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SOME POSSIBILITIES FOR THE IMMOBILIZATION OF ENZYMES IN POLY(VINYL ACETATE-CO-ETHYLENE) TUBES

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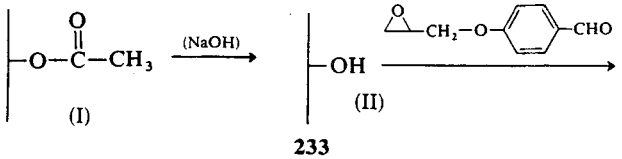
The inside surface of poly(vinyl acetate-co-ethylene) tubes was coated with poly(vinyl alcohol) in the absence as well as in the presence of terephthalaldehyde as crosslinking agent. A hydrophilic inside tube surface was obtained, which was modified by reaction with 2, 4, 6-trichloro-s-triazine resp. with 2-(3-aminophenyl)-1,3-dioxolane. The latter method gave reactive tube supports after diazotization resp. after activation with glutardialdehyde. Furthermore, the amino derivative could be reacted with enzyme aggregates formed by reaction of glucose oxidase with 4-(2,3-epoxypropoxy)-benzaldehyde. Trypsin and glucose oxidase were immobilized onto the activated inside tube surfaces, and the properties of the immobilized enzymes were studied. The flow patterns of tube-immobilized trypsin were studied. The application of tube-immobilized glucose oxidase for automated glucose analysis was tested.

INTRODUCTION

Reactive carriers suitable for covalent immobilization of enzymes can be synthesized starting from tubes consisting of vinyl acetate-ethylene copolymers (I). Tubes with an inner diameter of 0.2 cm were used, which contained about 10 mol% vinyl acetate. The inside surface of the tubes were hydrolyzed to vinyl alcohol groups (II) by treatment with 20% NaOH. In order to enlarge the inside hydrophilic surface, first the vinyl alcohol groups were reacted with 4-(2,3-epoxypropoxy)-benzaldehyde, giving derivate III, which contained free aldehyde groups. The aldehyde groups could be acetalized in acidic medium with poly(vinyl alcohol) (PVA), giving a PVA-coated inside tube surface (IVa). In the presence of terephthalaldehyde, the acetalization led to a hydrophilic crosslinked polymer covalently bound to the inside tube surface (IVb).



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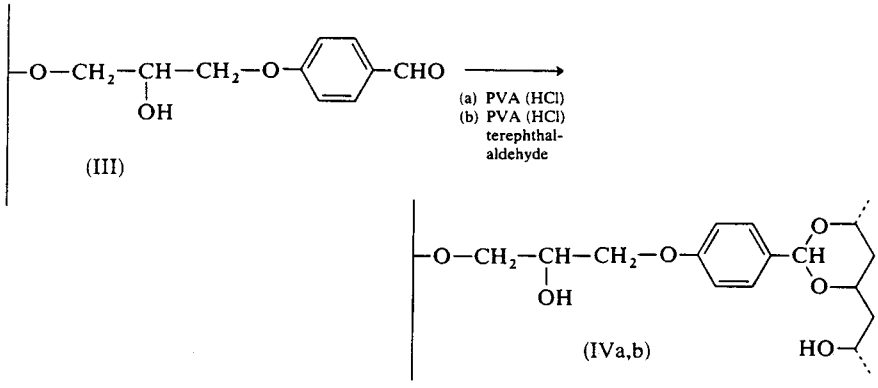
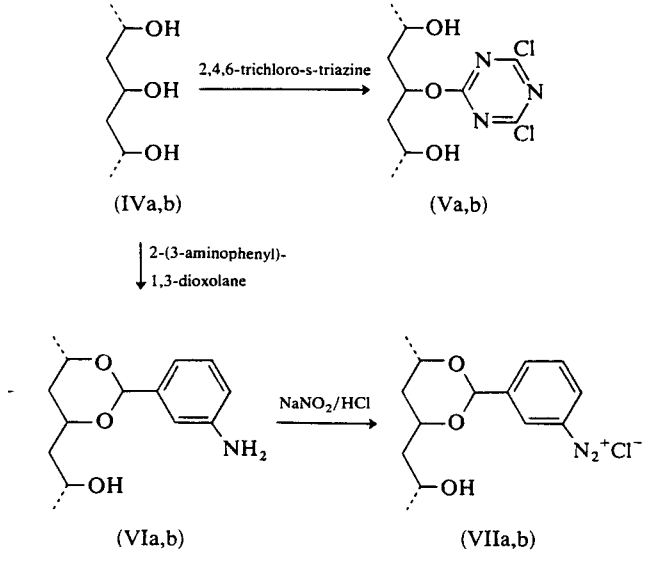


In the following scheme, the activation of the hydroxyl groups of tubes (IVa) and (IVb) with 2,4,6-trichloro-s-triazine to tube (Va) and (Vb), as well as the activation of the supports with diazonium groups to tubes (VIIa) and (VIIb), is described.



