Determination of Traces of Copper by Activation of Water-insoluble Apopolyphenol Oxidase

By **J. V. Stone** and **A. Townshend,*** Chemistry Dept., Birmingham University, PO Box 363, Birmingham B15 2TT

Polyphenol oxidase is insolubilised by chemical bonding to polyacrylamide. Copper is removed from the enzyme, and the resulting apoenzyme may be reproducibly and very selectively reactivated by incubation with 10—200 ng of copper(II), the extent of activation being a measure of copper concentration. The properties of the insoluble apoenzyme are described; the effect of other metal ions on copper activation or on the apoenzyme itself are minimal until their concentration is more than 1000 times that of copper.

THE activation and inhibition of enzymes by metal ions has been shown to provide an extremely sensitive means of detecting and determining traces of metals.[1-3] Perhaps the main disadvantage of some of these methods is their lack of selectivity. Although masking and separation procedures provide improvements in this respect, another approach has recently been pioneered.[4,5] This involves removal of the prosthetic metal ion from a metallo-enzyme. The resulting apoenzyme is activated very selectively by the prosthetic metal, and the degree of activation is a measure of the metal ion concentration.

In some initial studies, zinc was removed from alkaline phosphatase.[4,5] The activation process provided a reproducible method for the determination of as little as 6 ng of zinc. Alkaline phosphatase, however, is not ideal for this type of application as it is susceptible to inhibition by many heavy metal ions and to further activation or reversal of inhibition by other metal ions.[4,6] A further disadvantage of the use of apoenzymes for analytical purposes is their time-consuming preparation and their instability.

Here we describe a method which does not suffer these disadvantages. A search was made for metallo-enzymes that were relatively free from metal-ion inhibition and activation effects. One of these, a copper-containing polyphenol oxidase (E.C.1.10.3.1) from mushrooms, was chosen for the present investigation. Several workers have reported that only copper reactivates the apoenzymes of such enzymes from plant[7,8] and animal[9-11] sources. Moreover, it is reported that the native enzyme is not susceptible to inhibition or activation by metal ions.[12] It was anticipated that insolubilization of the enzyme would have several benefits. Removal of the prosthetic metal and subsequent collection and handling of the apoenzyme would be greatly facilitated. The stability of the apoenzyme should be enhanced by binding to the polymeric support. The enzyme could be reused. Most of these advantages were realised.

A preliminary report of this work has already been published.[13]

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[2] A. Townshend and A. Vaughan, *Talanta*, 1970, **17**, 299 and references therein.
[3] W. J. Blaedel and G. P. Hicks, *Adv. Analyt. Chem. Instrumen.*, 1964, **3**, 118.
[4] A. Townshend and A. Vaughan, *Analyt. Chim. Acta*, 1970, **49**, 366.
[5] A. Townshend and A. Vaughan, *Talanta*, 1970, **17**, 289.
[6] A. Townshend and A. Vaughan, *Analyt. Letters*, 1968, **1**, 907.
[7] F. Kubowitz, *Biochem. Z.*, 1937, **292**, 221.
[8] F. Kubowitz, *Biochem. Z.*, 1938, **299**, 32.
[9] T. H. Allen and J. H. Bodine, *Science*, 1941, **94**, 443.
[10] A. B. Lerner, T. B. Fitzpatrick, E. Calkins, and W. H. Summerson, *J. Biol. Chem.*, 1950, **187**, 793.
[11] A. B. Lerner, *Arch. Biochem. Biophys.*, 1952, **36**, 473.
[12] D. Kertész, *Nature*, 1951, **168**, 697.
[13] J. V. Stone and A. Townshend, *J.C.S. Chem. Comm.*, 1972, 502.

EXPERIMENTAL

Reagents. Polyphenol oxidase (from mushrooms) was purchased from the Sigma (London) Chemical Company. All water used in this investigation was distilled from an all-glass apparatus.

0·2M *Tris–acid maleate stock solution.* Tris-(hydroxymethyl)methylamine (Tris) (24·2 g) and maleic acid (23·2 g) were dissolved in 1 l of water. 40 mM Buffer solutions were prepared as follows.

pH 7·0. Stock solution (200 ml) and 0·2M-sodium hydroxide solution (192 ml) were diluted to 1 l with water.

pH 7·0, 0·5M in sodium chloride. Sodium chloride (29·2 g) was dissolved in and made up to 1 l with the above pH 7·0 buffer solution.

pH 7·6. Stock solution (200 ml) and 0·2M-sodium hydroxide solution (232 ml) were made up to 1 l with water.

