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# Enzyme Immobilization Techniques on Poly(Glycidyl Methacrylate-Co-Ethylene Dimethacrylate) Carrier with Penicillin Amidase as Model*

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## Summary

Two types of bead-form macroporous carriers based on glycidyl methacrylate with ethylene dimethacrylate copolymers were used for the immobilization of penicillin amidase either directly or after chemical modification. Direct binding through oxirane groups, which is equally efficient at pH 4.2 and 7, is relatively slow and brings about an activity loss at low enzyme concentrations. The most efficient immobilization was achieved on glutaraldehyde-activated amino carrier, irrespective of whether the amino groups were formed by ammonia or 1,6-diaminohexane treatment of the original oxirane carrier. Hydrazine treatment gave lower immobilization yields. The same is true of the azide method independent of the length of the spacer. Most enzyme activity was preserved by coupling the carbodiimide-activated enzyme to the carrier with alkyl or arylamino groups at the end of a longer substituent. Immobilization on diazo-modified carrier gave average results. Rapid immobilization by a lysine-modified phosgene-treated carrier resulted in an activity loss. It is suggested that multipoint and very tight attachment of the enzyme molecule to the matrix decreased the activity. The immobilized activity is quite stable in solution and very stable upon lyophilization with sucrose.

## INTRODUCTION

The combination of the unique functions of proteins with the good physical properties of synthetic and semisynthetic carriers have opened new horizons in biochemical technology. Many carriers, immobilization techniques, and applications have been described. From these data it is known that the immobilization method must be adjusted for each individual protein:[1] high pH and/or long

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incubation should be avoided with some enzymes, lysine amino groups are sometimes important for the function, a longer chain length between the carrier and the enzyme may improve activity, multipoint attachment increases the stability in some cases but reduces the activity in others, etc. Different proteins provide different immobilization groups: some are rich in terminal amino groups of lysine, others in carboxyls, or the aromatic residues are exposed favorably for coupling with diazonium salts. There are also practical considerations: a high enzymatic activity recovery is sought when using expensive enzymes. On the other hand, high specific activity is usually desired with inexpensive enzymes even if a great deal of the enzyme is lost during reaction.

It would greatly simplify the work with immobilized enzymes and other proteins if there was one type of a carrier that would meet most of the different demands expressed above.

In our previous papers[2,3] the reactive oxirane group containing copolymer of glycidyl methacrylate and ethylene dimethacrylate (GMA–EDMA) was reported as a very versatile carrier for protein immobilization. The reactivity of the oxirane ring not only allows direct covalent binding of proteins, but also provides the possibility of a whole spectrum of easy modifications, so that almost all known immobilization methods can be applied to this type of carrier.

In order to provide experimental data about the general features of different immobilization methods, we used one batch of the original GMA–EDMA polymer and carrier on various modifications that do not substantially change its physical properties (surface area, porosity, swelling, etc.). We proceeded with the most important immobilization principles. Although the results were obtained with one model enzyme of technical quality, some general conclusions can be derived therefrom.

## EXPERIMENTAL MATERIALS AND METHODS

The enzyme, penicillin amidase (PA) (EC 3.5.1.11), was a preparation of technical purity, isolated from a mutant strain of *Escherichia coli* in the Research Institute for Antibiotics and Biotransformations. The lyophilized powder was readily soluble in water. Its amino acid composition is given in Table I.

### *Activity Measurement*

The activity was measured by formation of 6-aminopenicillanic acid from sodium salt of benzylpenicillin using an automatic titrator.

TABLE I
Amino Acid Composition of the Crude Preparation of Penicillin Amidase Used

| Amino acid[a] | Amount (m*M*/g) | Amount (% (w/w)) | Assumed number of residues |
|---|---|---|---|
| Alanine | 0.974 | 8.68 | 25-26 |
| Arginine | 0.355 | 6.21 | 9 |
| Aspartic acid Asparagine | 1.034 | 13.75 | 26-27 |
| Glutamic acid Glutamine | 1.050 | 15.46 | 27-28 |
| Glycine | 0.736 | 5.53 | 19 |
| Histidine | 0.186 | 2.89 | 5 |
| Isoleucine | 0.456 | 5.98 | 12 |
| Leucine | 0.797 | 10.45 | 20-21 |
| Lysine | 0.620 | 9.00 | 16 |
| Methionine | 0.203 | 3.00 | 5-6 |
| Phenylalanine | 0.330 | 5.45 | 8-9 |
| Proline | 0.458 | 5.27 | 12 |
| Serine | 0.458 | 4.81 | 12 |
| Threonine | 0.534 | 6.37 | 14 |
| Tyrosine | 0.307 | 5.57 | 8 |
| Valine | 0.682 | 7.98 | 16-18 |

[a] Cysteine and tryptophan not determined.

