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MEMBRANE STRUCTURE: THE REACTIVITY OF TRYPTOPHAN, TYROSINE AND LYSINE IN PROTEINS OF THE MICROSOMAL MEMBRANE

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SUMMARY

1. A microsomal subfraction devoid of adsorbed soluble proteins has been prepared from rat liver by density gradient centrifugation. Through the use of group-specific reagents the reactivity of tryptophanyl, tyrosyl, and lysyl side chains of the membrane has been examined. Significant differences are noted when the results are compared to literature values for non-membrane proteins.

2. In the native microsomal membrane, tryptophan was unreactive toward *N*-bromosuccinimide. The results suggest that strong tryptophan interactions may exist which could likely have an important role in the maintenance of normal membrane structure. Furthermore, tryptophan appears to be involved at different levels of structural organization since extremely sharp transitions in reactivity were noted when sodium dodecyl sulfate was used as a perturbant.

3. 85% of the tyrosyl residues occupied an exposed position on the membrane surface and were characterized by their ease of nitration in the absence of denaturing agents. The unusual accessibility of tyrosine suggests that side chain interactions involving tyrosyl residues do not play a prominent structural role.

4. Carbamylation studies revealed that only 56% of the lysyl residues in the native membrane were available for reaction with cyanate while an additional 20–25% became reactive in the presence of urea. The remaining lysyl residues were unreactive under a wide variety of denaturing conditions.

5. Sodium dodecyl sulfate interacted with the membrane in such a way as to markedly decrease the apparent accessibility of tyrosyl and lysyl residues while substantially increasing the reactivity of tryptophan.

INTRODUCTION

The macromolecular interactions responsible for maintaining native membrane structure are poorly understood. Undoubtedly, the most prominent interactions are protein–protein, protein–lipid, and lipid–lipid in nature. The relative importance of each of these contributions remains unknown and represents a major area of investigation. Although membranes are composed of a variety of different proteins, their association with each other and with lipid generates a unique morphological structure which can be examined as a discrete entity. A wide variety of physicochemical techniques have been used to study membrane structure and these include infrared

spectroscopy[1], optical rotatory dispersion[2,3], nuclear magnetic resonance[4], electron paramagnetic resonance[5], and fluorescence measurements[6]. Valuable information has been obtained from these studies but certain interpretative problems have been encountered which are primarily the result of the multicomponent and particulate nature of most membrane systems.

An approach not used heretofore involves the use of group-specific reagents. Reagents exhibiting a high degree of selectivity for specific amino acid side chains have been employed to determine the accessibility or inaccessibility of side chains in proteins with a large degree of success. Results obtained from such studies have, in most cases, correlated well with the X-ray crystallographic findings[7]. Functional groups that are freely reacting usually are located on the surface of the protein while those that are unreactive are embedded in the polypeptide matrix and are shielded from the aqueous environment. Thus, it appears that specific chemical modification of various amino acid side chains in intact membranes would be useful in delineating the role of these groups in protein–protein and lipid–protein interactions within the membrane. In addition, information regarding the chemical nature of the membrane surface would also be obtained. The microsomal membrane was selected to explore some of these interactions since the findings can be correlated with a number of biochemical and morphological characteristics.

MATERIALS AND METHODS

Preparation of microsomal membrane

Rat liver microsomes were prepared from fasted Holtzman rats (190–210 g) by the method of KASHNIG AND KASPER[8]. The membrane isolated at the air–buffer interface was washed twice with 0.05 M sodium acetate. The yield of membrane was in the range of 3 to 4 mg protein per g of liver.

RNA was removed from the membrane by digestion with ribonuclease A and ribonuclease T_1 (Worthington) under standard conditions. The membrane was dispersed in water to give a protein concentration of approximately 5 mg/ml, and 10-ml aliquots were stored at —18° in individual vials.

Chemical analysis

Routine quantitative chemical analyses were carried out as previously described[8].

Amino acid analyses were performed on a Beckman model 120C automatic amino acid analyzer, equipped with Penton cuvettes having an optical path of 12 mm by the method of SPACKMAN *et al.*[9]. Standard 3-nitrotyrosine (K and K laboratories), homocitrulline (Nutritional Biochemical Co.), cysteic acid (Calbiochem), and *S*-carboxymethylcysteine (Mann Research Laboratories) were used without further purification. Cystine and cysteine were determined as cysteic acid after performic acid oxidation[10] and as *S*-carboxymethyl cysteine after reduction with mercaptoethanol followed by alkylation with iodoacetic acid[11]. Dithiothreitol was also used as the reducing agent in certain experiments.

