Preparation of a Water-insoluble *trans*-2,3-Cyclic Carbonate Derivative of Macroporous Cellulose and its Use as a Matrix for Enzyme Immobilisation †

By **John F. Kennedy,* S. Alan Barker,** and **Alan Rosevear,** Department of Chemistry, University of Birmingham, Edgbaston, Birmingham B15 2TT

The degree of substitution of macroporous cellulose with *trans*-2,3-cyclic carbonate groups has been controlled by moderating the reaction with water. Exercise of this control enabled the preparation of a matrix with physical and chemical properties which facilitated the covalent binding of chymotrypsin A in such a way that the activity of the insoluble enzyme was appreciable towards a high molecular weight substrate (casein) as well as towards a low molecular weight substrate (tyrosine ethyl ester). Under the optimum conditions of preparation the bound protein had a relative activity towards casein of 26% and towards the ester of 65% of the activity of the free enzyme.

MICROCRYSTALLINE cellulose has been treated previously with ethyl chloroformate in an anhydrous organic solvent mixture to yield a product containing a high proportion of *trans*-2,3-cyclic carbonate groups.[1] The suitability of this group for the binding of enzymic protein under mild conditions has been illustrated by the preparation of an active water-insoluble derivative of β-D-glucosidase.[2] The cellulose *trans*-2,3-carbonate has also been used for the insolubilisation of antibodies to human immunoglobulin E, the active product being suitable for the determination of human myeloma forms of immunoglobulin E by radioimmunoassay.[3] The actual covalent coupling reaction involved has been envisaged as a nucleophilic attack of free amino-groups in the proteins upon the carbonyl carbon atoms of the cyclic carbonate groups.[2,3]

However, it has been shown by several workers that the attachment of enzymes to high-density supports gives a derivative with a lower activity towards high molecular weight substrates than would be expected from their activity towards low molecular weight substrates.[4] Since the main enzymic reactions of interest to industry involve the hydrolysis of high molecular weight material by proteases and amylases,[5] this characteristic of bound enzymes is a significant drawback.

This group has endeavoured to find matrices, the enzyme derivatives of which show activities against high molecular weight substrates approaching those shown against low molecular weight substrates. Clearly the enzyme should not be attached within the matrix in such a way that it is inaccessible to the substrate.

In the case of enzymes attached to poly(allyl cyclic carbonate), it was not clear whether the lower activity of the enzyme derivatives against higher molecular weight substrate was due to inaccessibility or to disruption of the active site.[6,7] However, an excellent retention of activity against high molecular weight substrate has been achieved for glucoamylase attached to poly-(5-acrylamidosalicyclic acid) by co-ordination through a titanium bridge. The specific activity of the bound enzyme, with starch as substrate, was 55% of that of the free enzyme.[8]

It occurred to us that advantages could be gained in the formation of an insolubilised enzyme by attaching the enzyme to a highly porous matrix. We argued that a correctly chosen matrix would permit free access of high molecular weight substrates to the enzymes bound in the pores, whilst the stability of the bound enzyme could be enhanced by its being protected within the pores of the matrix for this purpose. We therefore chose a reconstituted cellulose [9] which possessed a very open structure and which was porous to macromolecules. This paper describes the examination of this cellulose for the preparation of active derivatives of an enzyme. Conditions have been identified which preserve this macroporosity during the introduction of *trans*-2,3-carbonate groups. Chymotrypsin A has then been covalently

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[1] S. A. Barker, H. Cho Tun, S. H. Doss, C. J. Gray, and J. F. Kennedy. *Carbohydrate Res.*, 1971, **17**, 471.

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

[3] P. McLaughlin, D. R. Stanworth, J. F. Kennedy, and H. Cho Tun, *Nature New Biol.*, 1971, **232**, 245.

[4] G. J. H. Melrose, *Rev. Pure Appl. Chem.*, 1971, **21**, 83.

[5] M. A. Sherwood, *Process Biochem.*, 1966, **1**, 279.

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

[7] J. F. Kennedy, S. A. Barker, and A. Rosevear, *J.C.S. Perkin I*, 1972, 2568.

[8] J. F. Kennedy and J. Epton, *Carbohydrate Res.*, 1973, **27**, 11.

[9] Pharmacia Fine Chemicals Ltd., B.P. 1,234,099/1971.

bound in such a way that it maintains much of its activity towards the high molecular weight substrate, caesin.