Thus, the enzyme or the immobilized preparation was added to 7 ml of $5 \times 10^{-2}M$ solution of the substrate containing $0.15M$ NaCl and $5 \times 10^{-3}M$ EDTA adjusted to pH 7.5 or another value when the effect of pH was checked. The pH value was kept constant by the titrator. The standard temperature was 25°C. The activity calculated from the volume of $0.1M$ NaOH consumed was expressed in units that gave the number of $\mu$mol hydrolyzed/minute. The units are expressed per dry weight of the preparation (mg or g).

### *Chemicals*

All chemicals were obtained from Fluka and were of p.a. quality. Glutaraldehyde (GA) was a 25% water solution and was used without further purification. Ethyl esters of *p*-aminobenzoic acid, glycine, and 6-aminohexanoic acid were prepared by the usual esterification method in ethanol using dry HCl. 4-Amino-*N*-(2-aminoethyl)benzamide was prepared by refluxing the ethyl ester of *p*-aminobenzoic acid with an excess of 1,2-ethanediamine. The residual diamine was distilled off, and the product was isolated as a monohydrochloride by crystallization from ethanol. The ethyl ester

of the dimer of 6-aminohexanoic acid was prepared from the *N-p*-nitrophenyl-sulfenyl-6-aminohexanoic acid,[4] which was coupled to the ethyl ester of 6-aminohexanoic acid by dicyclohexylcarbodiimide.[5] The protecting group was removed by hydrogen chloride in ethyl acetate, and the hydrochloride of the product was used. Macroporous polymeric carriers were prepared in water suspension by radical polymerization of glycidyl methacrylate with ethylene dimethacrylate in the presence of a mixture of aliphatic and cycloaliphatic alcohols as porogenic agents[6] in regular bead form. The properties of both basic carriers used are summarized in Table II.

### *General Immobilization Procedures*

If there are no specific modifications given for the particular method, 1 g dry carrier was suspended in 5 ml PA solution containing 50 mg (10 mg/ml) enzyme with a specific activity of 3.6 units/mg in 0.1*M* phosphate buffer or 0.05*M* borate buffer, pH 8.7. The immobilization proceeded at 25°C and was terminated by washing with 0.5*M* NaCl buffered with 0.01*M* phosphate to pH 7.0 and containing 0.5% Thesit (Boehringer, Mannheim GmbH). Without this detergent ($\omega,\omega'$-bis-methoxy(oligooxirane)) the adsorbed enzyme was not completely removed. The removal was checked by activity measurements in the filtrate after the first estimation of activity of the immobilized preparation. Any activity in the filtrate showed that there was a residual amount of sorbed enzyme that was solubilized by the substrate. A wet preparation was always used for the activity measurements and the units obtained were recalculated for the dry weight.

TABLE II
Properties of Basic Copolymers used for Immobilization and Modifications

| | G-60 | G-70 |
|---|---|---|
| Content of glycidyl methacrylate (wt %) | 49.7 | 63.2 |
| Pore volume ($cm^3/g$) | 0.969 | 1.214 |
| Most frequent pore radius (nm) | 25 | 109 |
| Specific surface area ($m^2/g$) | 60.2 | 27.5 |
| Upper GPC exclusion limit (Daltons) | $2 \times 10^5$ [a] | $3 \times 10^6$ [b] |
| Heat stability up to (°C)[c] | 235 | 210 |
| Beads fraction ($\mu$m) | 150–250 | |

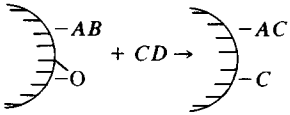
[a] For dextran in water.
[b] For polystyrene in THF.
[c] Temperature of onset of decomposition.