Cysteine was determined as *S*-carboxymethylcysteine after direct alkylation of the membrane and also by the procedure of ELLMAN[12].

Tryptophan was determined on native and acetone-delipidated membranes

as well as on tryptic digests of the membrane by the method of BENCZE AND SCHMID[13], using 0.2 M NaOH containing 0.05% sodium dodecyl sulfate.

Modification of amino acid side chains

Tyrosine

Nitration of the tyrosyl residues in the membrane using tetranitromethane (Aldrich Chemical Co.) was carried out by the method of SOKOLOVSKY *et al.*[14]. Stock solutions of reagent were prepared daily by dissolving 1.646 g of tetranitromethane in 10 ml of 95% ethanol (8.4 μmoles/μl) and aliquots diluted to a final concentration of 0.84 μmole/μl before use. In a typical nitration experiment, 0.2 ml of 0.5 M Tris–HCl (pH 8.0) buffer containing 0.5 M NaCl was added to 1 ml of a membrane suspension in water (5 mg of protein per ml). Appropriate amounts of urea or sodium dodecyl sulfate or both were then added and the volume made up to 2.0 ml with water. Tetranitromethane was added and the reaction mixture left at room temperature for 30 min. Excess reagent was removed by exhaustive dialysis against distilled water at 3°. After lyophilization the reaction mixture was hydrolysed (6 M HCl at 110° for 24 h) and analyzed for tyrosine and 3-nitrotyrosine.

Tryptophan

The oxidation of tryptophanyl residues was carried out with *N*-bromosuccinimide (Pierce Chemical Co.) by the method of WITKOP AND SPANDE[15]. A stock solution of N-bromosuccinimide was prepared by dissolving 4.45 mg of reagent in 2.5 ml of water or 8 M urea. 0.4 ml of membrane suspension (10 mg/ml) in 0.1 M acetate buffer, pH 4.0, was added to 7.6 ml of the 0.1 M acetate buffer. To one-half of the membrane suspension, solid sodium dodecyl sulfate was added to a final concentration of 0.5%. This solution was used to determine the initial absorbance at 280 nm. To the remainder of the membrane suspension an amount of *N*-bromosuccinimide solution (150 μl) equivalent to a 3-fold molar excess of reagent to tryptophan was added and the solution left at room temperature for the specified period of time (1 to 24 min). The reaction was terminated by the addition of a 2-fold molar excess of mercaptoethanol and 20 mg of sodium dodecyl sulfate were added. All solutions were passed through a medium porosity sintered glass filter prior to measuring the absorption at 280 nm. Individual solutions were examined for light scattering and the appropriate corrections were applied when necessary. From the decrease in absorption at 280 nm after treatment with *N*-bromosuccinimide, the μmoles of tryptophan oxidized were calculated using the molar absorbance difference value (ΔE) of $4 \cdot 10^3$ (see ref. 15). Absorbance values were corrected for the addition of reagents by assuming additivity of volumes.

Lysine

Carbamylation of lysyl residues in the membrane was carried out according to the procedure of SMYTH[16]. Potassium cyanate was obtained from Matheson, Coleman and Bell and sodium cyanate was obtained from Fairmont Chemical Co. The carbamylation reaction was carried out in a 1-ml volume using 0.1 M borate buffer, pH 8.8, and a protein concentration of 2 mg/ml. The desired amount of potassium cyanate was added and the reaction tubes were stoppered and incubated in a water bath at 30° $\pm$ 0.5° for the specified period of time. Excess cyanate was destroyed by the addition of glycyl-glycine (2 molar excess with respect to cyanate). By-products of the reaction were removed by exhaustive dialysis against distilled water at 3°.

The degree of carbamylation was determined by hydrolyzing the derivatized membrane with 6 M HCl at 110° for 22 h and measuring the amount of homocitrulline. A factor of 24% was used to correct for the hydrolytic reversion of homocitrulline to lysine under these conditions[17]. Sodium cyanate was employed when the carbamylation was conducted in the presence of sodium dodecyl sulfate.

