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The Inhibition of Pea-Seedling Diamine Oxidase by Chelating Agents

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Werle & Pechmann (1949) described an enzyme in extracts of higher plants that catalysed the oxidation of 1,4-diaminobutane, 1,5-diaminopentane and histamine, and suggested that it corresponds to the diamine oxidase (diamine-oxygen oxidoreductase, EC 1.4.3.6) of animal tissues. Kenten & Mann (1952) and Mann (1955) showed that the plant enzyme also catalyses the oxidation of phenylalkylamines and of aliphatic monoamines, and suggested the name 'plant amine oxidase' for the enzyme; this name was used later by Mann & Smithies (1955*a, b*), Clarke & Mann (1957, 1959) and Mann (1960, 1961). Since Fouts, Blanksma, Carbon & Zeller (1957) showed that animal diamine oxidase also catalyses the oxidation of monoamines and phenylalkylamines, this distinction between the two enzymes on the basis of substrate specificity seems unjustified and the plant enzyme is therefore called diamine oxidase in the present work. The isolation, from pig kidney, by Kapeller-Adler & Macfarlane (1962) of a histaminase acting specifically on histamine suggests that the use of the trivial name histaminase for diamine oxidases is incorrect.

Several workers have reported that the diamine oxidase of pea and clover seedlings is inhibited by chelating agents and have therefore suggested that the enzyme may be a metalloprotein. The enzyme is inhibited by sodium diethyldithiocarbamate, salicylaldoxime and potassium ethyl xanthane (Mann, 1955), and also by diphenylthiocarbazono and 8-hydroxyquinoline (Werle & Hartung, 1956) and by 2,2'-bipyridyl and 1,10-phenanthroline (Suzuki, 1959). Mann (1961) described a method yielding highly purified preparations of pea-seedling diamine oxidase and reported that the copper content increased during purification to 0.08–0.09% in the final preparations. The results of spectrographic analysis of one of the final preparations suggested that zinc was the only other trace

element present in possibly significant amounts. The removal of copper, by precipitation with sodium diethyldithiocarbamate, resulted in complete loss of the catalytic activity; most of the original activity was restored by the addition of Cu^{2+} ions, but not of Zn^{2+} ions, to the copper-free preparation. It was therefore suggested that the diamine oxidase is an enzyme containing copper.

The present work was started to find out if the inhibition of the enzyme by chelating agents is due to the presence of copper in the enzyme.

MATERIALS AND METHODS

Buffers. Orthophosphate buffers were prepared from KH_2PO_4 and KOH and freed from traces of copper by extraction with diphenylthiocarbazono in CCl_4 , as described by Hewitt (1952), and then freed from traces of diphenylthiocarbazono by repeated extraction with CCl_4 . Pyrophosphate buffers were prepared from $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$ and $\text{K}_4\text{P}_2\text{O}_7$.

Catalase. Crystalline ox-liver catalase (EC 1.11.1.6) was prepared as described by Clarke & Mann (1959).

Diamine oxidase. Diamine-oxidase preparations were made from pea seedlings by the method of Mann (1961). The assay was based on the initial rate of uptake of O_2 by reaction mixtures containing diamine oxidase, catalase (25 $\mu\text{g.}$), 10 mm-1,4-diaminobutane and 67 mm-orthophosphate buffer, pH 7, in a total volume of 3 ml. A unit of diamine oxidase is defined as the amount which will catalyse the oxidation of 1 μmole of 1,4-diaminobutane/min. at 28°, giving an initial rate of uptake of O_2 of 11.2 $\mu\text{l./min.}$ The specific activity is defined as units/mg. of protein. The protein content was calculated from the total nitrogen content by using the factor 6.25. The two preparations used contained 800 and 830 units/ml., 1920 and 2000 $\mu\text{g.}$ of N/ml., and 0.09 and 0.084% of copper, respectively. The specific activity of both preparations was 67. This corresponds to a value of 280000 for the specific activity (units/mg. of N) calculated from the units previously defined by Mann (1961).

The preparations were stored at -10° and were thawed about once weekly to take samples. The loss of activity was

slow; after 8 months the specific activity of one of the preparations had fallen to 58. For the manometric experiments the stock preparations were diluted 100-fold with water and 0.1 ml. of the diluted solution was used in each reaction mixture. Fresh dilutions were made at weekly intervals and stored at 0–5°.

Oxygen uptake. Oxygen uptake was measured in the Warburg apparatus, in air, at 28°. The volume of the reaction mixtures was 3 ml., and 0.2 ml. of 5*N*-KOH was present in the centre well.

Estimation of total nitrogen. The total nitrogen of the diamine-oxidase preparations was estimated by a micro-Kjeldahl procedure (Mann, 1961).

Estimation of copper. The copper content of the diamine-oxidase preparations was estimated colorimetrically with sodium diethyldithiocarbamate (Mann, 1961).

Spectrophotometry. Absorption was measured with a Unicam SP. 500 spectrophotometer.

