Hydrous Titanium Oxides—New Supports for the Simple Immobilisation of Enzymes

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Hydrous titanium oxides have been shown to be suitable as matrices on which enzymes may be immobilised with retention of enzymic activity. Various routes to the precipitation of the hydrous oxide have been evaluated in terms of the ability of the product to couple D-glucose oxidase with retention of activity. On account of the ease with which hydrous titanium(III) oxide is oxidised by atmospheric oxygen and other associated practical difficulties, hydrous titanium(IV) oxide was deemed preferable, and the optimum conditions of pH, time, and enzyme concentration in the coupling reaction were determined. Freeze drying of the immobilised enzyme was achieved with 74% retention of activity when sorbitol was included. The enzyme derivative was stable under conventional conditions for enzyme activity, and complete removal of bound protein could be achieved by using sodium carbonate–hydroxide solution. The ligand replacement system by which the enzyme is considered to be immobilised and associated factors are discussed in the light of the chemical and physical structures of hydrous titanium(IV) oxide. This support has the advantage over other matrices in that it can be easily, simply, and quickly produced *in situ*.

THE popularity of the derivatisation of enzymes to give active immobilised forms is well known, but most of the reactions employed are laborious and cumbersome [1,2] and the matrix in activated form may or may not be stable. We have attempted to circumvent such problems by using a chelation technique in which a matrix can be quickly activated for enzyme attachment by treatment with simple titanium compounds. Thus treatment of cellulose and nylon,[3,4] glass,[3–5] aliginic acid, chitin and Celite,[6] and poly-(4- and 5-acrylamidosalicylic acids) [7] with titanium chlorides gave derivatives to which enzymes could be attached as ligands replacing pre-existent ligands on the titanium atoms chelated to the support. We now report a new technique in which the support is formed as required virtually in the presence of the enzyme to be immobilised. The only requirement besides the enzyme is one reagent, which is commercially available and of adequate stability.

EXPERIMENTAL AND RESULTS

Assay for D-*Glucose Oxidase Activity*.—The general assay system employed was analogous to that described previously.[6,8] The assay reagent consisted of peroxidase (Boehringer, grade 1; 0.02 mg ml^{-1}) and diammonium 2,2′-azinobis-(3-ethyl-2,3-dihydrobenzothiazole-6-sulphonate) (ABTS) (0.5 mg ml^{-1}) in 0.1M-sodium phosphate buffer, pH 5.0. This reagent (2.5 ml) was mixed with a solution of D-glucose (100 mg ml^{-1}) 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 °C. Aliquot portions were withdrawn at intervals and centrifuged as necessary, and absorbances were read at 415 nm. The enzyme activity was calculated as before,[6] $\Delta E/(t/\text{min})$ being the gradient of the curve for optical density against time. The molar extinction coefficient (b) of oxidised ABTS was determined as before.[8] Where necessary, account was taken of the delay period which can occur when measuring the enzyme in the presence of titanium compounds, as observed and described previously,[8] the linear parts of the response–time curves being used for calculations.

Assay for Protein.—Protein was determined according to the method of Lowry *et al*.[9] Reagents consisted of (D)

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[2] J. F. Kennedy, in Chem. Soc. Specialist Periodical Report, Carbohydrate Chemistry, Part II, Macromolecules, vols. 4—7, 1971—1974.
[3] S. A. Barker, A. N. Emery, and J. M. Novais, *Process Biochem.*, 1971, **6**(**10**), 11.
[4] A. N. Emery, J. S. Hough, J. M. Novais, and T. P. Lyons, *Chem. Eng.*, 1972, 71.
[5] J. F. Kennedy and P. M. Watts, *Carbohydrate Res.*, 1974, **32**, 155.
[6] J. F. Kennedy and C. E. Doyle, *Carbohydrate Res.*, 1973, **28**, 89.
[7] J. F. Kennedy and J. Epton, *Carbohydrate Res.*, 1973, **27**, 11.
[8] J. F. Kennedy and I. M. Kay, *Carbohydrate Res.*, 1975, **44**, 291.
[9] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, 1951, **193**, 265.

