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EFFECTS OF TITANIUM COMPOUNDS ON A D-GLUCOSE–D-GLUCOSE OXIDASE ASSAY SYSTEM

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ABSTRACT

Titanium compounds affect the measurement of D-glucose oxidase (and therefore D-glucose) by the D-glucose–D-glucose oxidase–peroxidase–2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) assay system. The validity of measurement of the activity of D-glucose oxidase immobilised on supports based on titanium oxides is affected by complexation of the intermediate hydrogen peroxide with the support, and such supports may prove to be unsuitable for the immobilisation of D-glucose oxidase. The formation of titanic peroxides is among the reasons discussed for the various interactions encountered. The use of the assay system for the determination of D-glucose oxidase contaminated with catalase and for the determination of hydrogen peroxide is also described.

INTRODUCTION

D-Glucose oxidase[1] (β-D-glucose: oxygen oxidoreductase, EC 1.1.3.4) contains two moles of flavin adenosine diphosphate (FAD) per mole[2]. The FAD molecules play an important role in the enzyme action, and the oxidative and reductive half-reactions (*A* and *B*) are independent of one another[3]. A detailed discussion of the mechanism of D-glucose oxidase action is available[4].

$$\text{Enz-FAD} + \text{D-Glucose} \rightleftharpoons \text{Enz-FADH}_2 + \text{D-Glucono-1,5-lactone} \qquad (A)$$

$$\text{Enz-FADH}_2 + \text{O}_2 \rightleftharpoons \text{Enz-FAD} + \text{H}_2\text{O}_2 \qquad (B)$$

When D-glucose oxidase is used in conjunction with peroxidase (donor: hydrogen-peroxide oxidoreductase, EC 1.11.1.7) and a suitable electron-acceptor, the hydrogen peroxide is reduced by the peroxidase with a corresponding oxidation of the electron acceptor. The oxidised acceptor, which is usually highly coloured, can then be determined spectrophotometrically. Such systems have therefore been used extensively for the determination of D-glucose, and since peroxidase is not very specific with respect to the electron acceptor[5], various modifications using different chromogens as acceptors, *e.g.*, *o*-dianisidine[6], *o*-toluidine[7], 2,6-dichloroindophenol[8],

and the diammonium salt of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid)[9] (ABTS, Fig. 1), have been reported. Although *o*-dianisidine had been considered to be the most suitable[10], ABTS is now claimed to be superior[9,11], the sensitivity being four-times greater; unlike *o*-dianisidine, ABTS is not carcinogenic. We have adapted the D-glucose oxidase/peroxidase/ABTS-based assay of D-glucose for the determination of D-glucose oxidase activity[12].

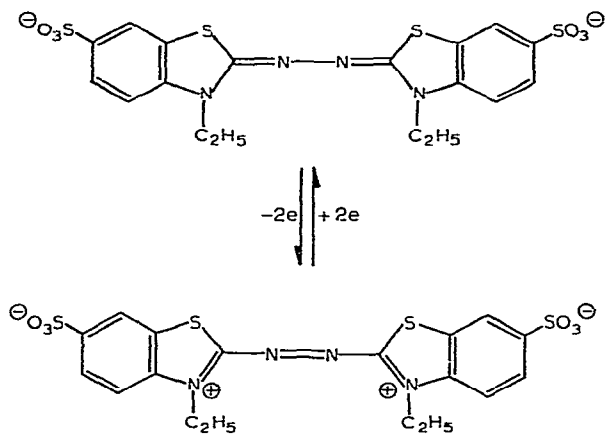

Fig. 1. Structure and redox reaction of ABTS.

In the course of our work on immobilised enzymes, we have produced active, immobilised forms of D-glucose oxidase by reaction with titanium chelates of alginic acid, chitin, and Celite[12]. This work, and the now considerable use of a number of matrices containing titanium (*e.g.*, titanium chelates of various materials[13–16], titania/titanium oxide[17–20], and titanium-hardened glasses) for enzyme immobilisation, prompted our investigation of the effects of titanium materials, both soluble and insoluble, on assays in which D-glucose oxidase, peroxidase, ABTS, and D-glucose are used in conjunction.

