ACTA CHEMICA SCANDINAVICA 27 (1973) 2769–2780

# The Use of Isocyanides for the Immobilization of Biological Molecules

PER VRETBLAD and ROLF AXÉN

*Group of Applied Biochemistry (Swedish Board for Technical Development), Institute of Biochemistry, University of Uppsala, Box 531, S-751 21 Uppsala 1, Sweden*

Isocyanides can be used for the preparation of immobilized enzymes and biospecific adsorbents. The technique is based on a four-component condensation involving an isocyanide, a primary amine, a carbonyl compound, and a carboxylate ion. The reaction proceeds in aqueous solution under physiological conditions.

The model substance human serum albumin has been immobilized on various carriers. The influence of reaction pH, temperature, and time on the immobilization has been studied. The immobilizations of several biological substances of various structure, *i.e.* nucleic acids, cofactors, peptides, steroids, and penicillins, are reported. The structures of the linkages between carrier and ligand are considered and also the possibilities to "direct" the reaction.

In recent years a number of methods for the preparation of immobilized biological activity have been developed and successfully applied to the preparation of immobilized enzymes and biospecific adsorbents. Several reviews have been written on the subject.[1–7]

As a part of our programme for development of methods for the covalent fixation of biologically active substances to polymers, we have investigated the four-component condensation of an isocyanide, a primary amine, a carbonyl compound, and an anion.[8] Many biological substances contain amino, carbonyl, or anionic groups or combinations of such groups and can thus be attached to a variety of polymeric carriers containing one or more of the other reaction components. In addition to ligand and carrier, the reaction mixture must contain the remaining components needed for the four-component condensation to take place. These are normally low-molecular weight substances.

The described procedure obviously implies a great flexibility in the choice of system for the immobilization of each individual compound. For example, the conditions of immobilization of biological molecules containing more than one class of the above mentioned reacting groups can be chosen so as to minimize modification of essential residues. Another feature giving the method a wide potential applicability is that the reaction proceeds readily, not only

in organic solvent systems, but also in aqueous solution at physiological pH values, thus decreasing the risk of denaturation or decomposition of pH-sensitive biological structures.

The mechanism of the condensation reaction has been thoroughly studied in homogeneous systems by Ugi and his associates.[9,10] A schematic representation is given in Fig. 1. The reaction is remarkable in the respect that a single end-product can be obtained from a reaction mixture containing all these reactive components.

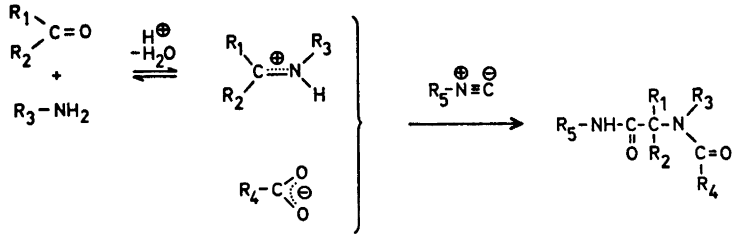

*Fig. 1.* Schematic representation of the four-component condensation exemplified by a primary amine and a carboxylate anion. The final product has a stable amide structure.

In this paper we present experiments performed in order to find conditions which allow high coupling efficiency. The protein human serum albumin (HSA)* has been used as a model substance. The properties of different carriers are discussed and comparison made between various low-molecular weight reagents which can be used as coupling mediators. The structures of the linkages between carrier and ligand are considered and also the possibilities to "direct" the reaction. The immobilizations of several biological substances of various structure, *i.e.* nucleic acids, cofactors, peptides, steroids, and penicillins, are reported.

In previous publications the preparations of immobilized enzymes of considerable catalytic efficiency and high stability have been described.[8,11,12]

## EXPERIMENTAL

*Preparation of carriers and immobilization of proteins.* Sepharose® 6B (Pharmacia Fine Chemicals, Uppsala, Sweden) was cross-linked with epichlorohydrin and converted to an amino derivative by reaction with MDA (Aldrich Chem. Co., Milwaukee, Wisc.) as previously described [12] except that 50 % higher MDA concentration was used. 5 ml MDA-Sepharose (sedimented in 0.1 M NaCl) was diluted with 0.1 M NaCl to a total volume of 7 ml, and 50 mg HSA (Kabi, Stockholm, Sweden) was added, followed by 10 $\mu$l acetaldehyde and 25 $\mu$l cyclohexyl isocyanide (EGA-Chemie KB, Steinheim/Albuch, West Germany). Identical mixtures were incubated at different pH's and temperatures and for various periods of time (see Figs. 2 – 3). Excess reagents were removed on a glass filter and the gel packed in a column. It was washed at 23° at 10 ml/h with the following buffers: 0.1 M Na phosphate, pH 7.5 (24 h) and 0.1 M Na acetate, 0.2 M NaCl, pH 4.0

