Insoluble Complexes of Amino-acids, Peptides, and Enzymes with Metal Hydroxides†

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Investigation of a number of gelatinous metal hydroxides has established that several (*e.g.* those formed from Ti^{IV}, Zr^{IV}, Fe^{III}, V^{III}, and Sn^{II}) are capable of forming with enzymes insoluble complexes which are enzymically active. From the practical viewpoint Ti^{IV} and Zr^{IV} proved the most satisfactory. Comparatively high retentions of enzyme specific activity may be achieved. Complexes of these metal hydroxides may also be formed with peptides and amino-acids. All these complexes are considered to be formed by the re-occupation of ligand sites on the metal ions by the incoming molecules. The metal hydroxide–amino-acid complexes can also subsequently bind an enzyme molecule, to give a product the pH–activity profile of which may differ from that of the soluble form of the enzyme. Thus by this technique it may be possible to alter the pH optimum of an enzyme. The peptide ligand can be displaced from the complex by phosphate or fluoride ions, or by any other such entity capable of forming a more stable complex with the metal ion involved.

RECENT research into means of insolubilising or immobilising enzymes has centred chiefly upon the formation of a stable, covalent bond between the enzyme and a water-insoluble support. The many methods of achieving this, and the supports used, have been extensively reviewed.[1-11] Whilst such techniques promise to be the best means of producing an insoluble enzyme for practical applications, they do not provide the closest analogues to naturally occurring immobilised enzymes.

In biological systems, enzymes often exist in close association with cellular membranes, to which they are

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bound by weak interactions such as adsorption, electrostatic attraction, or entrapment.[12,13] Such processes provide a simple route [14] to immobilised enzymes, often with retention of a large proportion of the enzymic activity. However, in the majority of cases the lack of stability of the conjugates renders them unsuitable for practical purposes, since it is impossible to prevent the enzyme from being leached into solution during use. Changes in temperature, pH, or ionic strength of the environment often accelerate this effect. On the other hand immobilisation by covalent binding often unavoidably involves reaction with the enzyme at more than one location in the enzyme molecule, thus reducing its specific activity in bound form unless special designs are brought into the matrix.[15]

Thus a matrix which permits a more loose type of binding but which is stable to conditions of normal use of enzymes is desirable, and this has been achieved in a novel but simple process by coupling the enzyme to various insoluble transition metal hydroxides or hydrous oxides. In the titanium system [16] it was shown that, in the production of the hydroxide when the ions were allowed to undergo hydrolysis (*i.e.* become co-ordinated by OH ligands) before the protein was added, the amount of protein bound was related to the amount of titanium hydroxide present. Thus a novel matrix could be formed as required without any pre-preparation, the only requirement being a solution of titanium chloride.

We now report studies in greater detail of the types of hydroxides which can be used, types of molecules which can be immobilised by such a process, and the potential applications of the process.

EXPERIMENTAL

Preparation of Metal Hydroxide Samples.—Metal hydroxides were prepared from the following: copper(II) chloride, iron(II) chloride (hydrated), tin(II) chloride, titanium(IV) chloride (15% w/v in 15% w/v hydrochloric acid), vanadium(III) chloride and zirconium(IV) chloride (B.D.H.Ltd.), chromium(III) chloride, cobalt(II) chloride, iron(III) chloride (hydrated), manganese(III) chloride, tin(IV) chloride, and zinc(II) chloride (Hopkin and Williams Ltd.) analytical or standard grade reagents. In a standard method for the preparation of the metal hydroxides, solutions were prepared by dissolving the requisite amount of metal chloride in 1.0M- [5.0M- for tin(II) chloride] hydrochloric acid to give a 0.65M-solution, except for titanium(IV) chloride which was used as supplied. The hydroxides were precipitated by slow addition of 2M-ammonium hydroxide to the metal chloride solutions (1.3 mmol) to give the desired pH and to ensure that the gelatinous suspension formed was homogeneous.

