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AFFINITY PURIFICATION METHODS IV. THE AMINO(2-HYDROXY)PROPYL ETHER-LEASH IN BIOSELECTIVE SORBENTS

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The amino(2-hydroxy)propyl ether derivative of agarose is obtained by treating the gel in 0.3 M NaOH with epibromohydrin at 30°C for 4 h, followed by a 16-h reaction with 1 M NH_4OH. The derivative is nonionic and chemically stable. Established coupling methods can be used to attach biochemical substances to this amino-leash. A variety of reaction parameters have been studied to optimize the epibromohydrin reaction step. It is shown that the amino-leash agarose gel can be readily used to prepare hydrophobic sorbents, affinity sorbents, immobilized enzymes, and immunosorbents.

INTRODUCTION

Recent studies suggest the need for inert, nonionic, and nonhydrophobic leash or tether structures by which ligands may be connected to insoluble carriers to yield useful affinity sorbents (1–4). The coupling of substances to agarose by the cyanogen bromide reaction is easy to perform, but is attended by two deficiencies: The isourea linkage that results from the reagent is cationic and is somewhat labile (5,6). These problems may be avoided by the use of coupling reagents that result in ether linkages to the carrier. Epihalohydrins and bisoxiranes (bifunctional epoxides) are two groups of reagents that have been found promising for this purpose.

Epichlorohydrin was employed by Schell and Ghetie as a cross-linking agent to improve the properties of agarose gels (7,8). Porath and coworkers have described the use of epichlorohydrin and bisoxiranes to cross-link agarose, as well as to immobilize biochemical substances onto it (9). A recent work by Sundberg and Porath has detailed the use of 1,4-butanediol diglycidyl ether (a bisoxirane) as a cross-linking and coupling agent to agarose (10).

In our search for alternatives to the cyanogen bromide coupling method, we were attracted to the epoxide reagents. The bisoxiranes were not appealing, however, because the two reactive ends of these molecules

lacked a differential selectivity. On the other hand, epihalohydrins could show differential reactivity depending on reaction conditions. For instance, the early work by Fairbourne et al. (11) suggested that in aqueous alkali, alkoxide anions attacked the epoxide ring of the epihalohydrin, and this attack was followed by an allylic rearrangement of the oxygen and expulsion of the halide ion. Since bromide ion is generally a better leaving group than chloride, it seemed that epibromohydrin should be more reactive than epichlorohydrin and a useful bifunctional reagent for both cross-linking and coupling to agarose. Our efforts were thus directed to a more detailed understanding of this reagent (with comparisons to epichlorohydrin and bisoxiranes where appropriate), as well as to its use in forming amino(2-hydroxy)propyl–agarose, a gel derivative of possibly wide utility in the preparation of affinity sorbents.

MATERIALS AND METHODS

Materials

Gel beads of 2%, 4% and 6% agarose density (respectively, Sepharose 2B, 4B, and 6B) were obtained from Pharmacia Fine Chemicals. The EBH,[1] PSA, and *m*-nitrobenzamidine hydrochloride were obtained from Aldrich Chemical Co. The ECH and 50% glutaraldehyde solution were from Eastman Organic Chemicals. The NMP was from Burdick and Jackson Laboratories. The DMF and dioxane were from major suppliers. Trypsin and chymotrypsin were obtained from Worthington Biochemical Corp. The β-lactoglobulin was from Sigma Chemical Co.; Miles Laboratories Research Products Division was the source of BSA. The *m*-nitrobenzamidine was reduced to the amino compound as described previously (12).

Methods

Amino Color Test. Free amino groups in gel derivatives were detected by treatment with PSA, according to Inman and Dintzis (13). Varying intensities of yellow-orange were observed in positive tests.

Epoxide Color Test. A quick qualitative test for the presence of epoxide groups in gels was obtained by treating about 0.5 to 1 ml of the derivative

[1] Abbreviations: *EBH*, epibromohydrin; *ECH*, epichlorohydrin; *NMP*, *N*-methylpyrrolidone; *DMF*, dimethylformamide; *PSA*, picryl sulfonic acid; *HSA*, human serum albumin; *anti*-HSA, immunoglobulin that binds HSA; *TMAH*, tetramethylammonium hydroxide; *BSA*, bovine serum albumin.

with 1 ml 1 M sodium thiosulfate at pH 7 and a drop of 0.1% phenolphthalein in methanol. A pink-colored supernatant liquid developed in a few minutes at room temperature to indicate the presence of epoxide groups in the gel.