2.9% w/v sodium carbonate in 0.3N-sodium hydroxide, (E) freshly prepared 1% w/v sodium potassium tartrate and 0.5% w/v copper sulphate in water, and (F) Folin-Ciocalteau phenol reagent (B.D.H. Ltd.) diluted with an equal volume of water. Protein samples in (D) (1 ml) were added to mixtures of (D) and (E) (25 : 1; 2.5 ml) and after 10 min (E) (1 ml) was added. Absorbances were read at 720 nm after a further 30 min, and concentrations of the unknowns were derived from a linear calibration curve obtained for standard solutions of D-glucose oxidase (0—250 μg ml^{-1}). The calibration curves of the initial standard protein solutions did not change with time at 25 °C or on heating to 70 °C for 1 h.

Co-precipitation of D-*Glucose Oxidase with Hydrous Titanium*(IV) *Oxide*.—Six methods were used for the precipitation at 25 °C of hydrous titanium(IV) oxide with D-glucose oxidase. In each method an aqueous solution of D-glucose oxidase (GOD 111, Boehringer; 20 units mg^{-1}; 2.8 mg ml^{-1}; 1.5 ml) (A), a 50% w/v solution of titanium(IV) chloride in 6N-hydrochloric acid (0.5 ml) (B), and 0.9N-ammonium hydroxide (7.1 ml) (C) were used; the final pH of each mixture was 5.0. All mixtures were stirred during the precipitations. (1) (C) was added to (B) until pH 0.4 was reached. This pH is the maximum attainable without precipitation of hydrous titanium(IV) oxide. (A) was added and immediately followed by the rest of (C) so that the enzyme was exposed to pH 0.4 for a minimum period but was present during the precipitation process. (2) (C) was added to (B) until pH 0.4 was reached and the mixture and (A) were added simultaneously to the rest of (C). The addition was made as quickly as possible to minimise the exposure of the enzyme to the high pH of (C). (3) (A) was added to (C) followed immediately by (B). This method was similar to method (2) expect that all the enzyme was present during the entire precipitation and was exposed to a pH of 14, whereas in method (2) fresh enzyme was being added right up to the end of the precipitation process and was not exposed to such an extreme pH. (4) (B) and (C) were added simultaneously to (A) and at such a rate that the ratio of (B) to (C) in the mixture was constant throughout, the two additions starting and finishing together, thus ensuring a constant pH of 5.0. (5) (C) was added to (B) and then (A), was added, thus ensuring that the enzyme, although not present during precipitation, was only exposed to a pH of 5.0. (6) (C) was added to (B) and the precipitate was washed with 0.1M-sodium phosphate buffer (pH 5.0; 2 × 10 ml). (A) was then added.

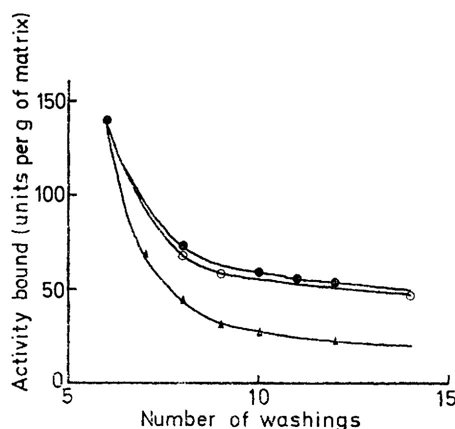


FIGURE 1 Effect on enzymic activity of D-glucose oxidase derivative of hydrous titanium(IV) oxide of washing with: 0.1M- sodium phosphate buffer pH 5.0 (—●—), 0.1M- sodium phosphate buffer pH 7.0 (—○—), 1.66% w/v D-glucose in 0.1M- sodium phosphate buffer pH 5.0 (—▲—)

After completion of additions, mixtures were stirred at 4 °C for 18 h, and the solids were removed by centrifugation and washed with 0.1M-sodium phosphate buffer (pH 5.0; 12 × 10 ml). This washing procedure yielded a product, further washing of which gave little further reduction in enzymic activity (Figure 1), thus suggesting that loosely adsorbed enzyme had been removed and that only tightly bound material remained. Analogous washing procedures with 0.1M-sodium phosphate buffer (pH 7.0) and 1.66% w/v D-glucose in 0.1M-sodium phosphate buffer (pH 5.0) were not completely effective in removing loosely adsorbed enzyme. The supernatant liquids and the washing were pooled and, along with the solids, assayed for D-glucose oxidase activity as above (Table 1). A hydrous titanium(IV) oxide blank was made with water (1.5 ml) instead of (A) by method (5). A portion of this blank was washed with water (12 × 10 ml) and used for dry weight determination.