* Abbreviations: HSA, human serum albumin; MDA, *p,p'*-diaminodiphenylmethane (4,4'-methylene dianiline); CM, carboxymethyl; ECD, epichlorohydrin cross-linked and desulphated.

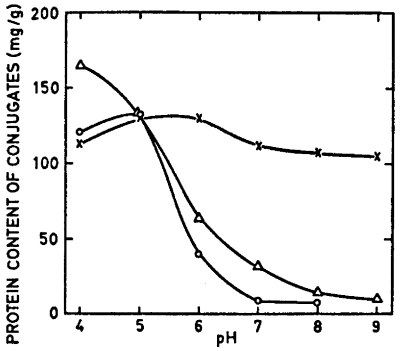


*Fig. 2.* The fixation of human serum albumin to various carriers as a function of reaction pH. ○, CM-Sephadex. ×, MDA-Sepharose. △, carbonyl-Sepharose. For composition of the reaction mixtures, see Experimental. Incubation for 6 h at 23°.

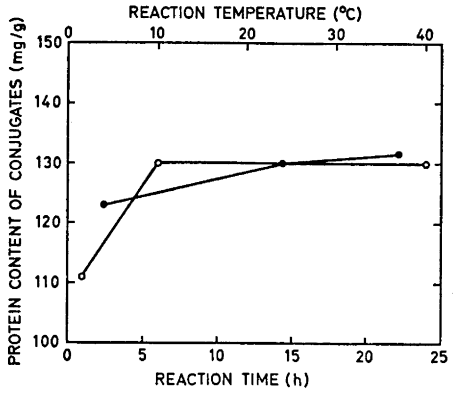


*Fig. 3.* The fixation of 50 mg human serum albumin to 5 ml MDA-Sepharose in a total volume of 7 ml 0.1 M NaCl at pH 6.0 as a function of reaction temperature (●) and reaction time (○). The immobilization was mediated by 25 μl cyclohexyl isocyanide and 10 μl acetaldehyde. The experiments performed at different temperatures were incubated for 6 h. The experiments performed for different periods of time were incubated at 23°.

(24 h). Samples of gel were treated with acetone, dried at 100° for 24 h, and subjected to amino acid analysis.[13] HSA was also coupled to 200 mg (dry weight) CM-Sephadex ® C-50 (Pharmacia) using the same amounts of reactants and the washing procedure as above; see Fig. 2.

A carbonyl polymer was prepared by dimethyl sulfoxide oxidation of ECD-Sepharose in the presence of $P_2O_5$ as previously described.[8] The product was washed in a column with 0.1 M NaCl at 10 ml/h for 24 h. To 1 ml sedimented gel in a total volume of 2 ml 0.1 M NaCl, were added 25 mg HSA, 25 μl cyclohexyl isocyanide, and 20 μl aniline. Incubation was carried out at various pH values at 23° for 6 h and the products washed as above; see Fig. 2.

A series of acetal- or aldehyde-substituted polyacrylamide carriers were used for the immobilization of α-chymotrypsin (3 × crystallized, Worthington Biochem. Corp., Freehold, N.J.). One acetal-polyacrylamide carrier, Enzacryl ® Polyacetal, is commercially available (Koch-Light, Colnbrook, Bucks., England). This polymer was hydrolyzed before application of the four-component reaction step. The hydrolysis was performed with 0.3 M HCl at 23° for 48 h. Chymotrypsin was attached to the hydrolyzed polymer as described in Table 2.