RESULTS

Physical and chemical properties of microsomal membrane

The membrane preparation described in this study is microsomal in origin but is devoid of essentially all soluble non-membrane proteins. The insoluble membrane matrix, however, possesses a variety of enzymic activities which are characteristic for the native microsomal membrane[8]. When the isolated membrane was examined in a CsCl gradient, it yielded a single band at denstiy 1.18 g per ml (unpublished studies by H. KUBINSKI AND C. B. KASPER). The composition of the membrane (Table I) bears a striking similarity to the composition of the nuclear envelope isolated from the same tissue[8]. The most pronounced difference resides in the neutral sugar to hexosamine ratio which is 1.7 and 15 for the microsomal and nuclear membranes,

TABLE I

COMPOSITION OF MICROSOMAL MEMBRANE FROM RAT LIVER AFTER TREATMENT WITH PANCREATIC AND T_1 RIBONUCLEASE

Component	%
Protein	62.4
Carbohydrate	2.8
Neutral sugars	1.7
Hexosamine*	1.0
Sialic acid	0.1
Lipid	34.4
Phospholipid	29.2
Cholesterol	5.2
RNA	0.4

* Glucosamine was the only amino sugar detected in acid hydrolysates of the membrane.

TABLE II

AMINO ACID COMPOSITION OF DELIPIDATED MICROSOMAL MEMBRANE

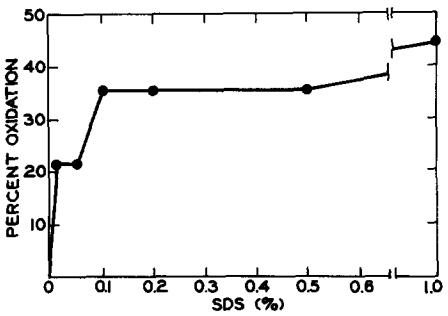
Amino acid	*μmoles* (%)	*Amino acid*	*μmoles* (%)
Lys	6.03	Ala	6.44
His	2.28	$^1/_2$ Cys	1.44
Arg	4.69	Val	6.67
Asp	8.75	Met	2.31
Thr	5.19	Ile	5.09
Ser	6.36	Leu	10.55
Glu	9.79	Tyr	3.27
Pro	5.11	Phe	5.10
Gly	6.61	Trp	4.35

respectively. The amino acid analysis of the microsomal membrane appears in Table II. Basic amino acids accounted for 13% of the total amino acid residues while aspartic and glutamic acids and their amides occurred at a combined level of 18.5%. Tyrosine, phenylalanine, and tryptophan accounted for 12.7% and leucine, isoleucine, and valine represented an additional 22.3%. Cystine was present in an amount corresponding to 8.5 residues per 160 000 g of protein. Cysteine was not detected in the membrane.

Reactivity of tryptophanyl residues

Preliminary experiments were performed with lysozyme to ascertain the effectiveness of mercaptoethanol as a quenching agent. The results indicated that (1) mercaptoethanol is effective at a molar ratio of mercaptan to *N*-bromosuccinimide of 2:1; (2) mercaptoethanol successfully competes with tryptophan for *N*-bromosuccinimide at a molar ratio of mercaptan to tryptophan of 4:1; (3) neither mercaptoethanol nor its oxidation product has significant absorbance at 280 nm; (4) addition of sodium dodecyl sulfate does not quantitatively or qualitatively affect the spectral characteristics of the tryptophan oxidation product. The possible interference of the reaction between *N*-bromosuccinimide and membrane lipid (*e.g.* allylic bromination of unsaturated fatty acid chains) with the spectral determination of tryptophan was also examined. *N*-Acetyltryptophan was analyzed in the presence and absence of a microsomal lipid extract with comparable results being obtained in each case.

Incubation of native microsomal membrane in the absence of denaturing agents with a 3-fold molar excess of *N*-bromosuccinimide to tryptophan for periods up to 10 min resulted in no tryptophan oxidation. Reaction times of 15 min or greater produced cloudy solutions after the addition of detergent as did increased levels of oxidizing agent. The effect of low concentrations of detergent on the reactivity of tryptophanyl residues was quite pronounced. The addition of sodium dodecyl sulfate to a final level of 0.01% prior to the introduction of *N*-bromosuccinimide resulted in the oxidation of 22% of the tryptophanyl residues in the membrane (Fig. 1).