### *Chemical Analysis*

The elemental composition was estimated using a Perkin–Elmer 240 elemental analyser. Amino acid analysis was performed at the Institute of Organic Chemistry and Biochemistry of the Czechoslovak Academy of Sciences. The samples were hydrolyzed at 105°C for 20 hr in a 6*M* HCl (ampules sealed at 1 Torr) and analyzed using an automatic two-column analyzer (type 6020, Development Workshops of CSAS). The IR spectra were run on a Perkin–Elmer 577 spectrophotometer in KBr pellets. Free hydrazine groups were detected by TNBS according to Inman.[7]

### *Calculation of Functional Group Conversion*

All modifications used resulted in a change in elemental composition, particularly in the nitrogen content. If the carrier used for modification contained some residual oxirane groups in addition to the functional group, the reaction can be described by eq. (1) and the conversion was calculated according to eqs. (2a) and (2b):




(1)

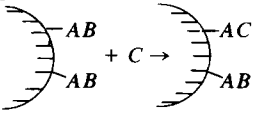
$$X_{AC} = \frac{nM_C - N_C}{M_C(N_A - N_C) + M_B - N_C \cdot Y_{AB}} \tag{2a}$$

$$X_C = \frac{N_A + n(Y_{AB} + M_C - M_B)}{M_C(N_A - N_C) + M_B - N_C Y_{AB}} \tag{2b}$$

where the symbols are expressed per gram of the final carrier: $X_{AC}$ is the number of equivalents of substituent $C$ that reacted with residue $A$ on the carrier, $n$ is the number of mol nitrogen found in the product, $N_C$ and $N_A$ are the numbers of nitrogen atoms in the residues $C$ and $A$, respectively, $M_C$ and $M_B$ are the molecular weights of residues $C$ and $B$, respectively, $Y_{AB}$ are the starting carrier that contain 1 mol group $AB$, and $X_C$ are equivalents of the group $C$ that reacted with the residual oxirane groups. This calculation can be applied in the cases where all $AB$ groups are able to react, which is the case in the hydrazinolysis of immobilized esters.

In reactions of carriers that do not contain residual oxirane groups and the conversion of $AB$ need not be complete, the following

expressions can be applied:




(3)

$$X_{AC} = \frac{N_C - nY_{AB}}{N_C M_C - N_{AB} Y_{AB}} \tag{4a}$$

$$X_{AB} = \frac{nM_C - N_C}{N_C \cdot M_C - N_{AB} Y_{AB}} \tag{4b}$$

where $N_{AB}$ is the number of nitrogen atoms in the group $AB$ and $X_{AB}$ are the equivalents of these groups that remained unchanged.

## EXPERIMENTAL MODIFICATION PROCEDURES AND IMMOBILIZATION TECHNIQUES

All procedures used are summarized in Figure 1.

### *Carrier Modification with Ammonia (G-70-$NH_2$ and G-60-$NH_2$) (II)*

The original carrier (G-70 or G-60) was treated with aqueous ammonia (25%) as described earlier.[6] The resulting carriers (G-70-$NH_2$ and G-60-$NH_2$) contained 2.66 and 2.48% nitrogen, which corresponds to 1.9 and 1.77 mequiv $NH_2$ groups, respectively, per gram. No oxirane rings were detected by infrared spectroscopy at 835 and 910 $cm^{-1}$.

### (a) Immobilization with glutaraldehyde (GA)

One g carrier (G-70-$NH_2$ or G-60-$NH_2$) was shaken with 30 ml 0.1*M* phosphate buffer, pH 6.9 and 4 ml GA at 25°C for 16 hr. After washing with 1 liter of the above buffer, the liquid was removed by suction and the wet cake added to 10 ml of the above buffer, which contained 5 mg PA/ml. The suspension was shaken at 25°C for 3 hr.

### (b) Immobilization with *N*-cyclohexyl-*N'*2-(4-morpholinyl)ethyl carbodiimide methyl-*p*-toluene sulfonate (CDI)

One g carrier (G-70-$NH_2$) in 20 ml water was titrated by 0.1*M* NaOH to pH 5.5 and this value was retained throughout the reaction by automatic titration. After sedimentation the volume of the liquid was reduced to 10 ml and after the addition of 50 mg PA the mixture was cooled by freezing. CDI (740 mg) was added in three portions

after each hour. One hour after the last portion was added, the temperature was raised to room temperature and the reaction was allowed to proceed overnight.

*Carrier Modification with 1,6-Diaminohexane (G-70-HMDA) (III)*

G-70 (21 g) was heated under reflux on a steam bath for 5 hr with 30 g 1,6-diaminohexane and 30 ml water. Washing was checked by ninhydrin reaction of the washing water. The resulting carrier (G-70-HMDA) contained 56.62% C, 8.02% H, and 3.95% N, corresponding to 2.82 mequiv amino groups. If there is no cyclization, half of them are primary and the other half are secondary amino groups. The carrier gave a deep color with ninhydrin reagent.

Immobilization of PA with GA and CDI was accomplished using the procedure described above (see II(a) and II(b)).