Enzacryl ® AA (Koch-Light) is a cross-linked polyacrylamide substituted by aromatic amino groups. Enzacryl AA was substituted with aldehyde or acetal groups using glutardialdehyde, *N*,*N*-bis(2,2-diethoxyethyl)amine, and 2,5-dihydro-2,5-dimethoxyfuran. The latter reagents were purchased from Aldrich Chem. Co. and Wolff and Kaaber, Farum, Denmark, respectively. The experiments were performed as follows: 50 mg polymer was suspended in 1.5 ml water and reacted with 50 mg Na acetate trihydrate, 50 μl cyclohexyl isocyanide, and 50 μl aldehyde or acetal reagent. The reaction mixture was stirred at 23° and pH 6.5 for 3 h. The product was washed free of soluble reagents with water on a glass filter and used for the attachment of chymotrypsin under the conditions given in Table 2. The conjugates were column washed as described earlier [14] and

*Table 1.* Immobilization of chymotrypsinogen A mediated by 10 μl acetaldehyde (omitted in the case of carbonyl polymer) and 25 μl cyclohexyl isocyanide. Incubation for 6 h at 23°.

| Carrier | Amount of carrier dry weight (mg) | Amount of carrier sedimented gel (ml) | Amount of protein (mg) | Total volume of reaction mixture (ml 0.1 M NaCl) | Reaction pH | Protein content of conjugates (mg/g) |
|---|---|---|---|---|---|---|
| CM-Sephadex | 200 | | 50 | 7 | 5.0 | 184 |
| CM-Sephadex | 200 | | 50 | 7 | 7.0 | 179 |
| MDA-Sepharose | | 5 | 50 | 7 | 6.0 | 134 |
| Carbonyl Sepharose | | 1 | 25 | 2 | 6.0 | 53 |

*Table 2.* Immobilization of α-chymotrypsin (20 mg) on acetal- or aldehyde-substituted polyacrylamide carriers (50 mg) mediated by cyclohexyl isocyanide (25 μl) at 23° and pH 6.5. Total reaction volume 2 ml. Reaction time 6 h. The specific activity of free chymotrypsin towards *N*-acetyl-L-tyrosine ethyl ester is 200 μmol $min^{-1}$ $mg^{-1}$.

| Carrier | Reagent for introduction of acetal or aldehyde groups | Esterolytic activity of chymotrypsin conjugates towards *N*-acetyl-L-tyrosine ethyl ester: Apparent pH-optimum | Specific activity (μmol $min^{-1}$ mg $conjugate^{-1}$) |
|---|---|---|---|
| Enzacryl Polyacetal | – | 9.6 | 6 |
| Enzacryl AA | Glutardialdehyde | 9.8 | 39 |
| Enzacryl AA | *N*,*N*-Bis(2,2-diethoxyethyl)amine | 9.8 | 42 |
| Enzacryl AA | 2,5-Dihydro-2,5-dimethoxyfuran | 9.8 | 32 |

*Table 3.* Immobilization of nucleic acids and nucleotides on 3 ml sedimented MDA-Sepharose or 200 mg (dry weight) CM-Sephadex in a total volume of 7 ml $H_2O$, mediated by 100 μl cyclohexyl isocyanide and 50 μl acetaldehyde (50 μl and 25 μl, resp., in the case of poly-A). Incubation for 6 h at 23°.

| Ligand | Amount of ligand (mg) | Carrier | Reaction pH | Ligand content of conjugates (mg/g) |
|---|---|---|---|---|
| RNA | 50 | CM-Sephadex | 5.0 | 0.1 |
| RNA | 50 | MDA-Sepharose | 6.0 | 39 |
| RNA | 50 | MDA-Sepharose | 6.0 | 1[a] |
| poly-A | 15 | MDA-Sepharose | 6.0 | 7 |
| AMP | 100 | MDA-Sepharose | 6.5 | 3 |
| NAD | 100 | MDA-Sepharose | 6.5 | 1 |
| NAD | 100 | CM-Sephadex | 6.5 | 0 |

[a] Isocyanide omitted.

characterized by their ability to catalyze the hydrolysis of *N*-acetyl L-tyrosine ethyl ester [14] at a substrate concentration of 15 mM.

Chymotrypsinogen A (Worthington) was immobilized and washed under the same conditions as given for HSA above. See Table 1.