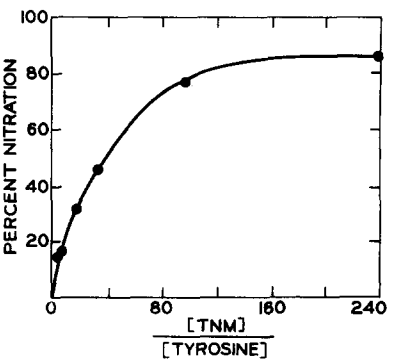


Fig. 1. Effect of sodium dodecyl sulfate (SDS) concentration on the reactivity of tryptophan in the microsomal membrane. The protein concentration was 0.5 mg/ml and the *N*-bromosuccinimide concentration was 0.067 mg/ml.

Fig. 2. The nitration of tyrosyl residues in the microsomal membrane with increasing concentrations of tetranitromethane (TNM). The concentration of TNM ranged from 2.1–154 μmoles/ml and the protein concentration was 2.5 mg/ml. The reaction was performed at pH 8.0 at room temperature.

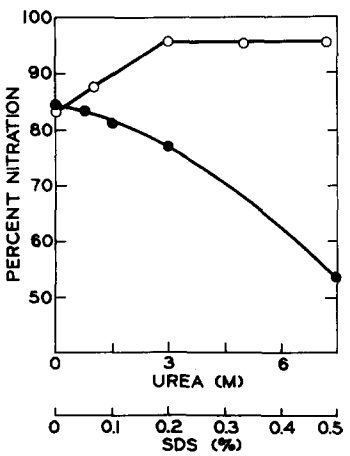
At 0.05% the degree of oxidation remained unchanged but increased to 35 % in the concentration range of 0.1% to 0.5% detergent. Maximum oxidation (44%) was obtained in 1% sodium dodecyl sulfate.

When the oxidation was carried out in 8 M urea at pH 4, the reaction proceeded slowly and required close to 30 min for completion with a given concentration of reagent. A maximum destruction of 27% of the tryptophan in the membrane was obtained at a molar ratio of *N*-bromosuccinimide to tryptophan of 4.5. This is in contrast to the results obtained with 0.5% sodium dodecyl sulfate where a molar ratio of 1.75 yielded maximum oxidation. The combined use of detergent and urea did not increase the reactivity of tryptophan.

In an effort to find conditions that would unmask all tryptophanyl residues, a tryptic digest of the membrane was treated with a 3-fold molar excess of *N*-bromosuccinimide to tryptophan in the presence of 0.5% sodium dodecyl sulfate. Under these conditions the extent of oxidation was identical to that obtained with the non-trypsin-treated membrane. Furthermore, if the reaction was carried out in the presence of 0.5% detergent and 8 M urea not more than 36% of the tryptophan was destroyed.

Reactivity of tyrosyl residues

Exposure of the microsomal membrane to increasing concentrations of tetranitromethane resulted in the reaction profile seen in Fig. 2. The plateau region corresponds to the derivatization of 85% of the tyrosine in the membrane and the continuity of the curve suggests that all of these tyrosyl residues possess essentially the same degree of reactivity. Urea was quite effective in disrupting the membrane structure in the region of tyrosine since essentially 100% nitration was obtained at urea concentrations of 3 M or greater (Fig. 3). Sodium dodecyl sulfate, however, appeared to reduce the accessibility of tyrosine as evidenced by a marked decrease



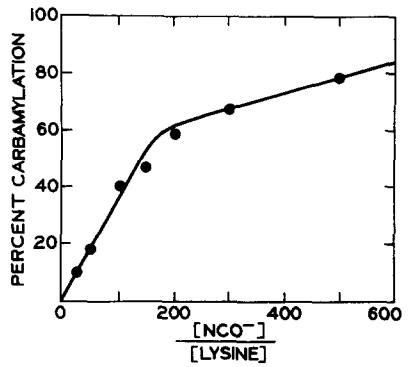


Fig. 3. Nitration of microsomal membrane as a function of increasing urea (○—○) and sodium dodecyl sulfate (SDS) (●—●) concentrations. A 100-fold molar excess of tetranitromethane to tyrosine was employed.