EXPERIMENTAL

Preparation of Macroporous Cellulose Carbonate.—An aqueous suspension of macroporous cellulose [9] (an experimental batch kindly supplied by Pharmacia Fine Chemicals A.B.), known to contain 1 g dry weight of cellulose, was filtered on sintered glass. The solid was stirred in a closed vessel with several changes of dry dimethyl sulphoxide (6 × 25 ml) for 18 h. The cellulose, filtered off at the pump on sintered glass, was then found to contain 9—10 ml of adsorbed dimethyl sulphoxide.

Batches of this material were suspended in dry dimethyl sulphoxide (10 ml; m.p. 18°) taken from a freshly opened bottle, and water (0—1 ml) was added to each. Each suspension was stirred in an ice–salt bath with dry 1,4-dioxan (1·5 ml) and dry triethylamine (8 ml). Ethyl chloroformate (16 ml) was added dropwise during 30 min and the mixture was stirred for a further 15 min at this low temperature. Dry 1,4-dioxan (20 ml) was then added, the suspension was filtered at the pump, and the solid was washed at room temperature with dry 1,4-dioxan (9 × 20 ml). The product was freed from triethylamine by washing with dry ethanol (10 × 20 ml) and was finally washed with dry diethyl ether (3 × 20 ml) and dried. The resulting off-white granular material showed $\nu_{max.}$ 1750 (CO_2Et), 1815, and 1850 cm^{-1} (*trans*-cyclic carbonate).

Several batches prepared in a similar manner were ground together lightly in a pestle and mortar and rewashed with 1,4-dioxan (3 × 15 ml), ethanol (3 × 15 ml), and diethyl ether (3 × 15 ml) to give a homogeneous batch for enzyme studies. The product was stored until required in a desiccator over phosphorus pentoxide and paraffin wax.

Determination of Cyclic Carbonate Content of Macroporous Cellulose Carbonate.—(i) *Estimation of ammonia uptake.* The uptake of ammonia by cellulose carbonate at 20° from 0·2N-ammonium hydroxide was shown to be complete after 2 h, yielding a product which showed only acyclic and no cyclic carbonate absorption.

The sample to be assayed (100 mg) was stirred with 0·2N-ammonium hydroxide (5 ml) for 2·5 h at 20°. The suspension was centrifuged and a sample (3 ml) of the supernatant was titrated against 0·1N-hydrochloric acid (Methyl Red). The cyclic carbonate content of the sample, expressed as mmol ammonia taken up per g of matrix, was plotted as a function of water added to the ethyl chloroformate reaction mixture (Figure 1).

(ii) *Estimation by quantitative i.r. spectroscopy.* Samples were dried *in vacuo* at 60° and *ca.* 2 mg of each, accurately weighed, was mixed with crystalline potassium bromide (300 mg) and compressed into a disc. Spectra were taken (Perkin-Elmer 125 spectrometer) and the intensity of the absorption at 1815 cm^{-1}, per mg of material, was plotted against water added to the reaction mixture (Figure 1).

Assay of Chymotrypsin A Activity.—(i) *Caseinolytic activity.* A modification of the assay described by Bergmeyer [10] was employed. A substrate solution was prepared from casein (Hammarsten, B.D.H. Ltd.; 1 g) dissolved in 0·1M-sodium borate buffer (pH 8·0; 95 ml) by warming. Calcium chloride solution (5% w/v; 1·1 ml) was added and the volume was made up to 100 ml with buffer.

Duplicate samples (15—40 mg) of each enzyme derivative to be assayed were weighed out. One was rigorously dried to determine the net weight of the solid. The other was suspended in 0·1M-sodium borate buffer (pH 8·0, 1 ml) and placed in a thermostatted bath at 37°. Substrate solution (1 ml), pre-warmed to 37°, was pipetted into each sample and the suspension was stirred for 30 min (20 min for wide range pH of coupling experiment). The reaction was then terminated by addition of aqueous 5% w/v trichloroacetic acid (3 ml). After a further 30 min the tubes were centrifuged, for 30 min. The optical density of each solution was then read at 280 nm (against water).