The activation with 2,4,6-trichloro-s-triazine was also studied on derivate II. In this case, the following coupling of trypsin gave a product lacking any detectable enzymatic activity. Starting from tubes IVa and IVb, both coated with PVA, the immobilization of trypsin was possible by the 2,4,6-trichloro-s-triazine method, as well as by azo coupling. Table 1 shows that in the case of both methods for enzyme coupling, tube IVb proved to be the more favorable starting material.

TABLE 1. **Activities of Trypsin Immobilized by the 2,4,6-Trichloro-s-Triazine Method resp. by Azo Coupling on Poly(Vinyl Acetate-co-Ethylene) Tube Derivatives**

Starting hydroxyl groups containing tube derivate	Immobilization method	Activity[a] of tube-immobilized trypsin in mU per 100-cm tube
II	2,4,6-Trichloro-s-triazine	0
IVa	2,4,6-Trichloro-s-triazine	10
IVb	2,4,6-Trichloro-s-triazine	90
IVa	Azo coupling	2
IVb	Azo coupling	100–150

[a]Substrate: 2.5×10^{-3} M D,L-BAPA, phosphate buffer, pH 7.8 ($\mu = 0.15$), 25°C.

Immobilized trypsin coupled by the 2,4,6-trichloro-s-triazine method to the inside tube surfaces was not very stable. After the first five assays with substrate, a decrease of activity of about 30% was observed without indicating any adsorbed enzyme. In contrast, the azo coupling of trypsin to tube VIIb gave a product with an excellent stability. In the case of repeated batchwise determinations of immobilized tryptic activity, as well as in the case of a continuous operating mode by perfusing the tube for 100 h with a substrate solution (Fig. 1), no loss of enzymatic activity could be observed.

The 100-cm tube VIIb could immobilize 0.6 mg trypsin. Toward BAEE in the presence of tris buffer, pH 8, the immobilized enzyme showed an activity of 4.8 U per 100 cm tube material (20% retained activity). The pH optima of trypsin immobilized in this way were determined using D,L-BAPA and BAEE as substrates (Figs 2 and 3). In the case of both substrates, the pH optima of the tube-immobilized trypsin differed only slightly from that of the native trypsin. Only a slight shift of the pH optima

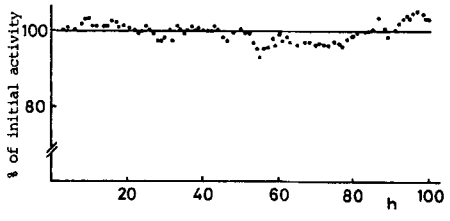


FIG. 1. Activity of tube VIIb-immobilized trypsin in dependence of reaction time with the following substrate: 0.001 M D,L-BAPA, phosphate buffer pH 8.0 ($\mu = 0.015$); flow rate, 10 $cm^3\ h^{-1}$.

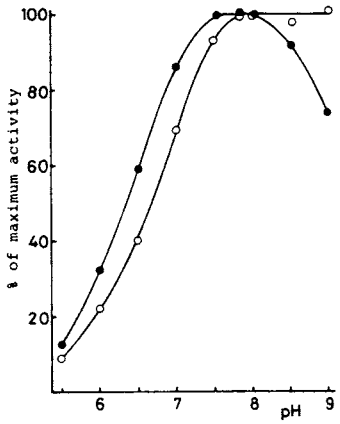


FIG. 2. pH Activity profile of native (●) and tube VIIb-immobilized trypsin (○). Substrate: 2.5×10^{-3} M D,L-BAPA, phosphate buffer $\mu = 0.15$, 25°C.

