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AN IMMOBILISED-ENZYME REACTOR–SEPARATOR FOR THE HYDROLYSIS OF MACROMOLECULAR CARBOHYDRATES

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ABSTRACT

Passage, in the pulsed mode, of dextran of molecular weight greater than the exclusion limit of Porasil E over dextranase (EC 3.2.1.11) immobilised by covalent reaction with porous titanium(IV) oxide particles coated with diazotised 1,3-diaminobenzene co-packed with Porasil E resulted in simultaneous hydrolysis of the dextran (reactor) and separation of the products of low molecular weight from unused substrate (separator). Operation of the reactor–separator in continuous and pulsed modes permitted the degree of degradation to be estimated. Kinetic aspects and the possibility that concomitant production and separation may improve the apparent reactivity of the enzyme are discussed.

INTRODUCTION

Recent work (*e.g.*, ref. 1) on the immobilisation of enzymes has been orientated toward their use as the chemically active ingredient of continuously operating reactors. An example is the conversion of D-glucose into fructose syrups by immobilised D-glucose isomerase (EC 5.3.1.18). However, in some instances, the fractionation of products may be required, and we have considered the possibility of combining an immobilised-enzyme reactor with a product-separation facility. Moreover, product removal might accelerate enzymic reaction by decreasing product inhibition.

As part of our programme[2] on the use of transition-metal chelation for immobilisation of enzymes on non-magnetic[3–7] and magnetic supports[6,8], amino acids and peptides[4], antibiotics[9], cells[10], and carbohydrates[11], an immobilisation matrix based on titanium(IV) oxide coated with diazotised 1,3-diaminobenzene[12] has been developed. This matrix is suited to column packing with good flow-rates, and dextranase immobilised upon it has been shown to degrade satisfactorily dextrans of a variety of molecular weights. The kinetics of the immobilised enzyme have been discussed[12].

We now report on the use of immobilised dextranase in combination with molecular permeation chromatography for the concomitant degradation of dextran and the separation of products of high and low molecular weight.

EXPERIMENTAL

Dual, automated assay for total and reducing carbohydrate. — Using modular equipment (Carlo Erba peristaltic proportionating pump, Fisons Vitatron Colorimeters, Leeds and Northrup 0–10 mV multipoint recorder), a system was set up as shown in Fig. 1, based on the 3,5-dinitrosalicylic acid assay for reducing sugars[5,13] and the L-cysteine–sulphuric acid assay for total carbohydrate[14,15]. The assay reagents consisted of 3,5-dinitrosalicylic acid (1 mg/ml) and sodium potassium tartrate (0.75 mg/ml) dissolved in 0.5M sodium hydroxide, and 0.07% (w/v) L-cysteine hydrochloride in 87% (w/v) sulphuric acid (acid–water, 1:6 by vol).

Immobilisation of dextranase. — The enzyme was immobilised as described previously[12], using porous titanium(IV) oxide (type M27, 0.3-mm particle diameter, large pore-size) (kindly supplied by Dr. A. R. Thompson, AERE, Harwell) treated with diazotised 1,3-diaminobenzene.

The solid (~2 g) was washed rapidly with 0.2M sodium acetate buffer (pH 5.0, 3 × 100 ml) at 0°. A solution of dextranase [(1→6)-α-D-glucan 6-glucanohydrolase, EC 3.2.1.11, Koch–Light Ltd.; 5 mg/ml] in the same buffer (20 ml) at 4° was added and the mixture was shaken at 4° for 2 h. The solid was then shaken with a saturated solution of 2-naphthol in saturated, aqueous sodium acetate (200 ml) at 4° for 4.5 h, collected by decantation, and washed with 0.2M sodium acetate buffer (pH 5.0,

