IMMOBILIZATION OF ENZYMES INTRODUCING SPACERS. A SYNTHESIS OF CARRIERS WITH SPACERS OF VARIOUS LENGTH

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A synthesis of spacers of various length and type, utilizing α,ω-dicarboxylic acids, α,ω-diaminoalkanes and succinic anhydride, condensation with carbodiimide, or introduction of —NCS functional groups is described. Carriers with a spacer were prepared by a method of binding the already synthesized spacer to the carrier (glass), and alternatively, by a stepwise synthesis of the spacer on the carrier surface. Carriers containing functional groups (37·2—39·3 μmol/g —NCS and 25—41·5 μmol/g —COOH) prepared in this way had total length of the spacer 0·62—3·92 nm. Whereas the length of the spacer is of no substantial importance for the reaction with low-molecular substances (L-valine, L-cysteine and 2-mercaptoethanol), the optimum length of the spacer for high-molecular compounds (albumin) is about 1·75—2·05 nm. The hydroxyl group adjacent to the functional group of the spacer (1,3-diaminopropan-2-ol) is also of noticeable influence.

Papers concerning the role of the so called spacer molecules involved in binding of biospecifically active groups or ligands at the surface of matrices are being more frequently reported in the last time[1-3]. Introduction of spacers enables to realize the basic structural principles of natural biologic systems[4], since spacers lenghten the distance between the ligand and the matrix surface; consequently, the flexibility of the ligands can be extended and/or the disturbing influence of the matrix restricted. Thus, the enhancement of the binding capacity of the affinity ligands due to the use of matrices with spacer was pointed out[1]. The introduction of spacers especially hydrophobic cannot exclude, on the other hand, the non-specific interactions between molecules of the spacer including the ligand and molecules of the linked biologically active substance, which can be dominant under specific conditions[5-7]. The criticism of the optimum spacer length (2 nm, 20 Å) reported in the literature[8] is needed, as far as the "brush effect" of the spacer molecules and/or ligands at the carrier surface can occur due to these mutual hydrophobic interactions. Hydrophilic spacers, however, reveal a really less pronounced dependence of the binding capacity on their length[6]. The effect of spacers on the catalytic activity of the immobilized ligands was investigated only sparingly, when compared with a wealth of papers dealing with spacers in affinity chromatography. The insolubilization

of chymotrypsin on a cellulose carrier by means of spacers of various length[9] and properties of trypsin immobilized through oligopeptides of various length[10] were published. It could be therefore anticipated that the increase of the spacer length is associated with the decrease of steric impediments and interaction of the matrix[11].

This paper is intended to investigate the influence of the spacer on the course of enzymic reactions. Aiming to generalize some conclusions, the effect of the spacer was examined not only with respect of its length, but also from the view points of chemical structure and the way of its synthesis. The presented paper deals with the preparation of spacers on the matrix of inorganic origin (macroporous glass) and with characterization of their reactions with low- and high-molecular substances.

EXPERIMENTAL

Materials

Macroporous glass (CPG-10-2000) of a mean pore size 210·8 nm, and 0·2—0·4 mm grain size (Electronucleonic, USA). γ-Aminopropyltriethoxysilane (γ-APT), thiophosgene, 1,6-diaminohexane and 1,12-diaminododecane were Ferak (West Berlin) products; 1,2-diaminoethane (Jenapharm, GDR); 1,3-diaminooctane and succinic anhydride (Merck, Darmstadt), ω-aminocaproic acid and *p*-phenylenediamine (Laborchemie Apolda, GDR); 2,4,6-trinitrobenzenesulfonic acid, N-cyclohexyl-N′-β-methylmorpholinoethylcarbodiimide-*p*-toluenesulfonate and 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB, Ellman's reagent, Serva Heidelberg, FRG) and 2-mercaptoethanol (Sigma, USA). (U-^{14}C)L-valine (0·2 MBq/mol) and (U-^{14}C)-L-cysteine (0·2 MBq/mol) were the products of the Radiochemical Centra, Amersham, England. ^{131}I human serum albumin (the starting specific radioactivity 0·5 MBq/mg) was donation of the Institute of Nuclear Research, Radioisotope Production and Distribution Centre, Otwock, Poland.