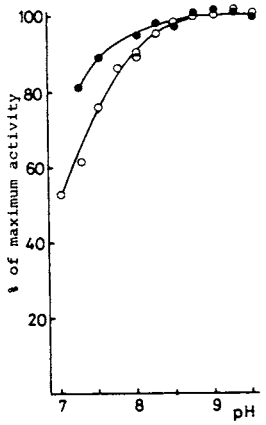


FIG. 3. pH Activity profile of native (●) and tube VIIb-immobilized trypsin (○). Substrate: 0.01 M BAEE, 0.2 M NaCl, 0.1 M tris buffer, 25°C.

toward the alkaline pH region could be observed, which was caused by product accumulation. The pH optima were determined in the presence of rather high buffer concentrations, which reduces proton accumulation in the microenvironment of the immobilized enzyme and results in an approximation of the pH activity profiles of the native and immobilized enzyme.

The Michaelis–Menten kinetics of trypsin, which was immobilized on tube VIIb, was determined with D,L-BAPA as substrate. The activities were assayed in the range of 3×10^{-4} to 5×10^{-3} M D,L-BAPA. The Lineweaver–Burk plots gave linearity in this range of substrate concentrations. The apparent Michaelis constant was evaluated to $K_{M(app)} = 1.6 \times 10^{-3}$ M. Under adaquate conditions the K_M value of native trypsin was determined to be 2.7×10^{-3} M.

The pH optima as well as the Michaelis–Menten kinetics of tube-immobilized trypsin were assayed by recirculation of the substrate solutions. Another possibility for assaying the enzymatic activity of tube-immobilized trypsin is to perfuse the tube with a substrate solution at a constant flow rate. This continuous operating mode is mainly used in systems made for automated analysis when working with enzymes that are immobilized on tubes. Therefore the characterization of the immobilized enzymes under these conditions is of interest for their possible application. Continuous flow characteristics have been described for packed bed reactors by Lilly et al. (1)

and for tube reactors by Hornby and Filippusson (2). Tube VIIb-immobilized trypsin was characterized by perfusing 20 cm of this tube with a solution of 0.001 M D,L-BAPA in phosphate buffer ($\mu = 0.015$) at constant flow rates in the range 4–60 $cm^3 h^{-1}$. The flow rate corresponds to a velocity of flow through the tube-immobilized trypsin of 2–30 cm min^{-1}. Figure 4 shows the relation between degree of substrate hydrolysis and flow rate.

The enzyme activity at each single flow rate can be calculated with the data of Fig. 4. The relation between the enzyme activity and the flow rate is shown in Fig. 5. Figure 5 shows that the enzyme activity increased with increasing flow rate and approximated a limiting upper value. In the investigated case, maximum activity was nearly reached at flow rates above 30 $cm^3 h^{-1}$. Furthermore, the relation between the degree of hydrolysis of BAEE and the flow rate was estimated for specimens of 20 cm and 100 cm of tube-immobilized trypsin (Fig. 6).

Compared with BAPA the same tube-immobilized trypsin caused a higher degree of hydrolysis if BAEE was used as substrate. This was caused by the higher turnover number of trypsin for the substrate BAEE. Complete hydrolysis of BAEE could be achieved by a 100-cm tube specimen at flow rates below 5 $cm^3 h^{-1}$.

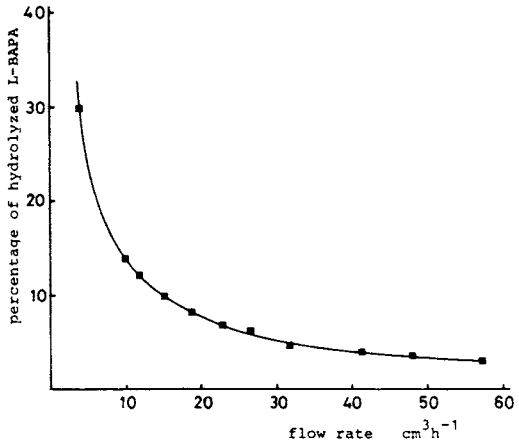


FIG. 4. Reaction of tube VIIb-immobilized trypsin with D,L-BAPA. Dependence of the flow rate on the percentage of hydrolyzed substrate is shown, using a 20-cm length of tube-immobilized trypsin. Substrate: 0.001 M D,L-BAPA, phosphate buffer, pH 8.0, μ = 0.015, 25°C. Activity of tube-immobilized trypsin (2.5×10^{-3} M D,L-BAPA, phosphate buffer, pH 8.0, $\mu = 0.15$, flow rate 500 $cm^3 h^{-1}$): 100 mU per 100-cm tube.

