H. (1968), *Arch. Mikrobiol. 60*, 326.
Medrano, L., Ruiz-Amil, M., and Losada, M. (1969), *Arch. Mikrobiol. 66*, 239.
Middelhoven, W. J. (1963), *Biochim. Biophys. Acta 77*, 152.
Middelhoven, W. J. (1964), *Biochim. Biophys. Acta 93*, 650.
Middelhoven, W. J. (1969), *Antonie van Leeuwenhoek; J. Microbiol. Serol. 35*, 215.
Middelhoven, W. J. (1970), *Antonie van Leeuwenhoek; J. Microbiol. Serol. 36*, 1.
Neilands, J. B. (1972), *in* Inorganic Biochemistry, Eichhorn, Gunther, Ed., New York, N. Y. ,Elsevier (in press).
Neilands, J. B., and Cannon, M. D. (1955), *Anal. Chem. 27*, 29.
Piotrowska, M., Sawicki, M., and Weglenski, P. (1969), *J. Gen. Microbiol. 55*, 301.
Pollack, J. R., Ames, B. N., and Neilands, J. B. (1970), *J. Bacteriol. 104*, 635.
Prieur, P. (1971), *C. R. Acad. Sci., Ser. D 272*, 1553.
Prozosky, O. W. (1967), *J. Gen. Microbiol. 49*, 325.
Prozosky, O. W. (1969), *J. Gen. Microbiol. 55*, 89.
Ramos, F., Thuriaux, P., Wiame, J. M., and Bechet, J. (1970), *Eur. J. Biochem. 12*, 40.
Ratner, S. (1957), *Methods Enzymol. 3*, 643.
Seifter, S., Gallop, P., Michaels, S., and Meilman, E. (1960), *J. Biol. Chem. 235*, 2613.
Smith, D. F., and Holmes, R. A. (1970), *J. Bacteriol. 104*, 1223.
Snell, E. E., and DiMari, S. J. (1970), The Enzymes, Vol. 2, 3rd ed, New York, N. Y., Academic Press, p 335.
Snow, G. A. (1954), *J. Chem. Soc. A*, 2588.
Srere, P. A. (1959), *J. Biol. Chem. 234*, 2544.
Strecker, H. J. (1965), *J. Biol. Chem. 240*, 1225.
Stevens, R. L., and Emergy, T. F. (1966), *Biochemistry 5*, 74.
Tateson, J. E. (1970), *Biochem. J. 118*, 747.
Tsuda, Y., and Friedmann, H. C. (1970), *J. Biol. Chem. 245*, 5914.
Udaka, S. (1966), *J. Bacteriol. 91*, 617.
Yorifuji, T., Misono, H., and Soda, K. (1971), *J. Biol. Chem. 246*, 5093.

Adsorbents for Affinity Chromatography. Use Of *N*-Hydroxysuccinimide Esters of Agarose†

Pedro Cuatrecasas*,‡ and Indu Parikh

ABSTRACT: Procedures are described for the preparation of the *N*-hydroxysuccinimide ester of succinylated aminoalkyl agarose derivatives. These active ester derivatives of agarose are stable for months when stored in dioxane. The reaction of the active ester of agarose with various amino acids and with trypsin has been studied in detail. Stable amide linkages are formed very rapidly (at 4°) with the unprotonated form of primary aliphatic or aromatic amino groups in the pH range of 6–9. Of the amino acid functional groups tested, only sulfhydryl groups compete effectively with free amino groups for reaction. The *N*-hydroxysuccinimide ester derivative of agarose can be used by very simple and mild procedures to immobilize proteins and complex amino group containing ligands to agarose. The coupled products are separated from the matrix backbone by lengthy hydrocarbon extensions. Similar procedures can be used to attach ligands and proteins to porous glass beads. These studies in addition demonstrate that agarose beads tolerate quite well certain organic solvents such as dioxane and methanol. The activated agarose derivatives, after lyophilization to remove dioxane, are quite stable in the powder form and readily swell and react with amino groups when suspended in aqueous medium.

Various procedures have been described for the preparation of selective agarose adsorbents for use in affinity chromatography of proteins (Cuatrecasas *et al.*, 1968; Porath *et al.*, 1967; Cuatrecasas, 1970, 1972; Cuatrecasas and Anfinsen, 1971a,b). There is abundant evidence that successful purification of enzymes frequently depends on utilizing an adsorbent in which the ligand is spatially separated from the matrix backbone (reviewed by Cuatrecasas, 1972). This is generally best accomplished by linking the ligand to agarose derivatives which contain lengthy hydrocarbon extensions terminating in a functional moiety such as a primary amino group or a carboxyl group. A variety of chemical reactions have been used to couple ligands to such "long-armed" agarose derivatives (Cuatrecasas, 1972). However, no generally applicable procedure is available for coupling ligands such as amino acids which contain more than one functional group without using complicated blocking and deblocking procedures of one or more of these groups. Furthermore, it has heretofore been very difficult to covalently attach proteins to any solid support which contains functional groups (*e.g.*, amino, carboxyl, diazonium, bromoacetyl) without coupling through the tyrosyl or histidyl residues of the protein or without introducing intermolecular protein cross-links and polymerization.

This report describes in detail the preparation and use of active carboxylic acid esters of long-armed agarose derivatives which are stable in anhydrous media over prolonged periods of time. Ligands and proteins which contain free amino groups can be coupled very rapidly through amide linkage to these activated derivatives under mild conditions (aqueous

† From the Department of Medicine and the Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205. *Received January 31, 1972.* Supported by Grant AM14956 from the National Institute of Arthritis and Metabolic Diseases.

‡ Recipient of U. S. Public Health Service Research Career Development Award AM31464.

medium, 4°, neutral pH). The general sequence of reactions (Figure 1) involves reaction in dioxane of a carboxylic acid derivative of agarose (Figure 1, A), which is prepared by succinylation of aminoalkyl agarose, with *N*-hydroxysuccinimide in the presence of *N*,*N*′-dicyclohexylcarbodiimide. The activated derivative (Figure 1B) can be stored in dioxane or used immediately for coupling of compounds which contain amino groups.

