The Use of a Poly(allyl carbonate) for the Preparation of Active, Water-insoluble Derivatives of Enzymes

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The optimum pH conditions have been determined for the covalent binding of the soluble enzymes α-amylase (E.C.3.2.1.1), β-D-glucosidase (E.C.3.2.1.21), and trypsin (E.C.3.4.4.4) to an insoluble poly(allyl carbonate). The resultant activity of these solid-phase enzymes varied considerably with the pH of the coupling media and could be increased at a given pH by raising the concentration of the soluble enzyme and extending the duration of the coupling. It was found that the pH at which the maximum amount of protein is bound is not the same as that at which protein is bound with the greatest retention of enzyme activity. The factors influencing the extent of the coupling reaction are discussed.

The extent of coupling of enzyme as a function of pH for poly(allyl carbonate) is compared with that for cellulose *trans*-2,3-carbonate, and it is concluded that the optimum conditions for coupling are unique for each enzyme and each insoluble matrix employed. The instability observed on storage of the insolubilised enzyme may be prevented by lyophilisation in the presence of a polyol, the latter presumably supplying a hydrophilic network in the solid state.

It has already been demonstrated that poly(allyl carbonate) holds potential for the insolubilisation of enzymes.[1] In this preceding work, the conditions employed for the reactions of the enzymes with poly(allyl carbonate) were based on those found to be optimum for coupling of β-D-glucosidase to cellulose

[1] S. A. Barker, J. F. Kennedy, and A. Rosevear, *J. Chem. Soc. (C)*, 1971, 2726.

trans-2,3-carbonate.[2] We have now investigated the degree of coupling achieved over a range of conditions and have found significant differences between the covalent binding properties of cellulose *trans*-2,3-carbonate, which contains five-membered carbonate rings, and poly(allyl carbonate), which is considered to contain eight-membered carbonate rings.

Insoluble derivatives of α-amylase, β-D-glucosidase, and trypsin were prepared at 4° by suspending poly(allyl carbonate) in buffered solutions of pH 3·0—10·0 containing soluble enzyme. The resulting insoluble enzymes were subjected to a rigorous washing procedure and activity measurements were performed by incubation of the solids with solutions of the appropriate substrate and spectrophotometric determination of the products of enzymic action (Figure 1a—c).