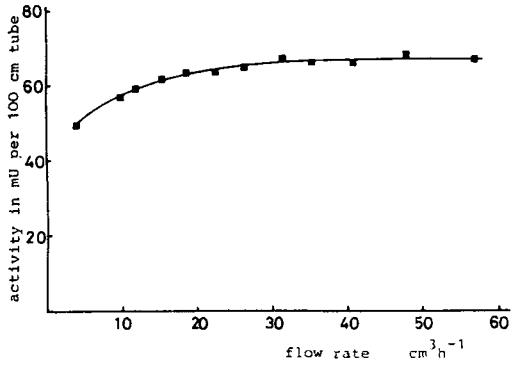


FIG. 5. Reaction of tube VIIb-immobilized trypsin with D,L-BAPA. Dependence of the flow rate on the activity of the tube-immobilized enzyme is shown. Substrate: 0.001 M D,L-BAPA, phosphate buffer, pH 8.0, $\mu =$ 0.015, 25°C; 20-cm tube VIIb-immobilized trypsin.

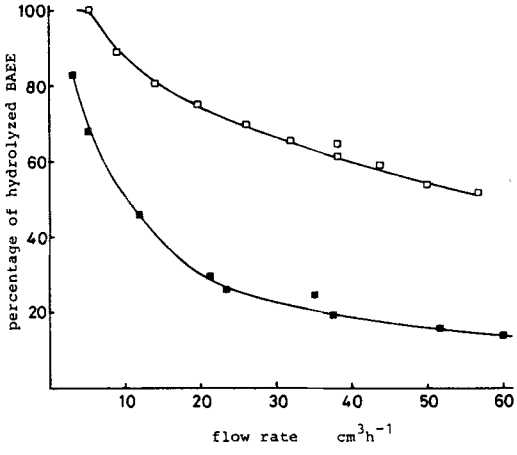
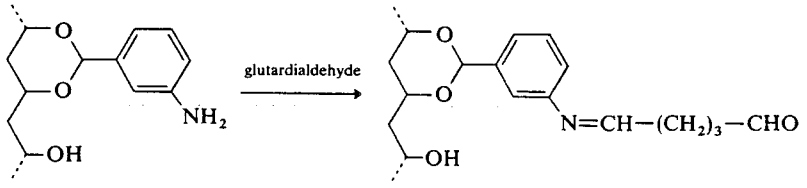


FIG. 6. Reaction fo tube VIIb-immobilized trypsin with BAEE. Dependence of the flow rate on the percentage of hydrolyzed substrate using a 20-cm (■) and a 100-cm (□) length of tube-immobilized trypsin. Substrate: 0.001 M BAEE, 0.02 M NaCl, 0.01 M tris buffer, pH 7.75, at 25°C. Activity of tube-immobilized trypsin (0.01 M BAEE, 0.2 M NaCl, 0.1 M tris buffer, pH 8.0; flow rate 500 $cm^3 h^{-1}$): 4.8 U per 100-cm tube.

Besides trypsin, glucose oxidase could also be immobilized on tube VIIb. The activity of the glucose oxidase was 160 mU per 100 cm tube material. Starting from tube VIb, enzymes could be immobilized by the glutardialdehyde method. The mechanisms of activation and enzyme coupling by this method are not yet completely clarified. In the following reaction scheme, a simplified activation step involving the possible formation of a Schiff's base is described.



(VIb) (VIIIb)

Glucose oxidase could also be immobilized on tube VIIIb, giving a product with an enzymatic activity of 70 mU per 100 cm tube material.

Another possible method for immobilizing glucose oxidase on tube surfaces, which also includes the formation of Schiff's bases, was performed as follows. Glucose oxidase was modified with 4-(2,3-epoxypropoxy)benzaldehyde, under conditions that have also been used by Jaworek (3) for enzyme modification with epoxy groups containing vinyl compounds. By use of this reagent under the chosen conditions, enzyme crosslinking can occur. Excessive aldehyde groups of the enzyme aggregates so formed then react with the amino groups of tube VIb.

Glucose oxidase immobilized on tube VIb gave products with a good reproducible activity of 1,200 mU per 100 cm tube material. The activity was assayed by the peroxidase/ABTS method (4). These immobilized glucose oxidase products were stable with regard to repeated determinations of the immobilized enzymatic activity. The possibility of the application of poly(vinyl acetate-co-ethylene) tube-immobilized glucose oxidase for automated glucose analysis was investigated by Jaworek (5) using a Technicon AutoAnalyzer System II. Under the chosen conditions, good reproducible results and good characteristics for the application in an automated glucose analyzing device were achieved.

