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SOME CARRIERS FOR THE IMMOBILIZATION OF ENZYMES BASED ON DERIVATIZED POLY(VINYL ALCOHOL) AND ON COPOLYMERS OF METHACRYLATES WITH DIFFERENT SPACER LENGTHS

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SUMMARY

Commercially available poly(vinyl alcohol) (PVA) was cross-linked with terephthalaldehyde, derivatized with 1-fluoro-4-nitrobenzene and reduced by means of sodium dithionite. The carrier obtained, containing N-substituted sulphamic acid groups, was applied to immobilize ribonuclease (E.C. 3.1.4.22). The influence of protein denaturing agents on the immobilization reaction and its pH dependence was investigated.

By copolymerizing suitable methacrylates, copolymers with different spacer lengths between the reactive groups and the polymer matrix were synthesized. After their reduction they showed the same swellability and the same content of amino groups. These products were transformed into reactive carriers by diazotization and coupled with ribonuclease, trypsin (E.C. 3.4.21.4), α-chymotrypsin (E.C. 3.4.21.1) and urease (E.C. 3.5.1.5). The amounts of bound proteins and the enzymatic activities of the immobilization products were determined and correlated with the spacer lengths of the applied carriers.

INTRODUCTION

The derivatization of polymers is important in the preparation of reactive carriers in studies of the immobilization of enzymes and in affinity chromatography. In addition to some methods using direct activation, *e.g.*, with cyanogen bromide[1], many of the carriers are synthesized by multiple-step polymer-analogous reactions. As suitable intermediates for azo coupling or the preparation of reactive isothiocyanates, often polymers containing aminophenyl groups are used, which can be synthesized by reducing the corresponding polymeric nitro compounds.

The synthesis of polymers containing nitrophenyl groups can be performed either by polymerizing appropriate monomers or by derivatizing available polymers by suitable methods. We have found that starting from cross-linked poly(vinyl alcohol) (PVA) the reaction with 1-fluoro-4-nitrobenzene is a good method for intro-

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ducing this group with a high degree of conversion. The formation of low-molecular-mass aromatic ethers by means of 1-fluoro-4-nitrobenzene was first described by Rarick *et al.*[2].

One of the most common methods for the preparation of polymers containing amino groups is the reduction of the corresponding nitro compounds with sodium dithionite under alkaline conditions. However, if sulphur-containing reducing agents, such as sodium hydrogen sulphite or sodium dithionite, are applied to the case of low-molecular-mass nitro compounds, N-substituted sulphamic acids can be produced[3]. In this paper we describe the formation of polymeric sulphamic acid carriers when sodium dithionite was used for the reduction of the above-mentioned derivatized PVA. These carriers are suitable for the direct immobilization of enzymes. The immobilization of ribonuclease and also the pH dependence of this immobilization reaction was investigated.

Normally the immobilization of an enzyme is carried out under conditions avoiding its denaturation. Reversible denaturation (unfolding) of ribonuclease has been reported[4]. We decided to immobilize an unfolded ribonuclease. This could be achieved in the presence of either urea or urea and 2-mercaptoethanol as denaturing agents. On washing out the denaturing agents, reactivation of the immobilized enzyme was observed.

The immobilization of enzymes is strongly influenced by the chemical properties and the structure of the applied carriers. The distance between the polymer matrix and the reactive groups seems to be important, as shown by investigations in affinity chromatography[5]. In this paper we describe the investigation of the influence of the length of the spacer arms of the polymeric carriers which were prepared from methacrylates of ethylene glycols with different numbers of oxyethylene units on the immobilization of enzymes.

