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HIGH-PERFORMANCE DISPLACEMENT CHROMATOGRAPHY OF NUCLEIC ACID FRAGMENTS IN A TANDEM ENZYME REACTOR–LIQUID CHROMATOGRAPH SYSTEM

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SUMMARY

Separation of the reaction mixture in the ribonuclease T_1-catalyzed synthesis of GpU from cyclic GMP in the presence of a large excess of uridine was carried out by displacement chromatography on octadecyl-silica columns with 0.25 *M* *n*-butanol in the carrier buffer (50 m*M* phosphate, pH 3.0) as the displacer. The tandem reaction and separation was performed with an instrument assembled from parts customary in analytical high-performance liquid chromatography (HPLC). The enzyme was immobilized on microparticulate amino-silica by glutaraldehyde coupling and used in a short packed-bed reactor. The conditions of the reaction that reaches equilibrium after approximately half of the cyclic GMP is converted and the results of displacement chromatography were evaluated by HPLC analysis. The synthesis took place in a 2.5 mm long × 4.6 mm I.D. packed-bed reactor used in the recirculating mode and the product was introduced directly into the chromatographic unit consisting of two 4.6 mm I.D. columns, 150 and 250 mm in lengths, in series. The bulk of the uridine separated by frontal chromatography in the first column was withdrawn and displacement development in the second column was used in order to isolate the product from other components. Nearly 100 mg of GpU with a purity of 99.7% could be achieved in a chromatographic experiment of 2.4 h. By returning unreacted starting materials to the reactor the process can be carried out in a continual fashion and the system readily leads itself to automation. The combination of similar enzyme reactors with the liquid chromatograph operated in the displacement mode offers an efficient means for laboratory-scale separation of complex biochemical substances.

INTRODUCTION

It has been shown recently that displacement chromatography may offer an efficient means to carry out preparative-scale separation with columns and equipment used customarily in high-performance liquid chromatography[1–4]. The renascent interest in this mode of chromatographic development stems from the availability of highly efficient columns that facilitate the separation of the sample components into adjacent bands of relatively high concentrations upon the action of the displacer

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front moving down the column. Although the theoretical basis of displacement chromatography is understood[5,6], the scope of applications is yet to be explored. The main advantage of the technique with respect to the most commonly used linear elution mode of chromatography is the relatively high sample loading. In fact, the technique is most efficient in separating relatively large quantities of the sample components[1].

Preparative chromatography in the elution mode is recognized as an efficient process to recover and purify the desired product from a reaction mixture. In order to expand the potential of displacement chromatography in such applications we constructed an instrument to carry out the enzyme catalyzed condensation of G>p* and U to GpU in tandem with the separation of the components of the reaction mixture.

Ribonuclease T_1 [ribonucleate guanosine nucleotide-2'-transferase (cyclizing), E.C. 2.7.7.26] from *Aspergillus oryzae* under appropriate conditions[7–14] catalyzes the esterification of G>p (phosphate donor) with the 5'-hydroxyl group of various nucleotides or oligonucleotides (phosphate acceptors) and can be used to synthetize various oligonucleotides containing guanosine-3'-phosphate moiety[13,14]. In this work we investigated the reaction:

$$G > p + U \overset{\text{RNase } T_1}{\rightleftharpoons} GpU$$

In the presence of a large excess of U about half of the G>p is converted under optimal conditions and G>p is also hydrolyzed to yield Gp:

$$G > p + H_2O \overset{\text{RNase } T_1}{\rightleftharpoons} Gp$$

However, the reaction is slow with respect to condensation and usually not more than 2% of G>p is hydrolyzed at the end of the reaction when excess U is present[14]. Thus, in the synthesis of GpU the reaction mixture contains besides the product and unreacted G>p a large excess of U and some Gp. Displacement chromatography on octadecyl-silica was used for the separation of these components.

The study was focused on the use of RNase T_1 immobilized on microparticulate silica and reactors packed with such catalyst were found to be superior to the enzyme in free solution. Moreover, the enzyme reactor was fully compatible with the chromatographic system used for displacement development by which the product was separated from unreacted reactants. Several reactor configurations were investigated in order to establish the most suitable mode of operation for the enzyme reactor *cum* liquid chromatograph system. The results suggest that such a combination based on hardware widely used in HPLC is suitable for synthetic preparations of complex biological substances on the laboratory scale, even as an automated instrument, and thus exploits the advantages of displacement chromatography.

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* Abbreviations: U = uridine (Urd); G>p = guanosine-2':3'-cyclic phosphate (cyclic GMP); Gp = guanosine-3'-phosphate (GMP); GpU = guanylyl-(3' → 5')-guanosine; GpGp = guanylyl-(3' → 5')-guanosine-3'-phosphate; GpG>p = guanylyl-(3' → 5')-guanosine-2':3'-cyclic phosphate; GpGpU = guanylyl-(3' → 5')-guanylyl-(3' → 5')-uridine; RNase T_1 = ribonuclease T_1; GpC = guanylyl-(3' → 5')-cytidine.

EXPERIMENTAL

Columns, materials and reagents

Supelcosil LC-18 (150 × 4.6 mm) and Zorbax C_{18} (250 × 4.6 mm) columns both packed with 5-μm octadecyl-silica, were obtained from Supelco (Bellefonte, PA, U.S.A.) and DuPont (Wilmington, DE, U.S.A.), respectively.

Octadecyl-Spherisorb having a carbon load of 15% (w/w) was prepared from 5-μm Spherisorb silica gel (Phase Separations, Happauge, NY, U.S.A.) by the procedure of Kováts and Boksányi[15] and packed into 30 × 4.6 mm and 150 × 4.6 mm stainless-steel columns. The support used for enzyme immobilization was spherical Vydac silica gel having particle size of 10 μm, specific surface area of 100 m^2/g and average pore diameter of 330 Å was a gift from Whatman (Clifton, NJ, U.S.A.). γ-Aminopropyltriethoxysilane was purchased from PCR Research Chemicals (Gainesville, FL, U.S.A.). Glutaraldehyde in 50% (w/w) aqueous solution, tetrabutylammonium bromide and Tris base were obtained from Eastman-Kodak (Rochester, NY, U.S.A.). U, G>p, GpU, Gp and ammonium sulphate were purchased from Sigma (St. Louis, MO, U.S.A.). RNase T_1 Grade V, Cat. No. R1003, having specific activity of 488.000 units per mg was also purchased from Sigma. One unit catalyzes the hydrolysis of RNA to acid-soluble oligonucleotides that increase absorbance at 260 nm by one absorbance unit in a reaction volume of 1.0 ml at 37°C and pH 7.5 in 15 min^{14}. Molecular sieve 3 Å, acetonitrile, methanol, *n*-heptane, *n*-butanol, H_3PO_4, NaH_2PO_4, Na_2HPO_4, acetic acid and sodium acetate were supplied by Fisher Scientific (Pittsburgh, PA, U.S.A.). Distilled water was prepared with a Barnstead distilling unit.