*Immobilization of nucleic acids and nucleotides.* Yeast RNA (Worthington), polyadenylic acid (poly-A, Type I, Sigma Chem. Co., St. Louis, Mo.), 5'-AMP (Type III, Sigma), and NAD (Grade III, Sigma) were immobilized as described in Table 3. The conjugates were washed under the conditions given for HSA but with the phosphate buffer replaced by 0.1 M sodium borate buffer, pH 8.0. The ligand contents of the conjugates were estimated from their phosphorus content determined using ammonium molybdate.[15] Blank couplings without isocyanide were performed and the products always found to have a negligible phosphate content. The coenzymatic activity of free and immobilized NAD was determined using lactate dehydrogenase and lactate in the presence of semicarbazide.[16]

*Immobilization of various low-molecular weight substances.* Biotin (Merck AG, Darmstadt, West Germany), benzyl penicillin (sodium salt, Sigma), and 6-aminopenicillanic acid (Nutritional Biochem. Corp., Cleveland, Ohio) were fixed as described in Tables 4 and 6. The ligand contents of the conjugates were estimated from their content of sulphur determined as methylene blue.[17] All values are corrected for the sulphur content of the matrix; no additional sulphur was introduced in blank couplings performed in the absence of low-molecular weight coupling mediators.

Immobilization of peptides and amino acids is exemplified in Table 5. These conjugates were washed as described for HSA. Penicillanic acid conjugates were washed with 0.1 M Na phosphate, pH 6.2, and 0.1 M Na phosphate, pH 5.5.

*Table 4.* Immobilization of biological substances mediated by 25 μl cyclohexyl isocyanide (3-dimethylaminopropyl isocyanide in the case of biotin). Incubation for 6 h at 23°.

| Ligand | Amount of ligand (mg) | Carrier | Total volume of reaction mixture (ml $H_2O$) | Amount of aldehyde (μl) | Additional reagent | Reaction pH | Ligand content of conjugates (μequiv./g) |
|---|---|---|---|---|---|---|---|
| Biotin | 25 | 50 mg Enzacryl AA | 2 | 25[a] | – | 6.5 | 25 |
| Benzylpenicillin | 25 | 50 mg Enzacryl AA | 2 | 25[a] | – | 6.5 | 10 |
| Benzylpenicillin | 25 | 1 ml carbonyl Sepharose | 2 | – | 20 μl butylamine | 6.5 | 16 |
| 6-Aminopenicillanic acid | 25 | 1 ml carbonyl Sepharose | 3 | – | 30 mg Na acetate | 6.0 | 34 |
| 6-Aminopenicillanic acid | 50 | 5 ml MDA-Sepharose | 7 | 25[b] | – | 6.5 | 16 |
| 6-Aminopenicillanic acid | 50 | 200 mg CM-Sephadex | 7 | 25[b] | – | 6.5 | 30 |

[a] Acetaldehyde. [b] Benzaldehyde.

*Table 5.* Immobilization of 35 mg amino acid ester or peptide on 50 mg (dry weight) CM-Sephadex mediated by 25 μl 3-dimethylaminopropyl isocyanide and 25 μl aldehyde in 2 ml $H_2O$ at pH 7.0. Incubation for 6 h at 23°.

| Ligand | Aldehyde | Amino acid content of acid hydrolysate of conjugate (μequiv./g conjugate) | |
|---|---|---|---|
| Phenylalanine ethyl ester | HCHO (35 %) | Phe: 84 | Gly: 9 |
| Phenylalanine ethyl ester | $CH_3CHO$ | Phe: 57 | Ala: 24 |
| Glycyl leucine | $CH_3CHO$ | Gly: 19<br>Leu: 275 | Ala: 32 |

*Table 6.* The effect of the addition of a five-fold excess of aniline (100 μl) or Na acetate (150 mg) on the immobilization of benzyl penicillin (75 mg) and phenylalanine ethyl ester (40 mg). Incubation with 1 ml carbonyl-Sepharose and 25 μl cyclohexyl isocyanide in 3 ml water for 6 h at pH 6.0 and 23°.

| Ligand | Ligand content of conjugates (μequiv./g) | | |
|---|---|---|---|
| | standard experiment | amine added | acetate added |
| Benzyl-penicillin | 12.2 | 15.0 | 0.5 |
| Phenylalanine ethyl ester | 5.3 | 0 | 3.7 |

Immobilization of cortisone (Sigma) was carried out as follows. 25 μl cyclohexyl isocyanide was added to 50 mg CM-Sephadex, 10 mg cortisone, and 25 μl aniline in 2 ml solvent mixture (50 % ethanol or dimethyl formamide in water). The pH was adjusted to 4.5 and the reaction allowed to proceed overnight at 23°. The product was washed on a glass filter and then in a column for 48 h at 10 ml/h with the same solvent mixture. IR spectra of dried samples in KBr were recorded and the steroid content of the conjugates estimated by comparisons with reference spectra recorded on mixtures of dry matrix and different amounts of steroid.

