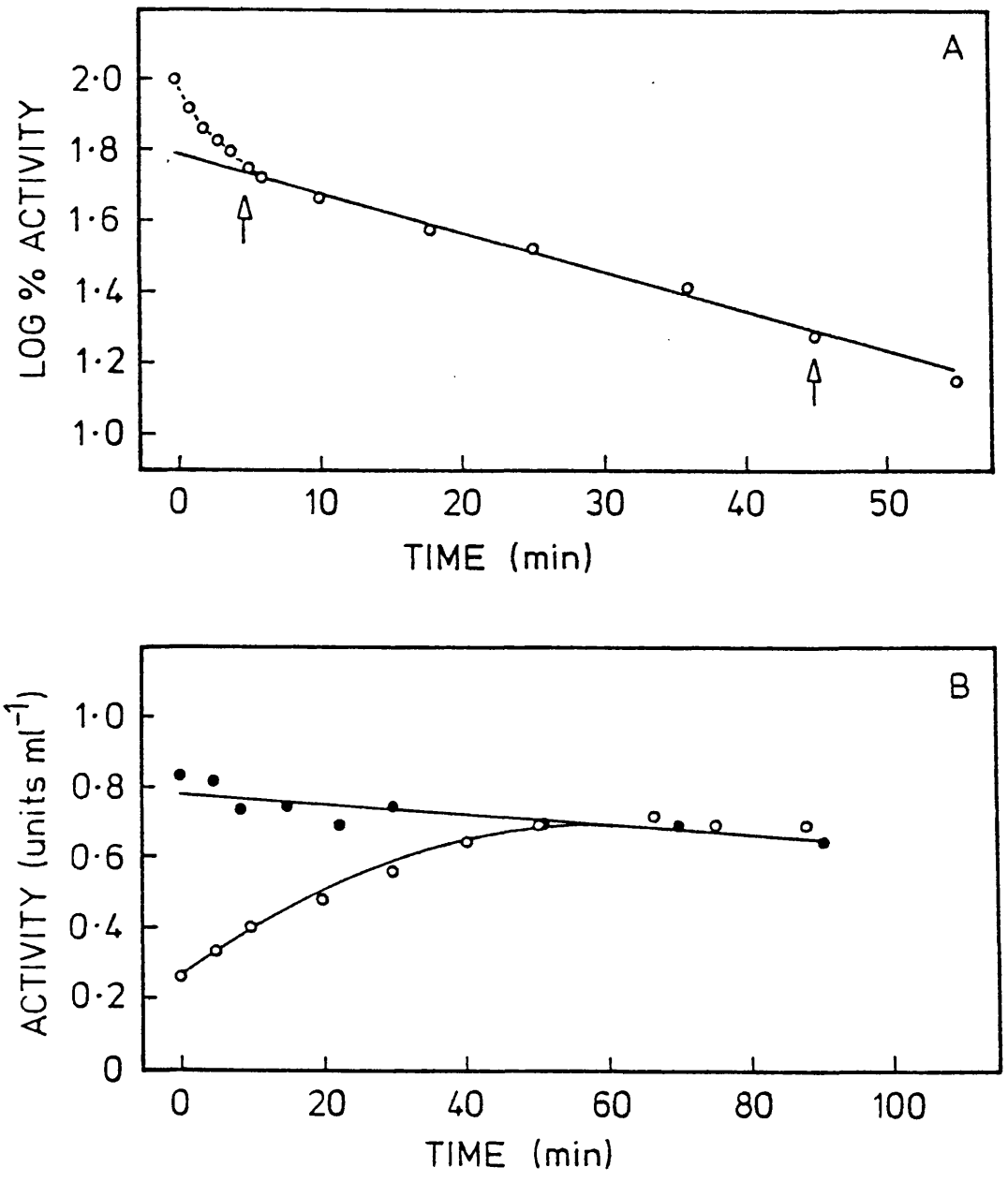


Fig. 44. Reactivation of DEPC-inactivated α-OGDH by hydroxylamine. 0·2ml aliquots were removed after incubating α-OGDH complex with DEPC for 5min and 45min. Further inactivation was prevented by the addition of excess imidazole and the effect of incubation in the presence of hydroxylamine was then determined. Experimental details are given in section IV.2·7.

A. Kinetics of DEPC inactivation.

B. Effect of incubating the 5min and 45min DEPC-inactivated samples with hydroxylamine.

(●——●), 5min sample. (○——○), 45min sample.

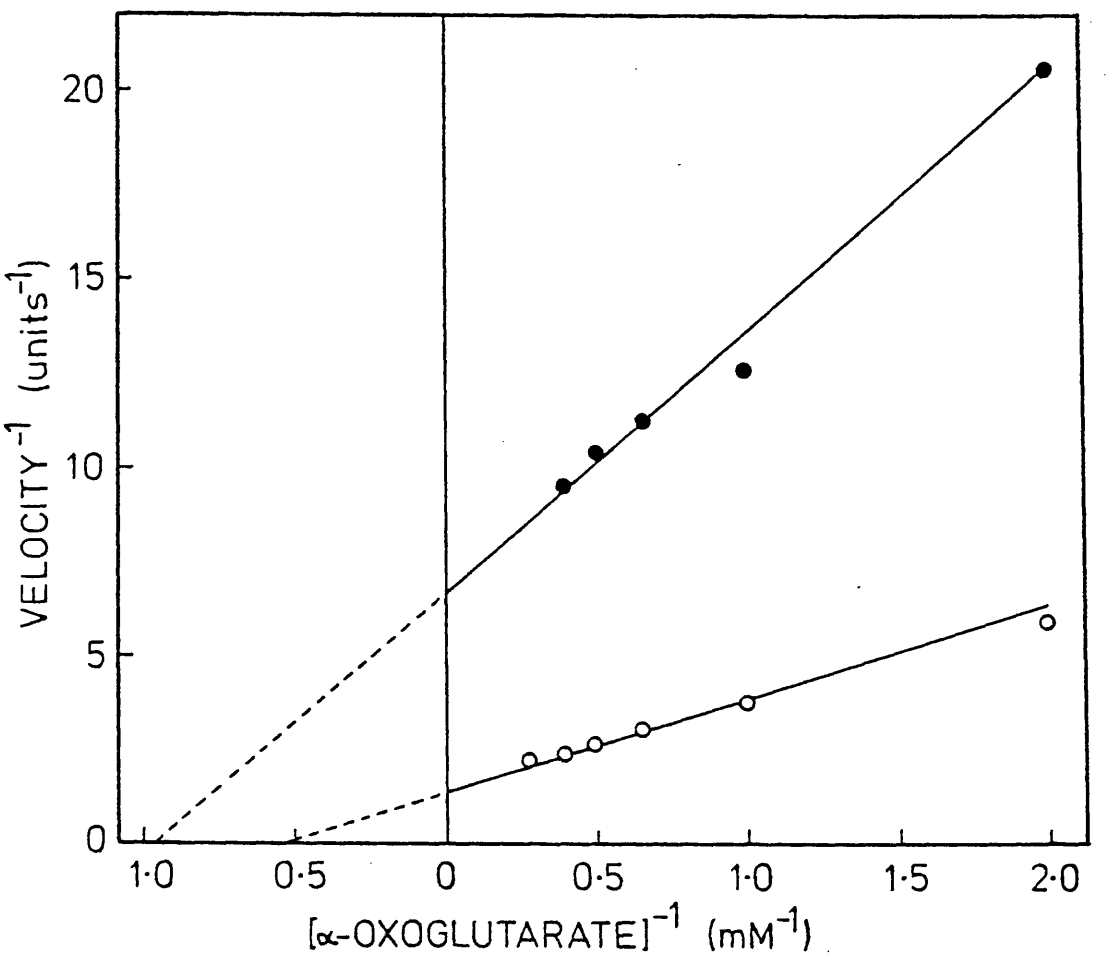


Fig. 45. Effect of DEPC modification on the K_m for α-oxoglutarate.

The K_m for α-oxoglutarate was determined before (o——o) and after (●——●) DEPC modification of A. lwoffi α-OGDH complex.

Modification was carried out as described in section IV.2.7 and the kinetics determined after 45min incubation with DEPC.

of the enzyme as might be expected, but surprisingly the K_m for α-oxo-glutarate was also decreased by this treatment (from 2·0 to 1·05mM). This suggests that modification of the enzyme may have increased its affinity for the substrate, possibly by a conformational change at the active site imposed by ethoxyformylation of the complex.

(b) Protection against DEPC inactivation. Various substrates and effectors (AMP, NADH, succinyl-CoA, lipoamide and CoA) were included in the enzyme-DEPC reaction mix in the hope that some protection against inactivation would be obtained; however, none was observed.

(c) Effect on the regulatory properties. Stimulation of complex activity by AMP was investigated before and during DEPC modification. It is apparent from Fig. 46A that although loss of complex activity occurred, the stimulatory effect of AMP was unchanged. Similarly the inhibitory effect of NADH was unaffected by treatment of the complex with DEPC (Fig. 46B).

In conclusion, it would appear that DEPC specifically modifies the catalytic site of the E2 component, with little or no effect on either the E1 and E3 active sites or the AMP and NADH regulatory sites.

IV.3·6 Chemical modification by HNBBr

Fig. 47 shows the inactivation of α-OGDH complex by HNBBr. However, this inactivation was not specific to one enzyme component; instead loss of all subunit activities was observed (Table 20).

IV.3·7 Inactivation by CoA

Although CoA is not generally thought of as a chemical modification agent it has been observed that this compound exhibits a time-dependent inactivating effect on very dilute solutions of *A. lwoffi* α-OGDH (100μg ml^{-1} protein), and has consequently been included in this section. Further investigation revealed that this CoA-directed inactivation was inversely dependent on enzyme concentration (Fig. 48), i.e. as the concentration of α-OGDH increased less inhibition by CoA was observed, and no inactivation

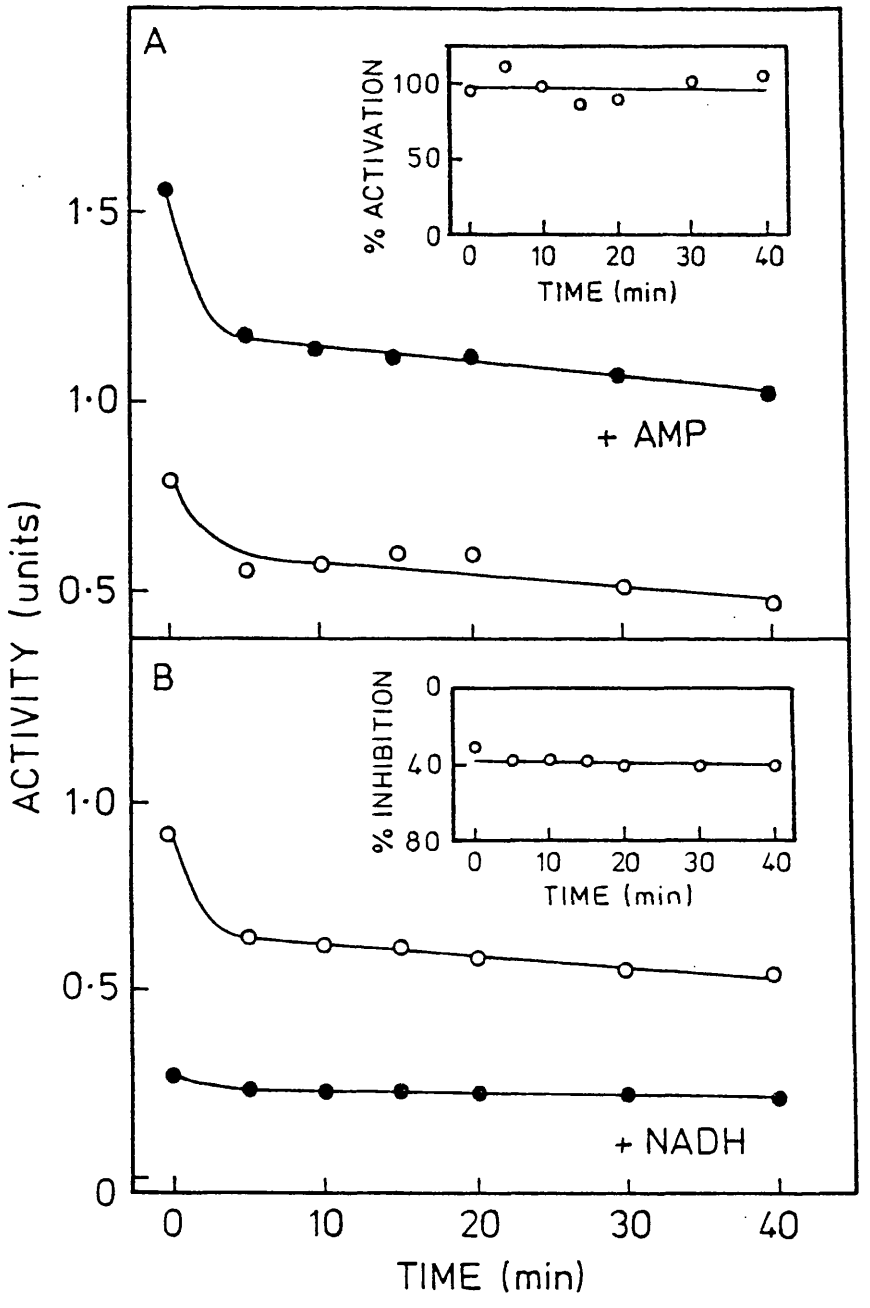


Fig. 46. The effect of DEPC modification on the regulatory properties of α-OGDH.