Complexation of Amino-acids by Zirconium Hydroxide.—Solutions (1.3M; 0.2—6.0 ml) of L-lysine and L-glutamic acid, adjusted to pH 7.0 with 2.0M-sodium hydroxide, were added to suspensions of zirconium hydroxide (pH adjusted 7.0; amount as above) to give a range of mixtures with an initial amino acid : zirconium(IV) molar ratio between 0.2 : 1 and 6 : 1. After dilution to 10.0 ml (total) with water, the mixtures were stirred at 22 °C for 2 h, and centrifuged. The amounts of amino-acid complexed were determined by spectrophotometry of the supernatant at 280 nm; standard solutions of the amino-acids were used for calibration (Table 1).

TABLE 1

Complexation of amino-acids with zirconium hydroxide

Amino-acid	Initial molar ratio of amino-acid to Zr	% Amino-acid complexed	Molar ratio of amino-acid to Zr in complex
L-Glu	6	38	2.3
	3	71	2.1
	2	78	1.6
	1	85	0.85
	0.5	>85	0.4
	0.2	>85	0.2
L-Lys	6	62	3.8
	3	65	1.9
	2	70	1.4
	1	74	0.7
	0.5	80	0.4
	0.2	82	0.2

Complexation of a Peptide by Various Metal Hydroxides.—To a suspension of metal hydroxide (methods and amounts as above; pH adjusted to 7.0) was added the peptide solution [aqueous (0.1% w/v) lathumycin, a cyclic peptide antibiotic [17] (1.0 ml)]. After dilution to 10.0 ml with water, the mixtures were stirred at 26 °C for 2 h. After centrifugation, the amount of peptide remaining in solution was determined by spectrophotometry at 336 nm (the wavelength of maximum absorbance of lathumycin under these conditions). The percentage of peptide insolubilised was then calculated (Table 2) by reference to a calibration curve for a lathumycin standard.

TABLE 2

Complexation of a peptide (lathumycin) by various metal hydroxides

Metal hydroxide	% Peptide insolubilised	Metal hydroxide	% Peptide insolubilised
Ti^{IV}	56	Fe^{III}	71
Zr^{IV}	92	Co^{II}	0
V^{III}	88	Cu^{II}	17
Cr^{III}	4	Zn^{II}	0
Mn^{II}	0	Sn^{II}	95
Fe^{II}	20	Sn^{IV}	4

Effect of Temperature and pH on the Complexation of Peptide by Titanium(IV) *Hydroxide.*—Suspensions of titanium(IV) hydroxide (method and amount as above) were adjusted to pH 6—8 and to each was added 0.1% w/v lathumycin solution (1.0 ml). After dilution to 10.0 ml as before, the mixtures were stirred at various temperatures for 2 h. After centrifugation, the amount of peptide remaining in solution was determined as above and the percentage insolubilised calculated therefrom (Table 3).

Release of Peptide from its Zirconium Hydroxide Complex.—Suspensions of zirconium hydroxide (method and amount as above; pH adjusted to 7.0) were treated with 0.1% w/v lathumycin solution (2.0 ml) and distilled water to give a total volume of 10.0 ml. The mixtures were stirred at

[12] R. H. Hopkins, Proceedings of the European Brewing Convention (Baden-Baden Congress), 1955, p. 315.
[13] J. O. Lampen, *Antonie van Leeuwenhoek J. Microbiol. Serol.*, 1968, **34**, 1.
[14] L. Michaelis and M. Ehrenreich, *Biochem. Z.*, 1908, **10**, 283.
[15] J. F. Kennedy, S. A. Barker, and A. Rosevear, *J.C.S. Perkin I*, 1973, 2293; J. F. Kennedy and A. Rosevear, *ibid.*, 1974, 757.
[16] J. F. Kennedy and I. M. Kay, *J.C.S. Perkin I*, 1976, 329.
[17] Dutch P. 106644/1963.

