Essential Role of Thiol Groups in Aldehyde Dehydrogenases

By A. O. M. STOPPANI and C. MILSTEIN
Department of Biochemistry, School of Medicine, University of Buenos Aires

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The thiol groups of some coenzyme-linked dehydrogenases, such as glyceraldehyde 3-phosphate and alcohol dehydrogenases, have been extensively studied and the evidence available points to an important function of the thiol group in the action of these enzymes (Racker, 1955*b*; Kaplan & Ciotti, 1954; Koeppe, Boyer & Stulberg, 1956; Van Eys & Kaplan, 1957). On the other hand little is known about the role of thiols in aldehyde dehydrogenases. A systematic study has therefore been carried out with the potassium-activated yeast aldehyde dehydrogenase (Black, 1951), the triphosphopyridine nucleotide (TPN)-specific yeast aldehyde dehydrogenase (Seegmiller, 1953) and the diphosphopyridine nucleotide (DPN)-specific aldehyde dehydrogenase from liver (Racker, 1949). These enzymes have been examined for (1) sensitivity to sulphydryl reagents, (2) activation by thiols, and (3) effect of coenzymes, acetaldehyde and cations on the sensitivity towards sulphydryl reagents. The potassium-activated enzyme from yeast and the liver aldehyde dehydrogenase are known to be thiols, as the first is inhibited by mercurial compounds and Cu^{2+} ions (Black, 1955) and the second by disulphide compounds (Racker, 1955*a*). The yeast TPN-specific aldehyde dehydrogenase has not been considered to be a thiol (Seegmiller, 1953, 1955) but the experiments reported below warrant its inclusion in the group of thiol enzymes.

A preliminary account of part of this work has been published (Stoppani & Milstein, 1957).

EXPERIMENTAL

Materials

The following were used: DPN (96% pure), TPN (90% pure), reduced diphosphopyridine nucleotide (DPNH) and 2-amino-2-hydroxymethylpropane-1:3-diol (tris), from Sigma Chemical Co.; adenylic acid (AMP), *N*-ethylmaleimide and glutathione (sodium salt), from Schwarz Laboratories Inc.; cysteine hydrochloride, thioglycollic acid and disodium ethylenediaminetetra-acetate (EDTA), from British Drug Houses Ltd.; glycylglycine from Hoffmann-La Roche and Co. Ltd.; L-histidine and glycine from Eastman Kodak Co. *p*-Chloromercuribenzoic acid, *o*-iodosobenzoic acid, iodoacetic acid, mapharside (3-amino-4-hydroxyphenylarsenoxide), and BAL (2:3-dimercaptopropanol) were the same as used previously (Stoppani, Actis, Deferrari & Gonzalez, 1953).

Methods

Preparation of potassium-activated aldehyde dehydrogenase from yeast. This was extracted from acetone-dried yeast and purified according to Black (1955), except that precipitation with NaCl was replaced by fractionation with acetone. The acetone-dried yeast has the following advantages over yeast treated with liquid N_2, as recommended by Black: (1) it can be prepared in bulk, stored indefinitely and extracted as required; (2) its preparation does not require equipment for handling large volumes of liquid N_2; and (3) the crude extracts obtained from them are free from TPN-specific aldehyde dehydrogenase. The fractionation with acetone gives enzyme preparations of low electrolyte concentration, which are required for experiments on SH protection by cations. However, only a two- or three-fold increase in purity is obtained, which is less than with the NaCl fractionation (cf. Black, 1955).

Baker's yeast (500 g.; starch-free) was washed twice in the centrifuge with 500 ml. of water, suspended in water and filtered with suction. The cake (280 g.) was cooled to 4°, blended for 1 min. in a Waring Blendor with 3 vol. of acetone previously cooled to −18°, and the solvent removed immediately with suction, on a Büchner funnel previously cooled to −18°. The acetone treatment was repeated twice. The cake was washed twice with 150–200 ml. of peroxide-free ether precooled to −18°, sucked dry and spread with a glass rod on a large sheet of paper, in a current of air. A nearly white powder was obtained which should be free from acetone or ether. The powder was kept in an evacuated desiccator, in the presence of liquid paraffin and solid NaOH.

Yeast powder (50 g.) was blended with 200 ml. of water and 100 ml. of 0·3M-K_2HPO_4 at 0–4°, and the pH of the suspension was adjusted to 8·6. Black's (1955) procedure was then followed as far as the precipitation of the enzyme at pH 4·7. In each step the volumes of reagents were adjusted to the initial amount of yeast in the proportions described by Black. The enzyme, dissolved in 0·025M-K_2HPO_4, was centrifuged at 2500 rev./min., the precipitate was washed in the centrifuge with 10 ml. of the same phosphate solution, and the supernatants were combined. These extracts, with or without mM-cysteine, were kept frozen at −18° for several weeks without much loss of activity. The enzyme preparations used in the experiments reported in this paper were obtained by acetone-precipitation of extracts in the absence of cysteine. For this purpose the enzyme solutions were centrifuged to remove denatured protein, the pH was carefully adjusted to 6·3 and 50% (v/v) of ice-cold acetone was added with continuous stirring. The precipitate was separated by centrifuging for 3–5 min. at 2500 rev./min. and −8°, dissolved in the original volume of 0·015M-tris buffer (pH 8·0) at 0°, and used shortly thereafter.

These preparations were very unstable and at -18° became inactive in 24 hr.

Preparation of TPN-specific aldehyde dehydrogenase from yeast. Yeast (200 g.), washed with water as described above, was added in small amounts to 1 l. of liquid N_2 in a Dewar flask. After all the N_2 had evaporated, the thawed yeast was poured slowly with stirring in 100 ml. of cold 0·3M-K_2HPO_4 and the pH was adjusted to 8·6 with aq. 6N-NH_3 soln., the temperature being kept at 3–5°. The suspension was mechanically shaken for 2 hr., left at 4° for 24 hr. with occasional stirring and centrifuged at 0° and 3000 rev./min. The supernatant was fractionated with $(NH_4)_2SO_4$ as described by Seegmiller (1955). The active precipitate was extracted several times with 4–5 ml. of 0·3M-K_2HPO_4, the extracts were mixed and the pH was adjusted to 6·3 with 3N-NH_3 soln. This preparation was submitted to a fractionation with acetone according to Seegmiller (1955). The precipitate was dissolved in 0·015M-tris buffer, pH 7·7, and used immediately for the experiments, as it was very unstable. In this method the liquid-N_2 treatment replaces the mechanical disintegration of the yeast with sand, suggested by Seegmiller (1953). It has the advantages of avoiding the need for the special shaking machine required by the original method and for the introduction of sand into the preparation, which may contaminate the enzyme with inhibitors (e.g. heavy metals). The enzyme extracts used in the experiments contained small amounts of Black's enzyme, but this did not interfere, as the latter is not active in the absence of K^+ ions and cysteine.