Metal ion solutions were prepared in the above pH 7·0 buffer solution from analytical grade reagents.

0·01% Phenol solution. Phenol (15 mg) was dissolved in water (150 ml) containing a few crystals of sodium acetate (pH $\simeq$ 6).

0·2M Phosphate buffer solution, 0·1M in potassium cyanide. Potassium cyanide (0·65 g) was dissolved in and made up to 100 ml with 0·2M phosphate buffer solution, pH 7·0 (final pH of solution $\simeq$ 9).

Procedures

Adsorption of Metal Ions on Glassware.—Traces of copper [14] and other metal ions [15,16] are readily adsorbed onto glass surfaces. To minimise this problem all glassware was routinely washed with Pyraneg detergent, soaked for several hours in a saturated aqueous solution of disodium EDTA, and then rinsed thoroughly with water.

As polyethylene is not so susceptible to these adsorption effects,[17] the experiment to determine the trace copper content of 2 l of water was carried out in a polyethylene container.

Measurement of Catecholase Activity of Polyphenol Oxidase.—The enzyme activity was measured by its catalysis of the oxidation by catechol by oxygen, and was monitored by an oxygen electrode. Enzyme samples (0·5—1 mg of soluble enzyme in 50 µl of buffer solution or 5—10 mg of insoluble enzyme in 0·5—1·0 ml of buffer solution) were pipetted into the reaction vessel and made up to 1·5 ml with pH 7·0 Tris–maleate buffer solution. Experiments to find the effect of metal ions on the enzyme activity were carried out using the buffer solution containing a metal ion in place of the normal buffer solution. Samples were equilibrated at 25 °C for 10 min in the thermostatted sample container of the oxygen measuring apparatus (Chark Electronics, Birmingham, England), after which the oxygen electrode was inserted and the reaction was started by the injection of freshly prepared 37·5 mM catechol solution (pH $\simeq$ 6, 20 µl). The initial rate of oxygen uptake expressed as µl of oxygen per min, was taken as the measure of enzymic activity. The samples were maintained at 25 °C and magnetically stirred throughout the whole procedure.

In experiments where prolonged incubation of the enzyme or apoenzyme with the metal ion was necessary, the mixtures of enzyme and metal ions, in a total volume of 1·5 ml, were incubated in a water-bath at 25 °C, or at 4 °C overnight and re-equilibrated to 25 °C, before transfer to the reaction vessel for assay. Insoluble enzyme samples were always stirred magnetically throughout these incubation periods.

Insolubilisation of the Enzyme.—Synthesis of Enzacryl AA. Enzacryl AN, a copolymer of acrylamide, 4-nitroacrylanilide, and *NN'*-methylenediacrylamide, was synthesised as described by previous workers; [18] 5 g batches of this polymer (in fine powder form) were selectively reduced to Enzacryl AA immediately before attachment of the enzyme.

Diazo-coupling of polyphenol oxidase to Enzacryl AA. The procedure developed by previous workers [18] was slightly modified. Enzacryl AA (1·5—2·0 g) was stirred with 2M-hydrochloric acid (75 ml) at 0 °C for 5 min. Ice-cold 2% sodium nitrite solution (60 ml) was added dropwise and stirring was continued for 15 min. The nitrous acid solution was decanted off and the diazo-derivative of Enzacryl AA was washed with ice-cold pH 7·6 buffer solution (4 × 250 ml). After decanting the final washings, a solution containing polyphenol oxidase (200 mg) in pH 7·6 buffer solution (10 ml) was added and coupling was allowed to proceed with stirring for 65 h at 4 °C. The 0·01% phenol solution (150 ml) was added to couple with unreacted diazo-groups and after 45 min the water-insoluble polyphenol oxidase was collected by centrifugation at 4 °C for 10 min at 25,000 g in an MSE High Speed 18 centrifuge. A control sample, containing Enzacryl AA preswollen in pH 7·6 buffer was not treated with nitrous acid but was put through the above coupling procedure.

Both the water-insoluble polyphenol oxidase and the control were washed alternately with pH 7·0 Tris–maleate buffer solution (100 ml) and the pH 7·0 buffer solution 0·5M in sodium chloride (100 ml). Each washing took 20 min at 0 °C with vigorous stirring. Four of these washing cycles was necessary to wash the control to negligible catecholase activity (<6% of that in the diazo-coupled derivative). The diazo-coupled polyphenol oxidase derivative ($\simeq$1 g) was finally suspended in pH 7·0 Tris–maleate buffer solution (100 ml).