Methods

γ-APT-glass. The macroporous glass (10 g) was silanized according to[12] with γ-APT (10% solution in toluene (100 ml)). The unreacted residues of the silanes to be adsorbed were removed by a two-fold cooking with excess toluene (200 ml). The reaction product was suction-filtered using a sintered-glass filter, washed with toluene, acetone and air-dried

Polyisothiocyanate of glass. Similarly as with γ-ATP, also other H_2N-derivatives of glass were reacted with thiophosgene in the presence of $NaHCO_3$ according to[13].

H_2N-Derivatives of glass. Polyisothiocyanate of glass (1·5 g) was treated with the respective α,ω-diaminoalkane (2·5%) in $CHCl_3$ (70 ml) at 80°C for 5 h. The product washed with hot $CHCl_3$, H_2O, and acetone, and left to air-dry was stored under diminished pressure.

Succinyl derivative of glass. The γ-APT-glass (or H_2N-derivatives of glass, 1 g) was reacted at 80°C with succinic anhydride (0·5 g) dissolved in $CHCl_3$ (50 ml) containing traces of water for 2 h. The product was washed with water, acetone and air-dried. Storage under diminished pressure.

Spacer of α,ω-dicarboxylic acid. ω-Aminocarboxylic acid, α,ω-diaminoalkane, or *p*-phenylenediamine (0·1 mol) (or alternatively 0·2 mol of diamino derivatives) were allowed to react with succinic anhydride (0·1 mol) added gradually to the ice-cooled reaction mixture at pH 6·5 under stirring. The pH value was kept constant by addition of aqueous (2%) NaOH.

Derivative of glass with a spacer from carboxylic acid. γ-APT-glass (1 g), α,ω-dicarboxylic acid (50 mg) and N,N'-dicyclohexylcarbodiimide (200 mg) reacted at room temperature and with stirring in CH_2Cl_2 (10 ml) for 40 h. The reaction product was filtered through a sintered glass filter, repeatidly washed with hot ethanol, acetone and air-dried. Storage in a desiccator.

Determination of —NH_2 *groups.* Procedure according to[14]. CPG-10-2000 glass after reaction with γ-APT (20 mg) was added into a pH 8 borate buffer (0·2M, 1 ml) containing 2,4,6-trinitrobenzenesulfonic acid (2 mmol) and reacted at 40°C for 1 h. The undissolved portion was centrifuged and the supernatant (100 μl) added to valine (20 mM) in 1% trichloroacetic acid (100 μl). After a 1 h reaction at 40°C, 0·5M-HCl (5 ml) was added. The —NH_2 content was calculated from the calibration graph for valine at 338 nm.

Determination of —COOH *groups.* The analyzed sample was titrated with 0·02, or 0·1M-NaOH after addition of $BaCl_2$ (10%) according to[15].

Determination of —NCS *groups*[16]. Isothiocyanate of glass (50 mg) was added under stirring to a 0·2M phosphate buffer (pH 8·1 ml), containing 2-mercaptoethanol (10 mM) at 25°C. The aliquot samples of the supernatant (50 μl) withdrawn after 10, 20, 30, and 60 min were transferred into a 0·1M Tis buffer (pH 8·5) with 20 mM EDTA. The free 2-mercaptoethanol was determined in the reaction with DTNB, and after 5 min the samples were diluted with a 4-fold volume of methanol. The absorbance was measured at 412 nm and the decrease of —SH groups was estimated from the calibration curve and the molar absorption coefficient $\varepsilon_{412} = 1{\cdot}25 \cdot 10^4\ mol^{-1}\ cm^{-1}$.

