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REACTIVE CARRIERS OF IMMOBILIZED COMPOUNDS

J. ČOUPEK [a], J. LABSKÝ [a], J. KÁLAL [a], J. TURKOVÁ [b] and O. VALENTOVÁ [b]

[a] *Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences, 162 06 Prague 6, and* [b] *Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague 6 (Czechoslovakia)*

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Summary

Spherical macroporous reactive carriers capable of forming covalent bonds with amino acids and proteins were prepared by the suspension copolymerization of 2-hydroxyethyl methacrylate, ethylene dimethacrylate and *p*-nitrophenyl esters of methacrylic acid and methacryloyl derivatives of glycine, β-alanine and ε-aminocaproic acid. The effect of the spacer length, pH and the type of the buffer used, concentration of reactive groups in the copolymer, concentration of the ligand and the participation of the hydrolytic and aminolytic reaction of *p*-nitrophenyl functional groups in the attachment of glycine, D,L-phenylalanine and serumalbumin was studied. Macroporous copolymers containing reactive functional groups can be used as active enzyme carriers, if their activity is not blocked by the presence of *p*-nitrophenol split off in the attachment reaction.

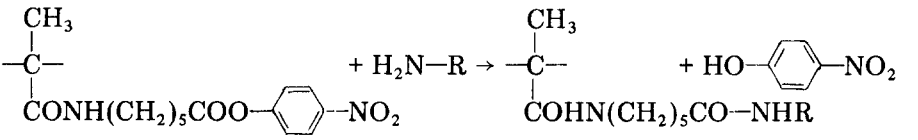
Introduction

The very good mechanical properties and resistence against hydrolysis of spherical particles of macroporous hydroxyalkyl methacrylate copolymers [1] led to their uses as carriers in affinity chromatography [2] and enzymatic catalysis [3]. The procedures employed for the activation of these carriers had a common denominator, namely, a chemical reaction modifying the already completed internal surface of the neutral hydrophilic material having a macroporous structure. This gave rise to reactive functional groups able to form covalent bonds with molecules of soluble compounds containing amine, carboxylic or sulphydryl groups.

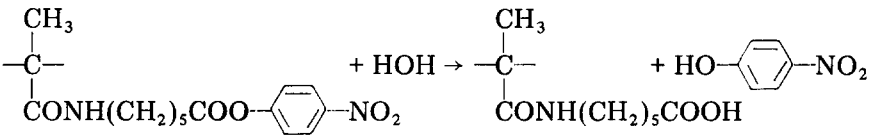
However, the procedures used so far, e.g. the reaction with CNBr, are for many reasons unsuitable for activation of major amounts of carriers for preparative or technological purposes. We therefore investigated the possibilities of

preparation of reactive carriers by direct ternary copolymerization with monomers containing reactive functional groups or their precursors, which either directly or after an uncomplicated polymer-analogous transformation make possible the attachment of peptides or proteins [4].

The use of aminolysis of reactive esters, in particular in peptide chemistry, enjoys a long tradition [5]. For the attachment of proteins or ligands containing primary aliphatic or aromatic amino groups, long-chain hydroxy succinimidic derivatives of agarose described by Cuatrecasas and Parikh proved to be useful [6]. The objective of this work was preparation of macroporous (for the description of physical properties see ref. 1) spherical particles of a polymeric carrier by the direct suspension copolymerization of a hydrophilic monomer containing neutral hydroxyl groups with a cross-linking agent and a third monomer represented by a methacrylic derivative containing the reactive *p*-nitrophenyl ester group situated at various distances of the vinylic double bond. An attachment of $-NH_2$ groups containing compounds by the aminolytic reaction may be schematically represented by the following example:



In aqueous media there is a competitive reaction of the *p*-nitrophenyl ester hydrolysis (hydrolytic reaction) depending on the pH value of the solution:



For the attachment of proteins it was necessary to determine basic reaction conditions, to verify the participation of the hydrolysis and aminolysis of the active ester bonded on the surface of the macroporous carrier and to determine the capacity of the carriers under investigation for the attachment of model compounds, that is, amino acid, protein and enzyme, which should not be deactivated by the *p*-nitrophenol released in the reaction.

Material and Methods

D,L-Phenylalanine and glycine were obtained from the firm Lachema Brno; papain preparation used for stabilization of beer was a product of the firm Enzymase Belgium. Lyophilized human serum albumin was produced by the firm Imuna Šarišké Michalany; 2-mercaptoethanol was a product of the firm Koch-Light Laboratories, Colnbrook, England.