EXPERIMENTAL AND RESULTS

General assay system. — The general assay system employed for measurement of D-glucose oxidase activity was analogous to that described previously[12]. The assay reagent consisted of peroxidase (Boehringer, Grade I, 0.02 mg/ml) and ABTS (0.5 mg/ml) in 0.1M sodium acetate buffer (pH 5.0; 2.5 ml). This assay reagent was mixed with a solution of D-glucose (100 mg/ml) in the same buffer (0.5 ml), aqueous solutions (suspensions) of D-glucose oxidase (derivatives) (25 µl) were added, and incubation was effected at 37°. Aliquots were withdrawn at various intervals, and centrifuged as necessary, and absorbances read at 415 nm. The enzyme activity was calculated as previously described[12], ΔE/min being the gradient of the curves obtained by plotting absorbance against time.

The molar extinction coefficient (b) of oxidised ABTS was determined by addition of aqueous solutions of hydrogen peroxide (100 vol., 0.5 ml) to solutions of ABTS (0–0.08 mg/ml) in 0.1M sodium phosphate buffer (pH as required, 2.5 ml) containing peroxidase (0.02 mg/ml). The absorbances at 415 nm were plotted against ABTS concentration, and the value of b was calculated from the gradient.

Omission of peroxidase from the assay reagent caused complete inhibition of colour formation, even when incubation was protracted. Addition of aqueous sodium azide (0.1 mg/ml, 10 μl) to the assay reagent gave no colour after incubation for 1 h. Incubation of D-glucose solution plus assay reagent alone for 1 h gave an increase in absorbance of 0.04 (10 mm), and this value was raised to 0.25 when azide had been added. Inclusion of sodium azide in the complete assay system gave the corresponding increased value, which could be normalised by application of the appropriate blank value. The azide effect was shown to be due to interaction between D-glucose and azide.

Effect of metal ions on the D-*glucose oxidase assay system.* — Just prior to the addition of D-glucose oxidase to the reagents as above, one of the following was added: (*a*) solutions of titanous citrate (20, 40, 100 μl) prepared by adding 0.1M sodium citrate buffer (pH 5.0, 7.0 ml) to 15% (w/v) titanous chloride in hydrochloric acid (B.D.H.), adjusting to pH 5.0 with 0.9M ammonium hydroxide (2.3 ml), and diluting to 10 ml with water; (*b*) an aqueous suspension of hydrous titanous oxide (35 mg/ml, 20 μl); (*c*) a solution of titanic citrate prepared as for titanous citrate using 50% (w/v) titanic chloride in hydrochloric acid (Hopkin and Williams); (*d*) an aqueous suspension of hydrous titanic oxide (46 mg/ml, 20 μl); and (*e*) a freshly prepared, aqueous solution of ferrous sulphate (13.9 mg/ml, 20 μl).

From graphs of the variation of absorbance with time, the effect of the added substance was found by comparison with the curve obtained for the assay of D-glucose oxidase alone (Fig. 2). Linear curves passing through the origin, and with gradients close to that for the untreated enzyme were obtained for (*c*) and (*d*). The apparent D-glucose oxidase activities as a percentage of the standard were (*a*) 113, 110, and 116, respectively; (*b*) 75; (*c*) 95; (*d*) 106; (*e*) 0. When peroxidase was omitted from the assay reagent, none of materials (*a*)–(*e*) produced any colour in the assay system.

Effect of metal ions on the peroxidase–ABTS assay reagents. — Titanous citrate solution (prepared as described above, 20 μl) was added to the assay reagent (2.5 ml) and D-glucose solution (500 μl), and the absorbance was measured after various times at 37° (Fig. 3). The experiment was repeated using ferrous sulphate (13.9 mg/ml); there was no colour change.