A. lwoffi α-OGDH complex (0·2mg in 1ml of 0·1M phosphate buffer, pH 5·8) was incubated at 20°C with 20µl of 100mM DEPC. At intervals samples were removed and added to cuvettes containing 50mM imidazole in MET-8 buffer. They were then assayed for whole complex activity.

A. Samples assayed in the presence (●——●) and absence (○——○) of 0·2mM AMP at 0·5mM α-oxoglutarate.

B. Samples assayed in the presence (●——●) and absence (○——○) of 0·2mM NADH at 5mM α-oxoglutarate.

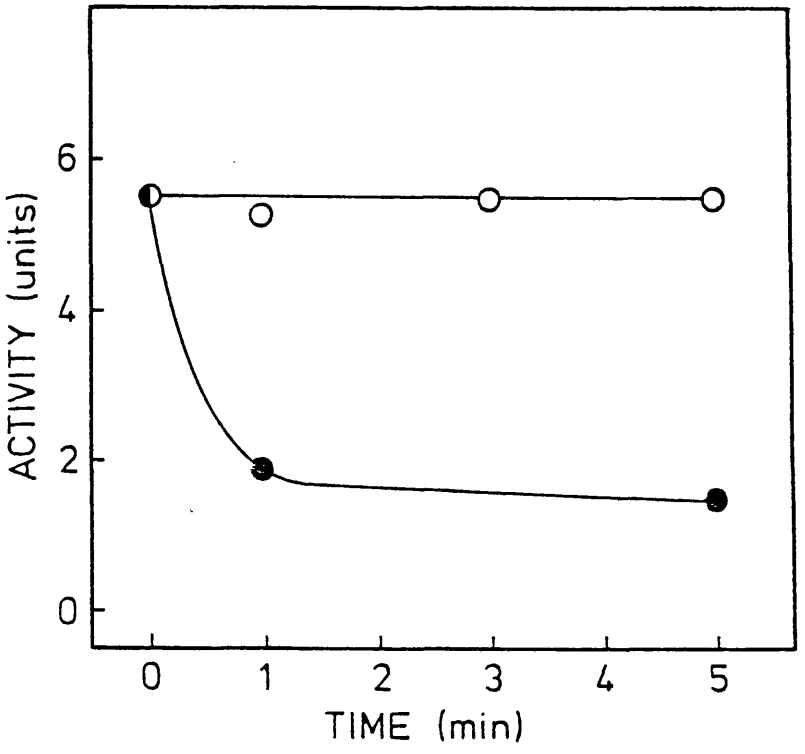


Fig. 47. Inactivation of A. lwoffi α-OGDH complex by HNBBr.

Experimental details are given in section IV.2·8.

(●—●), inactivation kinetics of α-OGDH by 10mM HNBBr; (○—○), control without HNBBr.

Table 20. The subunit specificity of inactivation by HNBBr.

The effect of HNBBr modification on the subunit activities of the A. lwoffi α-OGDH complex was investigated as described in section IV.2.8.

	Activity (absorbance units min^{-1} ml^{-1})	
	Before HNBBr treatment	After HNBBr treatment
Whole complex	375	10
E1 subunit	3.8	1.6
E2 subunit	1.5	0
E3 subunit	210	38

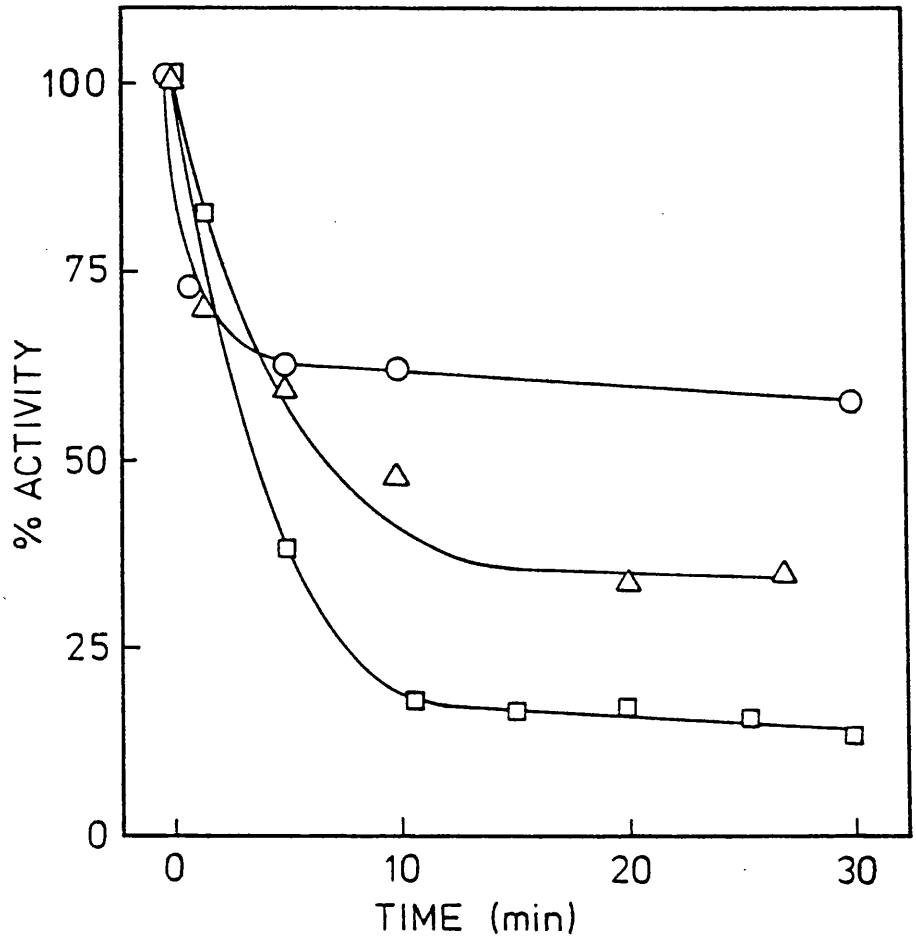


Fig. 48. Inactivation of A. lwoffi α-OGDH complex by CoA. 0·1ml of 5mM CoA was added to either 0·1mg (□—□), 0·2mg (△—△) or 0·5mg (○—○) of α-OGDH complex in 1ml of MET-8 buffer and incubated at 20°C. At intervals aliquots were removed and assayed for complex activity.

could be demonstrated with enzyme concentrations greater than 1·0mg ml^{-1}.

Similarly, dilute solutions of purified *E. coli* α-OGDH (200μg ml^{-1} of protein) showed time-dependent CoA inactivation (Fig. 49) but no loss of activity was observed with crude enzyme solutions.

(i) Effect of CoA on *A. lwoffi* α-OGDH

Fig. 50 illustrates the pH dependence of *A. lwoffi* α-OGDH inactivation by CoA. The optimum rate of inactivation was observed at a pH value of around 8·0.

Since the inactivation of α-OGDH by CoA can be slowly reversed by incubation with 4mM DTT (Fig. 51), the sulphydryl group of CoA would seem to be important for its effect. This is further supported by the observation that oxidised CoA (i.e. CoA-S-S-CoA) and acetyl-CoA showed no inactivating effect. Other sulphydryl compounds (e.g. cysteine or DTT) did not produce the same inactivating effect as did CoA.

Several difficulties were encountered when trying to determine the subunit specificity of CoA inactivation. First, this compound interferes with the E1 assay. Secondly, relatively large enzyme concentrations (at least 0·5mg ml^{-1}) are needed to obtain a reasonable measurement of E2 activity, concentrations at which the inhibitory effect of CoA was very much reduced. Consequently, although little or no inactivation of the E2 subunit by CoA was observed, this result is by no means conclusive. Finally, using dilute solutions of α-OGDH (100μg ml^{-1} of enzyme) it was possible to show that CoA had no effect on the E3 component activity.

No protection against inactivation of whole complex activity by CoA was seen in the presence of 10mM AMP, 0·5mM NADH or 5mM α-oxoglutarate but a slight protection was achieved by the addition of 0·5mM NAD^+.

It should perhaps be mentioned that inclusion of CoA did not appreciably alter the rate of photooxidation or DEPC inactivation of α-OGDH complex (sections IV.3·4 and IV.3·5). However, these studies were performed with

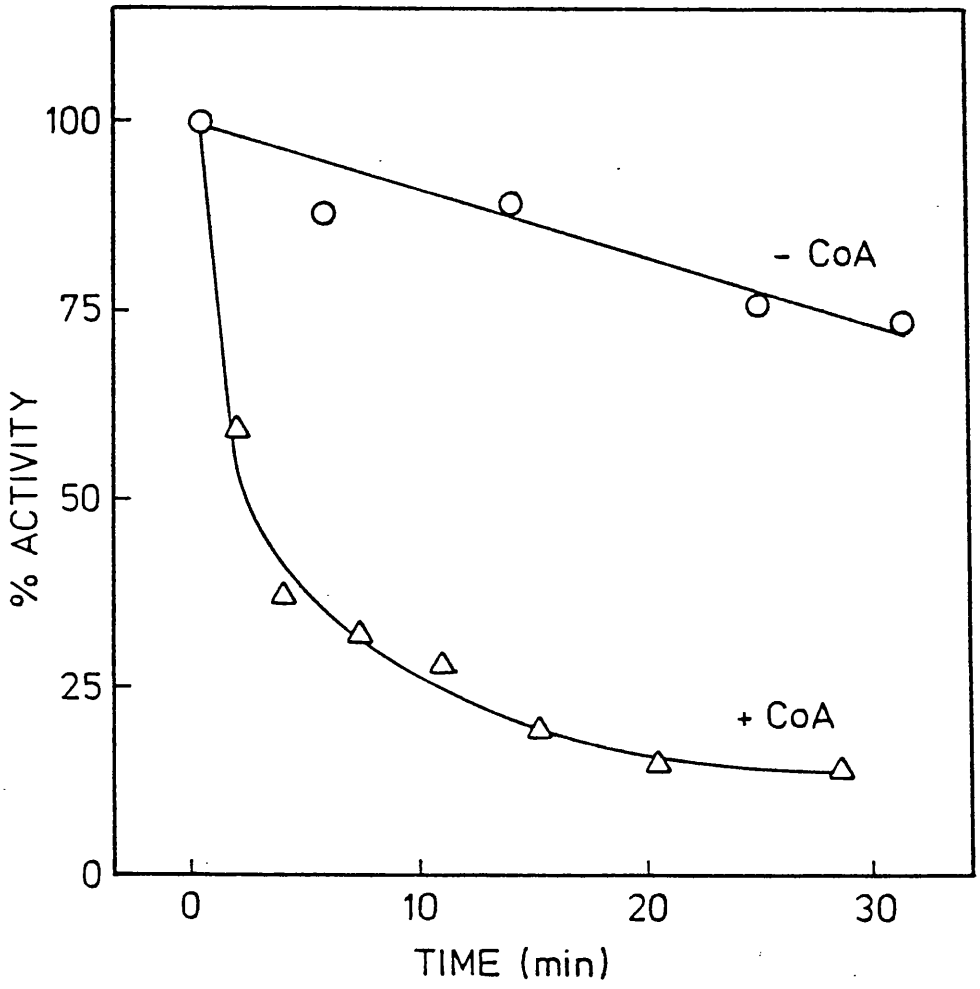


Fig. 49. Inactivation of E. coli α-OGDH complex by CoA.