Epoxide Reaction with Agarose. The general handling procedures for agarose were as described previously (14). Typically, 25 ml (well settled) of beads thoroughly washed in water were used in a given reaction. The other reagents in the reaction were added in appropriate concentrations, so that the concentrations described in the "Results" section were obtained in a final reaction volume of 50 ml. For the reaction-time study, one large reaction sample (150 ml agarose + 150 ml reagent solution) was prepared and aliquots were withdrawn at appropriate intervals.

Amino(2-hydroxy)propyl–agarose. The glycidyl ether derivative of agarose obtained in the previous step was mixed with NH_4OH (25 ml gel + 25 ml 2 M NH_4OH) and shaken for 16 h.[2] For convenience, this structure is henceforth referred to as the amino-leash.

Leash Analysis. The *in gel* concentration of amino-leash groups was determined by titration with standardized NaOH (0.5 N), using the Mettler Automatic Titration System. The gels were thoroughly washed in 0.2 M KCl before titration. For 2% agarose, gel samples of 5 to 7 ml were used; for 4%, gel from 4.4 to 6.2 ml; and for 6%, gel from 4.2 to 5 ml. In each case, 0.2 M KCl was added to a final volume of 15 ml for the titration. As the titration control blank, 15 ml 0.2 M KCl was used.

Leash concentration was also determined by elemental nitrogen analysis. The gel volume was determined with a well-settled amount of gel beads, which were then thoroughly washed with water, methanol, and acetone. After drying in a heating block at 50°C overnight, the residue was weighed. The 2% gel yielded dry weights of 9.2–12.9 mg/ml; the 4% gel, dry weights of 29.1–33.5 mg/ml; the 6% gel, dry weights of 39.8–43 mg/ml. the weights varied according to the degree of derivatization. The nitrogen analyses were performed by the Roche Microanalytical Laboratory. Noting that each amino-leash contains only one nitrogen atom, the calculation was as follows:

$$[\text{mg(dry wt)/ml wet gel}] \times [\%\,\text{N}] \times [1\ \mu\text{Eq}/14\ \mu\text{g}] \times 1{,}000 = \text{leash concentration, } \mu\text{Eq/ml wet gel}$$

Hydrophobic Sorbent. For this preparation, 25 ml 4% agarose containing 2.7 μEq/ml gel of amino-leash was treated with 1 ml isobutyloxycarbonyl caprylic anhydride (0.6 mmol/ml DMF) and 24 ml DMF for 2 h at

[2]Treatment for 24 h did not increase yield; after 7 h of treatment, the reaction was about 89% complete.

room temperature (15). A negative test with picryl sulfonate indicated that all amino-leash groups had reacted. Functional tests were carried out at room temperature on a column with 0.9 × 12 cm bed with 1.3 mg BSA and 1 mg β-lactoglobulin dissolved in 1 ml 0.05 M acetate buffer, pH 6, containing 1.0 M Na_2SO_4.

Affinity Sorbent for Trypsin. For this preparation, 25 ml 4% agarose containing about 20 μEq/ml glycidyl ether groups was reacted with 0.6 g *m*-aminobenzamidine hydrochloride (12) in 25 ml water, with pH adjusted to 11 with 0.5 N NaOH. Reaction at room temperature was carried out overnight. The sorbent was tested with 10 mg trypsin dissolved in 1 ml 0.05 M. Tris–acetate, pH 7.5, with 0.1 M NaCl. As expected, the inactive protein passed through the column bed (0.9 × 14 cm) unretarded (12). The active enzyme was desorbed by including 25 mM *m*-aminobenzamidine in the buffer (above). The affinity column effluent was directed to a 1.28 × 27 cm Sephadex G-25 column, which yielded the pure active enzyme well separated from the *m*-aminobenzamidine.

Immobilized Chymotrypsin. For this preparation, 10 ml 4% agarose gel containing 22 μEq/ml amino-leash was mixed with 10 ml 50% glutaraldehyde solution and shaken at 30°C for 90 min. After thorough washing with water, the gel beads were suspended in 10 ml 0.05 M Tris–HCl, pH 7.5, containing 100 mg chymotrypsin, and the mixture was shaken at room temperature for 1 h. The beads were recovered by filtration and washed thoroughly with water. The filtrate contained 42.7 mg protein and 1712 U (out of 4750 input) activity, as measured with benzoyl-L-tyrosine ethyl ester (16). Immobilized chymotrypsin (8 g moist weight was the total recovery) was assayed similarly to free enzyme. A quantity of 7 mg (moist) immobilized enzyme was added to 1.5 ml buffer and 1.4 ml substrate. After 1 min of mixing, the suspension was quickly filtered, and the absorbance at 256 nm was measured. A rate of 0.332 ΔÅ/min was observed, which corresponded to 0.148 U/mg immobilized enzyme, or a total of 1,181 U immobilized chymotrypsin.