Standard samples of the original soluble enzyme (0—16 μg) were similarly assayed and a calibration curve was constructed from the results. The initial slope of this curve was used to define the unit of activity (CU_{cas}) as that which causes the release of sufficient trichloroacetic-acid-soluble peptide to increase the optical density at 280 nm at a rate of 1·00 per min under the conditions of the assay.*

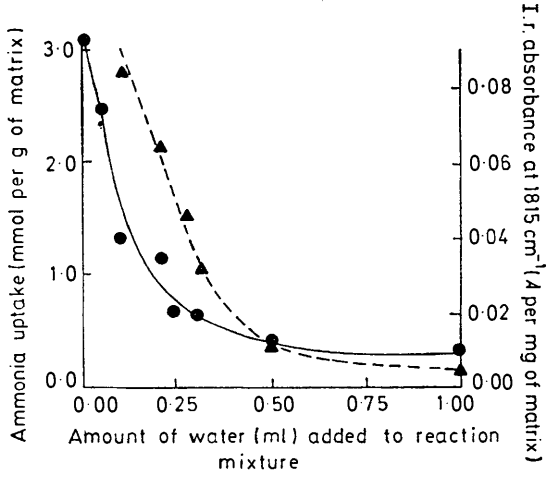

FIGURE 1 Dependence of the cyclic carbonate content of macroporous cellulose, as determined by ammonia uptake (——) and by i.r. absorption (- - - -), upon the amount of water added to the reaction mixture

(ii) *Tyrosine ethyl esterase activity.* A modification of the assay described by Bergmeyer [11] was employed. A substrate solution was prepared by dissolving L-tyrosine ethyl ester (49·2 mg) in 0·05M-tris(hydroxymethyl)methylamine buffer (pH 7·0; 90 ml). Calcium chloride solution (5% w/v; 4·4 ml) was added and the volume was then adjusted to 100 ml with buffer.

Duplicate samples of each enzyme derivative (10—20 mg) were weighed out. One was rigorously dried to determine the net weight of solid. The other was suspended in water (0·5 ml) and placed in a thermostatted bath at 37°. Freshly prepared substrate solution (3 ml), pre-warmed to 37°, was added and the suspension was stirred for 30 min. The reaction was terminated by centrifugation and transfer of a sample (1 ml) of the supernatant into an equal volume of ice cooled water. The optical density of this solution was read at 234 nm against a similarly treated sample of a solution of

* Control experiments conducted using unchanged cellulose carbonate in the assay system showed that the presence of cyclic carbonate groups did not extensively affect the value obtained for the enzyme activity. Blank values of <5% were obtained and corrections for these were made as appropriate.

[10] H. U. Bergmeyer, 'Methods of Enzymic Analysis,' Verlag Chemie, Weinheim, 1965, p. 800.

[11] Ref. 10, p. 802.

L-tyrosine (18·1 mg) in 0·05M-tris(hydroxymethyl)methylamine buffer (pH 7·0; 100 ml).

Standard samples of the original soluble enzyme (0—40 μg) were similarly assayed and the results were used to construct a calibration curve. The initial slope of this curve was used to define the unit of activity (CU_{tee}) as that which causes the optical density of the L-tyrosine ethyl ester relative to L-tyrosine to fall at a rate of 1·00 per min under the conditions of the assay.*

Determination of Bound Protein in the Insoluble Enzyme Derivatives.—Dry samples (20 mg) of each insoluble enzyme derivative and washed cellulose carbonate controls were weighed into individual ampoules, and water (0·1 ml) was added to each. Hydrochloric acid [concentrated S.L.R. grade (105 ml) diluted with water (95 ml); 2 ml] was added to each sample and the sealed ampoules were placed in a refluxing saturated salt bath at 108° for 18 h. After cooling and removal of the hydrolysates, the tubes were washed with 8M-sodium hydroxide (1·5 ml) and the hydrolysate and washing from each sample were combined and cooled. The pH of these solutions was adjusted to between 5 and 7 with dilute acid and alkali, and then the volumes were made up to 5 ml with water. The insoluble debris was cleared from the solutions by freezing, thawing, and centrifuging.

Samples of the hydrolysates (0·5—2·0 ml) were diluted with water (2—4 ml) and analysed by use of an automated ninhydrin assay for total amino-acid content.[12] The results were corrected for the non-specific colour developing from the cellulose carbonate controls.

Solutions of the original soluble chymotrypsin A (0·1 ml; 0—4 mg ml^{-1}) were simultaneously hydrolysed and analysed and a calibration curve was constructed from the results. The protein contents of the insoluble derivatives were calculated from this curve.