EXPERIMENTAL

Reagents

Poly(vinyl alcohol), molecular mass *ca.* 72,000 and degree of hydrolysis 97.5–99.5%, was a product of Schuchardt (Munich, G.F.R.). Terephthalaldehyde, 1-fluoro-4-nitrobenzene, 2-hydroxyethyl methacrylate, methacryloyl chloride and 2-hydroxy-1-naphthaldehyde were purchased from EGA-Chemie (Steinheim, G.F.R.). Ribonuclease, urease, cytidine-2′,3′-cyclic phosphate (barium salt), Nα-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) and N-acetyl-L-tyrosine ethyl ester (ATEE) were products of Merck (Darmstadt, G.F.R.). Trypsin and α-chymotrypsin were purchased from Boehringer (Mannheim, G.F.R.). All other chemicals were of the highest grade available.

Synthesis of the PVA-carrier

Cross-linking of PVA[6]. A 44-g amount of PVA was dissolved in 1800 ml of water at 65°C with stirring. After addition of the calculated amount (2.68 g) of terephthalaldehyde, the solution was cooled to 25°C and 20 ml of hydrochloric acid (32%) were added. Within *ca.* 30 min the solution turned to jelly, which was reduced to small pieces and stirred for 48 h. Then the suspension was filtered and the insoluble polymer was washed with water and resuspended. This procedure was repeated sev-

eral times in order to remove all of the water-soluble components. After treating the product with methanol it was dried thoroughly, ground and sieved. Particles with diameters in the range 0.1–0.2 mm were collected and used for further experiments.

Reaction with 1-fluoro-4-nitrobenzene. A 3.826-g amount of the cross-linked PVA was pre-soaked in 50 ml of water. With stirring, 8 g of potassium hydroxide in small amounts followed by 19 g of 1-fluoro-4-nitrobenzene were added. The resulting two-phase reaction mixture was then stirred vigorously and refluxed for 1.5 h. The polymer was separated in a funnel and washed with water and acetone until the filtrate was neutral and colourless. After drying, 7.173 g of a light yellow product were obtained. From the weight increase and the nitrogen content (5.1 %), a conversion of *ca.* 31 % was calculated.

This polymer had no detectable swellability whereas the starting product (cross-linked PVA) showed considerable swelling in water.

Reduction. A suspension of 4.00 g of cross-linked PVA containing nitrophenyl groups in 250 ml ethanol–water (1:1) was heated at 80°C with stirring. Within 1.5 h, 15 g of sodium dithionite and 2 g of sodium carbonate were added, then the liquid was removed and the procedure was repeated twice. Thus 45 g of sodium dithionite were used within *ca.* 5 h. The swellability of the polymer increased distinctly during the reduction. The polymer was collected on a Büchner funnel and was washed with water until no reducing agent could be detected in the filtrate by means of 5,5′-dithiobis-(2-nitrobenzoic acid). After drying, 4.5 g of a grey polymer were obtained. From the sulphur content (7.6 %) it was calculated that *ca.* 75 % of the nitro groups had been converted.

Synthesis of the spacer groups containing polymethacrylates

Preparation of the mono-substituted glycols. Ethylene glycol, diethylene glycol, triethylene glycol, tetraethylene glycol or Polydiol 400 was placed in a round-bottomed flask and potassium hydroxide was added and dissolved with stirring. Then an equivalent amount of 1-fluoro-4-nitrobenzene was added and the mixture was stirred for 4 h at 50–60°C. After cooling, extraction was performed using 1,2-dichloroethane. The organic layer was washed with small amounts of water, dried with potassium sulphate and evaporated to dryness under reduced pressure. Column chromatography using Kieselgel was to be found the best purification method in order to remove the starting materials and undesired by-products. The substances derived from ethylene glycol (m.p. 91°C), diethylene glycol (m.p. 88°C), triethylene glycol (m.p. 83°C) and tetraethylene glycol (m.p. 70–74°C) were crystalline, whereas the product synthesized from Polydiol 400 was a viscid liquid.