50µl of 5mM CoA were added to 200µg of E. coli α-OGDH complex in 1ml of MET-8 buffer and incubated at 0°C. At intervals samples were removed and assayed for complex activity.

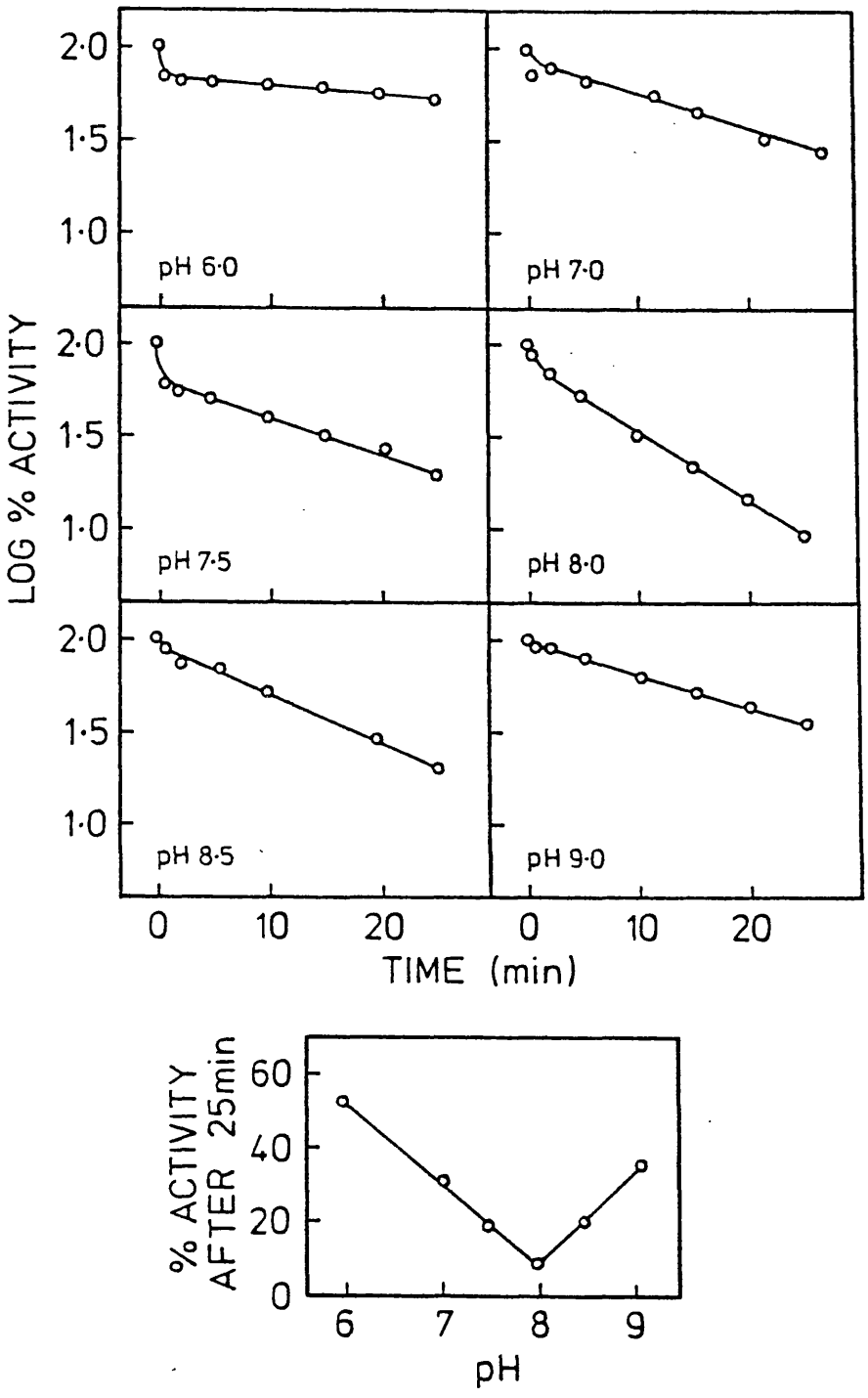


Fig. 50. The pH dependence of CoA inactivation of the A. lwoffi α-OGDH complex.

0·1mg of α-OGDH complex in 1ml of 20mM phosphate buffer at various pH values, was incubated with 0·5mM CoA at 20°C. At intervals aliquots were removed and assayed for complex activity.

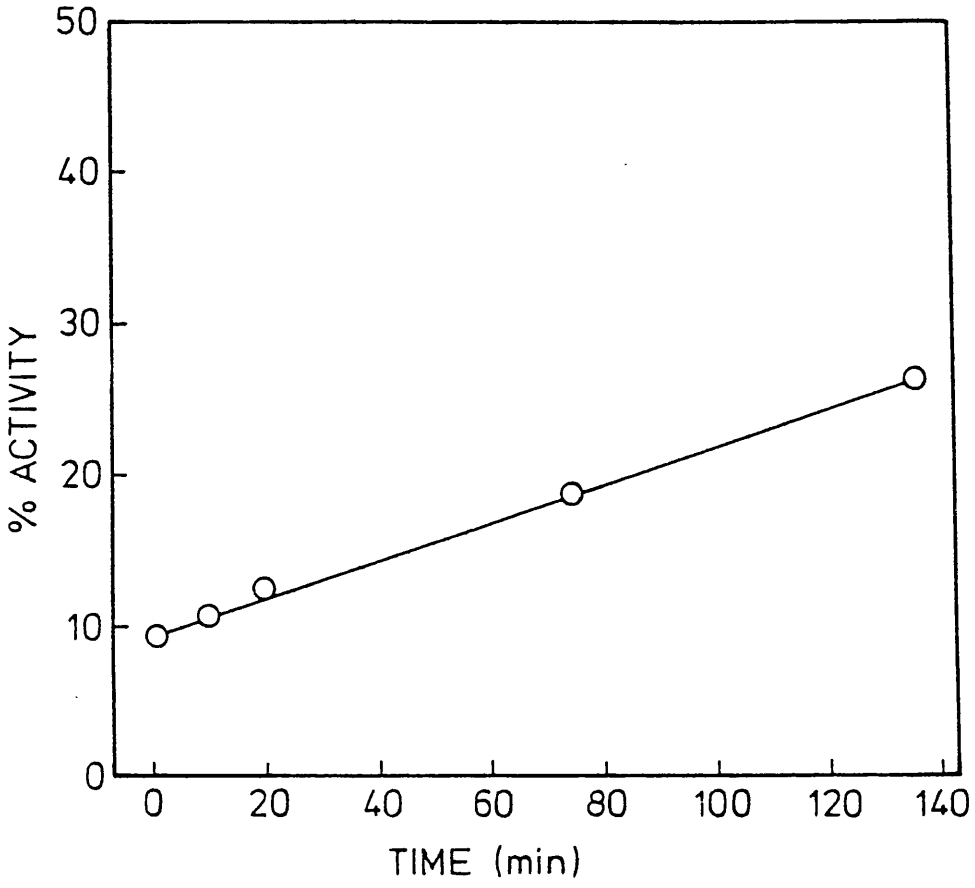


Fig. 51. The reversibility of CoA inactivation of A. lwoffi α-OGDH by DTT.

0·1mg of α-OGDH complex in 1ml of 20mM Tris-HCl, pH 8·0, was incubated at 20°C with·5mM CoA. After 40min incubation, 4mM DTT was added and incubation at 20°C continued. At intervals samples were removed and assayed for whole complex activity.

reasonably high enzyme concentrations (0·5mg ml^{-1}) and at pH values of 6·0 and 9·5, conditions under which the inactivating effect of CoA was much reduced.

(ii) Effect of CoA on E. coli α-OGDH

It was mentioned earlier in this section that CoA exerted a similar time-dependent inactivating effect on the E. coli α-OGDH as on the A. lwoffi enzyme. Similarly, it was shown that other sulphydryl compounds, e.g. DTT, did not have an inactivating action, nor was protection against CoA inactivation seen in the presence of α-oxoglutarate. However, in contrast to the A. lwoffi enzyme, complete protection was observed in the presence of 0·5mM NAD^+ and partial protection with 0·5mM NADH (Fig. 52). This suggests that in this case CoA exerts its effect on the E3 component. This was subsequently confirmed directly by studying the effect of CoA on the E3 subunit activity (Fig. 53), where it can again be seen that complete protection was obtained in the presence of NAD^+. What is perhaps more surprising is the observed stimulatory effect of NAD^+ on the E3 subunit; the activity of this component being 4 times greater in the presence of NAD^+ than in its absence.

Tracy & Kohlhaw (1975) have recently described a similar time-dependent inactivation of the biosynthetic condensing enzymes α-isopropylmalate synthase and homocitrate synthase by CoA. They suggested that CoA inactivation may provide a mechanism by which acetyl-CoA is channeled into the tricarboxylic acid cycle rather than into biosynthetic pathways. However, in view of the results obtained with the α-OGDH complexes, it is possible that inactivation by CoA may be a more general phenomenon than envisaged by these authors and consequently a more general explanation must be sought to explain these effects. From the results obtained with the α-OGDH complex of A. lwoffi it is clear that enzyme concentration is an important factor in this inactivation by CoA. This aspect does not seem to have been investigated by Tracy &

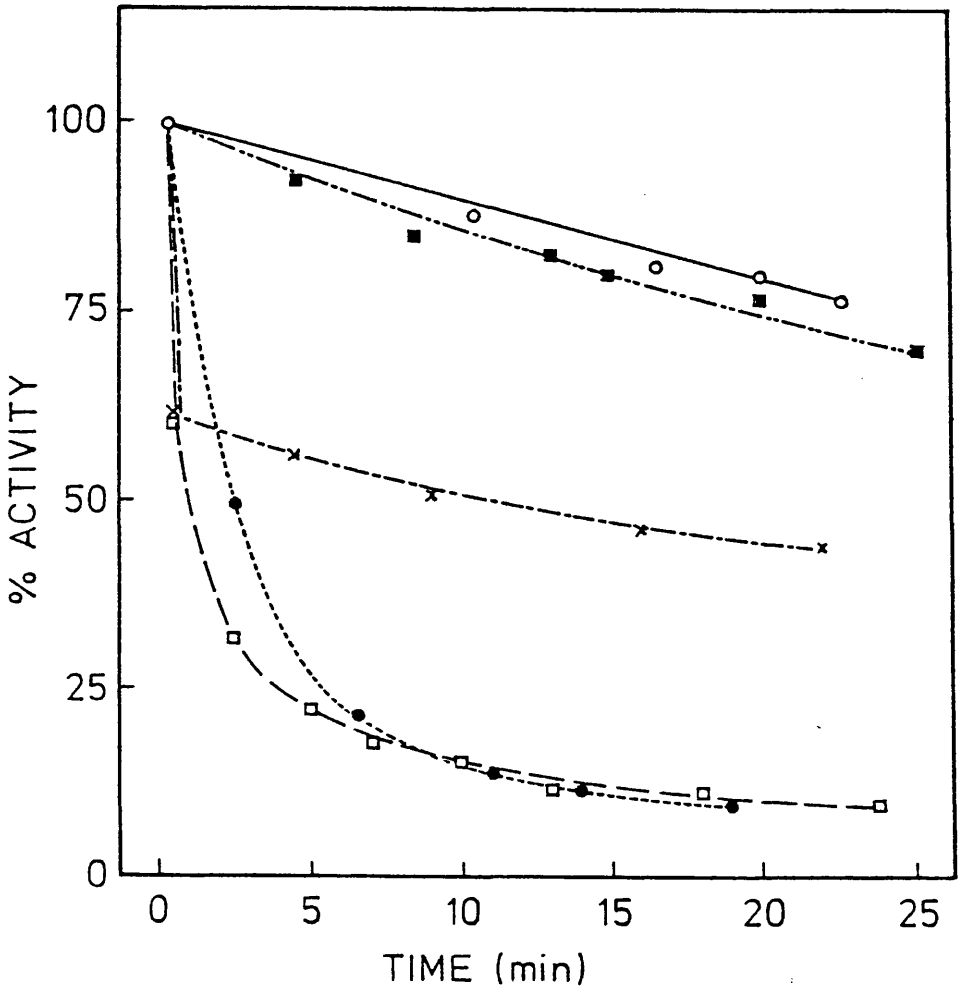


Fig. 52. Protection against CoA inactivation of E. coli α-OGDH by NADH and NAD^+.