## RESULTS AND DISCUSSION

*The choice of carrier.* Carriers containing amino, carbonyl, and carboxyl groups have been prepared and the isocyanide immobilization technique has been applied. A few commercially available derivatized polymers have also been used. The capacity of the polymers to take up ligands is important but the carrier must also satisfy requirements with regard to particle size and shape, mechanical rigidity, and permeability. As yet it has not been possible to prepare an isocyanide polymer with significant substitution.

The anionic polymers have exclusively been carboxyl-substituted. Carboxymethyl Sephadex has proved suitable in several cases (see tables, figures, and Ref. 8). Very high ligand uptakes can be obtained. We have earlier

used carboxymethyl agarose,[8] which has a lower capacity to take up protein. The relative activities of enzymes bound to these carriers, especially towards macromolecular substrates, are not very high which may be caused by intra- and intermolecular cross-linking effected by excess of low-molecular weight reagents (*cf.* below). Electrostatic repulsion between gel and substrate may also play a role in the case of casein hydrolysis.

The best amino polymers employed so far have been obtained by CNBr activation or bis-epoxide activation [18] of epichlorohydrin cross-linked Sepharose and subsequent coupling of aromatic diamines, *i.e.* MDA or occasionally *p*-phenylenediamine. The content of free amino groups in the MDA polymers is rather low,[12] but nevertheless good uptakes of proteins and other substances are obtained. Because of the low content of amino groups in the carrier, the proteins will be fixed by a small number of bonds which is advantageous since a large number of points of attachment may lead to destruction of the protein tertiary structure. Since aromatic amines are weak bases the charge density of the carrier is low at physiological pH values, which is important if the carrier is going to be used for the preparation of adsorbents for biospecific affinity chromatography. MDA-Sepharose has been used for the immobilization of pepsin,[11] β-amylase,[12] and several ligands discussed in this paper (see tables and figures).

The amino-substituted polymer Enzacryl AA has been used for the preparation of immobilized proteolytic enzymes with reasonable yields and with considerable activity in batch towards low-molecular weight substrates.[8] However, the proteolytic activity is low, probably because of steric hindrance. The powder-like conjugates are unsuitable for column use.

Structural proteins, *e.g.* keratin and wool, are examples of carriers containing both carboxyl and amino groups. They have been used for the preparation of catalytically active immobilized chymotrypsin.[8]

Carbonyl polymers can be prepared by dimethyl sulfoxide oxidation of alcoholic groups in polysaccharides in the presence of an activating electrophilic reagent such as acetic anhydride [19] or phosphorus pentoxide.[20] Using this reaction, carbonyl derivatives of ECD-Sepharose have been prepared which have been successfully used for the immobilization of biological molecules. The pH dependence of the fixation of HSA to such a polymer is shown in Fig. 2.

Aldehyde- and acetal-substituted polymers have been prepared by derivatizing Enzacryl AA with glutardialdehyde, *N*,*N*-bis(2,2-diethoxyethyl)amine, and 2,5-dihydro-2,5-dimethoxyfuran (see Experimental). These polymers, together with the commercial carrier Enzacryl Polyacetal, have been employed for the preparation of catalytically active immobilized chymotrypsin (Table 2).

Periodate-oxidized polysaccharide carriers have earlier been used for the immobilization of chymotrypsin.[8] In summary, proteolytic enzymes attached to carbonyl polymers exhibit high activities towards low-molecular weight substrates and, with the exception of Enzacryl derivatives, also have considerable proteolytic activities.

*The choice of isocyanide.* 3-Dimethylaminopropyl isocyanide, which is readily water-soluble but not commercially available, was used in early studies. Later cyclohexyl isocyanide has been used, which can be obtained commerci-

ally. This compound has a limited solubility in water, so that stirring or revolution of the reaction mixture must be efficient. Pepsin could be attached to Sepharose with equal yields using either of these isocyanides.[11] *t*-Butyl isocyanide and 1,1,3,3-tetramethyl butyl isocyanide (Kema Nord, Sweden) and also *N*-(3-isocyanidopropyl)-morpholine (prepared in our laboratory[21]) have also been used with no notable differences in the results.