TABLE 1

Activities of D-glucose oxidase derivatives of hydrous titanium oxides

Method of preparation [a]	Retention of enzymic activity in supernatant and washings (%)	Enzymic activity of immobilised enzyme and support (units g^{-1})
Ti^{IV}		
1	1	3.9
2	4	49.5
3	12	127
4	4	14.7
5	29	380
6	53	46.7
Ti^{III}		
1	0	0
2	13	94
3	11	92
4	3	40
5	37	205
6	39	118

[a] See text.

Co-precipitation of D-*Glucose Oxidase with Hydrous Titanium*(III) *Oxide*.—The preceding experiment was repeated but with solutions (B′) [15% w/v titanium(III) chloride in 6N hydrochloric acid] and (C′) [0.9N-ammonium hydroxide (2.55 ml)]. The pH to which (B′) was raised before precipitation occurred was 2.5. All manipulations and activity measurements were carried out under nitrogen to minimise oxidation of the hydrous titanium(III) oxide (dark blue) to the titanium(IV) form (white) (Table 1).

Optimisation of Conditions for Coupling of D-*Glucose Oxidase to Hydrous Titanium*(IV) *Oxide*.—D-Glucose oxidase was coupled to the oxide by method (5) and the effects of the pH and time of the reaction and the enzyme: oxide ratio were studied.

(a) *Variation of pH*. (C) was added slowly to (B) until the desired pH (3—9) was obtained. Aqueous D-glucose oxidase (GOD 1, Boehringer; 210 units mg^{-1}; 10 mg ml^{-1};

1.5 ml) was added and the mixture was diluted to 10 ml with water. The mixtures were stirred at 4 °C for 18 h, washed with 0.1M-sodium phosphate buffer (pH 5.0; 21 × 10 ml),

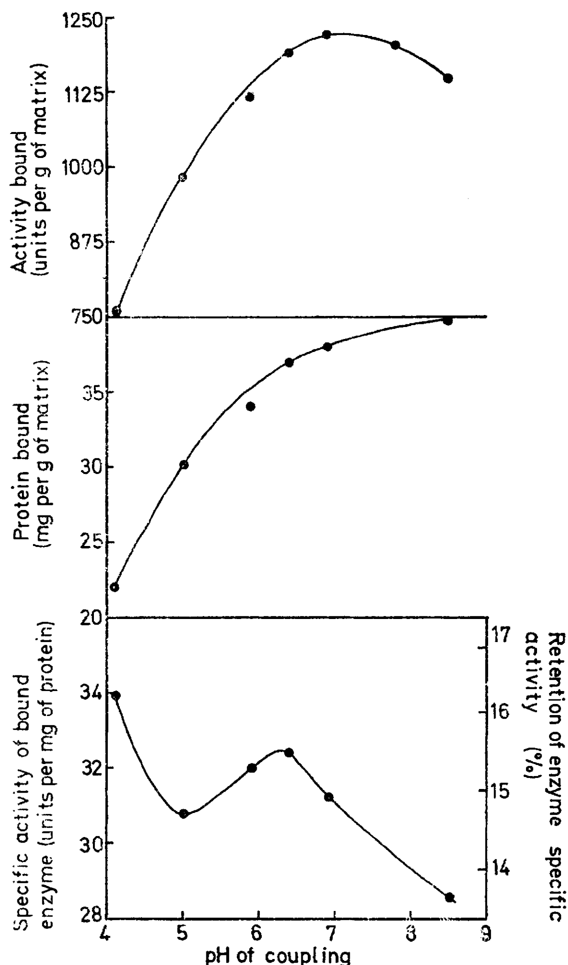


FIGURE 2 Effect of variation of pH on the coupling of D-glucose oxidase to hydrous titanium(IV) oxide

and assayed for enzyme activity as above and for protein content as above by using the optimum conditions defined below (Figure 2).

(b) *Variation of duration of reaction.* Eight mixtures were prepared as in (a) at pH 5.0. After the dilution stage the samples were stirred at 4 °C for various times (0—8 h) before proceeding with washing and assaying as in (a) (Figure 3).

(c) *Variation of enzyme:titanium ratio.* Eight mixtures were prepared as in (a) at pH 5.0 but with various enzyme concentrations (0—20 mg ml^{-1}). The mixtures were stirred, washed, and assayed as in (a) (Figure 4).

Freeze-drying of D-Glucose Oxidase Derivatives of Hydrous Titanium(IV) *Oxide.*—Samples of D-glucose oxidase derivative of hydrous titanium(IV) oxide prepared according to method (5) under optimum conditions were finally washed with water (12 × 10 ml), in place of buffer, and suspended in water or in 20% w/v sorbitol (10 ml). After freeze-drying samples were washed with water (3 × 10 ml) and assayed for activity. Freeze-drying in the presence and in the absence of sorbitol permitted 74 and 17% retention of activity, respectively.