Materials and Methods

Materials. Sepharose 4B was obtained from Pharmacia, 3-L-[^{3}H]alanine from New England Nuclear, *N*-hydroxysuccinimide from Aldrich. *N*,*N*′-Dicyclohexylcarbodiimide, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate, *N*-ethoxycarbonyl-2-ethoxyl-1,2-dihydroquinoline, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide were from Pierce Chemical Co. Trypsin was purchased from Worthington, 3,3′-diaminodipropylamine from Eastman, and *p*-aminophenyl β-D-thiogalactopyranoside, *N*-acetyl-L-histidine, and *N*-acetyl-L-serine from Calbiochem. *N*-Acetyl-L-tryptophan and *N*-acetyl-L-tyrosinamide were obtained from Schwarz/Mann, and *N*-acetyl-L-glutamine, *N*-acetyl-L-arginine, *N*-acetyl-L-methionine, *N*-acetyl-L-cysteine, and *N*-acetyl-L-phenylalanine from Fox Chemical Co., Los Angeles, Calif. Dehydrating granules added to the dioxane which was used for storage of the activated agarose were Molecular Sieve, type 4A, 4–8 mesh, Matheson, Coleman & Bell. Dioxane was dried by passage through columns of chromatographic neutral alumina (WN-3, activity grade 1, Sigma). The 3,3′-diaminodipropylamino agarose and the corresponding succinylated (Figure 1A) derivatives were prepared as described previously (Cuatrecasas, 1970); these gels can also be obtained commercially (Bio-Rad Laboratories, Richmond, Calif., and Affitron Corp., Rosemead, Calif.). Alkylamine glass (550 Å, 40–80 mesh) was a gift from Dr. Howard Weetall, Corning Glass Works.

Preparation of N-Hydroxysuccinimide Ester of Agarose. The long-armed aminoalkyl agarose is prepared by treating 3,3′-diaminodipropylamine with cyanogen bromide activated Sepharose 4B beads as described previously (Cuatrecasas, 1970). The derivatives used in these studies contained 2–5 μmoles of amino groups per ml of packed agarose. Succinylaminodipropylamino agarose (Figure 1A) is prepared as described by Cuatrecasas (1970) except that during washing of the coupled derivative a step is included which involves washing with 0.1 N NaOH at room temperature for 30–40 min. This step, not previously recognized, is necessary to remove labile carboxyl groups which, if not cleaved initially, will be released slowly at neutral pH during subsequent steps or during storage.

The washed, succinylated agarose (Figure 1A) is washed with distilled water followed by extensive washing with dioxane[1] to obtain anhydrous conditions. Washing is performed with suction in Buchner funnels equipped with coarse-grade fritted disks. Exposure of agarose to dioxane even over prolonged periods (more than 6 months) does not appreciably change the properties of the agarose beads.[2] The succinylated agarose is suspended in dioxane to achieve a total volume equal to three times the volume of the packed (settled) gel. Solid *N*-hydroxysuccinimide is added to the magnetically stirred suspension to obtain a concentration of 0.1 M. Solid *N*,*N*′-dicyclohexylcarbodiimide is added to adjust its concentration to 0.1 M, and the suspension is stirred gently for 70 min at room temperature. The activated agarose (Figure 1B) is washed over a Buchner funnel with six or eight volumes of dioxane over a 10-min period followed by three or four volumes of methanol (aided by stirring with a glass rod) over 5 min to remove all the precipitated dicyclohexylurea. This is followed by further washing (three volumes) with dioxane. After drying the gel briefly (1–2 min) under suction the slightly moist cake of activated agarose is used immediately in coupling reactions or is stored for future use. For storage the gel is suspended in dry dioxane[1] and the suspension is stored at room temperature over molecular sieves in tightly capped glass containers wrapped with tinfoil or otherwise protected from light. Before use the stored agarose need only be dried briefly as described above.

Coupling of Ligands and Proteins to the Activated Agarose. The slightly moist cake of activated agarose (Figure 1B) is weighed and added to a rapidly stirred buffered solution, maintained in an ice bath, which contains the ligand or protein to be coupled. Generally 1 g of the gel is added per 5 ml of ice-cold solution. The pH of the solution can be varied between 5 and 9, and the time of reaction (at 4°) can vary from 10 min to 6 hr, as will be described under Results. When it is desired to terminate the coupling reaction, glycine should be added to achieve a concentration of 1 M and the reaction should be permitted to continue at room temperature for 2 hr. This is important if termination is desired before the reaction has proceeded to completion. It is advisable to always include this step when proteins are being coupled to insure total masking of unreacted active ester groups. The completed adsorbent is washed extensively with appropriate buffers as described elsewhere (Cuatrecasas, 1970; Cuatrecasas and Anfinsen, 1971a).

The quantity of [^{3}H]alanine (10 Ci/mole) which has coupled to agarose[3] is determined by liquid scintillation. One milliliter of a slurry (10–30% agarose, v/v) of the suspension is counted at 50% efficiency using a mixture containing 10 ml of TLX toluene fluoralloy and 2 ml of Bio-Solv Solubilizer BBS-3 (Beckman). The accuracy of such estimations was confirmed by comparing the results of the determinations based on radioactivity with those calculated from amino acid analyses of acid hydrolysates of the same samples. The differences obtained were not statistically significant. The quantity of protein or of nonradioactive amino acid coupled was determined by amino acid analysis according to the procedure of Spackman *et al.* (1958) with the use of a Spinco Model 120 amino acid analyzer. Samples of agarose were lyophilized and hydrolyzed in constant-boiling HCl in evacuated, sealed tubes at 110° for 20 hr. After hydrolysis the samples were passed through Pasteur pipets containing glass wool plugs, or through Millipore filters, to remove particulate material.

Assay of Trypsin Activity. The activity of trypsin and trypsin agarose was determined by measuring the rate of hy-

[1] The dioxane used (Fisher, scintanalyzed) in the steps leading to the preparation of the active ester (Figure 1, B) need not be subjected to special drying procedures. The activated agarose, however, should be stored in dioxane which has been passed through an alumina column, and molecular sieve granules should be added during storage.

[2] Prolonged exposure to dioxane does not result in apparent dissolution, shrinkage, shattering, or change in the porosity or shape of agarose beads.

[3] Different preparations of aminoalkyl and succinylaminoalkyl agarose derivatives were used in the various experiments presented, which probably explains the apparent differences in the maximal substitution achieved in different experiments.

drolysis of benzoyl-L-arginine ethyl ester by the pH-Stat method (Schwert *et al.*, 1948; Laskowski, 1955; Goldstein *et al.*, 1964) using an automatic titrator (SBR-2c titrigraph and TTT-1c titrator, Radiometer, Copenhagen). The assays were performed at pH 8.0 in 0.1 N NaCl, and 0.1 N NaOH was used as titrant. The amount of alkali used to maintain the pH constant was considered equivalent to the amount of substrate hydrolyzed. The amount of trypsin was varied between 0.5 and 20 μg per ml, and the substrate concentration was varied from 1 to 100 mM. The data were plotted according to the method of Lineweaver and Burk (1934) and Goldstein *et al.* (1964). The concentration of native or agarose-bound trypsin was determined by amino acid analysis of acid hydrolysates as described above. In some experiments the amount of trypsin coupled was estimated by determining the disappearance of protein from the medium by the procedure of Lowry *et al.* (1951); this approach was only utilized for those derivatives which were not to be used for measurements of enzymic activity. Estimations of protein concentration in the medium or washes by absorbancy measurements are very inaccurate because of the release of ultraviolet-absorbing materials (*N*-hydroxysuccinimide) into the medium during the coupling reaction.