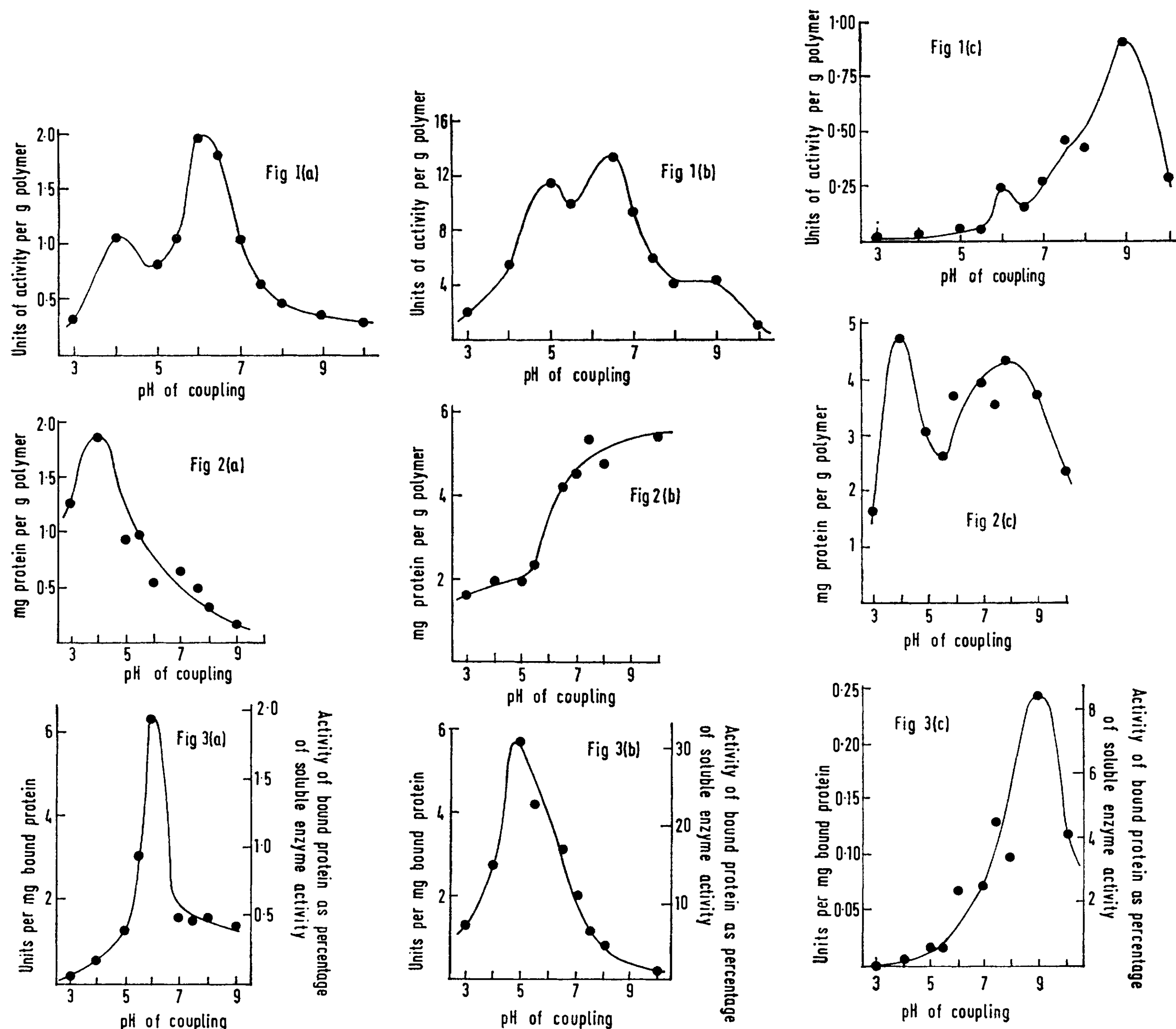


FIGURE 1 Effects of pH on the covalent coupling of enzyme to poly(allyl carbonate); pH–activity profiles: (a) α-Amylase (coupling time 4 h, soluble enzyme 5 mg in 5 ml); poly(allyl carbonate) takes up 3·33 mmol NH_3 per g (one unit of α-amylase activity is that which under the defined conditions releases reducing ends equivalent to one μmole of maltose in 1 min). (b) β-D-Glucosidase (coupling time 4 h, soluble enzyme 5 mg in 10 ml); poly(allyl carbonate) takes up 3·32 mmol NH_3 per g (one unit of β-D-glucosidase activity is that which under the defined conditions releases one μmol of *o*-nitrophenol in 1 min). (c) Trypsin (coupling time 4 h, soluble enzyme 5 mg in 5 ml); poly(allyl carbonate) takes up 1·48 mmol NH_3 per g (one unit of trypsin activity is that which under defined conditions liberates sufficient trichloroacetic acid-soluble hydrolysis products so that the optical density at 280 nm increases by 1·00 in 1 min)

FIGURE 2 Effects of pH on the covalent coupling of enzyme protein to poly(allyl carbonate): (a) α-amylase, (b) β-D-glucosidase, (c) trypsin

FIGURE 3 The relationship of pH of coupling to the specific activity of the resultant poly(allyl carbonate)-insolubilised enzyme: (a) α-amylase, (b) β-D-glucosidase, (c) trypsin

[2] S. A. Barker, S. H. Doss, C. J. Gray, J. F. Kennedy, M. Stacey, and T. H. Yeo, *Carbohydrate Res.*, 1971, **20**, 1.

The quantity of protein bound to the polymer was determined in each case by acid hydrolysis and ninhydrin determination of the amino-acid content of the neutralised hydrolysates (Figure 2a—c). The corresponding hydrolysate from poly(allyl carbonate) alone gave a non-specific ninhydrin reaction, but the relatively high protein contents of the insoluble β-D-glucosidase and trypsin enabled measurements to be made in the presence of hydrolysis products from the support polymer. However the smaller quantities of protein bound in the case of α-amylase required the separation of the amino-acids from the polymeric debris by paper chromatography before accurate measurements could be made.