MATERIALS

Materials were as follows: poly(vinyl acetate-co-ethylene) tubes (obtained from Boehringer/Mannheim); poly(vinyl alcohol) (Merck-Schuchardt); trypsin, EC 3.4.21.4 (Boehringer/Mannheim, Cat. No.

109819; Merck, Cat. No. 24579); glucose oxidase, EC 1.1.3.4 (grade I, Boehringer/Mannheim); peroxidase (POD, grade I, Boehringer/ Mannheim); $N\alpha$-benzoyl-L-arginine ethyl ester·HCl (BAEE, Merck); $N\alpha$-benzoyl-D,L-arginine-4-nitroanilide·HCl (D,L-BAPA, Boehringer/ Mannheim), 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate) (ABTS, Boehringer/ Mannheim).

METHODS

Introduction of Reactive Groups on Tubes Made from Vinyl Acetate-Ethylene Copolymers

Hydrolysis of the Inside Tube Surface to II. A 500-cm length of poly(vinyl acetate-co-ethylene) tube (I; 0.2 cm bore) was filled with 25 cm^3 of 20% (w/v) NaOH. The tube was incubated at 40°C for 2 h by recirculating the solution with a peristaltic pump.

Derivation of the Inside Tube Surface by Reaction with 4-(2,3-Epoxypropoxy)-Benzaldehyde to III. The hydrolyzed tube derivative II was filled with 25 cm^3 of 1 M 4-(2,3-epoxypropoxy)-benzaldehyde dissolved in dioxane, and the solution was recirculated for 2 h at room temperature. So prepared tube III was washed in an alternating mode with dioxane and water.

Coating of the Inside Tube Surface with PVA, Forming IVa. Tube III was reacted with an aqueous solution of 20 cm^3 2.2% (0.5 M with regard to vinyl alcohol units) PVA and 0.20 cm^3 conc. HCl for 15 h by recirculating the solution at 25°C, giving tube IVa. Thereafter the tube was washed with water.

Coating of the Inside Tube Surface with PVA in the Presence of Terephthalaldehyde as Crosslinking Agent Forming IVb. Tube III was reacted with an aqueous solution of 20 cm^3 2.2% PVA, with 0.20 cm^3 conc. HCl, and 1.00 cm^3 0.1 M terephthalaldehyde dissolved in dioxane was added. The tube was incubated for 30 min at 25°C by recirculating the mixture, giving tube IVb. Thereafter the tube was washed with water and incubated with 0.1 M HCl for 15 h; then the tube was washed with water again.

Activation of the Inside Tube Surface with 2,4,6-Trichloro-s-Triazine. According to an adapted method of Kay and Crook (6), a 100-cm length of tube derivatives II, IVa, and IVb was incubated with 10 cm^3 20% NaOH for 30 min at room temperature; thereafter the liquid was sucked off and 10 cm^3 10% (w/v) 2,4,6-trichloro-s-triazine in dioxane was added. The reaction was stopped after recirculating the solution through the tube for 10 min by addition of 10 cm^3 20% acetic acid. Thereafter the tube was washed with dioxane and water.

Derivation of Inside Tube Surfaces by Reaction with 2-(3-Aminophenyl)-1,3-Dioxolane, Giving VIa and VIb. A 500-cm length of tube derivatives IVa and IVb was reacted with an aqueous solution of 2.2% PVA, 2.00 cm^3 1 M 2-(3-aminophenyl)-1,3-dioxolane in dioxane, and 0.5 cm^3 conc. HCl. The mixture was recirculated at 40°C for 4 h; thereafter the tube was washed with 0.01 M HCl and with water.

Diazotization to VIIa and VIIb. A mixture of 10 cm^3 0.5 M HCl and 1 cm^3 1 M $NaNO_2$ was recirculated at 0°C for 30 min through a 100-cm length of tube VIa and VIb. Then the tube was washed with ice water and directly used for the immobilization of enzymes.

Immobilization of Enzymes
onto Activated Inside Tube Surfaces

Immobilization of Trypsin on Tube Va and Vb. Trypsin, 5 mg, was dissolved in 5 cm^3 phosphate buffer, pH 7.0 ($\mu = 0.15$), and coupled to a 100-cm length of tube containing activated dichloro-s-triazinyl groups. After 3 min of recirculating the enzyme solution, the immobilization reaction was stopped by addition of 3 cm^3 1 M NH_3/NH_4Cl (pH 9.2). The tube was then washed with phosphate buffer, pH 7.0, 0.5 M NaCl, and tridistilled water.