Elution of D-Glucose Oxidase from D-Glucose Oxidase Derivative of Hydrous Titanium(IV) *Oxide.*—Samples of D-glucose oxidase derivative of hydrous titanium(IV) oxide

TABLE 2

Elution of D-glucose oxidase from D-glucose oxidase derivative of hydrous titanium(IV) oxide

Eluant	Contact time	Contact temp. (°C)	Protein eluted (% of load)
H_2O	3 × 5 min	20	<6
Na_2CO_3–NaOH	3 × 5 min	20	<10
$NaHCO_3$	3 × 5 min	20	<5
Na_2CO_3–NaOH	1 × 24 h	20	62.5
Na_2CO_3–NaOH	1 × 60 min	70	97.5

prepared according to method (5) under optimum conditions were further washed with water (3 × 10 ml) and suspended in water (5 ml). Samples (1 ml) were treated and stirred with 3.5% w/v sodium carbonate in 0.36N-sodium hydroxide or with 4.5 w/v sodium hydrogen carbonate solution (5 ml)

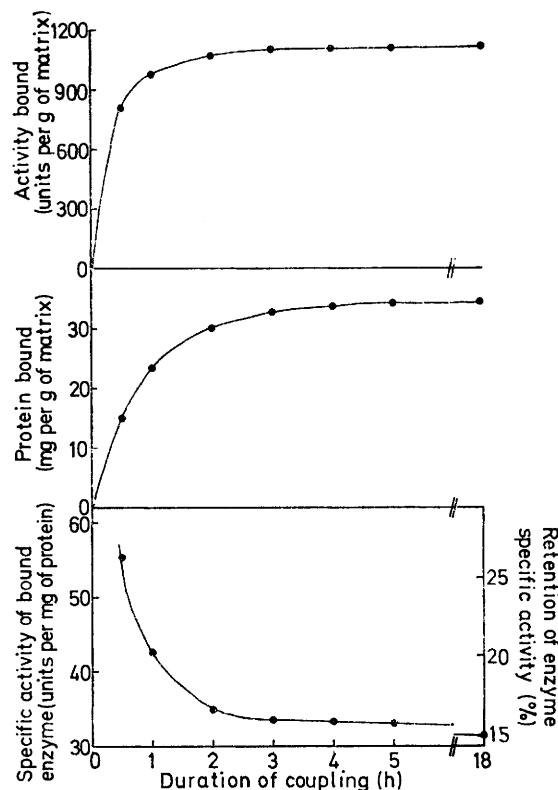


FIGURE 3 Effect of variation of the duration of coupling on the coupling of D-glucose oxidase to hydrous titanium(IV) oxide

at 25 or 70 °C for various times. After centrifugation, the supernatants were diluted and treated with 1.15N-sodium hydroxide as necessary to render them suitable for assay for protein contents (Table 2).

DISCUSSION

The gelatinous nature of freshly precipitated titanium(IV) oxide was first noted in the last century and later the first investigation [10] of the surface properties of active titanium(IV) oxide gels made by hydrolysing titanium salt solutions was reported. More recently, i.r. spectoscopy has detected Ti–OH bonds in the precipitate obtained from titanium(IV) chloride solutions with ammonia and dried over phosphorus pentaoxide,[11] and the formula $TiO(OH)_2$ was assigned to the precipitate on the basis of gravimetric analysis by calcination to titanium(IV) oxide. A more comprehensive study [12] of the fresh precipitate included determination of the hydroxy-groups by displacement with fluoride, acid titration of the released hydroxide ions, use of ^1H n.m.r. and i.r. spectoscopy to determine the state of the hydrogen ions in the precipitate, and determination of titanium in the precipitate by calcination and gravimetric analysis and of titanium in solution by reaction with hydrogen peroxide and spectrophotometry. From the observed changes which took place in the $TiCl_4$–NH_4OH–H_2O system with changes in the ammonia: titanium ratio (n) it was found that at n = 2—3.75 the system gave gelatinous, unfilterable precipitates, but when n was increased to 4 titanium was precipitated quantitatively as a white amorphous substance. At this point there was an inflection in the pH curve, a minimum in the electrical conductivity curve, and a maximum in the apparent volume of the precipitate, indicating that $n = 4$ represents the stoicheiometric quantities for the reaction. The precipitates contained very small quantities of chloride ions and much larger quantities of ammonia but the ratios Cl^- : Ti^{4+} and NH_4^+ : Ti^{4+} were not constant, and changes in these ratios did not show any regular features. The ratios also decreased sharply after the precipitates had been kept in air or washed with water, indicating that these ions were physically adsorbed on the precipitate surface. The OH^- : Ti^{4+} ratio, on the other hand, remained fairly constant for 5 days at a value around 2, when the precipitate was kept under the mother liquor. When the precipitate was aged in air, the OH^- : Ti^{4+} ratio had dropped to unity after 15 days, and remained at that value for 4 months. ^1H N.m.r. studies on the air- and heat-dried (190 °C) precipitates showed a value of *ca.* 1 for the ratio of OH^- to Ti^{4+}. The air-dried precipitates were amorphous, as shown by an *X*-ray study, and a polymeric structure was indicated by a broad i.r. absorption at 420—1 200 cm^{-1} and the absence of Ti=O absorption at 1 087 cm^{-1}. The i.r. spectrum also confirmed the presence of hydroxy-groups and molecular water.