Results

Formation of Active N-Hydroxysuccinimide Ester of Carboxylated Agarose. Although coupling of the alanine to agarose is clearly dependent on the reaction sequence described in Figure 1, it is notable that significant activation of unsubstituted agarose as well as of aminoalkyl agarose occurs after treatment with *N,N'*-dicyclohexylcarbodiimide and *N*-hydroxysuccinimide (Table I). This suggests that a certain number of carboxyl groups, or of other potentially reactive groups, exist in untreated agarose which can form active esters with these reagents. The minor reaction which occurs between dicyclohexylcarbodiimide and unsubstituted or aminoalkyl agarose in the absence of *N*-hydroxysuccinimide probably represents reaction with carboxyl groups of agarose since with the succinylated agarose derivative substantial coupling is observed in the absence of *N*-hydroxysuccinimide (Table I). This is not surprising since it probably reflects a certain stability of the carbodiimide-activated carboxyls groups when added rapidly to the aqueous medium containing alanine under the conditions used. Optimum coupling is achieved by preparing the *N*-hydroxysuccinimide ester as outlined in Figure 1.

Near maximal coupling effectiveness is obtained with derivatives prepared with 0.1 M *N,N'*-dicyclohexylcarbodiimide and *N*-hydroxysuccinimide (Table II). Higher concentrations of these reagents result in derivatives with slightly higher coupling capacity, but this increased coupling appears to result from a different form of linkage which is labile in alkali. For this reason all subsequent studies have been performed with 0.1 M concentration of these reagents.

Effect of pH on the Coupling of Amino Group Containing Ligands. The *N*-hydroxysuccinimide ester of succinylated agarose reacts optimally with the α-amino group of L-alanine at about pH 8.5 (Table III). The ϵ-amino group of L-lysine reacts at higher pH values, consistent with the higher p*K* value of this group and with the requirement for the unprotonated form of an amino group for reaction. Hydrolysis of the active agarose ester by water is substantially faster as the pH of the solution is raised so that coupling of L-alanine decreases at pH values above 8.5. This, as well as the marked reactivity of

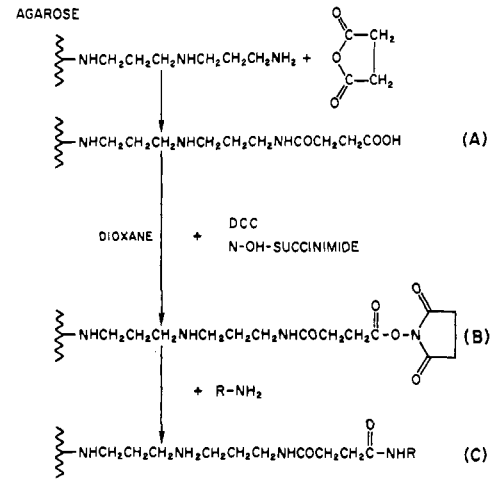

FIGURE 1: Scheme of reactions involved in the preparation and use of *N*-hydroxysuccinide esters of agarose. Diaminodipropylamino agarose is treated with succinic anhydride in saturated sodium borate buffer to obtain the corresponding succinylated derivative (A). The latter is reacted with *N,N'*-dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide in dioxane to yield the active agarose ester (B). After removing dicyclohexylurea and the unreacted reagents (dioxane and methanol washes) the active ester of agarose is reacted in aqueous medium with ligands or proteins to yield stable amide-linked derivatives (C).

TABLE I: Formation of *N*-Hydroxysuccinimide Ester of Succinyldiaminodipropylamino Agarose.[a]

Conditions for Synthesis	L-Alanine Coupled (μmoles/ml of Packed Agarose)
Unsubstituted agarose	
No additions	<0.001
DCC[b] alone	0.002
N-Hydroxysuccinimide alone	<0.001
DCC[b] + *N*-hydroxysuccinimide	0.08
Agarose–diaminodipropylamine	
No additions	<0.001
DCC[b] alone	0.003
N-Hydroxysuccinimide alone	<0.001
DCC[b] + *N*-hydroxysuccinimide	0.06
Agarose–succinyldiaminodipropylamine (Figure 1, A)	
No additions	<0.001
DCC[b] alone	0.03
N-Hydroxysuccinimide alone	<0.001
DCC[b] + *N*-hydroxysuccinimide	1.33

[a] Various agarose derivatives (Figure 1) were treated at room temperature for 70 min in dioxane with and without 0.1 M *N,N'*-dicyclohexylcarbodiimide and 0.1 M *N*-hydroxysuccinimide. The agarose suspension was washed with dioxane and methanol as described in the text and reacted for 6 hr at 4° with 0.1 M sodium bicarbonate buffer, pH 8.4, containing 10 mM L-[^{3}H]alanine (10 Ci/mole). The coupled derivatives were washed at room temperature with large volumes of 50 mM sodium bicarbonate buffer, pH 8.4, as well as with 0.1 M NaOH during a 40-min period to remove any alkaline-labile material. [b] *N,N'*-Dicyclohexylcarbodiimide.