Reactive monomers were prepared by methacrylation of glycine [7], β-alanine and ε-aminocaproic acid with methacrylic acid chloride giving rise to crystalline products. Methacrylic acid and methacryloylated amino acids were esterified in the further reaction step with *p*-nitrophenol in the presence of an equimolar of dicylohexylcarbodiimide. All monomers obtained were crystalline (Table I).

TABLE I

SURVEY OF MONOMERS CONTAINING CARBOXYLIC AND *p*-NITROPHENYL FUNCTIONAL GROUPS

Acid	m.p. (°C)	Ref.	*p*-Nitrophenyl ester m.p. (°C)	Ref.
$CH_2=C(CH_3)-COOH$	16.0	—	94—95	8
$CH_2=C(CH_3)-CONH-CH_2-COOH$	101—2	9	101	7
$CH_2=C(CH_3)-CONH-(CH_2)_2-COOH$	75—75.5	This work	63—65	This work
$CH_2=C(CH_3)-CONH-(CH_2)_5-COOH$	51	10	75—77	This work

To a mixture of 1 mol of amino acid with 1 mol NaOH in 250 ml of water a mixture of 1 mol of methacryloylchloride and 1 mol NaOH in 200 ml of water was added drop by drop under cooling. After 2 h stirring at room temperature the mixture was acidified with 100 ml of conc. HCl, the oily organic layer was extracted with ether and after evaporation recrystallized from ethanol. m.p. of amides are given in Table I.

0.2 mol of methacryloylamide was dissolved in 300 ml of $CHCl_3$, cooled down to −20°C and to this solution was added 0.2 mol of dicyclohexylcarbodiimide. After dissolving 0.2 mol of *p*-nitrophenol was added in small portions within 5 min. The mixture was stirred at −10°C for 3 h and after a further 3 h stirring at room temperature dicyclohexylurea was removed by filtration, chloroform was evaporated and the product formed was recrystallized from boiling ethanol.

The ternary copolymerization of 2-hydroxyethylmethacrylate, ethylenedimethacrylate and of the reactive monomer was carried out in a mildly acidic medium (pH 6.0) in an aqueous dispersion in the presence of cyclohexanol and dodecyl alcohol (7 : 3) the concentration of which controls the pore-size distribution of beads formed in the reaction. (Temperature of polymerization 80°C, catalyst azobisisobutyronitrile 0.1% w/w). The product thus obtained was thoroughly washed, dried and fractionated on sieves. The 100—200 μm fraction of perfect spherical particles was used in further work. The gel bed volume of 1 g of active carrier is about 4 ml.

Determination of the content of p-nitrophenyl groups in the carrier

The content of reactive groups was determined by means of a corrected spectrophotometric determination of released *p*-nitrophenol after aminolysis with a solution of ammonium hydroxide. 100 mg of dry gel were suspended each time in 10 ml of aqueous solution of ammonium hydroxide. The suspension was left to stand at room temperature for 4 h with occasional stirring. The volume was then made up with water to 100 ml and after sedimentation of the carrier particles the absorbance of supernatant was measured at 400 nm. The amount of

released *p*-nitrophenol was read off from the calibration curve determined by using spectrophotometric measurements of *p*-nitrophenol solutions in the presence of a similar binary copolymer of 2-hydroxyethyl methacrylate and ethylenedimethacrylate, which enabled errors due to the adsorption of *p*-nitrophenol to the macroporous gel matrix to be eliminated. The values thus obtained were expressed as the capacity of the gel in μmol/g of dry polymer.

Attachment of glycine and D,L-alanine on carriers containing p-nitrophenyl ester functional groups

250 mg of dry carrier was suspended each time in 4 ml of 0.5 M $NaHCO_3$, pH 8.8, containing dissolved glycine or D,L-phenylalanine in an amount corresponding roughly to a 20-fold capacity of the carrier (determined by aminolysis with ammonium hydroxide). The suspension was stirred at room temperature for 24 h. After that the gels were washed on a sintered glass filter, first with water, then decanted for 3 h with a solution of 6 M guanidine hydrochloride, pH 6.0, again washed with water and finally with acetone. On drying, the content of the respective amino acid was determined in the acid hydrolyzate by using the method of Spackman et al. [11] and related to 1 g of dry carrier.

pH and concentration-dependence for the coupling reaction of amino acids or protein to the support