Immunosorbent. For this preparation, 25 ml 4% agarose containing 3.2 μEq/ml amino-leash was mixed with 25 ml 20% glutaraldehyde solution (obtained by dilution of 50% solution) and shaken for 1 h at room temperature. The gel beads were recovered by filtration and washed thoroughly with water. The activated beads were mixed with 2 ml HSA solution (5% in isotonic saline)[3] and 23 ml 0.05 M sodium phosphate, pH 7, then shaken overnight. The HSA–agarose conjugate was recovered by filtration and washed with water, 0.2 M KCl, 2 M guanidine hydrochloride,

[3]This solution was generously provided by Mr. Ed Newman, Department of Immunology, Hoffmann–La Roche, Inc.

3 M ammonium isothiocyanate, and finally with 0.25 M NaCl in 0.05 M sodium phosphate buffer, pH 7. With the final washes, no UV, absorbing material was eluted from the gel.

The sorbent was packed into a 0.9×12 cm column and preequilibrated with 0.25 M NaCl in 0.05 M sodium phosphate buffer, pH 7. One milliliter of goat serum (anti-HSA)[4] was then percolated through the column. A large quantity of nonbinding proteins (e.g., hemoglobin) was washed out directly. The adsorbed anti-HSA fraction (5.8 mg) was recovered by eluting the column with 0.1 M glycine–HCl, pH 3. Further elution with 3 M ammonium isothiocyanate yielded trace amounts of UV-absorbing material.

RESULTS

The Amino(2-hydroxy)propyl-Leash

The reaction of EBH with agarose yields glycidyl ethers, in addition to cross-links. While these epoxide groups are capable of reacting directly with organic amines, hydroxyls, and thiols, we elected to convert them to amino(2-hydroxy)propyl leash structures which serve as connecting points for ligands either directly or via leash-extending structures. By treating the epoxides with excess ammonia, the amino (2-hydroxy)propyl leashes are readily obtained. The chemical analysis for such a group is very easy; we used both elemental nitrogen analysis and potentiometric titration values to evaluate our gel derivatives. The titrimetric analysis of the epoxide group in the presence of sodium thiosulfate (10,17) was slow and thus unattractive for evaluating a large number of samples. The amino-leash group was also easily detected by the PSA test (13), which produced a yellow-orange color in the gel. The phenolphthalein–thiosulfate test, which is also qualitative, is a convenient but indirect test for epoxides.

Elemental bromine analysis of EBH-treated agarose revealed little halogen (<0.01%). thus, the Fairbourne mechanism in aqueous alkali was confirmed. In the Lewis acid–catalyzed reaction in 1,2-dichloroethanol, Hjerten et al. (18) were able to follow the extent of reaction by elemental chlorine analysis, since a 3-chloro-2-hydroxypropyl ether is generated.

Comparison of Epoxide Reagents

The choice of EBH as a coupling agent was indicated in part by a comparative study of several reagents. The results shown in Table 1 indicate

[4]The anti-HSA was generously provided by Dr. Hans Hager, Department of Immunology, Hoffmann–La Roche, Inc.

TABLE 1. **Amino-Leash Derivatives Obtained with Various Epoxides**[a]

Coupling agent	Titration ([RNH_2], μEq/ml)	*N*-analysis		
		dry wt, mg/ml	%N	[N], μEq/ml
Epibromohydrin	14.7	30.06	0.65	13.9
Epichlorohydrin	10.5	29.66	0.44	9.3
1,2,7,8-Diepoxyoctane	8.5	26.08	0.46	8.6
1,2,3,4,-Diepoxybutane	7.3	26.0	0.41	7.6
Ethylene glycol diglycidyl ether	3.2	27.18	0.47	9.1

[a] Agarose gel beads, 4% (25 ml) were treated with 12 mmol (0.24 M final concn.) epoxide in a final reaction volume of 50 ml, which was 0.3 M in NaOH. After washing, the beads were mixed with 25 ml 2 M NH_4OH.

that EBH is more reactive than ECH. The diepoxyoctane and -butane are less reactive than ECH, and the ethylene glycol diglycidyl ether is the least reactive of the group. This last compound is similar to the 1,4-butanediol diglycidyl ether studied by Sundberg and Porath (10).

A perusal of our results reveals that titrimetric analysis of the gel derivatives generally correlates with concentration values obtained from nitrogen analysis. The one exception is that obtained with the diglycidyl ether. Repeat preparations and analyses have only reproduced the observation that nitrogen analysis yields an inexplicably higher leash content than titration analysis. The color reaction from the PSA test correlated with the titration analysis.