Preparation of liver aldehyde dehydrogenase. This was prepared as described by Racker (1955*a*), except that the fractionation with nucleic acid was replaced by a precipitation with $(NH_4)_2SO_4$. The fraction which precipitated from 51 to 60% saturation with $(NH_4)_2SO_4$ at pH 6·0 was collected, dissolved in a small volume of water and used in the experiments. This allowed a twofold increase in purity and the concentration of the enzyme in a small volume. The enzyme kept well at -18° for several weeks.

The protein concentration of the yeast aldehyde dehydrogenase preparations was determined according to Stadtman, Novelli & Lipmann (1951); with liver aldehyde dehydrogenase the spectrophotometric method of Warburg & Christian (1941) was used.

Enzyme activities. Aldehyde dehydrogenase activities were assayed at room temperature (17–25°) according to Black (1951), Seegmiller (1953) and Racker (1949) respectively, except that (1) 0·05M-KCl was used in the assay of the potassium-dependent yeast enzyme, as in our experiments this concentration gave the maximal enzyme activity, and (2) in most of the assays for the TPN-specific aldehyde dehydrogenase the glycylglycine buffer recommended by Seegmiller (1955) was replaced by 0·015M-tris, pH 7·7. Unless stated otherwise the final volume of the assay mixtures was 3·0 ml.

Enzyme activities are represented by $10^3 \times \Delta E_{340}$/min. where ΔE_{340} is the increase in optical density of the assay mixture at 340 mμ after the addition of the substrate. When the enzyme activity decreased with time (particularly with the potassium-dependent yeast enzyme) the change of optical density was plotted against time and the slope of the curve at zero time was taken for the calculation of the enzyme activity.

General procedure. Unless stated otherwise the inhibition experiments were carried out as follows. Water and the thiol reagent were successively added to the enzyme dissolved in tris buffer (final concn. 0·015M), pH 8·0, up to a total volume of 0·5 ml. After the required time measured from the addition of the inhibitor, a sample was quickly diluted with the rest of the reaction mixture, and the dehydrogenase activity was measured. A control, treated in the same way except for the addition of thiol reagent, was carried out simultaneously and the percentage inhibition (I_{tr}) was calculated. In the protection experiments, the thiol reagent was added to the enzyme in the presence of protector (coenzyme, acetaldehyde or cation) and the percentage inhibition ($I_{p,\,tr}$) was calculated in relation to a control treated with protector alone. The percentage protection (P) of the enzyme was calculated from the expression

$$P = 100\,(I_{tr} - I_{p,\,tr})/I_{tr}.$$

This procedure was imposed by the need to test the action of inhibitors in the absence of SH compounds, cations or substrates, which as shown in the results section may effect strongly the action of the SH reagents.

RESULTS

Sensitivity of aldehyde dehydrogenases to thiol reagents

Aldehyde dehydrogenases are inhibited by mapharside, *o*-iodosobenzoate, *N*-ethylmaleimide, *p*-chloromercuribenzoate and iodoacetate. The experiments summarized in Table 1 have not been conducted under identical conditions, but they allow the conclusion that Seegmiller's enzyme is the most strongly affected by *o*-iodosobenzoate, *N*-ethylmaleimide, *p*-chloromercuribenzoate and iodoacetate. On the other hand Black's enzyme is the most sensitive to mapharside. Among the thiol reagents tested, *p*-chloromercuribenzoate and iodoacetate were respectively the most and the least effective inhibitors of aldehyde dehydrogenases. The inhibition of Seegmiller's enzyme by *p*-chloromercuribenzoate and its partial reversal by cysteine are shown in Fig. 1. The action of iodoacetate developed slowly, incubation for 30 min. being required to obtain maximum inhibition of Black's enzyme.

Activation of aldehyde dehydrogenases by thiols and other metal-binding compounds

The activation by thiol compounds and other metal-complex-forming agents is a feature of many thiol enzymes. The addition of thiols is essential for the activity of the yeast potassium-activated enzyme and, when inactive, the aldehyde dehydrogenase from liver can be reactivated by addition of EDTA or thiols (Racker, 1955*a*). On the other hand there is no mention of a requirement by the yeast TPN-dependent enzyme for thiols, and this has been taken to differentiate this enzyme from the potassium-activated yeast aldehyde dehydrogenase (Seegmiller, 1953, 1955). On these grounds a systematic study of the effect of activators on aldehyde dehydrogenases seemed necessary.

Table 1. *Inhibition of aldehyde dehydrogenases by thiol reagents*

The enzymes were dissolved in 0·015M-tris buffer, pH 8·0.

Expt. no.	Enzyme	Amount of enzyme (mg.)	Inhibitor (concn.)	Duration of treatment with inhibitor (sec.)	Inhibition (%)
1	Black	0·6	Mapharside (0·032 mM)	30	97·3
	Seegmiller	0·3	Mapharside (0·032 mM)	30	79·8
	Racker	1·8	Mapharside (0·32 mM)	30	53·0
2	Black	0·8	*o*-Iodosobenzoate (0·06 mM)	30	85·0
	Black	0·8	*o*-Iodosobenzoate (0·02 mM)	30	58·2
	Seegmiller	4·4	*o*-Iodosobenzoate (0·06 mM)	30	94·0
	Seegmiller	1·4	*o*-Iodosobenzoate (0·02 mM)	30	94·4
	Racker	4·4	*o*-Iodosobenzoate (0·2 mM)	60	74·4
3	Black	1·9	*N*-Ethylmaleimide (0·024 mM)	60	50·0
	Seegmiller	4·0	*N*-Ethylmaleimide (0·024 mM)	60	71·4
	Racker	1·8	*N*-Ethylmaleimide (0·18 mM)	180	70·0
4	Seegmiller	0·2	*p*-Chloromercuribenzoate (8 μM)	30	61·0
	Racker	1·8	*p*-Chloromercuribenzoate (80 μM)	30	8·2
	Racker	1·8	*p*-Chloromercuribenzoate (80 μM)	120	24·2
	Racker	1·8	*p*-Chloromercuribenzoate (0·6 mM)	180	84·8
5	Black	—	Iodoacetate (0·01 M)	300	31·0
	Seegmiller	4·2	Iodoacetate (0·01 M)	300	94·3
	Racker	1·8	Iodoacetate (0·01 M)	300	18·0