22 °C for 2 h and centrifuged, and the amount (1.8 mg) of lathumycin complexed by the hydroxide was determined by difference as above. Various reagents (see Table 4) were added to the residues (final volume 10.0 ml). The mixtures were stirred at 22 °C for 1 h and centrifuged, and the amount of lathumycin released into solution was determined as before (Table 4).

TABLE 3

Complexation of a peptide by titanium(IV) hydroxide as a function of temperature and pH

	Amount of available lathumycin complexed at various temperatures (%)			
pH	5 °C	9 °C	15 °C	26 °C
6.2	77	76	71	58
7.0	80	75	67	56
7.8	74	54	47	44

TABLE 4

Release of peptide (lathumycin) from zirconium hydroxide–peptide complex

Releasing agent solution added	Amount of complexed peptide released (%)
Glycine (1.0M)	50
Sodium molybdate (1.0M)	72
Potassium fluoride (1.0M)	83
Sodium chloride (1.0M)	11
Disodium hydrogen phosphate (0.5M)	83
Borate buffer (0.1M) (pH 8.0)	22
Acetate buffer (0.05M) (pH 5.0)	6
Tris buffer (1.0M) (pH 8.8)	83

Complexation of Chymotrypsin, D-Glucose Oxidase, β-D-Glucosidase, and Trypsin with Various Metal Hydroxides.— Four samples each of the hydroxides of iron(III), tin(II), vanadium(III), and zirconium(IV) were prepared as above (pH adjusted to 7.0) and to them were added aqueous solutions of chymotrypsin (E.C. 3.4.21.1, Worthington Biochemical Co., activity 2.76 CU_{cas} mg^{-1}; 3.74 mg), D-glucose oxidase (β-D-glucose: oxygen oxidoreductase, E.C. 1.1.3.4, grade I, Boehringer Mannheim GmbH, activity 55 units mg^{-1}; 3.0 mg), β-D-glucosidase (β-D-glucoside glucohydrolase, E.C. 3.2.1.21, Koch-Light, activity 49 units mg^{-1}; 2.15 mg), or trypsin (E.C. 3.4.21.4, Koch-Light, activity 1.19 TU_{cas} mg^{-1}; 2.06 mg) and the total volume was made up to 10.0 ml in each case with distilled water. The samples were stirred for 2 h at 4 °C and centrifuged, and the solids were washed with distilled water (6 × 5.0 ml) at 22 °C. The solids were re-suspended in water (total volumes 5.0 ml) and aliquot portions (1.0 ml) were dried at 120 °C to constant weight. Further aliquot portions of the suspensions (diluted as necessary) were then assayed for enzymic activity as follows.

(*a*) Chymotrypsin: Bergmeyer [18] method (rate of production of trichloroacetic acid-soluble products from casein at pH 8.0; 37 °C). One CU_{cas} is the amount of enzyme which, under the defined conditions, liberates sufficient trichloroacetic acid-soluble products to cause an increase in O.D. at 280 nm of 1.00 unit per minute.

(*b*) D-Glucose oxidase: rate of oxidation of ABTS (ammonium salt of 2,2′-azinobis-[3-ethyl-2,3-dihydrobenzothiazole-6-sulphonic acid]) at 415 nm, pH 7.0, and 26 °C.[19,20] One unit of D-glucose oxidase activity is that which, under the defined conditions, transforms 1 μmol of D-glucose in 1 min.

(*c*) β-D-Glucosidase: the enzymic activity was determined from measurements of the rate of production of 2-nitrophenol from 2-nitrophenyl β-D-glucopyranoside at 37 °C and pH 5.0.[21] One unit of activity is the amount of enzyme which will liberate 1 μmol of 2-nitrophenol per minute under the assay conditions.

(*d*) Trypsin: Bergmeyer [22] method (rate of production of trichloracetic acid-soluble products from casein at pH 7.6; 37 °C). One TU_{cas} is the amount of enzyme which under the defined conditions, liberates sufficient trichloroacetic acid-soluble products to cause an increase in O.D. at 280 nm of 1.00 unit per minute.