By utilising the data on the activities of the insoluble enzymes and the corresponding amounts of bound protein, the specific activities of the bound enzymic protein and the specific activities relative to those of the original soluble enzymes were calculated (Figure 3a—c).

In the cases of α-amylase and β-D-glucosidase the curves depicting the variation of activity coupled as a function of pH showed dual maxima (Figure 1a and b). It can be seen that one of each pair of maxima (α-amylase, pH 4; β-D-glucosidase, pH 6·5) corresponds to the pH or pH range of maximum protein binding (Figure 2a and b). The second maximum of each pair of peaks (α-amylase, pH 6; β-D-glucosidase, pH 5) corresponds to the pH of coupling giving the greatest specific activity of bound enzymic protein (Figure 3a and b). (When β-D-glucosidase was coupled to cellulose *trans*-2,3-carbonate a single maximum at pH 7·8 was observed for the pH–activity curve.[2])

In the case of coupling of trypsin to poly(allyl carbonate), however, the activity–pH curve exhibits a major maximum corresponding to a pH of 9 for the coupling medium (Figure 1c). This coincides with a peak in both bound protein (Figure 2c) and relative activity of the bound protein (Figure 3c). A second peak in bound protein occurs at pH 4, where the relative activity of the bound protein is very low (Figure 3c). The minor maximum on the pH–activity curve does not correspond to any maximum in the bound protein or specific activity curves. Whether this is a true maximum is questionable, since the results with trypsin can be expected to be further complicated by the autolysis which can occur at pH values above 5·0.[3]

Many complex and interrelated factors would be expected to affect the coupling of the different enzymic proteins under the conditions of pH studied here. One factor of considerable importance is the stability of the cyclic carbonate groups of the poly(allyl carbonate). It has been reported that alkaline hydrolysis of five- and six-membered cyclic carbonate groups in aliphatic compounds occurs at rates much greater than does acid-catalysed hydrolysis.[4,5] As expected, poly(allyl carbonate) exhibited similar properties, the cyclic carbonate content being considerably more stable to N-hydrochloric acid than to N-sodium hydroxide (Table 1).

TABLE 1

Stability of poly(allyl carbonate)

Agent, *etc.*	Duration	Temp. (°C)	Ammonia uptake (% original)
Water	30 min	20	100
0·1N-HCl	15 min	20	105
N-HCl	15 min	20	105
Water	30 min	100	97
0·1N-NaOH	15 min	20	69
N-NaOH	30 min	20	24
Desiccator	2 weeks	20	90
β-D-Glucosidase at pH 6·25	48 h	4	63

Thus it is likely that during the covalent coupling of the enzymes to poly(allyl carbonate), considerable hydrolysis of the cyclic carbonate would occur at certain pH values. This suggestion was confirmed: during the coupling of β-D-glucosidase at pH 6·25 the ability of the poly(allyl carbonate) to react with ammonium hydroxide was reduced by 37% (Table 1). The amount of enzymic protein bound does not account for this reduction; furthermore, the proportion of soluble enzymic protein remaining unbound after the coupling reaction retained 100% of its original activity and therefore presumably had not reacted with the insoluble matrix in any way. It must therefore be concluded that the loss of cyclic carbonate was due to hydrolysis.

Thus the main reaction so far as covalent enzyme coupling is concerned, the nucleophilic attack of the terminal and ε-amino-groups of the enzymic protein on the cyclic carbonate groups, must be considered in conjunction with the hydrolysis. Clearly the competition between the two reactions will be dependent upon the pH of the system. Although under more acidic conditions, the hydrolytic process occurs to smaller extents, the availability of the groups on the protein suitable for coupling is reduced by protonation. This availability will to some extent depend upon the number and location of basic groups in the enzyme structure. However the only significant factor in the reported amino-acid compositions of α-amylase,[6] β-D-glucosidase,[7] and trypsin[3] is the high proportion of acidic residues in trypsin.