Apparatus

Displacement chromatography was performed with the system shown in Figs. 1 and 2 which consisted of a Model 110A solvent metering pump (Altex, Berkeley, CA, U.S.A.), and two chromatographic columns in series via a three-way valve, Model 7010 (Rheodyne, Berkeley, CA, U.S.A.). Columns I and II were Supelcosil LC-18 and Zorbax C_{18} respectively. The same pump was used to perfuse the column consecutively with the carrier, the displacer and the regenerant stored in their respective reservoirs. The column effluent was monitored at 254 nm by a Model 250A fixed-wavelength UV detector (Perkin-Elmer, Norwalk, CT, U.S.A.) and a Model SR-204 strip-chart recorder (Heath, Benton Harbor, MI, U.S.A.). Fractions containing 100 μl of column effluent were obtained with an Ultrarack II, No. 2070 fraction collector (LKB, Rockville, MD, U.S.A.). One or two Model No. 7010 sampling valves (Rheodyne) were used for introducing the reaction mixture into the chromatographic unit depending on the particular reactor arrangement used in the experiment. Particular attention was paid to minimize dead volume throughout the system and for this reason 0.25 mm I.D. connecting tubes were employed. When the enzyme reactor was in series with the chromatographic columns shown in Fig. 1, sample valve I was used to introduce the reactant mixture into the enzyme reactor and sample valve II to collect the reaction mixture and transfer it into the chromatographic columns. Not shown in Fig. 1 are two recirculating water-baths, Lauda Model K-2/R (Brinkman, Westbury, NY, U.S.A.) and Forma Model 2095 (Forma Scientific, Marrieta, OH, U.S.A.) used for independent temperature control of the

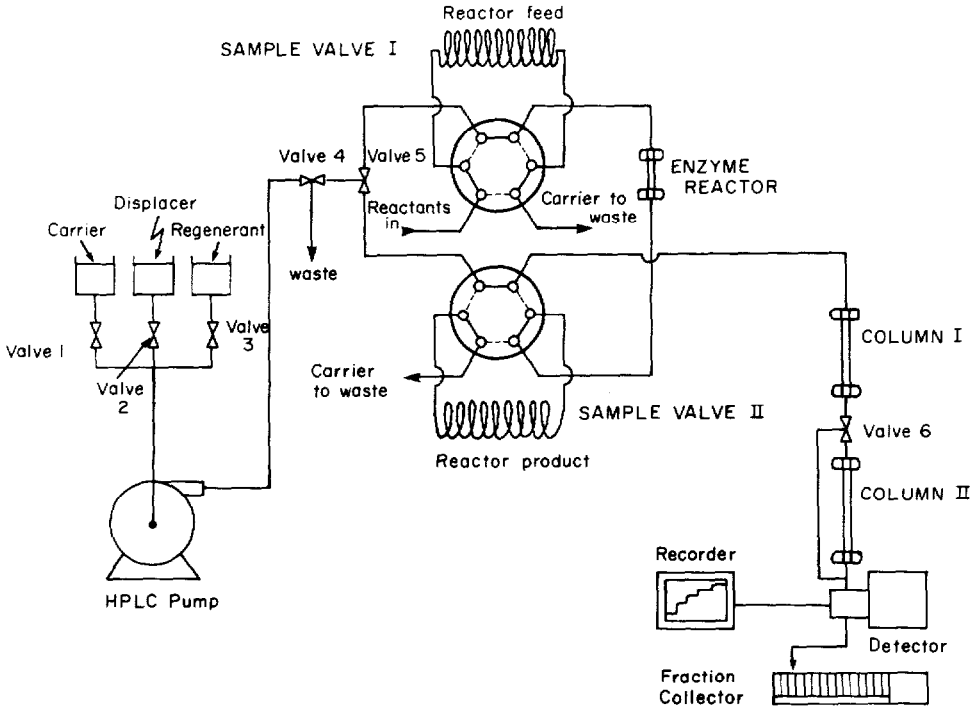

Fig. 1. Diagram of the single-pass reactor and chromatograph combination in series.

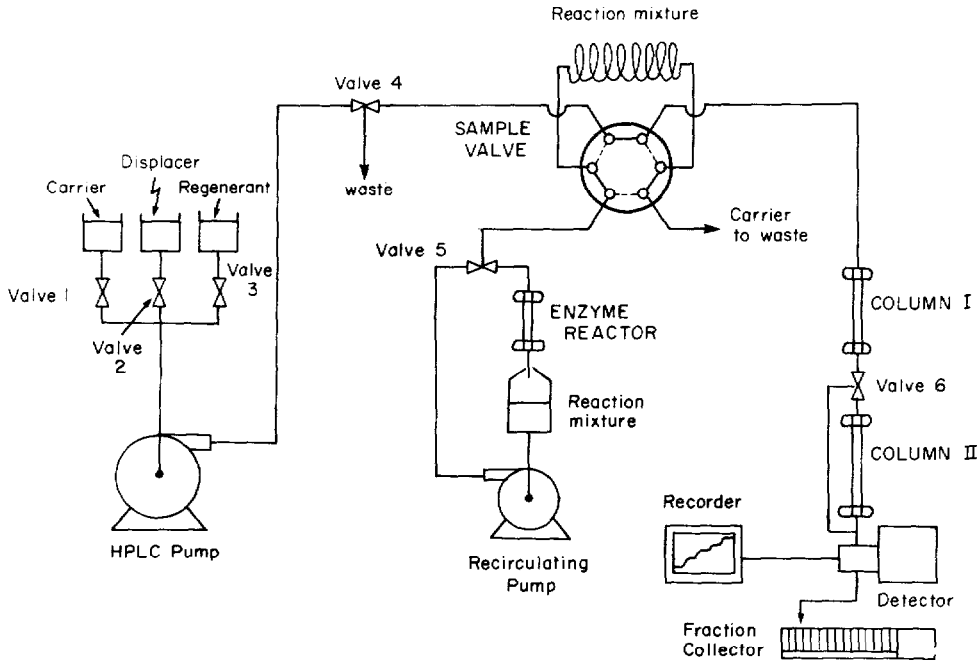

Fig. 2. Diagram of the recycling-enzyme reactor *cum* chromatograph.

enzyme reactor and chromatographic columns. Fig. 2 shows the flow sheet of the apparatus with the recirculating enzyme reactor. The reactor unit comprised of a reservoir, an Eldex pump Model A-30-S (Eldex, Menlo Park, CA, U.S.A.) and a three-way valve. The sampling valve was used to transfer an aliquot of the reaction mixture from the recirculating reactor system into the column. Not shown in Fig. 2 is the circulating bath used to maintain the enzyme reactor and the columns at the same temperature. In some experiments the enzymatic reaction was carried out in free solution. The apparatus was the same as that shown in Fig. 2 except that the recirculating enzyme reactor system was deleted and a 3-cm long filter column packed with octadecyl-Spherisorb was inserted between the sampling valve and the chromatographic column in order to hold back the protein. The reaction mixture was analyzed by HPLC. The liquid chromatograph was assembled from a Model LC 250/1 pump (Kratos, Westwood, NJ, U.S.A.), a Rheodyne Model 7010 sampling valve with a 20-μl sample loop and a Model SR-206 (Heath) dual-pen strip-chart recorder. All analytical separations were performed with an octadecyl-Spherisorb column (150 × 4.6 mm) at room temperature.

Microparticulate enzyme reactor. The reactor shown in Fig. 3 was made by packing the immobilized enzyme into a 1/4-in. union (Parker Hannifin, Huntsville, AL, U.S.A.). The length of the packing could be varied from 10 to 1 mm by elongating the 1/4-in. inlet and/or outlet bore in order to reduce the distance between the shoulders of the central space (4.6 mm I.D.) and thus diminish the space available for the catalyst. The microparticulate silica bearing the enzyme was packed between two 2-mm thick stainless-steel fritted disks, having a pore size of 2 μm. A Rheodyne gasket filter having 4.3-mm diameter and 2-μm pores was used in front of the frit to serve as a flow distributor at the inlet.