Fig. 4. Carbamylation of lysyl residues in the microsomal membrane as a function of cyanate concentration. The protein concentration was 2 mg/ml and the potassium cyanate concentration ranged from 2–40 mg/ml. The reaction was carried out at 30° at pH 8.80 for 24 h.

in nitration with increasing concentrations of detergent (Fig. 3). At a detergent concentration of 0.5% only 53%, of the tyrosyl residues were nitrated. This represented a reduction of 36% over that noted in the absence of added denaturant.

In order to ascertain if sodium dodecyl sulfate had a direct effect on the nitration reaction itself, the reaction of tetranitromethane with *N*-acetyltyrosine was examined in the presence and absence of detergent. No effect on the extent of nitration was detected. Thus, the effect of detergent is most likely on membrane conformation with the net result being a decrease in the reactivity of tyrosine. If the membrane was dissolved in 8 M urea prior to the addition of detergent to a final concentration of 0.5%, all tyrosyl residues were readily nitrated. The same result was obtained if the membrane was first solubilized in 0.5% sodium dodecyl sulfate before the addition of urea. These experiments indicate that urea not only reverses but prevents the effect of dodecyl sulfate.

Reactivity of lysyl residues

The effect of cyanate concentration on the extent of derivatization of lysyl side chains is illustrated in Fig. 4. The curve is characterized by a sharp break at a molar excess of cyanate to lysine of 200. These data suggest that two distinct classes of lysyl residue are present in the membrane which are distinguished by their ease of carbamylation. Using a 200-fold molar excess of cyanate, the carbamylation reaction was examined as a function of time in the presence and absence of 8 M urea (Fig. 5). Both the initial rate and the extent of the reaction were enhanced in the denaturing solvent. From control experiments, it was clear that the change in velocity of carbamylation was not due to the hydrolytic destruction of cyanate during the course of the reaction.

When sodium dodecyl sulfate was used as the denaturing agent, the rate of carbamylation was markedly diminished (Fig. 5). At the level of 0.05% sodium

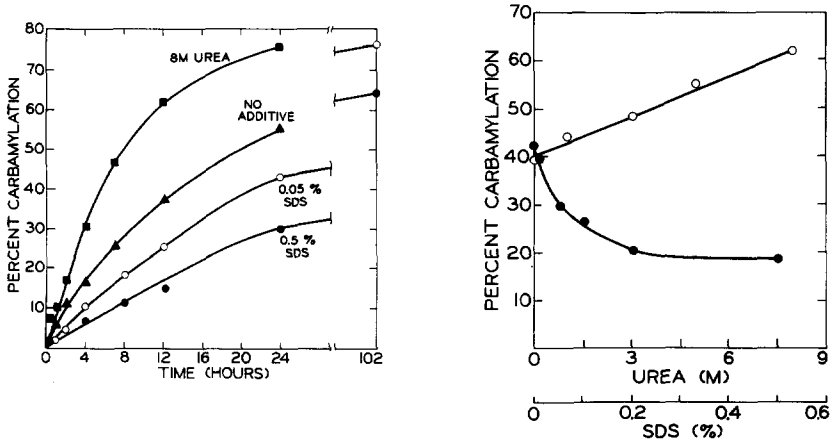


Fig. 5. The effect of urea and sodium dodecyl sulfate (SDS) on the time dependence of the reactivity of lysyl residues in the microsomal membrane. The molar ratio of cyanate to lysine was 200. The protein and potassium cyanate concentration was 2 and 16 mg/ml, respectively.

Fig. 6. Effect of urea (○—○) and sodium dodecyl sulfate (SDS) (●—●) concentration on the carbamylation of lysine in the microsomal membrane. The molar ratio of cyanate to lysine was 200 and the time of reaction was 12 h. The concentration of cyanate was the same as in Fig. 5.

dodecyl sulfate, 80% of the lysyl residues reacting in the absence of any denaturant were derivatized, but only 60% had reacted in the presence of 0.5% sodium dodecyl sulfate after 24 h. The results obtained after 102 h in the presence of 0.05% and 0.5% detergent indicate that the binding of the anionic alkyl sulfate to the membrane significantly reduces the rate of the carbamylation reaction.