RESULTS

Inhibition by chelating agents. The effect was tested of varying the time the chelating agent was incubated with the enzyme before adding the substrate. Fig. 1 shows that the inhibition by 50 μ M-potassium ethyl xanthate was incomplete and remained almost the same whether the enzyme was preincubated with the inhibitor for 1 min. or

Fig. 1. Effect of preincubation with potassium ethyl xanthate on the inhibition of the diamine oxidase. Reaction mixtures, of 3 ml. total volume, contained diamine oxidase (0.83 unit) and catalase (25 μ g.) in 67 mM-orthophosphate buffer, pH 7. Potassium ethyl xanthate (50 μ M) was added from the side arm after equilibration and the reaction mixtures were incubated for the times shown before adding the substrate (10 mM-1,4-diaminobutane) from the second side arm. The gas phase was air, and the temperature 28°. The preincubation times were: $\square$, 1 min.; $\triangle$, 6 hr. $\circ$, Control reaction mixture (potassium ethyl xanthate omitted) without preincubation.

6 hr. Fig. 2 shows that the inhibition by 100 μ M-sodium diethyldithiocarbamate also developed rapidly during the first minute of preincubation but thereafter increased slowly until after 6 hr. the inhibition was almost complete. The enzyme lost little activity when incubated for 6 hr. without inhibitor before adding the substrate. Sodium diethyldithiocarbamate is unstable at pH 7 (Hallaway, 1959), so the possibility was investigated of the secondary increase in inhibition being caused by a decomposition product. Control experiments showed that the initial partial inhibition by sodium diethyldithiocarbamate was not increased by incubating the sodium diethyldithiocarbamate in phosphate at pH 7 for 6 hr. before adding the enzyme and substrate.

The variation in inhibition with preincubation time was studied with six chelating agents. Table 1 shows that the inhibition by 8-hydroxyquinoline, like that by potassium ethyl xanthate, was independent of the preincubation time. With 1,10-phenanthroline, and more particularly with 2,2'-bipyridyl, the inhibition developed more slowly but showed little or no further increase when the preincubation time was extended beyond 30 min. The continued secondary slow increase in inhibition, leading eventually to the complete inhibition of the enzyme, was observed only with sodium

Fig. 2. Effect of preincubation with sodium diethyldithiocarbamate on the inhibition of the diamine oxidase. Reaction mixtures were similar to those of Fig. 1 except that sodium diethyldithiocarbamate (100 μ M) replaced potassium ethyl xanthate. The preincubation times were: $\square$, 1 min.; $\triangle$, 30 min.; $\blacksquare$, 2 hr.; $\bullet$, 6 hr. Control reaction mixtures without sodium diethyldithiocarbamate: $\circ$, no preincubation; $\blacktriangle$, 6 hr. preincubation.

diethyldithiocarbamate. The enzyme was not inhibited by *mm*-2,9-dimethyl-1,10-phenanthroline (neocuproine) even after preincubation for 6 hr. Table 1 also shows the effect of varying the concentration of the chelating agents with a preincubation time of 30 min. The concentrations required to give 50% inhibition increased in the order: 8-hydroxyquinoline, 1,10-phenanthroline, 2,2'-bipyridyl, potassium ethyl xanthate, sodium diethyldithiocarbamate; at higher concentrations all the chelating agents, except neocuproine, completely inhibited the enzyme.

Prevention by metal ions of the inhibition of the enzyme by chelating agents. Table 2 shows that, in reaction mixtures buffered with orthophosphate, the inhibition by all the chelating agents tested, except potassium ethyl xanthate, could be partially or completely prevented by a range of metal ions. The metal chloride or sulphate was added to the mixture of the chelating agent and the orthophosphate buffer before adding the enzyme, and the results may have been affected by the partial precipitation of some of the metal ions as insoluble phosphates. Generally Ni^{2+} and Co^{2+} ions appeared more effective than Fe^{2+} , Cu^{2+} and Zn^{2+} ions in preventing the inhibitions. The apparent ineffectiveness of Fe^{2+} ions in preventing the inhibition of the enzyme by 2,2'-bipyridyl and 1,10-phenanthroline

was surprising because Fe^{2+} ions form very stable complexes with these reagents. Warburg & Lüttgens (1946) reported that Fe^{2+} ions were less effective than Zn^{2+} ions in reversing the inhibition by 1,10-phenanthroline of the oxygen evolution of isolated chloroplasts. Arnon (1950) reported evidence that this happened because ferrous iron was oxidized to ferric iron under the experimental conditions. The same explanation probably applies to the present results; Fe^{3+} ions had no effect on the inhibition of the diamine oxidase by 2,2'-bipyridyl or 1,10-phenanthroline but partially prevented the inhibitions produced by 8-hydroxyquinoline and sodium diethyldithiocarbamate.