The curves realised for the degree of activity coupled as a function of pH therefore cannot be interpreted in simple terms but occur as a result of the compounding of these reactions. The coupling is also expected to be governed by physical properties of the system such as tertiary structure and molecular weight of the enzyme [α-amylase, 48,000 (ref. 6); β-D-glucosidase, 144,000 (ref. 7); trypsin, 24,000 (ref. 3)], and particle size and porosity of the poly(allyl carbonate), which will all determine the rate of diffusion of the enzyme to the

³ P. D. Boyer, H. Lardy, K. Myräck, and P. Desnuelle, 'The Enzymes,' 2nd edn., Academic Press, New York, 1960, vol. 4, p. 119.

⁴ J. Katzhendler, L. A. Poles, H. Dagan, and S. Sarel, *J. Chem. Soc.* (*B*), 1971, 1035.

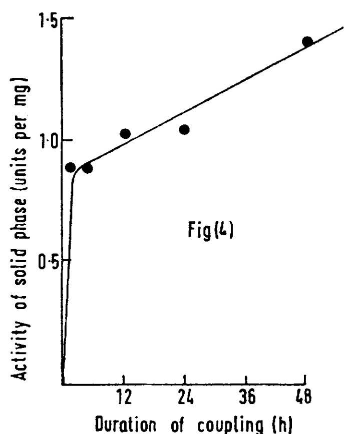
⁵ J. Katzhendler, L. A. Poles, and S. Sarel, *J. Chem. Soc.* (*B*), 1971, 1847.

⁶ C. T. Greenwood and E. A. Milne, *Adv. Carbohydrate Chem.*, 1968, **23**, 281.

⁷ F. J. Joubert and T. N. Vanderwalt, *J. S. African Chem. Inst.*, 1964, **17**, 79.

active groups in the poly(allyl carbonate), together with the cyclic carbonate content of the polymer and the coupling time and enzyme concentration employed.

By employing longer coupling times (Figure 4) and higher concentrations of soluble enzyme (Figure 5) more active products were produced. This indicates that the theoretical binding capacity of this polymer has not been reached and that the use of high soluble enzyme



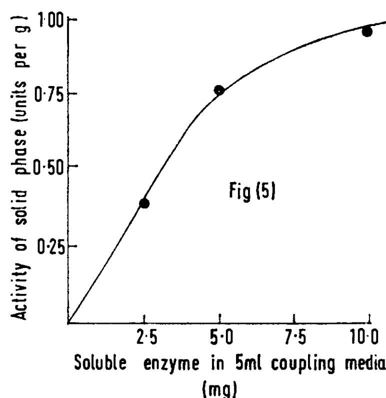


FIGURE 4 Effect of duration of coupling on the production of β-D-glucosidase insolubilised by attachment to poly(allyl carbonate)

FIGURE 5 Effect of concentration of soluble enzyme during coupling on the production of β-D-glucosidase insolubilised by attachment to poly(allyl carbonate)

concentrations and longer coupling times at the optimum pH would lead to a more active insolubilised enzyme if desired. However apart from the possibility of saturation of the cyclic carbonate groups available, overcrowding of enzyme molecules with concomitant reductions in activity, particularly towards high molecular weight substrates, must be taken into account.

The relative activity of the solid phase β-D-glucosidase towards the low molecular weight glucoside is appreciably higher than that of the trypsin and α-amylase derivatives towards their high molecular weight substrates. This is consistent with previous reports which indicate that insolubilisation of an enzyme depresses its activity towards high molecular weight substrates more than its activity towards low molecular weight substrates,[8] and that insolubilisation also depresses the activity towards substrates which are hydrolysed by endo-mechanisms.[9]

The activity of the solid-phase enzymes deteriorated on storage, especially when dehydration occurred (Table 2). However, when the insolubilised β-D-glucosidase was suspended in a solution of sorbitol and lyophilised, the insolubilised enzyme showed long-term activity retention properties similar to those of solutions of the soluble enzyme (Table 3). This is presumably due to the formation by the sorbitol of an involatile hydrophilic environment around the bound protein.