Upon examining the effect of urea and sodium dodecyl sulfate concentration on the derivatization of lysine, it was noted that carbamylation increased linearly with increasing urea concentrations while relatively low levels of detergent were found to be quite effective in retarding the reaction (Fig. 6). In order to determine if the inhibitory effect was due to the interference of sodium dodecyl sulfate with the carbamylation process itself, the reaction of α-*N*-acetyl lysine with cyanate was studied in the presence and absence of detergent. The results clearly showed that the presence of detergent did not affect the degree of carbamylation.

Since certain lysyl residues in the membrane were not available for reaction with cyanate, a variety of denaturing conditions were employed in an attempt to obtain complete derivatization with only limited success. The most effective set of conditions involved heating the membrane in 8 M urea at 60° for 30 min prior to carbamylation at 30°. This treatment resulted in the modification of 83% of the epsilon amino groups present in the membrane.

DISCUSSION

Side-chain reactivity

Tryptophan was unique in that all residues were completely buried in the native membrane structure. The finding of unreactive tryptophan is not unprecedented but is uncommon. Avidin, when in combination with biotin, exhibits little or no change in optical density when treated with *N*-bromosuccinimide[18]. However, in the absence of biotin, the equivalent of 16 of the 18 tryptophans in avidin are readily oxidized. The latter result is more in line with the general observation that tryptophan oxidation usually proceeds unhindered at pH 4 (*e.g.* all tryptophan residues in myoglobin, chymotrypsin, trypsin, and lysozyme are accessible to oxidation at this pH[19]).

Three classes of tryptophan were identified on the basis of their reactivity with *N*-bromosuccinimide in the presence of increasing concentrations of detergent. The marked inaccessibility of these side chains to low molecular weight solutes indicates that they may be involved in strong interactions within the membrane. Furthermore, the stepwise exposure of tryptophan by increasing levels of detergent strongly indicates that tryptophan residues are involved at different levels of membrane organization.

Nitration of tyrosyl residues clearly showed that 85% were freely reactive and thus occupied an exposed position on the membrane surface. In a variety of well-characterized proteins such as ribonuclease A[20], carbonic anhydrase A and B[21], subtilisin BPN (see ref. 22), subtilisin Carlsberg[22], and trypsin[23], 50% or more of the tyrosyl residues are involved in side chain interactions which confer an abnormal ionization behavior and a lack of reactivity toward tetranitromethane. The unusual accessibility of tyrosine in the membrane lipoprotein complex is in sharp contrast to that noted for these low molecular weight globular proteins.

Based on carbamylation studies, three different groups of lysyl residues have

been distinguished. It is of interest to note that in the case of pepsinogen[24] and subtilisin Novo[25] 90 and 100%, respectively, of all lysyl residues were carbamylated under conditions where only 55% of the ε-amino groups of the membrane were reactive. Furthermore, when the reaction was conducted in the presence of 8 M urea, 76% derivatization resulted, whereas complete reaction of all α- and ε-amino groups is usually obtained in the case of non-membrane proteins[16]. The membrane appears to be unique in having a high proportion of lysine that is unaccessible.

Effect of sodium dodecyl sulfate on side chain reactivity

Evidence is available which suggests that sodium dodecyl sulfate does not destroy all ordered regions in proteins and may actually induce the formation of new α-helical structure[27,28].

The detergent may bind to the membrane proteins in the vicinity of the tyrosyl side chains without completely unfolding the native structure. It is not unreasonable to envision micelles developing in these regions where a high ratio of detergent to tyrosyl side chains would exist. These micelles could either form a physical barrier to the approach of tetranitromethane or the electrostatic influence of the bound detergent may actually alter the pK_a of the phenolic hydroxyl group. Since one of the rate-determining steps in the nitration reaction involves the formation of a phenolate anion[14,29], suppression of this ionization would bring about the observed result. The reversal of the detergent inhibition by urea could be explained by the loss of all organized structure in the region of tyrosyl side chains with a concomitant decrease in the electrostatic interaction because of delocalization of bound anionic groups.

The carbamylation reaction showed a greater inhibition by relatively low concentrations of detergent (Fig. 6) than did the nitration reaction. Factors influencing this reaction are similar to those discussed for the nitration of tyrosine. It is of interest to note that the binding of fatty acids to serum albumin blocked the reactivity of certain epsilon amino groups with fluorodinitrobenzene[30]. From the observed effect of sodium dodecyl sulfate on the reactivity of various functional side chains in proteins, it is apparent that caution must be exercised in the interpretation of results when employing this anionic detergent.

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