Preparation of Insoluble Derivatives of Chymotrypsin A.—(i) *Variation of pH of coupling.* Samples (0·2 g) from one batch of cellulose carbonate were washed with water (3 × 5 ml) and suspended in buffered solutions (4·5 ml) of various pH values (pH 3·0—5·0, 0·1M-citric acid–0·2M-sodium phosphate; pH 6·0—8·0, 0·1M-sodium phosphate; pH 9·0—10·5, 0·1M-sodium carbonate–sodium hydrogen carbonate) at 4°. An aqueous solution of chymotrypsin A (E.C. 3.4.4.5., Worthington Biochem. Corp., Type CDI/OCC; 0·5 ml; 10 mg ml^{-1}) was added to each sample and the suspensions were stirred for 18 h at 4°. The samples were then centrifuged, the spent coupling media were removed and the solids were washed at 4° with 0·005M-sodium acetate buffer pH 5·0 (10 ml) followed by M-sucrose–M-sodium chloride (7·5 ml), the washing agent being removed at each stage after centrifugation. This washing procedure was repeated five times and the solids were finally washed with acetate buffer (2 × 10 ml) and were isolated on sintered glass at the pump. Surplus water was removed from the solids by pressing between sheets of filter paper, and the product was stored in a closed vessel at −8°.

The caseinolytic and esterase activities of the products were determined by the standard assays (Figure 2). The bound protein contents of the samples were also determined (Figure 3) and from these values the specific activities of the bound protein for the two substrates were determined (Figure 4).

* Control experiments conducted using unchanged cellulose carbonate in the assay system showed that the presence of cyclic carbonate groups did not extensively affect the value obtained for the enzyme activity. Blank values of <5% were obtained and corrections for these were made as appropriate.

Insoluble enzyme derivatives were similarly prepared but for the pH range 4·7—7·5, the esterase and caseinolytic

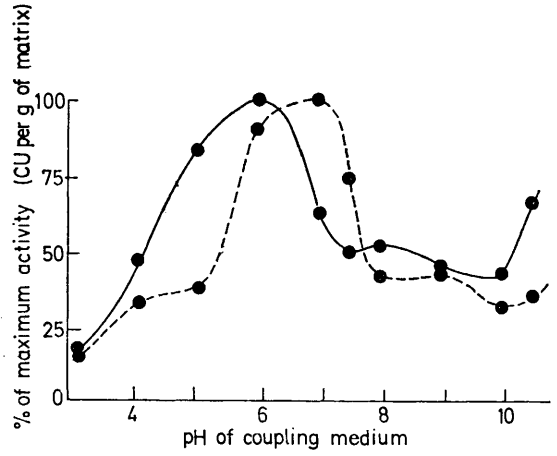

FIGURE 2 Dependence of the amount of chymotrypsin A activity bound to cellulose carbonate upon the pH of the coupling medium (cyclic carbonate content of matrix, 0·74 mmol NH_3 per g); caseinolytic activity (——), esterase activity (- - - -)

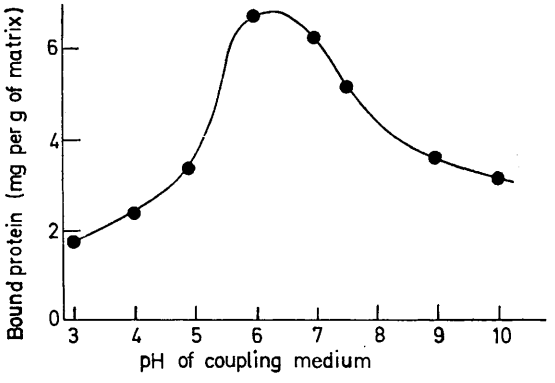

FIGURE 3 Dependence of the amount of chymotrypsin A protein bound to cellulose carbonate upon the pH of the coupling medium

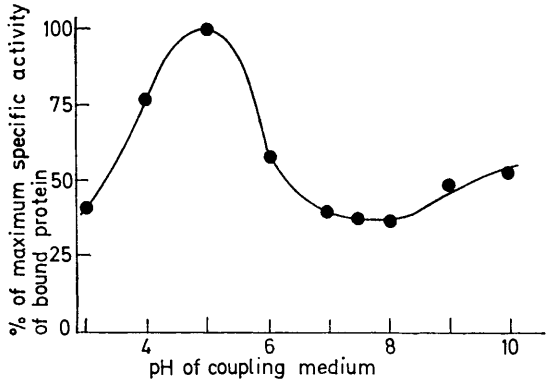

FIGURE 4 Relationship of the specific caseinolytic activity of chymotrypsin A bound to cellulose carbonate to the pH of the coupling medium

activity of each derivative being determined side by side. The ratios of these activities for the various samples were

[12] 'Techniques in Amino Acid Analysis,' Technicon Instruments Co. Ltd., Chertsey, England, 1966, p. 127.

compared with the ratio obtained for the soluble enzyme under identical conditions (Figure 5).