Effect of metal ions on the detection of hydrogen peroxide by the peroxidase–ABTS system. — Various volumes (0–250 μl) of the solutions of ferrous sulphate, and of titanous and titanic citrates, and the suspension of hydrous titanous oxide, described above, were added (separately) to hydrogen peroxide solutions [100 vol.; diluted 2×10^3 (Fig. 4) or 4×10^3 (Fig. 5)], and the volumes made up to 0.5 ml with water. After 4 min, the hydrous titanous oxide was removed by centrifugation for 45 sec, and aliquot samples (20 μl) of the supernatant solution and other liquids were

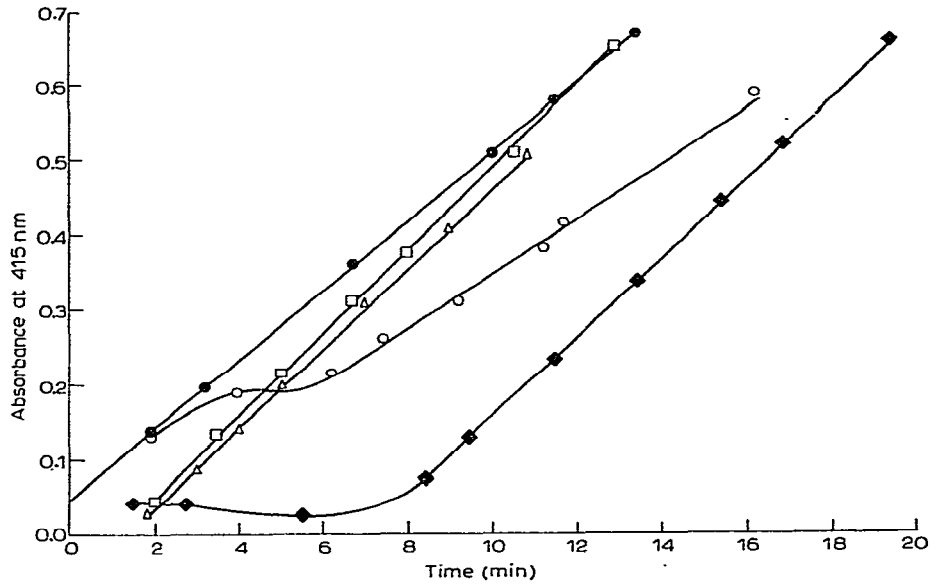

Fig. 2. Effect of titanous compounds on the D-glucose oxidase assay system. No additive, —●—; titanous citrate, 20 μl —□—; 40 μl —△—; 200 μl —◆—; hydrous titanous oxide, —○—; as per text.

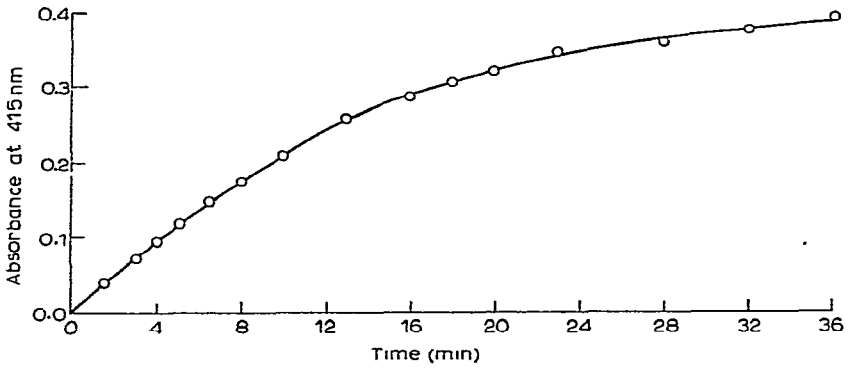

Fig. 3. Effect of titanous citrate on peroxidase–ABTS assay reagents plus D-glucose.

transferred to the assay reagent (2.5 ml) after a total of 5 min (unless otherwise stated). The effects of the additives were determined from plots of absorbance against volume of additive (Figs. 4 and 5).