Procedures

Preparation of the support for the immobilized enzyme. A 10-g amount of Vydac silica gel was heated at 150°C overnight. After it had cooled to room temperature in a dessicator, the product was transfered to a round-bottomed flask contain-

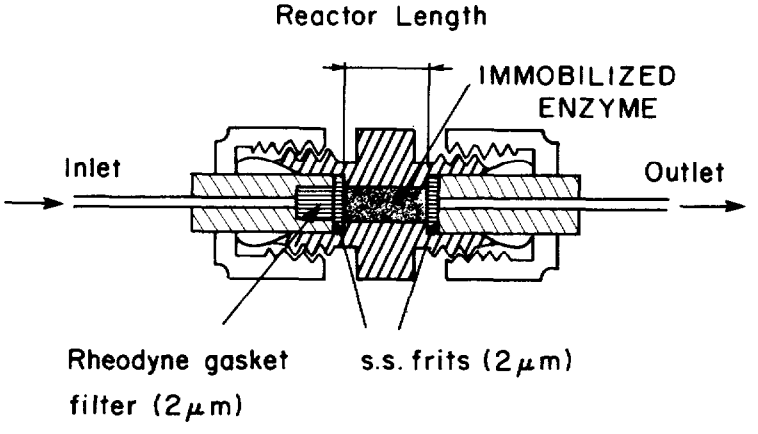

Fig. 3. Packed bed reactor with microparticulate immobilized enzyme constructed from a 1/4-in. stainless-steel (s.s.) tube fitting. The length of the catalyst bed can be varied from 1.0 to 10 mm.

ing 7.0 ml of γ-aminopropyltriethoxysilane in 70 ml of *n*-heptane which was previously dried with molecular sieve 3 Å. The suspension was refluxed at 110°C under dry nitrogen for 3 days. The resulting amino-silica was washed successively with dry *n*-heptane and methanol and dried at room temperature. Elemental analysis of the product showed 1% N and 2.84% C.

Enzyme immobilization. The amino-silica was slurry packed at 2000 p.s.i. into the reactor shown in Fig. 3 with methanol and thereafter washed with water. Then it was perfused with 20 ml of 10 m*M* phosphate buffer, pH 7.0, and subsequently with 10 ml of a 1% (w/w) solution of glutaraldehyde in the same buffer at a flow-rate of 1 ml/min. The reactive amino-silica packing thus prepared was washed with water and equilibrated with the above phosphate buffer. Then 5 ml of the buffer containing a known amount of RNase T_1 were recycled through the bed at a flow-rate of 5 ml/min for 1 h. Finally, the reactor was washed with 10 ml of 1.0 *M* Tris–HCl buffer, pH 7.5, in order to scavenge residual reactive functions at the support surface.

HPLC analysis. The analyzer unit was equipped with a 150 × 4.6 mm octadecyl-Spherisorb column and the eluent was 50 m*M* phosphate buffer, pH 3.0, containing 2% (v/v) acetonitrile and 5 m*M* tetrabutylammonium bromide. Chromatogram of the standard used in quantitative analysis of the reaction mixture is shown in Fig. 4. The fractions collected after displacement development were usually diluted 2000-fold with an aqueous solution of the internal standard, hippuric acid (0.28 mg/ml) and 20-μl aliquots were injected. In some cases even greater dilution was necessary. The data obtained by HPLC analysis were used to construct displacement chromatograms by plotting the concentrations of the species in the individual effluent fractions on a histogram (*cf.*, Figs. 13–15). The bars, representing fractions containing more than one substance, are broken into differently shaded areas to show the net concentrations of the individual species. A line is drawn to demarcate each product in mixed zones. In all cases the displacer front is represented by a vertical line.

Hydrolytic activity of the enzyme reactor. GpU was used as the substrate to measure the hydrolytic activity of immobilized RNase T_1. In the recycling reactor unit of the system illustrated in Fig. 2, but using an Altex 110 A pump instead of the Eldex pump the reactor was perfused with 5 ml of 0.2 m*M* GpU in 20 m*M* acetate buffer, pH 5.5, containing 0.5 *M* $(NH_4)_2SO_4$ at a temperature of 37.5°C and a flow-rate of 5 ml/min for 1 min. Thereafter, the reactor was washed with 20 m*M* acetate buffer, pH 5.5, containing 0.5 *M* $(NH_4)_2SO_4$ at the same flow-rate for 5 min. Aliquots of the reaction mixture were analyzed by HPLC in order to evaluate the conversion as per cent GpU hydrolyzed.

Study of enzyme reaction

The RNase T_1 catalyzed synthesis of GpU was studied under different conditions as described below. In each case the reaction mixture was analyzed by HPLC and the yield was calculated as the mole per cent of G>p converted into GpU.

Free solution. In a 2-ml vial 0.5 ml of 20 m*M* phosphate buffer, pH 7.0, containing 1.0 *M* U, 0.2 *M* G>p and 100 units of RNase T_1 were incubated at ice–water temperature for 20 h.

Single-pass reactor. In these experiments, first the 0.5-ml loop of sampling valve situated between the pump and the reactor was filled up with the solution

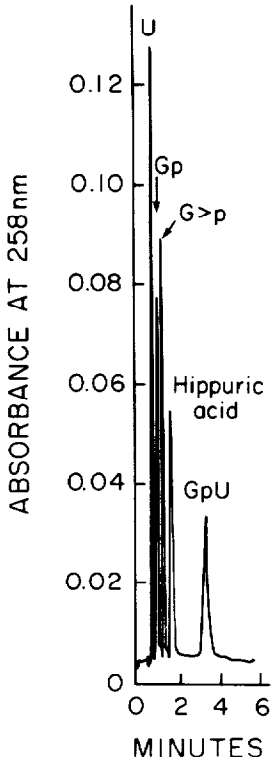

Fig. 4. Standard chromatogram of the components of the reaction mixture. Column: 150 × 4.6-mm octadecyl-Spherisorb 5 μm. Eluent: 50 m*M* phosphate buffer, pH 3.0, containing 2% (v/v) acetonitrile and 5 m*M* tetrabutylammonium bromide; flow-rate, 2 ml/min. Temperature: 22°C.

containing the reactants. Then the 0.5-ml slug of the reaction mixture was pumped through the reactor followed by an appropriate buffer solution at a flow-rate of 0.1 ml/min, unless otherwise indicated. Usually after 10 min the flow-rate was increased to 2.0 ml/min and pumping was continued until a total volume of 10 ml of reactor effluent was collected.

Recirculating reactor. In the recirculating reactor unit of the system shown in Fig. 2, 1.5 ml of the reaction mixture were placed into the reservoir and recycled at a flow-rate of 1.5 ml/min. From the reservoir 10-μl aliquots were taken at different times and analyzed.

Displacement chromatography

Sample introduction and development of diplacement train. First the column was equilibrated with the carrier, 50 m*M* phosphate buffer, pH 3.0. Then the loop of the sampling valve was filled with the reaction mixture. When the valve was turned to the "inject' position, the flow of the displacer, 0.25 *M* *n*-butanol in the carrier, simultaneously began through the sampling valve at 0.1 ml/min. Pumping continued in order to introduce the feed into the column and to bring about the separation by the displacer front moving down the column. The collection of 100-μl fractions of the column effluent started upon turning the sampling valve to the "inject" position.

In all experiments, unless otherwise indicated, the three-way valve No. 6 between the two chromatographic columns (see Figs. 1 and 2) was first in a position to allow the effluent of column I bypass column II and enter the detector, the outlet of which was connected to the fraction collector. After the front of U appeared in the detector, the bulk of U was collected over a period of *ca.* 22 min. Thereafter, the three-way valve No. 6 was turned to allow the effluent of column I to enter column II, and subsequently the detector that was used to monitor the steps of the process.

When the enzyme reactor was connected in series with the chromatographic columns as shown in Fig. 1, the 0.5-ml loop of sample valve I was filled with the solution of the reactants in 20 m*M* acetate buffer, pH 5.5, containing 0.5 *M* $(NH_4)_2SO_4$. This buffer was also used to equilibrate the enzyme reactor. Subsequently the slug of reactants from sample valve I was swept through the reactor by the same buffer at a flow-rate of 0.1 ml/min. The reactor effluent was collected in the 1.5-ml loop of sample valve II in load position. After 1.2 ml of buffer solution were pumped through the reactor, sampling valve II was turned to the "inject" position. At the same time the inlet of the pump was switched to the displacer reservoir and the three-way valve No. 5 was turned to allow the displacer to bypass the enzyme reactor and drive the reaction mixture into the chromatographic columns equilibrated with the carrier.

When the recirculating enzyme reactor was connected to the chromatographic system, as shown in Fig. 2, the reaction mixture was filled into the 0.5-ml loop of the sample valve by turning the three-way valve No. 5 between the pump and the reactor in the recirculation system. After loading the loop, the sampling valve was turned to inject position and the three-way valve No. 5 was turned to resume recycling. The displacer flow through the system swept the reaction mixture into the chromatographic column and subsequently brought about the separation.

