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Transition-metal Oxide Chelates of Carbohydrate-directed Macromolecules

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1 Introduction

Transition-metal oxides and related compounds have now been utilized in a wide variety of applications. The well-known large scale industrial routes to the production of parent metals have been used for some time. In most cases extreme specialist manipulation of the chemical and physical properties of the oxides *etc.* have resulted in development of materials and equipment now regarded as essential components of a modern community. Such applications of transition-metal oxides include pigmentation of latex paints, gloss paints, plastics, stabilization of glass, vitreous enamel, and pottery surfaces, catalysis of chemical reactions, production of solid state devices, display of defined magnetic properties in electronic components *etc.*, production of equipment stable to intense radiation for use in the atomic energy field, harnessing of amphoteric properties as cation and anion exchanges, extractants for large scale use, and production of synthetic gems.

2 Metal Complexes in Biological Systems

The occurrence of metal ions in living tissues is widespread and although they constitute a relatively small amount of the total weight of an organism, they are essential to many of the vital processes of both plants and animals.

The principal metals required by the animal body are sodium, potassium, calcium, and magnesium. Sodium is the major component of the cations of the extracellular fluid and potassium the principal component of the cations of the intracellular fluid. The oxides of these ions are of course unique since, as their hydroxides, they are water soluble. Calcium is the most abundant cation in the body, almost all of it being in the bones and teeth. Most of the magnesium in the body also occurs in the skeletal tissue but it is also one of the principal cations of soft tissue and functions in carbohydrate metabolism as an activator for many of the enzymes of the glycolytic systems. Although these two elements are

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capable of complex formation (*e.g.* chlorophyll is a magnesium complex) and play an important part in metabolism, their hydroxides are precipitated at a high pH.

The metals of interest in the particular context of this article, *i.e.* those which are associated with complex formation, are those which are referred to as trace elements. Six such metals have been identified in the body and a physiological role assigned to them:—iron, copper, manganese, cobalt, zinc, and molybdenum. Three other metals (aluminium, cadmium, and chromium) are also known to exist in living tissue but their functions, if any, are not clearly defined. These metals are usually associated, either directly or indirectly (*via* a 'prosthetic' group) with peptides and proteins and it is these types of interactions which are to be considered here.

The association of metals with proteins has been appreciated almost as long as the chemical identity of proteins has been recognized. Thus the presence of copper in the oxygen transporting haemocyanin of molluscs was first described more than a century ago.[1] The recognition of iron in horse and dog haemoglobin by Preyer,[2,3] Zinoffsky,[4] and Jaquet,[5] the studies of McMunn[6] on cytochrome and those of Spitzer[7] on iron in oxidative processes were the first suggestions which led to the discovery of the participation of metals in oxygen transport and enzymatic catalysis. No doubt the visible colours of these particular proteins accounts for the early interest in and recognition of their properties.

The complexes of metals and proteins have been classified as 'metalloproteins' or 'metal–protein complexes' on the basis of the apparent stability constants governing the associations.[8] The metal atoms of metalloproteins are bound so firmly that they are not removed from the protein by the isolation procedure; hence the highly purified protein ultimately contains stoicheiometric quantities of metal. This stability provides many of the advantageous practical features of these systems. When the metalloprotein is a catalytically active metalloenzyme, the stable association of a particular metal with an apoenzyme (the protein component of the metalloenzyme) suggests a specific biological role for the metal in the system. While essential for specific catalytic activities, metals may also serve to stabilize both enzymically active and inactive metalloproteins.[9,10] The role of metals in the tertiary and quaternary structure of proteins is becoming apparent[11] and requires further definition. When the metal is loosely bound, the association

[1] E. Harless, *Arch. Anat. Physiol.*, 1874, 148.
[2] W. T. Preyer, 'De Haemoglobino Observationes et Experimenta', M. Cohen and Sonn, Bonn, 1866, p. 27.
[3] W. T. Preyer, 'Die Blutkrystalle', Manke's Verlag (Hermann Dufft), Jena, 1871.
[4] D. Zinoffsky, *Z. Physiol. Chem.*, Hoppe-Seylers, 1886, **10**, 6.
[5] A. Jaquet, *Z. Physiol. Chem.*, Hoppe-Seylers, 1890, **14**, 289.
[6] C. A. McMunn, *Phil. Trans. Roy. Soc.* London, 1885, **177**, 267.
[7] W. Spitzer, *Arch. Ges. Physiol.*, Pfluegers, 1897, **67**, 615.
[8] B. L. Vallee, *Advan. Protein Chem.*, 1955, **16**, 401.
[9] B. L. Vallee, E. A. Stein, W. N. Sumerwell, and E. H. Fischer, *J. Biol. Chem.*, 1959, **234**, 2901.
[10] A. Rosenberg, *Archiv. Kemi.*, 1960, **17**, 25.
[11] D. E. Drum, J. H. Harrison, T. K. Li, J. L. Bethune, and B. L. Vallee, *Proc. Nat. Acad. Sci. U.S.A.*, 1967, **57**, 1434.

is both chemically and biologically more tenuous. The designation 'metal–protein complex' is intended to convey this subtle distinction from 'metalloproteins'.

The stabilities of metal–protein complexes span a considerable and continuous range depending on the nature, number, and geometry of the donor groups at the binding site. Only in a few instances have the interactions of metals with enzymically active proteins been measured with sufficient precision to yield dissociation constants. The values determined by equilibrium dialysis range from 2×10^{-5} M for zinc–enolase[12], a metal–enzyme complex, to 3.2×10^{-10} M for zinc in carboxypeptidase at pH 8[13] and carbonic anhydrase at pH 5.5[14] and 1×10^{-10} M for zinc in the alkaline phosphatase,[15] all of which are considered to be metalloenzymes.

Metalloenzymes containing stoicheiometric amounts of firmly bound, biologically active, metal atoms are amenable to investigative correlation of structure and composition with function. These systems are also well suited to the identification of the donor groups of the protein which account for specific metal-binding. In fact, metal ions may be considered to be site-specific, selective reagents for the identification of such loci which are part of active enzymic centres by definition. Moreover, since the chemical properties and reactivity of the metal ions differ distinctly from those of the amino-acid side-chains of proteins which may participate in catalysis, metallo-enzymes have provided convenient models for the study of the mechanism of enzyme action in general.

The identification of metal-binding sites of proteins has been based largely on the mode of interaction of metals with amino-acids, peptides, and their derivatives. Such studies have led to the general conclusion that the amino-acid side chains of proteins having dissociable hydrogen ions serve as the ligands for metal interactions, though peptide nitrogens can also participate. Virtually all of the potentially reactive groups of amino-acids have either been demonstrated or postulated to bind metals: the α-carboxy- and α-amino groups, the side-chain carboxy-groups of glutamic and aspartic acids, the ϵ-amino group of lysine, the imidazole group of histidine, the phenoxy group of tyrosine, the thiol group of cysteine, and the guanidinium group of arginine. Hydrogen ion titrations and chemical modifications have served as the primary experimental methods for the exploration and identification of such binding sites.

The interaction of any such sites with metal ions will inevitably lead to a change in the solubility of the protein molecule. In many instances, the effect is to decrease the solubility but in the case of the specific metal–protein complex, Fe^{III}_2-transferrin, the complex is more soluble than the metal-free protein under some conditions.[16] The processes which lead to a decrease in solubility may be explained simply in terms of various interactions of the metal ions with the protein molecule.

[12] B. G. Malmstrom, 'The Enzymes', ed. P. O. Boyer *et al.*, Academic Press, New York, 2nd edn. vol. V, p. 471.
[13] J. E. Coleman and B. L. Vallee, *J. Biol. Chem.*, 1960, **235**, 390.
[14] S. Lindskog and P. O. Nyman, *Biochim. Biophys. Acta*, 1964, **85**, 462.
[15] S. R. Cohen and I. B. Wilson, *Biochemistry*, 1966, **5**, 904.
[16] B. A. Koechlin, *J. Amer. Chem. Soc.*, 1952, **74**, 2649.

It is possible to distinguish between two general types of complexes between metal ions and binding sites in proteins: simple complexes in which each metal ion combines with a single ligand group, and chelate complexes in which each metal ion combines with two or more ligand groups. Three general processes can therefore be postulated in which the formation of metal–protein complexes may alter the solubility of the protein.[17] These are (*a*) binding of metal to individual groups, (*b*) binding of two or more ligand groups to form a cross-linked intermolecular complex, (*c*) cross-linking to form an intramolecular complex. The number of sites fulfilling the stereochemical requirements for the formation of a complex of type (*c*) will be very small compared with the number of single, metal-binding, ligand groups. The rigidity of the protein molecules and the steric properties of the metal–ligand bonds will also limit the number of linkages of type (*b*) but these may be present in sufficient number to render the protein insoluble by a process of aggregation.

The solubility of metal–protein complexes may also be affected by changes in pH, to an extent depending on the nature and concentration of the metal. As pH increases, a hydrated metal ion will hydrolyse[18] in accordance with the following general equation:—

$$[M(H_2O)_n]^{z+} + OH^- \rightarrow [M(OH)(H_2O)_{n-1}]^{(z-1)+} + H_2O$$

A similar equilibrium exists for the hydrated metal ions involved in complexes with proteins and this presents a major complication in the study of the interactions of metal ions with proteins at neutral pH. The extent of hydrolysis is dependent on the metal ion; some hydroxides do not begin to precipitate until pH 9 or above, *e.g.* those of calcium and magnesium. The hydroxides of iron(II), nickel(II), zinc(II), and copper(II) precipitate in the pH range 6—9 and the ions can be studied in the unhydrolysed form only over a very restricted range. For metals such as aluminium, vanadium(III), and iron(III) precipitation of the hydroxide takes place below pH 6, in a region where many proteins are unstable. It is significant that metals of this sort, if they occur in living systems at all, do so either as hydroxides (*e.g.* ferritin[19]) or in chelate complexes of very high stability (*e.g.* metal porphyrins), or in abnormally acidic media (*e.g.* V^{III} at pH 1—2 in the ascidian vanadocytes which contain sulphuric acid[20,21]).

A further significant factor is the tendency for the mono-hydroxides of some metals to associate in solution to form polynuclear complexes. This phenomenon occurs in a region of pH somewhat below the point at which the metal hydroxide begins to precipitate. Two types of linkages between metal atoms are found: partial covalent oxygen bridges of the type M—O—M, and hydroxy bridges of the type M—O—H—O—M.[22] Probably both types of bonds are effective in the

[17] F. R. N. Gurd, 'Ion Transport Across Membranes' ed. H. T. Clarke, Academic Press, New York, 1954, p. 246.
[18] C. W. Davies, *J. Chem. Soc.*, 1951, 1256.
[19] L. Michaelis, *Adv. Protein Chem.*, 1947, **3**, 53.
[20] H-J. Bielig and E. Bayer, *Experientia*, 1954, **10**, 300.
[21] E. Boeri and A. Ehrenberg, *Arch. Biochem. Biophys.*, 1954, **50**, 404.
[22] A. F. Wells, 'Structural Inorganic Chemistry', 2nd edn. Oxford University Press, 1950.

formation of polynuclear complexes. Aluminium(III), vanadium(III), chromium(III), and iron(III) all bond tightly to oxygen in preference to nitrogen and have a strong tendency to form polynuclear complexes at low pH values. Similar complexes are also formed by titanium(IV) and zirconium(IV). In complexes where the metal ions are only partially saturated with ligands, hydrolysis may occur at certain ranges of pH, followed by polymerization to give polynuclear structures with ligands attached.

When the amount of metal hydroxide is much greater than the amount of protein, then the protein may be completely removed from solution. This is common practice in certain procedures for the analysis of non-protein constituents in serum, and zirconium 'hydroxide' has been used to deproteinize serum in this way.[23] Similar procedures using the hydroxides of lead(II) and zinc(II) have been applied to the more selective separation of certain plasma proteins by precipitating some whilst leaving others in solution.[24] Enzymes may also be purified by these fractional adsorption techniques using hydrous alumina gel or zinc hydroxide gel,[25] although chromatographic methods have become more popular recently.

Whilst the above establishes a basis for the further study of insoluble metal–protein complexes/chelates, the metals which form 'hydroxides' at a low pH are seldom found in biological systems, except under unusual circumstances as mentioned earlier. The interactions of such metals with proteins has not, therefore, received much attention. However, since it has now been established that such metals, in the form of oxides *etc.*, are capable of binding biologically active species without total loss of biological activity, as described subsequently, a more thorough description of such metals is justified. It is obviously impossible to survey this comprehensively and so emphasis has been placed on those factors concerned with the formation, structure and properties of certain oxides and particularly hydrous oxides ('hydroxides')* so that some insight may be gained into the nature of the complexes formed from such compounds.