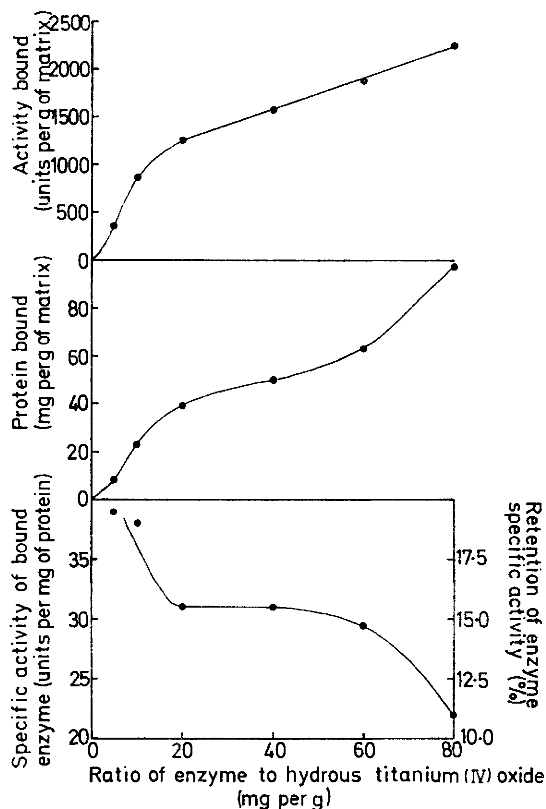


FIGURE 4 Effect of variation of the enzyme: oxide ratio on the coupling of D-glucose oxidase to hydrous titanium(IV) oxide

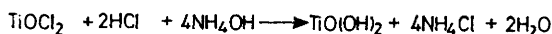
The structure proposed on the basis of these data is shown in the Scheme along with the equations for the precipitation reaction. No hydroxide [$Ti(OH)_4$] was detected; the species $TiO(OH)_2$ has been shown theoretically to be much more thermodynamically stable.[13] On the basis of this structure, it appeared that enzymes, *etc.* could be immobilised on the hydroxide by their acting as a ligand in the titanium complex, possibly replacing one of the hydroxy-groups as described [14] for chelation of enzymes, *etc.* However, although the freshly prepared precipitate was amorphous, after ageing at room temperature, a small amount of anatase is detectable.[11] Thus the possibility arises that the enzyme could become unbound when any such rearrangement occurs.

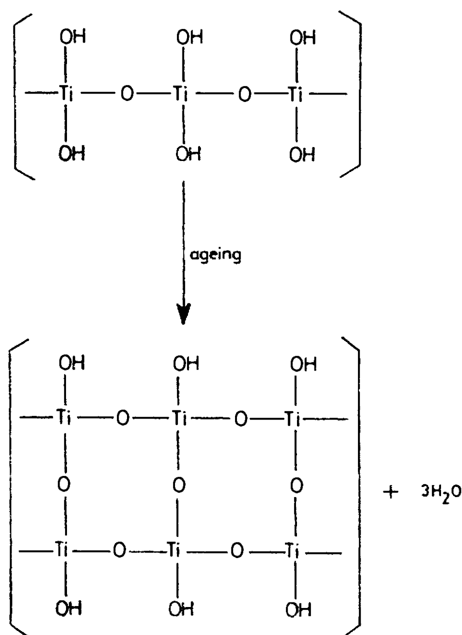
Hydrous titanium(IV) oxide was therefore produced directly by hydrolysis of titanium(IV) chloride. On the basis of experiments with attachment of enzyme by chelation to a titanium(IV) complex of polyacrylamide, in which D-glucose oxidase was coupled over a range of pH values, the optimum pH for the coupling was found to be *ca.* 5.0 and this was used for the preliminary experiments with hydrous titanium(IV) oxide.

