Improvement of the Enzyme Immobilisation Characteristics of Macroporous Cellulose *trans*-2,3-Carbonate by Pre-swelling in Dimethyl Sulphoxide

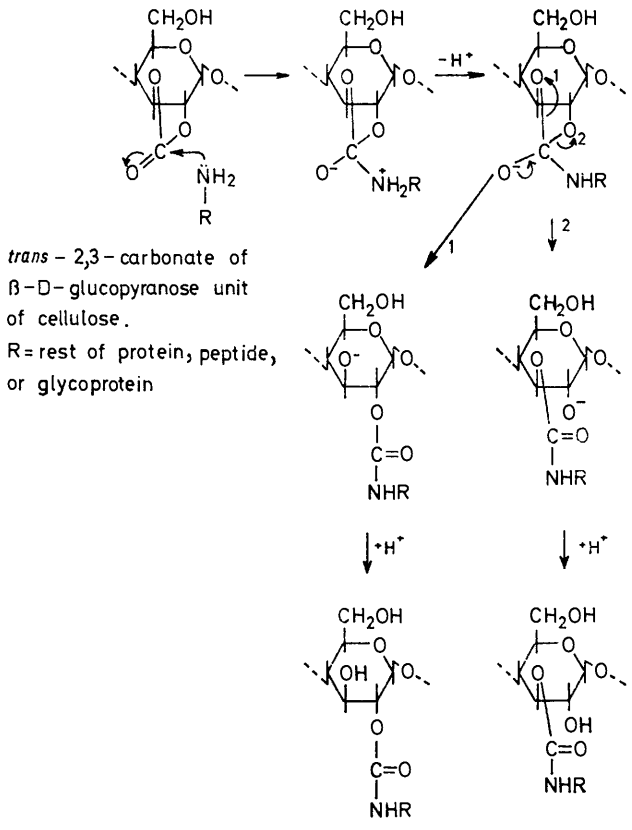
By **John F. Kennedy** * and **Alan Rosevear**, Department of Chemistry, The University of Birmingham, P.O. Box 363, Birmingham B15 2TT

The amount of chymotrypsin A protein covalently bound to macroporous cellulose *trans*-2,3-carbonate and the caseinolytic activity of the resulting immobilised enzyme have been substantially improved by swelling the matrix in dimethyl sulphoxide before coupling with the enzyme. This improvement has been attributed to an increase in the porosity of the insoluble support during the pre-swelling process which enables macromolecules to diffuse into the matrix. Storage of the cellulose carbonate under inappropriate conditions before coupling with protein resulted in an irreversible deterioration in the physical characteristics of the matrix.

THE use of polysaccharides as a matrix has proved very popular in the field of insolubilisation.[1] However, invariably the polysaccharide must first be converted into a more reactive form to permit a chemical bonding reaction with the molecule to be immobilised to occur. *trans*-2,3-Cyclic carbonate substituents have been introduced into microcrystalline cellulose by its reaction with ethyl chloroformate in anhydrous organic solvents, and the conditions for achieving a maximum degree of substitution have been defined.[2] The strain and electronic arrangement in the ground state of a *trans*-1,2-carbonate ring fused to a six-membered ring in the chair conformation is such that the carbonyl carbon atom of the carbonate ring is susceptible to nucleophilic attack. Thus the cyclic ester derivative of cellulose has been shown to exhibit suitable reactivity for the covalent coupling of enzymic protein under mild aqueous conditions to give an active insoluble derivative of β-D-glucosidase.[3] From a study of the reaction of simple compounds with cellulose carbonate,[4] it is envisaged that the coupling reaction involves the nucleophilic attack of a free amino-group in the enzyme protein on the strained *trans*-cyclic groups (Scheme). As can be seen, the carbonate ring may open in two ways to give products in which the D-glucopyranose ring is substituted at the 2- or 3-position. In view of the initial success in forming a stable covalent bond between cellulose *trans*-2,3-carbonate and an enzyme, further studies have been carried out to optimise the coupling conditions for the preparation of highly active derivatives of several enzymes.[5] Furthermore the use of cellulose *trans*-2,3-carbonate has been extended (*a*) to the insolubilisation of antigens for the purification of antibodies to immunoglobulins IgG and IgM [6] and for the radioimmunoassay of the fertility glycoprotein follicle-stimulating hormone,[7] (*b*) to the insolubilisation of antibodies for the radioimmunoassay of follicle-stimulating hormone [7] and immunoglobuilin IgE,[8] and (*c*) to the insolubilisation of antibiotics.[9]