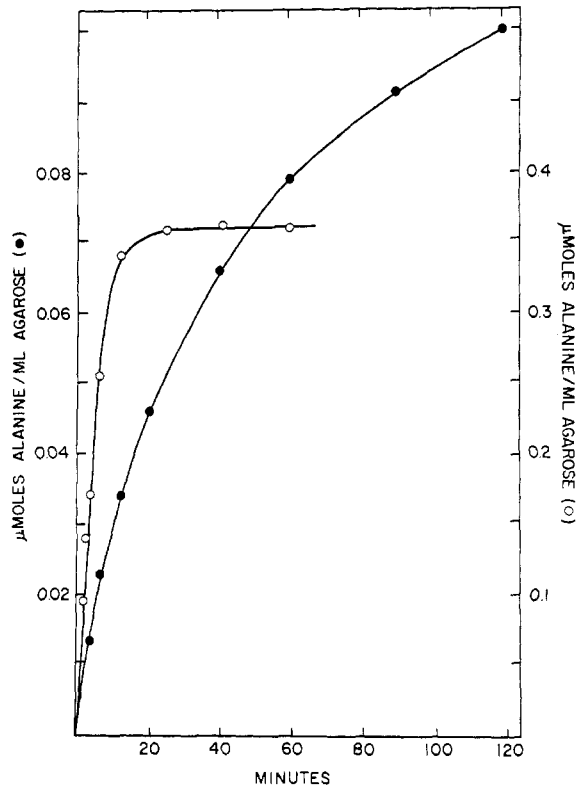

FIGURE 2: Rate of reaction of *N*-hydroxysuccinimide ester of agarose (Figure 1B) with L-[^{3}H]alanine at 4° in 0.1 M $NaHCO_3$ buffer, pH 8.6 (○), and in 0.1 M sodium acetate buffer, pH 6.3 (●). The reaction of *N*-α-acetyl-L-lysine in 0.1 M sodium acetate buffer, pH 6.3, yields 3nmoles at 20 min, 10 nmoles at 60 min, and 22 nmoles at 120 min (not shown in the figure).

the active ester toward nucleophiles, probably explain the relatively small changes in the amount of reaction observed over the broad range of pH. Coupling through an ε-amino group is not as effective as through the α-amino group, presumably because of the greater instability of the agarose ester at the higher pH values required for unprotonation of this group.

TABLE II: Effect of Concentration of Dicyclohexylcarbodiimide and of *N*-Hydroxysuccinimide on the Coupling Effectiveness of the Activated Ester Derivative of Agarose.[a]

Concn of DCC and of *N*-Hydroxy-succinimide (M)	L-Alanine Coupled (μmoles/ml of Packed Agarose)	
	Before NaOH	After NaOH
0.01	0.38	0.35
0.03	0.79	0.75
0.10	1.11	1.04
0.20	1.47	1.16
0.50	1.88	1.20

[a] Succinylated agarose (Figure 1A) was reacted for 70 min at 24° with varying concentrations of the reagents, as indicated. Alanine was coupled as described in Table I and in the text. The content of [^{3}H]alanine was determined before and after washing the agarose with 0.1 N NaOH during a period of 40 min.

TABLE III: Effect of pH on the Coupling of Amino Acids through α-Amino and through ε-Amino Groups.[a]

pH of Coupling	μmoles per ml of Packed Gel	
	L-Alanine	*N*-α-Ac-L-Lys
5.0[b]	0.24	0.06
6.0[b]	0.55	0.21
6.5[c]	0.63	
7.0[c]	0.70	0.36
8.0[d]	0.81	0.44
8.5[d]	0.85	0.56
9.0[d]	0.77	0.61
10.0[d]	0.60	0.48

[a] *N*-Hydroxysuccinimide ester of agarose (Figure 1, B), prepared as described in Table I and in the text, was treated with 10 mM L-[^{3}H]alanine or with *N*-α-acetyl-L-lysine in buffers of varying pH as described in Table I. The degree of lysine substitution was determined by amino acid analysis, as described in the text. [b] 0.1 M sodium acetate buffer. [c] 0.1 M sodium phosphate buffer. [d] 0.1 M sodium bicarbonate buffer.

Under the conditions described in Table III it is not possible to obtain good discriminative reaction between α- and ε-amino groups except at the lowest pH (5.0) tested. Highly preferential reaction of the α-amino group, however, can be obtained without severely compromising the total degree of substitution by using a relatively low pH (6.0) and by decreasing the time of reaction. In this way it is possible to take advantage of the relatively large difference in the *rates* of reaction of these two groups (Figure 2). Since at lower pH values the active agarose ester is quite stable (to be described shortly) the differences in the total degree of substitution at high and low pH will diminish with longer reaction periods. It should therefore be possible in certain cases to devise experimental conditions which result in the preferential coupling of polypeptides and proteins by their N-terminal residue rather than randomly through lysyl residues. With peptides the conditions for discriminative reaction may be even more favorable than suggested by the present data because the N-terminal group of a peptide chain is a much weaker base than the α-amino group of a free amino acid (alanine). An illustration of this is insulin, which can be selectively coupled to the activated ester of agarose through its N-terminal residues by reacting for 20 min (4°) in 0.1 M sodium acetate, pH 6.0, containing 5 M urea.

Time Course of Coupling Reaction. At a pH of 8.6 and at 4° the reaction of L-alanine with the active agarose ester is complete within 30–40 min (Figure 2). In contrast, at a pH of 6.3 the reaction requires more than 2 hr. The initial rates of reaction at these two pH values differ by about tenfold. Despite the rapidity of the reaction at the higher pH the total amount of substitution may be less than that observed at lower pH values, especially when relatively low concentrations of alanine are used. This is presumably due to the greater instability of the ester at the higher pH, as will be described shortly. It is notable that at pH 6.3 the initial rate of reaction of L-alanine is more than 20 times greater than the rate of reaction of *N*-α-acetyl-L-lysine (Figure 2).

Stability of N-Hydroxysuccinimide Ester of Agarose in Aqueous Medium. The active agarose ester is considerably

TABLE IV: Effect of Various *N*-Acetylamino Acids on the Coupling of L-Alanine to *N*-Hydroxysuccinimide Ester of Agarose.[a]

Addition	Concn (mM)	Ratio of Amino Acid: Alanine	% Alanine Coupled
None			100
N-Acetyl-L-cysteine	25	50	19
	5	10	33
	2	4	88
	0.5	1	97
N-Acetyl-L-histidine[b]	5	10	4
	2.5	5	7
	0.5	1	59
N-Acetyl-L-tyrosine	25	50	84
	5	10	97
N-Acetyl-L-methionine	25	50	99
	5	10	98
N-Acetyl-L-glutamine	25	50	92
	5	10	100
N-Acetyl-L-tryptophan	5	10	100
N-Acetyl-L-serine	25	50	86
	5	10	99
N-Acetyl-L-arginine	25	50	104
N-Acetyl-L-phenylalanine	25	50	90
p-Aminophenyl β-D-thiogalactopyranoside	5	10	7

[a] The *N*-hydroxysuccinimide ester of agarose (Figure 1, B) was reacted for 2 hr at 4° in 0.2 M $NaHCO_3$ buffer, pH 8.0, containing 0.5 mM L-[^{3}H]alanine (10 Ci/mole) and the indicated amino acid. The radioactive content of the substituted agarose was determined as indicated in the text after thorough washing with 0.1 M $NaHCO_3$ buffer, pH 8.4. The effect of *p*-aminophenyl β-D-thiogalactopyranoside is presented to indicate the reactivity of ligands containing primary aromatic amino groups. [b] Amino acid analyses of samples reacted with 25 mM *N*-acetyl-L-histidine revealed no significant coupling of this amino acid to agarose under the conditions described here.

more stable at pH 6.3 compared to pH 8.6 (Figure 3). After exposure of the agarose to an aqueous medium of pH 6.3 for 50 min (4°) its capacity for reaction with L-alanine is about 75% of that which is not preincubated in aqueous solutions. In contrast, only 30% of the active ester group appears to remain after exposing the agarose for 12 min to a solution having a pH of 8.6.