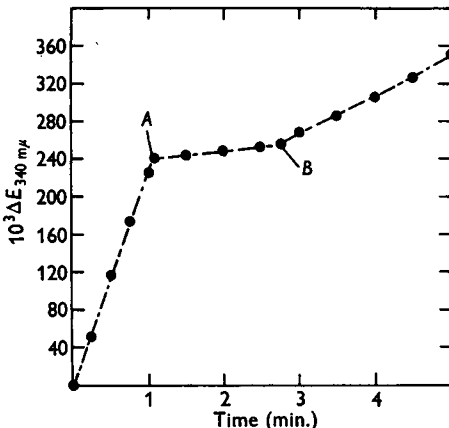

Fig. 1. Inhibition by *p*-chloromercuribenzoate and reactivation by cysteine of the TPN-specific aldehyde dehydrogenase from yeast. Reaction mixtures contained: 0·28 mg. of enzyme preparation; 0·015M-tris buffer, pH 7·7; 0·015M-$MgCl_2$; 0·12 mM-TPN; 0·5 mM-acetaldehyde; total vol., 3·0 ml. At *A*, addition of 0·02 ml. of *p*-chloromercuribenzoate (final concn., 0·12 mM); at *B*, addition of 0·03 ml. of cysteine (final concn., 0·10 mM).

Table 2. *Activation of aldehyde dehydrogenases*

Amount of enzyme: 80 μg. (Expt. 1), 13 μg. (Expt. 2), 0·44 mg. (Expt. 3) and 0·45 mg. (Expt. 4). The TPN-specific enzyme was assayed in 0·05M-glycylglycine buffer, pH 7·7. Unless stated otherwise cysteine was omitted in the mixture for the assay of the potassium-activated enzyme.

Expt. no.	Additions	Enzyme activity $10^3 \Delta E_{340}$/min.	Activity in relation to the enzyme treated with mM-cysteine (%)
	Yeast potassium-activated aldehyde dehydrogenase		
1	Cysteine (mM)	540	100
	Glutathione (mM)	530	98
	Thioglycollate (mM)	480	89
	BAL (0·016 mM)	620	115
	EDTA (0·6 mM)	630	116
	None	16	3
2	Cysteine (mM)	222	100
	Histidine (mM)	100	45
	Glycine (mM)	3	1
	None	0	0
	Yeast TPN-specific aldehyde dehydrogenase		
3	Cysteine (mM)	112	100
	None	52	46
	Liver aldehyde dehydrogenase		
4	Cysteine (mM)	57	100
	Glutathione (mM)	70	123
	EDTA (0·15 mM)	68	120
	None	66	114

Our observations can be summarized as follows: (*a*) cysteine was essential for the activity of the potassium-activated enzyme; it increased the activity of the TPN-specific aldehyde dehydrogenase but did not affect significantly the activity of the liver enzyme (Table 2). The partial activation of the TPN-specific enzyme differentiates it clearly from the essential activation of the potassium-activated dehydrogenase, the activity of which was negligible in the absence of cysteine. Glutathione and thioglycollate promoted the activity of this latter enzyme to the same extent as with cysteine (Table 2). (*b*) EDTA activated the potassium-requiring enzyme to the same extent as cysteine,

but did not affect the activity of the liver aldehyde dehydrogenase (Table 2). (*c*) Histidine promoted the activity of the potassium-activated enzyme, though less so than did cysteine, whereas glycine was completely ineffective (Table 2). (*d*) The actions of cysteine and EDTA were not additive (Table 3). Thus 0·06 mM-EDTA plus 0·002 mM-cysteine gave an enzyme activity of 530 units, that is only very slightly greater than the activity in the presence of EDTA alone, whereas the sum of their individual actions was 632 units. It is important that in this experiment both activators were at suboptimum concentrations. (*e*) The activation by histidine decreased with time, and hardly recovered the initial level by further addition of histidine. This was not due to enzyme inactivation, as the addition of cysteine reactivated the enzyme fully (Fig. 2).

Table 3. *Effect of cysteine and EDTA on the activity of the potassium-requiring aldehyde dehydrogenase*

Reaction mixtures contained 0·015M-tris buffer, pH 8·0, 0·44 mM-DPN, 0·05M-KCl and $1·7 \times 10^{-4}$M-acetaldehyde. Final vol., 3·0 ml. 80 μg. of enzyme preparation.

Additions	Enzyme activity $10^3\Delta E_{340}$/min.
Cysteine (mM)	545
Cysteine (2·0 μM)	117
EDTA (0·6 mM)	630
EDTA (0·06 mM)	515
EDTA (0·6 mM) + cysteine (2·0 μM)	626
EDTA (0·06 mM) + cysteine (2·0 μM)	530
None	16

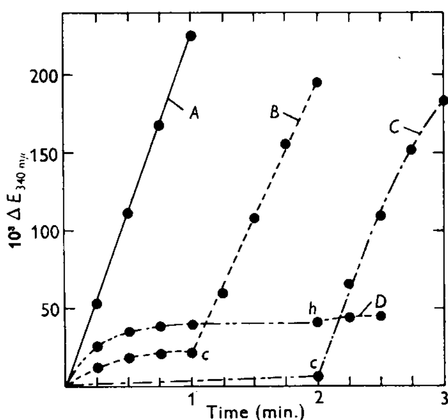

Fig. 2. Effect of cysteine, histidine and glycine on the potassium-activated aldehyde dehydrogenase from yeast. Reaction mixtures contained: 0·015M-tris buffer (pH 8·0); 0·05M-KCl; 0·44 mM-DPN; 0·17 mM-acetaldehyde; total vol., 3·0 ml. Additions: *A*, mM-Cysteine; *B* and *D*, mM-histidine; *C*, mM-glycine. At *c* and *h*, 0·03 ml. of cysteine and histidine respectively were added (final concn.: 1 and 2 mM respectively).

The activation experiments are consistent with the existence of essential thiol groups in the yeast aldehyde dehydrogenases. The lack of activation of the liver enzyme by the thiols or by EDTA, however, is not evidence against the function of thiols in this enzyme, as other thiol-containing proteins present in the relatively impure preparations could keep the thiol groups of the enzyme in the active state.

Effect of pyridine nucleotides on the inhibition of aldehyde dehydrogenases by thiol reagents

In some thiol enzymes, such as glyceraldehyde 3-phosphate dehydrogenase and alcohol dehydrogenase, the thiol groups of the enzyme are protected by the pyridine nucleotide coenzyme (Rapkine, Rapkine & Trpinac, 1939; Barron & Levine, 1952). These observations led us to test whether a similar protection is possible with aldehyde dehydrogenases. The Racker and Seegmiller enzymes are specific for DPN and TPN respectively. Black's aldehyde dehydrogenase reduces DPN and TPN, but the reaction with the DPN takes place ten times as fast as with TPN (Black, 1955).