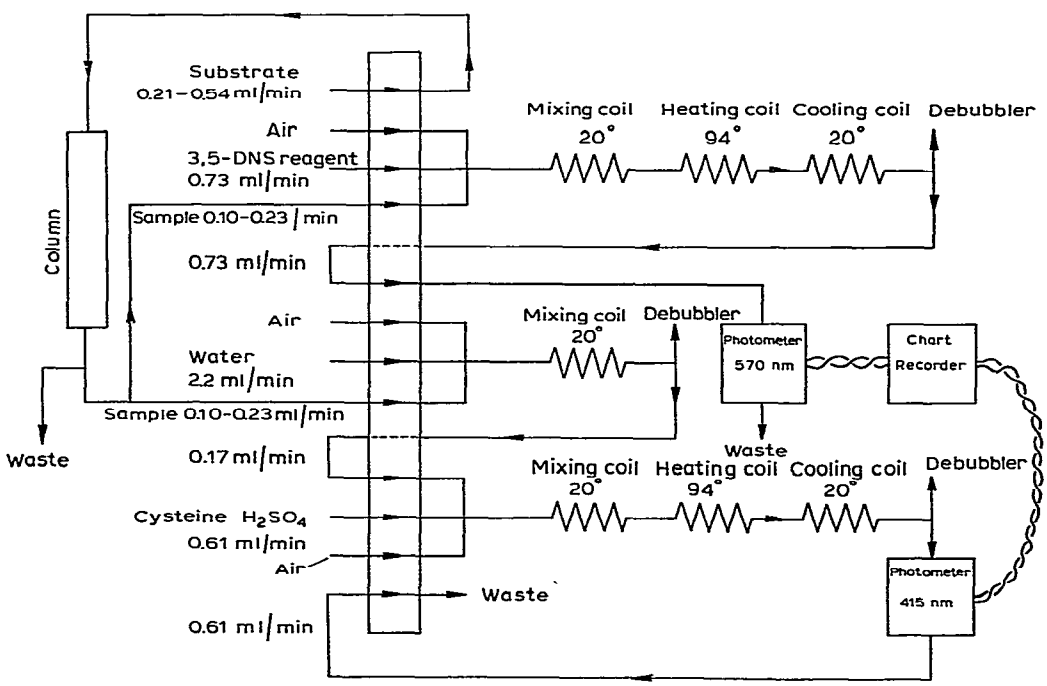

Fig. 1. Modular analytical system for dual, automated, total carbohydrate (L-cysteine–sulphuric acid) and reducing carbohydrate (3,5-dinitrosalicylic acid) assay of reactor–separator output.

5 × 100 ml) at 4°. The immobilised-enzyme derivative was stored at 4° in the same buffer. The material had 17 units[5]/g of dextranase activity, 4.08 mg/g of bound protein, and values for the specific activity and retention of activity of bound enzyme of 4.16 units/mg (original, 35.6 units/mg) and 11.7%, respectively.

Immobilised-dextranase reactor–separator. — Porasil E (Waters Associates Ltd) was boiled for 10 min with excess of M nitric acid, washed with water until neutral, and uniformly mixed with immobilised dextranase (1 g). The column (total volume, V_t) was continuously equilibrated with 0.1M sodium acetate buffer (pH 5.0) for 24 h.

Dextran solutions (0.5–5.0% w/v; molecular weights, 4.4×10^4 and 5.0×10^5; Pharmacia) in 0.1M sodium acetate buffer (pH 5.0) were passed down the column continuously at 0.21–0.54 ml/min and 25°. Recorder traces from product analysis (see below) were allowed to attain a steady value prior to changing the input concentration of dextran. Pulsed samples (0.1–0.4 ml) of various concentrations (1.25–20% w/v) of the same dextrans in the acetate buffer were also passed down the column, either singly, or in repeated pulses interspersed with buffer.

Products eluted from the column were monitored by the dual, automated

TABLE I

RETENTION FACTORS OF PRODUCTS FROM APPLICATION OF DEXTRAN TO AN IMMOBILISED-DEXTRANASE REACTOR–SEPARATOR

Carbohydrate type and weight (mg) loaded[a]	R_f *for reducing carbohydrate*	R_f *for total carbohydrate*	
		1st Peak	*2nd Peak*
D-*Glucose*			
4	0.88	—	0.88
4[b]	0.75	—	0.76
Dextran (mol. wt. 500,000)			
20[b]	0.83	—	0.84
20	0.75	0.43	0.76
15	0.75	0.43	0.76
10	0.70	0.41	0.70
5	0.75	0.43	0.76
2.5	0.76	0.43	0.76
1.25	0.76	0.45	0.77
0.625	0.76	0.43	0.77
Dextran (mol. wt. 44,000)			
20	0.75	—	0.76
15	0.76	—	0.76
10	0.75	—	0.76
5	0.75	—	0.76
2.5	0.76	—	0.77
1.25	0.76	—	0.77
0.625	0.76	—	0.77

[a]Load volume, 0.1 ml; column flow-rate, 0.25 ml/min. [b]Column flow-rate, 0.54 ml/min.