3 Structures of Some Transition-metal Oxides

Titanium.—Titanium is the first member of the *d*-block transition elements and has four valence electrons, giving an outer electron configuration of $3d^2\ 4s^2$. Titanium(IV) is the most stable and common oxidation state, compounds of lower oxidation states being readily oxidized by air, water, or other reagents, to the extent that oxidation states lower than Ti^{III} are rarely encountered. The ionization energy required for the formation of the Ti^{4+} ion is so high that this ion has no real existence, titanium(IV) compounds being generally covalent. The two most common co-ordination numbers of titanium(IV) are 4 and 6, giving rise to tetrahedral and octahedral geometries, respectively. Titanium(IV) chloride is

*The term 'hydrous metal oxide' is used preferentially where the formula of the metal 'hydroxide' cannot be simply depicted as $M^{n+}(OH^-)_n$.

[23] J. Erdos and J. Suru, *Biochem. Z.*, 1931, **231**, 6.
[24] K. Schmid, *J. Amer. Chem. Soc.*, 1953, **75**, 60 and 2532.
[25] M. Dixon and E. C. Webb, 'Enzymes' 2nd edn. Longmans, London, p. 42.

an example of the tetrahedral configuration; this colourless liquid behaves as a Lewis acid and is readily hydrolysed by water to give hydrous titanium(IV) oxide.

Titanium(IV) oxide is found in three crystalline modifications, rutile, anatase, and brookite, all of which contain an octahedrally co-ordinated titanium atom. Rutile and anatase are important white pigments which are produced on a large scale, whereas brookite is not very common and is only formed under hydrothermal conditions in the presence of sodium hydroxide.[26]

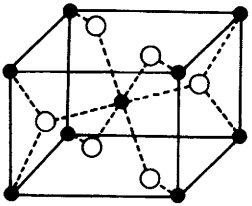
The rutile structure is based on a distorted hexagonal close packing of oxygen atoms, in which each oxygen atom is surrounded by three titanium atoms disposed towards the corners of an equilateral triangle (Figure 1). The unit of this structure is not a cube, since one of the axes is shorter than the other two by about 30%. The oxygen atoms occupy the corners of a slightly irregular octahedron,[27] in which four of the Ti—O bonds are shorter than the other two by 0.03 Å. Rutile is denser, harder, and has a higher refractive index than anatase.

The structure of the anatase form is based on cubic close packing of the oxygen atoms, and is essentially the same as a sodium chloride structure from which a number of sites have been left vacant (Figure 1). As with rutile, the basic unit is considerably distorted,[28] the ratio of the axes being 2.53, rather than the theoretical 2.00. Two of the Ti—O bonds are again larger than the other four by 0.002 Å.

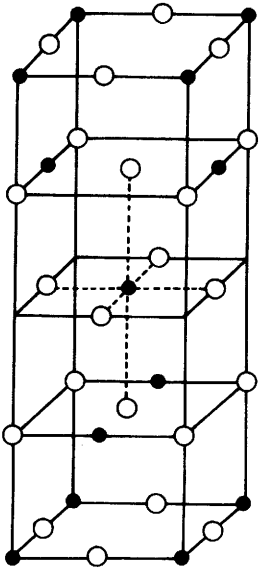
The Ti—O bond in titanium(IV) oxides has 63% ionic character, and one would therefore expect quite strong electrostatic forces to be present at the oxide surface. A clean (001) cleavage plane of anatase should have the structure shown in Figure 2*a*, with one O^{2-} ion missing from the co-ordination shells of each Ti^{4+} ion, and the third Ti^{4+} ion missing from the co-ordination shell of each O^{2-} ion. In the presence of water, the exposed Ti^{4+} ion would attract the dipolar water molecules, which would fill the vacant co-ordination sites on the surface (Figure 2*b*). A more stable situation could then be achieved by proton transfer from the chemisorbed water molecules to neighbouring oxygen ions, (Figure 2*c*) giving a fully hydroxylated surface. One would then expect two types of hydroxy groups to exist on the surface, exhibiting different reactivities.

A model proposed[29] for the double layer at the surface of rutile takes into account the ordering effect of the hydroxylated surface on the hydrogen bonding of liquid molecules adjacent to it, and the structure-promoting role of the potential-determining ions, OH^- and H_3O^+. Specific adsorption was related to the structure-promoting or disrupting influence of the adsorbed ion, the former species being preferentially adsorbed. A schematic representation of the model of the double layer is presented in Figure 3. The (100) rutile surface (A) and hydroxylated layer (B) are followed by an oriented layer of water molecules hydrogen bonded to the surface groups (B—C). C represents the proposed location of surface charge of the double layer, where potential-determining hydroxy ions

[26] I. Keesman, *Z. anorg. Chem.*, 1966, **346**, 30.
[27] W. H. Baur, *Acta Cryst.*, 1956, **9**, 515.
[28] D. T. Cromer and K. Herrington, *J. Amer. Chem. Soc.*, 1955, **77**, 4708.
[29] Y. G. Berubé and P. L. De Biuyn, *J. Colloid and Interfac. Sci.*, 1968, **27**, 305.



Rutile



Anatase

Titanium ●

Oxygen ○

Figure 1 *Structure of rutile and anatase forms of titanium*(IV) *oxide*

are co-ordinated with oriented water molecules. The closest distance to which a cationic structure-promoting counterion can approach is represented by D. In the upper part of the sketch a water-bridged ion pair is shown. E represents the beginning of the 'thawed' region separating the structured surface region from

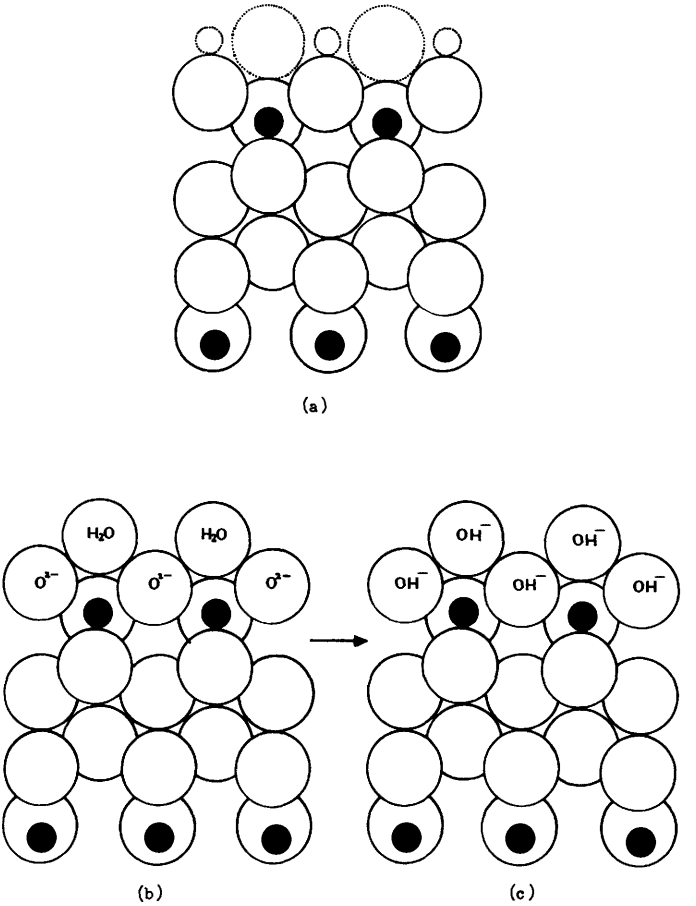


Figure 2 (001) *Cleavage plane of anatase form of titanium*(IV) *oxide.* (a) *anhydrous surface.* (b) *hydrated surface.* (c) *hydroxylated surface*

the bulk liquid in which the clustering of water molecules causes the ordering to increase slightly.

A wide variety of physical and chemical methods have been employed in providing evidence for the existence of surface hydroxy groups on titanium(IV) oxides. Using i.r. spectroscopy,[30] it can be shown that both anatase and rutile forms still contain some adsorbed molecular water after evacuation at 150 °C, as evidenced by a bending vibration at 1605 cm^{-1}. After outgassing at 350 °C, no free water is detectable, but there remain two OH stretching absorptions in the case of anatase (at 3715 and 3675 cm^{-1}) and one with rutile (at 3680 cm^{-1}), indicating the existence of two different types of hydroxy groups on anatase. This is sub-

[30] I. T. Smith, *Nature*, 1964, **201**, 67.

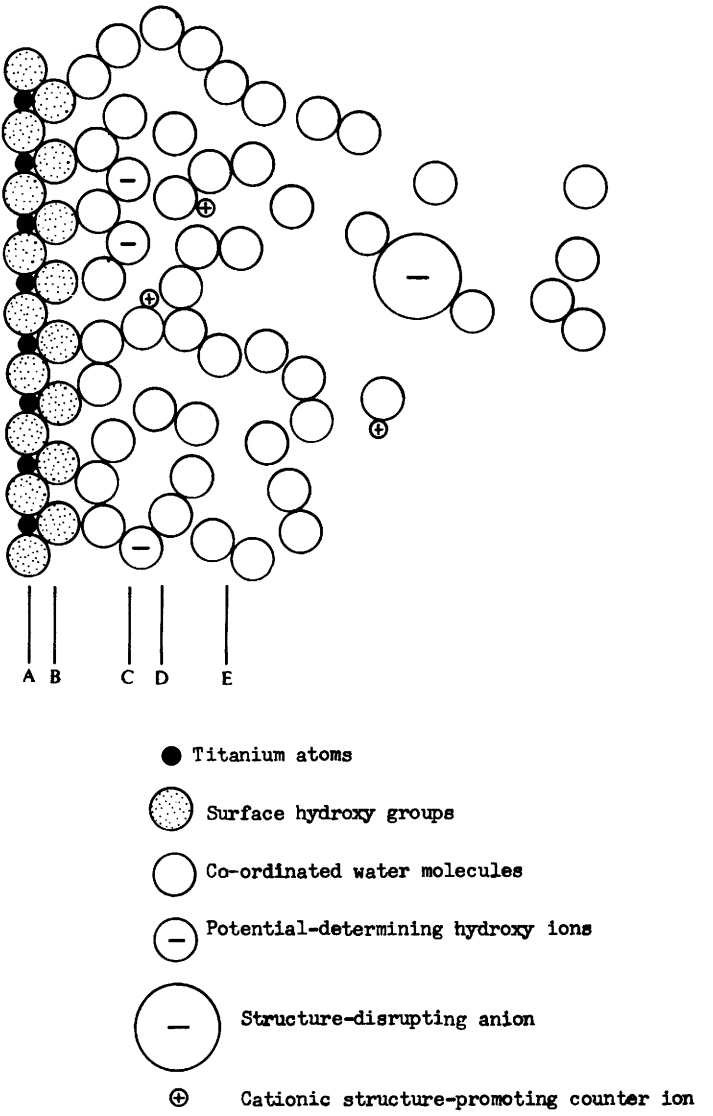


Figure 3 *A schematic picture of the interfacial region separating the* (100) *rutile surface of titanium*(IV) *oxide from the bulk liquid phase*

stantiated by the following facts: (*a*) sodium fluoride treatment of anatase indicates[31] that 50% of the hydroxy groups at the iso-electric point of anatase (pH 6.6) are basic in character and replacable, whilst the rest are acidic and not

[31] M. Herrman, U. Kaluza, and H. P. Boehm, *Z. anorg. Chem.*, 1970, **372**, 308.

replacable by fluoride until pH 4.6 is reached, and (*b*) 50% of the hydroxy groups determinable by deuterium exchange[32] are acidic with a pK_a of 2.9.

In the rutile form,[33] molecular water is removed by evacuation at temperatures above 200 °C. Above 300 °C the hydrogen-bonded hydroxy groups are progressively removed, and at 450 °C only isolated hydroxy groups with a stretching frequency of 3740 cm^{-1} remain. This bond suffers a marked reduction in intensity when the sample is evacuated at 610 °C. Thermogravimetric analysis shows two regions of high weight loss—the first (100—400 °C) corresponds to loss of physically adsorbed water and water formed by hydroxy group condensations, and the second (550—800 °C) is a more gradual loss of water resulting from the condensation of isolated hydroxy groups with very little lateral surface mobility. At 116—200 °C the dehydration is endothermic[34] and at 404—476 °C there is an exothermic effect due to changes in the crystal structure. Rehydration experiments[33] show a strong interaction of the evacuated surface with water molecules but it is not known whether surface hydroxy groups reform on a 450 °C-treated surface. The fact that there is a very slow uptake of hydroxy groups (or release of protons) on a negatively charged rutile surface, but no such effect when the surface is positively charged, is explicable by the presence of strongly hydrated ions in the double layer.[29]

Water physically adsorbed on anatase[35] covers the whole surface area with a monolayer at all temperatures (30—425 °C), whereas chemisorbed water increases from negligible at 30 °C to a maximum of 8% coverage at 280 °C. This leads to the conclusion that chemisorbed water is free to interact with water that is subsequently physically adsorbed, forming multiple layers over the oxide surface. The protons on the anatase surface are mobile and relatively strong hydrogen bonding with water molecules is possible.