As shown in Tables 4–6 the pyridine nucleotide coenzymes protected the thiol groups of aldehyde dehydrogenases. The effect was specific in the following respects: (*a*) TPN (but not DPN) protected Seegmiller's enzyme from inhibition by *N*-ethylmaleimide, *o*-iodosobenzoate, iodoacetate and *p*-chloromercuribenzoate. (*b*) DPN (but not TPN) protected the liver aldehyde dehydrogenase against *p*-chloromercuribenzoate and *o*-iodosobenzoate. It is remarkable that in the presence of DPN *o*-iodosobenzoate raised the activity of the enzyme to a level above that of the non-inhibited control preparations (Table 5). A smaller 'over-protection' was obtained also with DPNH but not with TPN or 2·9 mM-AMP. (*c*) Both DPN and TPN prevented the inhibition of Black's enzyme by mapharside and *o*-iodosobenzoate (Table 6), whereas in a control experiment 8·9 mM-AMP did not affect the action of this arsenical. TPN was far less effective than DPN in the protection against mapharside, but the difference was less significant with *o*-iodosobenzoate. It must be noted, however, that in the latter experiment (Table 6) the TPN concentration was higher and the concentration of *o*-iodosobenzoate lower than in the experiment on the protection by DPN; this prevents any quantitative conclusions being drawn from these experiments. The state of oxidation of DPN was unimportant for the protection of Black's aldehyde dehydrogenase, as DPN and DPNH had equal action on the inhibition of this enzyme by mapharside and *N*-ethylmaleimide (Table 6).

Table 4. *Effect of pyridine nucleotides on the inhibition of the yeast TPN-specific aldehyde dehydrogenase by thiol reagents*

0·3 mg. (Expt. 1), 0·8 mg. (Expt. 2), 0·7 mg. (Expt. 3) or 0·6 mg. (Expt. 4) of enzyme preparation in 0·5 ml. of 0·051 M-tris buffer, pH 8·0, was treated with additions as shown, for 30 sec. (Expts. 1, 2 and 3) or 5 min. (Expt. 4). Enzyme activity measured in 0·2 ml. of these extracts.

Expt. no.	Additions	Enzyme activity $10^3\Delta E_{340}$/min.	Enzyme Inhibition (%)	Enzyme Protection (%)
1	None	175	—	—
	N-Ethylmaleimide (0·02 mM)	2	98·6	—
	TPN (1·5 mM)	231	—	—
	TPN (1·5 mM) + *N*-ethylmaleimide (0·02 mM)	180	22·3	77·5
	DPN (2·7 mM)	160	—	—
	DPN (2·7 mM) + *N*-ethylmaleimide (0·02 mM)	3	98·1	0·9
2	None	187	—	—
	o-Iodosobenzoate (0·055 mM)	2	98·9	—
	TPN (0·7 mM)	185	—	—
	TPN (0·7 mM) + *o*-iodosobenzoate (0·055 mM)	73	60·5	38·8
	DPN (2·5 mM)	182	—	—
	DPN (2·5 mM) + *o*-iodosobenzoate (0·055 mM)	2	98·9	0·0
3	None	160	—	—
	Iodoacetate (0·004 M)	76	52·5	—
	TPN (0·75 mM)	168	—	—
	TPN (0·75 mM) + iodoacetate (0·004 M)	132	21·4	59·0
	DPN (2·7 mM)	162	—	—
	DPN (2·7 mM) + iodoacetate (0·004 M)	74	54·4	− 3·6
4	None	202	—	—
	p-Chloromercuribenzoate (8·0 μM)	78	61·3	—
	TPN (1·5 mM)	266	—	—
	TPN (1·5 mM) + *p*-chloromercuribenzoate (8·0 μM)	244	8·0	87·0
	DPN (2·7 mM)	225	—	—
	DPN (2·7 mM) + *p*-chloromercuribenzoate (8·0 μM)	104	53·8	7·5

Table 5. *Effect of pyridine nucleotides on the inhibition of liver aldehyde dehydrogenase by thiol reagents*

1·8 mg. (Expt. 1) or 4·3 mg. (Expts. 2 and 3) of enzyme preparation in 0·5 ml. of 0·015 M-tris buffer, pH 8·0, was treated with additions as shown for 30 sec. (Expt. 1) or 1 min. (Expts. 2 and 3). Enzyme activity measured in 0·05 ml. (Expt. 1) or 0·2 ml. (Expts. 2 and 3) of these extracts.

Expt. no.	Additions	Enzyme activity $10^3\Delta E_{340}$/min.	Enzyme Inhibition (%)	Enzyme Protection (%)
1	None	64	—	—
	p-Chloromercuribenzoate (0·6 mM)	8	84·8	—
	DPN (2·7 mM)	88	—	—
	DPN (2·7 mM) + *p*-chloromercuribenzoate (0·6 mM)	31	64·2	24·3
	TPN (1·5 mM)	75	—	—
	TPN (1·5 mM) + *p*-chloromercuribenzoate (0·6 mM)	11	85·3	− 0·6
2	None	64	—	—
	o-Iodosobenzoate (0·2 mM)	18	72·0	—
	DPN (0·3 mM)	64	—	—
	DPN (0·3 mM) + *o*-iodosobenzoate (0·2 mM)	75	− 16·7	123·0
	TPN (0·3 mM)	64	—	—
	TPN (0·3 mM) + *o*-iodosobenzoate (0·2 mM)	20	68·1	5·4
3	None	65	—	—
	o-Iodosobenzoate (0·2 mM)	33	49·0	—
	DPN (2·7 mM)	87	—	—
	DPN (2·7 mM) + *o*-iodosobenzoate (0·2 mM)	225	− 159·0	425·0
	DPNH (2·7 mM)	76	—	—
	DPNH (2·7 mM) + *o*-iodosobenzoate (0·2 mM)	120	− 58·0	218·0

Table 6. *Effect of pyridine nucleotides on the inhibition of the yeast potassium-activated aldehyde dehydrogenase by thiol reagents*

0·9 mg. (Expt. 1), 0·6 mg. (Expt. 2), 0·4 mg. (Expt. 3), 3·5 mg. (Expt. 5) or 0·6 mg. (Expt. 6) of enzyme preparation in 0·5 ml. of 0·015 M-tris buffer, pH 8·0, was treated with additions as shown, for 30 sec. (Expts. 1, 2, 5 and 6), 1 min. (Expt. 3) or 5 min. (Expt. 4). Enzyme activity was measured in 0·2 ml. (Expts. 1, 5 and 6), 0·1 ml. (Expt. 3) or 0·05 ml. (Expts. 2 and 4) of these extracts. The amount of enzyme used in Expt. 4 was not recorded.