For the determination of the pH-profile of the attachment reaction, 250 mg of carrier, capacity 68.5 μmol/g of releasable *p*-nitrophenol, was suspended in 40 ml resp. 4 ml of Britton-Robinson buffer (Solution A: 4.9 g 80% H_3PO_4, 2.4 g CH_3COOH and 2.747 g H_3BO_3 in 1 l. Solution B: 0.2 M NaOH. The buffer was prepared by addition of calculated volume of solution B to 100 ml solution A). The buffer pH ranged from 7 to 12 and the buffer containted each time 25 mg of dissolved D,L-phenylalanine at 25°C, resp. 195 mg of dissolved human serum albumin. The time course of the release of *p*-nitrophenol in the individual samples was followed by measuring absorbance at 400 nm. After 24 h at 25°C the gels with serum albumin were decanted in dilute ammonia (1 : 1), washed with water, 6 M guanidine, pH 6.0, water and acetone; on drying and hydrolysis the content of bounded protein was determined from the content of amino acids [11]. For the concentration dependence determination, the gel (250 mg) was suspended in 4 ml of 0.5 M $NaHCO_3$, pH 8.8, containing dissolved D,L-phenylalanine of increasing concentrations (0.43–87.5 μmol/ml). After 24 h at 25°C the content of attached amino acid was determined by using the method described above.

The effect of the medium on the rate of release of p-nitrophenol and on the amount of attached D,L-phenylalanine

The effect of the character of the buffer at constant pH (8.5) was examined for 0.5 M $NaHCO_3$, Britton-Robinson buffer, borate buffer and Britton-Robinson buffer containing 0.005 M $NaHCO_3$. 250 mg of gel, capacity 68.5 μmol/g, was suspended each time in 40 ml of the respective buffer containing 25 mg of D,L-phenylalanine, and change in absorbance at 400 nm with respect to time was recorded. The samples were washed as described above and the content of attached amino acid was determined after drying and hydrolysis.

Attachment of papain preparation on p-nitrophenyl derivatives

50 mg of papain preparation was dissolved in 5 ml of the Britton-Robinson buffer, pH 5.4, containing 5 l of 2-mercaptoethanol, and 1 g of active gel, capacity 68.5 μmol/g, was added to the solution. The suspension was stirred with a magnetic stirrer at 4°C for 2 h. The pH of the mixture was then adjusted to 8.8 with 0.2 M NaOH, and stirring was continued for another 20 h under the same conditions. On completion of the reaction, the gel was decanted three times with 50 ml of 0.5 M $NaHCO_3$ (pH 8) each time, after that with an 8.5 mM solution of cysteine and three times with 500 ml cooled acetone (−10°C). The gel was further washed with 0.01 M sodium acetate, 5 mM solutin of cysteine, pH 4.1, and stored in wet-cake form.

Determination of the proteolytic activity of free and bonded papain preparation

The determination was carried out using a modification [1] of the method of Ansen [12]. 200 μl of a solution containing 0.1 mg of enzyme resp. 5—50 mg of filtered gel with bound papain preparation was added to 12 ml of hemoglobin solution in 5 mM cysteine, pH 7. After incubation at 37°C for 10 min the cleavage was stopped by adding 5 ml of 5% trichloroacetic acid. The proteolytic activity is expressed in A_{280}/min · mg of enzyme or in A_{280}/min · g of dry gel. The amount of attached protein was again determined after acid hydrolysis of the conjugate [11].

Results and Discussion

The number of hydrolyzable functional groups was determined in all *p*-nitrophenyl ester derivatives, and their capacity for the attachment of D,L-phenylalanine and glycine in an alkaline medium was also determined. Table II shows that with increasing distance of the functional *p*-nitrophenyl ester group from the backbone chain its coupling yields also markedly increases; the reactivity of polymethacrylic acid ester is extremely low. This fact is related to the generally valid stability of acid esters with a tertiary α-carbon atom.

TABLE II

DEPENDENCE OF THE CONTENTS OF GLYCINE AND D,L-PHENYLALANINE ATTACHED TO *p*-NITROPHENYL ESTER CARRIERS ON THE SPACER LENGTH (INITIAL CONCENTRATION OF *p*-NITROPHENYL ESTER MONOMERS IN THE POLYMERIZATION MIXTURE 65.3 μmol/g)

Derivative	NH_3 capacity (μmol/g)	Attached glycine (μmol/g)	D,L-phenylalanine (μmol/g)
—COO—⟨O⟩—NO_2	2.52	Traces	Traces
—CONH—CH_2—COO—⟨O⟩—NO_2	32.1	5.7	3.5
—CONH$(CH_2)_2$COO—⟨O⟩—NO_2	61.5	15.6	18.2
—CONH$(CH_2)_5$COO—⟨O⟩—NO_2	68.5	34.8	44.9