*Additional Modification with 4-Aminobenzoic Acid (G-70-HMDApAB) (IV)*

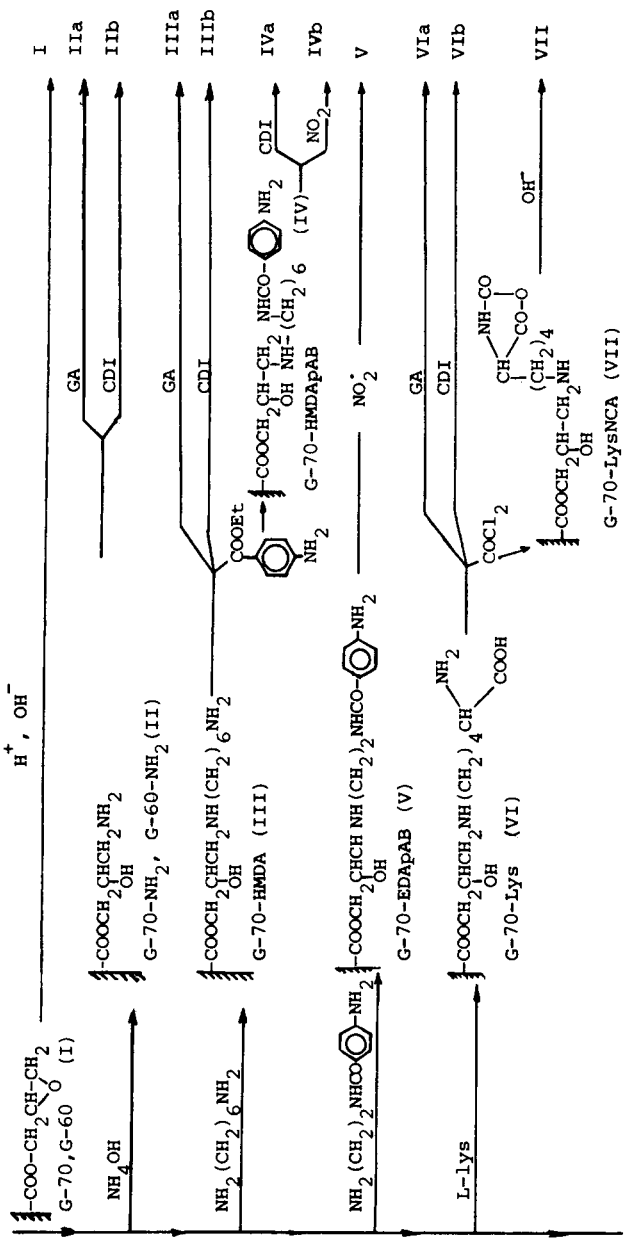
Carrier G-70-HMDA (9 g) was refluxed with 20 ml dioxane, 0.5 ml 1,2-dihydroxyethane, and 5.5 g (36.4 mmol) 4-aminobenzoic acid ethyl ester. After 8 hr the carrier was washed with dioxane (checked by diazotation and coupling with β-naphthol in the filtrate) and dried. The resulting carrier (G-70-HMDApAB) (57.5% C, 7.59% H, and 3.56% N) turned dark red upon diazotation and coupling with β-naphthol. The calculated amount of *p*-aminobenzoic acid residues was 0.62 mequiv/g and 0.69 mequiv 1.6 diaminohexane residues did not react.

Immobilization of PA with CDI followed the procedure described above (II(b)).

Immobilization by diazotation and coupling (IV(b)) were realized as follows: 1 g carrier G-70-HMDApAB was suspended in 4 ml 10% aqueous HCl, cooled in an ice-bath, and treated with 10 ml of a 2% $NaNO_2$ solution (1.5 mmol), added dropwise. After 1 hr the carrier was quickly filtered off, washed with 0.1*M* phosphate buffer, pH 7.0, with crushed ice, and transferred into 10 ml of the above buffer with 10 mg PA/ml. The suspension was then shaken in the ice-box overnight.