Preparation of the monomeric methacrylates. The monosubstituted glycols and 4-nitrophenol were dissolved in dry pyridine, the solution was cooled and methacryloyl chloride (*ca.* 10 % excess) was added in small amounts with stirring at temperatures below 5°C. The mixture was then acidified and extracted with 1,2-dichloroethane. The organic layer was washed several times with dilute hydrochloric acid and potassium hydroxide solution and finally with water. After drying with sodium sulphate and evaporating the solvent, the crude methacrylates were obtained and purified by chromatography on Kieselgel. The substances with $n = 0$ (m.p. 93°C), $n = 1$ (m.p. 90.5°C) and $n = 2$ (m.p. 149°C) (for explanation see Fig. 2) were crystalline, and all other products were syrupy liquids.

Polymerization of the methacrylates. According to the molar ratio given in Table III, the components were mixed and polymerized under nitrogen in sealed tubes at 70°C for *ca.* 10 h. The cross-linking agent ethylene glycol dimethacrylate and the inert monomer 2-hydroxyethyl methacrylate were purified by distillation. Recrystallized 2,2′-azobis(2-methylbutyronitrile) (0.3 %) was used as an initiator.

The glass-like polymers were crushed and extracted with dichloromethane by means of a Soxhlet extractor for 15 h. After thorough drying, nearly colourless products were obtained, which were ground and sieved (0.1–0.2 mm diameter).

Reduction of the polymeric methacrylates. Reduction was performed in *ca.* 1 *M* aqueous sodium hydroxide solution (50 % ethanol) using sodium dithionite at 70°C for 5 h. The reduction products were washed with water and treated with hydrochloric acid (4 *M*) with stirring for 4 h at 45°C. After further washing with water and drying, slightly reddish polymers were obtained that were free from sulphur and chlorine.

Diazotization of the polymeric methacrylates. A 30–50-mg amount of the products containing aminophenyl groups were stirred with 1 *M* hydrochloric acid overnight. The acid was removed and, after addition of 10 ml of 1 *M* hydrochloric acid, the mixture was cooled to 0–3°C and 4 ml of sodium nitrite solution (containing 500 mg) was added dropwise within 20 min with violent stirring. Stirring was continued for 10 min and, after sucking off the supernatant, the diazotized polymers were washed with cooled solutions of 30 ml of *ca.* 0.5 % hydrochloric acid, 25 ml of *ca.* 1 % sulphamic acid and 40 ml of *ca.* 0.5 % hydrochloric acid. The red polymers were coupled immediately after preparation, without delay.

Determination of the swellability

By means of a calibrated tube (I.D. 8 mm) the volume of a definite amount (100 mg, particle size 0.1–0.2 mm) of the dry polymer was measured. The appropriate buffer solution was poured on to the polymer and, after the swelling had finished, its volume was measured again. From the difference in the volumes the swellability could be calculated.

Determination of amino groups in the polymers

By means of 2-hydroxy-1-naphthaldehyde[7,8]. A 10-mg amount of the polymer was extracted with ethanol–dichloromethane (1:1) at 25°C with stirring for 48 h. The solvent was removed and a large excess (*ca.* 80-fold) of 2-hydroxy-1-naphthaldehyde in ethanol (5 ml) was added. After stirring the mixture for 24 h at 25°C, the polymer was carefully washed with ethanol and dichloromethane. Then a 0.4 *M* solution of benzylamine in dichloromethane was allowed to react with the polymer for exactly 30 min with stirring. The solution was sucked off and passed into a graduated flask. The absorption at 420 nm was measured spectrophotometrically and the amount of 2-hydroxy-1-naphthalylidenebenzylamine was calculated.

By means of titration with perchloric acid[9]. In a small flask a known amount of the polymer was suspended in 20 ml of glacial acetic acid and two drops of crystal violet were added as an indicator. The suspension was stirred and titrated with 0.1 *M* perchloric acid in glacial acetic acid.