TABLE 2

Stability of insolubilised β-D-glucosidase

Time after preparation (days)	% Water in polymer	% of original activity retained
0	31	100
1 [a]	16	27
10 [a]	9	7
1 [b]	0	5

[a] At 25°. [b] Lyophilised from water.

TABLE 3

Stabilisation of insolubilised β-D-glucosidase

	% of original activity retained	
	7 days, 0°	7 days, 25°
Lyophilised from 0·75% sorbitol solution	98*	78*
Damp solid	47	9
Solution of soluble enzyme (1 mg ml^{-1})	100	79

* Corrected for partial inhibition of enzyme activity by sorbitol.

In conclusion, the present work demonstrates that the optimum coupling conditions for each enzyme and solid-phase system are unique. There appears to be no universal set of optimum reaction conditions even when one enzyme (β-D-glucosidase) and similar supports [poly(allyl carbonate) and cellulose *trans*-2,3-carbonate] or the same insoluble support but different enzymes are employed, and thus the need to determine individually the correct conditions is highlighted. However, the potential of poly(allyl carbonate) as a matrix suitable for the insolubilisation of various types of enzymes with retention of enzymic activity has been confirmed. The non-biodegradable nature of the polymer is expected to render it generally applicable in the field of active insolubilised enzymes.

EXPERIMENTAL

Preparation of Poly(allyl carbonate).—Poly(allyl alcohol) (1 g) [1] was dissolved in dry dimethyl sulphoxide (10 ml),

[8] A. N. Glazer, A. Bar-Eli, and E. Katchalski, *J. Biol. Chem.*, 1962, **237**, 1832.

[9] S. A. Barker, P. J. Somers, and R. Epton, *Carbohydrate Res.*, 1969, **9**, 257.

dry 1,4-dioxan (1·5 ml), and dry triethylamine (8 ml). The solution was stirred on an ice–salt bath while ethyl chloroformate (16 ml) was carefully added dropwise during 45 min. Dry 1,4-dioxan (15 ml) was then added to ensure maximum precipitation of the product; the insoluble material was filtered off on sintered glass and washed with dry 1,4-dioxan (9 × 15 ml), water (3 × 15 ml), and diethyl ether (3 × 15 ml). Several such batches of poly(allyl carbonate) were ground together, washed further with water, dioxan, and ether, and stored in a desiccator (P_2O_5). The solid showed $\nu_{max.}$ 1820 (cyclic carbonate), 1750 (EtO_2C), and 1260—1300 and 1115 cm^{-1} (asym. C–O–C str.).[10]

Stability. Samples of the carbonate (100 mg) were treated with dilute acid or alkali (10 ml) at 20° for 15—30 min, and with water at 20° or 100° for 30 min. The samples were then washed with water (3 × 5 ml) at 25°. The cyclic carbonate contents of these polymers and of untreated material were assessed in terms of their ability to take up ammonia, by use of the titrimetric method described previously [1] (Table 1).

Preparation of Insoluble Enzyme Derivatives.—Samples of poly(allyl carbonate) (200 mg) were suspended in the appropriate buffer solution [0·1M-citric acid–0·2M-sodium phosphate for pH 3·0—5·5, 0·1M-sodium phosphate for pH 6·0—8·0, and 0·1M-sodium carbonate–hydrogen carbonate for pH 9·0—10·0 (4·5 or 5 ml)]. Aqueous solutions of α-amylase (E.C.3.2.1.1 Sigma Chemical Co., type IIa, *ex. Bacillus subtilis*), trypsin (E.C.3.4.4.4 Koch–Light Ltd., *ex.* bovine pancreas), or β-D-glucosidase (E.C.3.2.1.21 Koch–Light Ltd., *ex.* sweet almonds) were added to the suspensions such that 5 mg of soluble enzyme was contained in a final volume of 5, 5, or 10 ml, respectively. The suspensions were stirred for 4 h at 4° and the solids were isolated by centrifugation. The material was subjected to five washing cycles of 0·005M-sodium acetate buffer (pH 5·0; 10 ml) followed by M-sucrose in M-sodium chloride (7·5 ml) and was washed a further twice with acetate buffer before being collected on sintered glass. The excess of moisture was removed by pressing between filter papers and the product was stored at room temperature and assayed within 24 h.