*Carrier Modification with 4-Amino-N-(2-Aminoethyl)Benzamide (G-70-EDApAB) (V)*

4-Amino-*N*-(2-aminoethyl)benzamide hydrochloride (3 g) (13.9 mmol) and 1.5 g $Na_2CO_3$ (14 mmol) were dissolved in 6.5 ml water


I
IIa
IIb
IIIa
IIIb
IVa
IVb
V
VIa
VIb
VII
GA
CDI
CDI
GA
CDI
CDI
GA
CDI
(IV)
G-70-HMDApAB
G-70-EDApAB (V)
G-70-HMDA (III)
G-70-NH2, G-60-NH2 (II)
G-70-Lys (VI)
G-70-LysNCA (VII)
L-lys

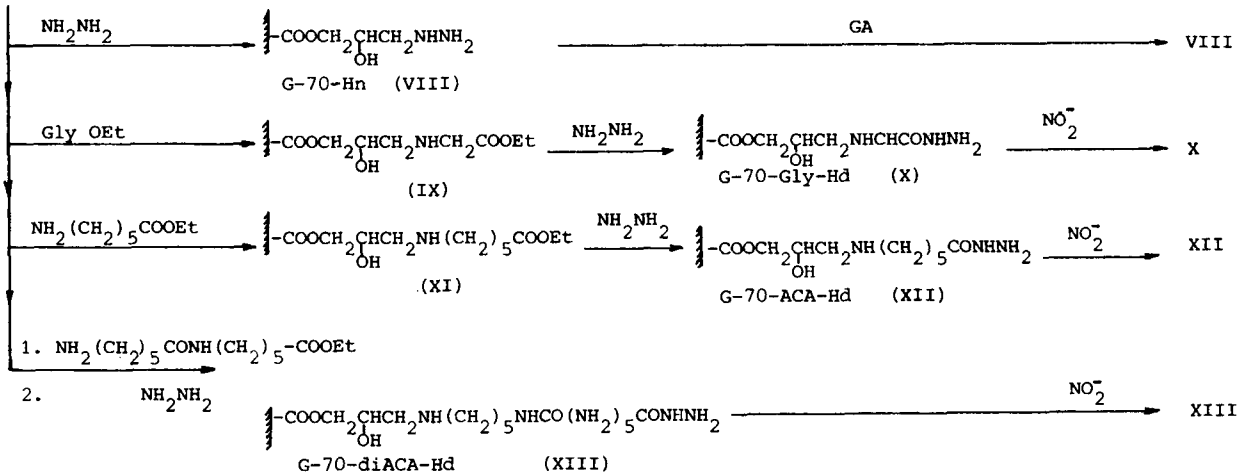



Fig. 1. Schematic pattern of poly(glycidyl methacrylate-co-ethylene dimethacrylate) carrier modifications and of immobilization reactions.

and 3 g G-70 were added. The suspension was refluxed 9 hr, washed (checked as with IV for completion), and dried. The resulting carrier (G-70-EDApAB) contained 53.0% C, 7.10% H, and 4.66% N, which indicated 1.11 mequiv 4-aminobenzoic residues/g. It turned dark red upon diazotation and coupling with β-naphthol.

Procedure IV(b) was followed when immobilization was tested by diazotation.

*Carrier Modification with L-Lysine (G-70-Lys) (VI)*

G-70 (50 g) was heated under reflux on a steam bath with 100 g L-lysine and 600 ml 4*M* NaOH. The carrier (G-70-Lys) contained 55.56% C, 7.32% H, and 1.14% N (corresponding to 0.4 mequiv lysine/g). Immobilization with GA and CDI was tested as above (II(a) and II(b)).

*Additional Modification with Phosgene (G-70-LysNCA) (VII)*

G-70-Lys (20 g) was suspended in 200 ml dry dioxane. Phosgene was bubbled (4–8 bubbles/sec) at 25°C for 2 hr. Then it was removed by dry nitrogen, the carrier was filtered off, and washed with dry dioxane. It was dried under vacuum directly in the reaction flask. The carrier contained 44.9% C, 6.73% H, 0.98% N, and 8.53% Cl. One g of this resulting G-70-LysNCA carrier was added to 5 ml of an ice-cool solution of 50 mg PA in a 0.05*M* borate buffer, pH 8.7, under vigorous mixing. The reaction was terminated after 20 min by addition of 10 ml 10% acetic acid.

*Carrier Modification with Hydrazine Hydrate (G-70-Hn) (VIII)*

G-70 (5 g) was refluxed for 7 hr with 25 ml 80% hydrazine hydrate and washed with water (washing checked by TNBS). The carrier contained 56.73% C, 8.31% H, and 5.23% N, corresponding to 1.85 mequiv/g hydrazine residues. The infrared (IR) spectra did not detect any residual oxirane rings. The modified carrier turned deep red with TNBS. Immobilization with GA was accomplished by the described procedure.

