Turkey Liver Xanthine Dehydrogenase

REACTIVATION OF THE CYANIDE-INACTIVATED ENZYME BY SULPHIDE AND BY SELENIDE

By WILLIAM F. CLEERE and MICHAEL P. COUGHLAN
Department of Biochemistry, University College, Galway, Ireland

(Received 13 May 1974)

1. Turkey liver xanthine dehydrogenase engaged in catalysing the oxidation of xanthine by dichlorophenol-indophenol was progressively inactivated by methanol. This inactivation was reversible by NAD^+ . 2. Reaction with arsenite and with cyanide, in each case first-order with respect to enzyme, resulted in characteristic alterations in the visible absorption spectrum of the enzyme. The rate of spectral change on reaction with either agent paralleled the rate of loss of enzyme activity. 3. Cyanide inactivation was accompanied by elimination from the enzyme of sulphur as thiocyanate. Partial restoration of activity was effected by incubation with sulphide or with selenide. The results suggest that turkey liver xanthine dehydrogenase, like milk xanthine oxidase (Massey & Edmonson, 1970), contains at the active centre a cyanolysable persulphide group essential to catalytic activity and that selenium may replace sulphur in this group to give an active enzyme. 4. Incubation of the native enzyme with sulphide or with selenide resulted in the rapid loss of half of the xanthine-oxidizing activity, apparently by disrupting the molybdenum and (Fe/S)II loci. This may indicate non-equivalence of the intramolecular electron-transfer systems.

The molybdoproteins, xanthine oxidase (EC 1.2.3.2), aldehyde oxidase (EC 1.2.3.1) and nitrate reductase (EC 1.6.6.3) are unique in their sensitivity to cyanide, arsenite and methanol (Rajagopalan & Handler, 1964). Evidence pointing to the enzyme-bound molybdenum as the site of action of these agents was supported by the altered molybdenum e.p.r. (electron paramagnetic resonance) signals of methanol-inactivated xanthine oxidase and aldehyde oxidase (Aleman *et al.*, 1965; Rajagopalan & Handler, 1968; Rajagopalan *et al.*, 1968; Pick *et al.*, 1971). Coughlan *et al.* (1969) showed that all three agents bound to the same site on chicken liver xanthine dehydrogenase, milk xanthine oxidase and rabbit liver aldehyde oxidase. That this site was the molybdenum group was again indicated by the altered molybdenum e.p.r. spectra of arsenite-inactivated and of cyanide-inactivated xanthine dehydrogenase. The accumulated evidence suggested that inactivation by these agents was due to complex-formation with the enzyme-bound molybdenum, thereby preventing substrate binding and electron transfer. However, failure to observe an isotope effect on the molybdenum signal of $[^{12}\text{C}]$ - and $[^{14}\text{C}]$ -cyanide-inactivated xanthine oxidase led Komai *et al.* (1969) to doubt the existence of a molybdenum–cyanide complex. Further work by Massey & Edmonson (1970) indicated the presence at the active centre of milk xanthine

oxidase of a persulphide group essential to catalytic activity. Cyanide-inactivation results from lysis of the persulphide group and removal of sulphur as thiocyanate. Regeneration of the persulphide accompanied by reactivation may be effected by treatment with sodium sulphide. This group is thought to be associated with the enzyme-bound molybdenum and to be the site of inactivation by arsenite and methanol (Edmonson *et al.*, 1972). That such a scheme might also obtain in the related enzyme, xanthine dehydrogenase from turkey liver, prompted the present investigation.

Experimental

Materials and reagents

Fresh-frozen turkey livers were obtained from Castlemahon Co-Op, Newcastle West, Co. Limerick, Ireland, and stored at -20°C until required. Enzyme was purified from this source by a slight modification of a previously published method (Cleere & Coughlan, 1974). Xanthine, trinitrobenzenesulphonate and phenazine methosulphate were obtained from Sigma Chemical Co., London S.W.6, U.K.; NAD^+ and NADH were from Boehringer, Mannheim, Germany, and sodium selenide was from Ventron-Hicol, N.V. Rotterdam, The Netherlands.

