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POLY(*N*-ACRYLOYL-4- AND -5-AMINOSALICYLIC ACIDS)

PART III. USES AS THEIR TITANIUM COMPLEXES FOR THE INSOLUBILISATION OF ENZYMES*

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ABSTRACT

The titanous and titanic complexes of the water-insoluble poly(*N*-acryloyl-4- and -5-aminosalicylic acids) have been prepared by several methods, and alpha-amylase, glucoamylase, and polygalacturonase (pectinase) have been coupled to the various preparations. The products from alpha-amylase and glucoamylase were enzymically active, but the alpha-amylase was washed off after only one use. With glucoamylase, the derivative withstood extensive washing and could be used continuously in a column. Particular advantages of the glucoamylase preparation were that maximal coupling of the enzyme was achieved in one hour and that a very high specific activity towards a macromolecular substrate was achieved. The polygalacturonase derivative was inactive, possibly because the polysalicylic acid acts as an inhibitor of the enzyme.

INTRODUCTION

One of the more-significant, recent advances in the chemistry of biologically active molecules has been the insolubilisation of enzymes, with retention of activity, by attaching them to water-insoluble, polymeric matrices. Since many matrices used for insolubilisation have one or more disadvantages, and since it appears that no one method of attachment gives active, insoluble derivatives of all enzymes, the search continues for suitable matrices and new modes of effecting the coupling. Chelation of titanium to cellulose yields[1,2] a complex which can be used as an insoluble matrix for the insolubilisation of such enzymes as alpha-amylase, glucoamylase, trypsin, D-glucose oxidase, and invertase. It has also been shown that salicylic acid forms co-ordination compounds in which both the carboxyl[3] and phenolic[4] hydrogen atoms are replaced by titanium. A further co-ordination compound, in which one titanium atom is co-ordinated to two molecules of salicylic acid, has also been prepared[5]. It therefore seemed that a poly(salicylic acid), in which the carboxyl and phenolic hydrogen atoms were available for co-ordination with titanium, could provide a useful, stable matrix for the insolubilisation of enzymes. Such polymers

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have been prepared by the polymerisation of *N*-acryloyl-4- and -5-aminosalicylic acids to give poly(*N*-acryloyl-4- and -5-aminosalicylic acids)[6]. These polymers are water-insoluble gels suitable for the insolubilisation of biologically active molecules. Thus, by using the ionic nature of these polymers, it is possible to form active, insoluble derivatives of gentamicin and streptomycin[7].

In this paper, the preparation of titanic and titanous complexes of poly(*N*-acryloyl-4- and -5-aminosalicylic acids) is described, and the potential of these complexes for the formation of active, insoluble enzyme-derivatives (as exemplified by certain polysaccharidases) is examined.

EXPERIMENTAL

Preparation of the titanium complexes of poly(N-*acryloyl-4- and -5-aminosalicylic acids.* — (*a*) *Titanous and titanic complexes I.* Poly(*N*-acryloyl-4- and -5-aminosalicylic acids) were prepared by a procedure based on one of the methods previously described[6]. *N*-Acryloyl-4-aminosalicylic acid (15 g) and sodium tetraborate (9.36 g) were dissolved in distilled water (180 ml), and the pH was adjusted to 9.0 with 10M sodium hydroxide. A solution of azobisisobutyronitrile (150 mg) in ethanol (50 ml) was added, and the mixture was heated at 80° for 48 h. The resulting solution was diluted with distilled water (200 ml), and the white polymer (11.1 g, 76%) was precipitated by the addition of 2M hydrochloric acid. After washing with distilled water (10 × 2 l) by decantation, the suspension was rotary-evaporated with methanol (3 × 250 ml) to remove any remaining borate as methyl borate. After a final washing with distilled water (200 ml), the polymer was stored as a suspension in distilled water (200 ml) at 4°. Poly(*N*-acryloyl-5-aminosalicylic acid) was prepared in a similar fashion, using *N*-acryloyl-5-aminosalicylic acid (15 g), to give a pink polymer (14.2 g, 94%).