*Carrier Modification with Glycine Ethyl Ester (IX)*

G-70 (5 g) was suspended in 25 ml ethanol solution containing 3.15 g (20m*M*) glycine ethyl ester hydrochloride and 2.22 g (22m*M*) triethylamine. The mixture was heated at 75°C for 24 hr under reflux. The carrier contained 54.84% C, 7.18% H, and 1.66% N,

which corresponded to 1.19 mequiv/g glycine ethyl ester residue. The IR spectra detected about 30% unreacted oxirane rings.

*Additional Modification with Hydrazine Hydrate (G-70-Gly-Hd) (X)*

The above treatment procedure with hydrazine hydrate was followed. The carrier contained 52.20% C, 7.31% H, and 7.49% N. According to eq. (2), the content of glycine hydrazide residues was estimated to be 1.18 mequiv/g, and that of hydrazine residues 0.91 mequiv/g. The IR spectra failed to show any oxirane rings; the product turned red with TNBS.

PA was immobilized by the conventional azide method: 1 g G-70-Gly-Hd was suspended in 15 ml 1*M* HCl, cooled with ice, and 0.3 g $NaNO_2$ was added. The suspension was shaken at 4°C for 30 min. Then the carrier was filtered off, washed with the borate buffer, pH 8.2, with crushed ice, and 15 ml of the above buffer containing 50 mg PA were added. Shaking at 4°C was continued overnight.

*Carrier Modification with the 6-Aminohexanoic Acid Ethyl Ester (XI)*

The procedure used with the glycine ester was followed. The analysis (54.40% C, 7.27% H, and 1.23% N) showed 0.88 mequiv/g immobilized residues. After treatment with hydrazine hydrate (55.63% C, 8.01% H, and 4.9% N), the substitution of the carrier was calculated as 0.88 mequiv/g hydrazide and 0.43 mequiv/g hydrazine residues, respectively. The carrier G-70-ACA-Hd (XII) gave a highly positive reaction with TNBS and was used in the azide immobilization method.

*Carrier Modification with the Ethyl Ester of the Dimer of 6-Aminohexanoic Acid (XIII)*

The above procedures resulted in a carrier with 57.4% C, 8.0% H, and 1.76% N before and 54.27% C, 7.7% H, and 5.49% N after hydrazinolysis. Such composition indicated 0.62 and 0.76 mequiv/g hydrazide and hydrazine groups, respectively. Immobilization by the azide method was reapplied.

## RESULTS AND DISCUSSION

The amino acid composition of the enzyme preparation used is given in Table I, where the probable ratio of individual amino acids

is also given. It can be seen that the enzyme has enough nucleophilic groups in the side chains available for the reaction with electrophilic groups in the carrier, as well as tyrosine residues for coupling with diazonium groups. The analysis did not reveal the content of cysteine and free carboxyls; however, we did not consider the reaction with sulfhydryl groups important for the immobilization techniques, since cysteine is either in the form of cystine or is part of the active center and therefore cannot be used for immobilization. The content of aspartic and glutamic acid residues is very high, so that a reasonable probability exists that there are enough carboxyls free for binding by CDI activation.

The stability of the enzyme in 0.1*M* phosphate buffers, pH 4.2 and 7.0, and 0.05*M* borate buffer, pH 8.7, was tested during a week. The lost of activity of room temperature was 2% at neutral pH and 6.7% at low and high pH, respectively. Thus, the enzyme can be used in long-term experiments and over a broad range of pH values. The Michaelis constant was found to be $K_m = 2.1 \times 10^{-5}M$ and $V_{max} = 3.62 \times 10^{-6}$ mol/mg/min, i.e., 3.62 U/mg. Since the phenylacetic acid, which is the product of the reaction, is also an enzyme inhibitor, we measured the inhibition constant and found it to be $K_i = 5 \times 10^{-4}M$. The activity increased with increasing pH, but due to the lability of the substrate, above pH 8 the optimum could not be measured. The results of direct immobilization at pH 4.2, 7.0, and 8.7 are given in Table III, which also shows that the washing procedure using the detergent safely removed all adsorbed enzyme (zero activity at zero time of incubation at all of pH values tested). The detergent did not affect the activity of the either the free or bound enzymes.