Of the metal ions tested Cu^{2+} ions were the only ones that prevented the inhibition of the diamine oxidase by potassium ethyl xanthate. In addition to the metal ions listed in Table 2, Ca^{2+} , Mg^{2+} , Mn^{2+} and molybdate ions (added as disodium molybdate) were without effect. But, in the orthophosphate medium, Cu^{2+} ions apparently only partially prevented the inhibition produced by potassium ethyl xanthate and appeared to be less effective than Ni^{2+} and Co^{2+} ions in preventing the inhibition of the enzyme by the other chelating agents. This partial prevention by Cu^{2+} ions of the inhibition by chelating agents was apparent only during the early stages of the enzyme-catalysed

Table 1. *Effect of variation in concentration of chelating agent and time of preincubation with the enzyme on the inhibition of diamine oxidase*

The reaction mixtures and conditions were similar to those of Fig. 1 except for the variation in the chelating agents used. The percentage inhibition was calculated from the uptakes of O_2 of the experimental reaction mixtures and of control reaction mixtures, without chelating agent, during the period 5–15 min. after adding the substrate.

Substrate.	Concn. of chelating agent (μM)	Preincubation time (min.)				
		1	5	30	120	360
		Percentage inhibition				
Chelating agent						
Potassium ethyl xanthate	10	—	—	32	—	—
	50	66	71	70	70	68
	100	—	—	87	—	—
	300	—	—	98	—	—
8-Hydroxyquinoline	3	—	—	28	—	—
	10	56	58	58	55	55
	30	—	—	94	—	—
2,2'-Bipyridyl	10	—	—	32	—	—
	20	21	31	56	63	64
	100	—	—	89	—	—
	200	—	—	97	—	—
1,10-Phenanthroline	5	—	—	26	—	—
	10	—	—	41	—	—
	20	46	60	65	64	65
	100	—	—	94	—	—
2,9-Dimethyl-1,10-phenanthroline	1000	0	0	0	0	0
Sodium diethyldithiocarbamate	17	—	—	15	—	—
	50	—	—	45	—	—
	100	52	58	70	84	99
	300	—	—	90	—	—
	500	—	—	99	—	—

reaction; Cu^{2+} ions appeared to delay the onset of the inhibition rather than to prevent it. Fig. 3 shows that, in the experiments with sodium diethyldithiocarbamate, in which the concentrations of metal ions and chelating agent were higher than in the other experiments of Table 2, this delayed inhibition was also observed in the reaction mixture containing Co^{2+} ions and the chelating agent. Mann (1961) reported that the diamine oxidase is inhibited by Cu^{2+} ions. Preliminary results of a separate investigation of this inhibition of the enzyme-catalysed reaction by Cu^{2+} ions suggest that it is due to inactivation of the enzyme. Fig. 4 shows that this inactivation is produced by Cu^{2+} ions at concentrations much lower than those used in Table 2; among those tested Co^{2+} ions were the only other ones that inhibited the reaction. These results suggested that Cu^{2+} ions appeared relatively ineffective in preventing the inhibition by chelating agents in the experiments of Table 2, where Cu^{2+} ions and chelating agents were mixed in equimolar proportions, because the concentration of free Cu^{2+} ions remained sufficient to inactivate the enzyme. In agreement with this suggestion Table 3 shows that Cu^{2+} ions usually appeared to be more effective in preventing such inhibition when added to the reaction mixtures in concentrations lower than those of the chelating agents.

In reaction mixtures buffered with 67 mM-pyrophosphate, pH 7, instead of orthophosphate,

added Cu^{2+} ions did not inactivate the enzyme, probably because an inactive copper-pyrophosphate complex was formed. Table 2 shows that, in this pyrophosphate medium, added Cu^{2+} ions almost completely prevented the inhibition by 2,2'-bipyridyl, 1,10-phenanthroline and sodium diethyldithiocarbamate, but still only partially prevented the inhibition by potassium ethyl xanthate and 8-hydroxyquinoline. Some of the other metal ions tested were less effective in preventing the inhibitions when the orthophosphate was replaced by pyrophosphate. When the complexes formed by the organic reagents with the metal ions were highly coloured or insoluble, it could be seen that the pyrophosphate prevented their formation. Thus the formation of the insoluble copper complex of 8-hydroxyquinoline and the coloured complexes of copper with potassium ethyl xanthate and of ferrous iron with 2,2'-bipyridyl and 1,10-phenanthroline were partially or completely prevented by pyrophosphate; pyrophosphate is known to interfere in the colorimetric estimation of ferrous iron with 2,2'-bipyridyl and 1,10-phenanthroline. This effect of pyrophosphate depended on its presence in very high concentrations relative to those of the organic chelating agents and was almost entirely eliminated by lowering the pyrophosphate concentration to 1 mM, although this concentration was sufficient to prevent the inactivation of the enzyme by Cu^{2+} ions at the concentrations used. Table 2

Table 2. *Prevention by metal ions of the inhibition of diamine oxidase by chelating agents*

Reaction mixtures, of 3 ml. total volume, were buffered with 67 mM-orthophosphate, pH 7 (Expt. A), 67 mM-pyrophosphate, pH 7 (Expt. B), or a mixture of 67 mM-orthophosphate and mM-pyrophosphate, pH 7 (Expt. C). The chelating agent was mixed with the buffer and the metal salt; diamine oxidase (0.83 unit) and catalase (25 $\mu\text{g.}$) were then added successively. The concentrations of the chelating agents and metal salts were 100 μM except in the experiments with sodium diethyldithiocarbamate where the concentrations were 500 μM . The metal salt and both metal salt and chelating agent were omitted from control reaction mixtures. The reaction mixtures were equilibrated for 30 min. before adding the substrate (10 mM-1,4-diaminobutane) from the side arm. The percentage inhibition was calculated from the uptakes of O_2 during the period 5–15 min. after adding the substrate.

