composes more than 90% of the islets of obese–hyperglycaemic mice (Christophe, 1965). The striking stimulation of glucose on β-cell respiration is in line with the observations of Keen *et al.* (1965), who reported a considerably higher oxidation of [1-^{14}C]glucose by isolated rat islets when the glucose concentration of the medium was increased. Friz, Lazarow & Cooperstein (1960) failed, however, to obtain a stimulating effect of glucose on the respiration of islet-tissue slices from toadfish. These results may reflect a significant metabolic difference between pancreatic β-cells in mammals and fish.

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The Enzymic Oxidation of Thiols

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Several observations have been recorded of thiols being able to reduce the coloured products formed by the action of peroxidase and hydrogen peroxide on substrates such as guaiacol and *p*-phenylenediamine, and the early observations have been reviewed by Randall (1946). Glock (1944) observed that thiourea and thiouracil inhibited the action of peroxidase and hydrogen peroxide on pyrogallol. Randall (1946) studied the action of peroxidase and hydrogen peroxide on several thiols and came to the conclusion that thiols do not inhibit peroxidase but are reducing agents acting as substrates for the peroxidase system and competing with other substrates for the available peroxide–peroxidase complex. He also found that thiols can reduce the coloured dyes formed from *p*-aminobenzoic acid and benzidine and thus they show an apparent inhibition of colour production by the peroxidase system.

Neufeld, Green, Latterell & Weintraub (1958) described an enzyme, thiol oxidase, that catalysed the oxidation of thiophenol and sodium diethyldithiocarbamate to the disulphide form, and the reactions catalysed by this enzyme were represented thus:

$$2R\text{–}\underset{\displaystyle X}{\overset{\displaystyle \|}{C}}\text{–SH} + \tfrac{1}{2}O_2 \rightarrow R\text{–}\underset{\displaystyle X}{\overset{\displaystyle \|}{C}}\text{–S–S–}\underset{\displaystyle X}{\overset{\displaystyle \|}{C}}\text{–}R + H_2O$$

(where X could be carbon, oxygen, nitrogen or sulphur). The enzyme was purified extensively by Aurbach & Jakoby (1962) and found to contain 0·01% of copper. They reported that the enzyme also catalysed the oxidation of phenols, e.g. catechol and resorcinol. These phenols have also been shown to be oxidized by laccase (*p*-diphenolase) (Benfield, Bocks, Bromley & Brown, 1964).

The oxidation of thiols by a copper oxidase is particularly interesting, since substances such as sodium diethyldithiocarbamate have been used as inhibitors of tyrosinase (*o*-diphenolase), which also contains copper as its prosthetic group (Hallaway, 1959). I have now prepared the thiol oxidase from *Piricularia oryzae* according to the method of Aurbach & Jakoby (1962) and have confirmed their

results with thiophenol, sodium diethyldithiocarbamate, resorcinol and catechol. This enzyme also catalysed the oxidation of other phenols oxidized by laccase. Pyrogallol was oxidized to purpurogallin, 2,6-dimethoxyphenol to 3,3′,5,5′-tetramethoxydiphenoquinone and 2,6-dimethylphenol to 3,3′,5,5′-tetramethyldiphenoquinone. The reactions were carried out under conditions identical with those described in a study of the oxidation of these substrates by laccase from *Polyporus versicolor* and *Rhus vernicifera* and the products were similarly identified (Benfield *et al.* 1964).

Since thiol oxidase from *Piricularia oryzae* resembled laccase in its activity towards phenolic substrates, it was considered likely that laccase would oxidize thiols, e.g. thiophenol and diethyldithiocarbamate, and this has been found to be so with laccase from two sources: a fungal enzyme from *Polyporus versicolor* and a plant laccase from *Rhus vernicifera* were obtained and purified according to methods previously described (Fåhraeus & Lundgren, 1961; Benfield *et al.* 1964). The reactions were carried out on a scale large enough to permit the isolation and identification of the product in each case: 5·0m-moles of the substrate in 100ml. of the appropriate buffer were incubated with 1000units of the enzyme. One unit of laccase caused a change of extinction of 1/min. when 2,6-dimethoxyphenol was oxidized to 3,3′,5,5′-tetramethoxydiphenoquinone, which has $\lambda_{max.}$ at 468mμ. The increase of extinction at this wavelength was used as a measure of laccase activity.

When incubated with thiophenol both enzymes catalysed its oxidation to diphenyl disulphide. The reaction was followed by the spectrophotometric method of Aurbach & Jakoby (1962). A white precipitate formed within 10min. in the incubation mixtures containing active enzyme, but not in the controls that contained boiled enzyme. Reaction mixtures containing *Polyporus versicolor* laccase were buffered at pH4·0 (10mM-sodium acetate) and the *Rhus vernicifera* laccase at pH7·0 (10mM-sodium phosphate). The incubations were carried out at 20° for 120min.; the insoluble precipitate was filtered off and, after recrystallization from ethanol, had an ultraviolet spectrum identical with that of diphenyl disulphide prepared by chemical oxidation of thiophenol with hydrogen peroxide. Each product and a mixture of the two had m.p. 60–61°.