In the precipitation of hydrous titanium(IV) oxide from the acidic solution of the chloride by ammonia a pH of 5.0 was obtained with a value of $n = 4.85$; however, this was based on a concentration of titanium(IV) chloride of 50% w/v, which is only an approximate value.

[10] F. Bischoff and H. Adkins, *J. Amer. Chem. Soc.*, 1925, **47**, 807.
[11] G. V. Jere and C. C. Patel, *J. Sci. Ind. Res.*, India, 1961, **20B**, 292.
[12] T. F. Limar, A. I. Savos'kina, V. I. Andreeva, and V. V. Mank, *Russ. J. Inorg. Chem.*, 1969, **14**, 1213.
[13] Y. Y. Bobyrenko, *Russ. J. Inorg. Chem.*, 1967, **12**, 931.
[14] J. F. Kennedy, S. A. Barker, and A. Zamir, *Antimicrobial Agents Chemother.*, 1974, **6**, 777.

The kinetics of coagulation of hydrous titanium(IV) oxide sols has been studied;[15] the type of electrolyte, the presence of impurities, and the pH of the medium all affect the coagulation type. The concentration required for the coagulation of hydrous titanium(IV) oxide sols is proportional to the salt concentration.[16] The conditions used in the present work yielded material the consistency of which appeared adequate. It was considered that precipitation of the hydrous oxide in the presence of enzyme might yield a more active product than adding the enzyme afterwards, owing to the higher surface area of the growing particles. Also, a one step insolubilisation process would be more desirable from an economic point of view. Four co-precipitation methods [(1)—(4)] were used, the routes being chosen to include means of minimising the risks of deactivation of the enzyme through exposure to extreme pH values. Two other methods, in which the enzyme was added immediately





SCHEME

after precipitation or after washing the precipitate with buffer, were used [methods (5) and (6), respectively].

Despite the attempts to prevent deactivation, none of the supernatant liquids from the co-precipitations showed more than 12% retention of enzymic activity (Table 1). The bound activities of the solids reflected to a certain extent the activities remaining in the supernatant liquids, indicating that the main factor affecting the efficiency of coupling was the amount of deactivation of enzyme resulting from exposure to extreme pH values, as opposed to the particular co-precipitation method used. With the other two methods (prior precipitation) deactivation was less, as expected, and that which did take place was probably caused by agitation of the enzyme during stirring. One would therefore expect much higher bound activities, and although this is the case for method (5), the bound activity obtained with method (6) is strikingly low. It is possible that this is related to the effect of phosphate ions on the surface structure of hydrous titanium(IV) oxide.

A parallel series of experiments were performed with hydrous titanium(III) oxide (Table 1) but when the enzyme assays were performed colour production was not linear with time, and only became so after a considerable delay period. But according to previous work[8] it could be safely assumed that the linear section of the curves represented the enzymic activity of the samples, and the bound activities resulting from the six methods were determined (Table 1). There is a striking similarity between these results and those obtained with hydrous titanium(IV) oxide.

The bound activities reflected the activity remaining in the supernatant liquids when co-precipitation was used [methods (1)—(4)], and none of these activities was very high. The highest activities in the supernatant liquids were obtained when enzyme was added after precipitation and this led to the highest bound activities, although when the precipitate was washed the bound activity was abnormally low. The activities generally were, however, lower than with hydrous titanium(IV) oxide. In view of this and the problems of oxidation of the hydrous titanium(III) oxide, and the complications arising as a result of oxidation caused by hydrogen peroxide, the hydrous titanium(IV) oxide was chosen as the better support material.

The effectiveness of the washing procedure used for enzyme coupling to hydrous titanium(IV) oxide was tested by performing a further series of washings at pH 5.0 and 7.0 and with D-glucose solution and assaying the residual bound activity (Figure 1). The results showed that enzyme which was not physically adsorbed was also removed at pH 7.0, and that D-glucose does not remove enzyme any more than buffer alone. Since the graphs level off after a number of washings, in subsequent experiments a total of twelve washings at pH 5.0 was employed.