Adsorption of water on the surface of the rutile form[36] extends to 30% of the surface area available for water adsorption when the sample has been heated to 450 °C. The surface density is 3.7 OH/100 Å as opposed to 11.4 OH/100 Å for a fully hydroxylated rutile surface. Hydroxy groups exist on the surface even after outgassing at high temperatures.[37]

Deuterium exchange experiments[38] show that roughly a third of the surface area of anatase is covered with hydroxy groups, the decomposition of which starts at 200 °C and is completed by 350 °C. Fifty per cent of the hydroxy groups are reformed on treatment with steam, and all are reformed with water.

The nature and composition of the gelatinous hydroxy oxide has been the subject of several investigations[39,40,41] but only recently has a comprehensive

[32] M. Herrman and H. P. Boehm, *Z. anorg. Chem.*, 1969, **368**, 73.
[33] K. E. Lewis and G. D. Parfitt, *Trans. Faraday Soc.*, 1966, **62**, 204.
[34] I. N. Belyaev and S. A. Artamonova, *Russ. J. Inorg. Chem.*, 1966, **11**, 253.
[35] J. J. Jurinak, *J. Colloid Sci.*, 1964, **19**, 477.
[36] C. M. Hollabrough and J. J. Cheswick, *J. Phys. Chem.*, 1961, **65**, 109.
[37] L. G. Ganichenko, V. F. Kiselev, and V. F. Murina, *Kinetika i Kataliz*, 1961, **2**, 877.
[38] M. Herrman and H. P. Boehm, *Z. anorg. Chem.*, 1967, **352**, 156.
[39] T. Graham, *Phil. Trans.*, 1861, **151**, 213.
[40] F. Bischoff and H. Adkins, *J. Amer. Chem. Soc.*, 1925, **47**, 807.
[41] G. V. Jere and C. C. Patel, *J. Sci. Ind. Res. India*, 1961, **208**, 292.

study been carried out.[42] The number of hydroxy groups present may be determined by displacement with fluoride (the Ti—F bond is very stable) and acid titration of the released hydroxy ions, insoluble titanium by calcination and gravimetric analysis, and titanium in solution by reaction with hydrogen peroxide and spectrophotometric measurement of the yellow chromophore produced; formulae of the type $TiO(OH)_2$ were assigned.

On addition of ammonia to a solution of titanium(IV) chloride, gelatinous, unfilterable precipitates are obtained at ammonia–titanium ratios (*m*) between 2 and 3.75. On increasing *m* to 4, titanium is precipitated quantitatively as a white, amorphous precipitate. At this point there is 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 a stoichiometric amount of precipitant has been used. The reaction equations are as follows:—

$$TiCl_4 + H_2O \rightarrow TiOCl_2 + 2HCl$$
$$TiOCl_2 + 2HCl + 4NH_4OH \rightarrow TiO(OH)_2 + 4NH_4Cl + 2H_2O$$

The precipitates contain very small quantities of chloride ions and larger quantities of ammonia (0.11—0.17 %) but the ratios Cl^-:Ti^{4+} and NH_4^+:Ti^{4+} are not constant and decrease sharply if the precipitates are kept in air or washed with water, indicating that these ions are physically absorbed on the precipitate surface. The OH^-:Ti^{4+} ratio, on the other hand, remains fairly constant for 5 days at a value of around 2, when the precipitate is kept submerged in the mother-liquor. When aged in air, the ratio decreases to 1 after 15 days, and remains at that value for 4 months.

A polymeric structure for the hydrous oxide is indicated by a broad absorption band in the i.r. spectrum at 420—1200 cm^{-1}, and the absence of a Ti=O double bond absorption band at 1087 cm^{-1}. The i.r. spectrum also confirms the presence of hydroxy groups and molecular water. The structures proposed on the basis of these data are shown in Figure 4. No hydroxide, $Ti(OH)_4$, is detectable, and the species $TiO(OH)_2$ has been shown, theoretically, to be thermodynamically much more stable.[43] To complete the octahedral co-ordination, water molecules may be adsorbed by polar ionic forces or form partial covalent bonds.

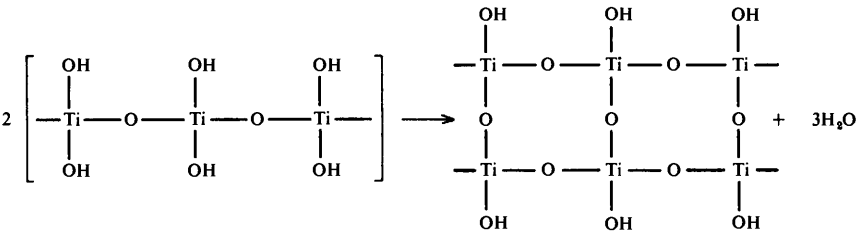


Figure 4 *Structure of hydrous titanium oxide and the structural changes which occur upon ageing*

[42] T. F. Limar', A. I. Savos'kina, V. I. Andreeva, and V. V. Mank, *Russ. J. Inorg. Chem.*, 1969, **14**, 1213.
[43] Y. Y. Bobryenko, *Russ. J. Inorg. Chem.*, 1967, **12**, 931.

Hydrous titanium oxide is in fact porous[44] and methods have been devised[45] for the preparation of active gels with any desired pore radius in the range 10—100 Å. Gelatinous hydrous titanium oxide freshly precipitated from titanium(IV) chloride and ammonia has pore radii in the region of 30 Å; when dried, the pore size increases to 100 Å, and the amorphous structure crystallizes into rutile.[46] The pore size dimensions do alter on heating (50—350 °C), but the specific pore volume remains constant, due to a decrease in surface area due to crystallization from amorphous to anatase.[47]

Zirconium.—Zirconium has an outer electron configuration of $4d^2 5s^2$ and occurs almost exclusively in its compounds in the oxidation state 4+. Bonding to the maximum possible extent results in a maximum co-ordination number of 8. The zirconium ion can use various hybrid orbitals to give strongly directional bonds. For eight-fold co-ordination the orbitals used are d^4sp^3, leading to a dodecahedral arrangement, or d^5p^3, giving a square antiprismatic configuration.[48] This latter configuration is found in $ZrOCl_2,8H_2O$ and $ZrOBr_2,8H_2O$.[49] In these compounds the zirconium is co-ordinated by four hydroxy groups and four water molecules. The square antiprismatic co-ordination indicates that the lone pairs of electrons in the ligands form co-ordinate bonds with the vacant 4*d* and 5*p* orbitals of the zirconium ion. These bonds are essentially covalent, involving the sharing of the lone pair of the oxygen atom.

The behaviour of zirconium complexes in aqueous solution is characterized by hydrolysis and polymerization. When zirconium(IV) chloride is dissolved in water a solution is obtained which behaves identically to the solution obtained from $ZrOCl_2,8H_2O$. The reactions which occur can be formulated as below:—

$$ZrCl_4 + 8H_2O \rightarrow [Zr(H_2O)_8]^{4+} + 4Cl^-$$
$$[Zr(H_2O)_8]^{4+} + H_2O \rightarrow [Zr(H_2O)_7(OH)]^{3+} + H_3O^+$$
$$[Zr(H_2O)_7(OH)]^{3+} + H_2O \rightarrow [Zr(H_2O)_6(OH)_2]^{2+} + H_3O^+$$

The final complex cation is then able to polymerize to give a tetrameric complex:—

$$4[Zr(H_2O)_6(OH)_2]^{2+} \rightarrow [Zr_4(H_2O)_{16}(OH)_8]^{8+} + H_2O$$

This tetrameric complex is present[50] in both aqueous solution and the solid zirconyl chloride octahydrate. The structure of the crystalline complex consists[49] of four zirconium ions lying at the corners of a square, each bound to its nearest neighbours by two hydroxide bridges, one above and one below the plane of the zirconium ions. The remaining four bonds are taken up by water molecules, there being no actual Zr—Cl bond.

The aqueous solution of zirconium(IV) chloride has acidic properties, the H^+

[44] M. R. Harris and G. Whitaker, *J. Appl. Chem.*, 1963, **13**, 198.
[45] M. R. Harris and G. Whitaker, *J. Appl. Chem.*, 1963, **13**, 348.
[46] A. A. Isirikyan, I. A. Kazmenko, and A. V. Kiseler, *Kolloid. Zhur.*, 1964, **26**, 675.
[47] A. P. Shtin, L. M. Sharygin, and V. F. Gonchar, *Zhur. fiz. Khim.*, 1977, **47**, 485.
[48] G. E. Kimball, *J. Chem. Phys.*, 1940, **8**, 188.
[49] A. Clearfield and P. A. Vaughan, *Acta Cryst.*, 1956, **9**, 555.
[50] J M. Muha and P. A. Vaughan, *J. Chem. Phys.*, 1960, **33**, 194.

activity of a 1 mM solution being almost equal to that of 1 mM-HCl.[50] This is due to the hydrolysis of the tetrameric complex:—

$$[Zr_4(OH)_8(H_2O)_{16}]^{8+} + 4H_2O \rightarrow [Zr_4(OH)_8(OH)_4(H_2O)_{12}]^{4+} + 4H_3O^+$$

The charge on the complex is thus reduced to 4+ and, depending on the acid concentration, this can be reduced further. Each of the four zirconium ions can hydrolyse separately:—

$$[Zr_4(OH)_8(OH)_4(H_2O)_{12}]^{4+} + H_2O \rightarrow [Zr_4(OH)_8(OH)_5(H_2O)_{11}]^{3+} + H_3O^+$$

This results in one neutral group in the tetrameric complex. Assuming that this neutral group is localized on one of the zirconium atoms, the other three retaining their single positive charges, then this can be the starting point for a polymerization reaction. The neutral site reacts with a singly-charged site of another tetrameric complex in such a way that the zirconium ions are connected by two hydroxide bridges (Figure 5). The degree of polymerization increases as the acid

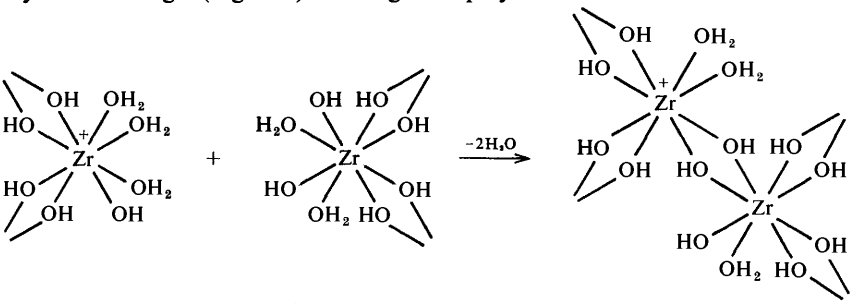


Figure 5 *The polymerization of zirconium hydrous oxides (hydrolysed zirconium complexes)*

strength decreases[51] and hence addition of alkali to neutralize the acidic solution formed upon dissolution of zirconium(IV) chloride also increases the degree of polymerization. When neutralization is continued to pH 3, a precipitate begins to form. At the equivalence point (pH 9) the precipitation is complete, the gelatinous precipitate being 'zirconium hydroxide', *i.e.* hydrous zirconium oxide (also known as hydrated zirconium dioxide or hydrous zirconia).