When diethyldithiocarbamate was used as substrate, both enzymes catalysed its oxidation to tetraethylthiuram disulphide. However, as diethyldithiocarbamate is unstable at pH values below 6·0, the reactions were carried out at pH6·0 with the *Polyporus versicolor* enzyme and at pH7·0 with the *Rhus vernicifera* enzyme; with each enzyme 10mM-sodium phosphate buffer was used. Here, too, a cloudy white precipitate formed after 10min. incubation at 20° in reaction mixtures containing active enzyme. After 180min. incubation, the insoluble white product was filtered off and recrystallized from ethanol. The product was identical with tetraethylthiuram disulphide prepared by oxidation of sodium diethyldithiocarbamate with potassium ferricyanide. The ultraviolet spectra of the two products were identical, and each product and a mixture of the two had m.p. 69–70°. It was concluded that both the aromatic compound thiophenol and sodium diethyldithiocarbamate, a non-aromatic compound containing thiol groups, were substrates of laccase from *Polyporus versicolor* and *Rhus vernicifera* or that a thiol oxidase was closely associated with both these enzymes and not separated by the procedures used for isolation.

The present results appear to support the findings of Aurbach & Jakoby (1962) for the *Piricularia oryzae* thiol oxidase, where the oxidation of thiols and phenols was catalysed by the same oxidase. They also reported a lack of activity towards thiophenol by a plant tyrosinase (*o*-diphenolase). This may be connected with the state of oxidation of copper in tyrosinase. Kertész (1957) has shown that copper is always present in the univalent state in purified mushroom tyrosinase, and similar results have been obtained for purified *Neurospora* tyrosinase (Fling, Horowitz & Heinnemann, 1963). Univalent copper forms very stable complexes with thiols, which could be very effective inhibitors of tyrosinase activity.

However, both *Polyporus versicolor* laccase and *Rhus vernicifera* laccase have been shown to contain copper in the bivalent state. Nakamura (1960), using electron-spin-resonance spectroscopy, has shown that all the copper in *Rhus vernicifera* laccase is present as Cu^{2+}, and by the same technique Malström, Mosbach & Vänngård (1959) have shown that copper exists partly as Cu^{+} and partly as Cu^{2+} and that a valency change (Cu^{2+} to Cu^{+}) occurs during the oxidation of the substrates. Hence the mechanism of oxidation catalysed by the blue laccase enzymes containing Cu^{2+} may be very different from that of the colourless tyrosinase enzymes containing Cu^{+}; any inhibition by thiols, of the oxidation of phenols or other substrates by laccase, may be complicated if thiols also act as substrates and as reducing agents for the coloured quinones produced during the oxidation of phenols and amines.

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Zinc as a Cofactor for Cephalosporinase from *Bacillus cereus* 569

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Crude preparations of β-lactamase from *Bacillus cereus* 569 show a selective loss of cephalosporinase activity on purification (Abraham & Newton, 1956) and during prolonged action on substrate (Sabath & Abraham, 1965). They also show a much greater loss of penicillinase (EC 3.5.2.6) activity than cephalosporinase activity on keeping at 60° (Crompton, Jago, Crawford, Newton & Abraham, 1962). During attempts to purify the crude preparation cephalosporinase activity was repeatedly lost although penicillinase activity was retained. It was therefore decided to investigate the possibility that a cofactor required only for cephalosporinase activity was removed during the purification procedures used.

Solutions of the crude enzyme from *B. cereus* 569 were prepared as described by Crompton *et al.* (1962) with cephalosporin C_A (pyridine) (10 μg./ml.) as inducer and an induction time of 3hr. β-Lactamase activities were measured manometrically (Henry & Housewright, 1947; Pollock, 1952) and related to μl. of CO_2/ml. of enzyme/hr., normally with substrate (cephalosporin C or benzylpenicillin) at a concentration of 2mg./ml.

Table 1 shows that some loss of cephalosporinase activity occurred when the crude enzyme was dialysed against running tap water, but that when EDTA (disodium salt) was added before dialysis (final concn. 0·27mM) the selective loss of cephalosporinase was much more striking. A similar extensive loss of cephalosporinase activity was observed when measurements were made with enzyme that had been treated with EDTA (at concentrations down to 2·7 μM) but not dialysed.

Cephalosporinase activity was restored to solutions of crude enzyme that had been subjected to treatment with EDTA and dialysis by the addition of $ZnSO_4$. When the latter was added in optimum amount (final concn. 1mM) the cephalosporinase activity rose to 4–5 times that of the original crude enzyme (Table 1). Addition of Zn^{2+} to the original crude enzyme, or to the dialysed enzyme, also produced a similar increase in cephalosporinase activity.

Several other metal-binding substances decreased the cephalosporinase activity of the crude enzyme to a relatively low level. After the addition of 1,10-phenanthroline (300 μM) the cephalosporinase activity was less than 10% of its original value and the penicillinase activity 95%. With 240 μM-quinalizarin (tetrahydroxyanthraquinone) the corresponding value for cephalosporinase activity was also less than 10%. After H_2S had been bubbled gently through the solution for about 7min. the values for cephalosporinase and penicillinase activities were less than 10% and 90% respectively. No significant loss of cephalosporinase activity was observed after treatment of the crude enzyme with ammonium aurin tricarboxylate (140 μM), titan yellow (160 μM), sodium azide (10mM) or 8-hydroxyquinoline (1mM). The use of 8-hydroxyquinoline (1mM) to terminate enzyme induction (Crompton *et al.* 1962) did not significantly affect the cephalosporinase activity of the crude enzyme obtained.

The logarithms of the equilibrium formation

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