Effect of Ligand Concentration on Substitution to Active Agarose Ester. Increasing the concentration of L-alanine results in proportionately greater coupling when this step is performed in a buffer of pH 6.4 compared to a buffer of pH 8.4 (Figure 4). These studies demonstrate that despite the limited stability of the ester in aqueous medium it is possible to achieve considerable degrees of substitution by increasing the concentration of ligand.

Reaction with Other Amino Acids. A variety of *N*-α-acetylated functional amino acids were examined for their capacity to interfere with the coupling of L-[^{3}H]alanine to the *N*-hydroxysuccinimide ester of agarose (Table IV). In these

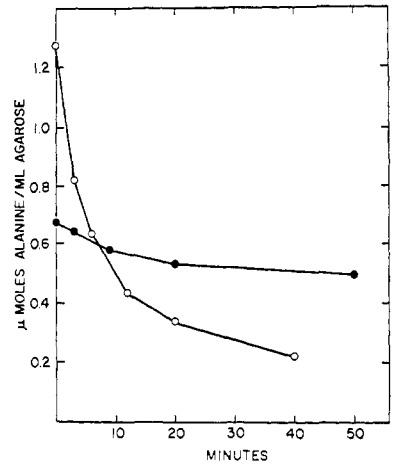

FIGURE 3: Stability of *N*-hydroxysuccinimide ester of agarose (Figure 1B) at 4° in aqueous medium at pH 8.6 (○) and at pH 6.3 (●). The active ester of agarose, prepared as described in Table I, was added to stirred solutions of 0.1 M $NaHCO_3$ buffer, pH 8.6, and 0.1 M sodium acetate buffer, pH 6.3. At the indicated times aliquots of the suspension were removed and reacted for 90 min at 4° with 10 nM L-[^{3}H]alanine.

experiments a very low concentration (0.5 mM) of L-alanine was used and the competing amino acid was used in very high concentration in order to detect even weakly competing reactions. The blocked derivatives of tyrosine, methionine, glutamine, tryptophan, serine, arginine, and phenylalanine demonstrate very little or no interference with the reaction of L-alanine even at concentrations 50 times greater than L-alanine.

Only the sulfhydryl and imidazole group of cysteine and histidine demonstrate significant reaction with the active ester

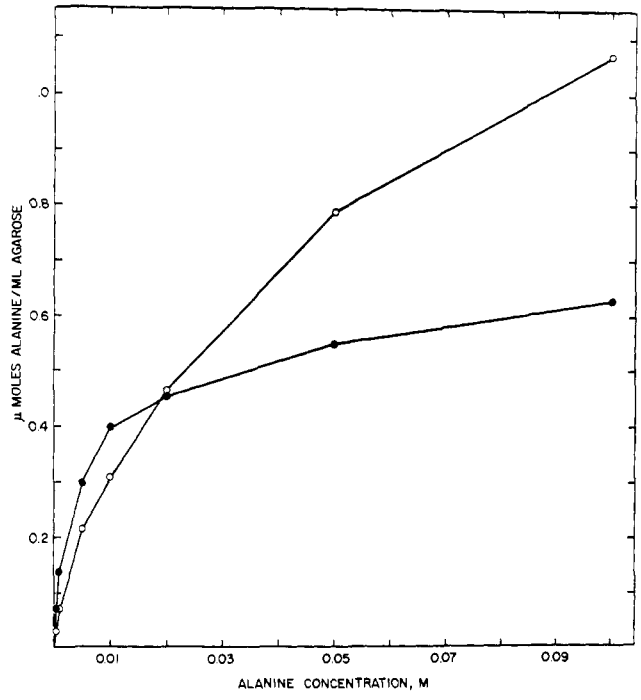

FIGURE 4: Effect of increasing the concentration of L-alanine on the coupling of this amino acid to *N*-hydroxysuccinimide ester of agarose (Figure 1B) at pH 8.4 (●) and pH 6.4 (○). The coupling reaction was performed for 90 min at 4° in 0.1 M $NaHCO_3$, pH 8.4, or in 0.1 M sodium acetate buffer, pH 6.4.

TABLE V: Use of Various Condensing Reagents in the Preparation of *N*-Hydroxysuccinimide Ester of Agarose.[a]

Coupling Reagent	L-Alanine Coupled (μmoles/ml of Packed Agarose)
N,N'-Dicyclohexylcarbodiimide[b]	1.5
1-Cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate[c]	4.2
N-Ethoxycarbonyl-2-ethoxyl-1,2-dihydroquinoline[d]	3.5
1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide[e]	4.0

[a] Succinylated agarose (Figure 1A) was incubated for 70 min at room temperature in dioxane with 0.1 M *N*-hydroxysuccinimide and 0.1 M of the indicated condensing reagent as described in detail in the text. The active agarose ester was reacted for 8 hr (4°) in 0.1 M $NaHCO_3$ buffer, pH 8.5, containing 50 mM L-[^{3}H]alanine (8 Ci/mole). The coupled derivatives were washed with bicarbonate buffer and with sodium hydroxide as described in Table I before determination of radioactive content. [b] Albertson (1962). [c] Sheehan and Hlvaka (1956). [d] Belleau and Malek (1968). [e] Sheehan *et al.* (1961).