The nature and composition of this precipitate in either its freshly precipitated or dried form have been the subject of some controversy in the literature in recent years. Magnetic susceptibility[52], differential thermal analysis and thermogravimetric analysis,[34,41,53–59] i.r. spectroscopy,[41,53,54,58–61] thermodynamic

[51] A. N. Ermakov, I. N. Marov, and V. K. Balyaeva, *Russ. J. Inorg. Chem.*, 1963, **8**, 845.
[52] M. F. Bourion and O. Hun, *Compte. rend.*, 1928, **187**, 886.
[53] C. Heitner-Winguin and A. Albu-Yaron, *J. Inorg. Nuclear Chem.*, 1966, **28**, 2379.
[54] C. Cabannes-Ott, *Ann. Chim.*, 1960, 2905.
[55] G. H. Nancollas and R. Paterson, *J. Inorg. Nuclear Chem.*, 1961, **22**, 259.
[56] R. Sh. Mikhail and R. B. Fahim, *J. Appl. Chem.*, 1967, **17**, 147.
[57] S. Takagi, *Nippon Kagaku Zasshi*, 1960, **81**, 1531.
[58] A. I. Lesnikovich and V. V. Sviridov, *Vesti Akad. Nauk Beloruss, S.S.R., Ser. Khim. Nauk.*, 1971, 4, 46.
[59] D. Vivien, J. Livage, and C. Mazieres, *J. Chim. Phys. Physicochem. Biol.*, 1970, **67**, (1), 198.
[60] J. Deabriges and R. Rohmer, *Bull. Soc. chim. France*, 1967, 1.
[61] C. W. F. T. Pistorius, *J. Inorg. Nuclear Chem.*, 1960, **15**, 187.

measurements,[62] chemical analysis,[63,64] measurement of electrical conductivity,[65] potentiometric study of precipitation,[66,67] and nuclear magnetic resonance spectroscopy[68] have led to the following formulae:— $Zr(OH)_4$ or $Zr(OH)_4,xH_2O$,[52,57,61,68] $Zr_4(OH)_{16}$[63] or $Zr_4(OH)_{16},xH_2O$,[65] $Zr_4O_2(OH)_{12}$,[63] $ZrO(OH)_2$[57,64,65] or $Zr_4O_4(OH)_8$,[63] ZrO_4H_4 (metazirconic acid),[34] $ZrO_{1.5}(OH)$,[64] $ZrO_{2-x}(OH)_{2x},xH_2O$,[59] and ZrO_2,xH_2O (hydrated zirconium dioxide only containing water physically adsorbed on the solid).[51,54,55,62,65,66] It has been concluded[69] that the water is loosely bound in non-stoicheiometric proportions, the compound being formed according to the following mechanism:—

$$ZrOCl_2 + H_2O \rightarrow ZrOOH^+ + H^+ + 2Cl^-$$
$$ZrOOH^+ + OH^- \rightarrow ZrO(OH)_2$$
$$ZrO(OH)_2 \rightarrow ZrO_2,xH_2O + (1 - x)H_2O$$

thus the formation of the hydrated oxide is preceded by a stage involving the production of a true hydroxide containing hydroxy groups. The conversion of the hydroxide into hydrous oxide takes place very rapidly and irreversibly, so that in practice the gelatinous precipitate is not the hydroxide.

However, freshly-precipitated hydrous zirconium oxide,[70] washed thoroughly with water, contains four hydroxo-groups for every zirconium atom. This material can apparently exist, without ageing, as an aqueous suspension or a gelatinous amorphous mass for several hours, depending on the temperature of the surrounding medium; the compound with the formula $Zr(OH)_4,xH_2O$ can also exist although its stability is low. When this hydroxide is kept under water for several days or is boiled, it ages and is converted into the so-called zirconyl hydroxide, containing two hydroxo-groups for every zirconium atom: $ZrO(OH)_2,xH_2O$.[70] When this is dried in the cold to a powder, it is converted into hydrated zirconium dioxide, having the formula ZrO_2,yH_2O and containing the hydroxy groups.

A feature of the reaction of freshly precipitated hydrous zirconium oxide with hydrochloric acid is that only two hydroxy groups per zirconium atom react with the acid. The other two remain inactive because double hydroxo- (or olate) bridges are formed between the zirconium atoms. The structure of the freshly-precipitated material is thus Figure 6*a*, together with an indefinite number of water molecules. This structural formula represents the main unit, but, under certain circumstances, some of these tetrameric molecules may be joined together

[62] A. G. Turnbull, *J. Phys. Chem.*, 1961, **65**, 1852.
[63] M. L. Zaitsev, *Russ. J. Inorg. Chem.*, 1966, **11**, 900.
[64] T. F. Limar', V. I. Andreeva, and K. A. Uvarova, *Bull. Acad. Sci. U.S.S.R., Inorg. Mat.*, 1965, **1**, 540.
[65] H. Th. Rijnten, 'Physical and Chemical Aspects of Adsorbents and Catalysts' ed. B. G. Linsen, Academic Press, 1970, p. 315.
[66] V. V. Gubbana and A. K. Bhattacharya, *Indian J. Chem.*, 1963, **1**, 1.
[67] R. Prasad and A. K. Dey, *Kolloid Z.*, 1961, **174**, 155.
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[69] W. B. Blumenthal, 'The Chemical Behaviour of Zirconium', Van Nostrand, New Jersey, 1958.
[70] L. M. Zaitsev and G. S. Bochkarev, *Russ. J. Inorg. Chem.*, 1962, **7**, 411.

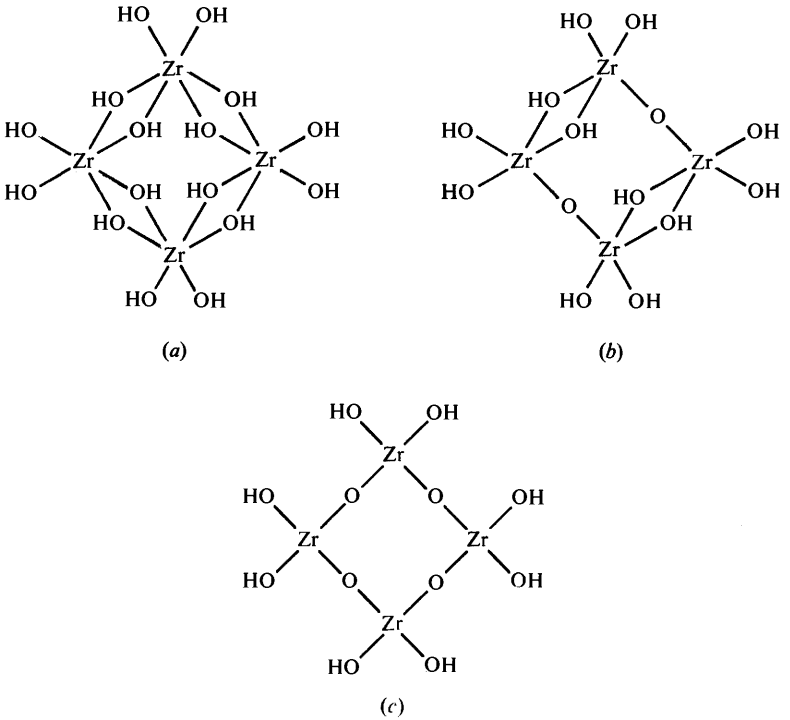
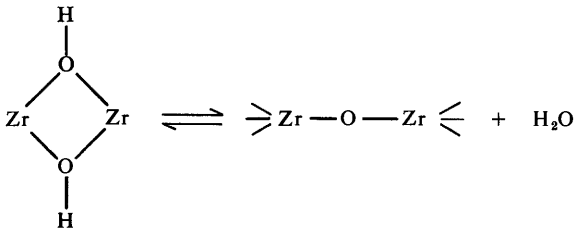


Figure 6 *Possible structures of hydrous zirconium oxide*[49]

by oxo-, olate, or other (—SO_4—, —C_2O_4—, —F—) bridges, may contain adsorbed or chemically combined ions, or may undergo a partial breakdown to trimers, dimers, or monomers.

Conversion of a double olate bridge into an oxo-bridge is possible[71] by the following mechanism:—



This suggests that two other zirconium hydroxides may exist having zirconium atoms joined alternatively by double olate and oxo-bridges or zirconium atoms joined only by oxo-bridges (Figures 6*b* and 6*c*). Structure *c* corresponds to the

[71] L. M. Zaitsev and G. S. Bochkarev, *Russ. J. Inorg. Chem.*, 1964, **9**, 1463.

formula $ZrO(OH)_2,xH_2O$ and structure *b* to $(OH)_3Zr—O—Zr(OH)_3,xH_2O$. This hydroxide can be precipitated from solutions of zirconium(IV) chloride or nitrate in methanol, at extremely high zirconium concentrations. The i.r. absorption spectra of these two other compounds indicate that they do not contain the Zr=O group and hence must be cyclic polymers of the type indicated.

According to reactions of these three hydroxides with hydrochloric, sulphuric, and oxalic acids,[63] their reactivity decreases in the order $a > b > c$. Neutral and acid salts and small quantities of acids, generally do not alter the nature of the fundamental ring of the hydroxide. However, reagents which are stronger complex-forming agents and give more covalent bonding with the zirconium atom produce greater changes in the original hydroxide ring, leading to an increase in its stability. Thus, chloride ion in solution does not alter the structure of the ring; sulphate ion, which forms ionic bonds with zirconium, does not alter the structure *a* and the structures *b* and *c* are only converted into *a* when the solution is strongly acidic. Oxalate ions bring about an increase in stability converting the *a* form into *b* or *c*.

It has also been reported[72] that zirconium hydroxide will form complexes with fatty acids and substituted benzoic acids to an extent which is dependent on the ionization constant of the acid. The presence of lactose, glucose, and fructose in zirconium solutions will prevent the precipitation of the hydroxide on addition of alkali.[73]

4 Chelation of Biopolymers by Hydrous Titanium and Zirconium Oxides

Enzymes have been used by man for hundreds of years in the preparation of food, drink, and clothing,[74] but it was not until after Payen and Persoz,[75] had isolated an individual enzyme, amylase, that full use could be made of the highly specific nature of most enzymes. Despite the tremendous advances that have been made in the isolation and purification of enzymes over the last few decades, the complex nature of the mixtures in which enzymes exist *in vivo* still makes purification of enzymes a lengthy and usually very expensive procedure. Re-isolation of the enzymes after use is generally impractical because of the low concentrations usually employed, and consequently enzymes have not been used widely for industrial purposes. In recent years much research has been directed into ways of overcoming these problems by immobilizing the enzyme onto an insoluble carrier. The chemical attachment of molecules, particularly biologically active macromolecules, to water-insoluble matrices has received considerable attention in the past decade, and the products of such attachments have been put to a number of uses, the most common of which is as a new phase-type of biologically active compound. However, one must not be led by all the extensive literature which has now been published on the chemical production of such compounds to think that the principle of insolubilization is something new. The overall principle of

[72] S. N. Chakrowarty and K. C. Sen, *Z. anorg. Chem.*, 1930, **186**, 357.
[73] K. C. Sen, *Z. anorg. Chem.*, 1928, **174**, 61.
[74] D. N. Kramer and R. Ford, *Sci. and Technol.*, 1967, **64**, 70.
[75] A. Payen and J. F. Persoz, *Ann. Chim. (Phys.)*, 1883, **53**, 73.

attachment of a biologically active molecule to an insoluble matrix is simple and simulates the natural mode of action and environment of enzymes, antibodies, antigens, *etc.* which are carried on the surfaces or in the interiors of cells, or which are embedded in biological membranes and tissues. Indeed, as is often discovered, 'Creation was there first'. In fact, it may be said that in the human the greater proportion of the biologically active molecules of the body exist at some time in insolubilized form. Apart from all the molecules present in the body known to have biological activity, many if not all of the others can be regarded as insoluble biological reactors of some description, *e.g.* the proteoglycans in their tissue matrix-forming role. However, perhaps less attention has been given to the insoluble forms rather than the soluble forms of such molecules since most chemical techniques, analyses, and manipulations are designed to be carried out in solution, and the chemistry of activity in the solid phase is less well developed.

In natural systems, the insolubilization of biologically active macromolecules such as enzymes and glycoprotein hormones may well be a reversible process, according to whether the macromolecule is originally synthesized in the solid or liquid phase. However, it is quite certain that insolubilized forms of such active macromolecules easily become converted into soluble forms to be transported to a new site at which they perform their function. In this respect the natural insolubilized molecules differ markedly from those produced in the laboratory. This is because synthetically insolubilized biologically active molecules are usually required to perform their biological function without being released into the surrounding solution and thereby contaminating it.

There are many applications of insolubilized, biologically active, molecules. Insolubilized enzymes are principally used to effect the reaction catalysed by the free enzyme, but in a simplified form since the enzyme (insoluble) can be very easily and simply removed from the substrate and products (soluble) by filtration or centrifugation, whereas use of the soluble enzyme in the conventional fashion requires subsequent laborious separation of the enzyme from the products by, for example, gel filtration and ion-exchange chromatography. Further advantages of insolubilized enzymes are that an enzyme is often stabilized to decomposition in storage and to heat on insolubilization, reactions may be rapidly terminated without the addition of foreign substances, the enzyme may be packed into a column and used for continuous conversion processes, the products of enzymic action are not contaminated with any unwanted biologically active material and immobilized enzymes lend themselves to easy re-use. Also, changes in stability and kinetic properties are sometimes found upon immobilization, and these may be put to good use. Uses include: simplification of reactors, industrial processes and clinical analyses, employment in analytical chemistry and biochemistry, sequence analysis syntheses, separation techniques, isolation of compounds related to enzymes, and use in membrane and column forms.