TABLE II

CONVERSIONS AND RECOVERIES OF DEXTRAN (MOL. WT. 500,000) FROM THE REACTOR–SEPARATOR

Dextran loaded[a] (mg)	*Reducing groups in product (mg D-glucose equivalents)*	*Converted dextran (%)*	*Carbohydrate recovery (%)*	*Average chain-length of converted product*	*Average chain-length of total product*
80	12.4	—	69	—	4.44
40	7.9	—	75	—	3.80
20	5.5	87	78	2.4	2.9
15	4.4	87	80	2.3	2.7
10	3.2	91	80	2.2	2.5
5	1.80	90	81	2.0	2.3
2.5	0.68	85	105	2.8	3.1
1.25	0.26	91	112	4.9	5.4

[a]Load volume, 0.1 ml; column flow-rate, 0.25 ml/min. [b]Calculated from the area of the first peak, total carbohydrate assay, and expressed as a percentage of the carbohydrate recovered.

TABLE III

CONVERSIONS AND RECOVERIES OF DEXTRAN (MOL. WT. 44,000) FROM THE REACTOR–SEPARATOR

Dextran loaded[a] (mg)	*Reducing groups in product (mg D-glucose equivalents)*	*Carbohydrate recovery (%)*	*Average chain-length of product*
60	11.4	71	3.7
40	10.3	79	3.1
20	6.0	82	2.7
15	5.2	85	2.5
10	3.9	85	2.2
5	2.22	90	2.0
2.5	1.17	107	2.3
1.25	0.64	104	2.0
0.625	0.20	111	3.4

[a]Load volume, 0.1 ml; column flow-rate, 0.25 ml/min.

assay described above. The elution times (t_e) were measured for the reducing "products" and "unconverted" dextran, and the elution volumes (V_e) and retention factors ($R_f = V_e/V_t$) were calculated after subtraction of the delay times for the assay system (Table I). R_f values were also determined for D-glucose by passage of a 1% w/v solution (0.4 ml) down the column (Table I). The amounts of unconverted dextran and oligosaccharide products (Tables II and III) were calculated conventionally from the relevant peak areas. Extrapolation and curve resolving were applied as necessary, and typical elution curves are shown in Fig. 2. Relationships between the product and substrate concentrations for continuous and pulsed modes of operation are shown in Figs. 3 and 4, respectively.