Effect of Dioxane–Water Composition

It was noted early that EBH has a limited solubility in water. Thus, it was of interest to examine the effects of a water-miscible solvent on the epoxide reaction. Figure 1 shows the results of varying the composition of dioxane in the reaction medium. While the solubility of EBH was apparently improved by higher dioxane levels (less or no turbidity due to EBH, especially at higher concentrations), the glycidyl ether yield declined above 30% dioxane. While this result was unexpected, a possible explanation evolved from further studies on the alkali effect on the EBH reaction (see below).

Effect of Alkali Concentration

The catalytic effect of strong bases on the EBH reaction is apparently due to the formation of alkoxide anions in the agarose, which then react with

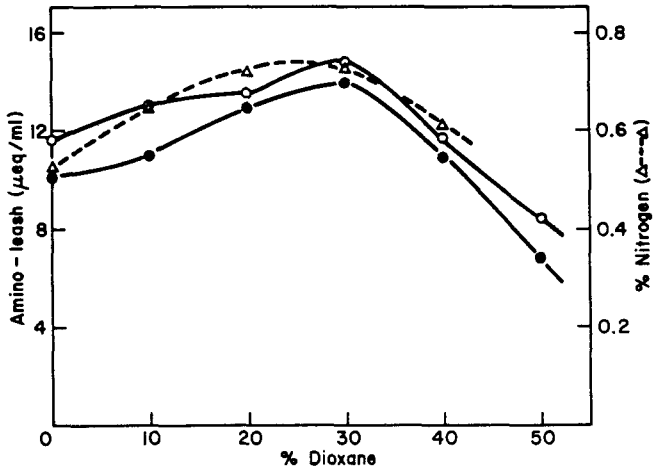


FIG. 1. Effect of dioxane–water composition. A quantity of 25 ml 4% agarose was washed with the mixed solvent, then treated with a fresh 25 ml of the same solvent containing 0.48 M EBH and 0.24 M NaOH. The samples were shaken for 4 h at 30°C. The beads were collected by filtration, washed with water, treated with ammonium hydroxide (see "Methods"), washed, and then analyzed. Here and in Figs. 2–5 (○) indicates capacity determined by titration; (●), capacity determined from elemental nitrogen data.

the reagent. In preliminary experiments, we observed that tetramethylammonium hydroxide (TMAH) was also effective, but triethylamine was not. It also appeared that the effects of TMAH were solvent-dependent—in pure dioxane, its presence caused a collapse of the agarose gel structure. Sodium hydroxide has a rather limited solubility in dioxane; hence, its effects could be studied only in water or aqueous solvent mixed media. The results of sodium hydroxide catalysis on the EBH reaction in 30% dioxane solution are in Fig. 2A. Qualitatively, they are similar to those in Fig. 2B, which are for the study done in water only.

The main difference in the results is that the presence of dioxane causes a shift of the optimum effect to lower concentrations of base. This shift is most likely due to an increase in the apparent basicity of NaOH in the presence of dioxane. Thus, a lower concentration of base suffices to catalyze the reaction of EBH to agarose. Above the optimum concentration, the base promotes more cross-linking or hydrolysis, or both, of the epoxide ether. Both these events would result in a lowering of the yield of the amino(2-hydroxy)propyl-leash.

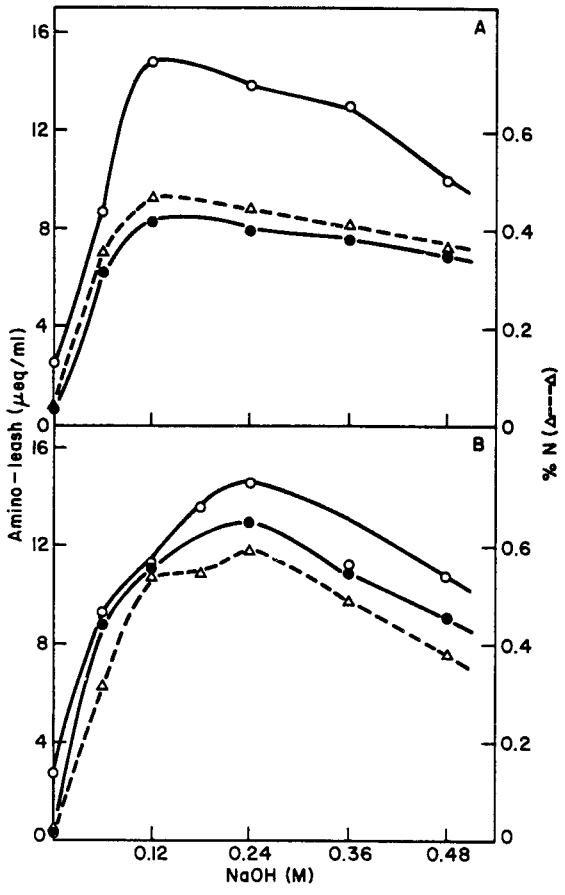


FIG. 2. Effect of NaOH concentration. A quantity of 25 ml 4% agarose (pre-washed in the solvent) was mixed with 25 ml solvent containing 0.48 M EBH and varying concentrations of NaOH. Samples were shaken at 30°C for 4 h, washed, and then treated with NH_4OH as in Fig. 1. (A) In 30% dioxane–water; (B) in water only.