*The choice of carbonyl compound.* The carbonyl function may be on the carrier (see above) or on the ligand, as exemplified by cortisone. Otherwise, a low-molecular weight aldehyde, such as acetaldehyde, is added to the reaction mixture. Several other aldehydes, *e.g.* formaldehyde, butyraldehyde, and benzaldehyde, have been used more occasionally. No significant differences in coupling yields have been found using these aldehydes. Ketones, however, give lower coupling yields.

*The choice of amine.* The amino component is usually present on the biological molecule to be immobilized or on the carrier. In other cases an amine is added as an additional component to make the system complete or to compete with an essential amino group. For such purposes we have used butylamine, ethanolamine, aniline, and *N*-(3-aminopropyl)-morpholine.

*The choice of anion.* Carboxylate anions have been regularly employed both on the carrier, on the ligand, and in the form of sodium acetate as an additional component. In a few cases anions of phosphate esters have been used (Table 3).

HSA is attached to MDA-agarose in exactly the same amount in 0.1 M Na phosphate buffer, pH 6.0 (131 mg/g) as in 0.1 M NaCl, adjusted to pH 6.0 (130 mg/g). This experiment indicates that phosphate ions do not participate in the immobilization reaction; no competition with carboxylate ions takes place.

The hydroxide ion is always present in the systems, although in low concentration at the pH at which the reaction is usually carried out. If this anion could compete efficiently with carboxylate anions it would not be possible to fix significant amounts of ligands to carboxylic carriers, or carboxylic ligands to amino carriers. However, at high pH the hydroxide ion may become important.

*Optimal conditions for the immobilization of proteins.* The attachment of HSA to MDA-Sepharose has been used as a model system for determing the optimal conditions for the immobilization of proteins. However, the results are probably representative for other classes of substances as well.

Identical reaction mixtures were incubated for 1, 6, and 24 h. The reaction was essentially complete in a few hours (Fig. 3). In earlier experiments with coupling of chymotrypsin to CM-Sephadex (unpublished) the protein uptake after 1 h was also 85 – 90 % of that after 6 h.

The temperature dependence of the immobilization reaction is not very marked. Only 8 % difference in protein content of the conjugates is observed if the coupling is performed at 4° or 37° (Fig. 3). This makes possible the immobilization of thermally unstable substances in good yields. Unless otherwise stated, a temperature of 23° has been used.

The pH-dependence of the extent of fixation of HSA to polymers substituted with amino, carbonyl, and carboxyl groups is illustrated in Fig. 2. The coupling of HSA to MDA-Sepharose proceeds with the same efficiency

over a wide range of pH. On the other hand, the fixation of HSA to CM-Sephadex and carbonyl-Sepharose is very dependent on pH. This is most likely due to electrostatic effects. MDA-Sepharose has a low charge density. CM-Sephadex carries many negative charges and repels the acidic protein HSA when the pH is significantly higher than the isoelectric point of the protein (pH 4.9). When the reaction pH is lowered, the protein loses its negative net charge and is attracted to the gel giving a high coupling yield. Contrary to HSA, the basic protein chymotrypsinogen A ($pI = 9.5$) is taken up in high yields by CM-Sephadex both at pH 5 and pH 7 (Table 1), which also may be explained by this charge effect. Chymotrypsin ($pI = 8.6$) has repeatedly been attached to CM-Sephadex at pH 6.5 in high yields.[8] When the carbonyl-Sepharose was synthesized, carboxyl groups were probably formed by oxidation of carbonyl functions. This gel thus repels HSA at neutral and alkaline pH analogous to CM-Sephadex. These results suggest that the four-component condensation is not very sensitive to pH but that electrostatic interactions between carrier and ligand can strongly affect the extent of coupling.

The reaction can be allowed to proceed in a pH-stat or in buffered solution. As stated above, some buffer anions (such as carboxylate) participate in the reaction, whereas others (such as phosphate) do not. This should be considered when an experiment is planned.

By varying the content of reactive groups in the carrier and the concentrations of soluble reactants, the uptake of ligand can be varied to a great extent.[8,11,12] However, high concentrations of reactants will not necessarily give the best products. A general observation is that there is an inverse relationship between the enzyme content of a conjugate and the relative activity of the fixed enzyme (compared to that of the free enzyme). For theoretical studies, the most desirable preparations may be those with high relative activity but, when practical applications are considered, the most important property of a conjugate is the percentage uptake of the total amount of catalytic activity added to the reaction mixture.[12]

*Immobilization of substances other than proteins.* The immobilization of nucleic acids and synthetic polynucleotides has received attention in the last few years. Such preparations, often with rather low ligand contents, have been employed as biospecific adsorbents.[6,22,23] The isocyanide method, used here for the immobilization of yeast RNA and polyadenylic acid (Table 3), should be a useful complement to the already existing methods.