Poly(*N*-acryloyl-4-aminosalicylic acid) (1 g) was stirred with 12.5% (w/v) titanous or titanic chloride in hydrochloric acid (10 ml) for 20 min, after which the solid was washed with distilled water (100 ml) and filtered off to give poly(*N*-acryloyl-4-aminosalicylic acid)–titanous and titanic complexes I, the colours of which were fawn and bright-yellow, respectively. On storage at 4°, the colour of the titanous complex changed from fawn to bright-yellow.

Similar treatment of poly(*N*-acryloyl-5-aminosalicylic acid) (1 g) yielded poly(*N*-acryloyl-5-aminosalicylic acid)–titanous and titanic complexes I, the colours of which were dark-brown and red, respectively. The dark-brown colour of the titanous complex turned dark-red on storing at 4°.

(*b*) *Titanous and titanic complexes II.* *N*-Acryloyl-4- and -5-aminosalicylic acids were polymerised as in (*a*), but the addition of sodium tetraborate was omitted and the pH was adjusted to pH 4.5 before the addition of initiator, to give the white poly(*N*-acryloyl-4-aminosalicylic acid) (13.2 g, 88%) and the pink poly(*N*-acryloyl-5-aminosalicylic acid) (12.1 g, 80.6%). The poly(*N*-acryloyl-4-aminosalicylic acid) (1 g) was treated with 12.5% titanous or titanic chloride (10 ml) to yield the poly-

(*N*-acryloyl-4-aminosalicylic acid)–titanous and titanic complexes II. Similar treatment of poly(*N*-acryloyl-5-aminosalicylic acid) (1 g) produced the poly(*N*-acryloyl-5-aminosalicylic acid)–titanous and titanic complexes II. The colours and colour stabilities of these four complexes were the same as those observed for the corresponding complexes I.

(*c*) *Titanous and titanic complexes III.* *N*-Acryloyl-4- and -5-aminosalicylic acids were polymerised on a scale 1/15 of that described in (*b*), up to the point where the polymer solution was diluted with distilled water. The titanium complexes were then precipitated by the addition of 12.5% titanous or titanic chloride (10 ml) to produce the poly(*N*-acryloyl-4-aminosalicylic acid)–titanous and titanic complexes III. The poly(*N*-acryloyl-5-aminosalicylic acid)–titanous and titanic complexes III were similarly prepared. The colours and colour stabilities of these four complexes were the same as those of the corresponding complexes I.

Determination of enzyme activities. — (*a*) *alpha-Amylase activity.* This was determined by a method based on that of Bernfeld[8]. The assay reagent was prepared by dissolving 3,5-dinitrosalicylic acid (2.5 g) in 2M sodium hydroxide (50 ml) and adding distilled water (125 ml). Sodium potassium tartrate (75 g) was dissolved in the solution, and the volume adjusted to 250 ml with distilled water. To remove carbon dioxide, nitrogen was passed through the solution for 20 min.

Suspensions of the test materials (1 ml) were incubated at 20° with a 1% solution of starch in 20mM phosphate buffer (pH 6.9, 9 ml) with constant stirring for 5 min. After centrifugation, aliquots (1 ml) were placed in the assay reagent (1 ml), the mixture was heated to 100° for 4 min, the tubes were rapidly cooled, and the absorbance at 520 nm was determined against the initial solution of starch.

The extent of liberation of reducing sugar was calculated by reference to a calibration graph derived from the use of standard solutions of maltose in the assay. One unit of alpha-amylase activity is defined as that which liberates reducing sugar equivalent to 1 μmole of maltose at 20° in 1 min.

(*b*) *Glucoamylase activity.* This was determined as for alpha-amylase activity, but using 1% starch in 20mM sodium acetate buffer (pH 4.5) and an incubation time of 40 min at 45°.