When the enzymic reaction was carried out in free solution, the operation was the same as that described here for the recirculating enzyme reactor except that the sampling valve was loaded with the reaction mixture by using a syringe.

Column regeneration. After the emergence of the displacer front at the outlet of column II, the columns were regenerated by being flushed with 100 ml acetonitrile through the column at a flow-rate of 4 ml/min. Thereafter the columns were reequilibrated by perfusion with the carrier at a flow-rate of 3 ml/min. Column regeneration was tested by elution chromatography of 20 μl of the reaction mixture with the carrier as the eluent. The reequilibration was considered adequate when the respective retention factors agreed within $\pm 2\%$ with the previously measured values.

RESULTS AND DISCUSSION

Enzymatic synthesis of GpU in free solution

The RNase T_1-catalyzed condensation of G>p and U in free solution has extensively been studied. Generally both the rate and yield of GpU production increase with the concentrations of the reactants[9,13,14]. At low temperature and with U in high molar excess (five-fold or more) formation of GpU proceeds much more rapidly than the hydrolysis of G>p[14]. Mohr and Thach[14] reported that the rate of GpU formation increases with the enzyme concentration without change in the extent of the reaction and that the final yield of GpU was the same over the pH

range from 5.5 to 7.5. Although phosphate ions are considered inhibitors for the enzyme the final yield was the same with phosphate, Tris or acetate buffers. No improvement in yield was observed by adding dimethyl sulfoxide or dimethylformamide to the reaction mixture. Because the synthesis is speeded up by increasing the ionic strength, the use of 1.0 *M* $(NH_4)_2SO_4$ was recommended. Generally, RNase T_1-catalyzed synthetic reactions are preferably carried out at low temperature[9,12,16]. The yield of GpC in a similar reaction[17] was found to decrease almost linearly when the temperature increased from 0 to 37°C. According to the literature on GpU synthesis with the enzyme in free solution, the final yield of GpU is most affected by the initial concentration and molar ratio of the reactants and by the temperature.

Several side reactions may take place besides the hydrolysis of G>p to Gp[9,13,14]. In the presence of RNase T_1 Gp may react with G>p[14] to produce GpGp:

$$G > p + Gp \overset{\text{RNase } T_1}{\rightleftharpoons} GpGp$$

GpGp may also be formed by the reaction[7,11,13]:

$$G > p + G > p \overset{\text{RNase } T_1}{\rightleftharpoons} GpG > p \overset{\text{RNase } T_1}{\rightleftharpoons} GpGp$$

On the other hand GpG > p also can act as phosphate donor and form GpGpU in the rection[9,13]:

$$GpG > p + U \overset{\text{RNase } T_1}{\rightleftharpoons} GpGpU$$

In free solution up to 10 mole per cent of G>p were incorporated into the three by-products Gp, GpGp and GpGpU.

For the reaction in free solution we used a five-fold molar excess of U with respect to 0.2 *M* G>p; the temperature was 2°C. The course of the reactin is illustrated in Fig. 5. In a 20-h reaction time more than 70 mole % of G>p were converted and

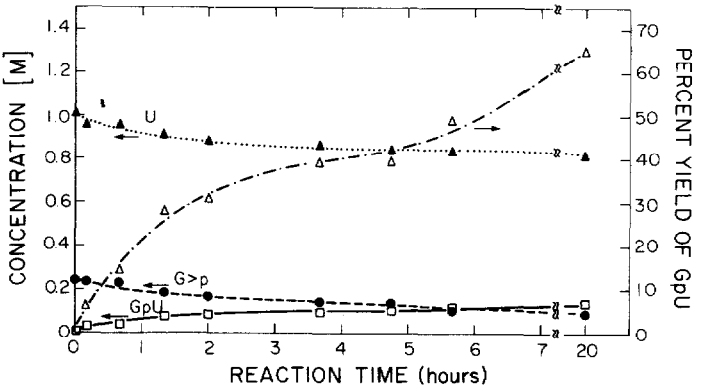

Fig. 5. Course of the reaction catalyzed by ribonuclease T_1 in free solution. 0.5 ml of 20 m*M* phosphate buffer, pH 7.0, containing 1.0 *M* U and 0.2 *M* G>p were incubated with 100 units of RNase T_1, at 2°C for 20 h.

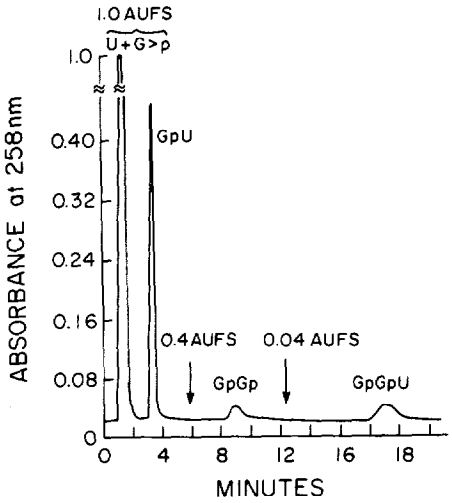

Fig. 6. Chromatogram of the reaction mixture containing the products of side reaction. Sample was taken after 20 h incubation under conditions given in Fig. 5 and analyzed under the conditions in Fig. 4.

more than 60 mole % of the originally present G>p were incorporated into GpU in agreement with earlier reports[11,13]. The chromatogram in Fig. 6 shows the by-products GpGp and GpGpU formed in the side reactions. As the column was overloaded by the large excess of U present, early peaks were not separated. It should be mentioned that the starting G>p contained *ca.* 0.5% Gp. Under our experimental conditions an estimated 2 and 8 mole per cent of the initial G>p were converted to Gp and the other two products, respectively. In all cases significantly more GpGp than GpGpU was formed in the side reactions.

Enzyme immobilization

Kuriyama and Egami[19] prepared water-insoluble RNase T_1 covalently bound to CM-cellulose that expressed 50% to 60% of the activity of the native enzyme in hydrolyzing G>p, but showed only 2% of the original activity in the hydrolysis of RNA. The pH optimum and the Michaelis constant (K_M) in the hydrolysis of G>p were the same for both the immobilized and soluble RNase T_1. Coupling to *p*-aminobenzyl-cellulose yielded a significantly less active immobilized enzyme[19]. Covalent binding of RNase T_1 to Sepharose or Sephadex activated by cyanogen bromide resulted in a stable conjugate without change in either substrate specificity of the enzyme or kinetic parameters[20]. RNase T_1 immobilized on cross-linked polyacrylamide by the azide method[21] exhibited about 45% and 77% of the original activity in hydrolyzing RNA and G>p, respectively. The immobilized enzyme was far more stable to heat and extremes of pH than the native enzyme.

In our application where the enzyme reactor had to conform with the flow conditions employed in HPLC, the above supports were unsuitable owing to their poor mechanical strength. Furthermore our design constraints mandated a reactor with high enzymic activity per unit volume and a minimum of priming and hold-up volume in order to be compatible with the analytical columns employed in dis-

placement development. Therefore, we selected a 10-μm amino-silica bonded phase, which has wide prevalence in HPLC, as the support. It was activated by glutaraldehyde[22] and then allowed to react with the enzyme. The average pore size and specific surface area of the starting material were 330 Å and 100 m^2/g, respectively. The pores of such a support are readily accessible to RNase T_1, mol. wt. 11,000 daltons, and the surface area is sufficiently large to obtain high immobilized-enzyme concentrations per unit volume of support. On the other hand, diffusion resistances that often reduce the effectiveness of immobilized enzymes[23] are greatly relaxed by the small particle diameter.

It was found convenient to carry out the various steps of the enzyme immobilization process *in situ*, with the amino-silica packed into the reactor-housing as shown in Fig. 3. The length of the packing ranged from 2.5 to 10 mm. With our flow system the reaction times could be easily controlled and the washing steps could be carried out rapidly without loss of microparticulate product. It was found that activation of the support with 1% glutaraldehyde solution resulted in higher activity of the immobilized enzyme than with glutaraldehyde solutions of higher concentrations.