Determination of Protein Bound to Enzacryl AA.—Aqueous samples (1 ml, containing *ca.* 10 mg of the diazo-coupled enzyme of the non-coupled carrier control) were lyophilised, weighed, and hydrolysed in 6M-hydrochloric acid (5 ml) in evacuated sealed tubes for 18 h at 110 °C. The soluble enzyme (2 mg) was also hydrolysed under identical conditions. The small amount of insoluble residue in the hydrolysates of coupled and non-coupled polymer was removed by centrifugation. Aliquots of the soluble enzyme hydrolysate (0·5 ml), the coupled polymer hydrolysate (2·0 ml), and the non-coupled polymer hydrolysate (2·0 ml) were lyophilised, then redissolved in a small volume of the appropriate buffer solution. A complete amino-acid separation and analysis was carried out on each of these samples using the Technicon amino-acid analysis system.[19] By this procedure the large amount of ammonia produced from hydrolysis of the Enzacryl AA was separated, so that it did not interfere with the estimation of the individual amino-acids by ninhydrin assay. The quantities of protein bound to the polymers in the original samples were calculated by comparison of these amino-acid colour yields with

[14] A. Gubin, E. Hass, H. Hoffman, and W. Reinmuth, *Mitt. ver. Grosskesselbesitzer*, 1955, No. 33, 435.

[15] F. Leutwein, *Zentralblatt f. Mineral. u. Palantol.*, 1940, *A*. 129.

[16] F. K. West, P. W. West, and F. A. Iddings, *Analyt. Chem.*, 1966, **38**, 1566.

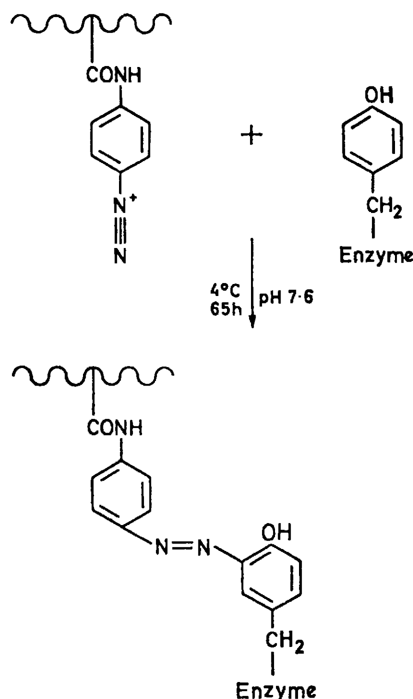
[17] R. E. Thiers, *Methods of Biochem. Analysis*, 1957, **5**, 273.

[18] S. A. Barker, P. J. Somers, R. Epton, and J. V. McLaren, *Carbohydrate Res.*, 1970, **14**, 287.

[19] 'Techniques in Amino-acid Analysis,' Technicon Monograph No. 1, 1966, Technicon Instruments Co. Ltd., Chertsey, Surrey, pp. 110—140.

those obtained from analysis of the soluble enzyme hydrolysate. These results are given in Table 4.

Preparation of the Soluble Apoenzyme.—Polyphenol oxidase (20 mg) dissolved in 0·2M-phosphate buffer, pH 7·0 (1 ml), was dialysed for 24 h at 4 °C against the pH 7·0



phosphate buffer solution, 0·1M in potassium cyanide (250 ml). The cyanide was removed by dialysis for three 3-h periods against 3 changes (each of 1 l) of pH 7·0 Tris–maleate buffer at 4 °C. A control sample of polyphenol oxidase was treated in the same way except that there was no cyanide in the phosphate buffer solution. The protein content of these dialysed samples was measured by the method of Lowry *et al.*[20] Aliquots containing 0·5 mg of protein were used in each of the reactivation experiments.

Preparation of the Water-insoluble Apoenzyme.—The water-insoluble enzyme was collected from 75 ml of its suspension in pH 7·0 Tris–maleate buffer solution by centrifugation, and vigorously stirred with the ice-cold phosphate buffer 0·1M in potassium cyanide (50 ml) for 30 min. After collection by centrifugation, the insoluble enzyme derivative was treated with more buffered cyanide solution (50 ml) and recovered by centrifugation. The resulting insoluble apoenzyme was washed with vigorous stirring at 0° for 20 min, six times with pH 7·0 Tris–maleate buffer (50 ml). Finally, the insoluble apoenzyme was resuspended in pH 7·0 Tris–maleate buffer solution (60 ml). Aliquots (0·5—1·0 ml) of this suspension were used for the reactivation experiments. A control sample of the water-insoluble enzyme suspension was treated and washed as above except that there was no cyanide in the phosphate buffer.