200µg of purified E. coli α-OGDH in 1ml of MET-8 buffer were incubated at 0°C with 0·1mM CoA (□——□), 0·1mM CoA + 5mM α-oxoglutarate (●------●), 0·1mM CoA + 0·5mM NADH (x——x), or 0·1mM CoA + 0·5mM NAD^+ (■----■). At intervals samples were removed and assayed for complex activity. (o——o), control (no additions).

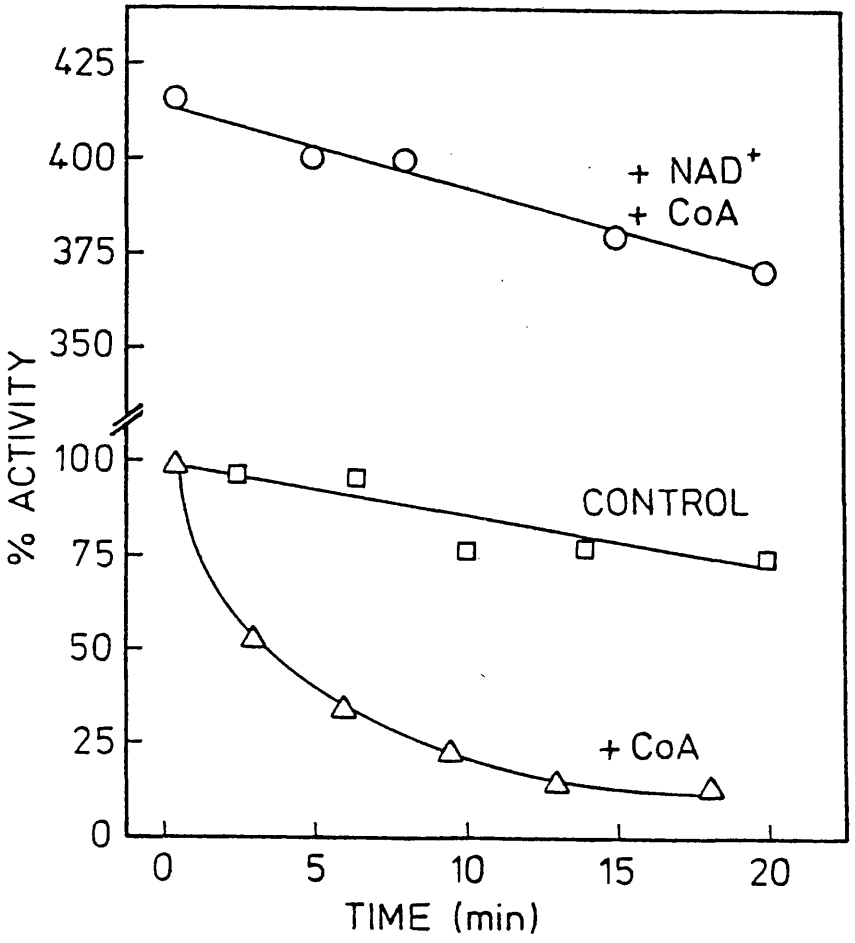


Fig. 53. The effect of CoA on E. coli E3 component activity. 200µg of purified enzyme were incubated at 0°C in the presence of 0·1mM CoA (△—△), or 0·5mM NAD^+ + 0·1mM CoA (○—○). At intervals samples were removed and assayed for E3 activity. Activities are expressed as % activity of enzyme which has been diluted for 30sec (without CoA or NAD^+).

Kohlhaw (1975).

In view of the enzyme concentration dependence of CoA inactivation it is difficult to assess the in vivo significance of this phenomenon.

CHAPTER V

ALLOSTERIC REGULATION OF THE α-OGDH COMPLEX

V.1 INTRODUCTION

Metabolism of food substances and biosynthesis of new compounds are regulated in the cell to satisfy the needs of cell maintenance and growth. Furthermore the cell must adapt its metabolic pattern to different growth conditions since the environment is rarely constant. Both of these are achieved by the control of certain "key" enzymes involved in metabolism, there being several ways in which regulation may operate:

(1) At the simplest level regulation occurs as a result of the catalytic properties of the enzyme protein, i.e. an enzyme-catalysed reaction may be regulated to some extent by the concentration of substrates, coenzymes and cofactors.

(2) Many key enzymes in procaryotes are inducible (i.e. regulation is achieved by the control of enzyme synthesis at the genetic level in response to the presence of inducers or derepressors). On the other hand, other enzymes are constitutive (i.e. their concentration is essentially constant, independent of growth conditions). In both cases the net enzyme concentration is a function of its synthesis and degradation. Control at the genetic level, although enabling the cell to adapt to a wide range of conditions, is nevertheless relatively slow and insensitive when compared with direct control of the catalytic properties of an enzyme.

(3) A much finer control of metabolism is achieved by the so called "regulatory enzymes". These enzymes usually catalyse an irreversible reaction at the beginning of a pathway or at a branch-point and are subject to regulation (which may be either stimulatory or inhibitory) by the interaction of various compounds with the enzyme, thereby altering its

catalytic properties. For example, the end-product of a metabolic sequence often causes inhibition of the first enzyme of that pathway ("feedback inhibition") and consequently prevents its over production. Furthermore, some regulatory enzymes may respond to "inhibitors" or "activators" from more than one metabolic sequence and thereby integrate the control of different pathways.

(4) The final mechanism of regulation is restricted to higher eukaryotic organisms and involves control by hormones.

Regulation of α-OGDH activity in bacteria serves two purposes. First, being situated in the tricarboxylic acid cycle, it is able to control energy production. Second, it may influence the fate of α-oxoglutarate which is situated at a branch-point and can either be converted to glutamate by glutamate dehydrogenase (L-glutamate:NAD oxidoreductase) or aspartate transaminase (L-aspartate:2-oxoglutarate aminotransferase) or to succinyl-CoA by α-OGDH. It has been noted that the α-OGDH complex of gram negative bacteria exhibits feedback control by NADH (Weitzman,1972; Parker & Weitzman, 1972, 1973; Parker, 1973). However a second level of control is observed in some strict aerobes, including A. lwoffi, where a high AMP concentration (reflecting a low ATP level in vivo) overcomes the NADH inhibition and in fact causes stimulation of enzyme activity (Weitzman, 1972). This dual control is likely to provide a more sensitive means of enzyme regulation.

It was mentioned earlier that it is usually the first enzyme in a pathway that is irreversible and shows the regulatory properties. This is indeed found to be the case for the sequence of reactions catalysed by the α-OGDH complex where it is the first component enzyme (E1) which is irreversible and is thought to be the site of AMP and NADH control.

Some information about the regulatory properties of the α-OGDH complex of A. lwoffi has been published by Parker & Weitzman (1973). These authors presented direct evidence that AMP activates the E1 component of the complex,

reducing its K_m for α-oxoglutarate 10-fold. However, the sites of NADH inhibition were more difficult to determine since, although it could be shown directly that NADH acts on the E3 subunit, its action on E1 could not be established (due to interference in the DCPIP assay by this nucleotide). However it was deduced from indirect kinetic evidence that inhibition of both the E1 and E3 components occurs. This evidence can be summarized as follows: First, the rate dependence of the complex on α-oxoglutarate concentration was determined at different NADH concentrations in the presence of saturating and non-saturating amounts of NAD^+. At non-saturating NAD^+ concentrations, although the extent of NADH inhibition decreased with increasing amounts of α-oxoglutarate, on extrapolation to infinite α-oxoglutarate concentration the NADH inhibition was not completely overcome. Complete loss of inhibition was achieved, however, in the presence of saturating levels of NAD^+. Secondly, a similar effect was seen when the rate dependence of the complex on NAD^+ concentration was determined at different NADH concentrations in the presence of saturating and non-saturating amounts of α-oxoglutarate. It was only at the higher α-oxoglutarate concentrations that strict competition with NAD^+ was observed. These results suggested that NADH inhibits the E1 component competitively with respect to α-oxoglutarate and the E3 component competitively with respect to NAD^+.

The present study sought to explore further the nature of this regulation and to examine, more directly, the effect of NADH on the E1 subunit. Since both AMP and NADH are structurally dissimilar to α-oxoglutarate it is not unreasonable to expect that they may interact with distinct sites on the E1 component, i.e. that the regulation would be allosteric rather than isosteric. The allosteric nature of many regulatory enzymes is becoming increasingly apparent. A classical example is aspartate transcarbamylase which shows the sigmoidal kinetics (of reaction velocity against substrate

concentration) frequently, but not always, associated with allosteric enzymes. This enzyme has been shown to be composed of discrete regulatory and catalytic subunits which may be dissociated from each other in the presence of mercurials, resulting in an enzyme which no longer shows feed-back inhibition and consequently possesses normal hyperbolic kinetics.

In view of the appearance of only three types of polypeptide chains after SDS gel electrophoresis of the α-OGDH complex of A. lwoffi (see section II.3·5) it seems unlikely that the E1 component is composed of separate regulatory and catalytic subunits. Nevertheless the regulatory and catalytic sites may be located in different regions of the polypeptide chain. This possibility was investigated using the techniques of enzyme desensitization towards effectors and multiple-inhibition analysis. In the former case the aim is to eliminate selectively the regulatory properties of the enzyme by treatment with a suitable reagent, without significantly affecting the catalytic properties, thereby indicating that substrate and effector bind at different sites. The method of multiple-inhibition analysis (Yonetani & Theorell, 1964) involves examination of the multiple inhibition of the enzyme by two inhibitors, each competitive with the same substrate. From graphical analysis of the results it is possible to deduce whether the two inhibitors act at the same or different sites on the enzyme. It has recently been shown that this method can distinguish between isosteric and allosteric nucleotide inhibition of diverse citrate synthases (Harford & Weitzman, 1975).

V.2 METHODS

V.2·1 Reversibility of the effect of urea on the AMP regulation of α-OGDH

A. lwoffi α-OGDH complex (0·2mg ml^{-1} in 10mM $MgCl_2$, 1mM EDTA, 0·1M Tris-HCl, pH 8·0) was assayed for whole complex activity in the presence and absence of 0·2mM AMP and at an α-oxoglutarate concentration of 0·5mM, under the following conditions:

(a) the enzyme solution and assay buffer contained no urea.

(b) the enzyme solution contained no urea but 1·5M urea was included in the assay cuvette.

(c) the enzyme solution was made 1·5M with respect to urea and immediately assayed in a cuvette containing 1·5M urea.

(d) the enzyme solution was made 1·5M with respect to urea and assayed immediately in a cuvette lacking urea.

Due to the time-dependent inactivation of the complex by urea, assays in the absence and presence of AMP were carried out simultaneously in the case of (c) and (d).

V.2·2 Reversibility of the effect of urea on the K_m for α-oxoglutarate

It was necessary to carry out all assays on the dependence of α-OGDH complex activity on α-oxoglutarate concentration simultaneously, due to the loss of activity in the presence of urea. This was achieved using a Unicam SP 800 spectrophotometer equipped with an automatic four place sample changer. Consequently this restricted the number of assays for each K_m determination to four.