[1] J. F. Kennedy, *Adv. Carbohydrate Chem. Biochem.*, in the press.
[2] S. A. Barker, H. Cho Tun, S. H. Doss, C. J. Gray, and J. F. Kennedy, *Carbohydrate Res.*, 1971, **17**, 471.
[3] S. A. Barker, S. H. Doss, C. J. Gray, J. F. Kennedy, M. Stacey, and T. H. Yeo, *Carbohydrate Res.*, 1971, **20**, 1.
[4] J. F. Kennedy and H. Cho Tun, *Carbohydrate Res.*, 1973, **29**, 246.
[5] J. F. Kennedy and A. Zamir, *Carbohydrate Res.*, 1973, **29**, 497.
[6] D. Catty, J. F. Kennedy, R. L. Drew, and H. Cho Tun, *J. Immunological Methods*, 1973, **2**, 353.
[7] J. F. Kennedy and H. Cho Tun, *Carbohydrate Res.*, 1973, **30**, 11.
[8] P. McLaughlan, D. R. Stanworth, J. F. Kennedy, and H. Cho Tun, *Nature (New Biology)*, 1971, **232**, 245.
[9] J. F. Kennedy and H. Cho Tun, *Antimicrobial Agents and Chemotherapy*, 1973, **3**, 575.

The preparation of cyclic carbonate derivatives of other polysaccharides and cycloamyloses has also been described [10] and a novel but analogous eight-membered carbonate ring system, poly(allyl carbonate), has also been used for enzyme insolubilisation.[11,12] More recently, we have applied the ethyl chloroformate reaction to macroporous cellulose.[13] The degree of substitution of macroporous cellulose with cyclic carbonate groups has been controlled by moderating the reaction of the carbohydrate and ethyl chloroformate through the addition of small quantities of water to the reaction mixture.[13] By comparing the esterase and caseinolytic activities of chymotrypsin A which had been covalently coupled to this matrix it was possible to make some estimation of the restriction imposed by the matrix on the approach of the high molecular weight casein substrate molecules to the bound enzyme molecules. It was proposed that as the degree of carbonate substitution of the matrix was increased the porosity of the matrix fell as a result of increased cross-linking by biscarbonate groups. The most suitable matrix for immobilisation of a protease with high caseinolytic activity was therefore one which was only partially substituted by carbonate groups and thus retained an appreciable porosity towards macromolecules. A method of further improving the enzyme binding characteristics of macroporous cellulose carbonate by pre-swelling the matrix in dimethyl sulphoxide is now reported.



SCHEME

DISCUSSION

Macroporous cellulose carbonate (type D), which had been stored in a desiccator prior to its coupling to chymotrypsin A, bound less enzymic protein and gave a less active enzyme derivative than the same matrix used immediately after its preparation as a dry powder (Table 1). This fall in protein binding ability could not be fully attributed to a deterioration in the chemical properties of the matrix since the reaction of the matrix with ammonia was only marginally impaired on storage (Table 1). It was therefore attributed to physical changes in the matrix and so a means of preventing this decline in the protein binding capabilities of the matrix was sought.

Dry macroporous cellulose carbonate was swollen in dimethyl sulphoxide for 8 and 13 days (type DS) before an enzyme derivative was prepared. During this time some deterioration occurred in the ammonia uptake of the matrix but the resulting enzyme derivatives contained larger amounts of bound protein with retention of higher specific caseinolytic activity than either the fresh or the stored samples of type D matrix (Table 1).

TABLE 1

Effect of storage of macroporous cellulose carbonate (types D and DS) before coupling to chymotrypsin A

Type of cellulose carbonate	Storage duration (days)	Ammonia uptake (mmol g^{-1} matrix)	Bound caseinolytic activity units g^{-1} matrix	Bound protein (mg g^{-1} matrix)	Retention of specific caseinolytic activity of bound protein (%)
D	0	3·1	0·22	7·9	0·9
D	13	3·0	0·22	4·2	1·6
DS [a]	13	2·3	2·97	15·4	6·0
D	0	1·6	0·48	1·7	8·9
D	8	1·6	0·29	1·7	5·4
DS [a]	8	1·4	4·39	10·7	12·6

[a] Swollen in dimethyl sulphoxide.

