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RIGID SUPPORT MATERIALS FOR THE IMMOBILIZATION OF ENZYMES

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ABSTRACT

The purpose of the work presented here was to prepare a support material for enzymes and "affinity ligands" with the following characteristics: low cost, durability, rigidity, and high capacity. Our study encompassed conjugates of porous and nonporous silicas with organic polymers and macroporous ion-exchange resins. Polyethyleneimine (PEI), polyacrylic acid (PAA), poly(methyl vinyl ether/maleic anhydride) were attached to porous glass and silica in various combinations. The composite of silica beads with PEI and PAA is a good support for the enzyme trypsin as judged by the activity against N-α-benzoyl-L-arginine ethyl ester.

Amberlyst (macroporous, sulfonated polystyrene) was activated by treatment with thionyl chloride; the resulting resin was either used directly or reacted with a diamine. The diamine derivative was used for enzyme coupling or transformed further to the succinyl or p-aminobenzoyl derivative. None of these derivatives were particularly good as supports for the enzyme trypsin. Duolite converted to a PAA, succinyl, or succinimide derivative was a good support. The enzyme-resin adduct has good activity and stability.

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The resin is quite durable and of low cost. The Duolite-trypsin has good activity against protein. In addition, this derivative was active in 7 M urea. The proteolytic activity was nearly doubled by urea, presumably as a result of substrate (casein) denaturation. The michaelis constants and pH dependences are compared for trypsin conjugates with Duolite A-7, Silica-PEI-PAA, agarose, and porous glass. A cost comparison reveals that the Duolite and silica derivatives are much less expensive than agarose and glass.

INTRODUCTION

Enzyme immobilization and affinity chromatography have had a dramatic impact on the application and isolation of enzymes [1-3]. Of obvious importance to both areas is the support material to which enzymes or affinity ligands are bound (for reviews, see Refs. 4 and 5). The ideal support would have all the characteristics listed in Table 1. The degree to which a carrier must meet these requirements is, of course, a function of the application for which it is intended. For a small-scale application which yields a valuable product, cost and durability are not important. For a large-scale process such as enzymatic treatment of industrial waste, the cost and life of the carrier might well represent the limiting factors. Porous glass and agarose are widely used supports. Both materials are expensive. Glass is friable unless coated with zirconium oxide which raises the cost still further. Agarose gel is compressible and susceptible to microbial attack.* Both glass and agarose are porous. It is our goal to develop an inexpensive, rigid support which is porous or of high surface area. Our approach was to attach various polymers to porous glass in the hope of retaining rigidity and amplifying binding capacity. Thus reducing the amount of carrier needed. Second, we bound organic polymers to colloidal silica (Figs. 1 and 2). This support is inexpensive, and

*Although the manufacturers of agarose recommend protection against microbial contamination with a disinfectant, Prof. Jerker Porath has suggested that the problem is not as severe as many believe.

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TABLE 1

Criteria for the Ideal Support Material for Enzyme Immobilization and Affinity Chromatography. Some Materials in Use Have Many of These Characteristics. For Some Applications a Carrier Which Meets Only a Few of these Requirements may be Acceptable.

---

1. Chemical functionality (or route thereto) sufficiently general to permit reaction with a variety of groups on proteins and ligands for affinity chromatography
2. Low cost
3. Resistance to microbial attack
4. Dimensional stability - the retention of physical shape following changes in pressure temperature, solvent composition, etc.
5. Durability - the support material should not erode, react chemically with the solvent, or break when used in suspension
6. Hydrophilic - many proteins denature at hydrocarbon-water interfaces
7. Regenerable - the feature of carrier reuse could be advantageous. However, if the support is of low initial cost, this may not be critical
8. High capacity for enzyme or ligand
9. Accessibility to solvent or high porosity

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suitable for use in suspension-type reactors. Finally, we studied rigid, macroporous ion-exchange resins which are of low cost (Figs. 3 and 4).