The liberation of reducing sugar was calculated by reference to a calibration graph derived from the use of standard solutions of D-glucose in the assay. One unit of glucoamylase activity is defined as that which liberates reducing sugar equivalent to 1 μmole of D-glucose at 45° in 1 min.

(*c*) *Polygalacturonase activity.* This was determined by a viscometric method. Suspensions of the test materials (1 ml) were incubated with a 0.5% solution of pectin (*ex* citrus fruit, Koch–Light) in 20mM sodium acetate buffer (pH 4.0, 9 ml) at 40° for 1 h. The sample was then centrifuged to stop the reaction, and the viscosity of the supernatant was determined by using a simple viscometer of the Ostwald type.

The viscosity of the original pectin was determined so that polygalacturonase activities could be calculated as a function of the decrease in viscosity.

Determination of protein. — Samples of enzymes (soluble, 100 μg; insoluble

12 mg) were hydrolysed in sealed tubes with 6M hydrochloric acid (2 ml) at 100° for 18 h. The combined hydrolysates and tube washings were treated with 8M sodium hydroxide (~1.5 ml) and centrifuged to remove any suspended material. After adjustment of the pH to 7.0 with dilute acid or alkali, the volumes were adjusted to 10 ml with distilled water. The total amino-acid contents of the solutions were determined by an automated ninhydrin assay[9]. Samples (12 mg) of the polymer–titanium complexes were similarly treated as controls for the samples of solid-phase enzymes. The absolute amount of protein present in insoluble enzyme-derivatives was derived from the corrected absorbances by reference to a calibration curve constructed from the hydrolysates of standard solutions of the enzymes.

Formation and assessment of insolubilised enzymes. — (*a*) *alpha-Amylase.* The polymeric, titanic complexes I (20 mg) were subjected to five washings with distilled water (5 ml) and were then suspended in a solution of alpha-amylase (α-1,4-glucan 4-glucanohydrolase, E.C. 3.2.1.1; ex. *Bacillus subtilis*, Sigma Chemical Co. Ltd., 1 mg) in distilled water (5 ml), and the mixture was stirred at 4° for 18 h. The solid was recovered by centrifugation and subjected to ten washings with 0.1M sodium acetate buffer (pH 5.0, 5 ml). Finally, the solid was suspended in 5mM sodium acetate buffer (pH 5.0, 2 ml) and stored at 4°. The enzymic activities of the insoluble materials, determined on aliquots as previously described, were 2.27 and 4.47 units/mg of matrix for the poly(*N*-acryloyl-4- and -5-aminosalicylic acid)–titanic complexes I, respectively.

(*b*) *Repeated use of the insolubilised alpha-amylases.* The suspensions of the solid-phase alpha-amylases were incubated with starch solution for 5 min, as described in the previous section. The solutions containing excess of starch and its degradation products were removed after centrifugation, and aliquots were assayed for reducing sugar content. The solids were washed with 0.1M sodium acetate buffer (pH 5.0, 5 ml) and then resuspended in 5mM sodium acetate buffer (pH 5.0, 1 ml). This cycle was then repeated twice. The enzymic activities of the solid-phase enzymes after one use were 20 and 10% for the poly(*N*-acryloyl-4- and -5-aminosalicylic acid)–titanic complexes I, respectively. After two and three uses, there was no residual activity.

(*c*) *Glucoamylase.* Samples (20 mg) of the titanous and titanic complexes of the polymers were subjected to 5 washings with distilled water (5 ml) and then suspended in an aqueous solution of glucoamylase [α-1,4-glucan glucohydrolase, E.C. 3.2.1.3; Agidex preparation, Glaxo Laboratories Ltd., which had been dialysed against distilled water (2 × 5 l) for 22 h; 1 mg/ml, 5 ml]. The mixture was stirred at 4° for 18 h, and the solid was recovered by centrifugation, subjected to ten washings with 0.1M sodium acetate buffer (pH 5.0, 2 ml), and stored at 4°.