Enzyme assays

All reactions, unless stated otherwise, were carried out at 30°C in 0.05M-potassium phosphate buffer, pH 7.8, containing 0.1mM-EDTA. Xanthine-NAD⁺ oxidoreductase activity was measured by following the increase in absorbance at 340nm caused by the formation of NADH in a reaction mixture containing 0.15mM-xanthine, 500µM-NAD⁺ and enzyme in a final volume of 1ml. Xanthine-dichlorophenol-indophenol and xanthine-ferricyanide oxidoreductase activities were measured at 600nm and at 420nm respectively, with reaction mixtures identical with the above except that NAD⁺ was replaced by 50µM-dichlorophenol-indophenol or 2.0mM-ferricyanide. The oxidation of xanthine by O₂ or by the autoxidizable dyes trinitrobenzenesulphonate (500µM), phenazine methosulphate (100µM) or Methylene Blue (20µM) was followed at 295nm. The oxidation of NADH (50µM) by dichlorophenol-indophenol (50µM) was followed at 600nm, by ferricyanide (2.0mM) at 420nm and by O₂ or autoxidizable dyes at 340nm. In all cases a change of 1 absorbance unit/min corresponded to 1 unit of enzyme activity. Assays were carried out in a Unicam SP1800 instrument, equipped with a thermostatically controlled cuvette holder and continuous chart recorder.

Protein determination

Enzyme concentration was determined by the u.v. method of Warburg & Christian (1942) or by the E_{450} value of 36550 litre·mol⁻¹·cm⁻¹ per equivalent of enzyme-bound flavin (Cleere & Coughlan, 1974).

Difference spectra

Difference spectra were recorded with the above spectrophotometer by using tandem cuvette holders to provide appropriate blanks in all procedures. All cuvettes used had a 1 cm light-path.

Dehydroalanine determination

Cyanide-inactivated turkey liver enzyme (Fig. 2), freed of excess of reagent by passage through Sephadex G-25, was hydrolysed under conditions in which the conversion of dehydroalanine into pyruvate occurs in good yield (Malkin & Rabinowitz, 1966). Pyruvate, and hence dehydroalanine, was then measured by a minor modification of the method of Malkin & Rabinowitz (1966). Cyanide-inactivated enzyme (20.8 nmol; see under 'Protein determination') was heated at 100°C in 3M-HCl (final volume 3.8 ml) for 1h. The solution was cooled and insoluble material removed by centrifugation. The pH of the supernatant was adjusted to 7.8 with NaOH and the volume adjusted to 5.5ml with 0.05M-potassium phosphate buffer, pH 7.8. The solution was then

assayed for pyruvate with lactate dehydrogenase and NADH (Malkin & Rabinowitz, 1966). Under the assay conditions used 10nmol of pyruvate gave a total absorbancy change at 340nm of 0.061.

Thiocyanate determination

Thiocyanate was measured spectrophotometrically as the rhenium complex by a modification of the method of Neas & Guyon (1969). Cyanide-inactivated enzyme (see Fig. 2; 20.4nmol) in 0.05M-potassium phosphate buffer, pH 7.8, was precipitated at room temperature by the addition of acetone to a final concentration of 66% (v/v). As a control, an equivalent amount of untreated enzyme was first precipitated with acetone and an appropriate amount of cyanide was then added. After centrifugation, the supernatant was collected as quantitatively as possible. Acetone was evaporated at 40°C under a stream of N₂. The remaining solution was freeze-dried and the residue dissolved in 0.225 ml of water. To this was added 0.025 ml of KReO₄ (4.0mM), 0.175 ml of conc. HCl and 0.025 ml of stannous chloride solution (Neas & Guyon, 1969). The mixture was incubated at 30°C for 30min. After addition of 0.75 ml of acetone, the solution was clarified by brief centrifugation and the absorbance of the supernatant at 390nm read immediately. Blank and standard solutions, containing the appropriate amount of cyanide, were treated in exactly the same fashion. Under these conditions, 26.75 nmol of thiocyanate (standardized by titration with AgNO₃ with iron alum as indicator) gave an absorbance reading at 390nm of 0.1. The concentrations of cyanide and of phosphate used, and the freeze-drying operation, did not interfere with the assay.