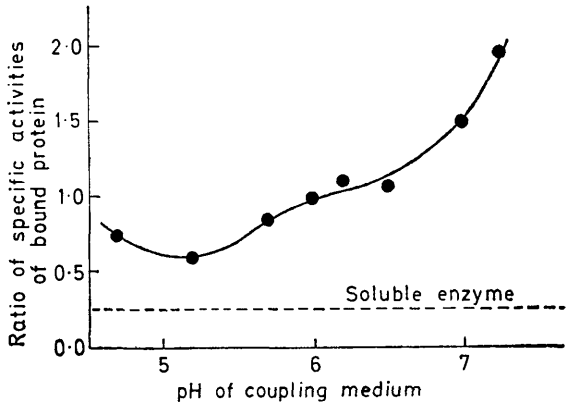

FIGURE 5 Ratio of the specific esterase and caseinolytic activities of chymotrypsin A bound to cellulose carbonate as a function of the pH of the coupling medium

(ii) *Variation of soluble enzyme concentration.* Insoluble enzyme derivatives were prepared at pH 5·4 in a similar way to that described in (i), but using an aqueous solution of the enzyme at various concentrations (0·5 ml; 5—100 mg ml^{-1}). The caseinolytic activities and the protein contents of the products were determined (Figure 6).

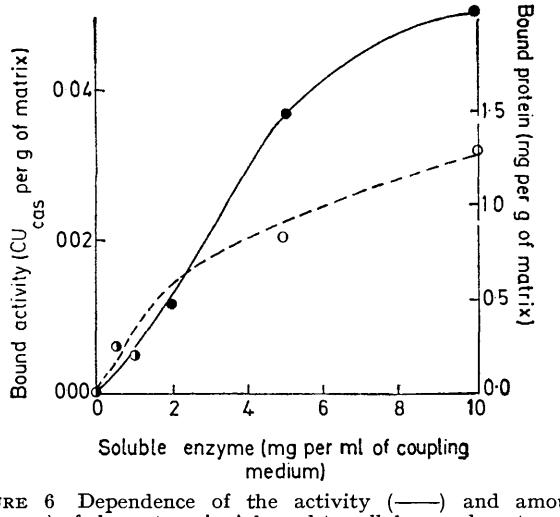

FIGURE 6 Dependence of the activity (——) and amount (- - - -) of chymotrypsin A bound to cellulose carbonate upon the concentration of soluble enzyme in the coupling medium (cyclic carbonate content of matrix 1·82 mmol NH_3 per g)

(iii) *Variation of coupling time.* Insoluble enzyme derivatives were prepared similarly at pH 5·4 but using an aqueous solution of the enzyme (0·5 ml; 20 mg ml^{-1}) and a reaction time of 1—45 h. The caseinolytic activities and the protein contents of the products were determined (Figure 7).

(iv) *Variation of cyclic carbonate content.* Insoluble enzyme derivatives were prepared similarly at pH 5·4 but using an aqueous solution of the enzyme (0·5 ml; 20 mg ml^{-1}) and cellulose carbonate samples with different cyclic carbonate contents. The esterase and caseinolytic activities of each sample were determined simultaneously (Figure 8) and the ratios of the two activities were compared with that of the soluble enzyme assayed under the same conditions (Figure 9). The protein content was determined (Figure 10), thus enabling the specific activity of the bound protein to be calculated (Figure 11).

Re-use of Insoluble Chymotrypsin A–Cellulose Carbonate.— Samples (40 mg) of the insoluble enzyme derivative prepared at pH 7·0 were incubated for a number of different times

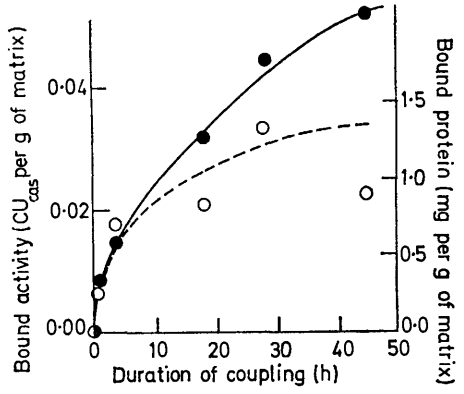

FIGURE 7 Dependence of the activity (——) and amount (- - - -) of chymotrypsin A bound to cellulose carbonate upon the duration of coupling (cyclic carbonate content of matrix 1·82 mmol NH_3 per g)