Insolubilized antibodies are principally useful for the purification of homologous antigens, usually by a type of column chromatography (immunoadsorption) in which the solution of impure antigen is passed through a bed of insolubilized antibody: the specific antigen is adsorbed by the antibody whilst im-

purities are washed through the column. Subsequently, the antigen may be desorbed from the column in pure form. Thus the lengthy conventional techniques of various types of column *etc.* chromatography are short-circuited. Immunoadsorption can of course also be applied in the reverse sense, using insolubilized antigen to purify an antibody. Immunoadsorption is a very versatile technique since many macromolecules are antigenic and therefore antibodies can be raised to them; but an important prerequisite is of course that the antigen that is to be insolubilized be obtained in pure form or that the antibody to be insolubilized be obtained in pure and/or highly specific form. Insolubilized antigens and antibodies are also of use in radioimmunoassay techniques.

Analogous to immunoadsorption is the technique of affinity chromatography, a technique which can be applied to the purification of enzymes, *etc.* Here the insolubilized molecule is usually one of low molecular weight but one for which the macromolecule to be purified has a specific affinity. Nucleic acids may also be used in an analogous way for the purification of complementary nucleic acids. Insolubilized molecules may be useful as enzyme substrates in the simplification of enzyme assays. The most recent innovation in the field of insolubilization has been the preparation of insolubilized antibiotics.

A number of methods of immobilization of biological molecules exist (Figure 7), and no one method is perfect for all molecules or purposes. When attaching a biologically active molecule to an insoluble support, it is important to avoid a mode of attachment that reacts with or disturbs the active site(s) of the molecule, as otherwise a loss of activity will result on binding. It is also important to avoid overloading the matrix when binding molecules, since overloading leads to overcrowding and hence reduced activity, by reason of steric hindrance of approach of the substrate *etc.* molecules to the active sites of the bound molecules. However, it does not follow that limited loading of the enzyme molecules on the matrix surface will be successful, since hydrogen bonding and hydrophobic forces may occur between immobilized enzyme and 'free' molecules thus causing the latter to block up the spaces between the immobilized molecules. Attention to the way in which the macromolecule can be attached to the insoluble matrix and the choice of matrix is also a matter of importance. A number of matrix types have been used in the field of insolubilization, and polysaccharide derivatives have been used extensively. Methods of insolubilization of enzymes, antigens, antibodies, nucleic acids, antibiotics, and affinants, and descriptions of the insolubilized molecules have been reviewed.[76]

Covalent type linkages between molecule and matrix are generally best but many of the recommended immobilization procedures require pre-derivatization of the matrix, long coupling reactions, and specialized conditions. We were searching for a method in which matrix derivatization after preparation could be avoided, and virtually instantaneous coupling could be achieved under simple conditions. It occurred to us that the chelation properties of transition metals could be employed to this end. Whereas there is a wide choice, the properties of

[76] J. F. Kennedy, *Adv. Carbohyd. Chem. Biochem.*, 1974, **29**, 305.

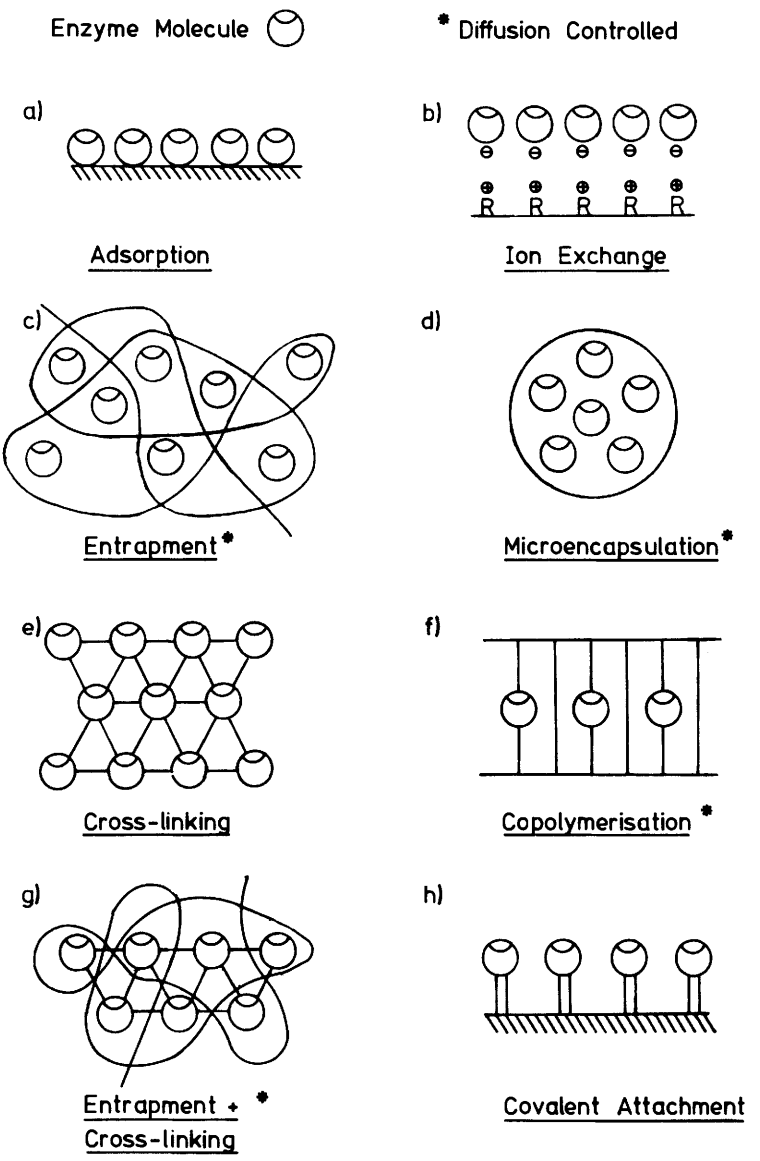
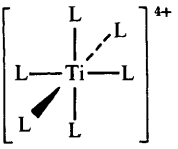


Figure 7 *Diagrammatic methods of enzyme immobilization*

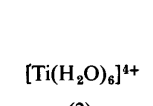
titanium and zirconium seemed particularly attractive on account of the non-toxicity of their oxides. The way in which we believe transition-metal compounds to chelate biopolymers *etc.* is illustrated from the viewpoint of a simple system of titanium(IV) chloride and cellulose.

A proportion of the titanium ions in the titanium chloride solution are octahedrally co-ordinated with molecules or ionic species that are essentially the ligands of the complex ion (1) (see Figure 8 for examples of complex ions). More



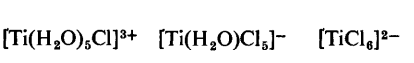
L = neutral ligand

(1)



$[Ti(H_2O)_6]^{4+}$

(2)



$[Ti(H_2O)_5Cl]^{3+}$ (3) $[Ti(H_2O)Cl_5]^{-}$ (4) $[TiCl_6]^{2-}$ (5)

Figure 8 *Titanium*(IV) *ions in titanium*(IV) *chloride*–HCl *solution*

specifically, these ligands may be water molecules or chloride ions. When a chloride ion acts as a ligand, its excess electron density may be utilized in the formation of a partially covalent bond with the titanium atom: concomitantly, the overall positive charge of the complex ion will be reduced by unity. When a water molecule acts as a ligand, an electron lone-pair may be utilized in the formation of a partially covalent bond with the titanium atom, the overall charge remaining the same. Whereas the water molecule could be bound alternatively solely *via* its action as a dipole, partial covalency at least is more likely for the transition metals. Concomitant release of a proton from the water molecule on its occupation of a ligand position will again reduce the overall positive charge of the complex ion by unity. This release of a proton is inhibited at low pH as in strongly acid solution, pH 0—2, but at comparatively higher pH, which is used for immobilization (pH 5.1), it occurs to a significant extent, particularly since highly polarizing cations are used. Thus, according to the type of ligand occupying each of the six sites, a whole series of six-co-ordinated complex titanium species may be considered to exist in solution, these species being the pure aquo complex (2) or chloroaquo complexes of various charges, *e.g.* (3) and (4), or the chloro complex (5).

These ligands are replaceable by other water molecules or chloride ions or by other ions or molecules containing electron-donating groups. Clearly the strength of the ligand–titanium co-ordination will depend upon the chemical character of the ligand. Hydroxy groups are effective ligands for the transition-metal ions, and therefore it is to be expected that transition-metal ions may complex with polysaccharides in which the hydroxy groups act as new ligands replacing others. Moreover, it is well known that glycols are very effective ligands that combine with transition-metal ions, replacing two ligand postions to form chelates (ring compounds), whereas other less suitable molecules may combine and replace

only one ligand to form further complexes (non-ring compounds). Certain polysaccharides such as cellulose contain vicinal diol groups not involved in the glycosidic linkages between residues and therefore are amenable to chelation by transition-metal ions, the chelate being formed by replacement of two of the titanium ion ligands by polysaccharide hydroxy groups. Thus, since cellulose is a polymer of β-1,4-linked D-glucopyranose units, the 2- and 3-hydroxy groups would be involved in chelate formation. The 6-hydroxy group of the D-glucopyranose residue can only be expected to participate in complexing of the cellulose chain by titanium, since sterically this hydroxy group is unable to become sufficiently close to any of the other hydroxy groups of the cellulose chain to be involved in chelate formation. Thus, titanium-treated cellulose material may be envisaged as being chelated and/or complexed along the length of its chain with the various aquo, chloroaquo, and chloro complex titanium species prevalent in solution.

For steric reasons, it is impossible for all the water or chloride ligands of the titanium ion to become replaced by other hydroxy groups of the polysaccharide chain. Furthermore, these remaining ligands will not be extensively replaced by hydroxy groups of adjacent cellulose chains on account of the insolubility and lack of mobility of the cellulose molecules. Thus, in the chelated form of the cellulose, there are many titanium centres with residual exchangeable water and/or chloride ligands. This exchangeable nature of residual original ligands imparts a reactivity to the derivatized cellulose, and on account of the insolubility of cellulose provides a matrix suitable for the immobilization of liquid-soluble molecules by covalent attachment. Covalent attachment may be achieved for any molecule containing groups appropriate to replace the ligands of the titanium bound to cellulose. The incoming molecule should be in aqueous solution and a near-neutral pH is expected to be adequate for the coupling. Thus for proteinaceous molecules such as enzymes, antibodies, and antigens, the molecular types most frequently immobilized, groups that can act as ligands will be the free carboxy groups from the *C*-terminus and acidic amino-acids, the phenolic hydroxy groups of tyrosyl residues, the alcoholic hydroxy groups of seryl and threonyl residues, free sulphydryl groups from any cysteinyl residues, and amino groups from the *N*-terminus of ϵ-amino groups of lysyl residues.

The production of such a bond between the titanium-treated polymer and the enzyme, to produce an enzymically active derivative, would be expected to depend on (*a*) the availability in the enzyme molecule of groups which can act as ligands, (*b*) steric factors which permit such groups to come in contact with the titanium atom, (*c*) the non-involvement of such groups in the region of the active site(s) of the enzyme, and (*d*) the close proximity of enzyme molecules already bound in the polymer matrix. The possibility of ionic binding is discounted because the enzymes can be bound to the titanium complexes of the polymers in ionic media.

In the case of hydrous metal oxides, it is clear that the chelating potential and chelating mechanism is analogous to that above. Thus the hydrous metal oxide itself chelates the incoming enzyme (*etc.*) molecule, which then occupies ligand

sites by displacing existing ligands. Enzymes best retain their activity on insolubilization when hydrophilic rather than hydrophobic matrices are used. The hydrophilic nature of hydrous metal oxides was rightly expected to be a major feature contributing to their suitability for use for enzyme insolubilization. Presumably, the hydroxy groups and water molecules of the hydrous oxide resemble water molecules sufficiently to provide the enzyme to be attached with an environment suited to its stability.

5 Use of Hydrous Titanium and Zirconium Oxides in the Field of Immobilization

The treatment of water-insoluble polysaccharides with titanium(IV) chloride has yielded products to which enzymes could be coupled simply and with retention of activity. This activation of the matrix involves its treatment with titanium(IV) chloride solution in hydrochloric acid for 15 minutes at 20 °C followed, after filtration, by drying at 45—60 °C for 16 hours. Enzyme is presented in buffered solution at pH 4.5. After agitation for the desired coupling time the product is removed by filtration. Thus in addition to work on cellulose, D-glucose oxidase (EC 1.1.3.4) an enzyme which is used extensively, for example in hospital laboratories, for the estimation of D-glucose, has been successfully immobilized on the alternative cheap, readily available, polysaccharide matrices alginic acid and chitin.[77] Higher enzyme activities are achieved when the treated support is not dried in the presence of the titanium(IV) chloride. As drying of the activated matrix proceeds, water may be driven off inducing cross-linking chelation and thereby decreasing the ability of the titanium to chelate enzyme. Clearly there is an optimum point, at which the degree of enzyme loading is balanced against overcrowding, to give a product of maximum activity per unit weight.