Immobilization

Immobilizations were carried out at 25°C with stirring under nitrogen for 22 h using 30 mg of the carriers containing sulphamic acid groups and 30 mg of ribonuclease dissolved in 3 ml of the appropriate Soerensen buffer.

The ribonuclease solutions in urea–2-mercaptoethanol were allowed to stand for *ca.* 1 h with stirring before use when immobilization under denaturing conditions was performed.

Azo coupling was carried out for 24 h at 4°C with stirring under nitrogen starting from 50 mg of the polymers containing aminophenyl groups and 50 mg of enzyme dissolved in 3 ml of Sorensen buffer. The immobilization products were washed with buffer solutions, 1 *M* sodium chloride solution and finally with water in all instances. Determination of protein was carried out according to the method of Lowry *et al.*[10].

Determination of enzymatic activities

The determination of the enzymatic activities was carried out by means of the pH-stat method under nitrogen at 25°C, with the following substrates: 22.2 m*M* cytidine-2′,3′-cyclic phosphate, barium salt, 0.1 m*M* EDTA in 0.1 *M* sodium chloride solution was used for ribonuclease; trypsin was assayed with 0.01 *M* Nα-benzoyl-L-arginine ethyl ester hydrochloride (BAEE), in 0.01 *M* Tris buffer, 0.1 *M* sodium chloride solution; N-acetyl-L-tyrosine ethyl ester (ATEE) (0.02 *M*) with 0.02 *M* calcium chloride in 0.02 *M* Tris buffer was used for the determination of α-chymotrypsin.

RESULTS AND DISCUSSION

The synthesis of the reactive carrier with N-substituted sulphamic acid groups was carried out starting from commercially available PVA (Fig. 1).

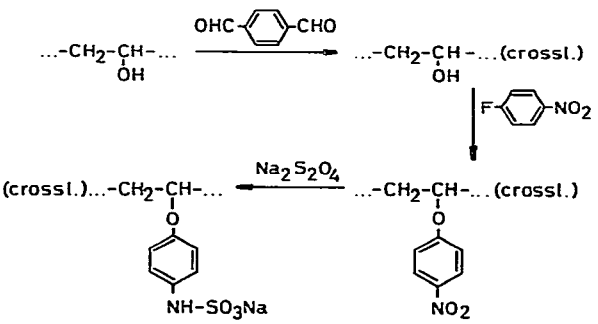


Fig. 1. Synthesis of the PVA carrier containing N-substituted sulphamic acid groups.

The first step was to cross-link the soluble PVA by means of the terephthalaldehyde[6], followed by a Williamson reaction with 1-fluoro-4-nitrobenzene, leading to an unsoluble polymer containing nitrophenyl groups. This we found to be a favourable method for obtaining PVA carriers with a high degree of substitution with a short reaction time. It also seems to be suitable for the derivatization of other hydroxyl-containing polymers.

In the next step this polymer was reduced with sodium dithionite in aqueous ethanol under slight alkaline conditions. For a high degree of conversion it is useful to change the reaction medium in between. This can be done by washing the polymer with water during the reduction and starting again with a small amount of sodium dithionite.

The presence of N-substituted sulphamic acid groups in this polymer could be shown by elemental analysis (C, H, N, S) and by quantitatively analysed decomposition with hydrochloric acid to the corresponding polymer containing amino groups. Further, comparative IR spectroscopy using a low-molecular-mass model substance showed that the polymer contained N-substituted sulphamic acid groups. The details of the investigation of this reaction will be the subject of another publication.

Because of the ionic groups, this carrier showed a pH-dependent swellability with a maximum between pH 8 and 9. Therefore, the immobilization reaction was carried out in the pH range 7.8–9.0 using appropriate Sorensen buffer solutions.

The carrier containing the sulphamic acid groups was reacted directly with ribonuclease. The mechanism of the immobilization reaction is still under investigation.