Results

Methanol inactivation

Turkey liver xanthine dehydrogenase subjected to prolonged methanol treatment retained full activity if, before assay, the unbound reagent was removed by gel filtration. However, enzyme engaged in catalysing the oxidation of xanthine by dichlorophenol-indophenol was rapidly inactivated by methanol (Fig. 1a). Thus inhibition by this agent is progressive with time and reaction-dependent. That increasing concentrations of xanthine protected, in competitive fashion, against methanol inactivation (Fig. 1c) suggested that inhibitor and substrate competed for the same site on the enzyme. Methanol inactivation of the turkey liver enzyme also took place during xanthine oxidation by phenazine methosulphate, trinitrobenzenesulphonate, ferricyanide and Methylene Blue. However, little or no inactivation was found when O₂ or NAD⁺ was used as the acceptor. In fact, the addition of NAD⁺ not only protected against but also

Fig. 1. Methanol inhibition of turkey liver xanthine-dichlorophenol-indophenol oxidoreductase activity

(a) Effects of methanol concentration. Curves 1, 2, 3 and 4 represent the activities of standard reaction mixtures in the presence of 0, 0.5, 1.0 and 1.5M-methanol respectively. Curve 5 represents the activity on addition of 0.3mM- NAD^+ to the reaction mixture of curve 4 at the time indicated by the arrow. (b) Reversal of methanol inhibition by NAD^+ . The curves represent the activities of standard reaction mixtures: 1, without addition; 2, +0.3mM- NAD^+ ; 3, +0.3mM- NAD^+ and 1.5M-methanol; 4, +1.5M-methanol; 5, 0.3mM- NAD^+ added to the reaction mixture of curve 4 at the time indicated by the arrow. (c) Effect of substrate concentration on methanol inhibition. All reaction mixtures contained 1.0M-methanol. Xanthine concentrations were: 1, 0.5mM; 2, 0.1mM; 3, 0.05mM; 4, 0.01mM. Other components of the reaction mixtures were as specified in the Experimental section. In the absence of methanol, experimental maximal velocity was attained at 0.05mM-xanthine.

Fig. 2. Difference spectra between arsenite- or cyanide-inactivated and native turkey liver xanthine dehydrogenase. Enzyme (final E_{450} 0.51) was incubated at 30°C in 0.05M-phosphate buffer, pH7.8, containing 0.1mM-EDTA and 0.2mM-sodium arsenite or 5.0mM-KCN, in a final volume of 1.0ml, until no further spectral change occurred. Difference spectra were recorded against the same concentration of untreated enzyme as described in the Experimental section.

reversed methanol inactivation of the transfer of electrons from xanthine to dichlorophenol-indophenol (Figs. 1a and 1b). The addition of electron acceptors other than NAD^+ did not reverse methanol inactivation.

Arsenite and cyanide difference spectra

Incubation of turkey liver xanthine dehydrogenase with arsenite or with cyanide resulted in characteristic alterations in the absorption spectrum of the enzyme (Fig. 2). The difference spectrum between arsenite-inactivated and native enzyme is largely positive, especially in the areas of maximal change, with a well-defined peak at 380nm, whereas that between cyanide-inactivated and native enzyme is completely negative with a well-defined trough around 320nm. The absolute extent of change is small compared with the initial absorbance of the native enzyme at these wavelengths. However, the change at 380nm in the arsenite difference spectrum and at 320nm in the cyanide difference spectrum represent molar-extinction-coefficient changes of 2250 and 4375 litre·mol⁻¹·cm⁻¹ respectively, per equivalent of flavin.

Kinetics of inhibition by arsenite and cyanide

The kinetics of the development of the difference spectra and the kinetics of inhibition of enzyme activity are compared in Figs. 3(a) and 3(b). Clearly,

Fig. 3. Kinetics of change in absorbance and of development of inhibition of xanthine- NAD^+ oxidoreductase activity accompanying arsenite and cyanide treatment

(a) Enzyme incubated with 5.0 mM-KCN as in Fig. 2. Decrease in absorbance at 320 nm ($\circ$) was compared with loss of activity ($\bullet$). (b) Enzyme incubated with 0.2 mM-arsenite as in Fig. 2. Increase in absorbance at 380 nm ($\circ$) was compared with loss of activity ($\bullet$).