Expt. no.	Additions	Enzyme activity $10^3\Delta E_{340}$/min.	Enzyme Inhibition (%)	Enzyme Protection (%)
1	None	181	—	—
	Mapharside (0·064 mM)	50	72·4	—
	DPN (4·0 mM)	211	—	—
	DPN (4·0 mM) + mapharside (0·064 mM)	131	37·8	47·8
	DPNH (4·0 mM)	105	—	—
	DPNH (4·0 mM) + mapharside (0·064 mM)	65	38·1	47·4
	DPN (1·1 mM)	223	—	—
	DPN (1·1 mM) + mapharside (0·064 mM)	136	39·0	46·3
2	None	330	—	—
	Mapharside (0·032 mM)	9	97·3	—
	TPN (1·5 mM)	270	—	—
	TPN (1·5 mM) + mapharside (0·032 mM)	40	85·2	12·4
3	None	149	—	—
	N-Ethylmaleimide (0·048 mM)	32	78·6	—
	DPN (1·3 mM)	179	—	—
	DPN (1·3 mM) + *N*-ethylmaleimide (0·048 mM)	100	44·3	43·6
	DPNH (1·3 mM)	108	—	—
	DPNH (1·3 mM) + *N*-ethylmaleimide (0·048 mM)	57	47·3	39·8
4	None	71	—	—
	Iodoacetate (0·01 M)	44	38·0	—
	DPN (2·5 mM)	68	—	—
	DPN (2·5 mM) + iodoacetate (0·01 M)	66	3·3	91·2
	DPN (0·25 mM)	77	—	—
	DPN (0·25 mM) + iodoacetate (0·01 M)	57	25·7	32·4
5	None	105	—	—
	o-Iodosobenzoate (0·06 mM)	26	75·2	—
	DPN (0·80 mM)	100	—	—
	DPN (0·80 mM) + *o*-iodosobenzoate (0·06 mM)	73	27·0	63·8
6	None	340	—	—
	o-Iodosobenzoate (0·02 mM)	58	83·0	—
	TPN (1·5 mM)	320	—	—
	TPN (1·5 mM) + *o*-iodosobenzoate (0·02 mM)	205	36·0	56·7

Effect of acetaldehyde on the inhibition of aldehyde dehydrogenases by thiol reagents

Protection of many thiol enzymes by the substrate is a well-known phenomenon, e.g. succinic dehydrogenase by succinate (Hopkins, Morgan & Lutwak-Mann, 1938), alcohol dehydrogenase by ethanol (Barron & Levine,1952), glyceraldehyde 3-phosphate dehydrogenase by glyceraldehyde 3-phosphate (Segal & Boyer, 1953) and carboxylase by pyruvate (Stoppani *et al.* 1953). On these grounds a similar protection of the aldehyde dehydrogenases by the substrate has been investigated. The results obtained were as follows: (*a*) Acetaldehyde diminished the inhibition of liver aldehyde dehydrogenase by mapharside, *o*-iodosobenzoate and *N*-ethylmaleimide, but did not affect the action of *p*-chloromercuribenzoate, although the enzyme inhibition was submaximal (Table 7). (*b*) Acetaldehyde also prevented, though to a smaller extent, the inhibition of Black's enzyme by mapharside, *o*-iodosobenzoate and *N*-ethylmaleimide, but not by iodoacetate (Table 8). (*c*) The inhibition (53·8 %) of Seegmiller's enzyme by 0·032 mM-mapharside was increased 23·3 % by 1·6 mM-acetaldehyde. On the other hand acetaldehyde, in the same concentration, did not affect the extent of inhibition of this enzyme by 0·055 mM-*o*-iodosobenzoate, 0·024 mM-*N*-ethylmaleimide or 8·0 μM-*p*-chloromercuribenzoate. A 14·3 % protection was, however, obtained against 0·004 M-iodoacetate as inhibitor (enzyme inhibition 52·5 %).

Effect of cations on the inhibition of aldehyde dehydrogenases by thiol reagents

The activity of yeast aldehyde dehydrogenase depends upon the cation composition of the medium. Potassium and rubidium are essential activators of

Black's enzyme (Black, 1951), whereas calcium and magnesium play the same role in regard to Seegmiller's aldehyde dehydrogenase (Seegmiller, 1953). Cations may influence the reactivity of the enzyme thiols and therefore their action on the inhibition of the enzymes by the thiol reagents was examined. Control experiments with NH_4^+, Na^+ and Li^+ ions were also carried out, the two last being known inhibitors of Black's enzyme. Cations were always introduced in the form of their chlorides.

In the experiment with Black's aldehyde dehydrogenase the following results were obtained: (*a*) Potassium ions diminished the inhibition by mapharside, *o*-iodosobenzoate, *N*-ethylmaleimide and iodoacetate (Tables 9 and 10). The protection against *o*-iodosobenzoate was quantitatively studied (Table 11) and the maximal effect was found with 0·06 M-K^+, i.e. near the concentration which produced maximal enzyme activity (unpublished work). (*b*) Rb^+ ions diminished the inhibition by

Table 7. *Effect of acetaldehyde on the inhibition of liver aldehyde dehydrogenase by thiol reagents*

4·3 mg. (Expts. 1 and 2) or 1·8 mg. (Expts. 3 and 4) of enzyme preparation in 0·5 ml. of 0·015 M-tris buffer, pH 8·0, was treated with additions as shown, for 3 min. (Expts. 1, 3 and 4) or 1 min. (Expt. 2). Enzyme activity was measured in 0·2 ml. of these extracts.