Collection of reactivated water-insoluble enzyme from 2 l of solution was achieved by filtration through a 100 nm pore diam. Millipore filter. The insoluble material was suspended in buffer pH 7·0 (1·5 ml) for assay.

[20] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, 1951, **193**, 265.

[21] D. Kertész and R. Zito, *Biochem. Biophys. Acta*, 1965, **96**, 447.

RESULTS

Effect of Metal Ions on the Enzymic Activity of Soluble Polyphenol Oxidase.—Native polyphenol oxidase is reportedly not activated or inhibited by metal ions.[12] This has largely been confirmed in the present study. Table 1

TABLE 1

Effect of metal ions on soluble polyphenol oxidase activity

Metal ion	Metal ion concn. (M) that had no effect when incubated with the enzyme at 25 °C: For 10 min	For 4 h
Fe^{III}, NH_4^+, Ca^{II}, Ba^{II}, VO^{2+}, Cu^{I}, Cu^{II}	1×10^{-3}; 2×10^{-6}	
Cd^{II}, Mg^{II}, Sr^{II}, Mn^{II}, Co^{II}, Hg^{II}	1×10^{-3}; 2×10^{-6}	1×10^{-3}
VO_3^-	2×10^{-6}	
Cr^{III}	9×10^{-4}; 7×10^{-7}	
Fe^{II}	2×10^{-6}	1×10^{-3}
Ni^{II}, Zn^{II}	1×10^{-3}; 2×10^{-6}	1×10^{-3}; 2×10^{-6}
Ag^{I}	1×10^{-3}; 2×10^{-6}	2×10^{-6}
Hg^{I}	3×10^{-4}; 2×10^{-7}	1×10^{-4}
Al^{III}	$1·4 \times 10^{-4}$; 7×10^{-7}	
Pb^{II}	9×10^{-4}; 2×10^{-6}	

shows the concentrations of metal ions which have no effect on the catecholase activity of polyphenol oxidase after incubation with the soluble enzyme for the times indicated. Chloride, nitrate, and sulphate ions up to 10^{-3}M also have no effect.

The only metal ion found to have an inhibitory effect on the activity of polyphenol oxidase after incubation with the enzyme for 10 min at 25 °C was the metavanadate ion. The effects of various concentrations of metavanadate are shown in Figure 1. It shows that *ca.* 10^{-3}M-metavanadate is required for appreciable inhibition to occur. Silver ions

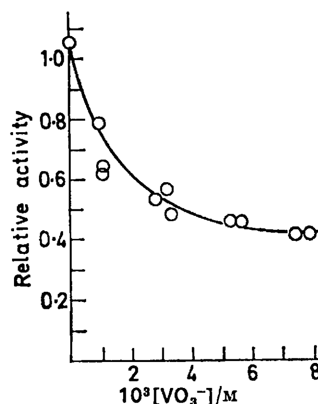


FIGURE 1 Inhibition of polyphenol oxidase by metavanadate ions

also inhibited the enzyme, but only when they had been incubated with the enzyme at 25° for 4 h (Figure 2). No ions activated the enzyme.

Polyphenol oxidase contains copper(I) ions,[21] which are quite strongly bound to the enzyme. Attempts to remove them by complexing with azide ions, EDTA, and some 1,10-phenanthroline derviatives were unsuccessful. Cyanide ions, however, were very effective for this purpose. The holoenzyme can be constituted by adding copper(II) ions to the apoenzyme, whence the enzyme protein immediately reduces the copper(II) to copper(I).[22] In the present

[22] D. Kertész, Proc. 4th Int. Cong. Biochem., Vienna, 1958, p. 65.

study it was confirmed that both copper(I) and copper(II) were able to reactivate the apoenzyme preparation but only reactivation by copper(II) was investigated in detail.

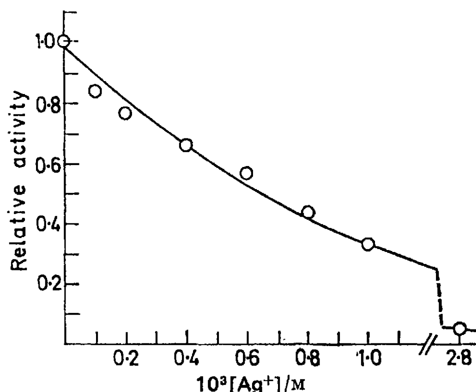


FIGURE 2 Inhibition of polyphenol oxidase by silver ions

Uptake of copper(II) (8 μg) by the apoenzyme was found to reach a maximum value within a few minutes of its being stirred with the apoenzyme (640 μg) at 25 °C. Further incubation up to 4 h gave no change in activity.