In order to obtain the highest bound activities possible it was necessary to optimise the immobilisation process with respect to some of the more critical parameters, *viz.* duration of coupling, pH of coupling, and enzyme: oxide ratio. Only one of these was varied at a time the other two being kept constant under the standard conditions. The samples were assayed for bound activity and for bound protein by an automated ninhydrin method but the blank values from the protein determinations were too high for reliable results to be obtained.

[15] S. Bandyopadhyay, *J. Indian Chem. Soc.*, 1963, **40**, 173.
[16] V. K. Srivastava and R. S. Rai, *Kolloid-Z.*, 1963, **190**, 138.

The most likely explanation for this is that ammonia which had been adsorbed on to the hydrous oxide surface during coupling was interfering with the assay. The ammonia contents of such precipitates are reported [17] to be small (0.11—0.17%) but such levels are sufficient to cause a problem in the present case. To overcome this problem it was necessary to use an assay method which did not respond to ammonia, and the widely used Lowry method [9] was considered suitable. Although this method does not measure absolute protein concentrations, the use of soluble enzyme standards prevented any major inaccuracies, and that the elution of enzyme from the matrix could be studied at the same time was an added advantage.

In an attempt to elute enzyme from the matrix for this protein estimation, various washing procedures were devised so that the final supernatant liquid would have the right concentrations of sodium hydroxide and sodium carbonate. The supernatant liquid and water washings from the preparation of the immobilised enzyme were assayed in the same way. At room temperature and for short treatment times sodium hydrogen carbonate and sodium carbonate–sodium hydroxide were ineffective, but the latter agent released more protein when applied for a longer period. However none of the methods used at room temperature removed the enzyme quantitatively, but, within the limits of experimental precision, treatment with sodium carbonate–hydroxide for 1 h at 70 °C removed the enzyme quantitively (Table 2). Treatment of soluble enzyme standards in the same way showed that heating at 70 °C for 1 h made no difference to the response obtained with the Lowry assay. This method was therefore used to determine the bound protein contents of the samples from investigation of effects of coupling parameters.

With respect to dry weight determinations, according to differential thermal analysis and thermogravimetry, on the loss of water from precipitates obtained from titanium(IV) chloride and ammonia, a constant composition is not achieved by drying at 60 °C for 150 h, but higher temperatures are required.[17]

The results for the variation of duration of coupling (Figure 3) show that both bound activity and bound protein levelled off after *ca.* 2 h, although there was still a slight increase in bound protein after this time. This is probably because all the surface was covered fairly quickly, although it may be simply a function of the amount of enzyme which was present in the solution, as this had decreased to only *ca.* 25% of the original after 2 h (but see later). The specific activity of the bound protein decreased rapidly at first, but more slowly after 2 h. This is probably due to deactivation of the enzyme caused by agitation of the suspension, so that although protein was still being adsorbed after 2 h, much of this was inactive. For coupling times less than 2 h, the higher specific activities may also be due to the lower amounts of enzyme coupled, resulting in less crowding at the matrix surface, and hence less deactivation. It is also possible that the aforementioned ageing of the hydrous oxide, resulting in a decrease in hydroxy-groups on the surface, may adversely affect the activity of enzyme immobilised on the surface.

The variation of bound activity with pH of coupling (Figure 2) shows a maximum at pH 7.0, but the profile is broad and a significant bound activity level is obtained even at pH 4.0. The bound protein increases slightly with pH, levelling off above pH *ca.* 7.0. The specific activity of the bound protein showed a maximum at pH 6.3. The factors affecting the coupling process at various pH values are numerous, and their relative importance is difficult to assess but they may be divided into the following categories.

(i) Deactivation of the enzyme due to exposure to solutions of different pH, for a prolonged period. Although no precise data are available, one might expect deactivation to increase at pH values above 8.0, as reflected in the lower specific activities at these pH values. However, D-glucose oxidase is fairly stable within the pH range studied, and this effect is probably not very significant.

(ii) The electrical state of the matrix surface. The isoelectric point of hydrous titanium(IV) oxide is at pH 6.6 so the surface has an overall negative charge at the activity maximum. One would therefore expect immobilisation to be taking place *via* an amino-acid residue on the protein which has a positive charge at this pH.