Immobilization of Trypsin by Azo-Coupling on Tube VIIa and VIIb. Trypsin, 5 mg, was dissolved in 5 cm^3 phosphate buffer, pH 7.0 ($\mu = 0.15$), and coupled at 0°C to a 100-cm length of tube VIIa and VIIb. After 2 h of recirculating the enzyme solution, the immobilization reaction was stopped and the solution was sucked off. The tube was washed with phosphate buffer, pH 8.0, 0.5 M NaCl, and tridistilled water. The washing solutions were collected in a 100-cm^3 measuring flask, their content of protein was determined with Folin's reagent (7), and the amount of immobilized enzyme was calculated. The tube was filled with water and stored at 4°C in a refrigerator.

Immobilization of Glucose Oxidase by Azo-Coupling on Tube VIIa and VIIb. A 50-cm length of tube VIIb was incubated for 1 h at 0°C with a solution of 2 mg glucose oxidase in 4 cm^3 0.5 M triethanolamine buffer, pH 8.0. The immobilization reaction was performed by recirculating the enzyme solution. The immobilization product was washed with 0.5 M triethanolamine buffer, pH 8.0, 0.5 M NaCl, and tridistilled water.

Immobilization of Glucose Oxidase on Tube VIb by the Glutardialdehyde Method. A 50-cm length of tube VIb was activated with a 5% solution of glutardialdehyde in phosphate buffer, pH 8.0 ($\mu = 0.15$), by recirculating the solution at room temperature for 1 h, giving tube VIIIb. The tube was then washed with water. Glucose oxidase, 3 mg, was dissolved in 3 cm^3 phosphate buffer, pH 8.0 ($\mu = 0.15$), placed in a 50-cm length of

tube VIIIb, and the enzyme solution was recirculated for 4 h at room temperature. The tube was then washed with phosphate buffer, pH 8.0, 0.5 M NaCl and tridistilled water.

Immobilization of Modified Glucose Oxidase on Tube VIb. Glucose oxidase, 3 mg, was dissolved in 0.3 cm^3 0.5 M triethanolamine buffer, pH 8.0, and 0.1 cm^3 2 M 4-(2,3-epoxypropoxy)-benzaldehyde in dioxane were addded. The reaction mixture was stirred for 30 min under N_2. Thus modified, the glucose oxidase was added to 10 cm^3 0.2 M acetate buffer, pH 4.6, and placed in a 100-cm length of tube VIb. After 1 h of recirculating the solution at room temperature, the immobilization reaction was stopped, and the tube was washed with 0.2 M acetate buffer, pH 4.6, 0.5 M NaCl, and tridistilled water.

Determination of the Activities of Enzymes Immobilized onto the Inside Tube Surfaces of Poly(Vinyl Acetate-co-Ethylene) Tubes

Determination of Enzymatic Activities by Recirculating the Substrate Solutions. The immobilized enzyme activities were assayed by recirculating the substrate solution at a flow rate of 500 $cm^3\ h^{-1}$. The substrate solutions were placed in a stirred reservoir and pumped through the tube-immobilized enzyme by a peristaltic pump; from there they went through a flow cuvette and back to the reservoir. The tube-immobilized enzyme was placed in a thermostated water bath. The activities of tube-immobilized trypsin were determined with 2.5×10^{-3} M D,L-BAPA under the conditions already described (8). With BAEE as substrate the activities were assayed in the presence of tris(hydroxymethyl)-aminomethane buffer as described in ref. (9). The activities of the tube-immobilized glucose oxidase were assayed by the peroxidase/ABTS-method (4) in a modified form (8).

Determination of Immobilized Tryptic Activities by a Continuous Operating Mode. A definite length of tube VIIb-immobilized trypsin was perfused by a substrate solution at a constant flow rate in the range 4–60 $cm^3\ h^{-1}$ and the absorption of the hydrolyzed substrate was registered. Under the following conditions, BAEE and BAPA were used as substrates: (*a*) 1×10^{-3} M BAEE, 0.02 M NaCl, 0.01 M tris buffer, pH 7.75, 25°C, 0.1-cm flow-through cuvette, 255 nm; (*b*) 1×10^{-3} M D,L-BAPA, phosphate buffer, pH 8.0 ($\mu = 0.015$), 25°C, 0.2-cm flow-through cuvette, 405 m,.

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cability of tube-immobilized glucose oxidase for automated glucose analysis.

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