Control experiments were also performed by adding uncomplexed metal hydroxides to these assay systems; there was no observable effect. The results of this investigation are presented in Table 5.

TABLE 5

Complexes of chymotrypsin, D-glucose oxidase, β-D-glucosidase, and trypsin with various metal hydroxides

Enzyme	Metal hydroxide	Activity (units g^{-1} of dried hydroxide)	Retention of activity* (%)
Chymotrypsin	Fe^{III}	7.76	23
	Sn^{II}	1.33	4
	V^{III}	8.82	27
	Zr^{IV}	1.40	4
D-Glucose oxidase	Fe^{III}	794	58
	Sn^{II}	250	26
	V^{III}	598	60
	Zr^{IV}	811	75
β-D-Glucosidase	Fe^{III}	289	44
	Sn^{II}	72	11
	V^{III}	76	11
	Zr^{IV}	202	31
Trypsin	Fe^{III}	6.54	43
	Sn^{II}	2.42	16
	V^{III}	13.1	86
	Zr^{IV}	0.87	6

* Retention of activity = [(total enzymatic activity of insolubilised sample)/(total enzymatic activity originally present in solution)] × 100.

Optimisation of the Conditions for Complex Formation between D-Glucose Oxidase and Zirconium Hydroxide.— Samples of zirconium hydroxide were prepared (amount and method as above) to give a range of complexing conditions by variation of the pH of the suspension, the time of contact between suspension and solution of D-glucose oxidase (activity 77.5 units mg^{-1} at pH 5.0), and the amount of enzyme added. The dry weights of the preparations were determined as described above and the immobilised D-glucose oxidase activities were determined essentially as above but at pH 5.0. On account of the alteration of enzyme specific activity on immobilisation, measurements of the activities of the insoluble enzyme preparations do not reflect the amount of bound protein present. Therefore protein contents of the insoluble complexes were determined

[18] W. Rick, 'Methods of Enzymatic Analysis,' ed. H. U. Bergmeyer, Verlag Chemie, Weinheim, 1963, p. 800.
[19] J. F. Kennedy and C. E. Doyle, *Carbohydrate Res.*, 1973, **28**, 89.
[20] J. F. Kennedy and I. M. Kay, *Carbohydrate Res.* 1975, **44**, 291.
[21] R. L. Nath and H. N. Rydon, *Biochem. J.*, 1954, **57**, 1.
[22] W. Rick, 'Methods of Enzymatic Analysis,' ed. H. U. Bergmeyer, Verlag Chemie, Weinheim, 1963, p. 811.

by an accurate independent method based on complete hydrolysis followed by paper chromatography [15] to separate the amino-acids from interfering materials. The amino-acids were detected by spraying with a solution of ninhydrin,

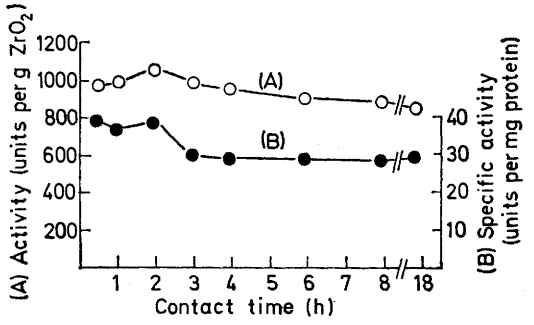

FIGURE 1 Variation of activity (A) and specific activity (B) of D-glucose oxidase immobilised on zirconium(IV) hydroxide with enzyme–zirconium hydroxide contact time. Ranges for the amount of enzymic protein complexed and the retention of specific activity on complexation of the enzyme were 25—34 μg per mg ZrO_2 and 37—51%, respectively. Enzymic activity was measured per g of zirconium hydroxide dried to constant weight (*i.e.* ZrO_2)