Table I shows that the greatest amount of ribonuclease was immobilized at pH 8.6. Concerning the retained activities of the immobilization products, it can be stated that with increasing amounts of bound protein a decrease in activity occurs. This is in good agreement with other investigations[11]. The activity of the immobilized ribonuclease showed an optimum at pH 8.6. At the optimal pH the immobilized ribonuclease showed an increase in the retained activity of *ca.* 50%. The storage stability was very good: after storage for 3 weeks under water at 4°C a loss of only *ca.* 5% of the original activity was observed. It is known that enzymes can be unfolded by breaking the stabilizing hydrogen bonds. This so-called denaturation is caused, *e.g.*, by urea[12]. In some instances the denaturation is reversible and the enzyme can be recovered without any loss of activity when the denaturing agent is removed[4].

TABLE I

pH DEPENDENCE OF THE IMMOBILIZATION OF RIBONUCLEASE ON A PVA CARRIER CONTAINING N-SUBSTITUTED SULPHAMIC ACID GROUPS

Immobilization pH	*Bound ribonuclease* (mg/g carrier)*	*Retained activity** (%)*
7.8	44.1	25.3
8.2	66.6	12.3
8.6	86.4	10.2
9.0	44.5	17.5

* For coupling conditions, see Experimental.
** Determined at pH 7.0.

Horton and Swaisgood[13] investigated the unfolding and refolding of trypsin and ribonuclease after their immobilization. In our study we investigated the immobilization of an enzyme which was unfolded prior to the immobilization reaction. Using the described carrier with N-substituted sulphamic acid groups the immobiliza-

tion of ribonuclease in the presence of increasing amounts of urea at pH 8.6 was studied.

The maximal amount of ribonuclease was bound when the immobilization was performed in 8 *M* urea solution. This can be explained by the fact that the most unfolded enzyme molecule provides the largest number of accessible reactive groups for the immobilization reaction[14]. Further, it was found that the retained activities of the thoroughly washed immobilized enzymes were strongly influenced by the concentration of urea during the immobilization reaction (see Table II).

TABLE II

IMMOBILIZATION OF RIBONUCLEASE ON A PVA CARRIER CONTAINING N-SUBSTITUTED SULPHAMIC ACID GROUPS UNDER DENATURATING CONDITIONS

Concentration of urea (M)	*Bound ribonuclease* (mg/g carrier)*	*Retained activity** (%)*
2	40.7	24.5
4	39.6	15.7
6	83.3	7.4
8	303.0	2.2
8***	345.0	0.34

* At pH 8.6 (for coupling conditions, see Experimental).
** Determined at pH 8.0.
*** In the presence of 2-mercaptoethanol (1 μl/mg enzyme).

By adding 2-mercaptoethanol to the enzyme–urea solution the disulphide bonds of the ribonuclease could be reduced. This led to complete unfolding of the enzyme molecule. Here a further increase in the bound amount of ribonuclease was observed. After removing the denaturing mixture from this immobilization product by washing, a very low retained activity of only 0.34% was found. However, after storage for 3 weeks a 3-fold increase in the activity of this product was observed. This shows that renaturation took place even in the case of totally unfolded immobilized ribonuclease.

The immobilization of enzymes is strongly influenced by the reaction conditions, the chemical structure and the physical properties of the carriers. Therefore, the amount and the concentration of the enzyme, the pH, the time and the temperature during the immobilization, the kind and content of reactive groups, the degree of cross-linking, the swellability and the particle size of the carriers are important immobilization parameters. It has been proposed that a spacer arm between the reactive group and the polymer matrix should have a favourable influence on the immobilization of biologically active molecules[5]. Many investigations have been carried out in affinity chromatography on the influence of spacers on the chromatographic properties of polymers[15,16]. Nearly all of the investigated polymers containing spacer arms were prepared by introducing the spacers by polymer-analogous reactions. Many of the spacers were aliphatic side-chains with different numbers of methylene groups, which caused increased hydrophobicity of the carriers and in some instances undesirable non-specific adsorptions of proteins by hydrophobic interaction[17]. In

order to avoid such disadvantages we decided to prepare carriers starting from monomers containing various numbers of oxyethylene units, which are hydrophilic. The attempt to synthesize such monomers on the basis of vinyl ethers was successful, but only polymers with inadequate mechanical properties were obtained when monomers containing more than four oxyethylene units were polymerized. Even the application of large amounts of cross-linking agents could not improve their properties. Therefore, another type of monomer based on methacrylates was synthesized, as shown in Fig. 2.