EXPERIMENTAL

Materials

Trypsin (lot TRL-20A) with specific activity of 40 units/mg against N-α-benzoylarginine ethyl ester at pH 8.0 was obtained from Worthington Biochemical. Agarose (Sepharose-4B) was a product of

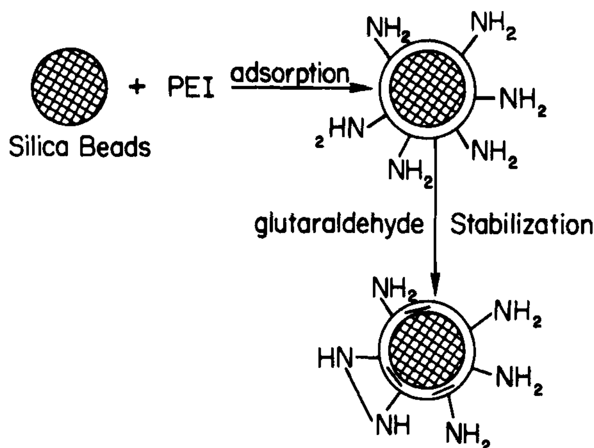

Fig. 1. Preparation of silica-PEI. An adsorption step is followed by a cross-linking step with a bifunctional reagent such as a glutaraldehyde.

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Pharmacia. The alkylamine porous glass (550 Å) was obtained from Pierce Chemical Co. Duolite A-7 was a generous gift of Dr. Richard Harris of the Diamond Shamrock Chemical Co. Amberlyst (16-50 mesh, 4.9 meq-SO_3H/g) was supplied by Mallinkrodt. PEI-600 and PAA (50,000 MW) were from Polyscience; GAN from GAF Corp. DMF (MCB, spectrograde) was treated with anhydrous $MgSO_4$ and molecular sieves. N,N-Dimethyl casein was prepared by the method of Lin et al. [6]. All other chemicals were reagent grade and used without further purification.*

Methods

Preparation of Supports

PEI was bound to glass through glutaraldehyde. The alkylamine porous glass (1 g) was added in increments to 20 ml of 2.5%

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*Abbreviations used: PEI, polyethyleneimine; PAA, polyacrylic acid; GAN, poly(methyl vinyl ether/maleic anhydride); CMC, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluenesulfonate; DMF, dimethylformamide; TNBS, trinitrobenzenesulfonate.

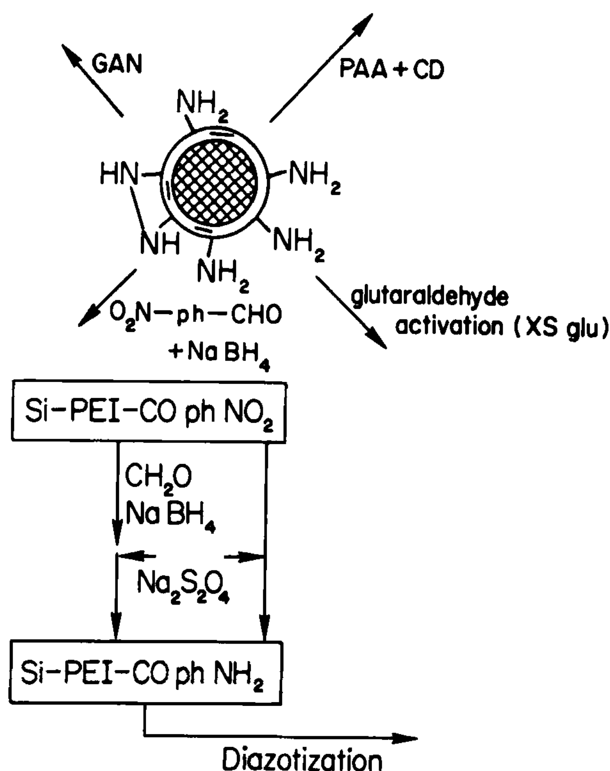

Fig. 2. Reaction pathways to various derviatives of silica-PEI.

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glutaraldehyde at 0°C. The suspension was stirred with an overhead mechanical stirrer and the pH was maintained at 7.0. Addition of glass was over a 40-min period. After an additional 30 min the glass was filtered and washed with 500 ml of cold distilled water. The derivitized glass was then added to 100 ml of PEI solution (1 mg/ml) adjusted to pH 8.0. This suspension was stirred for 2 hr. The glass-PEI (5% w/w) was acylated by refluxing in $CHCl_3$ with p-nitrobenzoyl chloride and triethylamine, both in 10-fold excess of the primary amine on the support. The nitro group was reduced by refluxing for 1 hr in 10% sodium dithionite. In an effort to improve the yield of enzyme coupling, we reductively methylated [7] the remaining amines prior to reduction of the nitro group.