The enzyme coupling process was carried out by recirculating 5.0 ml of RNase T_1 solution through the bed at a flow-rate of 5 ml/min and at room temperature for 1 h. The effect of enzyme concentration in the coupling solution on the effectiveness of the reactor in GpU production was investigated by using the single-pass method. A slug of the reactant solution containing U and G>p was swept through the reactor by the stream of buffer used in the reaction mixture, the effluent collected and the molar yield of GpU determined. Fig. 7 shows the results of the experiments with a 2.5-mm long reactor bed containing 25 mg of amino-silica. It is seen that GpU yield increased with the enzyme concentration in the coupling solution and reached

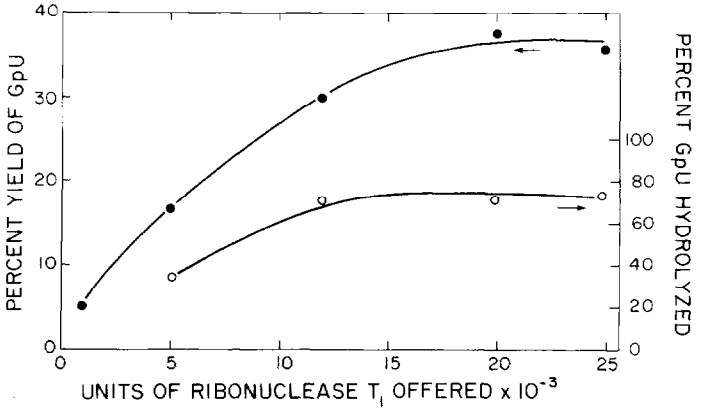

Fig. 7. Effect of enzyme concentration in the immobilized enzyme reaction on reactor activity. The abscissa shows the units of enzyme present in the 5 ml coupling solution. The reactor activity was measured in two ways (arrows indicate corresponding scales). (1) GpU formation by the single-pass method (●): 0.5 ml of 20 m*M* acetate buffer containing 0.5 *M* $(NH_4)_2SO_4$, 1.0 *M* U and 0.2 *M* G>p were forced through the enzyme reactor at a flow-rate of 0.1 ml/min. (2) GpU hydrolysis in the recirculating reactor (○): 5 ml of the above buffer containing 0.2 m*M* GpU were recirculated through the enzyme reactor at a flow-rate of 5 ml/min. In both cases the reactor was 2.5 mm long and the temperature was 37.5°C.

a plateau when 15,000 units RNase T_1 were offered in 5.0 ml. In further experiments 20,000 units were used for a 2.5-mm long reactor, or when the reactor length was varied, 800 units of RNase T_1 per mg of amino-silica packing were used in 5 ml of coupling solution.

The activity of RNase T_1 is frequently measured by the rate of hydrolysis of dinucleoside monophosphates containing guanosine at the 3′ terminus[24–26] to the corresponding nucleoside and G > p[25]. Here the hydrolysis of GpU at 37.5°C was also used to measure the effect of RNase T_1 concentration in the coupling reaction on the reactor activity as well as to follow changes in activity with time. With a 2.5-mm packing length in the recirculating reactor GpU was almost completely hydrolyzed, when 5 ml of the reaction mixture were recirculated five times, therefore only one recirculation was made. The effect of enzyme concentration used in the immobilization process is also shown in Fig. 7. It is seen that also the hydrolytic activity reaches a plateau when the amount of RNase T_1 in 5.0 ml of coupling solution is increased to 12,000 units. The reactor activity toward GpU hydrolysis decreased less than 30% after a 3-week period of frequent use and storage at 5°C between uses.

Enzyme reactor

In order to optimize the heterogeneous enzyme reaction in tandem with the chromatographic separation, several conditions were investigated. The single-pass method described above tests the use of the reactor in series with the chromatographic system and measures the yield of GpU under various conditions with a 0.5-ml reactant slug usually at a flow-rate of 0.1 ml/min. The recirculating mode tests the reactor activity either in GpU synthesis in the presence of large excess of U or in the hydrolysis of GpU. In the first case 1.5 ml of the reaction mixture were recirculated through the reactor at a flow-rate of 1.5 ml/min, whereas in the second case 5.0 ml of 0.2 m*M* GpU solution were recirculated at 5.0 ml/min.

Effect of reactor length and flow-rate

Reactors, 2.5, 5.0 and 10 mm in length, were prepared by using 800 units of RNase T_1 in the coupling solution per mg of amino silica packing. By connecting shorter lengths in series 15- and 17.5-mm long reactors were also prepared to study by the single-pass method the effect of reactor length on the GpU yield. The results in Fig. 8 show that by increasing reactor length from 2.5 to 17.5 mm the yield of GpU can be increased from 37 to 45% by increasing the residence time. It was found, however, that acidic components of the reaction mixture, G > p, Gp and GpU are bound to the amino-silica supported catalyst in proportion to the bed volume. Therefore, the transfer of the acidic components from long reactors to the chromatographic column required a large volume of buffer with concomitant deterioration of the separation. In order to keep the sample volume for chromatography relatively small 2.5-mm long reactors were used in single-pass experiments.

When the flow-rate was increased under otherwise identical experimental conditions from 0.1 ml/min to 0.6 ml/min, the yield of GpU decreased from 37% to 18% due to the decrease in contact time.

Effect of operating conditions on the yield of GpU

The pH profile of the immobilized RNase T_1 was measured by the single-pass

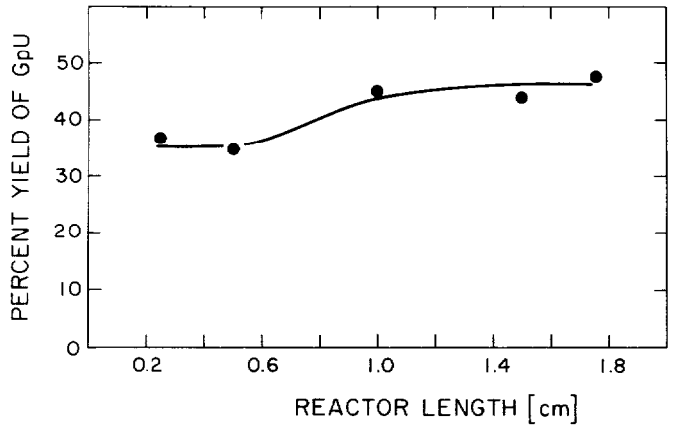

Fig. 8. Effect of reactor length on the yield of GpU. The reactors were prepared with 800 units of RNase T_1 per mg of silica support in 5 ml of coupling solution. Conditions were the same as stated in Fig. 7 for GpU formation.

method. Fig. 9 shows that the optimum for GpU synthesis lies at pH 5.4–5.6, *i.e.*, lower than reported for the free enzyme[17]. The shift is attributed to the weak cationic properties of the amino-silica that affects the enzymic microenvironment[27]. Binding by the positively charged support is believed to account also for the observation that the removal of the acidic products reactor required about 40 reactor volumes of buffer.

The effect of ionic strength on the yield of GpU was investigated at pH 5.5 at different concentrations of ammonium sulfate added to the reactant mixture in acetate buffer. Upon increasing the concentration of ammonium sulfate from zero to 0.5 *M* the percent yield of GpU increased in linear fashion from 16% to 37%. Besides the known rate enhancing effect of increased ionic strength or the salt induced renaturation observed with RNase T_1[28], a possible explanation is that the untoward effect of the positively charged matrix on the yield is attenuated by increasing the ionic strength[29].