The immobilization of various low-molecular weight substances (*i.e.* NAD, AMP, biotin, benzyl penicillin, 6-aminopenicillanic acid, glycyl leucine, and phenylalanine ethyl ester) is shown in Tables 3 – 6. The ligand uptakes of the carriers are low on a weight basis in comparison with the amounts of protein which can be taken up. However, an efficient biospecific adsorbent is not necessarily highly substituted. The immobilized NAD described in Table 3 was equally active as free NAD as coenzyme to lactate dehydrogenase.[16]

In addition to the substances mentioned above, matrix-bound cortisone has been prepared with a steroid content of approximately 20 mg/g conjugate. Because of the low water solubility of this substance the immobilization was carried out in mixtures of water and organic solvents. Immobilized hormones

and penicillins should be interesting objects for pharmacological investigations and studies on cellular metabolism. Immobilized peptide hormones have already been used for the latter purpose.[6]

*The structure of the carrier-ligand linkage.* The determinations of immobilized proteins, peptides and amino acids have been performed by amino acid analysis [13] of acid hydrolysates of the conjugates. It has been shown by hydrolyzing mixtures of carriers and amino acids that the presence of carrier does not normally influence the recovery of amino acids. However, the patterns of the chromatograms obtained from covalently linked ligands are often not identical with those of mere mixtures. The differences are understandable if it is assumed that attachment has taken place according to the four-component scheme and also that certain bonds are preferentially split upon hydrolysis.

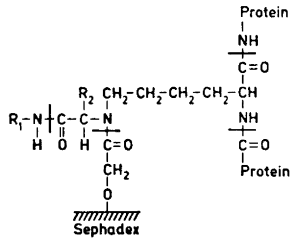


*Fig. 4.* Suggested structure and hydrolysis pattern of a CM-Sephadex-α-chymotrypsin conjugate. $R_1$: dimethylaminopropyl. $R_2$: H (formaldehyde). Hydrolytic cleavage indicated by heavy lines.

With the above assumptions, the study of amino acid chromatograms can give information about which functional groups participate in the condensation process. Fig. 4 gives an illustration. Chymotrypsin was attached to CM-Sephadex by means of formaldehyde and the conjugate analyzed. It was observed that the recovery of lysine was significantly reduced and that an extra peak had emerged. According to Fig. 4, the formation of carboxymethyl lysine could be expected and the extra peak was identified as this lysine derivative by comparison with a chromatogram of the products of hydrolysis of a bromoacetate-treated polylysine.

Analogous experiments have been performed with low-molecular weight substances (Table 5). When phenylalanine ethyl ester or glycyl leucine was immobilized on CM-Sephadex in the presence of acetaldehyde, alanine was formed in the hydrolysate; when phenylalanine ethyl ester was attached using formaldehyde, glycine was obtained. This is also in accordance with the model in Fig. 4, provided that all bonds of the tertiary amide nitrogen are susceptible to hydrolytic cleavage.

When proteins are attached to amino polymers, *e.g.* Enzacryl or MDA-agarose, and the linkage is mediated by acetaldehyde, the relative alanine content of the conjugates is significantly higher than in the native proteins. In experiments with carbonyl polymers no effects of this kind have been observed.

Blank couplings have been performed in the absence of isocyanide in order to estimate the amount of protein which becomes attached to different carriers by physical adsorption or electrostatic attraction. In all these experi-

ments, only very small amounts of protein have been found in the conjugates.

*Inter- and intramolecular cross-linking.* When isocyanides are used for the immobilization of biological molecules which contain more than one class of reactive group, and also all four reacting components are simultaneously present in the supernatant liquid, inter- and intramolecular cross-linking may occur. This can take place both in free solution and on the surface of the matrix. Such cross-linking will compete with the immobilization. Extensive cross-linking may affect the three-dimensional conformation of immobilized molecules, which can lead to destruction of the biological activity of these molecules.