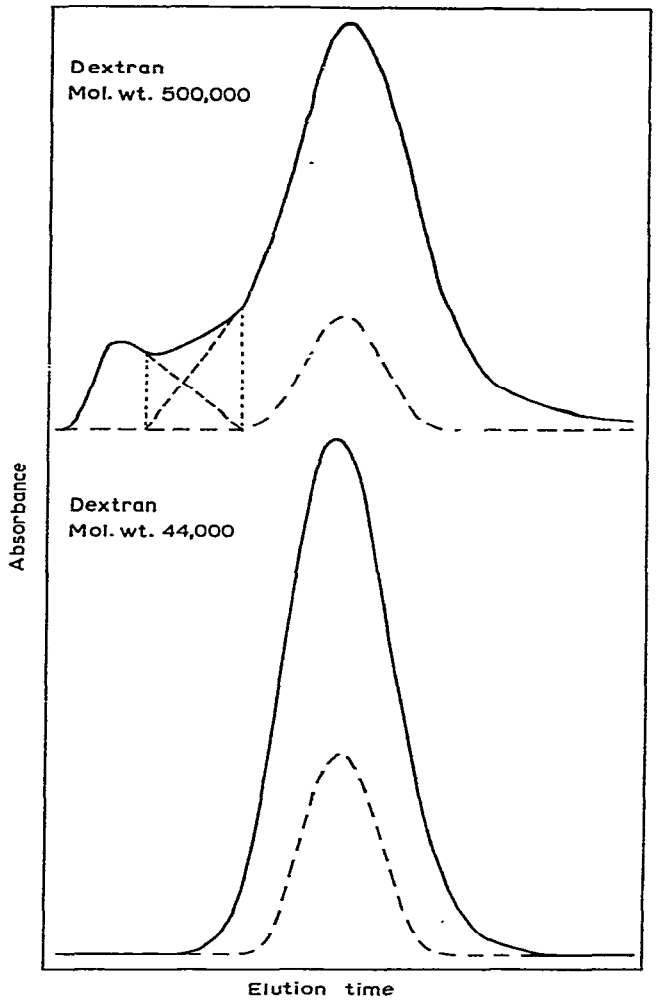

Fig. 2. Typical elution profiles for assay of products from application of dextran to the reactor–separator: ——, total carbohydrate; – – –, reducing carbohydrate (areas under overlapping peaks estimated by extrapolations, as indicated).

DISCUSSION

The substrate specificity of dextranase and the nature of the products of its action are particularly suitable for the achievement of a pilot reactor–separator. The large difference in molecular weight between the substrate and products enabled a molecular sieve to be used in the construction of a reactor that was capable of fractionation on the basis of molecular size. Porasil E was chosen because of its fractionation range, stability, and non-compressibility. The reactor was constructed simply by mixing the immobilised enzyme with the sieve.

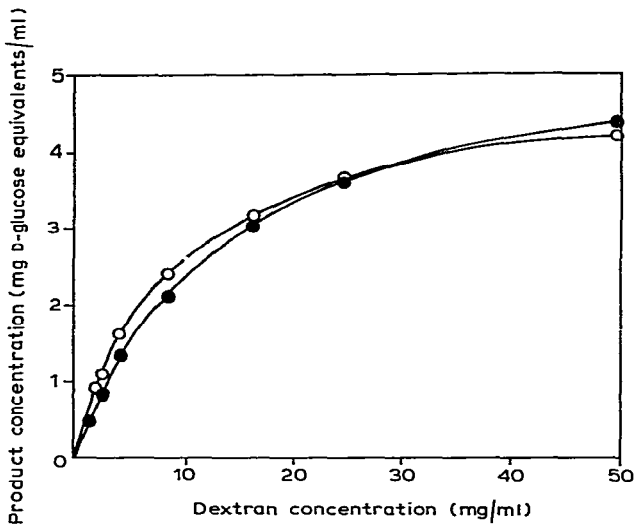

Fig. 3. Variation of product concentration with substrate concentration for the reactor–separator operated in the continuous mode: —○—, 4.4 × 10^4 dextran; —●—, 5.0 × 10^5 dextran.

Two dextran substrates were chosen, such that their molecular weights (4.4 × 10^4 and 5.0 × 10^5) lay on opposite sides of the molecular cut-off point of the permeable part of the column packing–Porasil E (av. pore diam., 800–1500 Å).

Whereas the column was incapable of separating dextran (mol. wt., 5.0 × 10^5) from the reaction products (Table I) at the higher flow-rate, on decreasing the flow-rate, a reasonable separation was effected for all the dextran concentrations used. There was some inevitable overlap between the peaks, because oligosaccharides were being produced throughout the length of the column.

When dextran was passed down the column continuously, the concentration of reducing groups in the eluate was marginally lower for the 5.0 × 10^5 dextran than for the 4.4 × 10^4 dextran (Fig. 2). A Lineweaver–Burk plot of the results was linear for both dextrans (Fig. 5). However, because of the long residence times with these columns and the large percentage conversions, an integral rate equation[12,16] is more appropriate:

$$P \cdot S_0 = K_m^{app} \cdot \ln(1 - P) + c/Q,$$

where P is the fraction of substrate reacted within the column, S_0 is the initial concentration of substrate, K_m^{app} is the apparent Michaelis constant, c is the reaction capacity of the column, and Q is the flow-rate through the column. If values of P are measured when various initial concentrations of substrate are passed through the same column at identical flow-rates (*i.e.*, Q is constant), then a plot of $P \cdot S_0$ *versus* $\ln(1 - P)$ should give a straight line if K_m^{app} and c are constant at this flow-rate, and the slope of the line will equal K_m^{app}. Such plots (Fig. 6) were linear for both dextrans. Any non-linearity obtained with the 5.0 × 10^5 dextran probably reflects the poor flow-