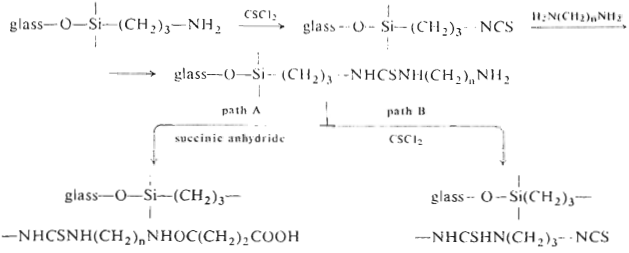
Reaction of glass isothiocyanate: Glass isothiocyanate (10 mg) was suspended in a 0·1M borate buffer (pH 8·2, 1 ml) and stirred with (U-^{14}C)L-valine, or (U-^{14}C)L-cysteine, or ^{131}I albumin (50, 100, 1 000 μg) at 35°C for 1, 5, 10, and 20 h. The derivative of glass was then washed with the above-mentioned borate buffer (5 ml), the respective L-valine, L-cysteine (5 ml, 8 mg/l), or albumin (5 ml), 1·5 mg/l), 20 mM NaCl, borate buffer, and finally it was dried in a vacuum drier. The incorporated radioactivity was measured with a Packard counter using a toluene scintillation liquid SLX-31.

Reaction of glass carboxylates. Carboxy derivatives of glass (10 mg) were treated with carbodiimide as already described, and allowed to react with (U-^{14}C)L-valine, or (U-^{14}C)L-cysteine, or ^{131}I albumin in amounts specific with glass isothiocyanates. The reaction proceeded at 0—2°C for 2 h. The further work-up as with glass isothiocyanate.

Calculation of the spacer lengths. The maximum distance between the Si atom of γ-APT and the reaction center of the linked spacer already made, *i.e.* up to carbon atoms of —NCS or —COOH groups was calculated from geometric parameters (bond lengths, valence angles) in the respective spacers[17].

RESULTS AND DISCUSSION

Molecules of the spacer can be linked in principle to the carrier *1*) by a distinct synthesis of the spacer molecule and linkage to the carrier in the next step, *2*) by a gradual construction of the spacer at the carrier by phase boundary synthesis. The first possibility was utilized with α,ω-diamines, or ω-aminocarboxylic acids, with succinic anhydride amining, at least with the lower C_2—C_4 diamines to approach requirements to be met with a spacer[4–7]. The second possibility was reached by two ways:



A) the amino derivative, obtained from the reaction of glass isothiocyanate with α,ω-diamines, was reacted with succinic anhydride, *B*) the amino derivative of glass was treated with thiophosgene; this reaction was up to three-times repeated when synthesizing the spacer from 1,6-diaminohexane.

Preparation of spacers of α,ω-dicarboxylic acid type: α,ω-Dicarboxylic acids of various length were succeeded to synthesize from succinic anhydride and α,ω-diamines:

$$(CH_2CO)_2O + H_2N(CH_2)_nNH_2 + O(OCCH_2)_2 \rightarrow$$
$$\rightarrow HOO(CH_2)_2CONH(CH_2)_nNHCO(CH_2)_2COOH$$
$$HOOC(CH_2)_nNH_2 + O(OCCH_2)_2 \rightarrow HOOC(CH_2)_nNHCO(CH_2)_2COOH$$

Products of the synthesis were characterized by their melting points, —COOH, and nitrogen contents (Table I).

TABLE I
Characterization of spacers of a dicarboxylic acid of HOOC—R—COOH type

—R—	Symbol	M.p., °C	Content[a]	
			COOH	N
$-(CH_2)_2CONH(CH_2)_2-$	R_1	122	106	94·5
$-(CH_2)_2CONH(CH_2)_5-$	R_2	103	99	98·5
$-(CH_2)_2CONH(CH_2)_2NHOC(CH_2)_2-$	R_3	197	99·5	99·1
$-(CH_2)_2CONH(CH_2)_6NHOC(CH_2)_2-$	R_4	176	99·8	101·0
$-(CH_2)_2CONH-C_6H_4-NHOC(CH_2)_2-$	R_5	250	94	95·2

[a] Per cent of the calculated value.

Derivatives of glass with a spacer of carboxylic acid: The α,ω-dicarboxylic acid type of spacer was linked to γ-APT-glass through N,N'-dicyclohexylcarbodiimide in CH_2Cl_2.

$$\mathrm{HOOC{-}R{-}COOH + H_2N(CH_2)_3Si(OH)_2{-}O{-}glass} \xrightarrow{\text{carbodiimide}}$$
$$\rightarrow \mathrm{HOOC{-}R{-}CONH(CH_2)_3{-}Si(OH)_2{-}O{-}glass}$$

This reaction does not proceed quantitatively as evident from Table II. Considering the —COOH groups content, the difference in the original $—NH_2$ groups content in the γ-APT-glass and the residual content of $—NH_2$ groups after the reaction, it is unlikely that the dicarboxylic acid would react through both —COOH groups. Noteworthy is the behaviour of dissuccinyl-*p*-phenylenediamine.