(i) K_m for α-oxoglutarate in the absence of urea

Purified α-OGDH was diluted with MET-8 buffer to a concentration of 100μg ml^{-1} and assayed for whole complex activity at four different α-oxoglutarate concentrations.

(ii) K_m for α-oxoglutarate in the presence of urea

α-OGDH complex was diluted to a concentration of 100μg ml^{-1} with MET-8

buffer containing 1M urea and assayed immediately at four different α-oxoglutarate concentrations in assay cuvettes containing 1M urea.

(iii) K_m for α-oxoglutarate after the removal of urea by dilution

After dilution to 100μg ml^{-1} in buffer containing 1M urea, the complex was assayed immediately at four different α-oxoglutarate concentrations in assay cuvettes lacking urea. In this way the urea concentration was reduced 100-fold in the assay cuvette.

V.2.3 The effect of non-ionic detergents on the AMP regulation of α-OGDH

(i) Desensitization of complex to AMP

2μg of purified α-OGDH complex were preincubated for 2min at 25°C in a cuvette containing assay buffer plus the appropriate concentration of Nonidet P40 or Triton X100 (usually 12%). The substrates and cofactors for the whole complex assay, and AMP where appropriate, were then added and the reaction started by the addition of α-oxoglutarate to give a total final volume of 1.0ml.

(ii) Reversibility of desensitization to AMP

The reversibility of desensitization to AMP by Triton X100 was investigated using an identical method to that described for urea (see section V.2.1) except that 12% Triton X100 was included in place of 1.5M urea.

(iii) Reversibility of the effect of Triton X100 on the K_m for α-oxoglutarate

The reversibility of the effect of Triton X100 on the K_m for α-oxoglutarate was studied using the method described for urea (section V.2.2), 12% Triton X100 being used in place of 1M urea.

V.2.4 Multiple-inhibition studies on α-OGDH

Whole complex assays on the α-OGDH complex of *A. lwoffi* were performed spectrophotometrically as described in section I.2.2. In all assays in which NADH was included the concentration of NAD^+ was raised to 2.0mM to overcome NADH inhibition of the E3 component enzyme (Parker & Weitzman, 1973). The

concentration of α-oxoglutarate was varied in some cases (as indicated in the relevant figure legends), but was maintained at 5·0mM for all multiple-inhibition experiments. Reactions were started by the addition of 2μg of enzyme to give a total volume of 1·0ml. Multiple-inhibition measurements were made in triplicate and the best fit to the data computed by linear regression analysis.

V.3 RESULTS AND DISCUSSION

For reasons outlined in the introduction to this chapter it seemed likely that AMP and NADH might act allosterically on the E1 component. Attempts were made to confirm this by desensitizing the complex to its regulatory effectors while retaining catalytic activity.

V.3.1 Effect of urea on the regulation of the α-OGDH complex

Preliminary results obtained by Parker (1973) indicated that the α-OGDH complex of A. lwoffi could be desensitized to AMP (but not NADH) regulation by urea. This effect has been studied more fully here. It can be seen from Fig. 54 that incubation of the complex with increasing amounts of urea resulted in a progressive loss of AMP stimulation but complete desensitization was not achieved. However, due to the irreversible inactivation of complex activity by this denaturant (see section III.3.1), the effect of high urea concentrations on AMP stimulation could not be determined, since virtually all complex activity was lost. Similar results were obtained with guanidine hydrochloride. Furthermore, incubation of the complex with urea prior to addition of AMP to the assay did not enhance the desensitization.

The K_m values for α-oxoglutarate in the absence and presence of 0.2mM AMP were found to be 2.5mM and 0.2mM respectively. After addition of 1.0M urea K_m values of 0.3-0.4mM in the absence of AMP and 0.12mM in the presence of AMP were obtained. Urea would therefore seem to affect both the catalytic and the regulatory properties of the complex.

In order to show that the effect of urea on the AMP regulatory properties of the complex is not simply due to the irreversible enzyme inactivation observed in section III.3.1 attempts were made to determine whether the effects of urea on regulation were reversible. It is clear from Table 21 that removal of urea by dilution resulted in a restoration of AMP activation showing that this effect is indeed reversible. However, the decrease in the K_m for α-oxoglutarate observed in the presence of 1.0M urea

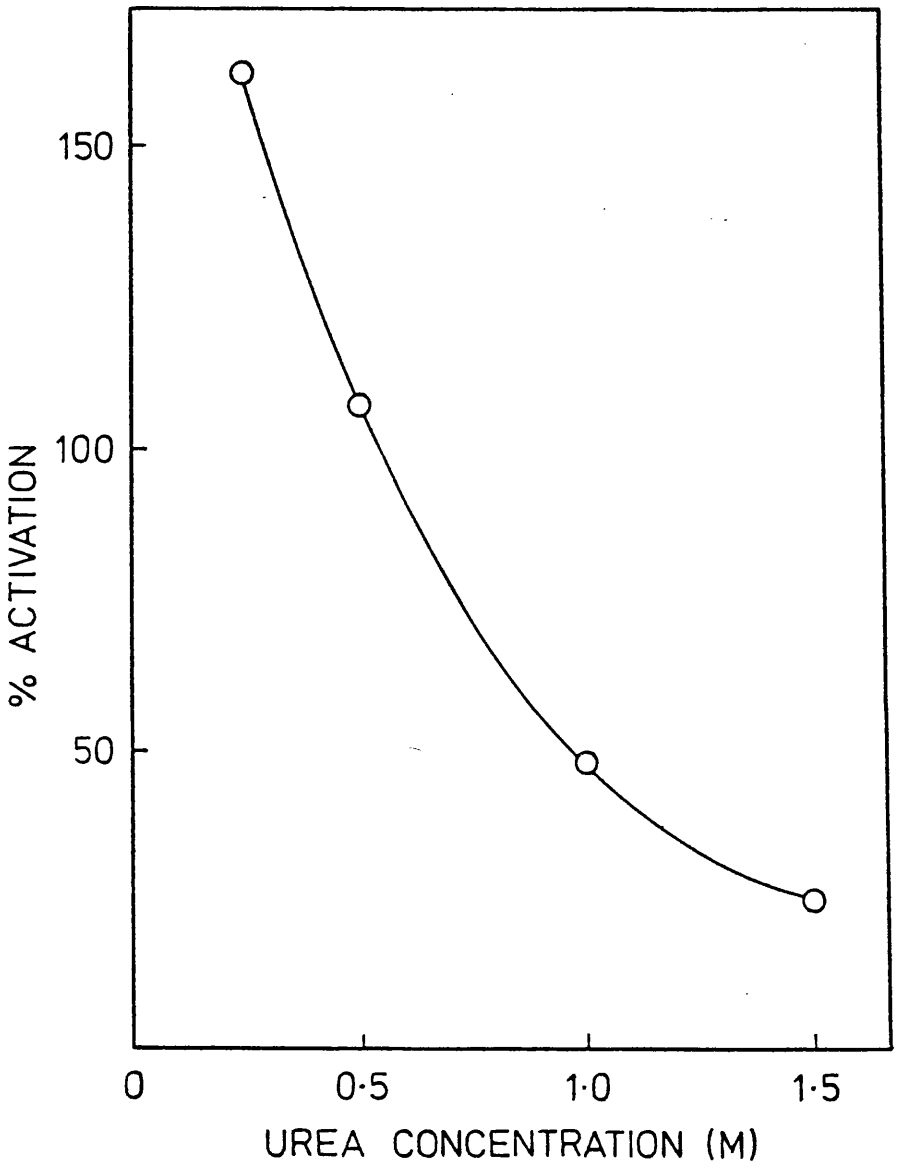


Fig. 54. Desensitization of A. lwoffi α-OGDH complex to AMP using urea.

5μg of purified α-OGDH were assayed for whole complex activity in the presence of varying concentrations of urea; in the presence and absence of 0·2mM AMP. The concentration of α-oxo-glutarate was 0·5mM.

Table 21. Reversibility of the effect of urea on the AMP regulation of α-OGDH.

Experimental details are described in section V.2.1

Whole complex assay conditions	Activity (absorbance units min^{-1} ml^{-1}) Without AMP	With AMP	% Stimulation
0.2mg of complex in 1ml of buffer; normal complex assay conditions.	1.825	5.025	275
0.2mg of complex in 1ml of buffer; assay cuvette containing 1.5M urea.	1.475	2.25	158
0.2mg of complex in 1ml of buffer containing 1M urea; assay cuvettes containing 1M urea.	1.075	1.425	132
0.2mg of complex in 1ml of buffer containing 1.5M urea; normal complex assay conditions.	1.525	4.0	261

was also shown to be reversible (K_m in the presence of 1·0M urea = 0·4 ± 0·1 mM [4 determinations]; K_m of untreated enzyme or after removal of urea by dilution = 2·3 ± 0·2mM [5 and 2 determinations respectively]; see section V·2·2).

In conclusion, urea causes a partial loss of AMP stimulation. However, attempts to remove specifically the regulatory properties of the complex without affecting the catalytic activity were not completely satisfactory and consequently it can not be concluded with any certainty whether or not the catalytic and regulatory sites are distinct.

V.3·2 Effect of non-ionic detergents on the regulation of α-OGDH complex

The non-ionic detergents Triton X100 and Nonidet P40 were investigated as potential agents for desensitizing the complex towards its regulatory effectors in a similar manner to urea. It can be seen from Fig. 55 that a progressively increasing degree of desensitization towards AMP was observed as the concentration of Triton X100 was increased. Similar results were obtained with Nonidet P40. However, this effect was confined to AMP regulation since complete NADH inhibition was retained throughout (Table 22); a situation analogous to that obtained with urea treatment. Unfortunately, due to the viscosity of Triton and Nonidet it was impossible to use detergent concentrations greater than 12% (v/v) without impairing efficient mixing, and hence complete desensitization could not be achieved. The time (up to 2h), temperature (from 25°C to 45°C) and pH (from 6·5 to 8·5) of enzyme preincubation in the presence of detergent was varied without any significant increase in desensitization of the complex to AMP stimulation. Inclusion of 1·0M urea in the preincubation mix was also without effect. Prolonged incubation of the enzyme with detergent resulted in partial loss of activity but this effect was not as pronounced as in the presence of urea.

Fig. 56 shows the effect of Triton X100 (12%) on the activation of the

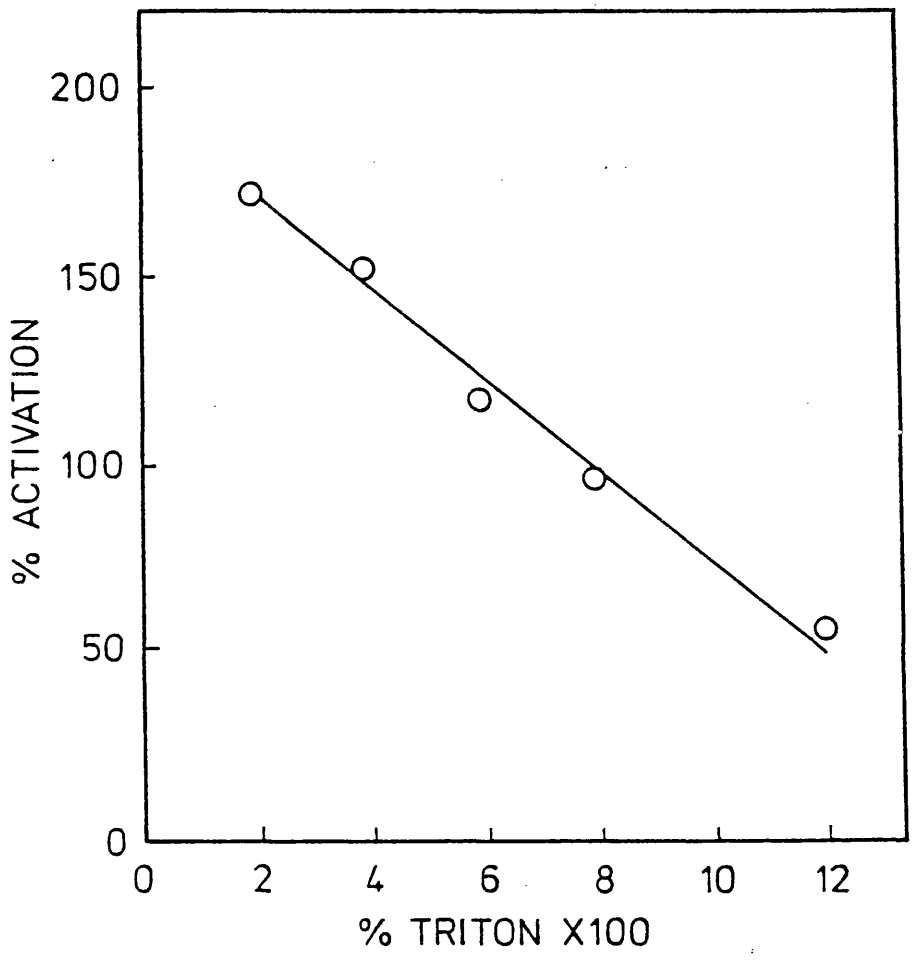


Fig. 55. Desensitization of A. lwoffi α-OGDH complex to AMP using Triton X100.