Variation of the coupling time showed that maximum coupling is achieved within one hour, longer times result in products of lower activity. The value for physical, non-chelative adsorption of enzyme to the supports is generally low. This is achieved by ensuring that coupled material is subjected to a washing procedure which includes buffered sodium chloride solution.

A ten-fold increase in the enzyme concentration employed in the coupling to the activated supports did not increase proportionally the activity of the product; this is presumably due either to overcrowding of the enzyme molecules or to saturation of the chelating sites on the support. The highest efficiency of coupling was achieved with chitin where, for the lower enzyme concentration, the enzyme activity as coupled enzyme represents 20% of the activity of the amount of free enzyme employed. This is a very acceptable economic factor, and supplementation of the supernatant from the coupling would permit re-use of uncoupled enzyme. The chelated supports did not give any significant, non-specific contribution to the assay of D-glucose oxidase.

The use of glass as a matrix for enzyme immobilization is of particular interest on account of its stability and inert nature. It also lends itself to the production of coils *etc.*, carrying active enzyme, suitable for continuous reactions *etc.* The

[77] J. F. Kennedy and C. E. Doyle, *Carbohydrate Res.*, 1973, **28**, 89.

potential of both glass and celite in particulate forms as enzyme immobilization matrices is significant again on account of stability, and the ease with which they may be used in column packings. Hence their non-compressibility contributes to sustained free running of the column without fear of the clogging of sinters, filters *etc.*

However, it has been shown that glass surfaces themselves will bind (enzymic) protein without pretreatment[78] and this phenomenon has probably been repeatedly overlooked when proteinaceous solutions are handled in glass vessels.

So far as activation of a matrix with titanium(IV) chloride is concerned, the inclusion of a drying step in the presence of the titanium salt may be predicted to give ultimately higher degrees of coupling of enzyme activity. Whilst it is arguable that such a drying stage permits more extensive chelation of titanium to the matrix, and hence greater attachment of enzyme, in fact, the drying in the presence of both titanium(IV) chloride and acid alone (before washing away of excess reagent) result in a 2—5-fold increase in activity of the coupled product in all cases. Thus, it appears that the increase in activity of the product effected by drying in the presence of titanium is not primarily due to increased chelation of titanium. Certainly, at the washing stage, all of the colour attributable to titanium is lost and the glass resumes its normal colour. It seems that the drying in the presence of acidic reagents induces sites, on the surface of the glass, which subsequently couple enzyme. Such observations are undoubtedly some function of the pre-existence of binding sites in the original glass. Generally the optimal pH for coupling of enzyme (*e.g.* β-D-glucosidase, EC 3.2.1.21) is 5.5—6.0.

Apart from the specific consideration of insolubilization of β-D-glucosidase, there arise some general implications in terms of the binding of proteinaceous materials to glass. Presumably, any such material may become bound to glass in a like manner, and problems of partial losses and inadvertent generation of biologically active surfaces may arise, particularly when small amounts are being handled. In this respect, whilst 'chromic acid', on account of its ionic and oxidative properties, is an effective cleansing agent for laboratory glassware, it should be noted that the cleansed surface is still susceptible to adsorption of proteinaceous materials from aqueous solution. Nevertheless a significant distinction between the titanium and non-titanium types of binding is the far greater ability of the former to resist decomposition by washing with salt and buffer solutions.

The commercial importance of the thiol-enzyme and protease, papain (EC 3.4.22.2), in beer chill-proofing and other applications has stimulated wide study of the enzyme's insolubilization, but many of the water-insoluble papains produced so far have been found to suffer from low specific proteolytic activity. We considered that it might be possible to bind papain to titanium(IV)-activated supports and obtain products with high specific proteolytic activity. It was also envisaged that the use of titanium(IV) activation would provide the further benefit of permitting papain to be insolubilized on inert non-particulate supports.

[78] J. F. Kennedy and P. M. Watts, *Carbohydrate Res.*, 1974, **32**, 155.

Titanium(IV) chloride-activated derivatives of polypropylene netting, zirconia-fibre sheet and glass-fibre paper have each been shown to bind papain.[79] The papain conjugate of each titanium(IV)-activated support exhibits a high ratio of specific proteolytic activity to specific esterolytic activity. These ratios range from 29—64% of the ratio for the soluble papain employed; the highest ratio obtained was for the papain conjugate of the titanium(IV)-activated glass fibre support. This conjugate is also very satisfactory with regard to its protein content, specific activities, catalytic stability, pH range of activity, mechanical stability, and ease of separation from substrate solution. The papain conjugate of titanium(IV)-activated polypropylene is more active and more stable than a papain conjugate of non-activated polypropylene. However, both preparations lose their activity during prolonged storage in substrate solutions, even though the activity of the papain conjugate of titanium(IV)-activated polypropylene is initially maintained for about 20 days. In each case the loss of activity is attributed to protein desorption during storage. The papain conjugate of titanium(IV)-activated zirconia is catalytically stable to storage in substrate solutions but suffers from poor wet strength. Nevertheless such results demonstrate that the titanium(IV)-activation method can prove successful even in the case of less-easily handled enzymes. Since papain retains high specific esterolytic activity on attachment to titanium(IV)-activated glass fibre it is concluded that the essential L-cysteine-25 and L-histidine-159 residues of papain are not significantly involved in binding the enzyme to the support. Therefore, it is predicted that titanium(IV)-activated supports may be useful for the insolubilization of other thiol-proteases and other enzymes dependent on the thiol group for activity.

Other biologically-important products produced *via* the action of titanium(IV) chloride on supports and subsequent chelation of a molecule include active insolubilized antibiotics based on cellulose–metal chelates.[80] Replacement of the ligands in titanium-treated cellulose by electron-donating groups of antibiotic molecules gives active immobilized antibacterial (ampicillin, gentamycin, kanamycin, neomycin, paromomycin, polymyxin B, and streptomycin) and antifungal (amphotericin B and natamycin) antibiotics. That the antibiotics have complexed with the cellulose–metal chelates is demonstrable in that the product cellulose–metal–antibiotic chelates exhibit antibiotic activities whereas the metal chelates of cellulose themselves were inactive. Of 140 tests conducted, cellulose–metal–antibiotic chelates were active in 102 cases. Since the antibiotic derivatives are water insoluble and in fact retain some of the antibacterial activities of the parent compounds, the chelation method provides a ready way of rendering cellulose surfaces, *etc.*, resistant to microbial attack over and above that degree of protection afforded by non-covalent adsorption of the antibiotic to cellulose itself. The data available indicate that the cellulose–metal–antibiotic chelates possess good storage characteristics.

This production of water-soluble antibacterial antibiotics may be applied in a

[79] J. F. Kennedy and V. W. Pike, *Enzyme Microb. Tech.*, 1979, **1**, 31.
[80] J. F. Kennedy, S. A. Barker, and A. Zamir, *Antimicrobial Agents and Chemotherapy*, 1974, **6**, 777.

number of ways according to the intended use of such derivatives. Where an antibacterial surface is required (*e.g.*, water storage tanks, industrial membranes, chromatographic columns), such surfaces could be realized by using cellulose-based paints, membranes, *etc.*, and insolubilization of the antibiotic by covalent attachment. In such cases, loss of the antibiotic would be minimal. Other applications which have come to light include provision of selective protection against microbial attack of paper and legal documents, of canvas and chromatographic media based on cellulosic materials, and of cellulose-based packings of cooling towers. The techniques also provide a novel form of sterility for sheets and other cotton-based fabrics and gauze, and for treating infected root canals in teeth before root filling. Also where it is required to have a slow continual release of antibiotics but a higher initial release (*e.g.*, bandages and surgeons' thread), immobilized antibiotics can be expected to be of use.

Since cellulose and other polysaccharides and their derivatives are used extensively in a number of forms as accessories to life, active insolubilized antibiotics could well be of great use in a number of other areas (*e.g.*, food packaging materials).

Poly(4- and 5- acrylamidosalicylic acids) have proved to be alternative, stable supports for the immobilization of enzymes *via* the titanium-type chelation process.[81] In this instance the titanium atoms are assumed to reside adjacent to the functional groups of the aromatic nuclei, with the hydroxy and carboxy groups acting as ligands. Immobilized α-amylase (EC 3.2.1.1) and glucoamylase (EC 3.2.1.3) produced in this way show promise on account of the high specific activity toward a macromolecular substrate which may be achieved.

Reconsideration of the chelation process in the case of titanium in the light of the known rapid hydrolysis of the chloride as the pH of the medium is raised has resulted in an alternative explanation of the chelation mechanism in terms of a partial coating, on the support, of hydrous titanium(IV) oxide.

It may be argued, since supports carrying hydroxy or carboxy groups, when exposed to titanium(IV) chloride solution, bind titanium(IV) either by forming adducts or by displacing chloride ion, that the subsequent exposure of such titanium(IV)-treated supports to buffer solutions at neutral pH hydrolyses any remaining T^{IV}—Cl bonds.

We have observed that both titanium(IV)-activated glass fibre and hydrous titanium(IV) oxide react with acidic hydrogen peroxide solution to produce a soluble yellow chromophore[79] known to be a titanium(IV) complex.[82] The rate of the release of titanium(IV) from titanium(IV)-activated glass fibre paper, during treatment with excess acidic hydrogen peroxide solution, was directly proportional to the weight of surface-bound titanium(IV)/unit surface area. In a control experiment, a much slower rate was found for the release of titanium(IV) from titanium(IV)-activated glass fibre in hydrochloric acid alone. The initial surface concentration of titanium(IV) was thereby estimated to be 27.7 $\mu g\ cm^{-2}$.

[81] J. F. Kennedy and J. Epton, *Carbohydrate Res.*, 1973, **27**, 11.
[82] F. Fiegl and V. Angdr, 'Spot Tests in Inorganic Analysis' 6th edn. Elsevier, Amsterdam, 1972, 489.

Thus, on considering the available evidence, titanium(IV)-activated supports are best described as materials that have thin films of hydrous titanium(IV) oxide. Since the ability of free hydrous titanium(IV) oxide to bind proteins is well established (see later), this description of titanium(IV)-activated supports accords well with their own ability to bind proteins.

In view of their probable surface–structural similarities, titanium(IV)-activated supports and free hydrous titanium(IV) oxide may be expected to offer similar surface microenvironments and similar possibilities for protein binding, *i.e.* the ligand exchange–chelation phenomena already described holds for the case of the hydrous oxide. In some cases the support may be chelated, as well as the bound molecule, according to the availability of suitable functional groups on its surface. Thus in the previously-described derivatization of papain, comparison of the stabilities of various papain conjugates of titanium(IV)-activated supports signifies that such films are weakly bound to non-porous materials of low hydrophilicity, such as polypropylene, but are strongly bound to porous polyhydroxylic supports, such as glass fibre. The latter, after titanium(IV) activation, therefore provide a most satisfactory support for the insolubilization of papain, and thus the papain conjugate of titanium(IV)-activated glass-fibre paper exhibits high protein loading, high specific activities, good catalytic stability and a broad profile of pH *versus* esterolytic activity. The physical form of this papain conjugate also permits its rapid separation from substrate solutions. Titanium(IV)-activated polypropylene and titanium(IV)-activated zirconia are less satisfactory as supports for papain: the former because of its chemical instability and the latter because of its mechanical instability.

On the basis of the foregoing consideration, we also turned our attention to the use of insoluble hydrous metal oxides *per se* for immobilization phenomena. Hydrous titanium(IV) oxide was produced directly by hydrolysis of titanium(IV) chloride.[83] 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 an ammonia: titanium ratio (*n*) value of $n = 4.85$. The kinetics of coagulation of hydrous titanium(IV) oxide sols has been studied;[84] 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.[85] The conditions used in our 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 insolubilization process would be more desirable from an economic point of view. However, four co-precipitation methods all resulted in extensive enzyme inactivation and it is undoubtedly best to precipitate the support, allow it to settle, remove the supernatant, and then add a solution of the molecule to be coupled.[83]

[83] J. F. Kennedy and I. M. Kay, *J.C.S. Perkin I*, 1976, 329.
[84] S. Bandyophadhyay, *J. Indian Chem. Soc.*, 1963, **40**, 173.
[85] V. K. Srivastava and R. S. Rai, *Kolloid-Z.*, 1963, **190**, 138.

Thus samples of the hydrous metal oxides for use in immobilization are prepared from solutions of their chlorides [titanium(IV) chloride, zirconium(IV) chloride] by the slow addition of 2.0 M—NH_4OH to neutrality (pH 7.0). The samples are washed with saline solution (0.9% w/v, 3 × 5.0 ml) to remove ammonium ions.

Thus investigation of a number of gelatinous, hydrous metal oxides established that several, including those formed from copper(II), iron(III), tin(II), titanium(III), titanium(IV), vanadium(III), and zirconium(IV) are capable of forming, with a variety of molecules, insoluble complexes.[86] Proteins, peptides, amino-acids *etc.* could all be effectively immobilized. Those hydrous oxides from cobalt(II), chromium(III), manganese(II), and tin(IV) do not necessarily behave in the same way.