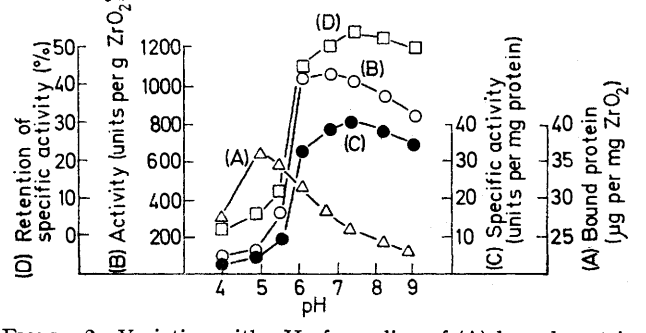

FIGURE 2 Variation with pH of coupling of (A) bound protein (B) activity, (C) specific activity, and (D) retention of specific activity of D-glucose oxidase immobilised on zirconium(IV) hydroxide

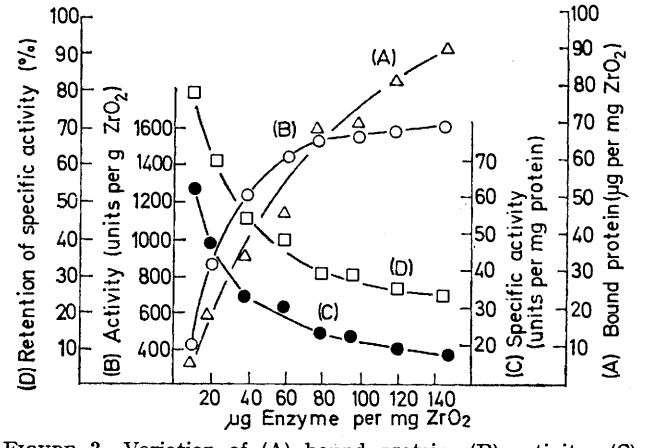

FIGURE 3 Variation of (A) bound protein, (B) activity, (C) specific activity, and (D) retention of specific activity of D-glucose oxidase immobilised on zirconium(IV) hydroxide with enzyme : zirconium(IV) hydroxide ratio

and the spots were eluted from the paper with ethanol and assayed quantitatively by spectrophotometry. The results are given in Figures 1—3. The amount of protein attached in coupling times of 0.5—18 h was 25—34 μg per mg ZrO_2, and the retention of specific activity of attached enzyme in coupling times of 0.5—18 h was 37—51%.

Investigation of the pH Dependence of the Behaviour of Dextranase–Zirconium Hydroxide and Dextranase–Zirconium Hydroxide–Amino-acid Complexes.—Samples of zirconium hydroxide suspension (amount as above; pH adjusted to 7.0) were treated with 0.65M-L-lysine (6.0 ml) or 0.65M-L-glutamic acid (6.0 ml) (molar ratio of amino-acid to zirconium, 3 : 1). Dextranase (α-1,6-glucan-6-glucanohydrolase, E.C.3.2.1.11, Koch-Light, activity 18.7 units mg^{-1}) solution (2.0 mg ml^{-1}; 2.5 ml) was added to each suspension and a further untreated zirconium hydroxide suspension, and the mixtures were stirred at 4 °C for 2 h. After centrifugation and removal of supernatant, the samples were washed with distilled water (3 × 5.0 ml) at 22 °C. Enzymic activity was determined over a range of pH values by the method of Bernfeld [23] (measurement of rate of

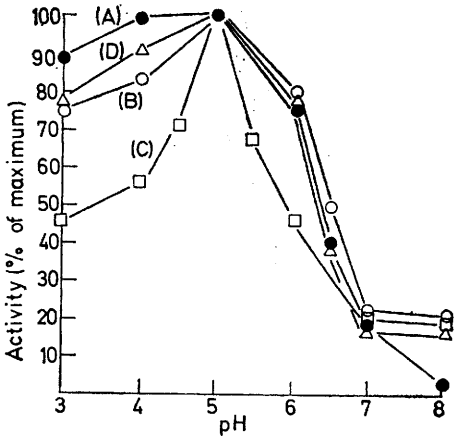

FIGURE 4 Effect of pH on the activity of dextranase (A) in soluble form, and immobilised (B) on zirconium(IV) hydroxide, (C) on zirconium(IV) hydroxide complexed with L-glutamic acid and (D) on zirconium(IV) hydroxide complexed with L-lysine

production of reducing groups at 25 °C, by reaction with 3,5-dinitrosalicyclic acid); one unit of dextranase activity is that which, under the defined conditions, causes an increase in O.D. at 570 nm of 0.01 unit per minute (Figure 4).