Matrices with a range of cyclic carbonate contents were stored in dimethyl sulphoxide for 16 days (type DS) before chymotrypsin A was coupled to them, and the results compared with those for enzyme derivatives

[10] J. F. Kennedy and H. Cho Tun, *Carbohydrate Res.*, 1973, **26**, 401.
[11] S. A. Barker, J. F. Kennedy, and A. Rosevear, *J. Chem. Soc.* (*C*), 1971, 2726.
[12] J. F. Kennedy, S. A. Barker, and A. Rosevear, *J.C.S. Perkin I*, 1972, 2568.
[13] J. F. Kennedy, S. A. Barker, and A. Rosevear, *J.C.S. Perkin I*, 1973, 2293.

prepared from fresh dry samples of the same matrix (type D). The maximum amounts of caseinolytic and

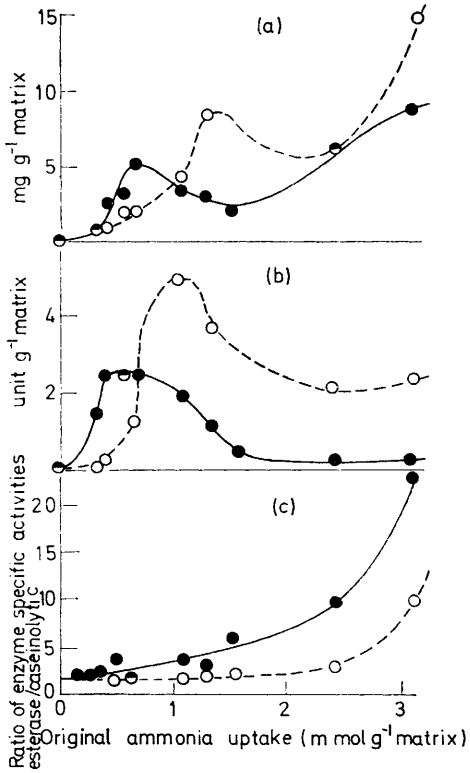


FIGURE 1 Dependence of (a) amount of protein bound, (b) bound caseinolytic activity, and (c) ratio of esterase to caseinolytic activity of bound chymotripsin A upon original cyclic carbonate contents of macroporous cellulose carbonates: type D (——), and type DS swollen in dimethyl sulphoxide for 16 days (— — —)

esterase activity and protein bound to type DS matrix were twice as large as those for type D matrix. However, the profiles of the dependence of these parameters on the cyclic carbonate contents of the matrices were similar for the protein [Figure 1(a)], the caseinolytic [Figure 1(b)], and esterase activities of types D and DS.

The storage of macroporous cellulose carbonate in dimethyl sulphoxide therefore apparently results in two types of modification of the matrix. First, the porosity of the matrix is increased by the swelling and thus a larger surface area is made available for reaction with macromolecules. This increases the protein bound and also contributes to the rise in its caseinolytic activity since the high molecular weight substrate can penetrate more freely to the active sites of the bound enzyme, hence the fall in the ratio of specific esterase to caseinolytic activities [Figure 1(c)]. Secondly, dimethyl sulphoxide also causes a slow deterioration in the cyclic carbonate content of the matrix thus reducing its potential for covalently binding enzymic protein. In matrices of high degrees of ester substitution, where the cyclic carbonate groups were in abundance, the improvement in activity and protein bound due to swelling would be the dominant factor, whereas in the more lightly substituted matrices, which would be expected to be fairly porous already, the deterioriation in the protein binding would be the dominant factor. Thus the overall change in the caseinolytic activity of the enzyme derivative prepared after swelling depended on the original cyclic carbonate content of the matrix (Figure 2).