Effect of hydrous titanic oxide and immobilised D-*glucose oxidase on the detection of hydrogen peroxide by the peroxidase–ABTS system.* — Aqueous suspensions of hydrous titanic oxide and of D-glucose oxidase (GOD III, Boehringer) immobilised in hydrous titanic oxide (active, and deactivated by storage for 1 h at 85°) (46 mg/ml,

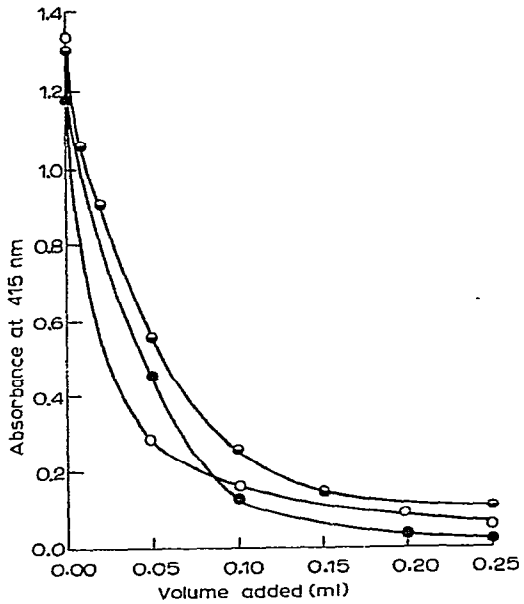

Fig. 4. Effect of metal ions on the detection of hydrogen peroxide by the peroxidase–ABTS assay system. Titanous citrate —●—; ferrous sulphate —○—; hydrous titanous oxide —◒—.

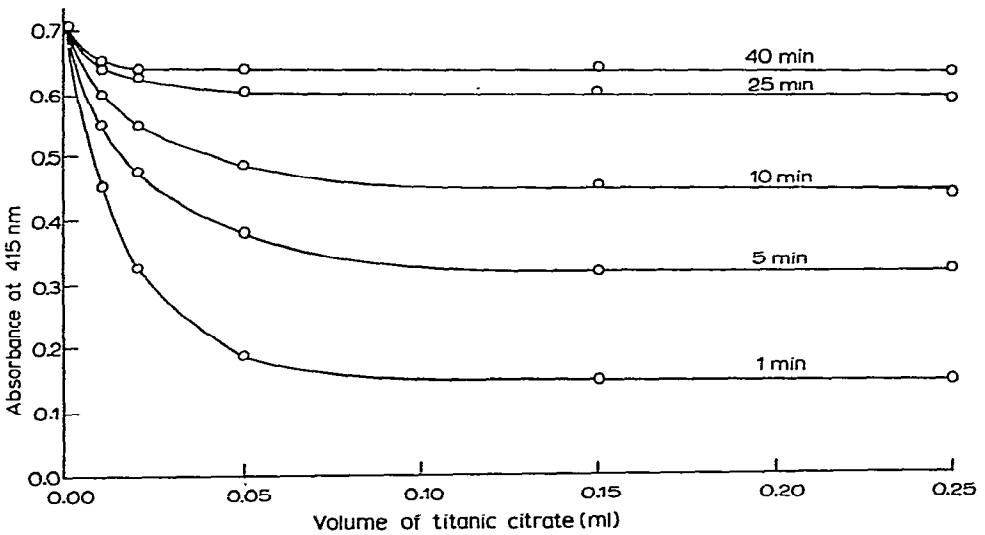

Fig. 5. Effect of time on the influence of titanic citrate on the detection of hydrogen peroxide by the peroxidase–ABTS assay system.

250 μl) were added to solutions of hydrogen peroxide (0–8mM, 250 μl). Generally after 5 min, aliquots (20 μl) were removed, before and after centrifugation, and assayed for peroxide as described above. Graphs of absorbance versus hydrogen peroxide concentration were all linear, passing approximately through the origin. Gradients were compared with those for untreated hydrogen peroxide solutions and solutions of D-glucose oxidase (GOD I and GOD III) in the presence and absence of sodium azide (0.01 mg/ml, 10 μl) added before the standing time (Table I).