Expt. no.	Additions	Enzyme activity $10^3\Delta E_{340}$/min.	Enzyme: Inhibition (%)	Enzyme: Protection (%)
1	None	77	—	—
	Mapharside (0·13 mM)	52	33·0	—
	Acetaldehyde (10 mM)	89	—	—
	Acetaldehyde (10 mM) + mapharside (0·13 mM)	79	11·0	67·0
2	None	66	—	—
	o-Iodosobenzoate (0·2 mM)	17	74·2	—
	Acetaldehyde (13 mM)	70	—	—
	Acetaldehyde (13 mM) + *o*-iodosobenzoate (0·2 mM)	36	48·6	34·4
3	None	80	—	—
	N-Ethylmaleimide (0·18 mM)	24	70·0	—
	Acetaldehyde (10 mM)	81	—	—
	Acetaldehyde (10 mM) + *N*-ethylmaleimide (0·18 mM)	37	54·3	22·4
4	None	64	—	—
	p-Chloromercuribenzoate (0·6 mM)	9	86·0	—
	Acetaldehyde (10 mM)	81	—	—
	Acetaldehyde (10 mM) + *p*-chloromercuribenzoate (0·6 mM)	9	88·9	−3·3

Table 8. *Effect of acetaldehyde on the inhibition of the potassium-activated yeast aldehyde dehydrogenase by thiol reagents*

1·6 mg. (Expt. 1), 3·5 mg. (Expt. 2), or 1·9 mg. (Expt. 3) of enzyme preparation in 0·5 ml. of 0·015 M-tris buffer, pH 8·0, was treated with additions as shown, for 30 sec. (Expts. 1 and 2), 1 min. (Expt. 3) or 5 min. (Expt. 4). Enzyme activity was measured in 0·05 ml. of these extracts. The amount of enzyme used in Expt. 4 was not recorded.

Expt. no.	Additions	Enzyme activity $10^3\Delta E_{340}$/min.	Enzyme: Inhibition (%)	Enzyme: Protection (%)
1	None	132	—	—
	Mapharside (0·064 mM)	32	75·8	—
	Acetaldehyde (1·6 mM)	108	—	—
	Acetaldehyde (1·6 mM) + mapharside (0·064 mM)	43	60·1	20·7
2	None	105	—	—
	o-Iodosobenzoate (0·06 mM)	26	75·2	—
	Acetaldehyde (1·6 mM)	79	—	—
	Acetaldehyde (1·6 mM) + *o*-iodosobenzoate (0·06 mM)	34	56·7	24·4
	Acetaldehyde (0·3 mM)	92	—	—
	Acetaldehyde (0·3 mM) + *o*-iodosobenzoate (0·06 mM)	29	68·5	8·9
3	None	104	—	—
	N-Ethylmaleimide (0·024 mM)	56	53·9	—
	Acetaldehyde (1·6 mM)	100	—	—
	Acetaldehyde (1·6 mM) + *N*-ethylmaleimide (0·024 mM)	55	45·0	16·5
4	None	122	—	—
	Iodoacetate (0·01 M)	87	28·8	—
	Acetaldehyde (1·6 mM)	118	—	—
	Acetaldehyde (1·6 mM) + iodoacetate (0·01 M)	84	29·5	−2·6

Table 9. *Effect of cations on the inhibition of Black's aldehyde dehydrogenase by mapharside and* o-*iodosobenzoate*

1·3 mg. (Expts. 1 and 3), 2·0 mg. (Expt. 2), 0·9 mg. (Expt. 4) and 2·4 mg. (Expt. 5) of enzyme preparation in 0·5 ml. of 0·015M-tris buffer, pH 8·0, were treated with additions as shown, for 30 sec. Enzyme activity was measured in 0·05 ml. (Expts. 1, 2 and 4) or 0·1 ml. (Expts. 3 and 5) of these extracts.

Expt. no.	Additions	Enzyme activity $10^3\Delta E_{340}$/min.	Enzyme: Inhibition (%)	Enzyme: Protection (%)
1	None	87	—	—
	Mapharside (0·064 mM)	23	73·5	—
	K^+ (0·12M)	91	—	—
	K^+ (0·12M) + mapharside (0·064 mM)	64	29·7	59·7
	Na^+ (0·12M)	89	—	—
	Na^+ (0·12M) + mapharside (0·064 mM)	14	84·3	−14·7
2	None	150	—	—
	Mapharside (0·064 mM)	36	76·0	—
	Rb^+ (0·10M)	118	—	—
	Rb^+ (0·10M) + mapharside (0·064 mM)	56	52·5	32·2
3	None	71	—	—
	o-Iodosobenzoate (0·02 mM)	32	55·0	—
	K^+ (0·4M)	95	—	—
	K^+ (0·4M) + *o*-iodosobenzoate (0·02 mM)	68	28·4	48·3
	K^+ (0·12M)	99	—	—
	K^+ (0·12M) + *o*-iodosobenzoate (0·02 mM)	66	31·3	42·7
	Na^+ (0·20M)	58	—	—
	Na^+ (0·20M) + *o*-iodosobenzoate (0·02 mM)	16	72·4	−31·6
4	None	60	—	—
	o-Iodosobenzoate (0·02 mM)	25	58·3	—
	Rb^+ (0·12M)	63	—	—
	Rb^+ (0·12M) + *o*-iodosobenzoate (0·02 mM)	22	65·0	−11·5
5	None	48	—	—
	o-Iodosobenzoate (0·02 mM)	15	68·7	—
	Li^+ (0·27M)	50	—	—
	Li^+ (0·27M) + *o*-iodosobenzoate (0·02 mM)	4	92·0	−34·0

Table 10. *Effect of cations on the inhibition of Black's aldehyde dehydrogenase by* N-*ethylmaleimide and iodoacetate*

1·9 mg. (Expt. 1) or 2·5 mg. (Expt. 2) of enzyme preparation in 0·5 ml. of 0·015M-tris buffer, pH 7·9, was treated with the additions as shown, for 1 min. (Expts. 1 and 2) or 5·5 min. (Expt. 3). Enzyme activity was measured in 0·05 ml. (Expts. 1 and 3) or 0·1 ml. (Expt. 2) of these extracts. The amount of enzyme used in Expt. 3 was not recorded.