2µg of purified α-OGDH were incubated for 2min at 25°C in cuvettes containing assay buffer and various concentrations of Triton X100. Substrates and cofactors needed for whole complex assays (with and without 0·2mM AMP) were then added, the reaction being started by the addition of 0·5mM α-oxoglutarate.

Table 22. Effect of Triton X100 on the inhibition of α-OGDH complex by NADH.

2μg of the purified complex were incubated in a cuvette containing assay buffer in the presence or absence of 12% (v/v) Triton X100 for 2min. The substrates and cofactors for the whole complex assay were then added together with 0·2mM NADH where indicated, and the reaction started by the addition of 5mM α-oxoglutarate. Each value is the mean of 6 independent determinations.

Concentration of Triton X100	Activity (units ml^{-1}) Without NADH	With NADH	% Inhibition
0	2·45	1·20	51%
12%	3·10	1·55	50%

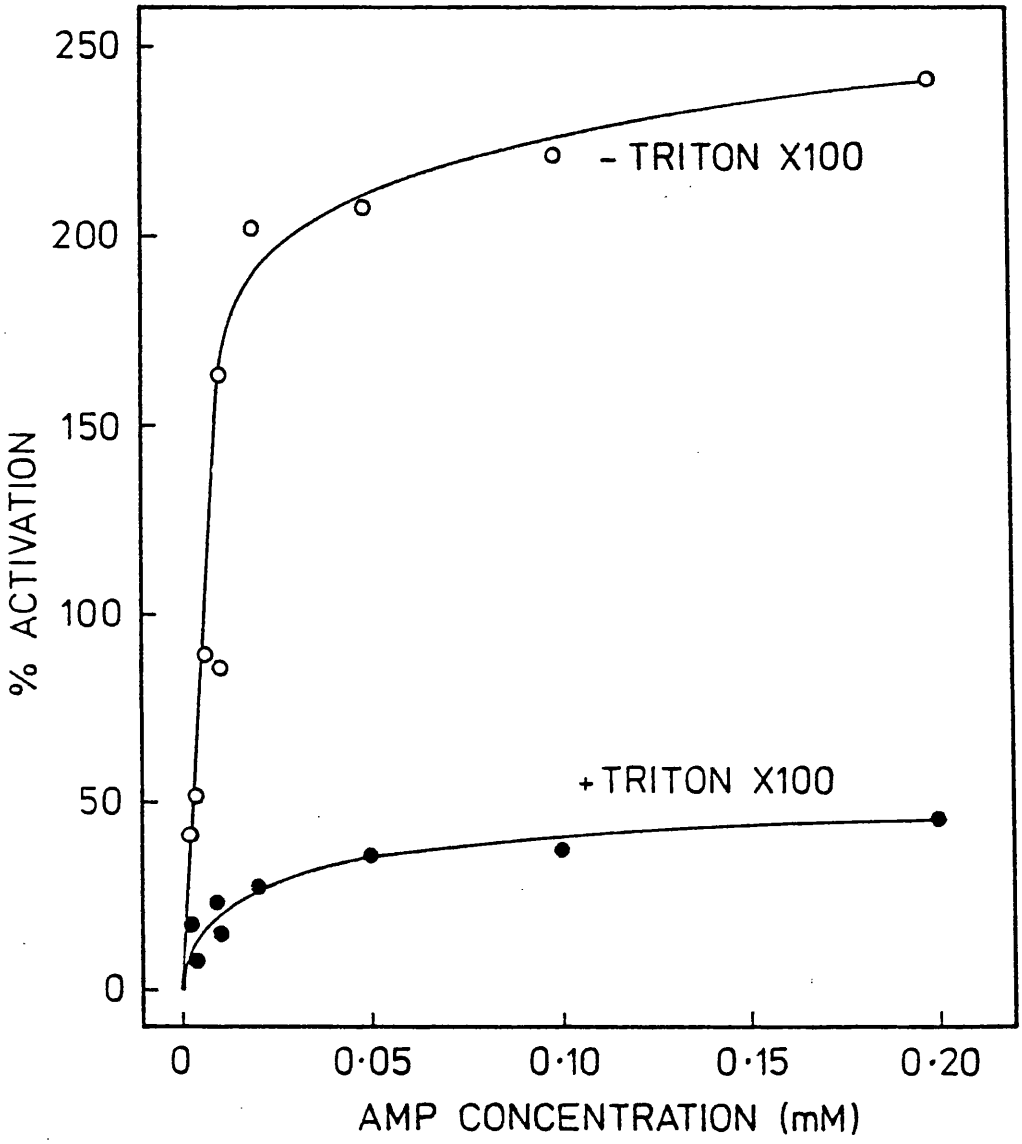


Fig. 56. The effect of Triton X100 (12%) on the activation of A. lwoffi α-OGDH complex at different AMP concentrations.

Experimental details are described in section V.2·3.

α-Oxoglutarate concentration was 0·5mM.

complex at different AMP concentrations. In the absence of detergent a curve very similar to that observed by Weitzman (1972) was obtained. In the presence of Triton X100 partial desensitization to AMP activation was observed even at the lowest AMP concentration investigated (2μM).

The mechanism of desensitization to AMP by non-ionic detergents appears to be similar to that by urea in that there is a reduction in the K_m for α-oxoglutarate. In the presence of the detergent (12% [v/v]) the K_m was decreased from 2·5mM to 0·5mM. Furthermore, the desensitization effect was found to be reversible when enzyme which had been preincubated in the presence of 12% Triton X100 (or Nonidet P40) was diluted 50-fold into a cuvette lacking detergent (Table 23). Similarly, the decrease in the K_m for α-oxoglutarate observed in the presence of the detergent was reversed on removal by dilution (K_m in the presence of detergent = 0·5mM [3 determinations]; K_m of untreated enzyme or after removal of detergent by dilution = 2·0-2·5mM [5 and 2 determinations respectively]; see section V.2·3).

In conclusion, therefore, as in the urea studies, partial desensitization of the complex to AMP regulation with a concomitant decrease in the K_m for α-oxoglutarate was observed in the presence of Triton X100 or Nonidet P40. Removal of these detergents resulted in a return to the normal K_m value and restoration of AMP sensitivity.

Although the effects of urea and non-ionic detergents on the regulatory properties of α-OGDH are not conclusive they show many striking similarities suggesting that they act in similar ways. In view of the results obtained it is tempting to speculate that the native A. lwoffi complex may be in a constrained conformation in which its affinity for α-oxoglutarate is reduced. In the presence of AMP this structural constraint may be released with a ~~sub~~consequent decrease in the K_m for α-oxoglutarate. This is supported to some extent by the observation that the complex from E. coli (which does not exhibit AMP regulation) has a K_m value similar to that of the A. lwoffi

Table 23. Reversibility of the effect of Triton X100 on the AMP regulation of α-OGDH.

Experimental details are described in section V.2.3

Whole complex assay conditions	Activity (units ml^{-1}) Without AMP	+0.2mM AMP	% Stimulation
0.2mg of complex in 1ml of buffer; 20µl assayed for whole complex activity.	0.327	1.040	218
0.2mg of complex in 1ml of buffer; assay cuvette containing 12% Triton X100.	0.44	1.05	136
0.2mg of complex in 1ml of buffer containing 12% Triton X100; assay cuvettes containing 12% Triton X100.	0.54	1.21	124
0.2mg of complex in 1ml of buffer containing 12% Triton X100; normal assay conditions.	0.40	1.19	198

enzyme in the presence of AMP and consequently lower than that in the absence of AMP. If this is the case it is not unreasonable that incubation of the complex with protein denaturing agents such as urea or detergents could also result in a partial release from the proposed constraint, causing a reduction in the K_m for α-oxoglutarate and an apparent loss of AMP sensitivity.

V.3.3 Effects of other reagents on the regulation of the α-OGDH complex

A number of reagents including acetone, ethanol, ethanediol, dioxane, toluene and sodium dodecyl sulphate were investigated to see if either desensitization of the complex to NADH or a more complete loss of AMP stimulation could be achieved. However, no convincing evidence of desensitization to either of these effectors was obtained.

V.3.4 Multiple-inhibition studies

To determine whether NADH inhibition of *A. lwoffi* α-OGDH is allosteric or isosteric in nature multiple-inhibition analysis of the complex was carried out. α-Oxoadipate, a structural analogue of α-oxoglutarate was first examined for its potential inhibitory effect on enzyme activity. Fig. 57 shows that α-oxoadipate is a strictly competitive inhibitor with respect to α-oxoglutarate and it is therefore reasonable to assume that these two α-oxo-acids compete for the same binding site on the enzyme. NADH also acts as a competitive inhibitor with respect to α-oxoglutarate (Fig. 58) when assayed in the presence of 2.0mM NAD^+ (to overcome inhibition of the E3 component enzyme; Parker & Weitzman, 1973). Multiple-inhibition studies were therefore carried out in the joint presence of these two inhibitors. The inhibition of activity by varying concentrations of α-oxoadipate was examined at several fixed concentrations of NADH, and the results are illustrated in Fig. 59. Yonetani & Theorell (1964) have shown that parallel lines in such plots indicate that the inhibitors interact with the same site on the enzyme, whereas different sites are indicated by a pattern of inter-

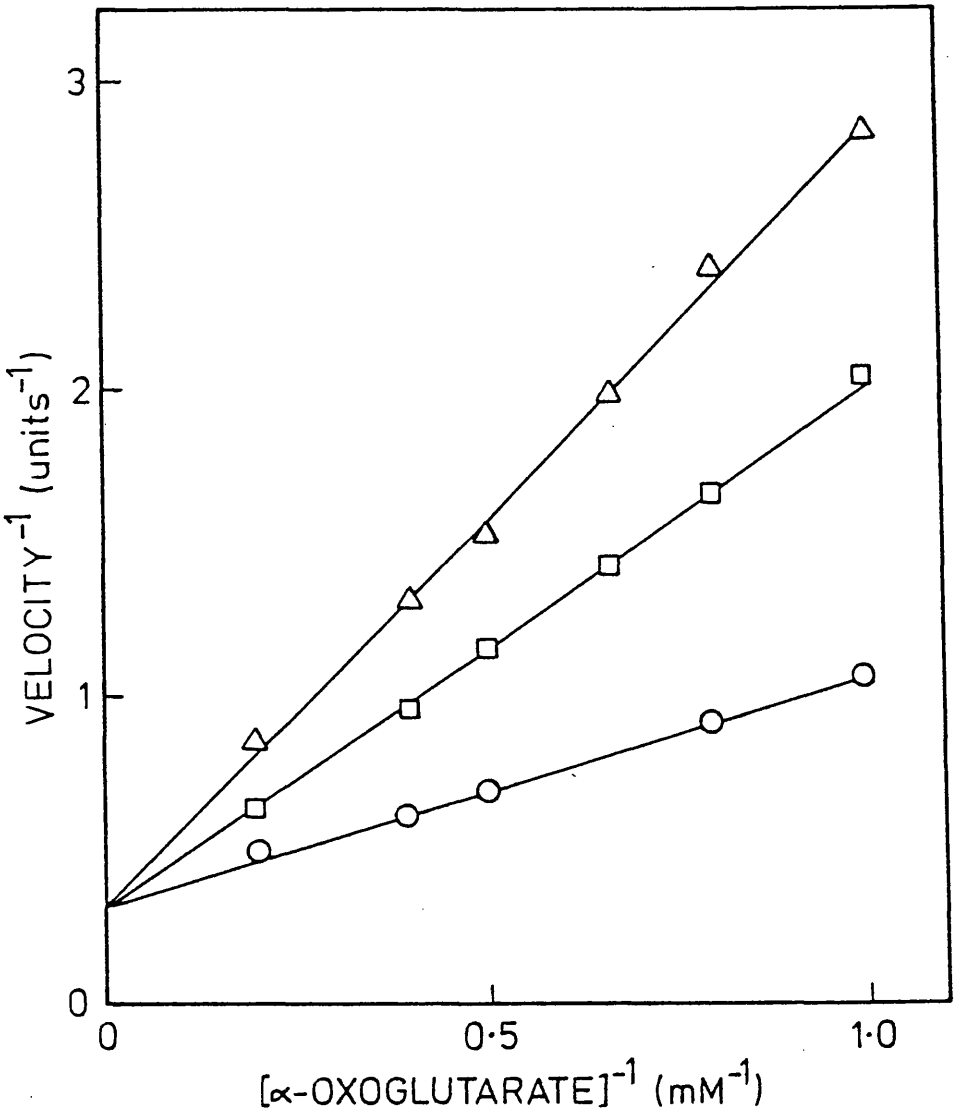


Fig. 57. Competitive inhibition of A. lwoffi α-OGDH complex by α-oxoadipate.