Fig. 4. Effect of heat on the xanthine- NAD^+ oxidoreductase activity of arsenite- and of cyanide-treated turkey liver xanthine dehydrogenase

Arsenite-inactivated ($\circ$) and cyanide-inactivated ($\bullet$) enzyme were prepared by incubation with 0.2 mM-arsenite and 5.0 mM-cyanide respectively, as described in Fig. 2. After removal of excess of inhibitor by passage through Sephadex G-25, the inactivated preparations and native enzyme were incubated at 50°C in 0.05 M-phosphate buffer, pH 7.8, containing 0.1 mM-EDTA, samples for assay being taken at various intervals. Activity in each case is expressed as a percentage of that of an equivalent amount of native enzyme which was stable at 50°C.

there is excellent correlation between the rate of change in E_{320} on cyanide treatment, and in E_{380} on arsenite treatment, and the rate of development of inhibition of xanthine- NAD^+ oxidoreductase activity. Thus the physical changes evident in the difference spectra are true reflections of the binding and inactivation of the turkey enzyme by arsenite and cyanide. Semilogarithmic replots of the data in Fig. 3 showed that inactivation by arsenite and by cyanide was pseudo-first-order with respect to enzyme in each case, with half-times of inactivation, under the experimental conditions, of 2.5 and 3.5 min respectively.

Heat treatment

Fig. 4 shows that incubation at 50°C effects a partial reactivation of arsenite-inactivated turkey liver xanthine dehydrogenase but does not reverse cyanide inactivation.

Sulphur elimination

Because of the many similarities between milk xanthine oxidase and turkey liver xanthine dehydrogenase (Cleere & Coughlan, 1974), it seemed likely that cyanide-inactivation of the latter might also be consequent on the removal from the enzyme of sulphur as thiocyanate (see the introduction). Accordingly, the amount of thiocyanate produced on inactivation of known amounts of the turkey liver enzyme

was estimated (Table 1). The measured release of 1.13 mol of thiocyanate per mol of enzyme is only half the predicted value of 2.0, assuming that the enzyme contains two active sites (as is generally assumed for these enzymes) and that one sulphur atom is associated with each active site.

It could be argued that the cyanolysable sulphur originates not from active-centre persulphide groups but from the labile iron sulphide chromophores. Assuming, that two of the eight iron sulphide chromophores could be disrupted without complete denaturation of the enzyme, one could have expected a molar-extinction-coefficient change at 450 nm of about $6100 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ per equivalent of flavin (Cleere & Coughlan, 1974). However, examination of Fig. 2 shows that although cyanide treatment does effect considerable bleaching of the visible absorption spectrum, especially at 320 nm, the calculated molar-extinction-coefficient change at 450 nm is only $700 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ per equivalent of flavin. Massey & Edmonson (1970), who have advanced similar reasoning, also obtained identical acid-labile sulphide analysis for native and cyanide-inactivated xanthine oxidase.

The possibility that the cyanolysable sulphur of the turkey liver enzyme might have originated from cysteine residues also appears to be ruled out (Table 1). The rationale behind this experiment was as follows. If the thiocyanate was derived from reaction of cyanide with cysteine as outlined in Scheme 1, dehydroalanine would be the other expected product.

Dehydroalanine is quantitatively converted into pyruvate during acid hydrolysis (see the Experimental section) and the latter was determined by using lactate dehydrogenase. Such analysis indicated that the cyanide-treated preparation did contain some dehydroalanine, approx. 1 mol/mol of enzyme (Table 1). However, analysis of the native enzyme gave exactly the same result, indicating that whatever the source of this dehydroalanine, it is unrelated to the action of cyanide in releasing thiocyanate.

Sulphide treatment

Massey & Edmonson (1970) have demonstrated that cyanide-inactivated xanthine oxidase can be re-activated by incubation with sodium sulphide. Moreover, the rate of reincorporation of ^{35}S into the protein, accompanying incubation with Na_2^{35}S , paralleled the rate of reactivation. Further treatment with cyanide resulted in the elimination from the enzyme of $[\text{S}^{35}]\text{thiocyanate}$. Accordingly, the possibility that sulphide treatment might effect reactivation of cyanide-inactivated turkey liver xanthine dehydrogenase was investigated. The results of one such experiment are shown in Fig. 5. Incubation with sulphide, at pH 7.0, 7.8 or 9.0, but especially at 7.8, does effect significant restoration of activity. The extent of reactivation of the cyanide-inactivated preparation obviously depends on whether comparison is made with native enzyme or with sulphide-treated enzyme. Thus 25 and 50% restoration of activity is reproducibly obtained at pH 7.8 (Fig. 5b). Fig. 5 also shows that sulphide treatment of native turkey xanthine dehydrogenase effected a rapid loss of 50% of the xanthine-NAD⁺ oxidoreductase activity, the

Table 1. Liberation of thiocyanate and formation of dehydroalanine accompanying cyanide inactivation of the turkey enzyme

Thiocyanate and dehydroalanine were measured as described in the Experimental section. The values given are the average of triplicate assays in each case.