Of the metal hydroxides exhibiting protein-binding ability the hydrous oxides of iron(III), tin(II), titanium(IV), vanadium(III), and zirconium(IV) are the most effective. Variation of some of the coupling parameters for formation of a titanium(IV) hydroxide–peptide complex shows that the preferred pH of coupling is *ca.* 7 and that the higher the coupling temperature the less easily is the complex formed.

However, during the production of a series of enzyme derivatives of the hydrous oxides of iron(III), tin(II), titanium(III), and vanadium(III), a number of problems were encountered. The vanadium(III)- and tin(II)-containing samples were especially awkward to deal with, the supernatant liquids being difficult to clarify and the solid undergoing rapid oxidation (as evidenced by colour changes). Titanium(IV) and zirconium(IV) give acceptable immobilized preparations.

Using D-glucose oxidase as the example for a more detailed study, variation of duration of coupling time shows a levelling off of both bound activity and bound protein after two hours.[83] For lesser coupling times higher specific activities of the protein (but smaller total activities) occur. These may 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 immobilized on the surface.

The variation of bound activity with pH of coupling 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.

[86] J. F. Kennedy, S. A. Barker, and J. D. Humphreys, *J.C.S. Perkin I*, 1976, 962.

(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 immobilization 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 immobilized enzyme is not easily removed by washing. The ammonia: titanium ratio, n, affects the apparent volume of the precipitate, this being a maximum at $n = 4$.[87] 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. As discussed previously, the conditions of preparation govern the pore size of the hydrous oxide. Clearly the pore effect may influence the immobilization 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.

(v) The age of the hydrous titanium(IV) oxide. As discussed previously, the hydrous oxides change their structures on ageing, but the aged structure retains chelating ability and there is no evidence to suggest that any ageing process over a matter of weeks is detrimental to the matrix–enzyme *etc.* bond. Clearly there is no need for any time gap between preparation of the matrix and coupling stages.

The effect of increasing the concentration of enzyme present for a constant amount of hydrous titanium(IV) oxide is that the bound activity increases rapidly at low enzyme concentrations, but at higher enzyme: oxide ratios the increase is less pronounced. 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.

Analogous work has shown that hydrous titanium(IV) oxide, on exposure to a dilute solution of papain (EC 3.4.22.2) at pH 7.0 binds the enzyme.[88] The resultant water-insoluble hydrous titanium(IV) oxide–papain conjugate exhibits substantial esterolytic and proteolytic activity, a broad profile of pH *versus* esterolytic activity, and stable activity during storage in aqueous suspension at 4 °C. The profile of pH *versus* esterolytic activity of hydrous titanium(IV) oxide–papain conjugate, compared with that of papain, shows a substantial shift in the alkaline

[87] T. F. Limar', A. I. Savos'kina, V. I. Andreeva, and V. V. Mank, *Russ. J. Inorg. Chem.*, 1969, **14**, 1213.
[88] J. F. Kennedy and V. W. Pike, *J.C.S. Perkin I*, 1978, 1058.

limb to higher pH. It has been suggested that hydrous titanium(IV) oxide consists of positively charged particles above pH 3.5.[89] This evidence indicates that hydrous titanium(IV) oxide perturbs the profile of pH by acting as a polyanionic surface in the pH range of papain activity, effecting a decrease in the local pH at the surface of the water-insoluble enzyme compared with that in the bulk solution. Chemical modification and conformational distortion of papain are also expected to contribute to the perturbation of the profile of pH *versus* esterolytic activity of the enzyme on binding to hydrous titanium(IV) oxide.

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

For the hydrous zirconium(IV) oxide–enzyme complexes, the retentions, on coupling, of activities assayed by using a high molecular weight substrate (trypsin activity 6%; chymotrypsin activity 4%) are much lower than those assayed with low molecular weight substrates (D-glucose oxidase activity 75%; β-D-glucosidase activity 31%).[86] This situation holds for enzyme immobilization on many matrices and is attributable to the inability of large substrate molecules to diffuse to the active site of the enzyme, owing to steric interactions with adjacent immobilized enzyme molecules and with the solid support.

A more detailed study of the complexation of D-glucose oxidase with hydrous zirconium(IV) oxide indicates that the contact time allowed for complexation is not critical provided that it is greater than 0.5 h; the maximum activity of the complex, amount of protein coupled, and specific activity of bound enzyme being reached after *ca.* 2 h.

As expected, variation of the initial pH of the hydrous oxide suspension has a noticeable effect upon enzyme complex formation. The maximum specific activity obtained was about 50% of that of the free enzyme. It has been claimed that the two hydroxy-groups are usually replaced by other ligands, but in the use of amino-acids as models for the study of the binding processes it was found that the L-glutamic acid– and L-lysine–hydrous zirconium(IV) oxide complexes contain a maximum of two and four molecules of amino-acid per zirconium atom, respectively. Clearly both the carboxy- and amino-groups are potential ligands, but physico-chemical investigation of the complexes is difficult since they are amorphous gels which undergo a permanent structural change on drying. However, the soluble zirconium(IV) ion, which has an identical tetrameric structure, is known to form a complex with glycine[90] in which there are two glycine molecules per zirconium atom, the zirconium atom being chelated by both oxygen atoms of the carboxy-group of the amino-acid. Competition between the amino- and carboxy-groups and the different molar ratio of these groups may be responsible for the higher L-lysine:zirconium ratios. However, the actual situation is

[89] A. M. El-Atrash, A. M. Azzam, and N. K. Ghattas, *J. Radioanalyt. Chem.*, 1974, **23**, 17.
[90] L. N. Pankratova and G. S. Kharitonova, *Zhur. neorg. Khim.*, 1972, **17**, 2653.

more complex since it was discovered that these zirconium complexes, although apparently containing a maximal amount of amino-acid can still complex significant amounts of enzyme. This secondary binding may be advantageous.

Dextranase (EC 3.2.1.11) immobilized on hydrous zirconium(IV) oxide possesses a near-normal pH-activity profile, as does the enzyme on the zirconium hydrous oxide–L-lysine complex.[86] Dextranase retains a high degree (61%) of its specific activity on direct immobilization and an even higher retention (85%) is achieved for the hydrous zirconium(IV) oxide–L-lysine complex. The L-lysine may act as a 'spacer' between the enzyme and the support, holding the enzyme away from the matrix and thus reducing the number of enzyme–hydrous oxide linkages and thereby facilitating access of substrate molecules to the active site. The pH-activity profile of dextranase was greatly sharpened on immobilization on hydrous zirconium(IV) oxide–L-glutamic acid complex, possibly owing to the effect of the acidic microenvironment (70% maximum activity achieved).

A logical extension of the immobilization of enzyme by attachment to water-insoluble materials, especially where multi-stage enzymic reactions are being considered, is the immobilization of micro-organisms, which are often the source of many enzyme preparations. The advantages of such an approach are immediately obvious. The tedious and time consuming procedures for enzyme extraction and purification are instantly eliminated, co-factors and co-enzymes are readily at hand, the cellular enzymes are often organized into the requisite metabolic pathways, and problems associated with enzyme instability may also be avoided. Furthermore, the use of immobilized cells would avoid the problem in industrial processes of separating the product from the enzyme. The immobilization of cells by the standard glutaraldehyde procedure, however, causes their decease, and entrapment in polyacrylamide gel is commonly used instead to achieve immobilization. This method produces a minimum of interaction between the microbial cell and the insoluble matrix, thus maximizing the probability of the cell's survival. On the other hand, the availability of the cell to any intended substrate is seriously reduced since it can reach the cell only by diffusion.

The procedure for cell immobilization on the hydrous metal oxides is equally as simple as for soluble entities.[91] In the case of *Escherichia coli*, the rate of oxygen uptake of the immobilized cells was ~30% of that of the same number of free cells, showing that respiration of the cells can continue when the cells are immobilized. The reduced rate of oxygen uptake is probably caused by the restriction, by the hydrous metal oxide, of access of aerated buffer to the cells and a decrease of the area of cell surface available for oxygen transfer.

To show that the cells were firmly attached to the surface of the metal hydrous oxide and not just loosely trapped in the gelatinous matrix, a number of types of cells (*e.g. Saccharomyces cerevisiae*) including coloured ones (*e.g. Serratia marcescens*) have been immobilized. Solutions of bicarbonate, phosphate, fluoride *etc.* ions are ineffective in any attempted release of the immobilized cells from the matrix.

[91] J. F. Kennedy, S. A. Barker, and J. D. Humphreys, *Nature*, 197, **261**, 242.

Cells may also be immobilized at low pH values (pH 2—5), hydrous titanium(IV) oxide being more suitable on account of its greater acid resistance. This phenomenon is useful since not all micro-organisms exist in a neutral pH environment (*e.g. Lactobacillus* and *Acetobacter*) and enabled us to produce a small scale immobilized cell reactor. *Acetobacter* cells, immobilized on titanium(IV) hydrous oxide, were able to produce acetic acid from an alcoholic medium in a continuous reactor, often at rates higher than those obtained when free cells were used [a basic rate 87 g acetic acid per day (96% conversion) increased to 263 g per day (99% conversion) after immobilization], thus demonstrating the practicability of this immobilization method.[91] Applications of this phenomenon have resulted in the successful establishment of lower fermentors for the continuous conversion of ethanol to acetic acid.[92] If this simple means of cell immobilization was applied to other micro-organisms it could well result in further immobilized cell reactors of this sort, for the selective production of commercially important biochemical and pharmaceutical compounds. Furthermore, we conclude from the results that immobilized cells can retain their activity for a matter of weeks at least.

The purposes and potential of immobilized antibiotics have already been described, and hydrous metal oxides have been used as supports for their preparation.[93] Chelation of lathumycin, a cyclic peptide antibiotic, with hydrous metal oxides of iron(III), tin(II), titanium(IV), vanadium(IV), and zirconium(IV) gives products retaining a number of antibacterial activities. Similar active products have also been prepared from ampicillin, chloroamphenicol, neomycin, penicillin G, polymyxin B, and streptomycin. It is interesting that the hydrous metal oxides themselves exhibit antibacterial activity—this may be due to a considerable degree of chelation and thereby immobilization and arrest of a bacterial cell upon contacting the hydrous oxide. When antibiotic is present, however, the action of the hydrous oxide *per se* is blocked.

In order to extract full benefit from the use of a water-insoluble enzyme to catalyse a chemical reaction it may be necessary both to be able to recover conveniently water-insoluble enzyme from treated substrate solution and, for many applications, to be able to treat a feed of substrate solution continuously. Columns and stirred tanks are both reactor-types specifically designed to meet these two requirements. However, freshly prepared hydrous titanium(IV) oxide is both amorphous and finely particulate and may therefore be unsuitable for use in either reactor-type. Although both filtration and centrifugation are adequate for the complete removal of hydrous titanium(IV) oxide from suspensions, both processes require substrate solution to be treated in batches, are unsuitable for rapid separations, and may be inconvenient for separations from large volumes of suspensions. Thus the ease of operation and recovery of hydrous titanium(IV) oxide–enzyme conjugates may be somewhat limited by their

[92] J. F. Kennedy, in 'Enzyme Engineering', Vol. 4, ed. G. B. Broun, G. Manecke, and L. B. Wingard, Plenum Press, New York, 1978, p. 323.

[93] J. F. Kennedy and J. D. Humphreys, *Antimicrobial Agents and Chemotherapy*, 1976, **9**, 766.

physical form, although some advantage may be achieved for columns by packing together with added spacer/filler. The rapid removal of immobilized enzyme from substrate solution at the end of reaction is feasible if the water-insoluble enzyme has the physical form of a flat surface-coating based on an inert support; the immobilized enzyme may then be mechanically withdrawn from substrate solution. Therefore, the possibility of binding papain to hydrous titanium(IV) oxide included in surface-coating materials attached to glass has been considered.

Hydrous titanium(IV) oxide may, in fact, be included in surfaces of emulsion paint or epoxy-resin and still retain an ability to bind papain.[88] This property of hydrous titanium(IV) oxide forms the basis of a novel procedure for the convenient preparation of surface-coating–enzyme conjugates exhibiting catalytic activity. Papain bound to hydrous titanium(IV) oxide that had been included in emulsion paint was found to possess substantial specific esterolytic activity and to maintain activity during storage, whereas papain bound to hydrous titanium(IV) oxide that had been included in epoxy-resin was found to possess lower specific esterolytic activity and to be less stable during storage. Such surface-coating–enzyme conjugates, having enzyme attached to a single surface, may be conveniently operated against continuous feeds of substrate solution and the surfaces (of any size or shape) may be pre-formed exactly to suit requirements. Further, since such conjugates are easily withdrawn from substrate solution, catalysis may be terminated rapidly. It has also been demonstrated that unmodified surfaces of glass, epoxy-resin, and emulsion paint form conjugates with papain by adsorption but that such conjugates are unstable to storage in substrate solution.