TABLE VII: Coupling of Trypsin to *N*-Hydroxysuccinimide Ester of Agarose.[a]

Conditions of Coupling	Trypsin Concn (mg/ml)	Trypsin Coupled to Agarose (mg/ml of Agarose)	Disappearance of Trypsin from Medium (%)
0.1 M Sodium bicarbonate, pH 8.5			
15 min	1	1.6	63
	5	7.8	59
30 min	1	1.5	59
	5	8.2	62
0.1 M Sodium phosphate, pH 6.5			
15 min	1	1.4	55
	5	7.4	56
	10	14.0	56
	20	13.4	25
	40	13.7	13
30 min	1	1.4	55
	5	8.2	60
	10	13.6	54
	20	14.0	28

[a] One gram of activated agarose ester (Figure 1, B) was added to 4 ml of a solution of trypsin and reacted at 4° under varying conditions, as indicated.

of agarose under the conditions examined. The reaction with cysteine appears to be less favorable than that observed with α-amino groups. For example, a fourfold excess of *N*-acetyl-L-cysteine over L-alanine results in a decrease of alanine coupling of only 12%. The imidazole moiety appears to interfere more seriously with the coupling of alanine, although the reaction with *N*-acetyl-L-histidine does not result in stable linkage of this amino acid to agarose (Table IV). This is consistent with the known rapid hydrolysis and thus instability in water of N^{im}-acetylimidazoles. The imidazole group of histidine therefore acts simply to accelerate the spontaneous hydrolysis of the hydroxysuccinimide ester of agarose, and proteins or peptides containing histidyl residues would not be expected to link covalently to agarose through this amino acid unless it exists in an N-terminal position of a polypeptide sequence.

These studies therefore suggest that, with the exception of cysteine sulfhydryl groups, native proteins and unblocked amino acids will probably couple with the activated agarose ester exclusively with α- or ε-amino groups. It is significant that ligands bearing primary aromatic amino groups will also couple very effectively to the active agarose derivative. This is illustrated (Table IV) by the reactivity of *p*-aminophenyl D-thiogalactopyranoside, an inhibitor of β-galactosidase which has been used effectively to purify this enzyme by affinity chromatography (Steers *et al.*, 1971). This ligand reacts at least as well as L-alanine in buffers of low pH, consistent with the relatively low p*K* of the aromatic amino group.

Use of Other Coupling Reagents. Other condensing reagents, such as those illustrated in Table V, are more effective than *N,N'*-dicyclohexylcarbodiimide at equimolar concentrations in formation of the *N*-hydroxysuccinimide ester of agarose. When very highly substituted agarose adsorbents are desired it may therefore be advisable to utilize one of these reagents rather than *N,N'*-dicyclohexylcarbodiimide.

Stability and Storage of N-Hydroxysuccinimide Ester of Agarose in Anhydrous Medium. It is notable that the activated agarose can be stored in dry dioxane for prolonged periods of time without serious loss in its capacity to undergo substitution reactions (Table VI). After storage for nearly 4 months at room temperature 80% of its capacity to react with L-alanine is retained.

Utilization of Dried Agarose Beads. Agarose beads which are lyophilized from aqueous suspension form a fine powder which does not swell upon addition of water (Cuatrecasas,

TABLE VI: Stability to Storage in Dioxane of *N*-Hydroxysuccinimide Ester of Agarose.[a]

Length of Storage (Days)	L-Alanine Coupled (μmoles/ml of Packed Agarose)
0	0.45
4	0.46
15	0.40
36	0.39
96	0.36
112	0.36

[a] The activated ester of agarose (Figure 1, B) was stored as a suspension in dry dioxane as described in the text. At certain intervals samples of the suspension were filtered to remove excess dioxane and 1 g of the gel was added to 5 ml of 0.1 M sodium acetate buffer, pH 6.8, containing 5 mM L-[^{3}H]alanine. The suspension was stirred for 2 hr at 4°.

TABLE VIII: Enzymic Activity of Trypsin Immobilized to Agarose by the Cyanogen Bromide and *N*-Hydroxysuccinimide Procedures.

Method of Linking to Agarose	pH Used in Coupling Reaction[a]	Trypsin Content of Adsorbent (mg/ml of Agarose)	Enzyme Activity	
			K_M (M)	V_{max} (mole/min per mg)
Native trypsin			6.2×10^{-3}	1.2×10^{-5}
N-Hydroxysuccinimide	6.5	4.0	1.1×10^{-2}	1.3×10^{-5}
ester of agarose[a]	8.5	5.1	5.8×10^{-3}	1.1×10^{-5}
Cyanogen bromide[b]	6.5	4.3	4.5×10^{-3}	0.9×10^{-5}
	8.5	5.7	1.2×10^{-2}	1.0×10^{-5}

[a] Prepared as described in Table VII, using 0.1 M sodium bicarbonate (pH 8.5) and 0.1 M sodium phosphate (pH 6.5). [b] Prepared by the cyanogen bromide procedure (Cuatrecasas, 1970), using 200 mg of CNBr/ml of agarose and 5 mg/ml of trypsin in 0.1 M sodium bicarbonate (pH 8.5) or 0.1 M sodium phosphate (pH 6.5).

1970). However, if the beads are washed thoroughly with dioxane before lyophilization, the dried powder swells immediately when introduced into aqueous solutions. The *N*-hydroxysuccinimide ester derivatives of agarose which are thus lyophilized appear to be quite stable and substitution of amino acids and proteins occurs readily when the powder is added to appropriate buffer solutions, as described for the use of agarose in dioxane. The amount of ligand coupled to the agarose which has been dried is slightly (20–30%) higher than that which is coupled to the same derivative before lyophilization. Upon swelling from the powder form, shrinkage of similar magnitude (20–30%) is observed when compared to the original bead volume in water or dioxane. One gram of dried derivative swells in water to about 15 ml. Compared to agarose beads which are not dried, the size of the swollen beads is not appreciably changed on microscopic examination and the flow rate of columns packed with these beads is essentially unchanged. It is not yet known whether small (20–30%) changes in the degree of porosity of the beads occur which may account for the slight shrinkage described above.

Stability of Ligand-Substituted Agarose. Agarose adsorbents are generally stored in aqueous suspension at 4° since lyophilization results in severe shrinkage and loss of physical properties of the beads (Cuatrecasas, 1970). The stability of the linkage between L-alanine and agarose was examined in derivatives prepared by coupling the amino acid directly to agarose by the cyanogen bromide procedure (Cuatrecasas, 1970) and in derivatives prepared by coupling L-alanine to the *N*-hydroxysuccinimide ester of agarose. It was clear that at room temperature these derivatives are of limited stability, and that the lability resides in the bonds resulting from the cyanogen bromide linkage step, which are presumably iminocarbonate and isourea linkages (Porath, 1968), rather than in the amide linkage present more distally. The half-life of the substituted ligand in 0.05 M sodium bicarbonate buffer, pH 8.0, at room temperature, is about 40 days.

Coupling of Trypsin to the Active Ester of Agarose. As suggested by the results described above with model compounds, the *N*-hydroxysuccinimide ester of agarose can be used very effectively for the coupling of proteins to the gel. This is illustrated for trypsin in Table VII. Coupling occurs very rapidly and the total quantity of protein immobilized to agarose is nearly the same if the coupling is performed at pH 6.5 or at pH 8.5.