TABLE III

ATTACHMENT CAPACITIES OF COPOLYMERS OF *p*-NITROPHENYL ESTERS OF *N*-METHACRYLOYL-ε-AMINOCAPROIC ACID AS A FUNCTION OF MONOMER CONCENTRATION

Monomer concentration (g/100 g carrier)	Capacity (NH_3) (μmol/g)	Attached glycine (μmol/g)	Attached D,L-phenylalanine (μmol/g)
0.5	5.8	0.9	0.7
1	18.2	3.4	3.6
2	68.5	34.8	44.9
4	80.0	52.7	83.7
8	82.2	—	—

The relationship between the attachment capacity and the initial concentration of the reactive monomer in the polymerization is illustrated in Table III. An increase in the monomer content does not cause a linear increase in the capacity of the carrier, which is obviously related with the copolymerization parameters of the reacting monomers.

Hydrolysis occurs along with aminolysis during the process of attachment of molecules on *p*-nitrophenyl ester carriers in an aqueous medium. The effect of various buffers having the same pH on the rate of release of *p*-nitrophenyl is shown in Fig. 1. The amount of attached amino acid at pH 8.5 in borate buffer was 0.9 μmol/g, in Britton-Robinson buffer 1.56 μmol/g, in Britton-Robinson buffer + 0.005 M $NaHCO_3$ 1.77 μmol/g and in 0.5 M $NaHCO_3$ 4.3 μmol/g. The amount of *p*-nitrophenol that could be hydrolytically released is not affected to any marked degree by the different quality of buffers. The yield of attached amino acid is highest in the presence of 0.5 M $NaHCO_3$; an addition of sodium bicarbonate somewhat favours aminolysis compared to hydrolysis, even if the Britton-Robinson buffer is used. The rate of release of *p*-nitrophenol as a function of pH of the reaction mixture is shown in Fig. 2. The presence of amino

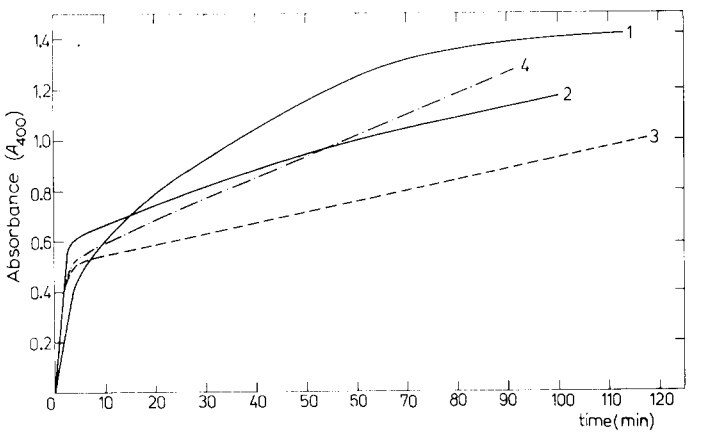


Fig. 1. Effect of the type of buffer having the same pH (8.5) on the rate of release of *p*-nitrophenol from covalently attached reactive ester: 1, 0.5 M $NaHCO_3$; 2, Britton-Robinson buffer; 3, acetate buffer; 4, Britton-Robinson buffer + 0.005 M $NaHCO_3$.

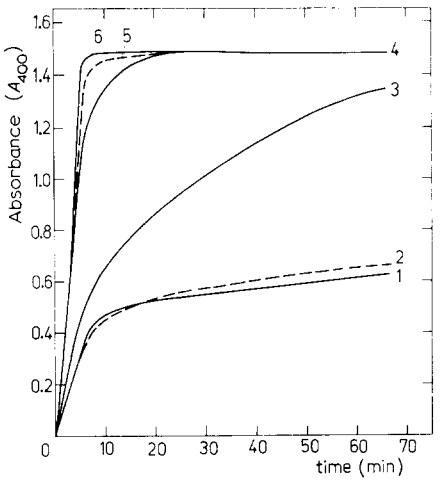


Fig. 2. Effect of pH of the Britton-Robinson buffers on the rate of release of *p*-nitrophenol from covalently attached reactive ester: 1, pH = 7; 2, pH = 8; 3, pH = 9; 4, pH = 10; 5, pH = 11; 6 pH = 12.