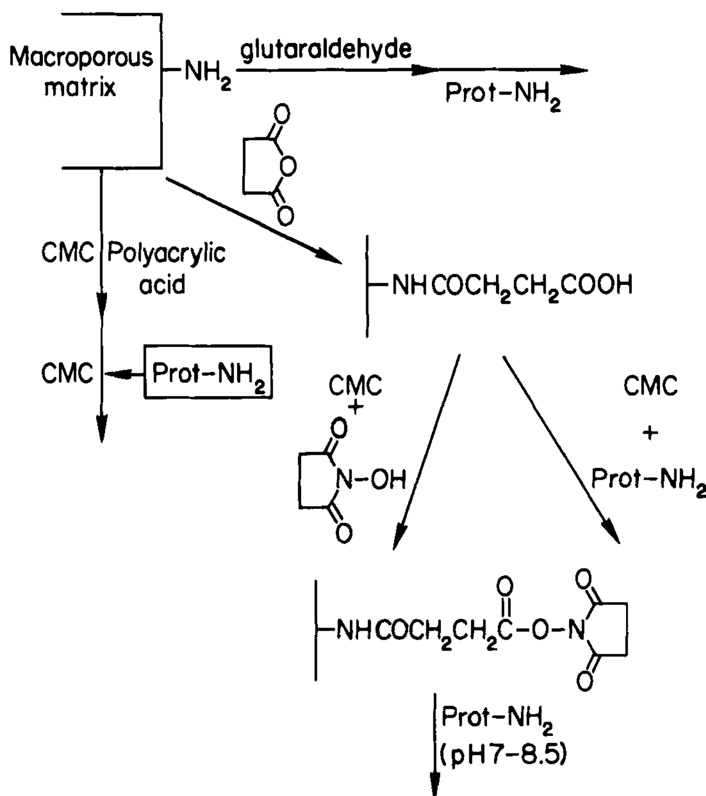

Fig. 3. Activation of Amberlyst. The initial step is the production of the sulfonyl chloride. This derivative may then be reacted with proteins or further derivitized by reaction with HDA (hexamethylenediamine). The resulting derivative may be treated still further with conventional techniques.

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Glass-PAA was prepared with a water-soluble carbodiimide. Alkylamine glass (1 g) and 125 mg of PAA was stired with 100 mg CMC in 10 ml H_2O. The pH was maintained at 4.0 for 30 min at room temp-

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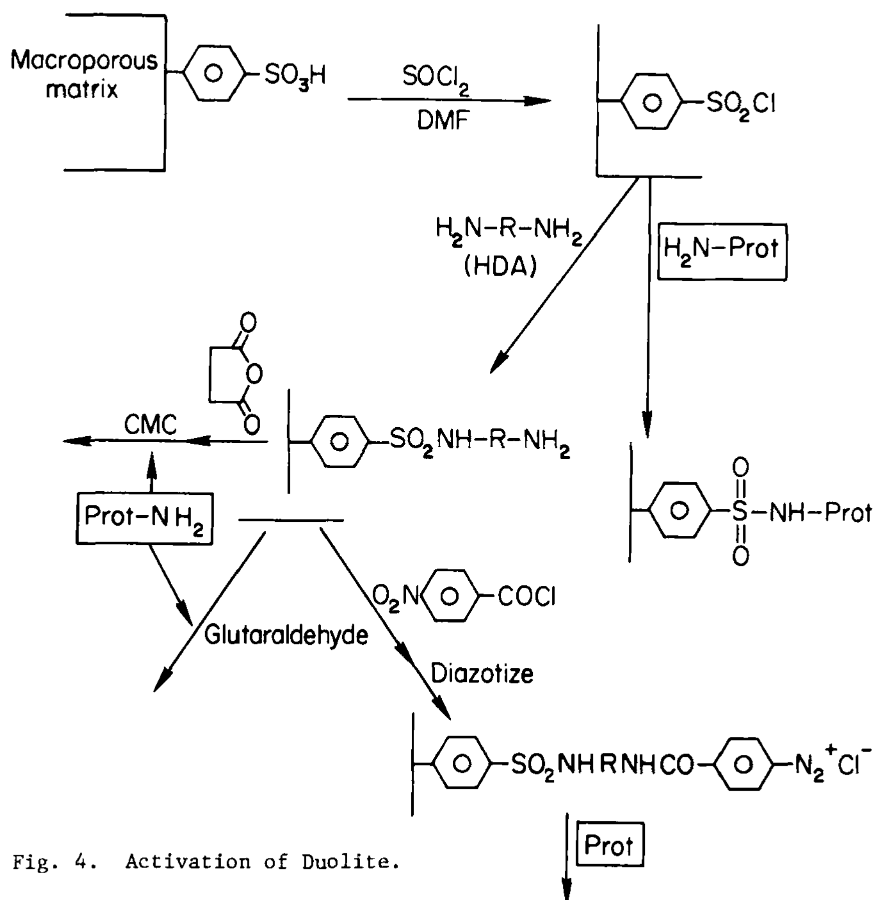

Fig. 4. Activation of Duolite.

erature. The suspension was then stirred overnight at 4°C. The glass-PAA was filtered and washed repeatedly with 4 M KCl and distilled water. This derivative bound 0.15 mmoles of glycine ethylester/g by the method of Line et al. [8].