Considerable enzymic activity is retained by the insoluble trypsin derivatives (Table VIII). It is interesting that despite the similarity in the total amount of immobilized enzyme in the derivatives coupled in buffers of different pH or coupled by different procedures, there appear to be qualitative differences in the kinetic parameters (K_M) of these derivatives. Differences in kinetic constants are also observed in derivatives of agarose prepared by coupling trypsin to cyanogen bromide activated agarose in buffers of different pH (Table VIII). More detailed kinetic analyses and chemical studies will have to be performed to ascertain if these differences are in part attributable to such factors as differences in the proportion of molecules coupled through N-terminal residues. Furthermore, it will be interesting to determine whether the biological behavior of immobilized enzymes is affected by the distance which separates the enzyme from the matrix backbone. The present procedures provide techniques which may be helpful in studies designed to examine these questions. Other proteins which have been successfully linked to the *N*-hydroxysuccinimide ester of agarose with retention of biological activity include insulin, wheat germ agglutinin, concanavalin A, and antibodies (unpublished).

Discussion

The procedures described in this report greatly facilitate the immobilization to agarose and other gels of virtually any protein or ligand containing primary aliphatic or aromatic amino groups. The activated *N*-hydroxysuccinimide ester of agarose, which is stable over many months when stored in dioxane, is very simple to use and coupling of ligands occurs rapidly under very mild conditions. The use of cyanogen bromide (Cuatrecasas, 1970) can be circumvented, and proteins and ligands are attached by a considerable distance from the matrix backbone. Since it appears that only sulfhydryl groups can seriously compete with primary amino groups for reaction, it is possible to utilize these techniques to directly immobilize ligands which contain multiple or complex functional groups provided that an amino group is present. For example, it has not heretofore been possible to couple simple or complex amino acids through their amino group to carboxyl group containing agarose derivatives without selectively blocking other functional groups of the amino acid. Furthermore, the principal alternative procedures for coupling amino group containing ligands to derivatized agarose, which involve the use of water-soluble carbodiimide reagents and alkylating groups (Cuatrecasas, 1970), require much longer reaction periods, are much less specific for amino groups

TABLE IX: Coupling of L-Alanine, Trypsin, and Insulin to *N*-Hydroxysuccinimide Esters of Glass Beads.[a]

Material Coupled	Amount Coupled (per g of glass)
L-Alanine	3.5 μmoles
Insulin	15 mg
Trypsin	25 mg

[a] Alkylamine-substituted (Weetall and Hersh, 1969) glass beads were succinylated (Cuatrecasas, 1970) with succinic anhydride in saturated sodium borate. The *N*-hydroxysuccinimide ester was prepared in dioxane using 0.1 M *N*,*N*′-dicyclohexylcarbodiimide and *N*-hydroxysuccinimide as described for the agarose derivatives. The activated glass beads (1 g/20 ml) were washed with dioxane and methanol and reacted at 4° for 3 hr with 0.1 M sodium phosphate buffer, pH 7.4, containing 10 mM L-[^{3}H]alanine; for 2 hr with 3 mg/ml of [^{125}I]insulin (0.1 μCi/mg) in 6 M urea, 0.1 M sodium acetate buffer, pH 6.5; and for 1 hr with 5 mg/ml of trypsin in 0.1 M sodium acetate buffer, pH 6.2. The reactions with insulin and trypsin were terminated by adding solid glycine to achieve a final concentration of 1 M followed by incubation at 4° for 4 hr. The content of trypsin was determined by hydrolysis and amino acid analysis as described in the text.

(especially alkylating agarose derivatives), result in complicating side reactions if the ligand contains other functional groups, and are less likely to proceed to completion.

The use of *N*-hydroxysuccinimide esters of agarose for the immobilization of proteins may also offer certain specific advantages other than those resulting from the rapidity (trypsin is coupled in 15 min at 4°) and simplicity of the procedures involved. If it is desired to place the protein at a distance from the matrix backbone by coupling to hydrocarbon extensions attached to the polymer, the current procedures (Cuatrecasas, 1970; Cuatrecasas and Anfinsen, 1971a) which can be used will (a) result primarily in reaction of tyrosyl or histidyl residues of the protein (diazonium derivatives); (b) necessarily lead to molecular cross-linking and polymerization reactions of the protein (carbodiimide reagents); and (c) result in reactions which are very slow, incomplete, and lacking in discrimination between amino, imidazole, phenolic, or sulfhydryl groups of the protein (bromoacetyl derivatives). The activated agarose ester, on the other hand, reacts rapidly and almost exclusively with amino groups of the protein under very mild conditions. These procedures should prove useful in studies designed to determine the specific contribution of the polymer microenvironment on the altered kinetic behavior so frequently observed with insolubilized enzymes (Silman and Katchalski, 1966; Katchalski *et al.*, 1971). With these derivatives it should be possible to systematically place an enzyme at varying distances from the matrix backbone without significantly altering the nature or distribution of the specific chemical bonds used for linking the protein to the polymer.

The procedures described in this report have also been used successfully to couple ligands and proteins to glass beads (Table IX). The activated glass beads can be stored for long periods of time in dioxane, and highly substituted derivatives can be obtained. These techniques should extend the potential uses of glass supports for the immobilization of proteins (Weetall and Hersh, 1969; Weetall, 1969a,b), especially in view of the restricted reaction of these ester derivatives with amino groups of proteins and the stability of the amide linkages formed. Attempts to extend these procedures to polyacrylamide supports have been unsuccessful because of the instability of the acrylamide beads in dioxane and other organic solvents.

The general versatility of the chemical reactions which can potentially be performed on agarose beads is greatly enhanced by the current demonstration that these gels tolerate certain organic solvents such as dioxane and methanol extremely well.[2] It is very simple, for example, to couple relatively water-insoluble substances such as fatty acids or other lipids to agarose in the presence of these organic solvents.

The demonstration that agarose beads lyophilized from dioxane are quite stable in powder form and that they swell rapidly when reintroduced into aqueous medium should prove very useful in a number of applications of insoluble agarose supports. For example, activated derivatives such as esters of *N*-hydroxysuccinimide or bromacetyl derivatives (Cuatrecasas, 1970) could be conveniently stored and dispensed in dry form. Furthermore, it is clear that many other agarose-substituted adsorbents which are moderately unstable in aqueous media can now be stored in dry form provided the substituted ligand tolerates the required dioxane-washing procedure. Until now the only available means of storing derivatives of agarose beads has been in aqueous suspension, which on prolonged storage may lead to complications such as bacterial contamination and hydrolytic reactions.