Expt.	Chelating agent	Addition						
		None	FeSO_4	FeCl_3	CoCl_2	NiSO_4	CuSO_4	ZnSO_4
		Percentage inhibition						
A	Potassium ethyl xanthate	82	80	82	81	82	63	82
	8-Hydroxyquinoline	91	76	77	33	17	49	71
	2,2'-Bipyridyl	91	68	92	19	4	57	74
	1,10-Phenanthroline	94	44	93	6	6	63	40
	Sodium diethyldithiocarbamate	100	71	86	31	11	90	24
B	Potassium ethyl xanthate	83	84	88	84	82	63	83
	8-Hydroxyquinoline	92	89	92	89	81	61	91
	2,2'-Bipyridyl	94	94	94	51	2	6	94
	1,10-Phenanthroline	93	91	95	18	0	5	95
	Sodium diethyldithiocarbamate	100	98	100	14	2	5	99
C	Potassium ethyl xanthate	80	80	80	81	81	4	83
	8-Hydroxyquinoline	89	73	84	61	25	0	82
	2,2'-Bipyridyl	95	85	94	24	2	2	78
	1,10-Phenanthroline	93	66	92	0	0	0	48
	Sodium diethyldithiocarbamate	99	78	89	5	1	3	23

shows that, in reaction media buffered with a mixture of 67 mM-orthophosphate and mM-pyrophosphate, Cu^{2+} ions almost completely prevented the inhibition of the enzyme by all the chelating agents used and were the only metal ions tested that prevented the inhibition of the enzyme by potassium ethyl xanthate. The results suggest that the inhibition of the enzyme by the chelating agents depends on the copper-complexing properties of these reagents.

Reversal by metal ions of the inhibition of the enzyme by chelating agents. Table 4 shows that, except with sodium diethyldithiocarbamate, the

metal ions which prevented the inhibition of the enzyme by the chelating agents (Table 2) also reversed the inhibition produced by preincubation of the enzyme with the chelating agents for 6 hr. The Co^{2+} , Ni^{2+} and Cu^{2+} ions were about equally effective in reversing the inhibition produced by 2,2'-bipyridyl and 1,10-phenanthroline though, with 2,2'-bipyridyl, these ions restored less than half of the initial activity. The Cu^{2+} ions were the most effective in reversing the inhibition by 8-hydroxyquinoline and were the only metal ions tested that reversed the inhibition by potassium

Fig. 3. Prevention by metal ions of the inhibition of the diamine oxidase by sodium diethyldithiocarbamate. Diamine oxidase (0.83 unit) and catalase (25 $\mu\text{g.}$) were added to mixtures of sodium diethyldithiocarbamate (500 μM) and metal salts (500 μM) in 67 mM-orthophosphate buffer, pH 7. Other conditions were as in Table 2. ●, No metal salt; △, CuSO_4 ; □, CoCl_2 ; ▲, NiSO_4 ; ○, control reaction mixture without sodium diethyldithiocarbamate or metal salt.

Fig. 4. Inactivation of the diamine oxidase by Cu^{2+} and Co^{2+} ions. Reaction mixtures, of 3 ml. total volume, contained diamine oxidase (0.83 unit) and catalase (25 $\mu\text{g.}$), with and without CoCl_2 or CuSO_4 , in 67 mM-orthophosphate buffer, pH 7. The substrate (10 mM-1,4-diaminobutane) was added from the side arm after equilibration. ○, No metal salt; ▲, 0.1 mM- CoCl_2 ; △, mM- CoCl_2 ; □, 0.01 mM- CuSO_4 ; ●, 0.1 mM- CuSO_4 .

Table 3. Prevention by cupric ions of the inhibition of the diamine oxidase by chelating agents

The amounts of CuSO_4 shown were added to mixtures of the chelating agent (100 μM) and 67 mM-orthophosphate buffer, pH 7. Diamine oxidase (0.83 unit) and catalase (25 $\mu\text{g.}$) were then added. Other conditions were as in Table 2.

Concn. of CuSO_4 (μM)	Chelating agent			
	Sodium diethyldithio- carbamate	Potassium ethyl xanthate	8-Hydroxy- quinoline	1,10-Phenan- throline
	Percentage inhibition			
0	77	87	92	96
33	23	18	57	85
50	15	40	27	76
67	41	49	43	71
83	53	63	56	63
100	59	65	58	61

Table 4. *Reversal by metal ions of the inhibition of the diamine oxidase by chelating agents*

Reaction mixtures, of 3 ml. total volume, containing diamine oxidase (0.83 unit), catalase (25 μ g.) and chelating agent (100 μ M) in a mixture of 67 mM-orthophosphate and mM-pyrophosphate, pH 7, were incubated for 6 hr. at 28°. The metal salts shown (100 μ M) were then added from the side arm and 15 min. later the substrate (10 mM-1,4-diaminobutane) was added from the second side arm. Other conditions were as in Table 2.