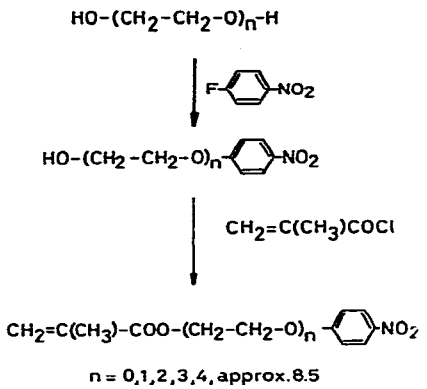


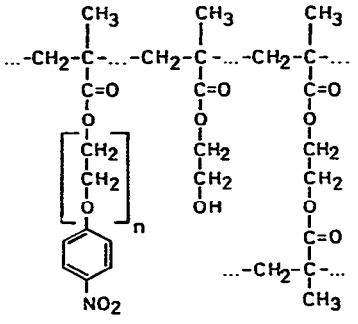
Fig. 2. Synthesis of methacrylates for the preparation of copolymers containing spacer arms.

Starting from mono-, di-, tri- and tetraethylene glycol and Polydiol 400, first a Williamson reaction with 1-fluoro-4-nitrobenzene was performed[2], leading to the corresponding mononitrophenyl ethers. In the next step, the remaining hydroxyl groups were esterified by means of methacryloyl chloride in pyridine. The monomers with $n = 0, 1, 2, 3$ and 4 were uniform substances, whereas the monomer with $n \approx 8.5$ was synthesized starting from Polydiol 400, a technical product which contained oxyethylene oligomers in the range $n = 3$–13. Nearly 75% of the product consists of oligomers with $n = 7, 8, 9$ and 10, as determined by gas chromatographic investigations of this product[18].

The prepared methacrylates could be polymerized by radical initiation. Using a constant proportion of ethylene glycol dimethacrylate as a cross-linking agent, copolymers with good mechanical strength were obtained. Further, 2-hydroxyethyl methacrylate was applied as an inert termonomer in order to obtain copolymers with a comparable swellability and a comparable content of reactive groups. The swellability and the content of reactive groups of a carrier influence the immobilization reaction and also the properties of the immobilized enzymes considerably. The structure of the prepared copolymers is shown in Fig. 3.

The compositions of the reduced copolymers, their contents of amino groups and their swellabilities are indicated in Table III. Table III shows that the contents of amino groups in all instances were nearly identical and that their swellabilities differed only very slightly. This was of great importance for the intended investigations.

Further characterization of the copolymers containing amino groups was car-



n = 0,1,2,3,4,approx. 8.5

Fig. 3. Structure of the prepared copolymers containing spacer arms.

TABLE III

CHARACTERIZATION OF POLYMERS CONTAINING SPACER ARMS ON THE BASIS OF METHACRYLATES

*Molar ratio of components**			*Number (n) of oxyethylene units, $-(CH_2-CH_2-O)_n-$*	*Content of amino groups (mmole/g carrier)*		*Swellability§ (ml/g carrier)*
Reactive	*Inert*	*Cross-linked*		*Calculated***	*Found****	
0.20	0.60	0.20	0	1.31	1.36	1.23
0.22	0.58	0.20	1	1.34	1.01	1.20
0.20	0.60	0.20	2	1.17	0.98	1.11
0.25	0.55	0.20	3	1.33	0.94	1.21
0.27	0.53	0.20	4	1.32	0.95	1.10
0.45	0.35	0.20	*ca.* 8.5	1.35	1.15	1.20