Effect of Temperature

Preliminary observations on the increased solubility of EBH in dioxane–water mixtures prompted an investigation of temperature effects on solubility. But while improved solubility of EBH in water could be noted at higher temperatures, the results shown in Fig. 3 revealed a reaction optimum. The lowness of this temperature optimum was surprising, even

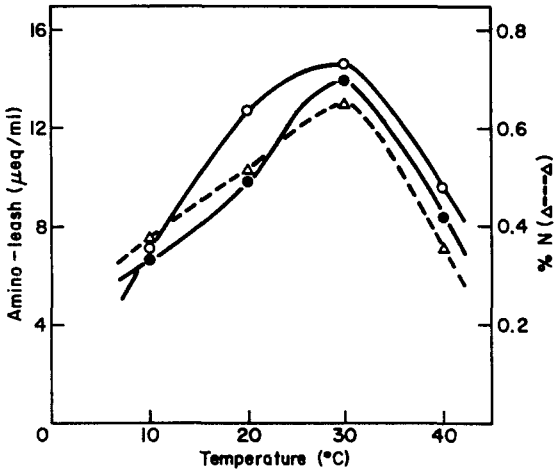


FIG. 3. Effect of temperature on EBH reaction. A quantity of 25 ml 4% agarose was treated with 25 ml 0.48 M EBH and 0.6 M NaOH, all in water. Mixtures were shaken 4 h at the appropriate temperatures, washed, and then treated with NH_4OH as in Fig. 1.

though it was similar to that reported for bisoxiranes (10). It is possible that the temperature optimum could be dependent on NaOH concentration, but this possibility was not investigated.

Time Course of the EBH Reaction

Compared to the reaction of cyanogen bromide on agarose, the EBH reaction is somewhat slower. As seen in Fig. 4, however, the reaction optimum occurs in a convenient 4 h. Since there is no requirement for careful pH monitoring, as in the BrCN reaction (14), it is easy to make several preparations simultaneously. It is surmised that at prolonged reaction times, decomposition of the epoxide ether by alkali results in lowered amino(2-hydroxy)propyl-leash yield.

Effect of EBH concentration and agarose gel density

It is useful to be able to derivatize gels to any predetermined leash-group concentration (19). As in the previously reported case of cyanogen bromide (14), one can obtain desired levels of leash structures in the gel by controlling the amount of reagent used. Figure 5 shows the results of varying EBH concentration with gels of different agarose density. A saturation limit

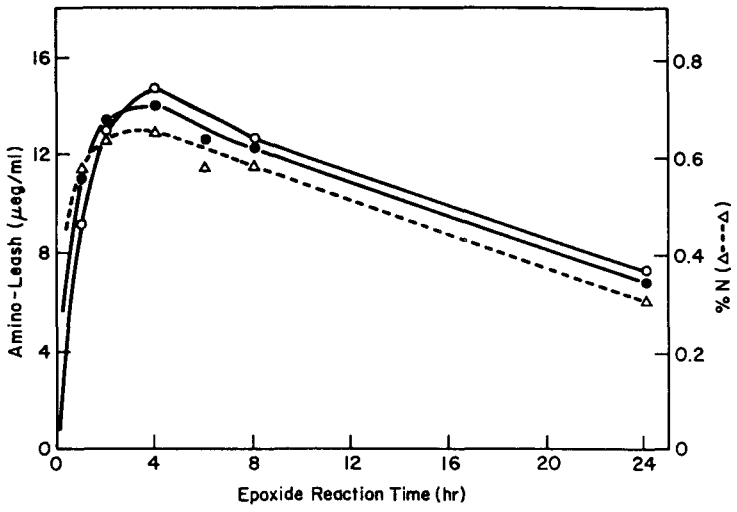


FIG. 4. Time course of EBH reaction. A quantity of 150 ml 4% agarose was added to 150 ml solution of 0.48 M EBH and 0.6 M NaOH. The mixture was swirled in shaker flasks at 30°C. Then 50-ml aliquots of the suspension were withdrawn at appropriate times, washed, and worked up with NH_4OH as described in Fig. 1.

in leash concentration is seen with each of the gels. The limits seem to be determined more by the gel density than by the reagent concentration. The limiting values of 9.5 μEq/ml for 2% agarose, 23 μEq/ml for 4% agarose, and 38–40 μEq/ml for 6% agarose are roughly similar to those obtained for 6-aminohexanoic acid coupled to agarose via cyanogen bromide (14). Both EBH coupling and BrCN chemistry yield leash concentrations of 20–22 μEq/ml for the 4% agarose. The EBH reaction in the 2% agarose, however, yields a leash content almost twice that obtained via BrCN. The 6% agarose with EBH yields only about 67–80% of leash groups incorporated via the BrCN reaction. Presently, it is not clear why these differences occur.