Expt. no.	Additions	Enzyme activity $10^3\Delta E_{340}$/min.	Enzymes: Inhibition (%)	Enzymes: Protection (%)
1	None	120	—	—
	N-Ethylmaleimide (0·024 mM)	52	56·7	—
	Rb^+ (0·4M)	83	—	—
	Rb^+ (0·4M) + *N*-ethylmaleimide (0·024 mM)	79	4·7	91·1
	K^+ (0·4M)	102	—	—
	K^+ (0·4M) + *N*-ethylmaleimide (0·024 mM)	58	43·1	24·0
2	None	116	—	—
	N-Ethylmaleimide (0·024 mM)	72	38·0	—
	Li^+ (0·27M)	92	—	—
	Li^+ (0·27M) + *N*-ethylmaleimide (0·024 mM)	65	29·3	22·8
	NH_4^+ (0·1M)	96	—	—
	NH_4^+ (0·1M) + *N*-ethylmaleimide (0·024 mM)	63	34·3	9·7
3	None	224	—	—
	Iodoacetate (0·01M)	150	33·0	—
	K^+ (0·4M)	211	—	—
	K^+ (0·4M) + iodoacetate (0·01M)	200	5·2	84·2
	K^+ (0·04M)	240	—	—
	K^+ (0·04M) + iodoacetate (0·01M)	202	16·0	51·5
	K^+ (0·004M)	198	—	—
	K^+ (0·004M) + iodoacetate (0·01M)	125	36·7	−11·2
	Na^+ (0·34M)	187	—	—
	Na^+ (0·34M) + iodoacetate (0·01M)	100	46·6	−41·7

mapharside and N-ethylmaleimide but potentiated the effect of o-iodosobenzoate. (c) Sodium ions increased the inhibition by mapharside and o-iodosobenzoate and, in a similar experiment, 0·34 M-Na^+ increased by 41·3% the effect of iodoacetate. (d) Li^+ ions decreased the inhibition by N-ethylmaleimide but increased the effect of o-iodosobenzoate. On the other hand 0·27 M-Li^+ did not affect the degree of inhibition (65·8%) by 0·064 mM-mapharside. (e) Ammonium ions slightly diminished the inhibition by N-ethylmaleimide, but in similar concentration did not alter the effect of 0·032 mM-mapharside (79·0% inhibition).

In the experiments with the TPN-specific yeast aldehyde dehydrogenase, Ca^{2+} and Mg^{2+} ions did not diminish the inhibition of this enzyme, and in some instances rather increased the inhibitory effects of the thiol reagents. Thus 0·076 M-Mg^{2+} and 0·076 M-Ca^{2+} increased by 13·4 and 10·9% respectively the inhibition by 0·032 mM-mapharside. At the same concentration Mg^{2+} ions increased by 7·0% the effect of 8·0 μM-p-chloromercuribenzoate and Ca^{2+} ions increased by 5·8% the inhibition by 0·055 mM-o-iodosobenzoate. On the other hand 0·066 M-Mg^{2+} reduced by 14·3% the inhibition (52·5%) by 0·004 M-iodoacetate, but the protection was not specific, as 0·1 M-K^+, which does not activate this enzyme, produced the same protection.

Although the liver aldehyde dehydrogenase does not require activating cations a few protection experiments were carried out with K^+ ions and mapharside as inhibitor. However, 0·12 M-K^+, i.e. the concentration which prevented the inhibition of Black's enzyme by the arsenical, did not affect significantly the inhibition of the liver enzyme.

Table 11. *Effect of* K^+ *ions on the sensitivity of Black's aldehyde dehydrogenase to* o-*iodosobenzoate*

The experimental conditions were the same as in Table 9. Amount of enzyme preparation: 0·8 mg. (Expt. 1) and 1·3 mg. (Expt. 2). Enzyme activity was measured in 0·1 ml. of these extracts. Inhibition (%) by o-iodosobenzoate in the absence of potassium ions: 84·5 (Expt. 1) and 55·0 (Expt. 2).

Expt. 1, 0·06 mM-o-Iodosobenzoate		Expt. 2, 0·02 mM-o-Iodosobenzoate	
K^+ (M)	Enzyme protection (%)	K^+ (M)	Enzyme protection (%)
0·400	21·8	0·120	42·7
0·120	26·9	0·060	73·2
0·060	30·6	0·012	57·2
0·012	9·1	0·006	40·3
—	—	0·0012	20·8
—	—	0·0006	−9·5

DISCUSSION

The systematic inhibition by different thiol reagents allows the inclusion of the TPN-specific aldehyde dehydrogenase from yeast in the group of sulphydryl enzymes and extends the evidence already existing on the role of thiols in the yeast potassium-requiring and liver aldehyde dehydrogenases. Except with mapharside, the TPN-specific aldehyde dehydrogenase appears to be the most susceptible towards thiol reagents. The fact that mapharside was more effective with the potassium-requiring enzyme rules out the possibility that its lesser sensitivity toward the other thiol reagents was due to protection by thiol impurities present in the enzyme preparation, for in that case all the thiol reagents should be less effective towards this enzyme than towards the TPN-specific aldehyde dehydrogenase.

The essential role of thiols in yeast aldehyde dehydrogenase is consistent with their activation by thiol compounds, an observation which might be interpreted as a removal of the inhibitory action of heavy metals. This is probably the mechanism for the non-essential activation of the TPN-specific enzyme, but the essential activation of the potassium-depending aldehyde dehydrogenase requires more careful examination. The role of thiols in preventing heavy-metal inactivation is supported by the following arguments: (1) EDTA, a metal-complex-forming agent, produces the same activation as, or higher activation than, cysteine even at lower concentration. (2) EDTA and cysteine act by the same mechanism, as shown by the non-additivity of their effects. (3) The effects of cysteine, histidine and glycine decrease in the order of their ability to form complexes with heavy metals (Albert, 1952). However, against this interpretation are the following facts: (1) EDTA and histidine activate enzyme systems, e.g. succinic oxidase, by a mechanism which does not involve removal of heavy metals (Bonner, 1954). (2) EDTA protects lactic dehydrogenase from inhibition by S^{2-} ions by a mechanism where the role of heavy metals is not evident (Terayama & Vestling, 1956). (3) The TPN-specific aldehyde dehydrogenase does not require an organic activator, although it is more sensitive to thiol reagents than is Black's enzyme. (4) The activation by histidine should be permanent and proportional to the histidine concentration if it is due to dissociation of an enzyme–mercaptide; this, however, is at variance with the experimental results. Although no mechanism can be proposed at present to explain the activation of Black's enzyme by cysteine and other metal-binding compounds, the evidence available does not warrant an essential role of heavy metals in this effect.