A comparison of the gel permeation and packing characteristics of the cellulose and cellulose carbonate (types D and DS) as evaluated using the materials for standard gel filtration experiments (Table 2) suggested that a difference existed between the macroporosities of the cellulose carbonate samples and cellulose itself. The porosity of the cellulose fell and the packing density of the matrix rose on conversion into the carbonate (type D), but storage in dimethyl sulphoxide reduced the packing density of the carbonate and increased the pore volume available to macromolecules, particularly those of a molecular weight $>2 \times 10^4$. These results therefore established that the swollen matrix is more accessible to chymotrypsin A and casein which both have molecular weights of *ca.* 25,000.[14,15]

A range of solvents were therefore examined as potential swelling agents but none proved superior to dimethyl sulphoxide for improving both the bound enzymic activity and bound protein (Table 3). Although dimethylformamide increased the porosity sufficiently to allow large amounts of enzymic protein to bind to the matrix, this bound enzyme did not appear to be as freely accessible to casein molecules as that bound to matrix which had been swollen in dimethyl sulphoxide. Ethylene glycol showed some potential as a swelling agent but acetone, 1,4-dioxan, and aqueous 4M-lithium

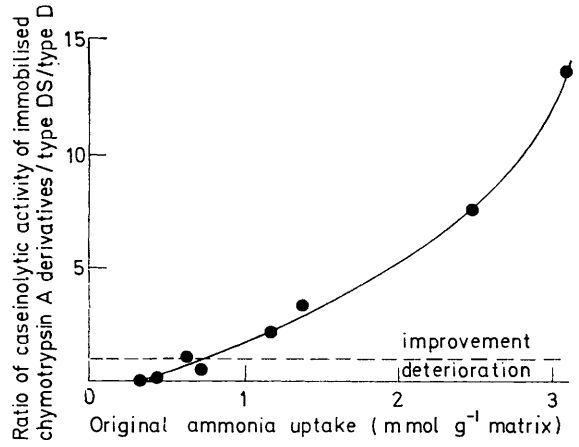


FIGURE 2 Dependence of the ratio of caseinolytic activities of chymotrypsin A bound to macroprous cellulose carbonates, type DS swollen in dimethyl sulphoxide for 16 days, and type D, upon original cyclic carbonate content of the macroporous cellulose carbonate

chloride caused a deterioration in the matrix as an enzyme support.

Thus, as expected, the overall enzyme binding characteristics of the matrix were found to depend on a

[14] T. E. Barman, 'Enzyme Handbook,' Springer-Verlag, Berlin, 1969.
[15] P. Jollès, *Angew. Chem. Internat. Edn.*, 1966, **5**, 558.

TABLE 2

Comparison of gel permeation characteristics of macroporous cellulose and macroporous cellulose carbonates (types D and DS)

Matrix	Original ammonia uptake (mmol g^{-1} matrix)	Bed volume (cm^3)	Density of packing (g cm^{-3})	Elution volume (ml): D-Glucose (G)	Elution volume (ml): 2×10^4 M.Wt. dextran (D_4)	Elution volume (ml): 2×10^6 M.Wt. dextran (D_6)	$(D_4 - D_6)$ per g of matrix (ml g^{-1})	$(G - D_6)$ per g of matrix (mol g^{-1})
Cellulose	0·0	30·3	0·09	28·2	18·2	13·4	1·74	5·5
Cellulose carbonate (type D)	0·7	30·0	0·30	24·1	15·7	15·2	0·06	1·0
Cellulose carbonate (type DS)	1·0	32·8	0·19	30·1	20·7	15·8	0·80	2·3

TABLE 3

Swelling of macroporous cellulose carbonate in various solvents

Type	Agent	Duration (h)	Bound caseinolytic activity (unit g^{-1} matrix)	Bound protein (mg g^{-1} matrix)	Retention of specific caseinolytic activity of bound protein (%)	Ratio of specific activities esterase/caseinolytic
D [a]	Dry	24	2·85	4·8	17·9	2·5
DS [a]	Dimethyl sulphoxide	6	4·40	11·9	11·3	2·5
		24	4·74	12·7	11·3	1·9
DS [a]	Ethylene glycol	24	3·75	6·3	17·9	2·0
DS [a]	4M-LiCl	24	0·17	1·0	6·1	9·2
D [b]	Dry	24	0·68	6·5	4·8	5·2
DS [b]	Dimethyl formamide	24	1·70	23·5	3·3	8·4
DS [b]	Dimethyl sulphoxide	24	3·00	24·0	5·8	5·6
D [b]	Dry	192	0·54	4·6	5·4	5·4
DS [b]	Acetone	192	0·46	3·9	5·4	10·4
DS [b]	1,4-Dioxan	192	0·38	4·5	3·9	9·5

[a] Ammonia uptake of matrix, 0·7 mmol g^{-1}. Activity of soluble enzyme, 3·27 casein unit mg^{-1}. [b] Ammonia uptake of matrix, 1·5 mmol g^{-1}. Activity of soluble enzyme, 2·17 casein unit mg^{-1}.