Succinyl derivatives of glass: The isothiocyanate of glass prepared from γ-APT-glass and thiophosgene reacted with diamines of a $HN_2(CH_2)_nNH_2$ type (n = 2, 4, 6, 8, 10, 12, and 1,3-diaminipropan-2-ol, as well); the obtained amines underwent a further reaction with succinyl anhydride to furnish the corresponding succinate. Reactions of diamines with —NCS derivatives proceeded almost quantitatively, whereas the following succinylation gave a 80–90% yield (Table III). The quantitative yield of this reaction was corroborated by determination of the $—NH_2$ and —COOH groups content.

$$\mathrm{glass{-}R_0{-}NCS + H_2N(CH_2)_nNH_2 \rightarrow glass{-}R_0{-}NHCSNH(CH_2)_nNH_2} \xrightarrow{\text{succinic anhydride}}$$
$$\rightarrow \mathrm{glass{-}R_0{-}NHCSNH(CH_2)_nNHCO(CH_2)_2COOH}$$

TABLE II
Content of $—NH_2$ and —COOH groups of glass derivatives with a spacer from carboxylic acid

Derivative[a]	NH_2— μmol/g	—COOH μmol/g	Spacer length nm
γ-APT—glass	42·1	—	0·5
$HOOCCH_2CH_2CO{-}NH{-}R_0$—glass	—	41·5	0·92
$HOOC{-}R_1{-}CONH{-}R_0$—glass	5·8	35·3	1·42
$HOOC{-}R_2{-}CONH{-}R_0$—glass	10·1	33·0	1·8
$HOOC{-}R_3{-}CONH{-}R_0$—glass	15·4	32·8	1·92
$HOOC{-}R_4{-}CONH{-}R_0$—glass	12·0	29·5	2·42
$HOOC{-}R_5{-}CONH{-}R_0$—glass	20·2	25·0	2·05

[a] $R_0 = —(CH_2)_3—Si(OH)_2—O—$; $R_1 - R_5$, see Table I.

TABLE III

Characterization of succinyl derivatives of glass

Derivative of glass with spacer[a]	Content[b] $-NH_2$ μmol/g	Content $-COOH$ μmol/g	L-valine μmol/g	L-cysteine μmol/g	Albumin mg/g	Spacer length nm
Glass—R_0—NH—succin	42·1	38·7	8·2	7·9	1·11	0·92
Glass—R_0—R_6—NH—succin	39·6	35·6	8·3	8·0	1·84	1·55
Glass—R_0—R_7—NH—succin	42·0	36·2	7·9	8·1	1·92	1·8
Glass—R_0—R_8—succin	39·2	34·2	8·6	8·5	6·4	2·05
Glass—R_0—R_9—NH—succin	38·4	33·6	8·1	7·9	2·3	2·3
Glass—R_0—R_{10}—NH—succin	39·3	33·2	7·6	7·4	1·24	2·58
Glass—R_0—R_{11}—NH—succin	38·6	33·3	7·2	7·0	0·91	2·8
Glass—R_0—$NHCSHNCH_2CHOHCH_2NH$— succin	39·0	34·1	7·6	7·7	5·2	1·67

[a] $R_0 = -(CH_2)_3-Si(OH)_2-O-$, $R_6-R_{11} = -NHCSHN(CH_2)_n-$ $n = 2, 4, 7, 8, 10, 12$; [b] the starting polyamine before reaction with succinic anyhdride.

Isothiocyanate derivatives of glass: The reaction product from the isothiocyanate of glass and diamines reacted with thiophospene. The binding capacity of reactive isothiocyanates of glass was determined in a reaction with L-valine, L-cysteine, and albumin. A simultaneous reaction with 2-mercaptoethanol served for determination of the —NCS content; as shown (Table IV), thiophosgenation proceeded in a 88 to 94% yield.