Silica-PEI was prepared by adsorption of PEI on silica followed by cross-linking with glutaraldehyde. To 2 liters of 10 mM sodium borate were added 20 g of silica beads (Sigma) and 8.75 g PEI. This suspension was stirred in a blender for 15 min. Glutaraldehyde (410 ml, 2.5%) was then added followed by 10 g $NaBH_4$. This mixture was blended for 10 min. The silica-PEI was collected by centrifugation and washed repeatedly with distilled water. The resulting

particles were 5 to 50 μm in diameter. Silica-PEI was reacted with GAN by rotation of 75 mg solid with 30 mg GAN and 5 ml dry pyridine in a closed tube for 2 hr. at room temperature. The solid was washed with methanol and dried. The existence of anhydride groups on the solid support was demonstrated by the hydroxamate test. Silica-PEI-PAA was prepared in a manner similar to Glass-PAA. Silica-PEI (250 mg), 125 mg PAA, and 50 mg CMC were reacted in 10 ml volume.

Derivatization of Amberlyst-15 was preceded by extensive washing with DMF under vacuum. One gram of resin was suspended in dry DMF, and 0.72 ml of freshly distilled thionyl chloride was added dropwise at 0°C with rapid stirring. The suspension was allowed to return to room temperature and stirred overnight. The beads were then washed several times with dry DMF and cooled to 0°C. Hexamethylenediamine (1.16 g in 10 ml of DMF, precooled) was added dropwise over a period of 1 hr. The suspension was then stirred overnight at room temperature. Succinylation of hexamethylenediamine-Amberlyst and Duolite A-7 was carried out with 5-fold excesses of succinic anhydride and triethylamine in DMF. The disappearance of amine may be followed with the ninhydrin procedure of Moore [6] using 5 mg of resin. The succinylated beads were washed with water and stirred for 45 min at room temperature in 0.1 N NaOH. The derivatives showed no base-sensitive groups remaining, and the ninhydrin reading remained negligible. The derivatives were stored under distilled water at 4°C. No evidence of microbial growth was apparent over a period of 6 months.

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The N-hydroxysuccinimide ester of succinyl-Duolite A-7 was prepared by reacting 2 g of damp resin with 2.3 g of N-hydroxysuccinimide and 8.4 g of CMC in 40 ml of dry DMF. After 4 hr. considerable insoluble urea appears. The resin is washed with cold, "super dry" ethanol and dry DMF. The product is stored under dry DMF in a dessicator.

Polyacrylic acid was bound to Duolite A-7 through CMC. Duolite A-7 was stirred with distilled water under reduced pressure for several hours. The hydrated resin (1 g) was mixed with 100 mg of

CMC and 200 mg of a 25% solution of polyacrylic acid. The pH was adjusted to 4.5 and maintained there for 3 hr. with 0.1 N NaOH. The derivative was washed with 2 N KCl and distilled water.

Enzyme Coupling

Arylamine derivatives were diazotized as previously described [9]. Coupling with trypsin was performed at pH 8.0 in 2 mM Tris HCl with 25 mM $CaCl_2$ at 0°C. Trypsin was bound to the carboxyl derivatives by the method of Line et al. [8]. Trypsin coupling via glutaraldehyde was performed by the same procedure used for attaching PEI to arylamine glass presented earlier. Reaction of trypsin with the N-hydroxysuccinimide ester of succinylated Duolite A-7 was done in 0.2 N Hepes HCl buffer, pH 8.0, containing 25 mM $CaCl_2$.

Enzyme Assays

Benzoyl-L-arginine ethyl ester (Fig. 3) is a synthetic substrate of low molecular which is hydrolyzed rapidly by the enzyme trypsin. The reaction can be conveniently followed titrimetrically. We employed the Radiometer SBR3 titrigraph, ABU12 autoburette with the TT11 titrator. The reaction vessels were stirred mechanically at speeds sufficient to minimize external diffusion. A nitrogen stream was blown over the surface of the reaction mixture. Conditions were as follows: 1 mM substrate, 25°C, 10 mM standard base, 10 ml reaction volume, and 5×10^{-4} M Tris HCℓ.