	Dehydroalanine (mol/mol of enzyme)	Thiocyanate (mol/mol of enzyme)
Native enzyme	1.02	0.00
Cyanide-inactivated enzyme	1.04	1.13
That due to cyanide inactivation	0.02	1.13

Scheme 1. A possible mechanism of thiocyanate formation accompanying cyanide treatment of turkey liver xanthine dehydrogenase

Fig. 5. Effects of sulphide treatment on the xanthine-NAD⁺ oxidoreductase activities of native and cyanide-inactivated turkey liver xanthine dehydrogenase

Enzyme was inactivated by incubation with 5.0 mM-cyanide as in Fig. 2, followed by removal of unbound inhibitor by passage through Sephadex G-25. Native and cyanide-inactivated enzyme were incubated in stoppered tubes at 45°C in 0.05 M-phosphate buffer (a, pH 7.0; b, pH 7.8) and citrate-phosphate-borate buffer (c, pH 9.0) prepared as described by Theorell & Stenhagen (1938). Each buffer contained 0.1 mM-EDTA and 0.01 M-sodium sulphide. Samples for assay of activity were withdrawn at intervals. The activity of the sulphide-treated cyanide-inactivated enzyme is expressed (○) as a percentage of that of the native untreated enzyme, and (●) of that of the sulphide-treated enzyme. Similarly, the activity of the sulphide-treated enzyme is expressed (□) as a percentage of that of the native enzyme. The native enzyme was stable at 45°C throughout the experiment.

Table 2. Effects of sulphide treatment on turkey liver xanthine and NADH oxidoreductase activities

Enzyme in stoppered tubes was incubated at 45°C for 1 h in 0.05 M-phosphate buffer, pH 7.8, containing 0.1 mM-EDTA and 0.01 M-sodium sulphide. After removal of excess of reagent by passage through Sephadex G-25, xanthine oxidoreductase and NADH oxidoreductase activities were measured and are expressed as a percentage of those of the untreated enzyme. Each value is the average of three separate experiments.

Electron acceptor	Oxidoreductase activity (% of control)	
	Xanthine	NADH
NAD ⁺	53.0	—
Phenazine methosulphate	47.6	—
Dichlorophenol-indophenol	48.2	69.0
Trinitrobenzenesulphonate	45.4	63.9
O ₂	51.7	97.9
Ferricyanide	51.3	98.3
Methylene Blue	52.0	97.0

remaining 50% being quite stable to sulphide treatment, especially at pH 7.8. When such enzyme was passed through Sephadex G-25, to remove unbound sulphide and left at 4°C, activity returned slowly, reaching a value of 80% of that of the untreated enzyme in 130 h. The effects of sulphide treatment on various other xanthine-oxidizing and

Fig. 6. Difference spectra between sulphide-treated and native turkey liver xanthine dehydrogenase

Enzyme (final E_{450} 0.68) was incubated in capped cuvettes at 45°C in 0.05 M-phosphate buffer, pH 7.8, containing 0.1 mM-EDTA and 0.01 M-sodium sulphide. Difference spectra against the same concentration of untreated enzyme were recorded thereafter at the times (min) indicated.

Fig. 7. Kinetics of change in absorbance on treatment of the turkey enzyme with sulphide

Data were obtained from experiments as described in Fig. 6, —, at 450nm; ----, at 550nm.

NADH-oxidizing activities of this enzyme are shown in Table 2. Incubation with sulphide results in the loss of half of the xanthine-oxidizing activity of the enzyme regardless of the electron acceptor used. On the other hand, whereas NADH-dichlorophenol-indophenol and NADH-trinitrobenzenesulphonate oxidoreductase activities were similarly decreased on sulphide treatment, the oxidation of NADH by O_2 , ferricyanide or by Methylene Blue was essentially unaffected.