The enzymic activities of the insoluble derivatives of glucoamylase were determined on aliquots of the suspensions, as previously described, and are shown in Table I.

(*d*) *Continuous use of insolubilised glucoamylase in a column.* Glucoamylase derivatives prepared from poly(*N*-acryloyl-4- and -5-aminosalicylic acid)–titanic

TABLE I

ENZYMIC ACTIVITIES OF INSOLUBILISED GLUCOAMYLASE

Titanous complex preparation	*Activity of solid-phase glucoamylase (units/mg)*	*Titanic complex preparation*	*Activity of solid-phase glucoamylase (units/mg)*
Poly-4-acid I	0.467	Poly-4-acid I	0.104
Poly-5-acid I	0.175	Poly-5-acid I	0.000
Poly-4-acid II	0.469	Poly-4-acid II	0.196
Poly-5-acid II	0.000	Poly-5-acid II	0.155
Poly-4-acid III	0.000	Poly-4-acid III	0.211
Poly-5-acid III	0.201	Poly-5-acid III	0.175

complexes I (5 mg) were mixed with glass beads (Ballotini No. 20, 53 μm, 1 g) and packed into jacketed columns (bed vol. 1 ml) at 37°. 0.1% Aqueous starch solution was continuously passed down the columns at a flow rate of 0.05 ml/min. Using Technicon modular equipment, the column eluates were continuously monitored for liberated, non-reducing D-glucose residues by mixing them with 15 mM periodic acid (neutralised with ammonia and buffered to pH 7.5 with M phosphate buffer). The liberated formaldehyde was assayed by the automated method of Samuelson and Stromberg[10] (Fig. 1).

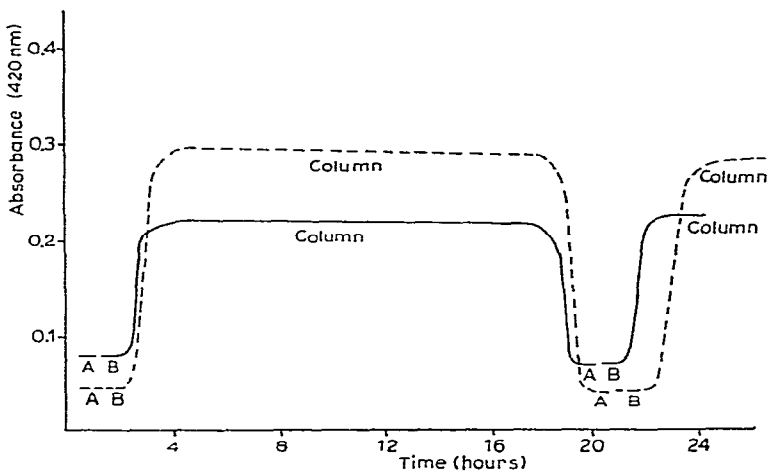

Fig. 1. Continuous use of a column of insolubilised glucoamylase formed by attachment to titanic complexes I. Assay for liberated, terminal, non-reducing D-glucose units: (——), enzyme attached to 4-isomer; (– – –), enzyme attached to 5-isomer. The horizontal sections *A* and *B* represent direct testing of the water input and the starch solution input, respectively, by passing these solutions through the analytical system but by-passing the column. The section denoted Column is the assay of the column eluate.

(*e*) *Coupling of glucoamylase under various conditions.* Glucoamylase was coupled to samples (20 mg) of poly(*N*-acryloyl-5-aminosalicylic acid)–titanic complex

III as described in (*c*), but in this case the enzyme solution (5 mg/ml, 1 ml) was buffered with 0.1M sodium acetate buffers (pH 3.0–7.0) or 0.1M sodium carbonate–hydrogen carbonate buffer (pH 9.0, 4 ml). The enzymic activities and the protein contents of these preparations were determined by the previously described methods, and the variations of these parameters, and that of specific activity, with the pH of coupling are shown in Fig. 2.