*Ways of directing the fixation.* Biological molecules may contain more than one class of reactive group which can participate in the four-component condensation. For example, most proteins can react through either amino or carboxyl groups, one of which may be essential for the biological activity of the protein. However, the immobilization reaction can be directed so as to eliminate or diminish inactivation caused by modification of essential groups. This can be done in at least two ways: A protein which contains an essential carboxyl group can be attached to a carboxylic polymer, thereby utilizing the amino groups of the protein for fixation. Alternatively, it can be attached to a carbonyl polymer and an excess of carboxylate added to the reaction mixture to compete with the protein carboxyl groups.

A model experiment which illustrates the second of these methods of "direction" is shown in Table 6. Benzyl penicillin, which carries a free carboxyl group, and phenylalanine ethyl ester with a free amino group were incubated with carbonyl-Sepharose and cyclohexyl isocyanide. The uptakes of these ligands were estimated separately by sulphur and amino acid analysis of the conjugates, respectively. The uptake of benzyl penicillin could be almost completely inhibited by a five-fold excess of sodium acetate, and the uptake of phenylalanine ethyl ester was eliminated by a five-fold excess of aniline. A blank experiment performed in the absence of isocyanide showed no background adsorption of amino acid, but the carrier contained some sulphur for which correction was made.

Another series of experiments demonstrates the quantitative effect of the addition of "directing" reagents to a second model system, *viz.* the fixation of HSA to carbonyl-Sepharose at pH 6.0. When only cyclohexyl isocyanide was present in addition to these reagents, the protein uptake was 63 mg/g conjugate. When an excess of aniline was added, the uptake was identical. When the aniline was replaced by sodium acetate, the uptake was 38 mg/g.

*Acknowledgements.* We are indebted to Professor Jerker Porath for his continuous support. Our thanks are also due to Mrs. Marianne Lindström for technical assistance. The work has been supported by grants from *Sven and Lilly Lawski's Foundation for Scientific Research* and the *Swedish Board for Technical Development.*

## REFERENCES

1. Katchalski, E., Silman, I. and Goldman, R. *Advan. Enzymol.* **34** (1971) 445.
2. Orth, H. D. and Brümmer, W. *Angew. Chem.* **84** (1972) 319.
3. Kay, G. *Process Biochem.* **3** (8) (1968) 36.

4. Lilly, M. D. and Dunnill, P. *Process Biochem.* **6** (8) (1971) 29.
5. Cuatrecasas, P. and Anfinsen, C. B. *Methods Enzymol.* **22** (1971) 345.
6. Cuatrecasas, P. *Advan. Enzymol.* **36** (1972) 29.
7. Porath, J. and Kristiansen, T. In Neurath, H., Ed., *The Proteins*, Academic, New York and London, 3rd Ed., Vol. 1. *In press.*
8. Axén, R., Vretblad, P. and Porath, J. *Acta Chem. Scand.* **25** (1971) 1129.
9. Ugi, I. *Angew. Chem.* **74** (1962) 9.
10. Ugi, I., Ed., *Isonitrile Chemistry*, Academic, N.Y. and London 1971.
11. Vretblad, P. and Axén, R. *FEBS Lett.* **18** (1971) 254.
12. Vretblad, P. and Axén, R. *Biotechnol. Bioeng.* **15** (1973) 783
13. Spackman, D. H., Stein, W. H. and Moore, S. *Anal. Chem.* **30** (1958) 1190.
14. Axén, R. and Ernback, S. *Eur. J. Biochem.* **18** (1971) 351.
15. Gustafsson, L. *Oikos. In press.*
16. Larsson, P.-O. and Mosbach, K. *Biotechnol. Bioeng.* **13** (1971) 393.
17. Gustafsson, L. *Talanta* **4** (1960) 227; 236.
18. Porath, J. and Sundberg, L. In Peeters, H., Ed., *Protides of the Biological Fluids*, Pergamon, Oxford 1971, Vol. 18, p. 401.
19. Albright, J. D. and Goldman, L. *J. Am. Chem. Soc.* **89** (1967) 2416.
20. Onodera, K., Hirano, S. and Kashimura, N. *Carbohyd. Res.* **6** (1968) 276.
21. Ugi, I., Betz, W., Fetzer, U. and Offermann, K. *Chem. Ber.* **94** (1961) 2814.
22. Wagner, A. F., Bugianesi, R. L. and Shen, T. Y. *Biochem. Biophys. Res. Commun.* **45** (1971) 184.
23. Rickwood, D. *Biochim. Biophys. Acta* **269** (1972) 47.

Received April 6, 1973.