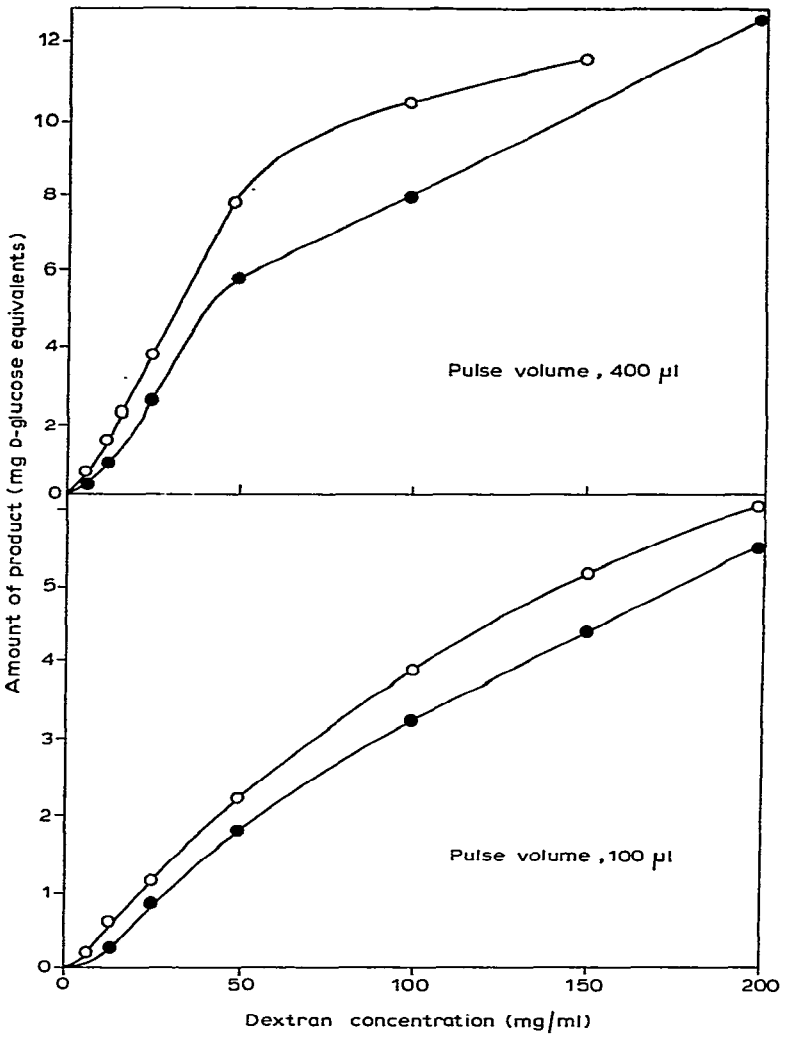

Fig. 4. Variation of product concentration with substrate concentration for the reactor–separator operated in the pulsed mode: —○—, 4.4×10^4 dextran; —●—, 5.0×10^5 dextran.

capacity already mentioned; this shows up more on an integrated plot than a Lineweaver–Burk plot, as plug flow conditions are necessary for the former to be valid.

The variation of reducing groups in the product with substrate concentration, for the pulsed mode of operation, shows a marked decrease at very low concentrations of substrate (Fig. 4). This may be due to diffusion of the pulse during its passage down the column. This effect was estimated for the 4.4×10^4 dextran, by subtracting from the peak-width the value obtained for the same sample when passed through the analysis system only. The sample was found to diffuse in the column ~4.8 times its original volume. When the substrate concentration is much greater than the Michaelis