The specific protection of aldehyde dehydrogenases by pyridine nucleotides shows the close connexion between coenzyme and enzyme thiols, which should be located near to or at the enzyme site that binds the coenzyme. The thiol(s) could (1) act as a link between enzyme and coenzyme whatever the state of oxidation of the latter (cf. Theorell & Bonnichsen, 1951; Van Eys & Kaplan, 1957), or (2) form a bond between enzyme and oxidized coenzyme subject to cleavage (aldehydolysis) by the substrate (cf. Racker, 1955*b*). Hypothesis (2) implies protection of the enzyme thiols by acetaldehyde and, in fact, this has been found with Racker's and Black's aldehyde dehydrogenases. However, the protection by acetaldehyde (1) is not absolute, as Black's enzyme and the liver aldehyde dehydrogenase were inhibited respectively by iodoacetate and *p*-chloromercuribenzoate in the presence of acetaldehyde and (2) could be non-specific on account of the high reactivity of the carbonyl group of acetaldehyde (Racker, 1955*b*). This is particularly important with Black's enzyme which, as shown by kinetic studies (unpublished work), can form two enzyme–substrate compounds. The first is catalytically active and contains one acetaldehyde molecule/enzyme active site, whereas the second is not active and contains two molecules of acetaldehyde/enzyme active site. In the second complex, acetaldehyde could bind the thiol group normally involved in the enzyme–coenzyme union and therefore (*a*) inhibit the enzyme and (*b*), in the absence of coenzyme, protect that thiol from inhibition by thiol reagents. These arguments, together with the protection by DPNH and the lack of protection of Seegmiller's enzyme by acetaldehyde, strongly support hypothesis (1), i.e. that thiols permanently bind enzyme to coenzyme. Nevertheless in Racker's enzyme there may be several thiol groups, and some could be involved in hemithioacetal formation. The problem resembles that presented by the role of thiols in glyceraldehyde 3-phosphate and alcohol dehydrogenases, and experiments with pure enzyme preparations are required for a final conclusion.

The action of cations on the sensitivity of aldehyde dehydrogenases to thiol reagents cannot be systematically related to the enzyme activation. In fact potassium and rubidium, two activators of Black's aldehyde dehydrogenase, protected the thiols of this enzyme, but calcium and magnesium, which activated Seegmiller's enzyme, rather potentiated the inhibition of the latter by the thiol reagents. Furthermore, potassium (activator) and lithium (inhibitor) gave similar protection to Black's aldehyde dehydrogenase against *N*-ethylmaleimide. The action of each cation varied also according to the nature of the thiol reagent. Thus rubidium decreased the effect of *N*-ethylmaleimide but increased that of *o*-iodosobenzoate, whereas potassium diminished the action of both but was more effective against *o*-iodosobenzoate.

Notwithstanding these discrepancies, in some instances the protection of the thiols seemed to be connected with enzyme activation (and *vice versa*) as those cations which inhibited Black's aldehyde dehydrogenase enhanced the action of the thiol reagents (e.g. *o*-iodosobenzoate), while potassium and rubidium (enzyme activators) protected the enzyme thiols. Further, with potassium a quantitative relationship could be established between activation and protection of the enzyme. These facts may be explained perhaps by those properties of the ions that are dependent on their size, since according to Kachmar & Boyer (1953) comparison of the ionic radii of hydrated alkali-metal and NH_4^+ ions shows that $K^+ \simeq Rb^+ \simeq NH_4^+ < Na^+ < Li^+$, which is the same order as that in which these ions (except NH_4^+) affect Black's aldehyde dehydrogenase.

Present knowledge on the binding of cations by proteins is limited (cf. Gurd & Wilcox, 1956) and except for Carr's (1956) recent observations there is no evidence for complex-formation between proteins and cations like Na^+ and K^+. In addition to a general action on the ionic atmosphere of the protein molecule, there must be other effects of cations on each particular reaction of the thiol groups, otherwise it would be very difficult to understand their variable influence on the inhibition of Black's aldehyde dehydrogenase by thiol reagents.

SUMMARY

1. The potassium-activated aldehyde dehydrogenase from yeast, the triphosphopyridine nucleotide (TPN)-specific aldehyde dehydrogenase from yeast and liver aldehyde dehydrogenase are inhibited by the following thiol reagents: mapharside, *N*-ethylmaleimide, *o*-iodosobenzoate, *p*-chloromercuribenzoate and iodoacetate.

2. Thiol compounds, ethylenediaminetetraacetate and histidine are essential activators of the potassium-activated enzyme, whereas cysteine is a non-essential activator of the TPN-specific aldehyde dehydrogenase.

3. Pyridine nucleotide coenzymes prevent specifically the inhibition of aldehyde dehydrogenases by thiol reagents.

4. Acetaldehyde prevents the inhibition of the potassium-activated aldehyde dehydrogenase and liver aldehyde dehydrogenase by some thiol reagents. The protection varies according to the inhibitor. The inhibition of the TPN-specific enzyme by thiol reagents is not significantly affected by acetaldehyde.

5. The sensitivity of the potassium-activated enzyme towards thiol reagents varies in the presence

of the cations. The effect of some thiol reagents is diminished by activators (K^+ and Rb^+ ions) and increased by inhibitors (Na^+ and Li^+ ions). There is no general correlation between the effect of ions on the enzyme activity and the reactivity of the thiol groups. Cations which activate the TPN-specific enzyme do not affect significantly the enzyme inhibition by thiol reagents.

6. Some convenient modifications have been introduced in the preparation of both yeast aldehyde dehydrogenases.

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Reaction of Ninhydrin in Acid Solution with Straight-Chain Amino Acids Containing Two Amino Groups and its Application to the Estimation of $\alpha\epsilon$-Diaminopimelic Acid

BY ELIZABETH WORK
University College Hospital Medical School, London, W.C. 1

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Certain basic amino acids, such as ornithine and lysine, were reported by Chinard (1952) to give coloured products when heated with ninhydrin in solution at about pH 1. With the exception of proline, the other common naturally occurring amino acids did not react significantly under these conditions. Since lanthionine also reacted (Newton, Abraham & Berridge, 1953) it was of interest to examine $\alpha\epsilon$-diaminopimelic acid, which is structurally related to both lanthionine and lysine and which is widely distributed among many species of bacteria (Work & Dewey, 1953; Hoare & Work, 1957). The product of reaction of acidic ninhydrin with diaminopimelic acid was yellow and thus resembled the reaction products from lysine or lanthionine, but differed from the red product given by ornithine.

Other similar amino acids containing two amino groups are the various naturally occurring sulphur-containing diaminodicarboxylic acids and also the higher and lower homologues of diaminopimelic acid. Their reaction products with acidic ninhydrin were all examined in order to determine whether the reaction is a general one for diamino acids. The reaction of acidic ninhydrin with diaminopimelic acid and with certain other naturally occurring amino acids has been investigated with a view to finding a specific method for estimating diaminopimelic acid.

MATERIALS AND METHODS

Unless otherwise stated, diaminopimelic acid and its homologues were unresolved synthetic samples; I am indebted to Dr H. Heath for diaminosuccinic and diaminoglutaric acids and to Dr D. Simmonds for other members of the homologous series. *S*-(β-Aminoethyl)cysteine was provided by Dr H. Lindley. LL- and *meso*-Diaminopimelic acid were prepared as described by Hoare & Work (1955). The other