acid in the hydrolysis does not raise the rate of release of *p*-nitrophenol to a marked extent at any pH value; consequently, no preference of aminolysis compared to hydrolysis could be detected in this arrangement. The dependence of the amount of amino acid actually bonded and of the model protein (human serum albumin M_r 60 000) on pH of the medium has a maximum at pH 9 (Table IV). The pronounced optimum of the amount of bonded D,L-phenylalanine is connected with p*K* of the α-amino group (7.6—8.4). Since the non-protonized form of the amino group participates in the reaction with the active ester, only a very low concentration of these groups is available at pH 7—8. On the other hand, at values above pH 10 the participation of the competitive hydrolytic reaction is very considerably due to the increased concentration of hydroxyl ions, and is reflected in an essential decrease in the attached amount of amino acid. The same decrease for the model protein (serum albumin) is not so pronounced owing to higher p*K* values of the ε-amino group of lysine (9.4—10.6), which one may assume to participate in the attachment [6].

TABLE IV

AMOUNT OF D,L-PHENYLALANINE (A) AND HUMAN SERUM ALBUMIN (B) ATTACHED TO THE COPOLYMER OF *p*-NITROPHENYL ESTER OF *N*-METHACRYLOYL-ε-AMINOCAPROIC ACID, NH_3 CAPACITY 68.5 μmol/g, AS A FUNCTION OF pH OF THE BRITTON-ROBINSON BUFFERS

pH	A (μmol/g)	B (μmol/g)
7	Traces	Traces
8	1.12	0.138
8.5	1.56	—
9	16.1	0.156
10	3.83	0.151
11	1.68	0.112
12	Traces	0.125

TABLE V

CONCENTRATION DEPENDENCE OF THE AMOUNT OF D,L-PHENYLALANINE (A) ATTACHED TO THE COPOLYMER OF *p*-NITROPHENYL ESTER OF *N*-METHACRYLOYL-ε-AMINOCAPROIC ACID, NH_3 CAPACITY 68.5 μmol/g

Concentration of D,L-phenylalanine (μmol/g)	A (μmol/g)	% of total capacity
87.5	44.9	65.6
43.0	30.2	44.1
8.75	8.5	12.4
4.3	4.8	7.0
0.43	0.76	1.1

It follows from the above that the amount of attached compound in the binding reaction of molecules in an aqueous medium will be affected in a conclusive way by the ratio of concentrations of primary nonprotonized amino groups and hydroxyl ions. Table V shows the dependence of the amount of covalently bonded D,L-phenylalanine on its concentration in the reaction mixture, which confirms the requirement of a high concentration of the compound used for attachment if high turnovers of the attachment reaction are needed.

Papain preparation was chosen in order to verify the possibility of using *p*-nitrophenyl copolymers with methacrylates for the attachment of enzymes; it was proven for enzymase that if did not lose its activity in contact with *p*-nitrophenol formed in the reaction. Several samples were prepared in which amount of attached enzyme varied from 1.5—30 mg/g. The relative proteolytic activity of these samples depends on the content of attached enzyme and reaches values from 26 to almost 100%. The relationship existing between the physical structure of gels, the content of reactive groups and the final activity of attached enzyme will be the object of a further study.

References

1 Čoupek, J., Křiváková, M. and Pokorný, S. (1973) J. Polymer Sci., Polymer Symposium No. 42, 185—190
2 Turková, J., Hubálková, O., Křiváková, M. and Čoupek, J. (1973) Biochim. Biophys. Acta 322, 1—9
3 Valentová, O., Turková, J., Lapka, R., Zima, J. and Čoupek, J. (1975) Biochim. Biophys. Acta 403, 192—196
4 Jelínková, M., Pokorný, S. and Čoupek, J. (1976) Angew. Makromol. Chem. 52, 21—30
5 Bodanzsky, M., (1960) Ann. N.Y. Acad. Sci. 88, 655—662
6 Cuatrecasas, P. and Parikh, J. (1972) Biochemistry 11, 2291—2297
7 Drobník, J., Kopeček, J., Labský, J., Rejmanová, P., Exner, J., Saudek, V. and Kálal, J. (July 1975) Reprint P-15, 4th IUPAC Conference on Modified Polymers, Their Preparation and Properties, Bratislava
8 Gaetjus, L. and Morawetz, H. (1961) J. Am. Chem. Soc. 83, 1738—1742
9 Hübner, K., Kollinsky, F., Markert, G. and Penewiss, H. (1970) Angew. Makromol. Chem. 11, 109—124
10 Glabisch, P., Becher, W. and Dennstedt, J. (1961) Chem. Abstr. 55, 20517a
11 Spackman, D., Stein, W.H. and Moore, S. (1958) Anal. Chem. 30, 1190—1206
12 Anson, M.L. (1939) J. Gen. Physiol. 22, 79—89