TABLE 4

Coupling of chymotrypsin A to undried, macroporous cellulose carbonate

Type	Bound caseinolytic activity (units g^{-1} matrix)	Bound protein (mg g^{-1} matrix)	Retention of specific activity of bound protein (%) [a]: Esterase	Retention of specific activity of bound protein (%) [a]: Caseinolytic	Ratio of specific activities esterase/caseinolytic	Proportion of soluble enzyme coupled (%)
D	2·85	4·8	45·0	17·9	2·5	10
UA	2·65	14·0	26·0	5·9	4·4	28
UB	7·12	26·9	13·5	8·1	1·7	54
UAS	4·20	7·4	36·9	17·4	2·2	15
UBS	9·08	18·7	18·8	14·9	1·3	37

[a] Activity of original soluble enzyme; caseinolytic 3·27 unit mg^{-1}; esterase 0·88 unit mg^{-1}.

TABLE 5

Effect of increasing the soluble enzyme concentration and coupling time in the binding of chymotrypsin A to macroporous cellulose carbonate type DS

Batch	Soluble enzyme (mg)	Volume of coupling media (ml)	Coupling time (h)	Bound caseinolytic activity (unit g^{-1} matrix)	Bound protein (mg^{-1} matrix)	Retention of specific caseinolytic activity of bound protein (%)	Proportion of soluble enzyme coupled (%)
1	10	5	18	2·92	6·6	13·5	13
	10	1	18	3·57	12·1	9·2	24
2	10	5	18	1·90	10·3	8·6	21
	10	1	18	2·85	17·5	7·6	35
	30	1	18	5·22	35·0	6·9	23
3	10	5	18	2·60	7·2	16·7	14
	30	1	18	5·18	19·2	12·5	13
4	10	5	18	4·17	7·4	17·4	15
	10	5	40	7·46	11·7	19·6	24
5	10	5	18	9·05	18·7	14·9	38
	10	5	40	10·39	22·8	13·9	46

combination of the two opposing effects of matrix swelling and cyclic carbonate deterioration. This resulted in a dependence of the swelling time for optimum bound protein and activity in the enzyme derivative upon the original cyclic carbonate content of the matrix. Swelling of the heavily substituted matrix in dimethyl sulphoxide gave a higher maximum bound protein and activity than the lightly substituted matrix, as might be expected from its greater protein binding potential. However, the maximum specific activity of protein bound to the heavily substituted matrix was only 18% compared with 35% for the less substituted matrix, presumably due to its lower porosity.

The protein bound to the macroporous cellulose carbonate declined after prolonged swelling and eventually fell below that for the dry stored matrix (type D), presumably as a result of the fall in the number of cyclic carbonate substituents. It was noted that the deterioration in the protein binding capabilities of the matrix was most serious in the early stages of storage (Figure 3). Batches of macroporous cellulose carbonate were therefore prepared by a modified procedure which kept the matrix suspended in a solvent for the period between its preparation and use for coupling enzyme. These batches were designated 'undried, solvent-retained' (types U). Undried macroporous cellulose carbonate was prepared by two different washing procedures (types UA and UB), and the enzyme was coupled to the matrices as soon as the final washing–storage process was complete. Type UA bound three times as much protein as a similar batch of carbonate which had been isolated as a dry, solvent free powder before coupling (type D) (Table 4). The low specific activity of enzyme bound to type UA matrix may have been the result of crowding of enzymic protein on the matrix surface. Type UB bound five times as much protein as type D matrix and again the specific activity of the bound protein was low. However, the overall bound activity of the derivative of undried (solvent stored) type UB matrix was appreciably higher than that of type D. The ratio of the esterase to caseinolytic activities of the bound enzyme was much lower for derivatives of type UB matrix than for those of preparations from type UA and D matrices, indicating that this matrix interfered least with the diffusion of high molecular weight substrate.

These results suggested that when all solvent was removed, rapid irreversible deterioration of the physical properties of the matrix needed to produce a suitable enzyme support occurred. The superior enzyme binding properties of type UB matrix also indicated that the solvents used in the washing procedure could also modify the reactivity of the surface and make it a less acceptable microenvironment for the bound protein. This was due particularly to 1,4-dioxan which had already been shown to have poor matrix swelling properties (Table 3).