Sulphide difference spectra

The difference spectra between sulphide-treated and native turkey xanthine dehydrogenase at various incubation times are shown in Fig. 6. From the extent of the decrease in the visible absorption spectrum it is clear that both the flavin and the iron sulphide chromophores were being reduced. Semilogarithmic plots of the time-course of the decrease in absorbance at 450 and 550nm (Fig. 7) show that more than one reaction (pseudo-first-order with respect to enzyme) had taken place. Since the time required for completion of spectral change and that for loss of the sulphide-sensitive activity (Fig. 6) were the same, it would seem that the sulphide difference spectra are true reflexions of the binding and inactivation of the enzyme by this agent.

Fig. 8. Effects of selenide treatment on the xanthine- NAD^+ oxidoreductase activities of native and cyanide-inactivated turkey liver xanthine dehydrogenase

Enzyme was inactivated by incubation with 5.0mM-cyanide as in Fig. 2. After removal of unbound cyanide by passage through Sephadex G-25, the inactive preparation and native enzyme were incubated at 45°C in capped vials containing 0.05M-phosphate buffer, pH 7.8, 0.1mM-EDTA and 0.01M-sodium selenide. The activity of the selenide-treated cyanide-inactivated enzyme is expressed (○) as a percentage of that of the native enzyme and (●) of that of the selenide-treated enzyme. Similarly, the activity of the selenide-treated enzyme is expressed (□) as a percentage of that of the native enzyme.

Selenide treatment

An experiment in which cyanide-inactivated turkey enzyme was incubated with sodium selenide is shown in Fig. 8. Although the presence of a red precipitate in the incubation mixture attested to the formation of elemental selenium, such treatment resulted in significant reactivation of the inactive enzyme. Analysis of the selenide used showed that this reactivation was not due to sulphide contamination. However, proof that reactivation resulted from incorporation of selenium into the enzyme must await radioactive studies. Fig. 8 also shows that selenide treatment effects the loss of 50% of the xanthine- NAD^+ oxidoreductase activity of the native enzyme. However, the rate at which this activity is lost is greater on treatment with selenide than with sulphide.

Discussion

The effects of methanol treatment on turkey liver xanthine dehydrogenase are similar to those reported for aldehyde oxidase and xanthine oxidase (Aleman *et al.*, 1965), xanthine dehydrogenase from *Micrococcus lactilyticus* (Smith *et al.*, 1967) and chicken liver

xanthine dehydrogenase (Rajagopalan & Handler, 1967). The latter investigators have shown that reversal of methanol inactivation of chicken liver xanthine dehydrogenase by NAD^+ requires prior reduction of the latter in the incubation mixture. Surprisingly, methanol-inactivated milk xanthine oxidase is not reactivated by NAD^+ (Rajagopalan & Handler, 1967). Why xanthine- O_2 and xanthine- NAD^+ oxidoreductase activities should be the only two xanthine-oxidizing activities of the turkey liver enzyme resistant to methanol inhibition is unclear. However, it may be significant that O_2 and NAD^+ , unlike other acceptors, react with the flavin chromophores (Komai *et al.*, 1969).

Since the reaction catalysed by these enzymes is essentially one of hydroxylation, it has been proposed that inhibition by methanol results from its substituting for water as a ligand of molybdenum (Aleman *et al.*, 1965). However, Pick *et al.* (1971) suggested that methanol is oxidized to formaldehyde at the active centre and that the latter, rather than methanol, is the actual inhibitor. They concluded that a formyl residue becomes attached to a group in the active centre and that interaction of molybdenum with this residue traps the metal in the reduced form thereby preventing turnover. Later work by Edmonson *et al.* (1972) would suggest the proposed active-centre persulphide as the site of attack by formaldehyde.

Massey & Edmonson (1970) found that sulphide treatment of cyanide-inactivated or of native milk xanthine oxidase (a mixture of functional and non-functional enzyme as normally isolated) resulted initially in an increase in activity presumably because of regeneration of the persulphide groups. Prolonged treatment (>4h) of native xanthine oxidase with sulphide resulted in the incorporation of extraneous sulphur into the protein, possibly by forming extra persulphides on reaction with disulphides (Massey & Edmonson, 1970). The latter may well be the cause of the observed loss of activity on sulphide treatment of the native turkey enzyme (see below). Thus loss of activity and bleaching of the absorption spectrum may be consequent on incorporation of extraneous sulphur into the enzyme to form new persulphides. Bond formation between the new persulphide and a neighbouring group could effect reduction of the enzyme. Alternatively, conformational changes on binding sulphur might explain the decrease in absorbance in the visible region. Restoration of activity on standing may be due to disruption of the extra persulphide, loss of the newly incorporated sulphur followed by reformation of the original disulphide.