The effect of temperature on the yield of GpU was examined in the range from

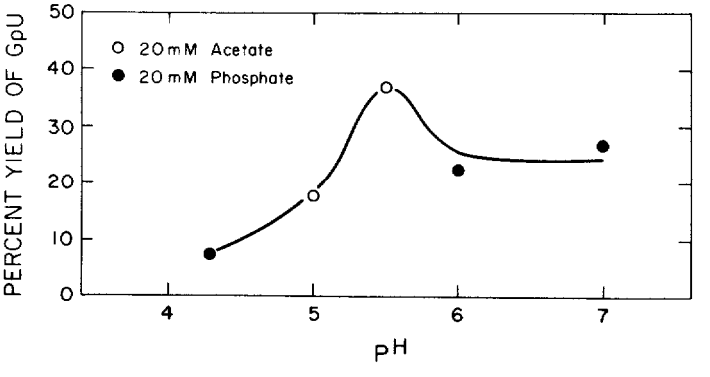

Fig. 9. Effect of pH on the yield of GpU. Conditions for the 2.5-mm reactor were the same as in Fig. 8.

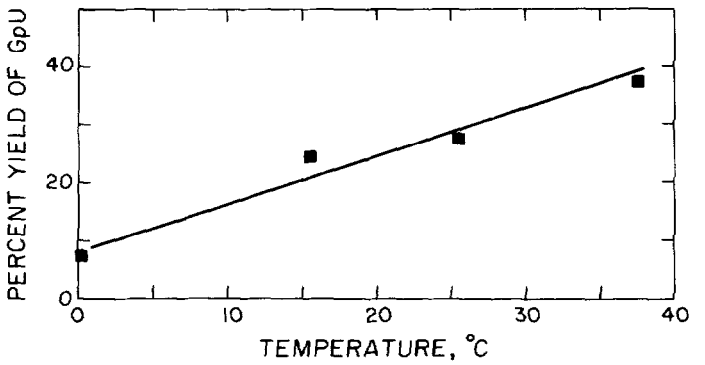

Fig. 10. Effect of temperature on the yield of GpU. Conditions for the 2.5-mm reactor were the same as in Fig. 8.

2 to 37.5°C by the single-pass method. The results are shown in Fig. 10. The yield was found to increase almost linearly with the temperature in contradistinction to a reported increase in the yield with decreasing temperature for RNase T_1-catalyzed synthetic reaction in free solution[9,14,17,18]. The adverse effect of increasing temperature on the reaction in free solution is due to the side reactions which become more pronounced during the long reaction times employed. With immobilized enzyme of high activity the reaction time was relatively short and the effect of side reactions on the yield was small.

In another set of experiments the effect of the initial concentration of reactants was investigated. The results in Fig. 11 show that with increasing G>p concentration and a fixed five-fold molar excess of U the yield of GpU first increased then reached a plateau at *ca.* 0.27 *M* G>p. The molar excess of U was varied at two G>p concentrations fixed at 0.1 and 0.2 *M*. As shown in Fig. 11 the yield increased with the magnitude of U excess in agreement with literature data[9,13,14] on the reaction in free

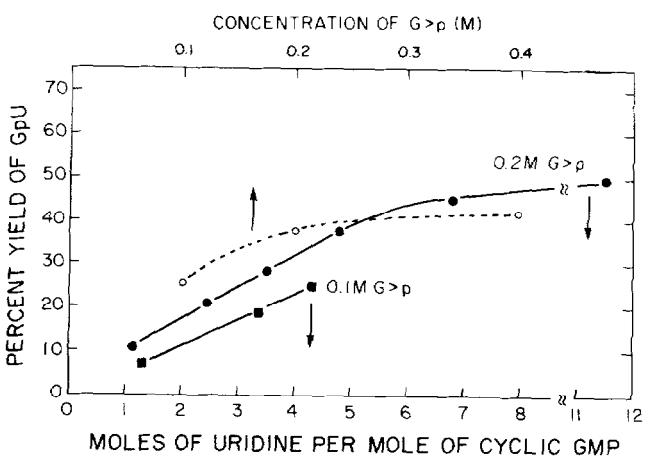

Fig. 11. Effect of reactant concentration on the yield of GpU. Conditions for the 2.5-mm reactor were the same as in Fig. 8. Arrows indicate corresponding scales.

solution. Yet practical considerations limit the molar excess of U that was kept five-fold in other experiments with the enzyme reactors.

The recirculating reactor offers a greater control over the reaction conditions than the single-pass reactor. The time course of the reaction obtained with a 2.5-mm long reactor in the recirculation system at relatively low flow-rate is illustrated in Fig. 12. Comparison of the results with those obtained with the enzyme in free solution and given in Fig. 5 shows that the reaction took place much faster in the recirculating reactor, due to the greater amount of enzyme present, than in free enzyme solution. The reaction rate is expected to be proportional to the length of the reactor and to increase with the flow-rate[30]. Therefore with longer reactors operated at higher flow-rates, higher reaction rates would be expected. The final yield of GpU, however, was higher in the reaction with the free enzyme. The relatively low yield with the reactor indicates that equilibrium had not been reached, but accumulation of the reaction product in the microenvironment of the enzyme immobilized on amino-silica might also have affected the results.

Displacement chromatography

The reaction mixture was separated in the chromatographic units of the systems shown in Figs. 1 and 2 on two reversed-phase columns, column I and column II containing Supelcosil LC-18 and Zorbax C_{18} stationary phase, respectively. As shown in Table I the retentive power of the stationary phase in column I for the components of the reaction mixture was about twice as high than that in column II, as measured by the k' values obtained with the carrier as the eluent. The three-way valve No. 6 between the two columns made it possible to skim off U that was separated in column I from other, more strongly retained components by frontal chromatography. The bulk of U was recovered to avoid its dilution in column II and to facilitate the separation of the 170-mg or 340-mg quantities with relatively short analytical columns.

The carrier was 50 m*M* phosphate buffer, pH 3.0, that afforded relatively strong retention by the hydrocarbonaceous bonded stationary phase[31,32] and served

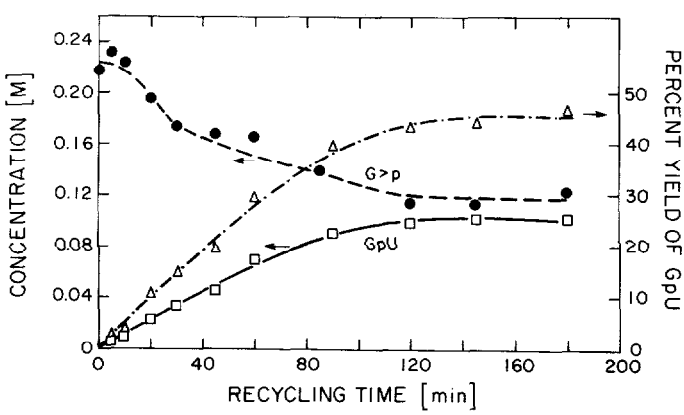

Fig. 12. Time course of the reaction with the recirculating reactor system. 1.5 ml of 20 m*M* acetate buffer pH 5.5 containing 0.5 *M* $(NH_4)_2SO_4$, 1.0 *M* U and 0.2 *M* G>p were recirculated through a 2.5-mm long reactor, at a flow-rate of 1.5 ml/min and at temperature of 2°C. Arrows indicate corresponding scales.

TABLE I

RETENTION FACTOR OF THE REACTANTS AND PRODUCTS ON TWO DIFFERENT OCTADECYL-SILICA COLUMNS UNDER THE SAME CONDITIONS

Columns: 250 × 4.6 mm. Eluent: 50 m*M* phosphate buffer, pH 3.0; flow-rate, 2 ml/min. Temperature: 25°C.

Compound	*Retention factor, k'*	
	Zorbax C_{18}	*Supelcosil LC-18*
U	2.4	4.7
Gp	3.2	6.4
G>p	5.0	9.7
GpU	>30	>30

as background electrolyte. The solubility of all components was sufficiently high in the carrier to avoid precipitation in the course of displacement development[2].