DISCUSSION

Titanium citrates were selected for providing titanium ions in solution, as titanic citrate is one of the few forms of Ti(IV) known to be soluble at pH 5.0. Both titanic citrate and hydrous titanic oxide did not significantly affect the assay of D-glucose oxidase (Fig. 2). However, the presence of titanous ions (Fig. 2) caused a delay in the response to D-glucose oxidase, and the delay time before the linear increase of absorbance with time increased with the volume of titanous citrate added. This delay in the production of colour may be explained by postulating the reduction of hydrogen peroxide by titanous ions, which are in turn oxidised to Ti(IV). When all the titanous ions have been oxidised, the decomposition of hydrogen peroxide by peroxidase can proceed normally, and the curve becomes linear. Using ^{18}O-tracer methods[21], it has been deduced that the reduction of hydrogen peroxide by titanous ions proceeds *via* a one-electron transfer mechanism. The production of hydroxyl radicals has been demonstrated[22], and a free-radical breakdown of hydrogen peroxide (Equations *1* and *2*) was therefore suggested.

$$Ti^{3+} + H_2O_2 \rightarrow Ti^{4+} + OH^{\bullet} + OH^{-} \quad (1)$$

$$Ti^{3+} + OH^{\bullet} \rightarrow Ti^{4+} + OH^{-} \quad (2)$$

The effects of various amounts of titanous citrate and hydrous titanous oxide on hydrogen peroxide solutions (Fig. 4) show that the hydrogen peroxide concentrations, as measured by peroxidase–ABTS, decrease with increasing concentration of titanous citrate or hydrous titanous oxide. The effect is not linear. The fact that titanous citrate went yellow and then colourless immediately it was added to the hydrogen peroxide, and hydrous titanous oxide went white, confirmed that reduction of hydrogen peroxide was taking place.

The effect of titanous citrate on the assay reagent alone was investigated in the hope of explaining the curious hump which arose in the graph showing the effect of hydrous titanous oxide (and possibly a high concentration of titanous citrate) on the measurement of D-glucose oxidase (Fig. 2). Since no colour was formed in the presence of additive but absence of peroxidase, it is clear that the hump effect is caused by a reaction that takes place at a stage prior to peroxidase attack on hydrogen peroxide and therefore is not a result of a direct reaction between Ti^{3+} ions and ABTS, *i.e.*, there is no case for oxidation of the metal ion. Titanous citrate produces colour with the assay reagent plus D-glucose (Fig. 4), and this effect increases with time, levelling

off as the reaction proceeds. The humps can now be explained by superimposing this phenomenom on the delay effect already mentioned. Colour is at first produced by reaction of Ti^{3+} ions and peroxidase–ABTS alone, but when the Ti(III) is oxidised, the reaction proceeds normally. The linearity of the curve in the latter stages indicates that oxidised ABTS is not reduced by Ti^{3+} ions.

The production of colour by titanous ions and peroxidase–ABTS can be explained in two ways. (*i*) Titanous ions cause the reduction of the enzyme at the flavin moiety. The stoichiometry of the reduction reaction is the same as that of D-glucose oxidation[23], *i.e.*, the addition of two hydrogen atoms to each flavin group, and the reduced D-glucose oxidase is identical to that formed using D-glucose. The reduced enzyme is then available for the conversion of dissolved oxygen into hydrogen peroxide, which produces colour with peroxidase–ABTS as usual. When the titanous ions have all been oxidised, the colour production ceases. (*ii*) Titanous ions are readily oxidised by dissolved oxygen, according to Equations *3* and *4*. The accumulation of hydrogen peroxide as an intermediate is not great, as reaction *4* is much more rapid[24] than reaction *3*.