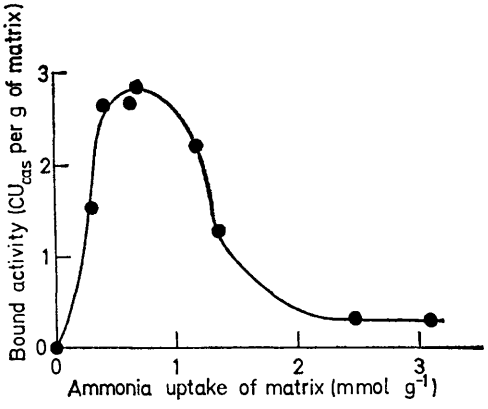

FIGURE 8 Dependence of the caseinolytic activity of chymotrypsin A bound to cellulose carbonate upon the cyclic carbonate content of the matrix

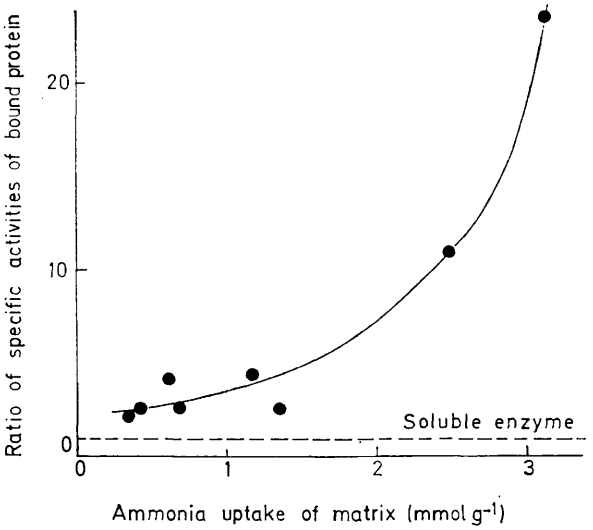

FIGURE 9 Ratio of the specific esterase and caseinolytic activities of chymotrypsin A bound to cellulose carbonate as a function of the cyclic carbonate content of the matrix

with the casein reagent under the conditions of the assay. After each incubation the substrate was removed and the solids were washed with water (2 ml). Fresh substrate was added and the incubation and washing process was repeated

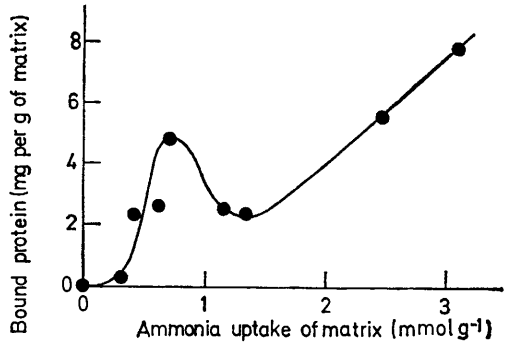

FIGURE 10 Dependence of the amount of chymotrypsin A protein bound to cellulose carbonate upon the cyclic carbonate content of the matrix

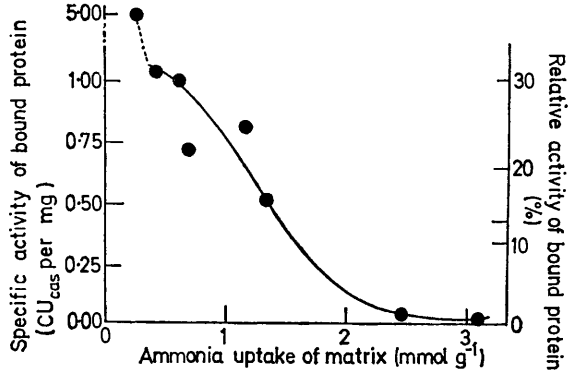

FIGURE 11 Dependence of the specific caseinolytic activity of chymotrypsin bound to cellulose carbonate upon the cyclic carbonate content of the matrix

the desired number of times. Before the final incubation the solids were washed with water (4 × 2 ml) and 0·1M-sodium borate buffer (pH 8·0; 2 × 2 ml), and the normal assay procedure was followed for the determination of the activity of each sample (Figure 12).