(iii) Other surface properties of hydrous titanium(IV) oxide. Ammonium ions adsorbed on the surface are expected to be more abundant at higher pH values, and this may be the cause of the increase in bound protein with pH. However, as is clear from the earlier discussion, the ammonium ions are easily removed by washing, so it is unlikely that they play an important role once the coupling has taken place, particularly as the immobilised enzyme is not easily removed by washing. The ammonia : titanium ratio, n, affects the apparent volume of the precipitate, this being a maximum at $n = 4$.[12] This value of n gives a pH of 5.0, a pH of 7.0 being obtained with $n = 4.3$, and pH 8.5 with $n = 4.5$. The surface area would therefore be less at $n = 4$, and hence the capacity to adsorb enzymes would decrease, and deactivation due to crowding would increase.

(iv) Pore size of hydrous titanium(IV) oxide. A study [18] of the pore size of hydrous titanium(IV) oxide prepared by steam hydrolysis of several titanium alkoxides over a wide range of pH showed that the pore size was a maximum at the isoelectric point, and fell sharply for gels prepared at pH values above 8.5 Pore radii in the region of 30 Å have been found for gels freshly precipitated from titanium(IV) chloride and ammonia,[19] and for hydrous titanium(IV) oxide freshly precipitated from titanium(IV) chloride and sodium hydroxide values

[17] A. F. Tischenko and I. F. Kokot, *Izvest. Vyssh. ucheb. zaved., Khim. i khim. Tekhnol.*, 1970, **13**, 461.

[18] M. R. Harris and G. Whitaker, *J. Appl. Chem.*, 1963, **13**, 198.

[19] A. A. Isirikyan, I. A. Kazmenko, and A. V. Kiselev, *Kolloid-Z.*, 1964, **26**, 675.

of 60 Å have been found increasing to 90 Å after 24 h and 400 Å after 48 h. When the material was dried, the pore size increased to 100 Å, and the amorphous structure crystallised into rutile. Although the pore dimensions of hydrous titanium(IV) oxide alter on heating (50—350°), the specific pore volume remains constant.[20] This is due to a decrease in surface area as a result of crystallisation from amorphous to anatase; up to 50 °C the surface area increases owing to fragmentation of the primary particles. Clearly the pore effect may influence the immobilisation of the enzyme, particularly *via* the surface area available, and also provide some protection of the bound enzyme, although pore diffusion will be operable at both coupling and substrate-approach stages.

The effect of increasing the concentration of enzyme present for a constant amount of hydrous titanium(IV) oxide (Figure 4) was that the bound activity increased rapidly at low enzyme concentrations, but above the enzyme:oxide ratio of *ca.* 20 mg g^{-1} the increase was less pronounced. This was reflected in the profile for the bound protein. The less efficient recoveries of activity at high enzyme concentrations are probably due to overcrowding causing deactivation, since the specific activities of the bound enzyme show an overall decrease with increasing enzyme:oxide ratio.

From the retention of enzymic activity by the immobilised enzyme on subjection to the various conditions of environmental ionic strength, pH, substrate concentration, *etc.* it is evident that the immobilised enzyme is stable once it has been fully washed. Furthermore in all this work no evidence was obtained to suggest that the ageing process undergone by hydrous titanium(IV) oxide is detrimental to the retention of bound enzyme.

So far as applications of enzymes immobilised on hydrous titanium(IV) oxide are concerned, there is no difficulty in removal of the material from suspension by centrifugation or by filtration. Alternatively the material may be used in a column by packing together with added spacer/filler.

Freeze-drying provides a convenient means of storing insoluble enzyme derivatives, but often suffers from the disadvantage that enzymic activity is lost owing to denaturation of the protein structure. This was found to be the case with D-glucose oxidase derivatives of hydrous titanium(IV) oxide, prepared by method (5), which lost 83% of their activity on freeze-drying. However, when sorbitol was present only 26% activity was lost, and this effect is attributed to the stabilising hydrophilic environment provided by the sorbitol.

In conclusion hydrous titanium(IV) oxide is an effective matrix for the immobilisation of enzymes to give active products, and the methods described herein constitute a new mode of enzyme immobilisation wherein the support preparation and enzyme coupling may be simply carried out *in situ* and on site. The method could be conveniently extended to the immobilisation of other proteins, glycoproteins, peptides, amino-acids, antibodies, antigens, antibiotics, *etc.*

We thank Professors S. A. Barker and M. Stacey, C.B.E., F.R.S., for their interest and the S.R.C. for a scholarship (to I. M. K.).

[5/1545 *Received, 5th August*, 1975]

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