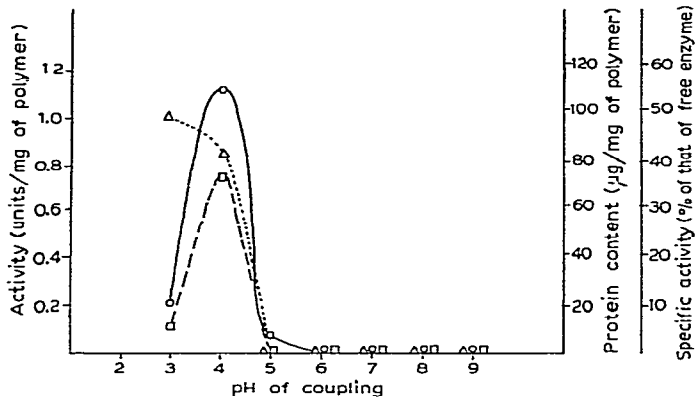

Fig. 2. Enzymic activity (O—O), protein content (□ – – □), and specific activities (△... △) of the solid-phase enzyme prepared by coupling glucoamylase to poly(*N*-acryloyl-5-aminosalicylic acid)–titanic complex III at various pH values.

The coupling process was repeated at a series of enzyme concentrations (0–20 mg/ml, 1 ml). All couplings were carried out with 0.1M sodium acetate buffer (pH 4.0, 4 ml). After 18 h at 4°, the solids were recovered and washed as before. Activities and protein contents were determined as before (Fig. 3).

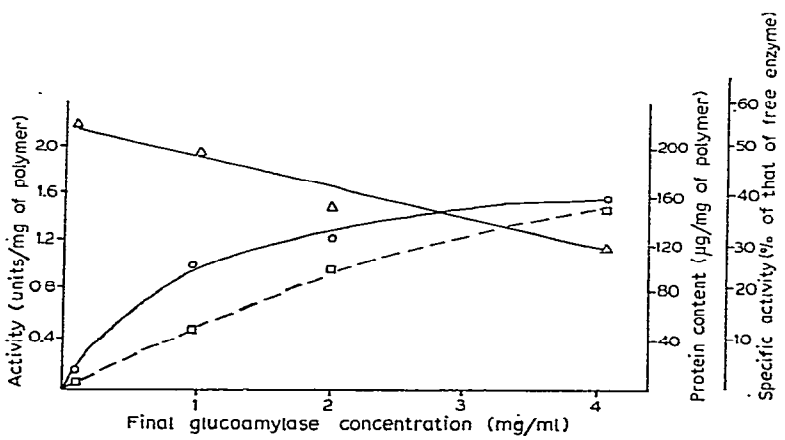

Fig. 3. Enzymic activity (O—O), protein content (□ – – □), and specific activities (△ — △) of the solid-phase enzyme prepared by coupling glucoamylase to poly(*N*-acryloyl-5-aminosalicylic acid)–titanic complex III at various enzyme concentrations.

The coupling process was also repeated for a series of coupling times (1–48 h). All couplings were carried out with 0.1M sodium acetate buffer (pH 4.0, 4 ml) and an enzyme concentration of 5 mg/ml (1 ml). Washings and assays were effected as usual, and it was found that, throughout the period 1–48 h, the enzyme activity and protein loading were 1.0 unit and 41 μg, respectively, per mg of matrix.