In this set of experiments catecholase activity was determined in the presence of a low concentration of catechol ($6{\cdot}6 \times 10^{-6}$M) and a large excess of reducing agent, hydroquinone ($9{\cdot}4 \times 10^{-3}$M), to minimise reaction inactivation by the quinone (see below). However, this reduces the sensitivity of the method to copper, for the enzyme is not working at its maximum rate ($K_m = 1{\cdot}7 \times 10^{-4}$ mol l^{-1}).[23] Consequently in all studies of the insoluble enzyme, enzymic activity was measured in the presence of a high concentration of substrate and in the absence of hydroquinone. The extent of reactivation of the apoenzyme was proportional to the amount of copper(II) added over the range 25—200 ng copper ($2{\cdot}5 \times 10^{-7}$—2×10^{-6}M); this is illustrated in Figure 3. The enzymic activity of the fully reactivated apoenzyme

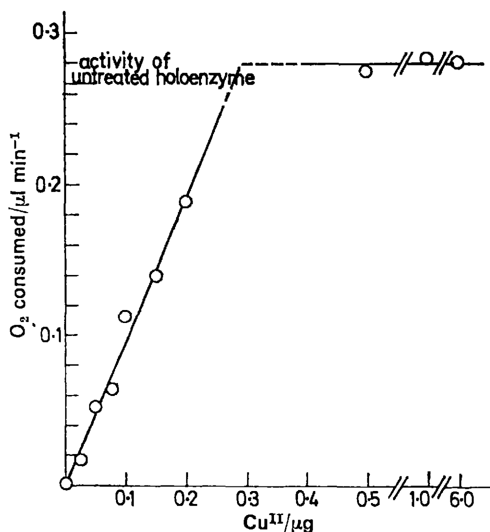


FIGURE 3 Activation of soluble polyphenol oxidase by copper(II) ions. Each sample contained 480 μg apoenzyme protein

was almost identical to that of an equivalent amount of untreated holoenzyme. It is evident, therefore, that the soluble apoenzyme could be used in a method for the determination of traces of copper, especially if the sensitivity was enhanced by use of higher concentrations of catechol, as outlined above.

Activation of the Insoluble Apoenzyme by Copper.—The insoluble apoenzyme achieved maximum reactivation by small quantities of Cu^{II} after incubation of the metal ion with the apoenzyme for several hours at 25 °C (Figure 4). In most instances, however, it was more convenient to incubate the metal ion overnight at 4 °C and then allow the preparation to equilibrate to 25 °C before assay. Similar results were obtained from both procedures. The rate of

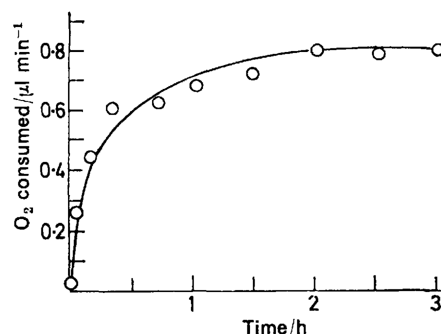


FIGURE 4 Rate of uptake of 100 ng of copper(II) ions by insoluble apopolyphenol oxidase

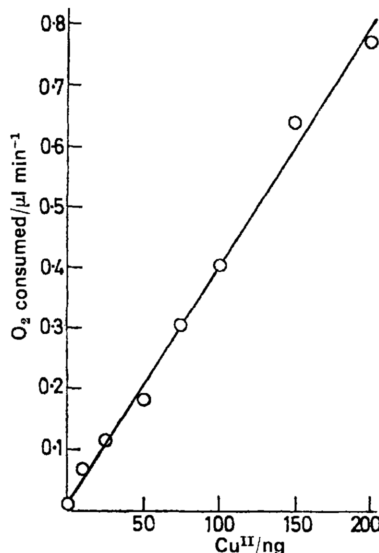


FIGURE 5 Activation of insoluble apoenzyme by copper(II) ions. (Each point is the mean of two results)

uptake of copper was markedly slower than with the soluble apoenzyme.