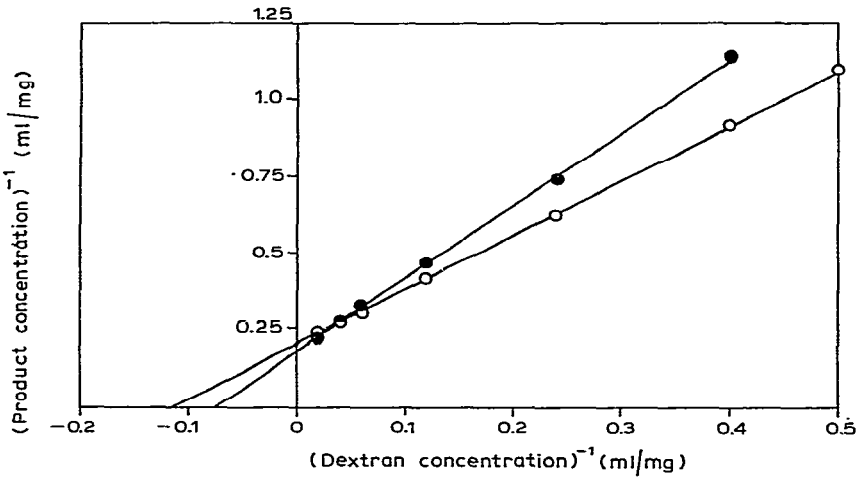

Fig. 5. Lineweaver–Burk plots for the reactor–separator operated in the continuous mode: —○—, 4.4 × 10^4 dextran; —●—, 5.0 × 10^5 dextran.

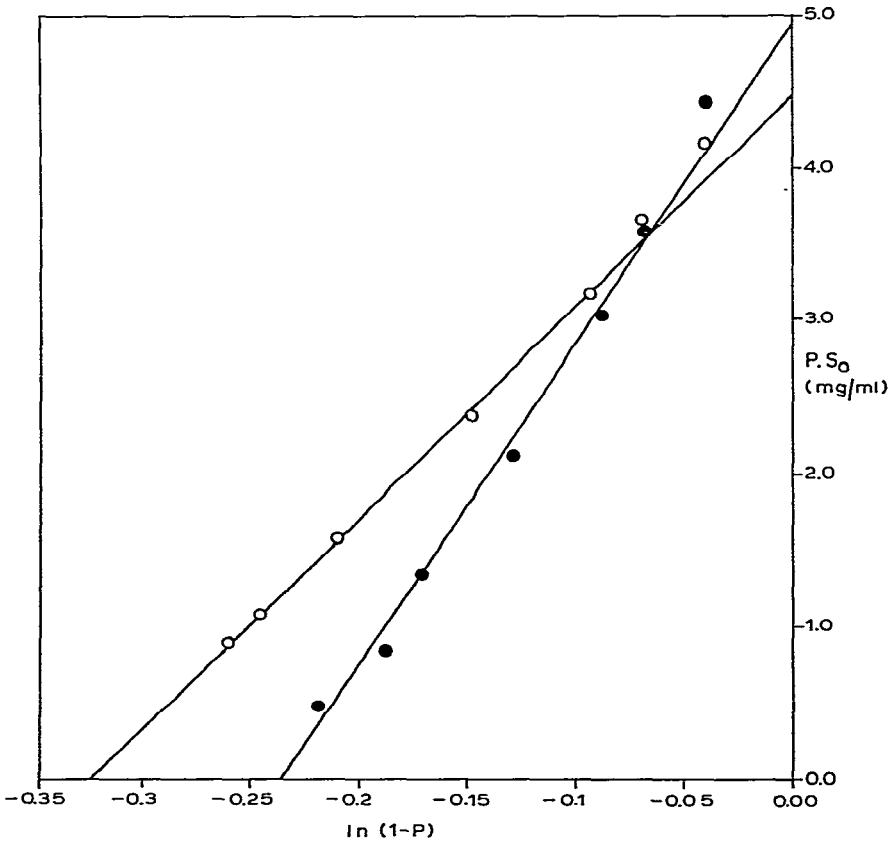

Fig. 6. Variation of $P{\cdot}S_0$ with ln(1 − P) for the reactor–separator operated in the continuous mode: —○—, 4.4 × 10^4 dextran; —●—, 5.0 × 10^5 dextran.

constant, a decrease in concentration due to diffusion does not have much effect on the reaction rate. The overall conversion will therefore increase, or remain the same, as the substrate diffuses. However, at low concentrations, a decrease in substrate concentration by diffusion causes a rapid drop in reaction rate, which may not be compensated by the rate-increasing effect of the peak spreading. This is the cause of the shape of the curves (Fig. 4) in the region of low concentrations of substrate.