Stability of Epoxide-Treated Agarose (Glycidyl–Agarose)

As with Schell and Ghetie (8), our early applications of EBH to agarose were prompted by a desire to obtain agarose beads that were stable to warming and to treatment with polar, water-miscible organic solvents. As expected, EBH was very effective in rendering agarose beads more stable to solvent and elevated temperatures.

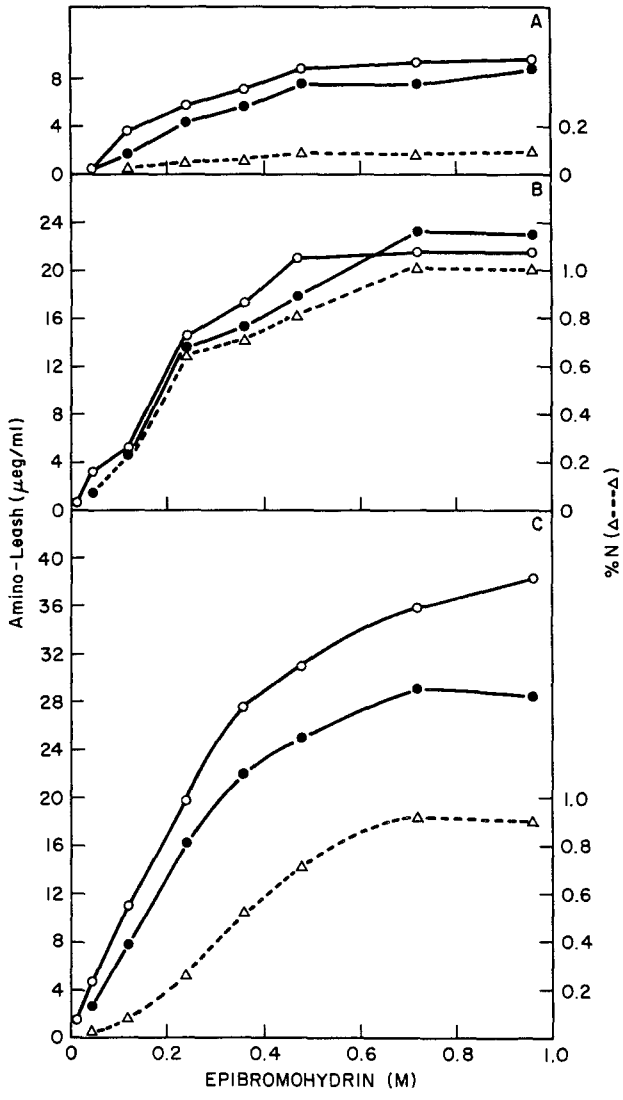


FIG. 5. Effect of EBH concentration on various agarose gels. Agarose in 25-ml samples was mixed with 25 ml 0.6 M NaOH containing various amounts of EBH so that the final concentrations indicated in the abscissa were obtained. The mixtures were shaken at 30°C for 4 h, washed, and then treated with NH_4OH as in Fig. 1. (A) 2% agarose; (B) 4% agarose; (C) 6% agarose.

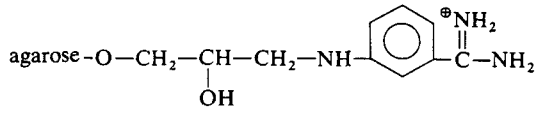
On mixing the various gel samples (taken from the studies indicated in Fig. 5) with NMP, it was observed that agarose gels subjected to 0.048 M or higher concentrations of EBH were stable and did not dissolve. A caprylamido(2-hydroxy)propyl ether derivative of 4% agarose was held at 40°C for 6 h, and was found not to lose any of its hydrophobic property. This result was in marked contrast to caprylyl hydrazide derivatives of agarose obtained with BrCN (see below).

Usage Examples

The utility of the amino(2-hydroxy)propyl derivative of agarose is best illustrated by converting it into a few useful forms, as described below.