It has been pointed out[2] that in displacement chromatography of ampholytes, such as nucleotides, the displacer should have no ionic charges. Therefore a solution of 0.25 *M* *n*-butanol in the carrier was selected as the displacer. Since the 0.25 *M* butanol concentration used is significantly lower than the 0.87 *M* saturation limit it would be possible to use higher displacer concentrations to bring about faster separation and to recover the components at higher concentrations provided none of them drops out of solution. The flow-rate was 0.1 ml/min in all experiments in order to minimize zone overlap due to mass transfer resistances and slow displacement kinetics[2]. The column temperature was kept at 2°C in order to safeguard against possible degradation of GpU and the unreacted G>p.

Samples for displacement development were prepared in three different ways. (1) When the enzymic reaction was carried out in free solution, the arrangement was the same as shown in Fig. 2, but without the recirculating reactor and with a precolumn ahead of column I. The 0.5-ml loop of the sample valve was filled with the reaction mixture and the displacer was pumped through the loop to sweep the sample through the precolumn into column I. The 3-cm long precolumn acted as a filter to remove the protein from the mixture before it could enter the separating column. In fact some separation of the other components of the reaction mixture also took place in the precolumn with corresponding increases in retention volume. (2) With the reactor in series with the chromatographic column, as illustrated in Fig. 1, the reaction mixture was first washed out of the reactor with 1.2 ml buffer and into the 1.5-ml loop of sample valve II in order to effect a quantitative transfer of acidic components bound to the catalyst from the reactor to the chromatographic columns. (3) With the recirculating reactor, a direct sampling of the reaction mixture was effected by making use of the 0.5-ml loop of sample valve I as shown in Fig. 2.

The separation of the mixture obtained after enzymic reaction in free solution is illustrated in Fig. 13. The chromatogram in Fig. 13A shows the results of displacement development without skimming off of U through the three-way valve between columns I and II. Fig. 13B shows the results when U was skimmed off. The arrow indicates the time when the three-way valve was turned to terminate the withdrawal

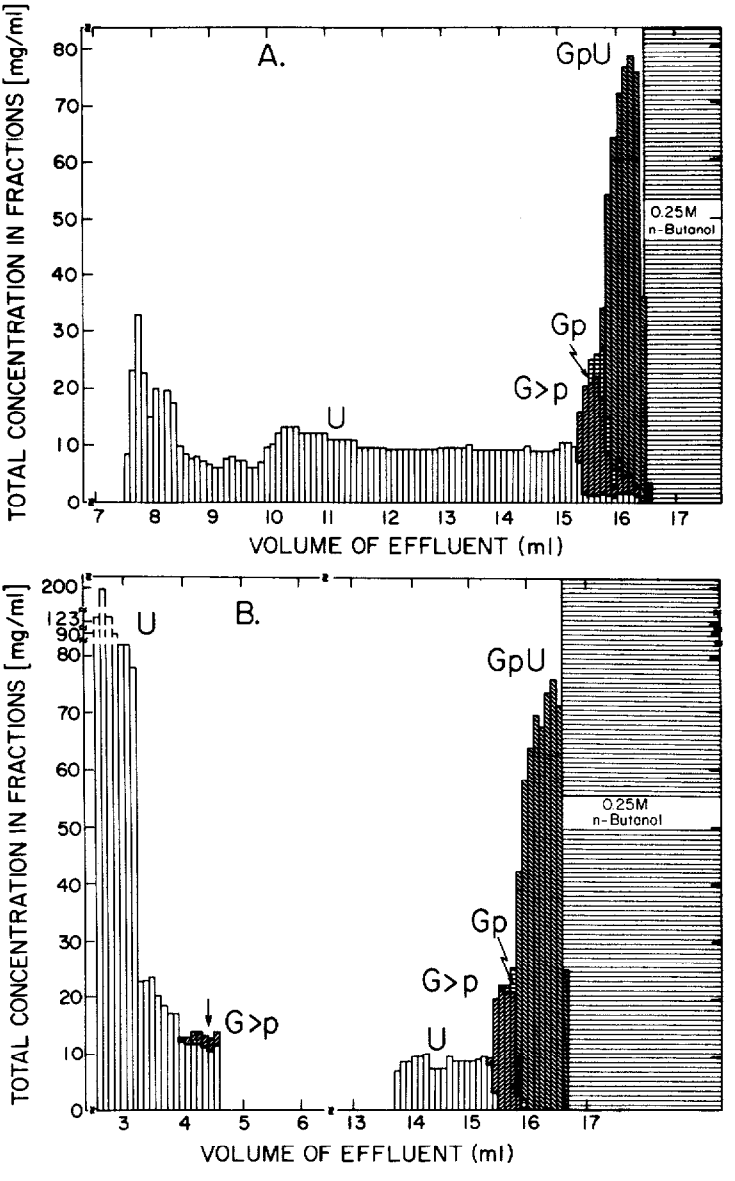

Fig. 13. Displacement chromatograms of the mixture obtained after reaction in free solution. A, Whole sample passed through both columns I and II. B, The bulk of U separated by frontal chromatography in column I was withdrawn before entering column II. Column I, 5-μm Supelcosil LC-18 (150 × 4.6 mm); column II, 5-μm Zorbax C_{18} (250 × 4.6 mm). Carrier: 50 m*M* phosphate buffer, pH 3.0. Displacer: 0.25 *M* *n*-butanol in carrier; flow-rate 0.1 ml/min. Sample: *ca.* 170 mg in 0.5 ml. Temperature: 2°C. Fraction size: 100 μl.

of U and to direct the effluent of column I into column II. The gap in the middle of the chromatogram corresponds to the breakthrough volume of U in column II. Comparison of the two chromatograms shows that the separation of GpU from Gp and G>p is superior when U is skimmed off. The breakthrough volume of the displacement front is almost the same in both cases and U is recovered at higher concentrations when withdrawn after leaving column I. As both U and G>p, the starting materials for the synthesis, are returned to the enzyme reactor, the contamination of U by G>p shown in Fig. 13B is acceptable. It is noted that the retention order of G>p and Gp is reversed in the displacement development with respect to that observed in elution development with the same chromatographic system (*cf.*, Table I). GpGp and GpGpU were not found in the fractions analyzed, although they were present in the reaction mixture as shown in Fig. 6. Presumably, their recovery would have required a stronger displacer than 0.25 *M* butanol.

The chromatogram shown in Fig. 14 illustrates the separation of the reaction mixture prepared in the single-pass reactor. Since U was skimmed off the results are similar to those shown in Fig. 13B. The breakthrough volume of U is somewhat larger in Fig. 14 than in Fig. 13B because with the single-pass method the sample volume was 1.5 ml instead of 0.5 ml as in other experiments. On the other hand the breakthrough volume of the displacer, which was significantly retained in the precolumn, was greater in the experiment with the precolumn (Fig. 13A) than without it (Fig. 14).

The separation of the mixture prepared by the recirculating reactor at two reactant concentrations is shown in Fig. 15A and B. In both cases U was skimmed off. The results illustrated in Fig. 15A were obtained with the same sample load as

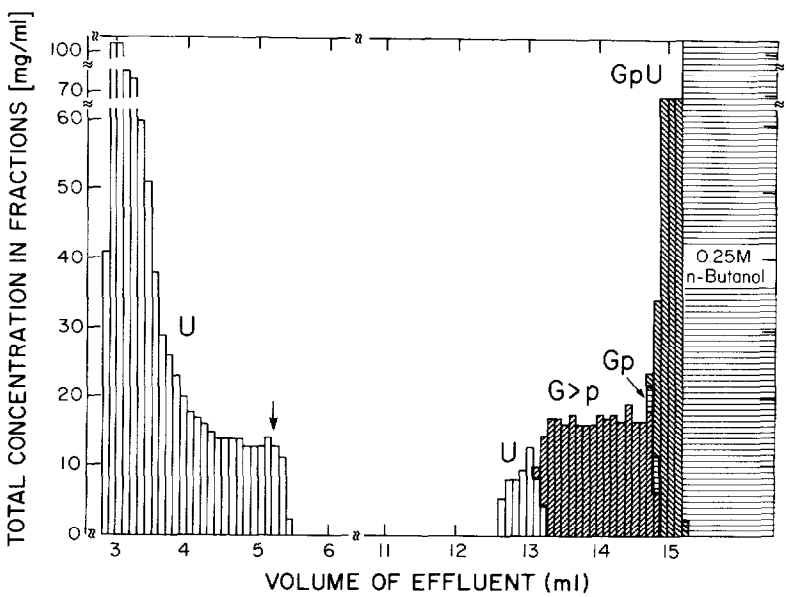

Fig. 14. Displacement chromatogram of the mixture obtained with the single-pass reactor and the chromatograph in series. Reactor length: 2.5 mm. Sample: *ca.* 170 mg in 1.5 ml. Other conditions of displacement development were the same as in Fig. 13B.