The results of cyanide and of sulphide treatments reported in the present paper are consistent with the idea that turkey liver xanthine dehydrogenase, like milk xanthine oxidase (see the introduction), contains an active-centre persulphide, essential to catalytic

activity, that may be removed by cyanide treatment and regenerated on incubation with sulphide. Massey & Edmonson (1970) have postulated that arsenite inactivation of milk xanthine oxidase may result from its forming a complex with the active-centre persulphide and a neighbouring thiol group. That the difference spectrum between arsenite-inactivated and native turkey liver xanthine dehydrogenase is so similar to those reported earlier (Coughlan *et al.*, 1969), that cyanide is known to interfere with the generation of the arsenite difference spectra of these enzymes (Coughlan *et al.*, 1969), and that heating the inactivated enzyme restores activity (Fig. 4) suggest that arsenite inhibition of the turkey enzyme may operate through a similar mechanism.

Komai *et al.* (1969) proposed a triangular sequence of electron-transfer groups for xanthine oxidase which has subsequently been modified somewhat by Edmonson *et al.* (1973). By using this sequence of intramolecular electron flow and knowing that the molybdenum atom is the site of egress of electrons to dichlorophenol-indophenol (Gurtoo & Johns, 1971) and to phenazine methosulphate (see Bray & Swann, 1972), the differential effects of sulphide treatment on the various activities of the turkey liver enzyme (Table 2) may be reconciled as in Scheme 2.

Sulphide treatment obviously disrupts the electron-transferring ability of the molybdenum atom and possibly the associated substrate-binding site. Consequently the oxidation of xanthine by any electron acceptor and of NADH by dichlorophenol-indophenol would be decreased markedly. From the results shown in Fig. 7, it is clear that sulphide reacts with the enzyme in more than one way. Thus we suggest that the (Fe/S)II chromophore is the site of egress of electrons to trinitrobenzenesulphonate and that sulphide treatment also disrupts this particular locus. As a consequence the NADH-trinitrobenzenesulphonate oxidoreductase activity would be decreased considerably. Disruption of the molybdenum and (Fe/S)II loci could, of course, leave the oxidation of NADH by O_2 , ferricyanide and Methylene Blue unchanged, as was observed.

Whether these effects are due to incorporation of sulphur into the molybdenum and (Fe/S)II regions of the enzyme or to conformational changes on binding sulphur at any region in such a way as to disrupt these loci is not known as yet. That the sulphide difference spectrum (Fig. 6) bears such a striking resemblance to the anaerobic difference spectrum between cyanide-inactivated and native xanthine oxidase (Massey & Edmonson, 1970) is interesting but does not necessarily indicate that the molybdenum locus or its immediate vicinity is directly attacked by sulphide.

It has long been assumed, perhaps for reasons of simplicity, that xanthine oxidase and related enzymes contain two equivalent intramolecular electron-transfer systems each containing one molybdenum,

Scheme 2. Proposed routes of electron transfer in turkey liver xanthine dehydrogenase

The iron sulphide chromophores of the turkey enzyme may, as in xanthine oxidase, exist in two discrete populations that are distinguishable by e.p.r. (see Edmonson *et al.*, 1973). Abbreviations: PMS, phenazine methosulphate; DCI, dichlorophenol-indophenol; TNBS, trinitrobenzenesulphonate; MB, Methylene Blue.

one flavin and four acid-labile iron sulphide prosthetic groups (Bray & Swann, 1972). Despite this assumption, various investigators have reported evidence suggesting non-equivalence of the flavin chromophores of these enzymes (Bray, 1969; Kanda & Rajagopalan, 1972; Coughlan & Johnson, 1973). The observed loss of half of the xanthine-NAD⁺ oxidoreductase activity on sulphide treatment of the native turkey enzyme suggests a number of possibilities. On the one hand, the electron-transport chains *per se* may be non-equivalent. Alternatively, they may be identical but reaction of sulphide with the enzyme may bring about a conformational change (e.g. by disruption of disulphide bridges) that effects one of the electron-transfer systems but not the other. However, it could be argued that these findings were due to the use of preparations containing significant amounts of non-functional enzyme (see Komai & Massey, 1969). The release of only half of the expected amount of sulphur on cyanide-inactivation (Table 2) and unpublished findings indicate that turkey liver xanthine dehydrogenase, as normally isolated, does contain up to 50% non-functional enzyme. Only the functional enzyme exhibits xanthine-NAD⁺ oxidoreductase activity. The results of sulphide treatment of the native enzyme (Fig. 5) may be due to concurrent activation of the non-functional molecules by regeneration of the persulphides and inactivation of the functional enzyme by formation of extraneous persulphides. Distinction between these various possibilities must await the isolation of 100% functional enzyme.