Casein was reductively methylated to reduce the background absorbance in the trinitrobenzenesulfonate (TNBS) assay [6] (Fig. 5). We found this assay for proteolytic activity superior to titrimetric or ninhydrin procedures. In the proteolytic assays the amount of bound enzyme used was based on a standard activity against the low molecular weight substrate, benzoylarginine ethyl ester. The activities reported in Table 4 are therefore relative activities which could reflect the accessibility of the enzyme bound to the solvent. A representative plot of caseinolytic activity versus enzyme concentration is shown in Fig. 6. The shape of this curve is quite similar to that of the free enzyme.

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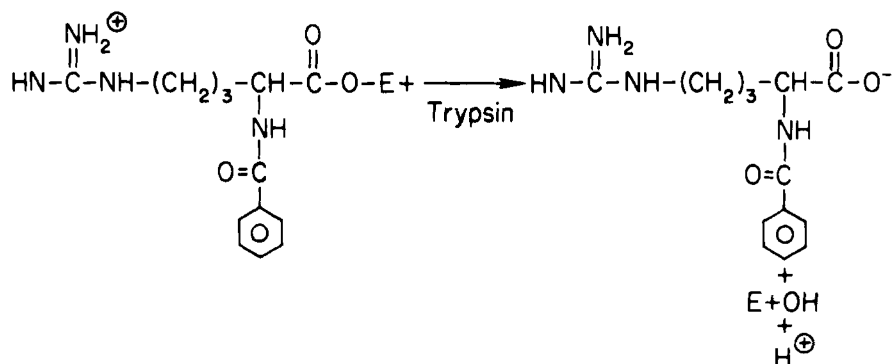

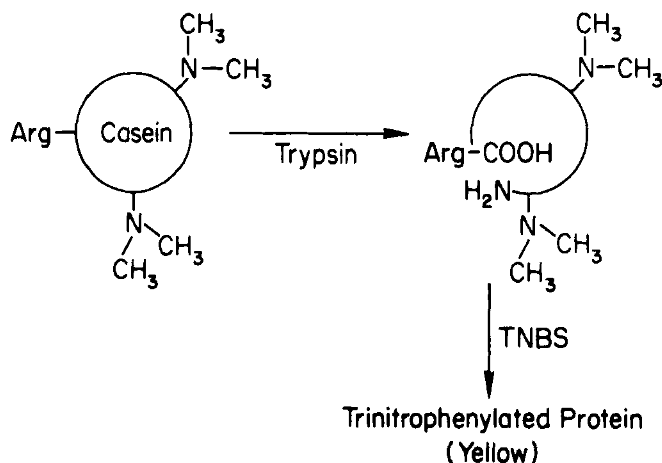

Fig. 5. Assays of trypsin. In the top reaction the trypsin-catalyzed hydrolysis of benzoylarginine ethyl ester is shown. One equivalent of acid is produced. In the bottom reaction the hydrolysis of N,N-dimethyl Casein is shown. Peptide bond hydrolysis produces a free amino group which may be derivatized by reaction with trinitrobenzene sulfonate.

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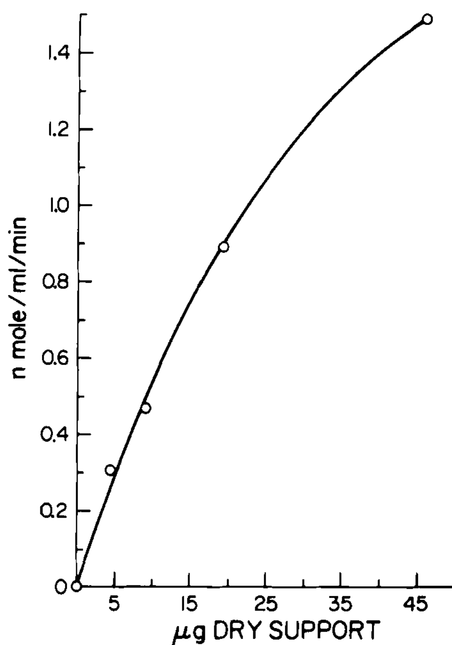

Fig. 6. A representative plot showing the dependence of the activity of agarose-trypsin on concentration of enzyme. The substrate is N,N-dimethyl casein. Use of the TNBS assay permits the spectrophotometer determination of the nmoles of amides broken/mg/min [6].