Assays of Activity.—(a) *α-Amylase*. This was measured as the rate of release of new reducing ends from starch by use of a suitable modification of the method reported by Bernfield.[11] The reagent was prepared by dissolving 3,5-dinitrosalicyclic acid (1 g) in 2N-sodium hydroxide (20 ml.) This solution was diluted to 50 ml with water, sodium potassium tartrate (30 g) was added, the solution was diluted to 100 ml, and nitrogen was passed through to remove carbon dioxide.

Two identical samples (100 mg) of each damp preparation of insoluble α-amylase were taken; one was dried rigorously to determine the actual solid weight. The other was suspended in a solution (0·5% w/v) of starch in 0·02M-sodium phosphate buffer (pH 6·9; 10 ml) and vigorously stirred. Samples (1 ml) were withdrawn at intervals (0·5 h) and each was added to an equal volume of the dinitrosalicylate reagent. The samples were heated for 3 min at 80° to develop the red chromophore and after rapid cooling were centrifuged for 3 min to remove the polymer. The optical density of each solution with respect to a reagent blank incubated with starch was determined at 520 nm. Standard solutions of the soluble enzyme and spent coupling solutions were also assayed.

Calibration was provided by the simultaneous incubation of standard solutions of maltose (0·1—2·0 mg ml^{-1}) with the reagent and the α-amylase activities of the insoluble enzyme preparations were determined as the initial rate of production of reducing ends, as equivalents of maltose, in the starch incubation (Figure 1a).

(b) β-D-*Glucosidase*. This was measured as the amount of free *o*-nitrophenolate ion released from the corresponding glucoside under standard conditions. Two identical samples (20 mg) of the damp insoluble enzyme were taken and the dry weight of one was determined. The other was suspended in 0·005M-sodium acetate buffer (pH 5·0; 1 ml) by stirring. A solution of *o*-nitrophenyl β-D-glucopyranoside (12 mg) in the same buffer (5 ml) was added and the suspension was incubated at 37° for 12 min. The reaction was terminated by addition of 0·2M-sodium carbonate (5 ml) and the tubes were rapidly cooled and centrifuged to remove the polymer. The optical densities of the supernatants were determined with respect to water at 420 nm. Standard solutions of the soluble enzyme were also incubated with the substrate and the enzyme activities were calculated from a calibration curve constructed for standard solutions of *o*-nitrophenol (Figure 1b).

(c) *Trypsin*. This was measured as the amount of trichloroacetic acid-soluble peptide released from casein in 20 min by a modification of the method described by Bergmeyer.[12] Two identical samples (40 mg) of insoluble enzyme were taken and the dry weight of one determined. The other was suspended by stirring in 0·05M-sodium phosphate buffer (pH 7·6; 1 ml) and incubated at 37° with a solution (1% w/v) of Hammarsten casein (B.D.H. Ltd.) in 0·05M-sodium phosphate buffer (pH 7·6; 1 ml). The reaction was terminated after 20 min by addition of aqueous 5% w/v trichloroacetic acid (3 ml). The suspension was set aside for 30 min, and was then centrifuged for 30 min; the optical densities of the supernatants were determined at 280 nm with respect to water. Standard solutions of soluble trypsin (0·40 μg) in 0·05M-sodium phosphate buffer (pH 7·6; 1 ml) were similarly treated to provide a calibration curve and the activities of the unknowns were calculated (Figure 1c).