Measurements of activity were made as described in section V.2·4 at various concentrations of α-oxoglutarate in the absence (○—○) or presence of 1mM (□—□) or 2mM (△—△) α-oxoadipate.

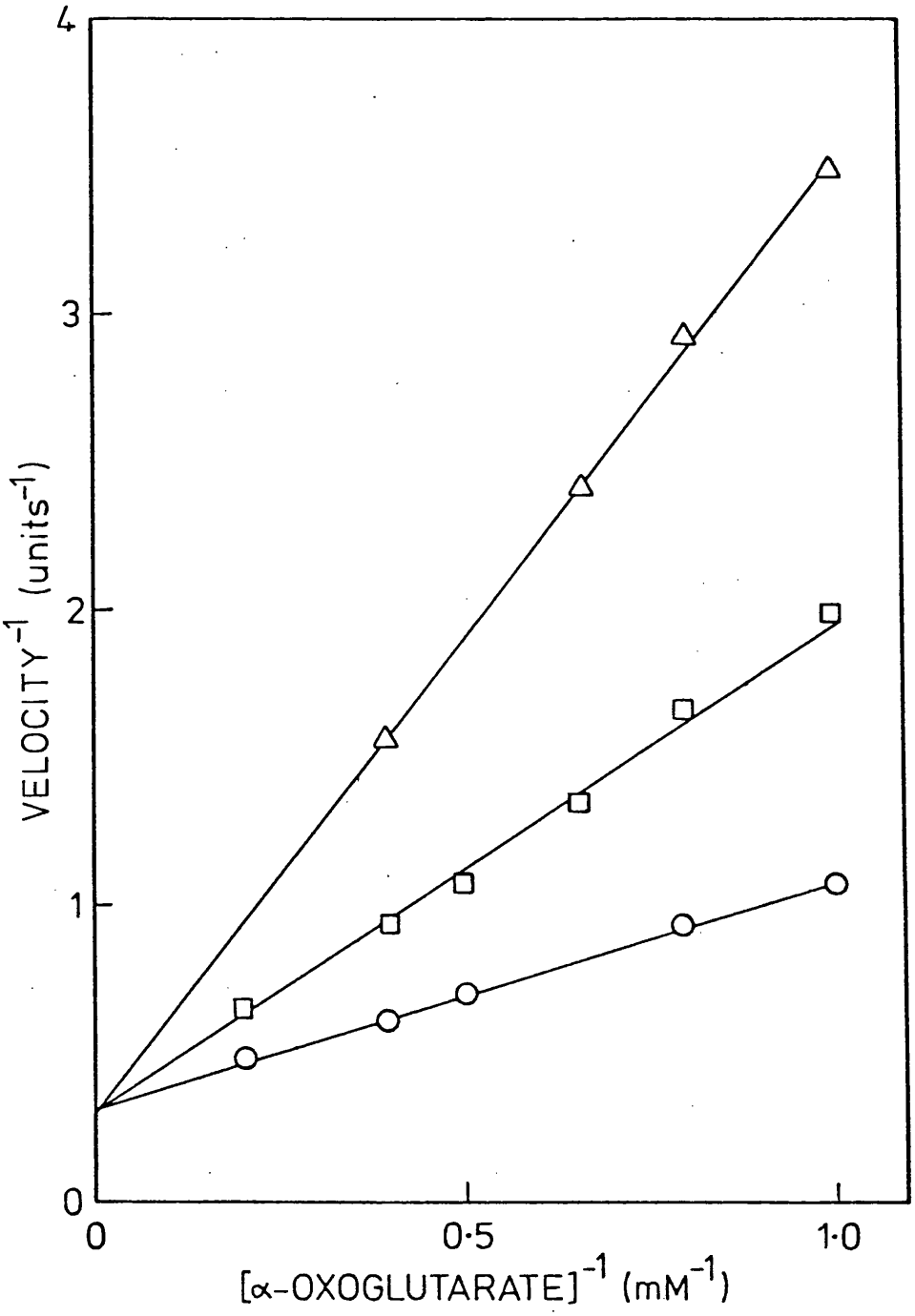


Fig. 58. Competitive inhibition of A. lwoffi α-OGDH complex by NADH.

Measurements of activity were made as described in section V.2·4 at various concentrations of α-oxoglutarate in the absence (○—○) or presence of 0·1mM (□—□) or 0·2mM (△—△) NADH.

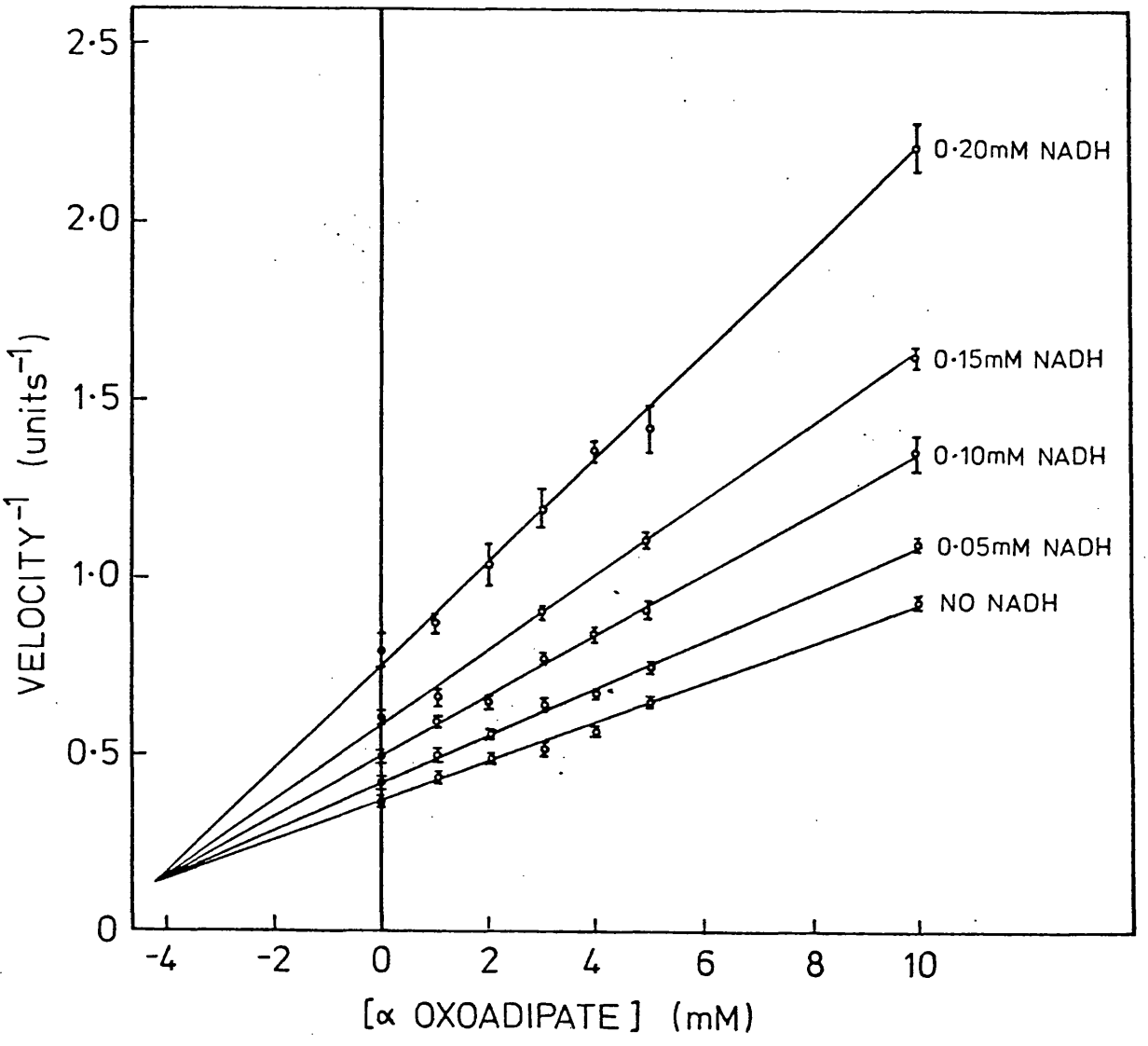


Fig. 59. Multiple inhibition of A. lwoffi α-OGDH complex.

Measurements of activity were made as described in section V.2·4 at varying concentrations of α-oxoadipate in the presence of several fixed concentrations of NADH. All measurements were made in triplicate and subjected to linear regression analysis.

secting lines. From Fig. 59 it can be seen that α-oxoadipate and NADH clearly do not share a common interaction site on the enzyme. In view of the close structural similarity between α-oxoadipate and α-oxoglutarate it is reasonable to conclude that NADH does not bind at the α-oxoglutarate site; in other words inhibition by NADH is exerted allosterically.

V.3.5 Subunit specificity of AMP regulation

It has been shown directly that the E1 component is stimulated by AMP (Parker & Weitzman, 1973) whereas the E3 subunit is unaffected. The E2 subunit, on the other hand, had not been investigated as a possible site of AMP regulation due to the lack of a convenient E2 assay. Consequently this was tested using the new assay described in section I.2.6 but no increase in rate was observed in the presence of AMP. Thus the E1 component appears to be the only site of AMP sensitivity.

V.3.6 Investigation of the regulatory properties of α-OGDH by the radiochemical assay.

Direct evidence for the interaction of NADH with the E1 component could not be obtained using the DCPIP assay since NADH reduces DCPIP in the absence of enzyme; hence information had to be obtained by indirect methods (Parker & Weitzman, 1973, see section V.1). The results of these studies suggested that NADH acts on both the E1 and E3 components. However, with the establishment of the radiochemical assay for the E1 component (section I.1.3) direct examination of the regulatory properties of this component could be obtained. Initially, the regulatory effects of AMP and NADH on the whole complex activity were examined by the radiochemical assay and it can be seen from Table 24 that both AMP stimulation and NADH inhibition were observed.

Fig. 60 shows the kinetics of CO_2 production by the E1 component in the absence and presence of effectors. It is again clear that AMP stimulates this component. However, 0.2mM NADH did not inhibit the E1 assay but,

Table 24. Effect of NADH and AMP on α-OGDH complex activity.

Whole complex radiochemical assays were performed as described in section I.2·5, in the presence of NADH or AMP. Reaction time = 3min. α-Oxoglutarate concentration = 1mM.