DISCUSSION

With a cyclic peptide as the entity to be immobilised, our results indicated that the hydroxides of copper(II), iron(II) and (III), tin(II), vanadium(III), and zirconium-(IV) behave similarly to titanium(IV) [16] and are able to remove appreciable amounts of peptide from solution at neutral pH and at ambient temperature, whereas cobalt(II), chromium(III), manganese(II), tin(IV), and zinc hydroxides do not behave in this way (Table 2). It therefore appeared that the former metal hydroxides were suitable for the production of stable, active, insoluble enzyme derivatives.

Of the metal hydroxides exhibiting protein-binding ability in addition to titanium(IV) hydroxide, the hydroxides of iron(III), tin(II), vanadium(III), and zirconium(IV) were the most effective (Table 2). The results (Table 3) of variation of some of the coupling

[23] P. Bernfeld, *Methods Enzymol.*, 1955, **1**, 149.

parameters for formation of a titanium(IV) hydroxide–peptide complex show 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 metal hydroxides of iron(III), tin(II), vanadium(III), and zirconium(IV), a number of problems were encountered, as may be adduced from the various degrees of retention of enzymic activity on coupling of four enzymes (Table 5). The vanadium- 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 a colour change). Although none of the metal hydroxides interfered with the redox assay system (soluble titanium species have been shown to affect the assay [20]), it was felt that this instability of oxidation state was undesirable. Of these four metal hydroxides tested, only zirconium(IV) gave an acceptable immobilised preparation and so further investigations were concentrated on this metal, which has a single, well-defined, oxidation state. The utility of hydroxides of titanium has already been reported.[16]

For the zirconium hydroxide–enzyme complexes, the retentions, on coupling, of activities (Table 5) 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%). This situation holds for enzyme immobilisation 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 immobilised enzyme molecules and with the solid support.

A more detailed study of the complexation of D-glucose oxidase with zirconium hydroxide indicated that the contact time allowed for complexation was not critical provided that it was greater than 0.5 h (Figure 1), the maximum activity of the complex, amount of protein coupled, and specific activity of bound enzyme being reached after *ca.* 2 h. The subsequent slight decreases in activity and specific activity (protein content constant) on prolonged stirring is attributable to denaturation of the protein caused by mechanical shearing forces.

As expected, variation of the initial pH of the hydroxide suspension had a noticeable effect upon enzyme complex formation (Figure 2). The amount of D-glucose oxidase activity bound to the zirconium hydroxide rose dramatically in the region of pH 5.5, giving a maximum at pH 6.5 which coincides with the reported optimum pH for this enzyme.[24] The maximum specific activity, however, was obtained at pH 7.5 and was about 50% of that of the free enzyme. This suggests that the slightly alkaline microenvironment provided for the enzyme by the hydroxide gel at this pH is just sufficient to counteract the 'unfavourable' assay conditions employed (pH 5) and thus produce an optimum pH environment for the enzyme.

The maximum amount of protein was coupled at pH 5, and since the gelatinous hydroxide is not produced unless the pH of the suspension is greater than 4 (equivalence point is pH 9), protein binding seems to be dependant upon the physical structure of the hydroxide.

Increasing the amount of enzyme offered to a fixed amount of hydroxide showed that the percentage of protein complexed remained constant at 90% until *ca.* 80 μg of enzyme per mg (dry weight) of oxide was reached (Figure 3). After this, the percentage decreased, although no limiting value for the amount of protein complexed was obtained. The curves for activity and specific activity levelled off at the highest amounts of enzyme offered (samples containing *ca.* 10% w/w protein); this is due to limitation of reaction velocity by the aforementioned steric effects. In support of this, the retention of specific activity on binding of the enzyme gradually decreased (from 80 to 20%) with increase in the offered enzyme : hydroxide ratio. However, such retentions of specific activity are all higher than those obtained for the majority of matrices used for enzyme immobilisation.