(*f*) *Polygalacturonase.* Samples of the titanous complex III (20 mg) were subjected to five washings with distilled water (5 ml) and then suspended in solutions of polygalacturonase (poly-α-1,4-galacturonide glycanohydrolase, E.C. 3.2.1.15; *ex Aspergillus niger*, Koch–Light; 5 mg/ml, 1 ml) in 0.1M sodium acetate buffers (pH 3.0–6.0; 4 ml). The mixtures were stirred at 4° for 16 h, after which the solids were recovered by centrifugation and subjected to ten washings with 0.1M sodium acetate buffer (pH 4.0, 5 ml). The solids were finally suspended in 20mM sodium acetate buffer (pH 4.0, 3 ml) and assayed for enzymic activity as previously described (Table II).

TABLE II

ENZYMIC ACTIVITIES OF SOLUBLE AND INSOLUBILISED POLYGALACTURONASE

Coupling pH for insoluble samples	*Percentage decrease in the original, relative viscosity*	*Solvent for soluble samples*	*Percentage decrease in the original, relative viscosity*
3.0	4.20	Acetate buffer (pH 4.0)	39.5
4.0	3.50	Titanium citrate (pH 4.0)	59.0
5.0	0.44	Citrate buffer (pH 4.0)	57.8
6.0	3.55	Polymer solution (pH 4.0)	15.1

The effect of certain ions on the activity of free polygalacturonase was investigated as follows. Solutions of polygalacturonase (0.5 mg/ml) were prepared in 0.65M sodium citrate buffer (pH 4.0) and in 20mM sodium acetate buffer (pH 4.0). Titanous chloride (12.5%, 10 ml) was adjusted to pH 4.0 with 0.65M sodium citrate buffer (pH 5.0, ~90 ml), and an aliquot (1 ml) was added to a solution of polygalacturonase in 20mM sodium acetate buffer (pH 4.0; 1 mg/ml, 1 ml). Aliquots (1 ml) of the three enzyme solutions so prepared were assayed as described previously (Table II).

The effect of soluble poly(*N*-acryloyl-5-aminosalicylic acid) on the activity of free polygalacturonase was investigated as follows. An aliquot (4 ml; 50 mg) of the polymer suspension was centrifuged, and 0.1M sodium hydroxide (4 ml) was added to the solid. On heating to 100°, the solid gradually dissolved. The pH of the resultant solution was adjusted very cautiously to 4.0–4.5 with dilute hydrochloric acid, and an aliquot (1 ml) was added to a solution of polygalacturonase in 27mM sodium acetate buffer (pH 4.0; 1 mg/ml, 1 ml). The enzymic activity was assayed in the usual way (Table II).

DISCUSSION

In aqueous solution, titanous and titanic ions exist as octahedrally co-ordinated complexes in which the tri- and tetra-valent titanium atoms are surrounded by six molecules of water. These six ligands are easily replaced by others, and on reaction with a salicylic acid residue two of the water molecules are replaced by the hydroxyl groups from the phenol and carboxylic acid groups. Alternatively, the titanium ions may become attached to the polymers by replacement of one water ligand only, by either of the hydroxyl groups. This situation presumably extends to the present poly(salicylic acids). In all three cases, water ligands are still held by the titanium atoms, and we envisage that these can be replaced by suitable groups present in enzyme molecules. Of particular use here would be any *N*-terminal amino and lysyl ε-amino groups. 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 to the polymer matrix. The possibility of ionic binding was discounted because the enzymes could be bound to the titanium complexes of the polymers in ionic media.

The use of three polysaccharidases for investigation of the utility of the titanium complexes of poly(*N*-acryloyl-4- and -5-aminosalicylic acids) for the preparation of active, insoluble derivatives of enzymes has revealed that different situations can be attained with different enzymes.

With alpha-amylase, it appeared initially that highly active derivatives had been formed. Nevertheless, on further examination, it was found that this activity was not maintained on re-use of the preparation. This was somewhat surprising since, prior to testing, the preparation had been extensively washed to remove any physically adsorbed enzyme. However, the affinity of enzymes for their substrates is well known, and it appears that the critical bond between the enzyme and the titanium was insufficiently strong to withstand substrate desorption. The weakness of the binding could well involve the factors mentioned above, and the initially high activities were probably due to free enzyme released by the substrate. That the weakness of the bond lay in instability of the titanium chelation of the salicylic acid matrix was discounted in the light of the results obtained with glucoamylase.