The increase in enzymic activity of the apoenzyme after 16 h incubation with increasing concentrations of Cu^{II} is shown in Figure 5. There is a linear relationship between copper concentration and enzyme activity. Between 10 and 200 ng of copper in a total volume of 1·52 ml (10^{-7}—2×10^{-6}M) could be determined accurately and reproducibly by their activation effect.

This method has even greater potential sensitivity. A small amount of the insolubilised apoenzyme can be exposed to large volumes of very dilute aqueous copper(II) solutions.

[23] M. A. El-Bayoumi and E. Frieden, *J. Amer. Chem. Soc.*, 1957, **79**, 4854.

For example, 2 ml of the apoenzyme suspension stirred continuously in 2 l of pH 7·0 Tris–maleate buffer solution containing 50 ng of copper(II) ions (25 pg per ml; $4\cdot2 \times 10^{-10}$M) for 24 h collected 42 ng of the copper (84%) as measured by enzyme reactivation. A similar experiment with a blank solution (containing no added copper) indicated a background level of copper(II) of 20 pg per ml. It is believed that the most important source of this copper is air-borne dust.

Effect of Metal Ions on Reactivation of the Insoluble Apoenzyme.—Table 2 shows that relatively large concentrations of some metal ions reactivate the insoluble apoenzyme to some extent after incubation overnight at 4° with the apoenzyme. Of these, iron(II), strontium(II), nickel(II), and magnesium(II) are the most potent activators. In most instances, however, these activators are at least 1000 times less effective than copper(II); Fe^{III}, Zn^{II}, Cd^{II}, Mn^{II}, Ca^{II}, Hg^{II}, Ba^{II}, Ag^{I}, NH_4^+ (all 10^{-3}M), Pb^{II} (3×10^{-4}M), VO_3^- ($1\cdot7 \times 10^{-4}$M), and Hg^{I} (10^{-4}M) did not reactivate the apoenzyme. At the 2×10^{-6}M level, no metal ion other than copper gave any measurable reactivation.

The effects of metal ions, when added ***with*** copper to the apoenzyme are summarised in Table 3. Relatively large concentrations of calcium, magnesium, barium, and lead do not appear to affect activation by copper, but similarly large concentrations of zinc, cadmium, mercury, and lead completely prevent copper activation. Manganese(II), vanadate(V), iron(III), and ammonium ions (all 10^{-3}M) and aluminium (5×10^{-5}M) reduce the extent of activation by copper. As aluminium at this level has a slight activating effect, the actual reduction in copper activation may be greater than is apparent from the enzyme activity measured. Strontium, iron(II), nickel(II), and vanadium(IV) (all 10^{-3}M) and chromium(III) (3×10^{-4}M) in the presence of copper give activities in excess of those measured for copper alone. For nickel, chromium(III), and vanadium(IV), the activity is the sum of the activities expected for activation by copper and the other metal ion. This indicates a lack of effective competition between copper and these particular metal ions for enzyme sites. In the presence of strontium, however, and also of magnesium, the activity of the reconstituted enzyme is exactly that found in the absence of copper possibly indicating a complete exclusion of copper by these ions. Iron(II) shows intermediate behaviour in that the enzyme activity is greater than that expected for iron or copper alone, but is less than the sum of their activities, indicating some competition for enzyme sites. Cobalt(II) behaves uniquely. Although it alone does not activate the apoenzyme, it enhances activation by copper. The reason for this effect has not been established, but it could be that cobalt ions occupy non-active sites on the apoenzyme in preference to copper.

At the 2×10^{-6}M level, only silver ions have any effect on activation by 5×10^{-7}M copper(II) ions. They reduce the activity of the regenerated enzyme by 50%.

Oxidation of Catechol by Metal Ions.—It has been shown that traces of metal ions, including copper, catalyse the oxidation of catechol.[24] Therefore the abilities were tested of various metal ions to catalyse the oxidation of catechol in the absence of enzyme. At the concentrations used in the enzyme experiments, iron(II) (10^{-3}M) was the only metal that catalysed the oxidation of catechol to any extent. Appropriate corrections were made, therefore, in assays involving iron(II).