Additions	CO_2 production (nmol)	Effect on activity
None	33·6	-
2mM NADH	22·4	33% inhibition
0·5mM AMP	49·8	48% stimulation

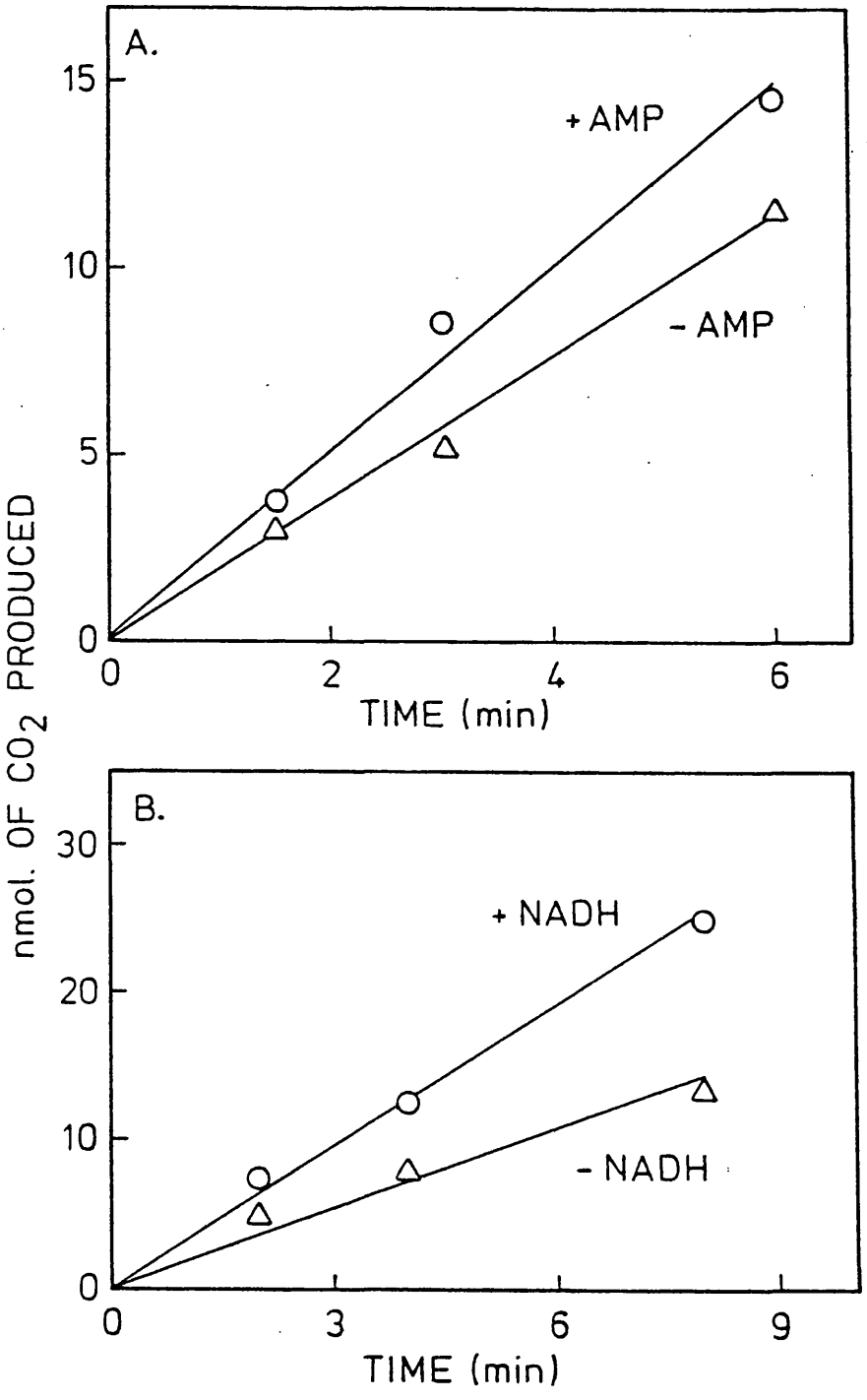


Fig. 60. The effect of AMP and NADH on E1 activity examined with the radiochemical assay.

The assay procedure is described in section I.2·5, using a [1-^{14}C]α-oxoglutarate concentration of 1·0mM (200,000 cpm μmol^{-1})

A. E1 activity in the absence and presence of 0·5mM AMP.

B. E1 activity in the absence and presence of 0·2mM NADH.

contrary to expectation, it had a stimulatory effect. This result was obtained consistently even at NADH concentrations as high as 2·0mM. This unexpected result appears to be in conflict with the indirect evidence obtained by Parker & Weitzman (1973) which suggested that NADH inhibited the E1 subunit. Although at first the direct radiochemical approach may seem more convincing, it should be borne in mind that the E1 activity measured under these conditions is only 18% of that of the whole complex. It was suggested in section I.3·2 that this may be due to the utilization of free TPP being rate limiting. Consequently under these conditions any inhibitory effect of NADH could conceivably be masked, although of course this would not explain its observed stimulatory effect. It should be pointed out that this stimulation by NADH does not appear to be a non-specific nucleotide effect since NAD^+ had only a marginal effect (Table 25).

In view of the problem associated with the E1 assay, radiochemical assays were performed on the whole complex in the absence and presence of NADH with the inclusion of a large excess of NAD^+ (2·0mM). At this NAD^+ concentration product inhibition of the E3 component by NADH is effectively overcome (Parker & Weitzman, 1973). It is evident from Table 26 that under these conditions NADH inhibition is not solely restricted to product inhibition of the E3 component in agreement with the results of Parker & Weitzman (1973).

In conclusion, indirect evidence has suggested that the E1 subunit is subjected to NADH regulation in apparent contrast to the direct results obtained by the radiochemical analysis of E1 activity presented in this section. Both these approaches have their disadvantages, so clearly a better direct method needs to be devised to clarify the situation.

Table 25. The effect of NAD^+ on the E1 radiochemical assay.

E1 radiochemical assays were performed in the presence or absence of NAD^+ as described in section I.2.5, using an α-oxoglutarate concentration of 1.0mM.

Additions	Reaction time (min)	CO_2 production (nmol)
None	2.0	6.13
0.5mM NAD^+	2.0	6.40
None	3.0	7.38
0.5mM NAD^+	3.0	7.88

Table 26. Effect of NADH on α-OGDH in the presence of NAD^+.

Whole complex radiochemical assays were performed in the presence of 1mM α-oxoglutarate plus 2mM NAD^+, as described in section I.2.5. The reaction time was 2min.

Additions	CO_2 production (nmol min^{-1})	Inhibition (%)
None	37.1	-
0.5mM NADH	16.25	56

CONCLUDING REMARKS

There were a number of justifiable reasons for studying the α-OGDH complex of A. lwoffi - it catalyses a fundamentally important reaction in intermediary metabolism and it is a multienzyme complex composed of three distinct subunit types. However, perhaps the most important reason lay in its interesting regulatory properties (NADH inhibition and AMP stimulation) which ~~is~~ are only shared by a relatively small group of bacteria.

Comparison of the properties of the α-OGDH complex from A. lwoffi with those from E. coli and mammalian tissues has yielded both differences and similarities. Application of polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate has confirmed that the α-OGDH complex from A. lwoffi is indeed composed of three distinct subunit types, two of which differ in molecular weight from their counterparts in E. coli and mammalian tissues. Furthermore, the whole complex is somewhat smaller in both diameter and sedimentation coefficient and consequently molecular weight.

By chemically modifying the lipoic acid moiety its involvement in the reaction mechanism could be confirmed. In addition the success of the E2-E3 interaction test and the E2 assay were also consistent with the proposed mechanism of catalysis. It seems likely that the E3 subunit of A. lwoffi is similar to that of the E. coli and mammalian enzymes in that it contains a disulphide bond at its active site, which is important in the reaction mechanism. Thus loss of E3 activity was observed when this S-S bond was reduced.

Studies into the allosteric nature of the regulation of the complex yielded some indication as to the mode of action of the nucleotide effectors. Using the technique of multiple-inhibition described by Yonetani & Theorell (1964), it was shown that NADH acts at a locus other than the α-oxoglutarate

binding site. In the presence of protein denaturing agents a partial desensitization to AMP was observed. However, it could not be established with any degree of certainty whether it was the catalytic or regulatory site which was affected, since a reduction in the K_m for α-oxoglutarate was observed. These results are consistent with the hypothesis that the native α-OGDH complex is subjected to a constrained conformation in which its affinity for α-oxoglutarate is reduced. In the presence of AMP this constraint may become released causing an apparent decrease in the K_m for α-oxoglutarate. This is not inconceivable since the E. coli enzyme which does not exhibit AMP regulation has a low K_m for α-oxoglutarate, very similar to that of the A. lwoffi complex in the presence of AMP. It is not unreasonable to suppose that the presence of protein denaturing agents, such as urea or non-ionic detergents, also cause partial release from the proposed constraint and hence a reduction in the K_m for α-oxoglutarate and loss of AMP sensitivity.

Finally, there now exists some doubt as to whether the E1 subunit is involved in regulation by NADH. Previous indirect evidence had suggested that NADH acted on this subunit (Parker & Weitzman, 1973) but this could not be confirmed directly using the radiochemical assay. However, both of these methods have their disadvantages and are open to criticism and consequently a better method needs to be found to resolve this dilemma.

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ABBREVIATIONS

α-OGDH	α-oxoglutarate dehydrogenase complex
PDH	pyruvate dehydrogenase complex
E1	α-oxoglutarate dehydrogenase subunit (EC 1•2•4•2)
E2	dihydrolipoamide transsuccinylase (EC 2•3•1•12)
E3	dihydrolipoamide dehydrogenase (EC 1•6•4•3)
TPP	thiamine pyrophosphate
CoA	coenzyme A
DCPIP	dichlorophenol-indophenol
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
TNB^-	5-thio,2-nitrobenzoate anion
NTCB	nitrothiocyanobenzoic acid
NEM	N-ethylmaleimide
DEPC	diethylpyrocarbonate
HNBBr	2-hydroxy-5-nitrobenzyl bromide
PPO	2,5-diphenyloxazole
dimethyl POPOP	p-bis-[2-(4-methyl-5-phenyloxazoyl)] benzene
MET-8 buffer	10mM $MgCl_2$, 1mM EDTA, 20mM Tris-HCl, pH 8•0

SUMMARY

A study has been made of the α-oxoglutarate dehydrogenase (α-OGDH) multi-enzyme complex from the bacterium Acinetobacter lwoffi, in order to obtain a more detailed understanding of its molecular, catalytic and, in particular, its regulatory properties. This complex, which catalyses the oxidative decarboxylation of α-oxoglutarate to produce succinyl-CoA is composed of three subunit types (E1, E2 and E3), each possessing a different enzyme activity. Comparison with the analogous complexes from E. coli and mammalian tissues has yielded both differences and similarities.

The native A. lwoffi α-OGDH complex was found to have a sedimentation coefficient of 29·9S, corresponding to a molecular weight of $1{\cdot}82 \times 10^6$. Electrophoresis under completely denaturing conditions revealed that the complex is composed of three discrete polypeptide chain types with molecular weights of 55000, 60080 and 80900.

Using specific chemical modification it seems likely that the E3 subunit of A. lwoffi α-OGDH is similar to those of the E. coli and mammalian complexes in that they all contain a disulphide bond at the active site, which is important in the reaction mechanism. Consequently, a loss of E3 activity was observed when this S-S bond was reduced. Furthermore, by chemically modifying the E2-bound lipoic acid moiety its involvement in the reaction mechanism was also confirmed.

Attempts to dissociate the complex with retention of the individual subunit activities met with only limited success.

Studies into the allosteric nature of regulation of the complex yielded some indication as to the mode of action of the nucleotide effectors (NADH and AMP). Using the technique of multiple-inhibition it was shown that NADH acts at a locus other than the α-oxoglutarate binding site. In the presence of protein denaturing agents a partial desensitization to AMP was observed. A possible explanation which is consistent with these results is discussed.

Finally, some doubt now exists as to whether the E1 subunit is involved in NADH regulation, since previously reported indirect kinetic evidence that NADH acts on this subunit could not be confirmed directly using a radiochemical assay for the measurement of E1 activity.