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RESULTS

One goal of this study was the amplification of the enzyme-binding capacity of porous glass. We reasoned that a polymer coating should provide more functional groups through which to bind enzyme with no significant loss in the rigidity of the support. The arylamine derivative of PEI-glass proved inferior to unmodified porous glass as an enzyme support (Table 2). The possibility of reaction within the particle during the activation and coupling steps was addressed by methylation of the remaining primary amines

TABLE 2

Summary of Activities of Trypsin Conjugates with Polymer-Coated Inorganics

Support	Activity (units/g derivative)
Porous glass	110
Glass-PEI[1]-Ph-NH_2	69
Glass-PEI(CH_3)-Ph-NH_2	55
Glass-PAA[2]	59
Silica-PEI-GAN[3]	82
Silica-PEI-PAA	300

1. PEI; polyethyleneimine.
2. PAA; polyacrylic acid.
3. GAN; poly(methyl vinyl ether/maleic anhydride), GANTREZ-AN 119, GAF Corp.

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prior to reduction and diazotization of the arylamine groups. The methylation provided a derivative less effective than the unmethylated one.

PEI is a globular polymer; we decided to investigate a linear polymer, PAA. The resulting glass-PAA was also inferior to porous glass as a support for trypsin. Our attention then turned to Silica-PEI. We coated silica beads with a mantle of PEI which was subsequently stabilized by cross-linking with glutaraldehyde (Fig. 1). Linear polymers were then added in hopes of producing a rigid bead with projecting linear polymers as sites for enzyme binding. The PAA derivative was superior to GAN derivative (Table 2). The activity of the trypsin complex of Silica-PEI-PAA was initially about 3 times greater than porous glass. After extensive washing and repeated use, the activities became about equal (Table 4). The Michaelis constant for the enzyme on silica-PEI-PAA, however, is more than double that for enzyme on glass.

Amberlyst is a cross-linked polystyrene with a macroporous, rigid structure. Our primary concern at the outset was the apolar character of polystyrene supports. As shown in Table 3, Amberlyst is not a particularly good support for trypsin. Reactions of the Amberlyst in DMF went smoothly. However, the use of water as a solvent was not successful for enzyme binding. This result was not surprising since it is well known that many proteins denature at hydrocarbon-water interfaces [10]. In an investigation of an insoluble Edman reagent, Dowling and Stark used glucosaminol to reduce the hydrophobicity of an isocyanatopolystyrene [11]. This approach applied to our system failed to promote protein binding or enzyme

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TABLE 3

Activity of Trypsin Bound to Derivatives of Macroporous Ion Exchange Resins

Support	Derivative	Coupling Reaction	Specific Activity[1]
Amberlyst-15	$-SO_2Cl$	Direct-sulfonamide	1.7
	$-SO_2-NH(CH_2)_6NH_2$	Glutaraldehyde	4.9
	$-SO_2NH(CH_2)_6NHCO-CH_2CH_2COOH$	CMC[2]	1.7
	$-SO_2NH(CH_2)_6NH-COphNH_2$	Diazonimum salt	0.6
Duolite A-7	Unmodified	Glutaraldehyde	0.9
	$-NH(CH_2CH_2)_nCOOH$	CMC	11.4
	$-NHCOCH_2CH_2COOH$	CMC	21.7
	$-NHCOH_2CH_2COOH$	N-Hydroxysuccinimide ester	10.3 (23)[3]
Porous glass	$-Si(OR)_2R'NHCO-phNH_2$	Diazonium salt	100 (110)[3]

1. μ mole/min/g carrier, substrate is 1 mM benzoylarginine ethyl ester, at pH 8.0, 25 mM $CaCl_2$ and 25°C.

2. 1-Cyclohexyl-3-(2-morphollinoethyl)-carbodiimide metho-p-toluene sulfonate.

3. Values in parentheses were determined at pH 9.5.

stability. Although these derivatives of Amberlyst are not satisfactory for the enzyme trypsin, they should be good supports for enzymes which naturally occur in a hydrophobic environment.

The results shown in Table 3 indicate that Duolite-A7 derivatives of trypsin have activities of the same order of magnitude as porous glass. These rates were determined at saturating levels of benzoylarginine ethyl ester with new derivatives washed with dilute buffer only. The succinylated Duolite activated by a water-soluble carbodiimide or by an N-hydroxysuccinimide ester is the most promising form of enzyme carrier, at least for trypsin.