Hydrophobic Sorbent. In a previous report, it was shown that caprylyl hydrazide bonded to agarose via cyanogen bromide resulted in a useful, nonionic hydrophobic sorbent (20). We have observed since then that this sorbent is unstable above 30°C in that the caprylate groups wash out and the hydrophobic properties are lost (Nishikawa and Bailon, unpublished observations). The new sorbent made by reacting isobutyl–oxycarbonylcaprylate with the aminopropyl–agarose is as stable as the gel itself. A sorbent containing 2.7 μEq caprylamido group/ml gel was equilibrated with 1 M sodium sulfate in 50 mM acetate buffer, pH 6. Under these conditions, β-lactoglobulin percolated through the column without adsorption, while BSA was adsorbed. The latter was desorbed when the column was eluted with buffer only. The behavior of these two proteins on this sorbent was comparable to that observed with a hydrophobic sorbent prepared from caprylyl hydrazide and BrCN (20).

Affinity Sorbent. An affinity sorbent selective for trypsin (EC 3.4.21.4) was prepared by reacting *m*-aminobenzamidine with the glycidyl ether derivative of 4% agarose:



A column packed with this sorbent readily adsorbed the active trypsin fraction from a highly purified commercial preparation. The inactive enzyme passed through the column unretarded. The active enzyme was recovered by desorption with 0.025 M benzamidine solution. The results were comparable to a previous study where the sorbent was prepared via BrCN coupling (12).

Enzyme Immobilization Support. Chymotrypsin (EC 3.4.21.1) was easily immobilized via glutaraldehyde on 4% agarose containing 22 μEq/ml of the amino-leash. By measuring the decrease in enzyme activity of the supernatant fluid from the coupling mixture, it was estimated that 57% of the protein and 63% of the activity was immobilized. Direct assay of the immobilized enzyme yielded about 25% of the original activity used in the coupling step.

Immunosorbent. Human serum albumin immobilized with glutaraldehyde on 4% agarose containing 3.2 μEq/ml of amino-leash was an efficient immunosorbent for anti-HSA from goat serum. Based on dry weight (30.9 mg/ml wet vol) and nitrogen analysis (1.8%) with corrections for the leash–matrix nitrogen (0.09% N; 26.11 mg/ml wet vol), the HSA content was 3.5 mg/ml gel. To a 0.9×12 cm column packed with this sorbent pre-equilibrated with 0.25 M NaCl in 0.05 M phosphate, pH 7, was added 1 ml goat anti-HSA serum. Continued elution with the pre-equilibration buffer removed nonbinding proteins. The anti-HSA IgG fraction (5.8 mg protein) was recovered by washing the column with 0.1 M glycine–HCl buffer, pH 3. This recovery of specific IgG was unexpectedly high. Yet immunoelectrophoretic analysis[5] indicated the material to be of high purity.

A further purification study was carried out on anti-HSA IgG (from the same lot of goat serum), which was freshly prefractionated by 3 cycles of ammonium sulfate precipitation (21). As seen in Fig. 6, 20.9 mg partially purified IgG yielded 3.5 mg anti-HSA IgG fraction from the immunosorbent column. This amount of column-purified IgG obtained from an equivalent of 1 ml goat serum was still higher than anticipated.

DISCUSSION

The Amino(2-Hydroxy)Propyl-Leash

The glycidyl ether group obtained after treatment of agarose with EBH can be directly reacted with organic amines, alcohols, and thiols. The relatively slow reaction rates of these compounds with epoxides, however, may require concentrations that are too high to be practical—especially with scarce or low-solubility compounds. By contrast, the treatment of epoxides with 1 M NH_4OH is inexpensive and fairly rapid. The amino(2-hydroxy)propyl-leash obtained is easy to analyze (elemental nitrogen and titrimetric data correlate reasonably well). Furthermore, this structure is

[5]The immunoelectrophoretic analysis was kindly provided by Mr. K. Kolinsky of Dr. Hager's laboratory.