Enzyme Activity Retained on Insolubilisation of Polyphenol Oxidase.—A reduction in enzymic activity per unit of enzyme protein as a result of the insolubilisation of an enzyme is a widely observed phenomenon. However, the extent of the activity loss varies widely and depends on both the method of immobilisation and the nature of the enzyme itself. For example, Barker *et al.*,[18] who developed the insolubilisation method described in this paper, showed that α-amylase retained 6% of its activity when coupled to Enzacryl AA through a diazo-link, and 9·5% of its activity when coupled through an isothiocyanato-group to the same polymer (which couples the enzyme through its lysine residues). Another enzyme, β-amylase, retained only 1·5% of its activity when diazo-linked to Enzacryl AA, and even less (0·8%) when linked through the isothiocyanato-group.[18]

The immobilisation procedure used in the present study is considered a suitable one for this particular polyphenol oxidase enzyme as it is found that 25% of the soluble enzymic activity is retained when the enzyme is covalently linked to the carrier (Table 4). Amino-acid analysis indicated that linkage occurs predominantly to tyrosine residues. It is noteworthy, however, that after the extensive washing procedures, the control sample contains a considerable quantity of adsorbed protein, although this protein is relatively inactive. This inactivity is probably due to the vigorous stirring over a long period of time of the soluble enzyme with the carrier resulting in extensive denaturation of the enzyme, whereas diazo-linkage of the enzyme to the polymer presumably makes the enzyme less susceptible to this type of effect.

Prolonged storage of both the insoluble holoenzyme and the insoluble apoenzyme in Tris–maleate buffer solution,

TABLE 2

Reactivation of insoluble apopolyphenol oxidase by metal ions

Metal ion	Cu^{II}	Al^{III}	VO^{2+}	Co^{II}	Cr^{III}	Mg^{II}	Sr^{II}	Ni^{II}	Fe^{II}
Concentration (M)	5×10^{-7}	5×10^{-5}	2×10^{-4}	10^{-3}	3×10^{-4}	10^{-3}	10^{-3}	10^{-3}	10^{-3}
Relative activity	1·00	0·13	0·19	0·26	0·32	1·00	1·17	1·17	1·25

TABLE 3

Effect of other metal ions on reactivation of insoluble apopolyphenol oxidase by 5×10^{-7}M copper(II)

Metal ion		Sr^{II}	Mn^{II}	Al^{III}	Fe^{II}	VO_3^-	Fe^{III}	NH_4^+	Ni^{II}	Cr^{III}	VO^{2+}	Co^{II}
Concentration (M)		10^{-3}	10^{-3}	5×10^{-5}	10^{-3}	2×10^{-4}	10^{-3}	10^{-3}	10^{-3}	3×10^{-4}	10^{-3}	10^{-2}
Relative Activity	1·00	1·17	0·45	0·63	1·80	0·60	0·65	0·70	2·17	1·32	1·19	1·40

10^{-3}M-Ca^{II}, Ba^{II}, Mg^{II}, and 3×10^{-4}M Pb^{II} give relative activity = 1·00. 10^{-3}M-Zn^{II}, Cd^{II}, Hg^{II}, Ag^{I}, and 10^{-4}M Hg^{I} give relative activity = 0·0.

[24] K. Bhagvat and D. Richter, *Biochem. J.*, 1938, **32**, 1397.

pH 7·0 at 4 °C, for 3 months did not have any serious effects. The holoenzyme lost no activity over this period, whereas the apoenzyme could be reactivated by Cu^{II} to 81% of the maximum reactivation level of the freshly prepared apoenzyme. In addition, the holoenzyme and apoenzyme were unaffected by lyophilisation. After four days storage, the lyophilised apoenzyme could be reactivated by copper to its original activity.

TABLE 4

Enzyme activity retained on insolubilisation of polyphenol oxidase

	Bound or adsorbed protein (mg/100 mg polymer)	Enzyme units */ mg bound and adsorbed protein	% Activity retained on polymer
Diazo-linked enzyme	4·2	1 550	25·2
Enzyme-polymer mixture	1·4	165	2·7

* Each mg of soluble enzyme contained 6 150 enzyme units. 1 unit is that which takes up 1 nmol of O_2 per min at 25°.

Recovery and Reuse of the Insoluble Enzyme.—After incubation of the insoluble holoenzyme with catechol (20 µl of a 37·5 mM solution per ml of suspension) at 25 °C with continuous stirring in the presence of air, the insoluble enzyme

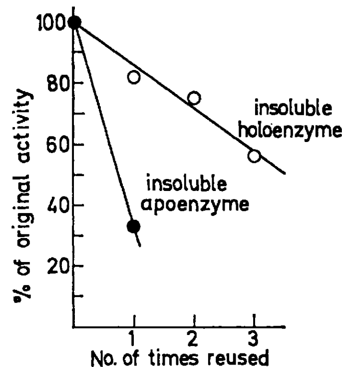


FIGURE 6 Variation of enzyme activity with reuse

derivative was collected by centrifugation, washed, resuspended in buffer, and aliquots taken for enzyme assay by the usual method. This procedure was repeated twice, and the activity retained after successive reuse compared to the original activity of the suspension, after correction for losses during washing. The results are shown in Figure 6. After reuse three times, the enzymic activity falls to almost half its initial value.