Determination of Bound Protein.—(a) *α-Amylase*. The samples were hydrolysed and the amino-acid contents of the hydrolysates were determined by the quantitative paper chromatographic method.[13]

Dried samples of the insolubilised α-amylase (50 mg) and the soluble enzyme (1—2 mg) were hydrolysed with 6N-hydrochloric acid (2 ml) for 18 h at 108°. The hydrolysates and tube washings (2 × 2 ml water) were evaporated to dryness with an excess of water. The residues were kept in a desiccator ($CaCl_2$ and KOH) for 12 h before being dissolved in water (200 μl). Strips of Whatman No. 1 paper (3·5 × 50 cm) which had been soaked for 10 min in 1 : 1 ethanol–1·2N-acetic acid were washed three times in distilled water and finally with ethanol before being allowed to dry. Samples of the hydrolysates (5—100 μl) from soluble and insoluble enzymes were applied to separate paper strips in bands 2·5 cm long. The components were separated by ascending chromatography (24 h) with n-butanol–acetic acid–water (4 : 1 : 5; organic phase) as solvent. The strips were dried, sprayed with ninhydrin reagent [nin-

[10] R. A. Nyquist and W. J. Potts, *Spectrochim. Acta*, 1961, **17**, 679.

[11] P. Bernfield, *Methods Enzymol.*, 1955, **1**, 149.

[12] H. U. Bergmeyer, 'Methods of Enzymic Analysis,' Verlag Chemie, Weinheim, 1965, p. 811.

[13] R. Epton and T. H. Thomas, 'An Introduction to Water-insoluble Enzymes,' Koch–Light Ltd., Colnbrook, 1971.

hydrin (0·5 g) in ethanol (75 ml), water (25 ml), and M-sodium hydroxide (0·5 ml)] and heated at 65° for 22 min. Bands were found with R_F values 0·07—0·14, 0·20, 0·26, 0·41, and 0·52 (relative intensities 16 : 24 : 43 : 4 : 13). The two bands with highest intensity were extracted with ethanol–water (3 : 1). The optical densities of the solutions were read at 575 nm and the protein contents of the solid-phase enzymes determined by comparison with similarly treated soluble enzyme standards (Figure 2a).

(b) β-D-*Glucosidase and trypsin.* Dried samples of the solid-phase preparations (20—30 mg) and the soluble enzyme (2—4 mg) were hydrolysed with 6N-hydrochloric acid (2 ml) for 18 h in sealed tubes on a refluxing saturated salt bath. The hydrolysates were removed from the tubes, which were then washed with 8M-sodium hydroxide (1·5 ml) and the pH of the combined hydrolysates and washings was adjusted to 7·0 with dilute acid or alkali. The solutions were diluted with water to a final volume of 5 ml. Each sample was frozen and thawed to aid precipitation of insoluble debris and was finally centrifuged to give a clear solution. The solid-phase hydrolysates were diluted 3—4 times and the soluble enzyme hydrolysates 100 times with water before being analysed for their total amino-acid content by an automated ninhydrin method [14] (Figures 2b and c).

Variation of Soluble Enzyme Concentration and Duration of Coupling.—β-D-Glucosidase was coupled to samples of poly-(allyl carbonate) (200 mg) by stirring suspensions of the polymer in solutions of β-D-glucosidase (2·5—10·0 mg) in sodium phosphate buffer (pH 6·25; 5 ml) for 4 h. The soluble enzyme (10 mg) was also coupled to samples of the polymer in the same buffer for 1—48 h. In each case, the resultant solids were washed and assayed for enzymic activity as before (Figures 4 and 5).

Stability of Solid Phase Enzyme.—Samples of damp insolubilised β-D-glucosidase were stored under various conditions while others were lyophilised. After storage, *etc.*, the water contents of the samples were determined by weight difference, and the enzyme activities remaining were determined in the usual manner (Table 2).

Stabilisation of Solid-phase Enzyme.—Samples of damp solid phase β-D-glucosidase (40 mg) of known activity were lyophilised from a solution of sorbitol (7·5 mg ml^{-1}; 1 ml). Duplicate samples of damp solid-phase enzyme were stored in covered tubes at 25 and 0° for 7 days and assayed in the usual way (Table 3). The activities were compared with those of solutions of the soluble enzyme (1 mg ml^{-1}) stored under identical conditions.

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[14] D. H. Spackman, W. H. Stein, and S. Moore, *Analyt. Chem.*, 1958, **30**, 1190.