When type UA and UB matrices were swollen in dimethyl sulphoxide for 8 days before coupling, the resulting matrices, type UAS and UBS respectively, had a higher bound activity and lower ratio of specific esterase to caseinolytic activities than any previous preparations (Table 4). The bound protein of these samples was, however, lower than for types UA and UB presumably as a result of a loss in cyclic carbonate groups.

In an attempt to define the upper limits of the protein binding capacity of the swollen macroporous cellulose carbonate, higher concentrations of soluble enzyme were used in the coupling media and longer coupling times were employed. Increases in both these parameters led

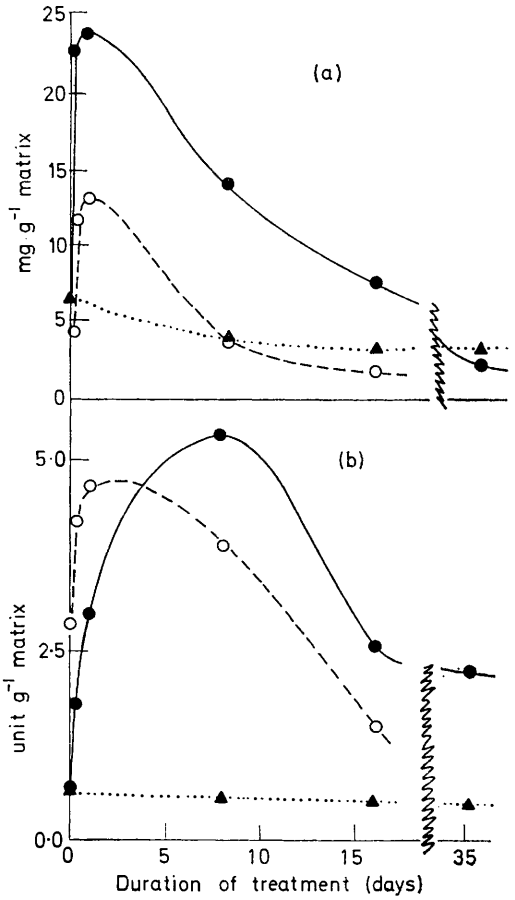


FIGURE 3 Dependence of amount of chymotrypsin A (a) protein and (b) caseinolytic activity bound to macroporous cellulose carbonate upon degree of swelling in dimethyl sulphoxide of the carrier macroporous cellulose carbonate type DS: (——) original ammonia uptake 1·46 mmol g^{-1} matrix, (— — —) original ammonia uptake 0·69 mmol g^{-1} matrix, and upon storage (· · · ·) of macroporous cellulose carbonate type D

to enzyme derivatives with higher bound protein and activity (Table 5). It was therefore concluded that the limits of protein binding to the swollen matrix had not been reached. However, there was a slow decrease in the specific caseinolytic activity of the matrices as more protein was bound, probably as a result of increased mutual interference between bound enzyme molecules. When enzyme coupling was performed with a smaller volume of enzyme solution, a higher proportion of the

enzyme was coupled, thus giving a more economic use of the soluble enzyme.

From these studies it was concluded that the drop in macroporosity of cellulose carbonate owing to biscarbonate cross-linking could be partially overcome by swelling the matrix in dimethyl sulphoxide. Since the physical characteristics of the matrix deteriorated irreversibly when all solvent was removed, it was necessary to suspend the matrix in dimethyl sulphoxide during the period between its preparation and its use. Because a slow deterioration in the chemical properties of the matrix occurred under these conditions reducing the amount of protein which could be covalently bound to the support, the period between preparation of the carbonate and the enzyme binding reaction was limited. This work illustrates the need in the field of insolubilisation to define closely the physical as well as the chemical properties of a matrix to which protein is to be bound.

EXPERIMENTAL

Preparation of Dry, Solvent-free, Macroporous Cellulose Carbonate Samples.—Macroporous cellulose carbonate was prepared as described previously,[13] using various amounts of water to give a range of cyclic carbonate contents. The material was either used directly in this dry, solvent-free form (type D) or was swollen by suspension (0·2 g) in the appropriate solvent [usually dimethyl sulphoxide (5 ml)] for 6—35 days (type DS).