Chelating agent	Addition				
	None	CoCl ₂	NiSO ₄	CuSO ₄	ZnSO ₄
	Percentage inhibition				
Potassium ethyl xanthate	81	83	81	14	84
8-Hydroxyquinoline	96	61	40	2	90
2,2'-Bipyridyl	97	63	60	58	78
1,10-Phenanthroline	97	19	21	14	59
Sodium diethyldithiocarbamate	100	100	98	50	100

ethyl xanthate. But, whereas Co²⁺, Ni²⁺, Cu²⁺ and Zn²⁺ ions partially or completely prevented the inhibition of the enzyme by sodium diethyldithiocarbamate (Table 2), only Cu²⁺ ions reversed the inhibition by this reagent. Under the conditions specified in Table 4 the enzyme inhibited by preincubation with sodium diethyldithiocarbamate was only partially reactivated by Cu²⁺ ions. Table 5 shows that this was because pyrophosphate inhibited the reactivation. The reactivation was increased by adding more Cu²⁺ ions; the concentration of Cu²⁺ ions required for maximum reactivation increased with the increase in pyrophosphate concentration. The reactivation by Cu²⁺ ions of the enzyme preincubated with 2,2'-bipyridyl and 1,10-phenanthroline was not inhibited by pyrophosphate. Table 2 shows that pyrophosphate at these concentrations did not prevent the formation of the copper-diethyldithiocarbamate complex. Mann (1961) reported that the copper can be removed from diamine-oxidase preparations by precipitating it with sodium diethyldithiocarbamate. This suggested that, during the preincubation with sodium diethyldithiocarbamate, the copper was removed from the enzyme and that the reaction inhibited by the pyrophosphate was the combination of the copper-free protein with Cu²⁺ ions to form the enzyme.

Effect of dialysis against chelating agents on the copper content and activity of the diamine-oxidase preparations. Table 6 shows that when a diamine-oxidase preparation was dialysed for 24 hr. against mM-solutions of the chelating agents at pH 7 the copper was removed by sodium diethyldithiocarbamate only. As reported by Mann (1961), most of the copper was precipitated inside the dialysis sac as the copper-diethyldithiocarbamate complex. The supernatant solution contained little or no copper. No precipitate formed when the enzyme preparation was dialysed against the other chelating agents and the copper content of the solutions inside the dialysis sacs changed very little.

Table 5. *Effect of variation in pyrophosphate concentration on the reversal by cupric ions of the inhibition of the diamine oxidase by chelating agents*

Reaction mixtures, of 3 ml. total volume, containing diamine oxidase (0.83 unit), catalase (25 μ g.) and chelating agent (100 μ M) buffered with 67 mM-pyrophosphate, pH 7, or with mixtures of 67 mM-orthophosphate with mM- and 10 mM-pyrophosphate, were incubated for 6 hr. at 28°. The CuSO₄ additions shown were then made from the side arm and 15 min. later the substrate (10 mM-1,4-diaminobutane) was added from the second side arm. The percentage inhibition was calculated from the uptakes of O₂ of the experimental reaction mixtures and of control reaction mixtures, without chelating agent or CuSO₄, during the period 5–15 min. after adding the substrate.

Chelating agent	Concn. of CuSO ₄ (mM)	Pyrophosphate concentration (mM)		
		1	10	67
		Percentage inhibition		
Sodium diethyldithiocarbamate	0	100	100	100
	0.1	45	84	94
	0.2	25	—	—
	0.3	30	67	—
	1.0	74	41	73
	3.0	—	19	—
	10.0	—	—	53
2,2'-Bipyridyl	0	89	—	91
	0.1	50	—	54
	0.2	52	—	—
	0.3	59	—	—
1,10-Phenanthroline	0	97	—	95
	0.1	13	—	15

Table 7 shows that, except with sodium diethyldithiocarbamate and 2,2'-bipyridyl, the inhibition of the enzyme by the chelating agents was reversed when the chelating agents were removed by dialysis against orthophosphate buffer, pH 7. The inhibition produced by sodium diethyldithiocarbamate was not reversed by such dialysis but most of the original activity was restored by adding Cu²⁺ ions to the dialysed solution, as

reported by Mann (1961). The inhibition of the enzyme by 2,2'-bipyridyl was not completely reversed by dialysis; the lost activity was not restored by Cu^{2+} ions.

Valency state of the copper. Fig. 5 shows that, when a solution of 2,2'-biquinolyl in acetic acid was added to a diamine-oxidase preparation, part of the copper reacted rapidly with the reagent to form the purple cuprous chelate, as shown by the increase in absorption at $540\text{ m}\mu$ during the first minute after the addition. Thereafter the absorption at $540\text{ m}\mu$ increased very slowly. When hydroxylamine hydrochloride was later added the remainder of the copper rapidly reacted to form the

cuprous chelate. Taking ϵ to be 6160 (Riley & Sinhaseni, 1958), the copper content of the enzyme preparation was calculated to be $10.7\text{ }\mu\text{g./ml.}$ compared with a value of $10.8\text{ }\mu\text{g./ml.}$ obtained by the sodium diethyldithiocarbamate estimation method.