That the results of treatment with selenide and sul-

phide are so very similar, qualitatively, suggests that selenium may have replaced sulphur in the active-centre persulphides. If this is so, new avenues of investigation of these complex enzymes are opened up. This would of course require proof of the incorporation of selenium into the enzyme and the finding of conditions under which greater reactivation and stabilization could be effected. Separation of the selenium-containing active preparation from inactive enzyme should theoretically be possible by using the affinity-chromatography procedure of Edmonson *et al.* (1972).

W. F. C. is in receipt of a maintenance grant from the Department of Education, U.K.

References

- Aleman, V., Smith, S. T., Rajagopalan, K. V. & Handler, P. (1965) in *Non-Haem Iron Proteins: Role in Energy Conversion* (San Pietro, A., ed.), pp. 327-348, Antioch Press, Ohio
- Bray, R. C. (1969) in *Flavins and Flavoproteins* (Kamin, H., ed.), pp. 385-398, University Park Press, Baltimore
- Bray, R. C. & Swann, J. C. (1972) *Struct. Bonding* **11**, 107-144
- Cleere, W. F. & Coughlan, M. P. (1974) *Comp. Biochem. Physiol.* in the press
- Coughlan, M. P. & Johnson, D. B. (1973) *Biochim. Biophys. Acta* **302**, 200-204
- Coughlan, M. P., Rajagopalan, K. V. & Handler, P. (1969) *J. Biol. Chem.* **244**, 2658-2663
- Edmonson, D., Massey, V., Palmer, G., Beacham, L. M., III & Elion, G. B. (1972) *J. Biol. Chem.* **247**, 1597-1604

- Edmonson, D., Ballou, D., Van Heuvelen, A., Palmer, G. & Massey, V. (1973) *J. Biol. Chem.* **248**, 6135–6144
- Gurtoo, H. L. & Johns, D. G. (1971) *J. Biol. Chem.* **246**, 286–293
- Kanda, M. & Rajagopalan, K. V. (1972) *J. Biol. Chem.* **247**, 2177–2182
- Komai, H. & Massey, V. (1969) in *Flavins and Flavoproteins* (Kamin, H., ed.), pp. 399–423, University Park Press, Baltimore
- Komai, H., Massey, V. & Palmer, G. (1969) *J. Biol. Chem.* **244**, 1692–1700
- Malkin, R. & Rabinowitz, J. C. (1966) *Biochemistry* **5**, 1262–1268
- Massey, V. & Edmonson, D. (1970) *J. Biol. Chem.* **245**, 6595–6598
- Neas, R. E. & Guyon, J. C. (1969) *Anal. Chem.* **41**, 1470–1473
- Pick, F. M., McGartoll, M. A. & Bray, R. C. (1971) *Eur. J. Biochem.* **18**, 65–72
- Rajagopalan, K. V. & Handler, P. (1964) *J. Biol. Chem.* **239**, 2027–2035
- Rajagopalan, K. V. & Handler, P. (1967) *J. Biol. Chem.* **242**, 4097–4107
- Rajagopalan, K. V. & Handler, P. (1968) in *Biological Oxidations* (Singer, T. P., ed.), pp. 301–337, Interscience Publishers, New York
- Rajagopalan, K. V., Handler, P., Palmer, G. & Beinert, H. (1968) *J. Biol. Chem.* **243**, 3784–3796
- Smith, S. T., Rajagopalan, K. V. & Handler, P. (1967) *J. Biol. Chem.* **242**, 4108–4117
- Theorell, H. & Stenhagen, E. (1938) *Biochem. Z.* **299**, 416–419
- Warburg, O. & Christian, W. (1942) *Biochem. Z.* **310**, 384–421