Preparation of Undried, Solvent-retained Macroporous Cellulose Carbonate Samples.—Macroporous cellulose [16] was treated with ethyl chloroformate in a mixture containing water (0·25 ml) as described previously.[13] The insoluble product was washed either with dry 1,4-dioxan (10 × 20 ml), dry ethanol (10 × 20 ml), and dry dimethyl sulphoxide (3 × 20 ml) (type UA) or with 1 : 1 v/v dry dimethylsulphoxide–dry 1,4-dioxan (10 × 20 ml), 1 : 1 v/v dry dimethyl sulphoxide–dry ethanol (12 × 20 ml), and dry dimethyl sulphoxide (3 × 20 ml) (type UB). The products, from which solvent at no time had been removed, were isolated at the pump on sintered glass. Samples of types UA and UB were swollen in dimethyl sulphoxide as above for 8 days (types UAS and UBS respectively).

Coupling of Chymotrypsin A to Macroporous Cellulose Carbonates.—Samples of dry solvent free macroporous cellulose carbonate (type D; 0·2 g), which had been stored over phosphorous pentoxide and paraffin wax at 20° for 6 h—35 days, and of dry, solvent-free, macroporous cellulose carbonate (type DS; 0·2 g) which had been swollen in organic solvent for the same period, were washed with water (5 × 5 ml). Samples were suspended in 0·1M-citric acid–0·2M-sodium phosphate buffer (pH 5·4; 4·5 ml) and stirred with an aqueous solution of chymotrypsin A (Worthington Biochem Corp.; CDI/OCC; from bovine pancreas; 20 mg ml^{-1}; 0·5 ml) for 18 h at 4°. Further samples of type DS material (0·2 g) were also stirred with a solution of the enzyme in the citric acid–phosphate buffer (10 and 30 mg ml^{-1}; 1 ml). Freshly prepared samples of undried, macroporous cellulose carbonate (from which the solvent had not been removed) (types UA, UB, UAS, and UBS; 0·2 g) were washed with water (5 × 5 ml), suspended in the citric acid–phosphate buffer (4·5 ml), and stirred with an aqueous suspension of the enzyme as above.

Immediately after coupling, all insoluble enzyme samples were washed alternately with 0·005M-sodium acetate buffer (pH 5·0; 5 × 10 ml) and M-sodium chloride–M-sucrose (5 × 7·5 ml) over 2—3 h. The samples were finally washed with the acetate buffer (2 × 10 ml) and were isolated on sintered glass at the pump. The excess of water was removed by pressing the samples between filter papers, and the caseinolytic and esterase activities of aliquot portions of each sample were determined simultaneously by the previously described modifications [13] of the methods of Bergmeyer.[17] Further portions of the samples were rigorously dried and the protein contents determined by the method previously described.[13]

Gel Permeation Properties of Matrices.—Aqueous suspensions of macroporous cellulose, macroporous cellulose carbonate (type D), and of macroporous cellulose carbonate which had been swollen in dimethyl sulphoxide for 1 day (type DS) and washed with water, were allowed to settle under gravity in columns (*ca.* 30 cm × 0·6 cm). The columns were eluted overnight for 16 h to achieve equilibrium. Individual samples of dextrans of known molecular weights (Pharmacia Ltd.; 1 mg ml^{-1}, 0·1 ml) were eluted separately at a constant rate from each column and the carbohydrate content of the eluate was continuously monitored by an automated version of the cysteine sulphuric acid assay.[18] Part (0·175 ml min^{-1}) of the column eluate was mixed with 86% v/v sulphuric acid containing L-cysteine hydrochloride (700 μg ml^{-1}; 0·614 ml min^{-1}) using a Carlo Erba peristaltic proportionating pump, heated at 100° for 5 min by passage through a coil submerged in a heating bath, cooled, and the absorbance at 415 nm was taken using a Fisons Vitatron colorimeter with a 10 mm path length flow through cell. The elution volume of each dextran was calculated, plotted graphically, and the gel permeation properties of each matrix determined (Table 2).

We thank Professors S. A. Barker and M. Stacey for their interest in this work which was supported by an S.R.C. research studentship to A. R.

[3/1370 *Received*, 29*th June*, 1973]

[16] Pharmacia Fine Chemicals AB., Uppsala, B.P. 1,234,099/1971.

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

[18] J. F. Kennedy and W. R. Butt, *Biochem. J.*, 1969, 115, 225·