A typical loss of activity of a bound enzyme as a function of washing with concentrated salt solutions is shown in Fig. 7. After extensive washing with concentrated salt solutions and numerous assays, "plateau" values of specific activity (act/g resin) were determined. The results are shown in Table 4. All four derivatives are in the same range of activity. The comparison is admittedly only an approximation since the ages of the preparations were not exactly identical. It may be noted that Duolite A7, activated by N-hydroxysuccinimide ester, is better than porous glass as a support for trypsin.

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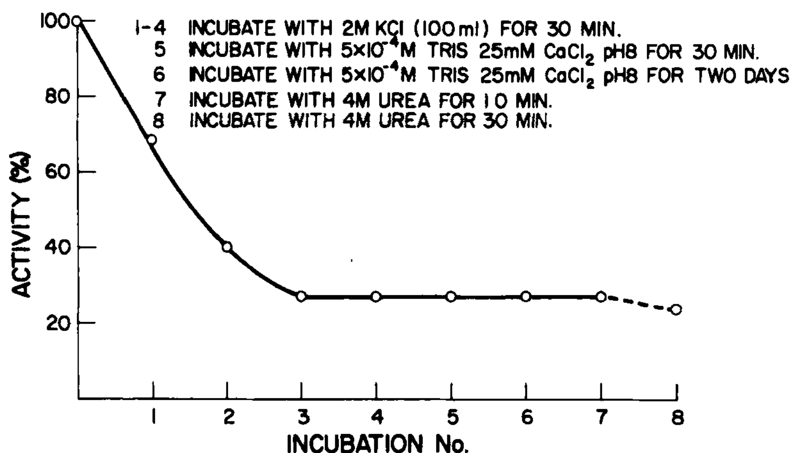

Fig. 7. Washing of agarose-trypsin with concentrated salt solutions.

TABLE 4

A Comparison of Duolite and Silica-PEI-PAA with Agarose and Glass as Supports for the Enzyme Trypsin. All Derivatives Were Extensively Washed with Concentrated Salt Solution and Assayed Many Times until a Constant Level of Activity Was Reached

Support	BAEE[1] Activity	Apparent K_m	Apparent pH opt.	Casein[2] Activity
Duolite A-7 (SIE)	1.01	0.8×10^{-4}	9.5	0.5 (0.9)[3]
Silica-PEI-PAA	0.79	1.4×10^{-3}	10	0 (0.7)[4]
Agarose	1.59	0.4×10^{-3}	10	0.9
Glass (AZO)	0.88	0.6×10^{-3}	9.5	0.5

1. Activity against N-benzoyl-L-arginine ethylester at pH 9.0, μ mole/min/g support.
2. Activity against N,N-dimethyl casein normalized to a constant BAEE activity, pH 9.0 (nmole amine released/ml/min).
3. Determined with reaction mixture which contains 7 M urea.
4. Determined at pH 8.0.

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The apparent Michaelis constants which appear in Table 4 were determined under conditions where external diffusion was not limiting. The pH of the bulk solution for these determinations was 9. It is clear, however, from the data in Fig. 8 that "local" pH effects are operating. In the hydrolysis of benzoylarginine ethyl ester, a proton is produced (Fig. 5). Accumulation of protons within the porous matrix would tend to raise the apparent pH optimum. Electrostatic effects would also contribute to the apparent K_m (see next section).

The proteolytic activities of the various bound enzyme derivatives are shown in Table 4. The assay employing TNBS and N,N-dimethylcasein is very sensitive and more acceptable than the assays based on TCA-soluble peptides of ninhydrin. Figure 6 is a representative plot showing the dependence of activity on enzyme concentration. The soluble and insoluble trypsins show very similar concentration

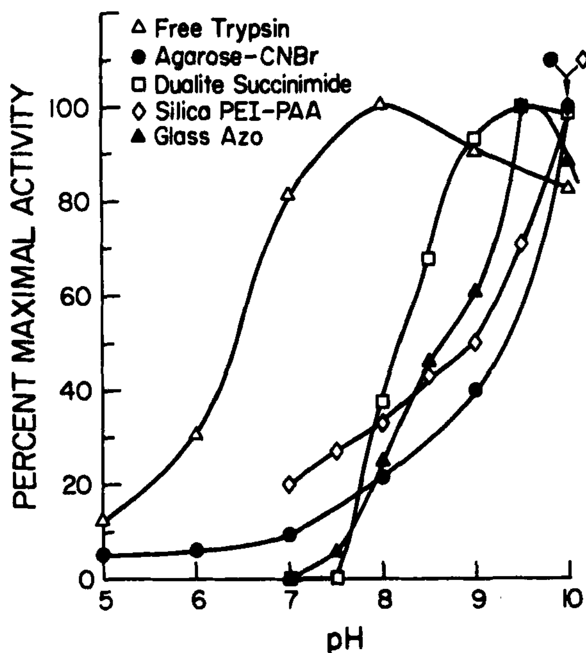

Fig. 8. The pH-dependence of the esterolytic activity of various insoluble derivatives of trypsin. Notice that the pH optima of all the derivatives, regardless of charge, are increased from that of free trypsin.