* Calculated under the assumption of complete polymerization.
** Under the assumption of complete reduction.
*** Determined with perchloric acid under anhydrous conditions in acetic acid.
§ Determined at pH 8.5 in Sorensen buffer (see Experimental).

ried out using the method illustrated in Fig. 4. This procedure, developed by Esko and co-workers[7,8], is used for the determination of free primary amino groups in solid-phase peptide synthesis.

After the formation of the Schiff base with a large excess of 2-hydroxy-1-naphthaldehyde the unbound reagent was washed out completely. In order to compare the accessibility of the aldimine groups of different polymers to chemical reactions the amount of 2-hydroxy-1-naphthaldehyde which was released during the first 30 min when reacting the polymeric Schiff base with a large excess of benzylamine was determined. The results of this investigation are shown in Fig. 5.

The curve in Fig. 5 shows that the accessibility of the aldimine groups in this reaction increases with increasing spacer length. This is in agreement with the assumption that even when reactants of small molecular dimensions are applied, the reactivity of a carrier is strongly influenced by the length of the spacer arms.

The next step was to transform the copolymers containing amino groups into

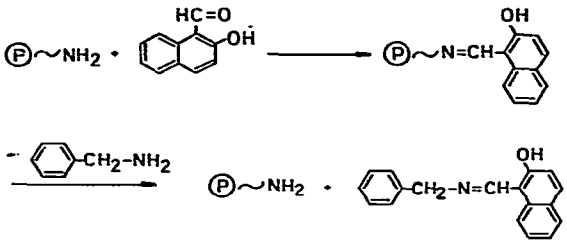


Fig. 4. Scheme for the determination of amino groups in polymers by means of 2-hydroxy-1-naphthaldehyde[7].

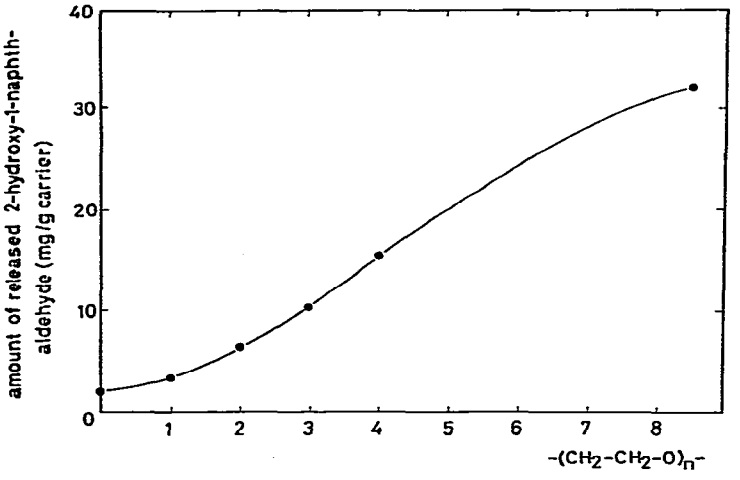


Fig. 5. Dependence of the amount of polymer-bound 2-hydroxy-1-naphthaldehyde released during the first 30 min of reaction with benzylamine on the spacer length.

reactive carriers with diazonium groups. The diazotized copolymers were then coupled with several enzymes under comparable conditions. The dependence of the amounts of bound ribonuclease, trypsin, α-chymotrypsin and urease on the spacer length of the reactive carriers is shown in Fig. 6.

Table IV gives the molecular masses and the dimensions of the enzymes.