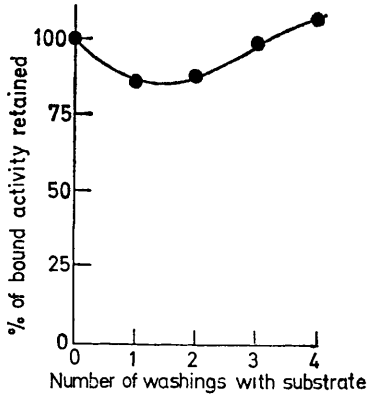

FIGURE 12 Examination of the caseinolytic activity of chymotrypsin A bound to cellulose carbonate on re-use

DISCUSSION

The method previously reported for the preparation of carbonates of cellulose [1] and other polysaccharides [13] was applied to samples which could be pre-dried to eliminate water, the presence of which in the reaction mixture can reduce extensively the degree of conversion into cyclic carbonate. Although in these cases the removal of water was not expected to have any effect on the physical form of the polysaccharides, it was necessary to modify the drying process for macroporous cellulose in order to maintain the open structure during derivatisation. Failure to maintain the cellulose in the extended state could be expected to give rise to high degrees of cross-linking, destruction of the macroporosity, and little derivatisation within the pores. Solvent exchange with dimethyl sulphoxide was therefore used to remove water from the cellulose and to maintain it in a swollen and macroporous form for and during the reaction. Complete removal of triethylamine hydrochloride after the derivatisation reaction could only be accomplished by increasing the number of washings with ethanol from the normal three [1] to ten. Presumably this was necessary because some of the hydrochloride was produced within the pores of the matrix.

It can be argued that the best insoluble derivative will not necessarily come from the cellulose sample which contains the highest degree of substitution with the cyclic carbonate groups. Overcrowding and multiple attachment of the enzyme molecules is most likely to occur in cases of high substitution, causing distortion or lack of availability of the active sites. It was found that the cyclic carbonate content of the cellulose carbonate could be closely controlled by the addition of water to the solvent mixture used for the derivatisation reaction. The fall of the cyclic carbonate content of the product, as determined by ammonia uptake and by quantitative i.r. spectroscopy at 1815 cm^{-1}, with increase in the water content of the reaction mixture is shown in Figure 1. A linear relationship existed between i.r. absorption and ammonia uptake of the various samples.

From samples of cellulose carbonate prepared in this way, a series of insoluble derivatives of chymotrypsin A was produced, and the effect of a number of parameters on the degree of covalent coupling of the enzyme to the cellulose carbonate was investigated. Variation of the pH of coupling for a fixed enzyme concentration and cyclic carbonate content (Figure 2) showed that a product with high activity towards casein was produced with a coupling pH of 5·0—6·0. This range is lower than that at which the derivative with the highest activity towards tyrosine ethyl ester was produced (pH 6·0—7·0). The highest degree of protein binding for a constant enzyme concentration and cyclic carbonate content was found to occur at pH 6·0—7·0 (Figure 3).

A more detailed investigation of the dependence of activity coupled upon the pH of coupling near the maximum narrowed the range to pH 5·2—5·7 and combination of this with the protein bound–pH curve showed that the highest specific caseinolytic activity of the bound enzyme was achieved by coupling at pH 5·2 (Figure 4). A plot

[13] J. F. Kennedy and H. Cho Tun, *Carbohydrate Res.*, 1973, **26**, 401.

of the ratio of esterase activity to caseinolytic activity (Figure 5) shows that the ratio is a minimum at pH 5·2, *i.e.* the caseinolytic activity is closest to the esterase activity at this point. Since maximum activity coupled was exhibited over the range pH 5·2—5·7 it was decided that the most suitable coupling pH would be 5·4. Under these conditions the specific activities of the bound enzyme towards casein and ester were 26 and 65%, respectively, of the specific activities of the free enzyme.

These results show that the amounts of activity and of protein bound during coupling are highly dependent upon the pH at which the coupling is carried out. The coupling observed is in fact the result of the operation of several factors; moreover, enzyme binding through nucleophilic attack of the free amino-groups of the protein is a minor reaction compared with the hydrolysis of the cyclic carbonate groups, as borne out by the fact that, after coupling for 18 h at pH 5·4, the ammonia uptake value of a sample of cellulose carbonate had fallen from 1·6 to 1·4 mmol per g of matrix, while only 1·7 mg of protein became bound per g of matrix. Although the attack on the cyclic carbonate groups by the protein could be expected to be favoured at higher pH values, so also would the hydrolysis reaction, and this may explain the low degree of coupling observed at higher pH values. Although autolysis of chymotrypsin A occurs to a maximum extent at pH 9—10 (ref. 14), any alternative explanation of the low degree of coupling at the higher pH values in terms of autolysis is probably of little significance on account of the low temperature used for the coupling. However it appears that when the hydrolysis reaction is slowed down by dropping the pH, the protein binding reaches a maximum. This maximum protein binding at a pH where nucleophilicity is expected to be low has already been noted for enzyme coupling to poly(allyl cyclic carbonate).[6,7]

The difference in the activity of the enzyme derivatives towards the two substrates may be due to chemical or physical effects. Chemical effects would arise from the different size and involvement of the active sites for the two enzyme activities, and from different degrees of distortion of the active sites on binding insofar as their binding abilities for the two substrates are altered. Physical effects can arise from the inability of the larger substrate to diffuse into the matrix and through an excess of attached protein compared with the ability of the lower molecular weight substrate to diffuse.