Papain protein bound to emulsion paint, modified to contain hydrous titanium(IV) oxide, was found to possess both greater specific esterolytic activity and greater specific proteolytic activity than papain protein bound to modified epoxy-resin. A possible explanation of these observations is that emulsion paint provides a more hydrophilic microenvironment than does epoxy-resin. Even so, both the specific esterolytic and specific proteolytic activities of papain protein bound to modified emulsion paint are substantially lower than the corresponding values of papain protein bound to free hydrous titanium(IV) oxide and this suggests that extra steric restrictions on the diffusion of substrate also prevail in the emulsion paint microenvironment. The exceptionally good stability of the esterolytic activity of the papain conjugate of modified emulsion paint during storage in substrate solution is further evidence that the emulsion paint microenvironment provides some hydrophilicity.

In fact the use of a surface of hydrous metal oxide based on a conformable support is not confined to incorporation of the active material within the support as above. Provided the correct conditions are used, the hydrous metal oxide can be directed to be coated, during the precipitation process, upon surfaces.[94] It appears that surfaces of particles, in particular, are amenable to the coating process, and by careful selection the bulk density of the hydrous oxide may be increased and

[94] J. F. Kennedy, S. A. Barker, and C. A. White, *Die Stärke*, 1977, **29**, 240.

thereby the precipitation and sedimentation characteristics improved. This is certainly important where colloidal suspensions or undissolved particles other than the immobilized enzyme particles are present.

A logical extension of the foregoing is the hydrous metal oxide coating of magnetic materials, since enzymes immobilized on magnetic supports will have the added advantage that they can be held back against any liquid flow by an external, non-disrupting force. The speed and ease of removal by a magnetic field ensures that any washing or incubation process can be finished promptly and efficiently. This is useful where a strict control of solution temperature or reaction time is required. Also no internal stirrer is necessary. However, known magnetic materials do not in their own right react with proteins *etc.* Under certain conditions, hydrous titanium(IV) oxide forms a coating on magnetic iron oxide[94] [iron(II) di-iron(III) oxide]. Since freshly prepared hydrous titanium(IV) oxide adsorbs ammonium chloride to give a very gelatinous precipitate,[87] which was considered to be unacceptable in the present instance, liquid titanium(IV) chloride was used in place of titanium(IV) chloride solutions in hydrochloric acid for making the freshly prepared hydrous oxide; the amount of ammonium chloride produced is thereby kept to a minimum. This coating is stable, *i.e.* is not easily stripped off, under conditions used in enzyme-catalysed reactions. Yet the coating retains all the chelating ability displayed by hydrous titanium(IV) oxide *per se* and thus the coated particles are effective, high density, magnetic matrices for immobilization phenomena.

In the case of α-amylase K (see EC 3.2.1.1),[94] this method of immobilization produces a product with a greater stability of the enzyme, allowing its use at elevated temperatures.[94a] For these reasons the method of producing α-amylase immobilized on hydrous titanium(IV) oxide-coated magnetic iron oxide was considered to be one of the best of the methods reported herein for the industrial degradation of starch. Its use will result in preparation and purification of degraded starch by a route which is easier than the traditional chemical degradation methods.

Coatings of hydrous titanium(IV) oxide have also shown promise for the improved abstraction of carbohydrates from solution by an immobilization process.[95] Cellulose and magnetic iron oxide so coated readily adsorb branched polysaccharides, such as glycogen, but have low affinities for monosaccharides. The hydrous oxide, as a dried powder, has a lower maximum adsorption showing the beneficial effect of its coating on cellulose. Conversely, freshly-prepared hydrous titanium(IV) oxide, which has been neither washed nor dried, has a greater maximum adsorption of glycogen, whilst the generation of the hydrous oxide in solutions of glycogen further increases the maximum adsorption of glycogen.

The uptake of carbohydrates by hydrous titanium(IV) oxide, either alone, or as coatings on cellulose or magnetic iron oxide, most probably proceeds by the above described ligand replacement mechanism by hydroxy groups from the

[94a] J. F. Kennedy, C. A. White, and C. L. Riddiford, *Die Stärke*, 1979, in the press.
[95] J. F. Kennedy, S. A. Barker, and C. A. White, *Carbohydrate Res.*, 1977, **54**, 1.

carbohydrate molecules (Figure 9). Since D-glucose, glycogen, and starch possess vicinal-diol groups, it is probable that they form chelates. Whereas glycogen and starch can form only one chelate per D-glucose residue, a number of chelates can be formed with D-glucose. Complexation may also occur with single hydroxy

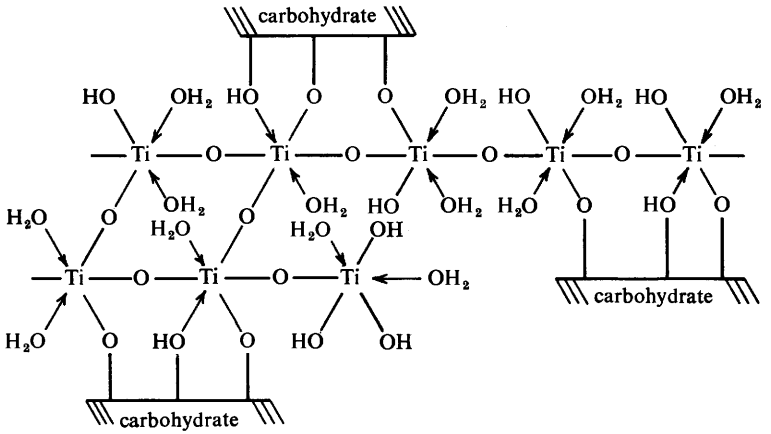


Figure 9 *Representative structures of hydrous titanium*(IV) *oxide–polysaccharide chelate*

groups in the carbohydrate. Furthermore, the formation of one chelate bridge will result in a greater adsorption of polysaccharides than monosaccharides. This explains why the maximum adsorption for D-glucose (0.09 mg mg^{-1} of titanium) was ~90 times lower than that for glycogen (8.40 mg mg^{-1} of titanium) when the respective carbohydrate solutions were used in the initial hydrolysis of titanium(IV) chloride. The adsorption of D-glucose is, however, less subject to diffusion factors than that of glycogen, as shown by the difference in the maximum adsorption of D-glucose and glycogen for oven-dried hydrous titanium(IV) oxide.

The pH dependence of the adsorption of glycogen by hydrous titanium(IV) oxide-coated iron oxide can be explained in terms of the precipitation of the hydrous oxide. At low pH (~3.0), little hydrous oxide is precipitated, as can be seen by the colloidal nature of the solution during precipitation. As the pH of the solution is increased above 4.0, precipitation takes place, a coating is formed, and the adsorption increases. Increasing the pH above 7.0 causes complete precipitation of the hydrous oxide and the coating becomes thicker, allowing more cross-linking of the hydrous oxide chains to occur; this results in a lower surface area:volume ratio and a consequent lowering of the maximum adsorption. By increasing the amount of titanium(IV) chloride present, precipitation and coating formation starts at a lower pH because of the increased concentration of titanium(IV) species; however, the thickening of the coating and consequent dehydration also starts at a lower pH, resulting in a shift in the maximum adsorption to a lower pH for higher titanium(IV) chloride:iron oxide ratios.

The adsorption of carbohydrates from solution by these means is to some

extent analogous to the adsorption of polysaccharides by polyaromatic surfaces.[96] However, the adsorption by hydrous titanium(IV) oxide is less specific than that by polyaromatic surfaces, as D-glucose is also adsorbed. Accordingly, it is envisaged that the hydrous titanium(IV) oxide will be used for purification purposes by removal of carbohydrate contamination, rather than the analytical process of affinity chromatography envisaged for the polyaromatic surfaces.

As an aside to this work with hydrous zirconium(IV) oxide, we discovered that the gelatinous precipitate can catalyse the hydrolysis of 4-nitrophenyl phosphate although the rates of hydrolysis of a range of phosphate esters and related compounds are not altered.[97] This reaction appears to involve an initial interaction between the insoluble hydrous oxide and inorganic phosphate ions produced by hydrolysis, followed by heterogeneous catalysis of the hydrolytic reaction by the mixed hydroxy–phosphate complex so produced. The reaction thus seems to be a specific one between the metal hydrous oxide and 4-nitrophenyl phosphate and in this respect could be regarded as pseudoenzymic.

6 Use of Titanium Oxides *etc.* in the Field of Immobilization

It is clearly predictable from all the foregoing theories of chelation that the corresponding metal oxides *per se* cannot be expected to bind proteinaceous *etc.* species to the same degree, on account of their inability to chelate. However, as described earlier, the oxides can in fact possess a water layer and this may in part give rise to hydrous oxide centres. Such a situation is in fact borne out by the fact that titanium(IV) oxide will bind enzyme.[98] Treatment of particles of porous titanium(IV) oxide with dextranase (EC 3.2.1.11) in the presence and absence of ammonium ions showed that the presence of ammonia induces a greater coupling of protein, whereas a greater retention of enzyme specific activity is achieved in the absence of ammonia. However, unlike the hydrous oxide case, the coupling between enzyme and support is easily reversible, indicating the necessity of the chelating-type support for production of a stable product, and the comparative non-utility of oxide *per se*.

Titanium(IV) oxide *per se*, by virtue of its high stability and relatively inert character does, nevertheless, provide a basic support for immobilization phenomena. Thus coating titanium(IV) oxide particles with diazotized 1,3-diaminobenzene yields a product which can be substituted covalently with enzyme *etc.* according to conventional organic chemistry. Dextranase has been successfully immobilized in this way.[99] Alternatively, a more porous, polar surface may be provided for the aromatic coating, leading to greater adsorption properties, by using hydrous titanium(IV) oxide. An example of this is the immobilization of α-amylase on diazotized 1,3-diaminobenzene, coated on hydrous titanium(IV) oxide, coated on magnetic iron oxide.[94]

Titanium(IV) oxide may also be used in the immobilization of enzymes on

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inorganic supports – such are activated by the surface deposition of an imperfectly crystallized film of titanium(IV) oxide.[100] The stability of the preparations is strongly dependent on the integrity of the support-oxide linkage which is in turn dependent on the pretreatment of the solid support. The enzyme (*e.g.* glucoamylase EC 3.2.1.3) may be linked to the film using 5-aminosalicylic acid (with or without diazotization), tannic acid, or tin(II) chloride, or by direct contact. Basic support material such as glass gives products with glucoamylase and β-D-glucosidase (EC 3.2.1.21) possessing excellent activities and operational stabilities.[100,101]

Thus, once the hydrous metal oxides have been heated and dehydration effected, they no longer possess their former chelating (adsorptive) powers. The resultant oxides *per se*, however, do provide useful core materials, along with other inert materials, on which to build up reactive immobilization-orientated matrices.

7 Conclusions

In conclusion, it may be stated that hydrous oxides of titanium and zirconium in various forms are effective matrices for immobilization. Their advantages include their low cost, their convenient preparation (which may be conducted in any location without specialized facilities), the absence of any need for pre-preparation, their ability to couple enzyme at neutral pH, the high retentions of specific activity of the enzyme on immobilization, and the ability of modification to exert microenvironmental effects on, and thereby alter the characteristics of, the immobilized enzyme. The success of the preparation and activity of the matrix is guaranteed and, unlike the majority of immobilization matrices, does not require use of spectroscopy, titration, *etc.* to confirm the reactivity of the preparation in hand. The form of the hydrous oxide may be modified with ease to give high or low density, magnetic or non-magnetic, products of a variety of sizes, shapes, and uses.

Although much of the work conducted hitherto has employed (carbohydrate-directed) enzymes as models, on account of the additional, biological testing parameter afforded thereby, it is clear that the hydrous metal oxide matrices are equally suitable for the immobilization, removal, and recovery of antibodies, antigens, affinants, amino-acids, antibiotics, carbohydrates, cells, enzymes, glycoproteins, lectins, peptides, *etc.* This necessarily gives rise to a number of possible investigations of the properties of such compounds *etc.* in the fields of pure, biological, and clinical chemistries.

Inevitably, this article must describe only a part of my research interests in the field of carbohydrates and associated molecules. Other interests in carbohydrate

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chemistry which broadly come under the umbrella of polysaccharides, glycoproteins, and proteoglycans have been described elsewhere.[76,102–108]

I do indeed regard it a very great honour that I should receive the Carbohydrate Award and am very grateful to Tate and Lyle Ltd. and the Chemical Society for giving it to me. I am also grateful to the numerous colleagues, relatives, friends, and research students who have participated in and encouraged me in the work which has led to this success.

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