The effect of temperature and enzyme concentration on the efficiency of the direct immobilization on oxirane carrier is shown in Table IV. It shows that with increasing enzyme concentration the

TABLE III
Immobilization of PA on Unmodified G-70 at Various pH[a]

| Hour / pH | 0 | | 73 | | 293 | |
|---|---|---|---|---|---|---|
| | (U/g) | (%) | (U/g) | (%) | (U/g) | (%) |
| 4.2 | 0 | 0 | 6 | 3.3 | 16.2 | 9.0 |
| 7.0 | 0 | 0 | 5.2 | 2.9 | 19.2 | 10.6 |
| 8.7 | 0 | 0 | 3.8 | 2.1 | 8.8 | 4.8 |

[a] Carrier (1 g) shaken at 25°C in 5 ml buffer containing 50 mg (= 180 U) PA. Results are given in U/g and in % of immobilized activity; time of incubation in hr.

TABLE IV
Immobilization of PA on Carrier G-70 at Various Temperatures and with Various Amounts of Enzyme[a]

| PA (mg in 5 ml) | Immobilization conditions: temp (°C) | Immobilization conditions: time | Immobilized activity: (U/g) | Immobilized activity: (%) | Immobilized protein: amount (mg/g) | Immobilized protein: amount (%) | Immobilized protein: activity (%) |
|---|---|---|---|---|---|---|---|
| 50 | 25 | 240 | 19.1 | 10.5 | – | – | – |
| 50 | 30 | 240 | 20.2 | 11.2 | – | – | – |
| 50 | 37 | 240 | 23.0 | 12.7 | – | – | – |
| 50 | 40 | 240 | 20.0 | 11.0 | – | – | – |
| 50 | 50 | 240 | 10.1 | 6.1 | – | – | – |
| 4 | 25 | 92 | 0.52 | 3.6 | 1.96 | 49 | 7.4 |
| 15 | 25 | 92 | 2.62 | 4.85 | 4.1 | 27.3 | 17.75 |
| 50 | 25 | 92 | 7.86 | 4.40 | 10.4 | 20.8 | 20.95 |
| 300 | 25 | 92 | 42.00 | 3.9 | 25.7 | 8.6 | 45.3 |

[a] Carrier (1 g) shaken at given temperature with 5 ml phosphate buffer, pH 7.0, containing the given amount of enzyme. Immobilized activity is given in U/g dry product and in % of total activity used. Amount of immobilized proteins is calculated from the analysis of amino acids in the final product and the activity of immobilized protein is calculated in % of the original value (3.6 U/mg).

efficiency of protein binding decreased, but the enzyme retained more activity.

The results of other immobilization techniques are given in Table V. Some general conclusions can be derived from them. With the carrier that gave the most rapid immobilization (VII) enough protein is immobilized, but the enzyme activity is almost lost. The result is similar to that obtained with a low enzyme concentration and direct binding (Table IV). In the latter case the loss of activity is partially reversed by extended incubation, which did not increase the amount of immobilized protein but partially restored its activity. We explained this effect by the multipoint binding of the enzyme molecule, which destroyed the activity.

The effect of the spacer is also very clearly demonstrated. G-70-$NH_2$ and G-70-HMDA both provided amino groups for direct coupling of the enzyme by CDI or spacered binding via the GA residue. With a carrier with no intrinsic spacer (G-70-$NH_2$) the former method (II(b)) resulted in very tight binding of the enzyme molecule, whereas with G-70-HMDA (III(b)) there is a six-atom-chain separating the enzyme from the carrier. This steric difference was reflected in the activity of the immobilized protein by the factor of

TABLE V
Immobilization of PA on Carriers G-70 and G-60[a] and Their Modifications by Various Techniques[b]

| IP[c] | Binding groups (mequiv/g) | Immobilized activity (U/g) | Immobilized activity (%) | Immobilized protein (mg/g) | Immobilized protein (mg/mequiv) | Immobilized protein activity (%) |
|---|---|---|---|---|---|---|
| I[d] | 4.45 | 7.9 | 4.4 | 10.4 | 2.34 | 21 |
| I[e] | 4.45 | 19.2 | 10.6 | 11.0 | 2.47 | 48.6 |
| I+[f] | 3.50 | 20.0 | 11.1 | 27.3 | 7.8 | 20.4 |
| IIa | 2.66 | 66.6 | 37.0 | 44.6 | 16.8 | 41.5 |
| IIb | 2.66 | 2.6 | 1.44 | 14.6 | 5.5 | 5.0 |
| II+a | 1.77 | 70.3 | 39.0 | 46.5 | 26.2 | 42 |
| IIIa[g] | <1.4 | 44.4 | 24.5 | – | – | – |
| IIIa[h] | <1.4 | 51.2 | 28.3 | 45.1 | >32 | 31.5 |
| IIIb | <1.4 | 45.8 | 25.4 | 22.5 | >16 | 56.5 |
| IVa | 0.62 | 43.2 | 24.0 | 18.8 | 30.3 | 63.7 |
| IVb | 0.62 | 0 | 0 | – | – | – |
| V | 1.11 | 43.0 | 23.8 | 35.1 | 31.6 | 34 |
| VIa | 0.4 | 13.4 | 7.4 | 6.8 | 17 | 54.7 |
| VIb | 0.4 | 0 | 0 | 2.13 | 5.3 | 0 |
| VII | ? | 0.88 | 0.48 | 17.8 | ? | 1.4 |
| VIII | 1.85 | 6.2 | 3.5 | 6.7 | 3.6 | 25.6 |
| X | 1.18 | 2.0 | 1.1 | 4.9 | 4.2 | 11.3 |
| XII | 0.88 | 7.3 | 4.0 | 11.9 | 13.5 | 17 |
| XIII | 0.62 | 3.1 | 1.7 | 5.4 | 8.7 | 16 |