Felsenfeld & Printz (1959) and Felsenfeld (1960) modified the 2,2'-biquinolyl estimation method for cuprous copper in proteins by adding sodium ethylenediaminetetra-acetate (EDTA). This prevents the reduction of cupric copper by SH groups when the protein is denatured by acetic acid but does not prevent the reaction between cuprous copper and 2,2'-biquinolyl. Fig. 5 shows that no cuprous copper was found in the diamine oxidase when the modified estimation method was used; it is therefore concluded that all the copper in the diamine-oxidase preparation is in the cupric form.

Table 6. *Effect of dialysis against chelating agents on the copper content of a diamine-oxidase preparation*

The diamine-oxidase solution (1 ml. containing 800 units and $10.8\text{ }\mu\text{g.}$ of copper) was mixed with 0.1 ml. of 10 mm-chelating agent and dialysed for 24 hr. at $0-5^\circ$ against 250 ml. of mm-chelating agent in 10 mm-orthophosphate, pH 7. The copper content of the dialysis residues was then estimated. In the experiment with sodium diethyldithiocarbamate a precipitate formed in the enzyme solution during the dialysis; this precipitate was removed by centrifuging and the copper content of the supernatant solution was estimated.

Chelating agent	Copper content of dialysis residue ($\mu\text{g.}$)
Potassium ethyl xanthate	10.7
8-Hydroxyquinoline	10.6
2,2'-Bipyridyl	10.0
1,10-Phenanthroline	11.7
Sodium diethyldithiocarbamate	1.5

Table 7. *Reversal by dialysis of the inhibition of the diamine oxidase by chelating agents*

Diamine oxidase (2.7 ml. containing 0.83 unit/ml.) was mixed with 0.3 ml. of 10 mm-chelating agent and dialysed for 24 hr. at $0-5^\circ$ against 250 ml. of mm-chelating agent in 10 mm-orthophosphate, pH 7, and then for a further 24 hr. against 10 mm-orthophosphate, pH 7. In control experiments the enzyme was dialysed under the same conditions except that the chelating agent was omitted. The diamine-oxidase activities of the experimental dialysis residues, in the presence and absence of $\mu\text{M-CuSO}_4$, and of the control dialysis residues were assayed manometrically.

Chelating agent	Diamine-oxidase activity	
	Without CuSO_4	With $\mu\text{M-CuSO}_4$
	Percentage of control	
Potassium ethyl xanthate	92	92
8-Hydroxyquinoline	101	101
2,2'-Bipyridyl	77	69
1,10-Phenanthroline	99	88
Sodium diethyldithiocarbamate	2	66

DISCUSSION

The present results show that the inhibition of the diamine oxidase by the chelating agents used depends on the copper-complex-forming properties of these reagents and illustrate the lack of specificity of most of these metal reagents. From a survey of chelating agents for trace metals Albert & Gledhill (1947) concluded that there were too few

Fig. 5. Valency state of the copper. Reaction mixtures containing diamine oxidase (0.5 ml.; 400 units) with and without 0.15 ml. of 200 mm-EDTA were diluted with water to 1.5 ml. 2,2'-Biquinolyl was then added (1.5 ml. of a solution in acetic acid containing 0.5 mg./ml.) and the subsequent changes in absorption at $540\text{ m}\mu$ were measured. The control reaction mixture contained diamine oxidase (0.5 ml.), water (1 ml.) and acetic acid (1.5 ml.). O, Without EDTA, 0.1 ml. of 10% (w/v) hydroxylamine hydrochloride added at arrow; ●, with EDTA.

reagents specific for particular metals. Of the chelating agents used in the present work sodium diethyldithiocarbamate, which had previously generally been considered a specific inhibitor of enzymes containing copper, and 8-hydroxyquinoline were shown by Albert & Gledhill (1947) to form complexes with a wide range of metal ions at pH 7. Potassium ethyl xanthate was a more specific metal reagent, and 2,2'-bipyridyl and 1,10-phenanthroline gave coloured complexes only with Fe^{2+} ions of the metal ions tested. It was pointed out that where colour tests were used as evidence of chelation, only positive results were significant because a reagent and metal ions may chelate without visible colour change. This qualification has sometimes been overlooked and the results have been cited as evidence that 2,2'-bipyridyl and 1,10-phenanthroline are specific reagents for ferrous iron. Later studies showed that the stability of complexes formed by bivalent ions of the first transition series with ligands containing nitrogen or oxygen donor groups is generally in the $\text{Mn}^{2+} < \text{Fe}^{2+} < \text{Co}^{2+} < \text{Ni}^{2+} < \text{Cu}^{2+} > \text{Zn}^{2+}$, irrespective of the ligand. According to Irving (1959) this order of stability, which was derived by Irving & Williams (1948, 1953), has now been shown to hold rigidly, apart from a few predictable exceptions, for about 80 ligands containing nitrogen or oxygen donor groups. The metal complexes of 2,2'-bipyridyl and 1,10-phenanthroline do not follow the Irving-Williams sequence; the overall stabilities of the iron and nickel complexes may equal or exceed those of the copper complexes (Bjerrum, Schwarzenback & Sillén, 1957). These reagents are now known to inhibit enzymes containing zinc (Vallee, 1955) and, as they form more stable complexes with Cu^{2+} than with Zn^{2+} ions, would be expected to inhibit enzymes containing cupric copper, insofar as such inhibition is determined by the stabilities of the complexes formed with metal ions. It is not yet clear how far the Irving-Williams stability sequence is followed by complexes of ligands with sulphur donor groups, though it is known not to apply where the donor groups are nitrogen and sulphur, as in cysteine, glutathione and mercaptoacetic acid. This dependence of the stability sequence on the nature of the ligand was used by Williams (1960), Vallee, Williams & Coleman (1961) and Coleman & Vallee (1961) to identify the zinc-binding groups in carboxypeptidase A (EC 3.4.2.1). The present results confirm the findings (Albert & Gledhill, 1947) that potassium ethyl xanthate is a more specific copper reagent than sodium diethyldithiocarbamate. It also forms complexes with molybdate ions, and Nason & Evans (1953) reported that it inhibits NADPH_2 -nitrate reductase (EC 1.6.6.3), an enzyme containing molybdenum. Under the conditions used