Differences in the shapes of the curves obtained for product concentration *versus* substrate concentration will arise from different pulse volumes. Since the smaller pulse diffused to a greater extent (~4 times) than the larger pulse, the effects of diffusion are more pronounced in the lower set of curves in Fig. 4. Thus, there was a slightly greater depression of the reaction rate at the lower concentrations of substrate, and the curves did not level off at high concentrations.

Similarly, the differences between the curves (Fig. 4) for the two dextrans can be attributed to the greater spreading of the 5.0×10^5 dextran peaks. This effect is caused mainly by the separation of unconverted dextran from the hydrolysis products, which, in turn, can act as substrates for further hydrolysis. Also, exclusion from the Porasil E matrix may increase diffusion. Thus, the curve for the 5.0×10^5 dextran was lowered more than that for the 4.4×10^4 dextran at low concentrations of substrate, and did not level off so much at high concentrations.

The carbohydrate recoveries (Tables II and III) were determined from the areas under the peaks (extrapolated as necessary; Fig. 2) and, for the 5.0×10^5 dextran, the recoveries of unconverted dextran and of hydrolysis products. Good degrees of conversion and recoveries were achieved. It is possible to calculate the number-average molecular weight of the total product; for the 5.0×10^5 dextran, where separation is possible, the same parameter for the converted product may also be obtained (Tables II and III). Although the results demonstrate unequivocally that extensive breakdown of the dextran is achieved, there are shortcomings in the precise number obtained when the calculation is applied to such short-chain oligosaccharides as isomaltose and isomaltotriose[17]. Greater attention should be given to the relative variations of average chain-lengths than to their absolute values. The average chain-length of the total product (unconverted dextran and hydrolysis products) varied in the same way with dextran concentration as did that of the hydrolysis products, the values of the latter being slightly lower in every case, as expected. Comparison of the average chain-lengths of the total product for the 5.0×10^5 dextran with those of the 4.4×10^4 dextran (Tables II and III) shows that the latter was lower in almost every case. This result is also as expected, since for a particular degree of reaction (*i.e.*, % of intersaccharide linkages broken), the substrate of smaller molecular weight would yield a smaller product. Rapid increases in the average chain-lengths at low concentrations of substrate are found in all cases, because of the low rates of reaction in this concentration range. However, it is clear that the immobilised-dextranase reactor–separator is able both to degrade the substrate and to separate, simultaneously, products of high and low molecular weight.

The effect of separation of the products on reaction rate could not be evaluated

by a direct comparison of the K_m^{app} values obtained with and without Porasil E, as other factors, *e.g.*, the physical parameters of the column and the microenvironment surrounding the enzyme, also affect K_m^{app}. A comparison between continuous and pulsed modes of operation using only the immobilised-dextranase reactor–separator column would overcome this problem, since, in both cases, the product would be retarded by its inclusion in the Porasil E matrix, but with the continuous mode this would not cause separation along the length of the column, whereas it would with the pulsed mode. It was anticipated that the main source of difficulty in measuring K_m^{app} for the pulsed mode of operation would be due to diffusion of the pulse as it passed down the column, and control experiments were therefore performed using the dextran of lower molecular weight (4.4×10^4). This did not separate from the reaction products (Table I); therefore, in this case, there is no reason to expect any difference in the K_m^{app} values for the continuous and pulsed modes. If the diffusions of the two dextrans are the same, the effect of diffusion could, hopefully, be eliminated in the determination of K_m^{app} for the 5.0×10^5 dextran pulsed-mode.

Several factors make the evaluation of K_m^{app} in the pulsed mode extremely difficult. These are as follows. (*a*) As the pulsed sample is diluted by diffusion, the rate of reaction changes in a complicated way, depending on the initial substrate concentration. This may even change the shape of the concentration profile of the pulse, as the peak will have a higher concentration than the edges, and the rate of reaction at the peak will therefore be effected less by dilution if the concentration is in the middle (non-linear) region of the curve of reaction rate *versus* substrate concentration. (*b*) The effects described in (*a*) change throughout the length of the column, as the extent and rate of diffusion change, and as the substrate concentration is lowered by the enzymic reaction. (*c*) The hydrolysis products can act as substrates, which may have different affinities for the active site of the enzyme, and therefore different K_m values. These products may diffuse at rates different from that of the unconverted dextran and, in addition, will be separated from it by molecular sieving. The effect on the rate of this separation cannot be disentangled from the other effects without a greater knowledge of the exact hydrolysis pattern.