[a] Indicated by super plus.

[b] PA (50 mg) (= 180 U) was added to 1 g carrier at 25°C (if not specified) and kept with gentle shaking. Immobilized activity is calculated per g dry preparation and in % of starting activity (= 180 U). Immobilized protein and its residual activity is calculated as in Table IV, and mg immobilized protein/mequiv binding groups is calculated in addition.

[c] Immobilization procedure, see Figure 1.

[d] pH 7, 92 hr.

[e] pH 7, 293 hr.

[f] pH 7, 72 hr.

[g] 4°C, 16 hr.

[h] 25°C, 16 hr.

ten (II(b) and III(b)), whereas the amount of immobilized protein calculated per one binding group was affected only by a factor of three. When the GA method, which provides additional spacer, is used, the carrier with an intrinsic spacer again binds more protein (III(a)), but its activity is lowered; which could be due to the mul-

tipoint binding facilitated by long flexible chains. On the other hand, this method preserved the activity with G-70-$NH_2$ carrier well (II(a)).

The important role of the surface area is apparent from the comparison of the results obtained with carriers based on G-70 (27.5 $m^2$/g) and G-60 (60.2 $m^2$/g) polymers. By direct binding, the latter carrier binds about three times more protein and by ammonia modification–in the GA method the immobilization is more than 50% higher when calculated per binding group. It is important that the activity of the immobilized protein is the same with either carrier.

The results with G-60 also showed that the measurement of the activity was not diffusion limited, at least with G-70, since the most frequent pore radius in G-60 is 25 nm, compared with 109 nm in G-70, thus the diffusion limitation should be more expressed with the former carrier. Instead, the highest immobilized activity was found with G-60-$NH_2$ after the GA method (II(a)).

The stability of the immobilized enzyme was measured in wet and lyophilized preparations. In the former case, 1 g (recalculated for dry weight) G-60-$NH_2$ GA-immobilized preparation (II(a)) was resuspended in 0.5*M* NaCl solution in a 0.01*M* phosphate buffer, pH 7, with traces of Thesit (it prevents bacterial growth). The suspension was kept at room temperature. The activity dropped after two months from 70.3 down to 42.5 U/g. No activity was found in the liquid. For the latter test, 1 g G-70-$NH_2$ GA-immobilized preparation (II(a)) (66.6 U/g) was resuspended (when wet) in 5 ml 25% sucrose solution, the excess liquid was poured off, and the rest lyophilized using ethanol–solid carbon dioxide cooling. After two months the activity remained unchanged. Similar results were obtained with all preparations listed in Table III.

The aim of this study was the evaluation of various immobilization procedures. It follows from the results that with stable nonexpensive enzymes direct immobilization is the method of choice. The amount of the enzyme should be higher than 100 mg/g carrier, the incubation longer than three days, and the temperature as high as the stability of the enzyme allows. Azide can be added to prevent microbial growth.

With less stable or more expensive enzymes the carrier should be modified by ammonia and activated by glutaraldehyde. This method is very efficient, giving yields of immobilized activity above $\frac{1}{3}$ of the activity added. However, some enzymes are inactivated by the reaction with glutaraldehyde groups (e.g., thiol proteases; un-

published results). In such cases more expensive methods using water-soluble carbodiimides or special spacer and diazotation have to be used.

Surprisingly, the azide method recommended for other carriers was relatively inefficient with respect to the spacer length. Also the combination of hydrazine treatment and glutaraldehyde activation remained far below the ammonia-modified carrier.

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