in the present work molybdate ions did not prevent the inhibition of the diamine oxidase by potassium ethyl xanthate. Arnon (1950) reported that the inhibition by 1,10-phenanthroline of the evolution of oxygen by isolated chloroplasts was reversed by Fe^{2+} , Co^{2+} , Ni^{2+} , Cu^{2+} and Zn^{2+} ions, but, of these, only Cu^{2+} ions reversed the inhibition of the reaction by potassium ethyl xanthate. The possibility was suggested that copper may be one of the metals concerned in the light-reaction of photosynthesis.

It is suggested that the primary reaction involved in the inhibition of the diamine oxidase by the chelating agents is the combination of the reagents with enzyme-bound copper to form inactive enzyme-inhibitor complexes. These complexes are dissociable and usually break down on dialysis, or on addition of metal ions with which the inhibitor forms a complex, to liberate the enzyme. The enzyme-diethyldithiocarbamate complex dissociates to give the copper-free protein and the copper-diethyldithiocarbamate complex. This type of dissociation is responsible for the secondary increase in inhibition observed on preincubation with sodium diethyldithiocarbamate which is reversible only by Cu^{2+} ions. The secondary increases in inhibition on preincubation with 2,2'-bipyridyl and 1,10-phenanthroline were not associated with removal of copper from the enzyme and may have been caused by changes in the nature of the enzyme-inhibitor complexes. The finding that sodium diethyldithiocarbamate was the only chelating agent that removed the copper from the enzyme may have depended on the experimental conditions used. Lindskog & Malmstrom (1960) found that the reversible removal of zinc from carbonic anhydrase (EC 4.2.1.1) required dialysis for several weeks against mM-1,10-phenanthroline at pH 5.

The copper is firmly bound to the enzyme protein and the diamine oxidase may therefore be classified as a metalloenzyme according to the terminology of Vallee (1955). The copper is present in the cupric state, and the fact that neocuproine, a reagent specific for cuprous copper, does not inhibit the enzyme suggests that the reaction mechanism does not involve valency change in the copper. The diamine oxidase of animal tissues will also probably prove to be an enzyme containing copper; Werle & Hartung (1956) have already found that it is inhibited by chelating agents. Gorkin (1959, 1961) reported that the monoamine oxidase (EC 1.4.3.4) and spermine oxidase (EC 1.5.3.3) of animal tissues are inhibited by chelating agents, and concluded that bivalent metal ions participate in the action of both these enzymes. Gorkin (1962) reported evidence that spermine oxidase is an enzyme containing zinc. Highly purified preparations of the enzyme contained 0.1–0.2% of zinc.

Dialysis of the enzyme against EDTA decreased the enzyme activity and zinc content. The lost activity was restored by adding Zn^{2+} ions to the dialysed preparation.

The pea-seedling enzyme belongs to the group of amine oxidases inhibited by carbonyl reagents such as hydrazine and semicarbazide. Zeller (1940) concluded that animal diamine oxidase is inhibited by cyanide because this compound acts as a carbonyl reagent; the inhibition of the plant enzyme by cyanide was attributed to the same cause by Kenten & Mann (1952). The present finding that the plant enzyme contains copper suggests the possibility that the inhibition by cyanide may be due, at least in part, to the properties of this compound as a metal reagent.

In the absence of catalase the pea-seedling diamine oxidase is rapidly inactivated during the reactions it catalyses. This inactivation was attributed by Werle & Pechmann (1949) and Mann (1955) to the hydrogen peroxide formed as a reaction product. The present results suggest that, with added Cu^{2+} ions, such inactivation also takes place in the presence of catalase. It is not yet clear whether this is due to catalysis by Cu^{2+} ions of the inactivation of the enzyme by hydrogen peroxide. Rapid reaction-inactivation is a characteristic of other copper-containing oxidases (Dawson, 1950). Tokuyama & Dawson (1962) attribute such reaction-inactivation of ascorbate oxidase (EC 1.10.3.3) to traces of hydrogen peroxide formed in a secondary reaction. The reaction-inactivation of ascorbate oxidase is increased by Cu^{2+} ions (Frieden, 1952; Benhamou, Magee & Dawson, 1959).