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dependences. The proteolysis rates shown in Fig. 4 are from the linear parts of the plots of activity vs enzyme concentration.

It is significant that the Duolite-trypsin is activated by the presence of 7 M urea. The activity increases from 0.5 nmole amine released/ml/min in dilute buffer to 0.9 nmole amine released/ml/min in 7 M urea. The most reasonable explanation for this observation is that the casein is unfolded by urea and made more susceptible to proteolysis. Since casein is relatively accessible in dilute buffer, one would expect even larger effects with more compact proteins. Prolonged exposure of the Duolite-trypsin, unfortunately, leads to inactivation of the enzyme.

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DISCUSSION

The pH profiles shown in Fig. 8 cannot be examined by the conventional formulations of electrostatic effects based on equilibrium thermodynamics (Ref. 12 and references therein). In the simplest case the pH optimum of an enzyme will be unchanged when the enzyme is on a neutral support, raised when on a negatively charged support, and lowered when on a positively charged support. The rationale for these observations may be expressed both in qualitative and quantitative terms. When the enzyme is in the midst of negative charges, the "local" pH is lower near the enzyme than in the bulk solution. For the enzyme to reach maximal activity, therefore, the pH of the bulk solution must be higher than in the case of an enzyme free in solution or on a neutral carrier. For an enzyme bound to a positively charged carrier, the "local pH" is above that of the bulk solution (H^+ is repelled). This condition of course results in a decrease in the apparent pH optimum based on the pH of the bulk solution.

Using the concept of electrochemical potential, it can be shown that

$$\frac{a'_{H^+}}{a_{H^+}} = e^{-\varepsilon\psi/kT} \qquad (1)$$

in which a'_{H^+} is the activity of the proton within the matrix and a_{H^+} is the activity of the proton in the bulk solution. The charge on the electron is represented by ε, ψ is the electrostatic potential, k is Boltzmann's constant, and T is the absolute temperature. Taking logs and rearranging, Eq. (1) becomes

$$pH' - pH = \Delta pH = 0.43\varepsilon\psi/kT \qquad (2)$$

The ΔpH is reflected by the change in pH optimum of the bound enzyme. At ψ = 150 mV, ΔpH would be about 2.5 units.

It is clear that the pH profiles shown in Fig. 8 cannot be explained by the forgoing treatment. Agarose, for instance, is neutral or slightly positively charged after CNBr treatment. However, the ΔpH shown in Fig. 8 would suggest a very high degree of

negative charge. In stronger buffer at about the same ionic strength, we see a much smaller ΔpH. The large effect we see in Fig. 8 may be explained by proton accumulation within the matrix. Knights and Light have recently reported the same observation [13]. In addition, for the trypsin-catalyzed hydrolysis of substrates such as amides where protons are not produced, very little change is pH optimum is observed for glass and agarose derivatives of trypsin.

Using glass and agarose as standards, it appears that the silica-PEI-PAA and the succinyl derivative of Duolite-A7 have considerable promise as support materials. In small quantities, glass and agarose are orders of magnitude more expensive than the silica-PEI-PAA or the Duolites. The Duolite has the advantages that it is rigid, durable, and macroporous. It can be stirred with any type of stirring device including a magnetic stirrer bar.

We have recently tested carbohydrate adducts of porous glass as enzyme supports. Glycophase-G and CPG/Dextran are produced for gel permeation and affinity chromatography by Corning Glass and Distributed by Pierce Chemical. The Glycophase-G is prepared by coating porous glass with a glyceryl silane. We have activated this material with CNBr and immobilized trypsin. The resulting derivative had 75% of the activity of agarose-trypsin. We found this encouraging since the Glycophase-G could be used in columns at high flow rate. The lack of nonspecific adsorption could be an advantage in many cases. In addition, the hydrophylic nature of the surface should make it compatible with a wide range of enzymes. With the exception of cost, this material fulfills every criterion listed in Table 1.

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