As K_m^{app} cannot really be determined for the pulsed mode, it is not possible to use K_m^{app} values to ascertain whether the reactor–separator causes any improvement in the degrees of conversions. However, a plot of the ratio (conversion of 4.4×10^4 dextran)/(conversion of 5.0×10^5 dextran) for the two dextrans against dextran concentration provides a guide to improvement (Fig. 7). A lowering of this ratio on changing from continuous to pulsed mode would indicate an improvement in the conversion of 5.0×10^5 dextran relative to 4.4×10^4 dextran. The width (spread) of the peaks was calculated from the total-carbohydrate assay, assuming that the products spread to the same extent as the original dextran would have done had it not been converted. The spread was independent of concentration of substrate. As the widths of the hydrolysis-product peaks from the two dextrans were nearly the same, the main difference in the effect of pulsing on the conversions may be attributed to the separation of the products from substrate with one dextran and not the other.

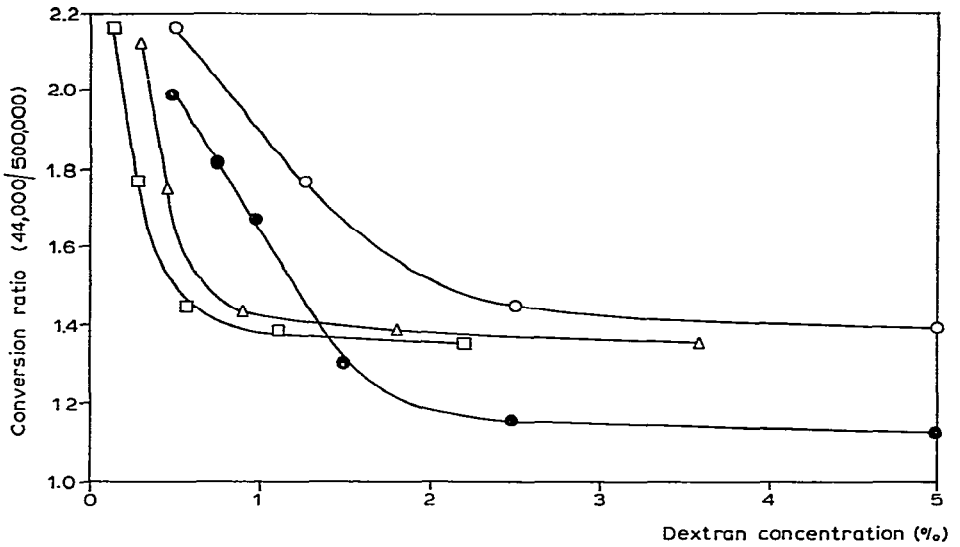

Fig. 7. Variation of dextran conversion ratio with dextran concentration for the mode of operation of the reactor–separator: —●—, continuous; —○—, pulsed; —△—, pulsed (mean); —□—, pulsed (adjusted).

For the continuous mode (Fig. 7), the dextran of lower molecular weight reacts faster at low concentrations, but the ratio of the rates tends to unity as the concentration is increased. The pattern is the same for the pulsed mode. However, the concentration of substrate is changing throughout the column because of diffusion and consumption. The dilution of substrate throughout the column affects the rate of reaction at high concentrations of substrate more than at low concentrations, and therefore some adjustment must be made. The unadjusted concentration is that of the original pulse, and the adjusted value is that of the pulse at the bottom of the column, calculated from the width (spread) of the peaks. Thus, the two curves shown for the pulsed mode (Fig. 7) represent the extremes within which the actual curve (represented by the mean) lies. Below dextran concentrations of 1.4% w/v, it is probable that separation of the products on pulsing caused an improvement in the conversion of 5.0×10^5 dextran, as represented by a lowering of this curve relative to that of the continuous mode.

Thus, dextranase action may be coupled with product separation, to achieve the two processes in a single stage. The system would be equally applicable, in principle, to other enzyme–substrate systems where the product and substrate are separable by molecular sieving, and our investigations are continuing.

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