As already indicated, the full binding potential of the cyclic carbonate groups of the cellulose carbonate is not being employed in the coupling reaction. In addition to the fact that cyclic carbonate groups still remain after coupling, this incomplete reaction is illustrated by the continued rise in activity and in protein bound as the enzyme concentration (Figure 6) and reaction time (Figure 7) are increased. Presumably the increments are non-linear on account of increased crowding of the bound enzyme molecules.

The preparation of insoluble enzyme derivatives from cellulose carbonates of differing cyclic contents showed that the maximum coupling of caseinolytic activity occurred for samples with cyclic carbonate contents equivalent to 0·5—1·0 mmol of ammonia per g of matrix (Figure 8). The bound protein was generally found to rise with cyclic content, but there was a peak in protein binding for derivatives which showed the high enzyme activity (Figure 10). The specific activity of the bound protein showed a fall as the protein binding increased (Figure 11),* being highest at low levels of protein binding. This could again be the result of crowding of the bound enzyme molecules; alternatively it could be an indication that the matrix was of a poorer quality. It seemed likely that these types of factors were involved since the ratio of activities of the derivatives towards the ester and casein rose as the cyclic carbonate content of the matrix increased (Figure 9).

It was noted that as the water content of the reaction medium for the preparation of the cyclic carbonate was reduced, so the product (cellulose carbonate) particles were more compact. This is most probably due to the formation of carbonate cross linkages. Similar cross-linking has already been noted in dextrans undergoing treatment with cyanogen bromide to generate iminocarbonate groups.[15] This shrinkage must result in a loss of macroporous structure and this will result in the product being unable to bind large enzyme molecules within its matrix and therefore being of less use for enzyme insolubilisation than the porous matrices. This fall of activity of the bound enzyme product with increase in the initial cyclic carbonate content of the cellulose was found to be more serious for casein than for the ester (Figure 9). This is in agreement with the theory that diffusion effects are manifested on account of the closed structure and comparatively short assay times. Thus it is proposed that the peak in the bound protein curve (Figure 10) marks the point at which the degree of protein binding has reached a maximum under the coupling conditions used for a cellulose carbonate which is still macroporous. As the cyclic carbonate content is further increased, the matrix loses its gel structure and becomes increasingly granular. Since the specific caseinolytic activity of the insoluble enzyme continually fell as the cyclic carbonate content of the matrix was raised, the most suitable carbonate content is that which corresponds to maximum caseinolytic activity (Figure 8).

The experiments on re-use of the insoluble chymotrypsin A showed that after a slight fall in apparent activity, the activity of the material rose marginally with further re-use. The initial fall could be due to a slight loss of enzyme, *e.g.* removal of traces of physically bound

* In assessing the curve, it must be realised that calculation of specific activity may be subject to an amplified error arising from compounding of the errors inherent in the two contributing experimentally determined parameters. The value of the specific activity calculated for the cellulose carbonate of lowest ammonia uptake is relatively high, and this situation presumably arises from a low density of enzyme molecules on the surface of the matrix.

[14] B. H. J. Hoftsee, *Arch. Biochem. Biophys.*, 1965, **112**, 224
[15] L. Kågedal and S. Åkerström, *Acta Chem. Scand.*, 1971, **25**, 1855.

material by the substrate. The subsequent rise could be due to reorganisation, by the action of substrate as a template, of enzyme molecules reversibly disorganised or distorted on coupling. However, it is clear from the results that the insoluble enzyme derivative is stable to use.

In conclusion, the macroporous cellulose has advantages for the preparation of cellulose carbonate suitable for the insolubilisation of enzymes. Maximum activity of the enzyme derivative is achieved by controlling the cyclic carbonate content of the matrix and by using a coupling pH of 5·4. The enzyme derivative so produced exhibited high specific activity toward a high molecular weight substrate (25% of that of the free enzyme) and could be used many times without loss of activity.

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