Acknowledgment

The valuable technical assistance of Miss Lily Lee Wu is gratefully acknowledged.

References

Albertson, N. F. (1962), *Org. React. 12*, 205.
Belleau, B., and Malek, G. (1968), *J. Amer. Chem. Soc. 90*, 1651.
Cuatrecasas, P. (1970), *J. Biol. Chem. 245*, 3059.
Cuatrecasas, P. (1972), *Advan. Enzymol. 36* (in press).
Cuatrecasas, P., and Anfinsen, C. B. (1971a), *Methods Enzymol. 22*, 345.
Cuatrecasas, P., and Anfinsen, C. B. (1971b), *Annu. Rev. Biochem. 40*, 259.
Cuatrecasas, P., Wilchek, M., and Anfinsen, C. B. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 636.
Goldstein, L., Levin, Y., and Katchalski, E. (1964), *Biochemistry 3*, 1913.
Katchalski, E., Silman, I., and Goldman, R. (1971), *Advan. Enzymol. 34*, 445.
Laskowski, M. (1955), *Methods Enzymol. 2*, 26.
Lineweaver, H., and Burk, D. (1934), *J. Amer. Chem. Soc. 56*, 658.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Porath, J. (1968), *Nature (London) 218*, 834.
Porath, J., Axén, R., and Ernbäck, S. (1967), *Nature (London) 215*, 1491.
Schwert, G. W., Neurath, H., Kaufmann, S., and Snoke, J. E. (1948), *J. Biol. Chem. 172*, 221.
Sheehan, J. C., Cruickshank, P. A., and Boshart, G. L. (1961), *J. Org. Chem. 26*, 2525.

Sheehan, J. C., and Hlavka, J. J. (1956), *J. Org. Chem. 21*, 439.
Silman, I. H., and Katchalski, E. (1966), *Annu. Rev. Biochem. 35*, 873.
Spackman, D. H., Moore, S., and Stein, W. H. (1958), *Anal. Chem. 30*, 1190.
Steers, E., Cuatrecasas, P., and Pollard, H. (1971), *J. Biol. Chem. 246*, 196.
Weetall, H. H. (1969a), *Science 166*, 615.
Weetall, H. H. (1969b), *Nature (London) 223*, 959.
Weetall, H. H., and Hersh, L. S. (1969), *Biochim. Biophys. Acta 185*, 464.

Effect of Fatty Acids on the Binding of 1-Anilino-8-naphthalenesulfonate to Bovine Serum Albumin†

Elsa C. Santos and Arthur A. Spector*

ABSTRACT: The fluorescence of 1-anilino-8-naphthalenesulfonate (ANS) bound to bovine serum albumin was reduced by the presence of medium or long-chain fatty acids. This occurred over the pH range of 4–10. Quenching increased as the molar ratio of fatty acid to albumin was raised, the maximum reductions being in the range of 45–55%. The degree of quenching produced by a given amount of fatty acid was dependent upon the ANS–albumin molar ratio. Fatty acid induced quenching was not accompanied by any change in the wavelength of maximum fluorescence. Effects on ANS fluorescence similar to those noted with bovine albumin were observed when palmitate was added to human, rabbit, or equine albumins. Equilibrium dialysis studies revealed that ANS binding to bovine albumin was decreased when either palmitate or laurate were present. Therefore, the fatty acid induced quenching of ANS fluorescence probably was due to displacement of ANS from albumin binding sites. These results suggest that variations in the plasma free fatty acid concentration may be important in regulating the capacity of albumin to transport other nonpolar organic compounds.

Fluorescent probes such as 1-anilino-8-naphthalenesulfonate (ANS)[1] have been employed to examine the structure and interactions of proteins, particularly the nature of their nonpolar binding sites (Stryer, 1965; Turner and Brand, 1968). Bovine serum albumin has been studied extensively by this technique (Weber and Young, 1964; Daniel and Weber, 1966; Weber and Daniel, 1966), and albumin often has served as a model protein for structural interpretations.

A major physiological function of albumin is to transport long-chain free fatty acid in the plasma (Dole, 1956; Gordon and Cherkes, 1956), and the binding of fatty acids to albumin has been investigated in detail (Boyer *et al.*, 1946; Teresi and Luck, 1952; Goodman, 1958; Reynolds *et al.*, 1968; Spector *et al.*, 1969, 1971). In the course of studies with albumin, we found that addition of palmitate altered the fluorescence of albumin-bound ANS. We thought it of interest to explore this observation in order to better understand the effect of lipids on the binding of fluorescent probes to proteins and to gain insight into possible interactions between fatty acids and other nonpolar ligands that are transported by serum albumin.

Methods

ANS, obtained from Eastman Kodak Co., was recrystallized from water and then dried at 110° for 8 hr (Weber and Young, 1964). Serum albumins, purchased from Miles Laboratories, were treated with charcoal and then dialyzed to remove inherent fatty acids (Chen, 1967; Spector *et al.*, 1969). Sodium phosphate was added to the protein solutions so that the final concentration was 0.05 M (pH 7.4). Protein concentration was determined by the biuret method with dried, crystalline albumin as the standard (Gornall *et al.*, 1949). Fatty acids were purchased from either Applied Science Laboratories or the Hormel Institute and were of the highest purity available commercially (99%). Fatty acid–albumin complexes were prepared by adding slowly a warm solution of the sodium salt of the fatty acid to the albumin solution. The concentration of the fatty acid was determined by titration (Trout *et al.*, 1960).

Fluorescence measurements were made with a Hitachi Perkin-Elmer Model MPF-2A fluorescence spectrophotometer equipped with a recorder. A thermostatically controlled Lauda K-2/R water circulator (Brinkmann Instruments) maintained the temperature in the cell holder at 25°. The ratio recording mode was used to eliminate fluctuations of the source output. The excitation wavelength was 380 nm, and the fluorescent emission was recorded between 400 and 600 nm. Fluorescence measurements were taken at least 1 hr after the incubation mixtures were prepared.

ANS binding to albumin was measured at 37° by equilibrium dialysis (Spector and Imig, 1971). Equilibrium was attained in this system within 16 hr. Preliminary studies indi-

† From the Departments of Biochemistry and Internal Medicine, University of Iowa, Iowa City, Iowa 52240. *Received February 14, 1972.* These studies were aided by grants from the National Heart and Lung Institute (HE-14,781) and the American Heart Association (71-895), the latter being supported in part by the Iowa Heart Association.

* Research Career Development awardee of the National Heart and Lung Institute (K4-HE-20,338).

[1] Abbreviation used is: ANS, 1-anilino-8-naphthalenesulfonate.