It has been proposed that freshly precipitated zirconium hydroxide is a tetrameric complex.[25] Since the maximum co-ordination number of the zirconium atom is eight, the maximum number of ligand binding sites available is therefore four. Two of these sites are occupied by hydroxy-groups and the other two may, depending on conditions, be occupied by loosely bound water molecules.

In the formation of the protein (*etc.*)–zirconium (and other metal) hydroxide complexes, the incoming molecules are considered to occupy ligand sites by displacing existing ligands on the zirconium ions or atoms as described [16] and depicted [26] for the complexation of enzymes, antibiotics, *etc.* to a variety of metal chelates of cellulose and to titanium hydroxides. 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–zirconium hydroxide and L-lysine–zirconium hydroxide complexes contained a maximum of two and four molecules of amino-acid per zirconium atom, respectively (Table 1). Clearly both the carboxy- and amino-groups are potential ligands, but physicochemical 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,[27] is known to form a complex with glycine [28] in which there are two glycine molecules per zirconium atom, the zirconium atom being chelated by both oxygen atoms of the carboxy-group of

[24] G. Brown, E. Selegny, S. Avrameas, and D. Thomas, *Biochim. Biophys. Acta*, 1969, **185**, 260.
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[26] J. F. Kennedy, S. A. Barker, and A. Zamir, *Antimicrobial Agents Chemother.*, 1974, **6**, 777.
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[28] L. N. Pankratova and G. S. Kharitonova, *Zhur. neorg. Khim.*, 1972, **17**, 2653.

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 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 an ion-exchange effect.

The ability of the hydroxide to bind amino-acid and enzyme in a two-stage process permitted investigation of variation of the microenvironment of an enzyme immobilised on a metal hydroxide. Dextranase was chosen for this purpose since its pH-activity profile is susceptible to certain microenvironmental changes.[29] Dextranase immobilised on zirconium hydroxide possessed a near-normal pH–activity profile, as did the enzyme on the zirconium hydroxide–L-lysine complex (Figure 4). Dextranase retained a high degree (61%) of its specific activity on direct immobilisation and an even higher retention (85%) was achieved for the zirconium hydroxide–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–hydroxide linkages, and thereby facilitating access of substrate molecules to the active site. The pH–activity profile of dextranase was greatly sharpened on immobilisation on the zirconium hydroxide–L-glutamic acid complex, possibly owing to the effect of the acidic microenvironment. The retention of enzymic activity at the maximum was 70% of that of the free enzyme. In all three cases examined, the pH of maximum enzymic activity was the same as that of the soluble enzyme (pH 5.0).

With a view to regenerating the carrier material after the enzyme *etc.* complex had lost its activity, the reactivation of the surface by removal of complexed material was investigated. With lathumycin as the model, treatment of the complex with various agents (Table 4) released the peptide. Release was considered to occur by displacement of the peptide ligand in favour of formation of a stronger complex.

It is concluded, therefore, that gelatinous zirconium hydroxide is an effective matrix for enzyme *etc.* immobilisation. Its advantages include its low cost, its convenient preparation (which may be conducted in any location without specialised facilities), the absence of any need for pre-preparation, its ability to couple enzyme at neutral pH, the high retentions of specific activity of the enzyme on immobilisation, and the ability of modification to exert microenvironmental effects on and thereby alter the characteristics of the immobilised enzyme. All other types of matrix for immobilisation require more complex methods of preparation. Zirconium(IV) hydroxide matrices are also suitable for the immobilisation of antibodies, antigens, affinants, antibiotics, glycoproteins, proteins, *etc.*

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[29] C. M. Livingstone, Ph.D. Thesis, University of Birmingham, 1974.