With urease, a high-molecular-mass enzyme, no significant influence of the spacer length on the bound amounts of the enzyme was observed. It seems that because of the dimensions of the very large molecule of urease the relatively short spacer arms have no influence on the amounts immobilized. In contrast, all other enzymes used showed optimal binding when the carriers contained three oxyethylene units in the spacer arms. From Table IV it can be seen that these enzymes have a lower molecular mass and therefore smaller dimensions than urease. With trypsin another large increase in binding was observed when a carrier with *ca.* 8.5 oxyethylene units was applied. The reason for this might be the non-uniform length of the spacers introduced (see explanation above).

The enzymatic activities of the immobilized enzymes were determined. Unfortunately, no activity could be detected for the urease conjugates. Maximal activity was found for the trypsin and α-chymotrypsin conjugates when the spacers of the

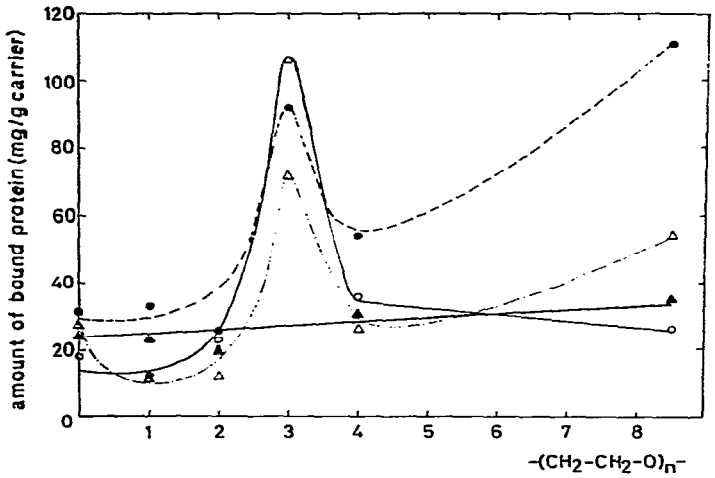


Fig. 6. Dependence of the immobilization of ribonuclease (○—○), trypsin (●--- ●), α-chymotrypsin (△-·--△) and urease (▲—▲) on the spacer length of diazotized copolymers. For coupling conditions, see Experimental.

TABLE IV

MOLECULAR MASSES AND DIMENSIONS OF THE ENZYMES USED

Enzyme	*Molecular mass*	*Dimensions (Å)*	*Reference*
Ribonuclease	13680	38 × 28 × 22	19
α-Chymotrypsin	25000	51 × 40 × 40	20
Trypsin	23600	54.8 × 58.7 × 67.6	21
Urease	483000	—	

TABLE V

ACTIVITIES OF IMMOBILIZED RIBONUCLEASE, TRYPSIN AND α-CHYMOTRYPSIN BY MEANS OF DIAZOTIZED CARRIERS BASED ON METHACRYLIC POLYMERS WITH DIFFERENT SPACER LENGTHS

Number of oxyethylene units in the applied carrier	*Activity of immobilized enzyme [U/mg of bound enzyme (% retained)]*		
	*Ribonuclease**	*Trypsin***	*α-Chymotrypsin****
0	0.47 (3.9)	0.44 (2.3)	2.21 (0.7)
1	0.19 (1.6)	0.32 (1.7)	5.93 (2.0)
2	0.23 (1.9)	0.68 (3.6)	5.8 (1.9)
3	0.22 (1.8)	1.5 (7.9)	8.0 (2.7)
4	0.20 (1.7)	1.08 (5.7)	10.9 (3.6)
ca. 8.5	0.29 (2.4)	0.21 (1.1)	2.6 (0.9)

* Determined at pH 8.2.
** Determined at pH 8.5.
*** Determined at pH 8.2.

carriers contained three or four oxyethylene units (see Table V). With ribonuclease no significant increase in the activity with increasing spacer length was observed. This might indicate that if an enzyme with a low molecular mass is bound in the described manner the amount of binding but not the retained activity of the immobilized enzyme is effected by the spacer length of the carrier.

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