SUMMARY

1. The inhibition of pea-seedling diamine oxidase by 2,2'-bipyridyl, 1,10-phenanthroline, 8-hydroxyquinoline, potassium ethyl xanthate and sodium diethyldithiocarbamate was investigated. At concentrations of 30–300 μM all these compounds completely inhibited the enzyme.

2. The degree of inhibition by sodium diethyldithiocarbamate, 2,2'-bipyridyl and 1,10-phenanthroline depended not only on the inhibitor concentration but also on the time of preincubation with the enzyme.

3. The enzyme was inactivated by Cu^{2+} and Co^{2+} ions during the course of the catalysed reaction in orthophosphate buffer, pH 7; this inactivation was prevented by pyrophosphate buffer.

4. The inhibition of the enzyme by all the chelating agents except potassium ethyl xanthate was partially or completely prevented by Fe^{2+} , Co^{2+} , Ni^{2+} , Cu^{2+} and Zn^{2+} ions; Cu^{2+} ions were the only metal ions tested that prevented the inhibition by potassium ethyl xanthate.

5. The same metal ions that prevented the inhibitions also reversed the inhibitions produced by preincubating the enzyme with all the chelating agents except sodium diethyldithiocarbamate; Cu^{2+} ions were the only metal ions tested that reversed the inhibition by sodium diethyldithiocarbamate.

6. When the enzyme was dialysed against the chelating agents the copper was removed only by sodium diethyldithiocarbamate. Subsequent dialysis against orthophosphate buffer, pH 7, partially or completely reversed the inhibitions except that produced by sodium diethyldithiocarbamate, which was reversed by adding Cu^{2+} ions to the dialysed solution.

7. No cuprous copper was found in the diamine oxidase by the biquinolyl estimation method, and neocuproine did not inhibit the enzyme.

8. It is concluded that the diamine oxidase is a metalloenzyme containing cupric copper.

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The Denaturation of Acetic Acid-Soluble Calf-Skin Collagen

CHANGES IN OPTICAL ROTATION, VISCOSITY AND SUSCEPTIBILITY TOWARDS ENZYMES DURING SERIAL DENATURATION IN SOLUTIONS OF UREA

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The thermal conversion of collagen into gelatin is accompanied by dramatic changes in the physical and biological properties of the parent protein, e.g. a marked decrease in the values of the optical rotation and viscosity and an increase in the ease with which proteolytic enzymes can hydrolyse the derived gelatin as compared with the original collagen (Boedtker & Doty, 1956). These authors suggested that the highly organized structure of the collagen triple-helix was converted into the random-coil configuration of gelatin by a process of thermal depolymerization. This process carried out at neutral pH takes place with the minimal rupture of peptide bonds (Steven & Tristram, 1962b).

Gustavson (1956) indicated the mechanism by which concentrated solutions of urea at neutral pH caused the depolymerization of collagen. Courts (1958) has also drawn attention to the importance of various hydrogen-bonding agents, including urea, in the conversion of collagen into gelatin. Work on the soluble collagens by Orekhovich & Shpikiter (1957, 1958) has shown that treatment with urea induced physical depolymerization and formation of gelatin, the latter protein having the physical properties associated with the corresponding gelatin produced by thermal shrinkage of the original collagen. Piez (1961) reported the isolation of two types of both the α - and β -components from collagen depolymerized by either heat- or urea-pretreatment.

It seemed worth while to investigate the physical changes brought about by serial urea-denaturation

of acetic acid-soluble collagen. The normal effect of concentrated solutions of urea on soluble globular proteins is to increase the laevorotation and viscosity (Neurath, Rupley & Dreyer, 1956; Schellman, 1958; Hamaguchi, 1958; Rovery, Gabeloteau, Vernejoul, Guidoni & Desnuelle, 1959). Schellman (1958) suggested that these changes reflected an alteration from a helical structure to a random-coil configuration. The physical changes which accompany urea-denaturation of collagen are in the opposite sense to those observed in globular proteins. The high values of laevorotation and viscosity which are typical of collagen are converted to the lower values found in gelatins (Boedtker & Doty, 1956). The present work describes these changes during the serial urea-denaturation of acetic acid-soluble collagen.

MATERIALS

Acetic acid-soluble calf-skin collagen was prepared as described by Steven & Tristram (1962a). The protein contained 18% of total nitrogen on an ash- and moisture-free basis.

Crystalline salt-free trypsin (Armour, batch no. 22191) and crystalline pepsin (Armour, batch no. 22306) were used in this study.

Urea obtained from British Drug Houses Ltd. was recrystallized from ethanol.

Physical measurements were made on a Hilger standard polarimeter by using the sodium D line, and an Ostwald viscometer with a flow-time of 69 sec. for water was used to determine the viscosity of the collagen solutions, relative to water. All physical measurements were made at 18°.