The possibility of reusing the apoenzyme after reactivating it with copper was also investigated. An excess of copper [2 µg of copper(II) per ml of suspension] was added to the apoenzyme preparation and stirred overnight at 4 °C. After incubating this reactivated enzyme with catechol, then washing it, as described above for the holoenzyme, the copper was removed by treatment with cyanide, and the apoenzyme washed, as described in the Experimental section. The percentage of original activity retained after the reactivation of this apoenzyme with excess copper was determined by the usual enzyme assay after correction for washing losses (Figure 6).

Clearly, the copper-containing insoluble enzyme can be used several times without too much loss of activity. However, the insoluble apoenzyme loses about two thirds of its activity after removal and readdition of the copper for a second time, thus limiting its useful lifespan. The susceptibility of the apoenzyme to activation was also reduced, since at non-saturating concentrations of copper, approximately twice as much was needed to produce the same amount of activation as with the unused apoenzyme.

DISCUSSION

Copper is by far the most powerful activator of the apoenzyme, and therefore, as expected, is probably bound strongly by the enzyme protein. Those other metal ions which can cause some reactivation of the apoenzyme (Al^{III}, VO^{2+}, Co^{II}, Cr^{III}, Mg^{II}, Ni^{II}, Sr^{II}, and Fe^{II}) must also be bound by the apoenzyme. In addition, the fact that sufficient concentrations of metal ions such as zinc, cadmium, mercury, and silver do not activate the apoenzyme yet completely prevent copper-activation, implies that these metals, too, compete with copper for the metal-binding site on the enzyme. A similar inhibition of the copper activation of a mammalian apotyrosinase in the presence of larger concentrations of mercury, silver, and gold,[11] has been explained in the same way.

Since silver is the only metal that exerts any effect on copper reactivation when present in low concentrations, this metal may be bound more strongly than any of the other metal ions except copper. This is also suggested by the fact that silver inhibits the holoenzyme, but only when incubated with it for several hours. Presumably a slow silver-copper exchange takes place when silver is present in sufficiently high concentrations (10^{-4}—10^{-3}M). Lower concentrations of silver have a measurable effect only when silver and copper compete directly for a free apoenzyme site.

Native polyphenol oxidase becomes inactive during the oxidation of its substrate.[25-29] Binding of the product, quinone, to the enzyme has been shown to account for at least part of this inhibition.[30] In the present study it was noted that on successive reuse of the insoluble holoenzyme and on reuse of the apoenzyme, the polymer particles became progressively darker brown. It is possible that this darkening could be the result of binding of the product, thus making some of the active sites of the enzyme inaccessible to the substrate. This effect could account partly for the reduction in activity observed on reuse of the holo- and apo-enzyme preparations. Some denaturation could also have occurred.

The fact that more copper is needed to produce the same degree of reactivation of the regenerated apoenzyme suggests that the copper is actually bound by many of the active sites of the enzyme, but a smaller proportion of these sites are catalytically active in the presence of

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copper than in the original apoenzyme preparation. Alternatively, denaturation of the enzyme may have exposed additional, but non-active binding sites for the copper, so that a greater copper concentration is required to regenerate the required degree of activity.

Analytical Applications.—The method developed here provides a highly selective means of detecting and determining copper at ultra-trace levels, since other metal ions only interfere with the determination when present in considerably higher concentrations than copper. The sensitivity of the method is very high, for as little as $6{\cdot}7 \times 10^{-9}$ g Cu^{II} per ml of sample can be determined with reasonable accuracy and precision. Moreover as this insoluble apoenzyme is easily collected from large volumes of sample by filtration or centrifugation, the method can be applied to the collection from and determination of copper in even more dilute solutions. Preliminary investigations here show that $2{\cdot}5 \times 10^{-11}$ g Cu^{II} per ml in a large volume of sample may easily be determined, and such sensitivity could well be increased.

The apoenzyme preparation is extremely stable both to lyophilisation and to prolonged storage in solution. As it has often been shown that covalent linkage of enzymes to insoluble matrices reduces their susceptibility to inactivation by denaturation,[31-34] this stability is probably at least partly the result of chemical bonding of the apoenzyme to the insoluble polymer. Preparation of the apoenzyme is also quicker and easier because of its insoluble nature.

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