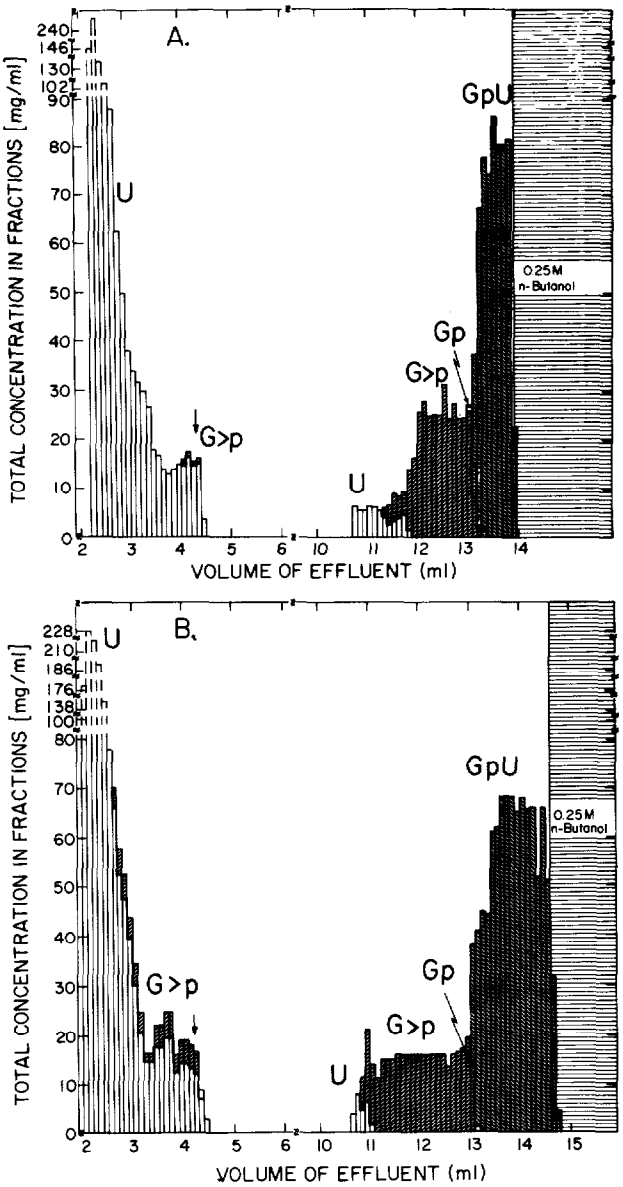

Fig. 15. Displacement chromatograms of the mixture obtained with the recycling enzyme reactor and chromatograph combination. The initial concentrations in the reactor were: A, 1.0 *M* uridine and 0.2 *M* G>p; B, 2.0 *M* uridine and 0.4 *M* G>p. Thus, samples were 170 mg and 340 mg in A and B, respectively. Chromatographic conditions were identical to those in Fig. 13B.

in the experiments represented by Figs. 13 and 14 and as expected they are similar. However, comparison of Fig. 15A and B clearly demonstrates the advantages of higher sample load in our chromatographic system as far as the efficacy of the separation is concerned. Recovery of U is similar; the slight difference in the "peak"

TABLE II

RECOVERY OF GpU BY DISPLACEMENT CHROMATOGRAPHY

Sample origin	*Product, GpU*	
	% Purity	*Amount recovered per run (mg)*
Free solution*	97.7	47.4
Single-pass reactor*	95.5	24.0
Recirculating reactor*	96.5	58.4
Recirculating reactor**	99.7	93.0

* Initial concentrations of U and G > p in the reaction mixture were 1.0 *M* and 0.2 *M*, respectively.
** Initial concentrations of U and G > p in the reaction mixture were 2.0 *M* and 0.4 *M*, respectively.

concentration of U may be due to error in HPLC analysis for which this fraction had to be diluted 40,000 times. As mentioned before the poor separation of the two reactants U and G > p is acceptable as they are usually returned to the reactor.

The amount of product recovered and its purity are given in Table II for displacement chromatography of the samples prepared by different methods. The duration of the chromatographic runs ranged from 140 to 165 min. Best results were obtained with the sample prepared in the recirculating reactor by using high reactant concentrations; 93 mg of GpU at a purity of 99.7% could be recovered in one run with analytical-size columns.

The need for removing the displacer from the column in a subsequent regeneration step may be considered a drawback of displacement chromatography, although a column clean-up step after any type of preparative run is expedient in elution chromatography as well. In the present work no attempt was made to regenerate the column in a shorter time than the time span of the chromatographic run proper. In routine work, such separation is preferably carried out with two sets of chromatographic columns alternating: while one separates, the other regenerates.

CONCLUSIONS

The results indicate that tandem operation of the recirculating enzyme reactor with the displacement chromatograph offers a powerful means for effecting product recovery. The instrument we have described can easily be developed for laboratory-scale production by recycling the unreacted reactants, U and G > p, into the reservoir of the enzyme reactor and by supplementing the amounts converted to GpU. In a fashion similar to advanced liquid chromatographs, the system can be automated to yield a continually operating, self-contained reactor-*cum*-chromatograph. The employment of two sets of chromatographic columns in parallel, which alternately separate and regenerate, would secure maximum throughput rate without taxing instrumentation.

According to the data in Table II, at sufficiently high feed concentration the system described can produce in one chromatographic run of 2.4 h 93 mg of GpU in >99% purity. Assuming that scaling up the process by increasing the column

from 4.6 to 25 mm I.D. and accordingly the flow-rate from 0.1 ml/min to 2.5 ml/min will not affect adversely the efficacy of the separation, one chromatographic experiment would yield *ca.* 2.3 g of product. An instrument with a battery of two parallel column sets having such diameters could then produce *ca.* 23 g of GpU over a period of 24 h.

The combination of a recirculating enzyme reactor with a chromatograph to form a single unit may serve as a model for similar instruments suitable for the *ad hoc* preparation or continual supply of complex biochemical substances in the laboratory. The reactor containing the enzyme immobilized on a microparticulate support is assembled from parts commonly used in HPLC and may, therefore, be considered as an accessory to the liquid chromatograph. The use of such immobilized-enzyme reaction offers high reaction rates and therefore high rates of conversion to the desired product can be achieved with short contact times that may attenuate the effect of side reactions. In the present case the hydrolysis of G > p to Gp was negligible in the reaction with the immobilized enzyme as satisfactory yield of GpU could be obtained rapidly. Another advantage of the immobilized enzyme is that it can be used with only a slight decrease in enzymic activity over several weeks of operation.

The recirculating reactor affords control of reaction conditions much more independently from the sampling requirements for chromatographic separations than the single-pass reactor. Yet it is fully compatible with the chromatograph. The results of this work strongly suggest that a microparticulate immobilized enzyme reactor in the recirculating mode offers the greatest potential for similar reactor-*cum*-chromatograph systems.

Displacement development with high-efficiency columns augments the scale of chromatography with respect to elution development for columns having similar dimensions. The concentration of the separated components in the effluent is significantly higher because the process does not require and is not subject to sample dilution to the extent usual in elution chromatography. Thus, product recovery and recycling of unreacted substances is greatly facilitated.

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