In view of the instability of the alpha-amylase derivatives, attention was turned to glucoamylase. Most of the polymer preparations gave active derivatives (Table I), and testing of two of these showed that the activity was firmly bound, was not removed by the substrate, and was stable under conditions of continuous use (Fig. 1). These attributes are among the ideals of solid-phase enzymes and were a great improvement over the alpha-amylase product. Thus, the situation warranted further investigation, particularly with respect to optimisation of the attachment of the enzyme. The various activity values shown in Table I presented little indication of the

polymer of choice. There appeared to be little difference between the products from the various methods of preparation of the polymers and chelation of titanium. The 5-isomer of the polymer was therefore chosen since, industrially, 5-aminosalicylic acid is easier and cheaper to make than the 4-isomer. The titanic form of the complex was chosen since the titanous forms were oxidised in a matter of hours to the corresponding titanic forms, and the type III complex was selected since this avoided the two-step process of pre-isolation of the polymer and subsequent formation of the complex.

The pH for optimal coupling of glucoamylase to the poly(*N*-acryloyl-5-aminosalicylic acid)–titanic complex was critical, with very much higher activity occurring at pH 4 than at 3 and 5. It was also interesting to find that the degree of protein binding followed the same curve, in contrast to experiences with poly(allyl carbonate)[11,12] where the greatest protein-binding occurred at quite a different pH from that giving maximal activity. As might be expected, on the basis of some degree of crowding of the enzymic active-sites at high protein-binding, the maximum specific activity of the bound enzyme corresponds to a very low degree of protein binding.

From an investigation of the variation of enzyme concentration in the coupling process (Fig. 3), it appeared that maximal activity and protein-contents were achieved at a concentration of ~4–5 mg/ml under the conditions used [*cf.* poly(allyl carbonate) 2 mg/ml[12]]. This maximum is presumably reached when all the titanium sites are saturated, or shielded by existing enzyme ligands. Since the protein-content curve followed the activity curve, it appears that little overcrowding occurred. This is confirmed by the fact that, although specific activity dropped on greater binding of the enzyme, it did not do so dramatically, and by the very high percentage retention of specific activity of the enzyme against a macromolecular substrate on insolubilisation. Both these properties are always sought after in preparation of insolubilised enzymes, but are not easy to achieve. Insolubilised enzymes are usually less active against substrates of high molecular weight because of diffusion effects. Presumably, in the present case, the gel-like nature of the matrix is such that the enzyme is bound to the surface only, thus obviating the need for the substrate to diffuse into the gel. Furthermore, in view of the high specificity of the derivative, co-ordination of one enzyme molecule through more than one titanium atom is not expected.

The coupling reaction was complete within one hour. This is advantageous and contrasts with the much longer times necessary to reach maximal coupling of enzymes to other matrices, where different principles of attachment are involved, *e.g.*, poly(allyl carbonate)[11,12] and cellulose carbonate[13].

The very low activities obtained with the polygalacturonase derivative were disappointing. It was found that, although the enzyme was not inhibited in either sodium citrate or titanium citrate buffers, its activity was considerably reduced when determined in an aqueous solution of poly(*N*-acryloyl-5-aminosalicylic acid) (Table II). The way in which this inhibition occurs is unknown, but it is possible that a carboxyl group in the polymer binds to the enzyme, the natural substrate of which is a poly-

carboxylic acid, and thereby the active site of the bound enzyme is unavailable for catalytic activity.

In conclusion, it may be said that the poly(salicylic acid) matrices have an advantage over many others used for insolubilisation of enzymes, in that they are synthetic and are not bio-degradable. They could well be suited to technological transformation for the ultimate production of enzymes attached to fibres and membranes.

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