$$2Ti^{3+} + O_2 + 2H_2O \rightarrow 2TiO^{2+} + H_2O_2 + 2H^+ \quad (3)$$

$$2Ti^{3+} + H_2O_2 \rightarrow 2TiO^{2+} + 2H^+ \quad (4)$$

However, the large excess of peroxidase ensures a virtually instantaneous production of colour on addition of hydrogen peroxide to peroxidase–ABTS, so it is possible that some of the hydrogen peroxide formed in reaction *3* could be attacked by peroxidase in preference to undergoing reaction *4*. Again, colour production should cease when all the titanous ions has been oxidised. Attempts to distinguish between the two possibilities using ferrous sulphate in place of titanous citrate were unsuccessful; although ferrous sulphate had an effect similar to that of titanous citrate on hydrogen peroxide concentration (Fig. 4), the ferrous ions being oxidised by the hydrogen peroxide, ferrous sulphate completely abolished colour production in the D-glucose oxidase assay. Ferrous ions (peroxide absent) reduce the oxidised ABTS, thus reversing the production of the oxidised form. Thus, advantage could not be taken of the slower oxidation of ferrous ion by dissolved oxygen.

However, because of the problems associated with oxidation of the titanous state, the titanic form was given further attention. That Ti^{4+} ions do not oxidise ABTS was apparent. Titanium peroxide is known and has neither a skew nor a linear configuration, as indicated by the absence of the appropriate i.r. absorption[25], but the proposed ring structure[26] has a high degree of decreased overlap of the bonding orbitals of the O–O group, due to ring strain and repulsion of the lone pairs, thus accounting for the orange colour and thermal instability of titanium peroxide. In the effects of hydrous titanic oxide on the measurement of hydrogen peroxide by the peroxidase–ABTS system (Table I), a yellow colour therefore indicates the presence of a titanium peroxide complex, and the fact that the apparent concentration of hydrogen peroxide was lowered by only 5% suggests that either the complex was being attacked

TABLE I

EFFECT OF TITANIUM COMPOUNDS ON THE MEASUREMENT OF HYDROGEN PEROXIDE CONCENTRATION

Additive	*Duration (min)*	*Hydrogen peroxide detected (%)*	*Colour observed*
No additive	5	100	Bluish green (normal)
Hydrous TiO_2 (centrifuged prior to analysis)	5	84	Supernatant and solid, yellow
Hydrous TiO_2 (in suspension)	5	95	Suspension, yellow
Hydrous TiO_2 (centrifuged prior to analysis)	180	80	Supernatant and solid, yellow
D-Glucose oxidase derivative of hydrous TiO_2 (centrifuged prior to analysis)	5	13	Solid, slightly yellow
D-Glucose oxidase derivative of hydrous TiO_2 (centrifuged prior to analysis)	80	0	No colour
Deactivated D-glucose oxidase derivative of hydrous TiO_2 (centrifuged prior to analysis)	5	70	Supernatant and solid, yellow
D-Glucose oxidase (GOD III)	5	21	Bluish green
D-Glucose oxidase (GOD III) + sodium azide	5	97	Bluish green
D-Glucose oxidase (GOD I)	5	96	Bluish green
D-Glucose oxidase (GOD I) + sodium azide	5	98	Bluish green

by peroxidase almost as efficiently as hydrogen peroxide, or that only a small amount of the complex was formed. However, for the centrifuged solution, the apparent concentration of hydrogen peroxide was 16% less than the theoretical value, indicating that the complex was being formed in significant quantities, and that it was being adsorbed onto the surface of the hydrous titanic oxide. The apparent concentration of hydrogen peroxide was lowered only a further 4% after storage at room temperature for 3 h, probably because the entire surface of the oxide was covered after a few minutes.

Peroxy titanium complexes of dicarboxylic acids have a larger, less-strained ring that incorporates the acid, and hence are more stable[27]. It is therefore acceptable that titanic citrate forms a similar complex with hydrogen peroxide, and this proved to be the case (Fig. 5). This peroxy titanic citrate affected the measurement of hydrogen peroxide and, in relation to the determination in a D-glucose oxidase or D-glucose assay, caused a slight decrease ($\sim$5%) in the value obtained; again the utilisation of the peroxide as it is released is rapid. When the time interval after addition of the complex was one minute, the curve of apparent concentration of hydrogen peroxide versus volume of titanic citrate added (Fig. 5) resembles that found with titanous citrate (Fig. 3). However, unlike the titanous citrate system, the absorbance increased