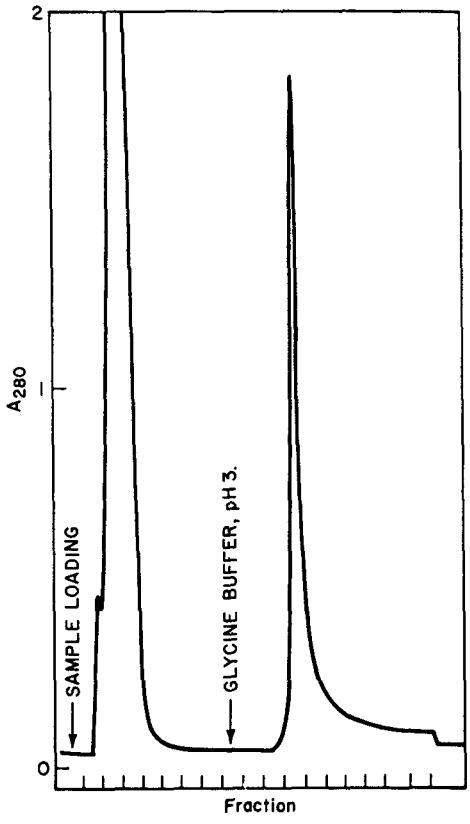


FIG. 6. Affinity purification of goat anti-HSA. Sorbent containing 5.3 mg HSA/ml gel (see text) was packed into a 0.9 × 10 cm column. A quantity of 1 ml partially purified goat anti-HSA (20.9 mg protein) was applied to the column, which was preequilibrated with 0.25 M NaCl in 0.05 M sodium phosphate buffer, pH 7. Nonbinding immunoglobulins are washed out immediately. The specific anti-HSA (3.5 mg) is recovered by elution with 0.1 M glycine–HCl buffer, pH 3.

hydrophilic and chemically stable. When bonded to a ligand by amide linkage, this leash affords a nonionic structure. By comparison, the direct reaction of an organic amine with an epoxide yields a prototropic secondary amino group, which could become a site for nonspecific adsorption of proteins (4).

In terms of the architectural strategy for affinity-sorbent preparation, this amino-leash offers great convenience for monitoring the completeness of coupling, since the PSA test can directly reveal any unreacted amino groups still remaining in the gel. With regard to ligand coupling, the amino-leash, of course, can be the receptive partner for a wide array of reagents developed in peptide-coupling chemistry.

Epibromohydrin as a Reagent

Compared to cyanogen bromide, EBH is much easier to handle and far less obnoxious. It is cheaper than BrCN, but more costly than ECH. Although EBH is not so reactive as BrCN, it reacts rapidly enough with agarose to be convenient and affords the same benefits without some of the drawbacks. Because it reacts with proteins, EBH should be handled in a fume hood for safety.

In this study, EBH has been found to react readily with agarose and does not require the more strenuous conditions of temperatures in excess of 60°C (autoclaving) reported for ECH (9). The facile reaction of EBH in aqueous alkali media is easier to carry out than the ECH reaction with boron trifluoride catalyst. Lewis acid–catalyzed reaction requires a tedious solvent exchange from water to 1,2-dichloroethane (18).

Functional-Group Yields

Compared to the bisoxiranes, the EBH reaction with agarose affords a higher yield of functional group in the gel relative to the amount of reagent expended. For instance, with 6% agarose, Sundberg and Porath obtained ligand concentrations of about 24 μEq/ml, using a reaction mixture containing 50% (by volume) bisoxirane. In this work with 6% agarose, a capacity of 38 μEq amino(2-hydroxy)propyl ether/ml gel was obtained with a reaction mixture containing only 8% (by volume) EBH. The difference in apparent yields may be due to the difference in reactivities between EBH and bisoxiranes. As expected from earlier studies (14), agarose gels of lower density yielded correspondingly lower capacities.

Reaction Time

While a number of requirements for the reaction of EBH with agarose were qualitatively similar to that observed for bisoxiranes, the optimum time period was unexpectedly short. This may be a consequence of the difference in reactivity between the first step, involving bromide expulsion due to allylic epoxide rearrangement on alkoxide attack, and the second, where epoxide

ring is opened by alkoxide ion. The latter step, of course, leads to cross-linking of the agarose gel network, with the consequent result of a more chemically stable matrix. It is surmised, however, that if this cross-linking is allowed to proceed unchecked, no useful amounts of epoxide groups will be left for conversion to the amino(2-hydroxy)propyl-leash structures. The decline in functional-group yield at extended EBH reaction times may also be due in part to hydrolysis of the epoxides in alkaline medium (17).

Some Applications of the Leash

The amino(2-hydroxy)propyl ether–leash structure affords a useful starting point in the preparation of affinity sorbents devoid of the unwanted cationic groups that can arise from cyanogen bromide coupling (3,4). Furthermore, this leash structure provides a stable anchoring point for the preparation of nonionic hydrophobic sorbents. The caprylamido gel derivative described here is vastly superior to the hydrophobic gels obtained with cyanogen bromide and caprylyl hydrazide as described previously (20). The caprylyl hydrazide–leash structures have been found to be quite labile above 30°C (Nishikawa and Bailon, unpublished observations).

The examples presented in this paper are intended only to be illustrative. Once the amino(2-hydroxy)propyl-agarose gel is at hand, a wide variety of coupling methods may be employed for the attachment of ligands, as evident from the vast available literature—e.g., Guilford (22).

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