As it follows from the content of bound 2-mercaptoethanol and L-cysteine, also the latter is apt for —NCS group analysis, since in the given reaction it reacts as an SH-compound (L-valine with $—NH_2$ groups reacted in a considerable lesser measure). The same activity of L-cysteine and L-valine, but in a lower extent as with —NCS, showed these amino acids when linked to a polycarboxyl derivative, where they evidently reacted through $—NH_2$ (Table III). No one of the homologous series of spacers was favourized in reactions with either —COOH, or —NCS derivatives of glass with low-molecular compounds ($—NH_2$, —SH). In reactions with albumin on the other hand, species from 1,6-diaminohexane, or 1,3-diaminopropan-2-ol were unambiguously favourized. The amount of the linked albumin increases with the increasing length of the spacer up to 1;6-diaminohexane spacer derivative;

TABLE IV
Characterization of glass isothiocyanates

Derivatives of glass[a]	L-valine μmol/g	L-cysteine μmol/g	2-Mercapto-ethanol μmol/g	Albumin[b] mg/g	Spacer length nm
Glass—R_0—NCS	13·3	39·8	38·2	0·6	0·62
Glass—R_0—R_6—NCS	13·1	39·2	39·0	2·7	1·24
Glass—R_0—R_7—NCS	13·2	39·6	38·4	6·3	1·5
Glass—R_0—R_8—NCS	12·8	38·6	37·9	14·7	1·75
Glass—R_0—R_9—NCS	12·4	37·2	37·4	7·1	2·0
Glass—R_0—R_{10}—NCS	13·1	38·9	39·6	5·05	2·25
Glass—R_0—R_{11}—NCS	12·4	37·2	37·2	4·0	2·5
Glass—R—$—NHCSHNCH_2CHOHCH_2NCS$	13·3	39·2	39·3	13·4	1·37
Glass—R_0—R_8—R_8—NCS	13·0	38·5	39·1	4·4	2·82
Glass—R_0—R_8—R_8—R_8—$—NCS$	13·4	39·2	38·7	4·2	3·92

[a] $R_0 = —(CH_2)_3—Si(OH_2)—O—$, $R_6—R_{11} = —NHCSHN(CH_2)_n$ $n = 2, 4, 6, 8, 10, 12$; [b] nonspecific sorptions of albumin to the starting CPG-10-2 000, 1·1 mg/g were substracted.

further extension of the chain resulted in a decrease of the amount (Tables III and IV) equally with isothiocyanate, or carboxylated derivatives of glass. The amount of the bound albumin is, however, noticeably greater with —NCS glass. The spacer from 1,3-diaminopropan-2-ol should be judged separatedly with respect to the presence of an —OH group. The lower binding capacity of —COOH derivatives of glass, when compared with that of —NCS, can be rationalized by steric hindrance of the bulky condensation reagent.

$$\text{glass—R}_0\text{—NCS} + \text{H}_2\text{N(CH}_2)_6\text{NH}_2 \cdots \text{glass—R}_0\text{—NHCSNH(CH}_2)_6\text{NH}_2 \xrightarrow{\text{CSCl}_2}$$

$$\rightarrow \text{glass—R}_0\text{—NHCSNH(CH}_2)_6\text{NCS} \xrightarrow{\text{H}_2\text{N(CH}_2)_6\text{NH}_2} \text{glass—R}_0\text{—(NHCSNH(CH}_2)_6)_2\text{NH}_2 \rightarrow$$

$$\xrightarrow{\text{CSCl}_2} \text{glass—R}_0\text{—(NHCSNH(CH}_2)_6)_2\text{NCS} \xrightarrow{\text{NH}_2\text{(CH}_2)_6\text{NH}_2}$$

$$\rightarrow \text{glass—R}_0\text{—(NHCSNH(CH}_2)_6)_3\text{NH}_2 \xrightarrow{\text{CSCl}_2} \text{glass—R}_0\text{—(NHCSNH(CH}_2)_6)_3\text{NCS}$$

SCHEME 1

A stepwise binding of molecules of 1,6-diaminohexane to —NCS group of the activated carrier of isothiocyanate derivatives of glass resulted in a formation of spacer 3·92 nm in length (Scheme 1). The amount of the bound albumin further decreased by more than 70% due to this intervention (Table IV):

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