with time until, after 40 min, the apparent concentration of hydrogen peroxide was 72% of the theoretical value for all concentrations of titanic citrate. This can be explained in two ways. (*i*) The complex is attacked directly by peroxidase, but at a lower rate than hydrogen peroxide. If this is so, then the rate of attack on titanium peroxide should be intermediate, as its structure is much closer to that of hydrogen peroxide than is that of peroxy titanium complexes of carboxylic acids[27]. Peroxidase is highly specific for hydrogen peroxide, but it also attacks dimethyl and diethyl peroxides very slowly[4]. It is conceivable, therefore, that it could attack peroxy complexes of titanium. (*ii*) The complex is first decomposed to hydrogen peroxide, which is then attacked by peroxidase. Such a decomposition would have to be brought about by sodium phosphate buffer, ABTS, or some non-functional part of the protein of peroxidase, since it is known that titanium peroxide is stable for at least 3 h (Table I) and that peroxy titanium citrate is more stable than titanium peroxide, so that decomposition cannot be assumed to be spontaneous. As in (*i*), the rate of reaction of titanium peroxide should be greater than that of peroxy titanium citrate. Whichever explanation is correct, the low rate of attack of peroxidase accounts for the slight decrease in apparent activity of D-glucose oxidase in the presence of titanium citrate.

When D-glucose oxidase derivatives of hydrous titanic oxide were added to hydrogen peroxide, the apparent concentration of the latter was decreased dramatically (Table I). After 3 h, all the hydrogen peroxide had disappeared, as had the slight yellow colour which appeared after 5 min. D-Glucose oxidase that had been deactivated by heating at 85° for 1 h did not have this effect on the hydrogen peroxide, and caused only 30% lowering of the peroxide concentration and the production of a yellow colour, as would be expected from untreated, hydrous titanic oxide. This result shows that the effect of D-glucose oxidase on the hydrogen peroxide is a consequence of enzymic activity and not merely of the presence of protein. However, the activity was attributed to the presence of catalase (hydrogen peroxide: hydrogen peroxide oxidoreductase EC 1.11.1.6), and this was demonstrated by use of a catalase inhibitor, sodium azide, or of a catalase-free grade of D-glucose oxidase (GOD I). The catalase reaction did not affect the assay of D-glucose oxidase, presumably because the rate of peroxidase attack on hydrogen peroxide is much higher than that of catalase, even in the presence of titanic citrate.

Additional experiments in our laboratory, in which D-glucose was continuously passed through a column of D-glucose oxidase having effectively zero catalase activity and immobilised on hydrous titanic oxide, showed that only 10% of the peroxide formed was eluted from the column. It must therefore be concluded that matrices based on titanium oxide may prove to be unsuitable for the immobilisation of D-glucose oxidase.

In view of the clinical importance attached to D-glucose levels in blood or serum, D-glucose oxidase is used extensively in clinical chemistry. With the increasing demand for simplified tests using the immobilised enzyme, the advent of a number of titanium-based matrices for enzyme immobilisation, and the use of titanium-hardening to

improve the characteristics of glass, it is important to recognise that titanium compounds may affect the value recorded for the measurement of D-glucose. It is therefore clear that care must be exercised in the use of D-glucose oxidase where there is a possibility of forming a complex titanium peroxide. Furthermore, effects that might not be apparent from controls performed at the original assay of a preparation of immobilised enzyme may appear as the titanium matrix is very gradually solubilised by its fluid environment.

This work also confirms that the peroxidase–ABTS system can be used to measure hydrogen peroxide and serves to demonstrate that, where it is necessary to avoid use of the most highly purified form of D-glucose oxidase on account of its high cost, a catalase contaminant is acceptable, as the inhibitor, sodium azide, can be used in the presence of D-glucose, D-glucose oxidase, hydrogen peroxide, peroxidase, and ABTS without interrupting their function.

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