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Effect of sodium dodecyl sulfate on immuno-electrosyneresis between normal human erythrocyte membrane and sera of systemic lupus erythematosus patients*

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Abstract

An anti-membrane antibody was present in the sera of systemic lupus erythematosus patients in immunoelectrosyneresis with sodium dodecyl sulfate (SDS) solubilized erythrocyte membrane as antigen. The SDS bound to protein was detected by chromatography at 10(-3)M concentration under U.V. light, at 10(-5)M concentration by the distilled water spray method and at 10(-6)M concentration by using rosaniline hydrochloride colorimetry. SDS was removed from the membrane protein at a concentration of 10(-3)M by the first gel filtration of Sephadex G-25 column and at a concentration of 10(-6)M by rechromatography of the same column. More than 99% of SDS in the solubilized erythrocyte membrane was removed by gel filtration. The antigenicity was still positive in the refiltrated fractions of systemic lupus erythematosus patients. Therefore, all precipitates in the gels were antigen-antibody aggregates.

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EFFECT OF SODIUM DODECYL SULFATE ON IMMUNO-ELECTROSYNERESIS BETWEEN NORMAL HUMAN ERYTHROCYTE MEMBRANE AND SERA OF SYSTEMIC LUPUS ERYTHEMATOSUS PATIENTS

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Abstract: An anti-membrane antibody was present in the sera of systemic lupus erythematosus patients in immunoelectrosyneresis with sodium dodecyl sulfate (SDS) solubilized erythrocyte membrane as antigen. The SDS bound to protein was detected by chromatography at 10^{-3}M concentration under U.V. light, at 10^{-5}M concentration by the distilled water spray method and at 10^{-6}M concentration by using rosaniline hydrochloride colorimetry. SDS was removed from the membrane protein at a concentration of 10^{-3}M by the first gel filtration of Sephadex G-25 column and at a concentration of 10^{-6}M by rechromatography of the same column. More than 99% of SDS in the solubilized erythrocyte membrane was removed by gel filtration. The antigenicity was still positive in the refiltrated fractions of systemic lupus erythematosus patients. Therefore, all precipitates in the gels were antigen-antibody aggregates.

We have previously reported (1) that anti-membrane antibody was present in sera of patients of various autoimmune diseases, especially systemic lupus erythematosus (SLE), against normal human erythrocyte membrane as antigen. In the immunoelectrosyneresis response, the sodium dodecyl sulfate (SDS) concentrations in the solubilized erythrocyte membrane may be critical (2) for the specificity of this antigen-antibody reaction. To determine that the precipitates in the gels of immunoelectrosyneresis represent genuine antigen-antibody reactions and not non-specific interactions of SDS and serum protein, we removed the SDS by gel filtration of Sephadex G-25 column from solubilized erythrocyte membrane and examined the SDS effects on the antigen-antibody reaction.

MATERIALS AND METHODS

Erythrocyte membranes were prepared according to the procedure of Dodge, Mitchell and Hanahan (3) with the following modifications. The human

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erythrocytes were collected from blood group O normal subjects with heparinized syringe and washed with saline three times at 4°C. The erythrocytes were hemolysed with 0.01M phosphate buffer (pH 7.4). The specimen was washed and centrifuged at 2000×g repeatedly with distilled water until the supernatant was clear. The final pellet of hemoglobin-free erythrocyte membrane was suspended in distilled water and lyophilized.

Solubilization of isolated erythrocyte membrane (*4*): A 2.4% (W/V) SDS solution was prepared for dissolving erythrocyte membrane using 0.01M phosphate buffer (pH 7.4) containing 0.14M 2-mercaptoethanol and 20% (V/V) glycerol. The lyophilized erythrocyte membrane was dissolved in this solution at a concentration of 10.0 mg/ml (dry weight). Samples of 2ml were allowed to stand 48 hours at 37°C under nitrogen atmosphere and were centrifuged at 100,000×g for 60 minutes. The supernatant was placed in a cellulose tube and dialyzed overnight against 0.1M phosphate buffer (pH 7.8) to remove 2-mercaptoethanol and SDS. The total recovered protein was measured at about 5,500 μg by the Lowry-Folin method using bovine serum albumin as the standard material (5).

Extraction of SDS from thin-layer chromatography (*TLC*). Chromatoplates (20×20 cm) coated with 0.25mm layer of Kieselgel G or H (Merck, Type 60) were prepared. The plates were activated at 110°-120°C for 2 hours prior to use. Solvent for the development was composed of chloroform, methanol and 0.1N sulfuric acid in a volume of 80 : 19 : 1 (6). Four μl of fractionated membrane materials was applied on TLC and developed for 40 minutes at 20-25°C. The developed chromatoplates were sprayed with a 0.2% alcoholic solution of 2′, 7′-dichlorofluorescein or distilled water and observed under U.V. (2536Å) or fluorescent light to detect the SDS spots. Fractionated erythrocyte membranes containing SDS were developed on TLC. Alcoholic solution (0.2%) of 2′, 7′-dichlorofluorescein or distilled water was sprayed on TLC. The Rf value of the SDS spot was referred to the SDS-containing sample spot, and the corresponding part of the adsorbent was extracted with 100% methanol and filtered by Toyoroshi No. 51 filter paper. The dried adsorbent was transferred into another tube and the same procedure was repeated three times. The extract was collected and evaporated under reduced atmosphere. The residue was resuspended and solubilized in 4.0 ml of 0.025M phosphate buffer (pH 6.1). The product in each core was added to 1 ml of 4×10^{-4}M rosaniline hydrochloride solution (7). The solvent (ethylacetate : chloroform = 50 : 50 V/V) was added to the extracted SDS-rosaniline hydrochloride complex. The solvent layer was separated by centrifugation at 2,000×g for 10 minutes. SDS content in the solvent was assayed by a Shimazu spectrophotometer (Model QV-50) at 550 nm absorption. The standard curve for SDS was drawn from 0.1 μg/ml to 1.2 μg/ml.

Gel filtration method (*8*): To remove SDS from the dialyzed membrane solution, Sephadex G-25 was charged onto a plastic column. The gel was buffered by 0.025M Tris-HCl buffer (pH 7.4) at 4°C. The membrane solution was loaded on top of the column and eluted in 3 ml fractions at a flow rate of 30 ml/h. Quantitation of aliquots of each protein fraction was carried out by both the Lowry-Folin method and ultraviolet absorbance at O.D. 280nm.

Immunoelectrosyneresis (9): The 1.5% Noble agar in pH 8.6 veronal buffer (μ=0.025) was spread on a slide glass (1 mm thickness) and 10 pairs of wells were placed into the agar medium. Each well was 2 mm in diameter and the distance between the wells was 3 mm. The power supply was kept constant at 2 mA/cm, 90 to 100 volts for 25 minutes. The solubilized membrane solution diluted five-fold was placed in the wells on the cathode side while the SLE patient sera were placed on the anode side. The precipitin band was observed immediately and 24 hours after electrophoresis.

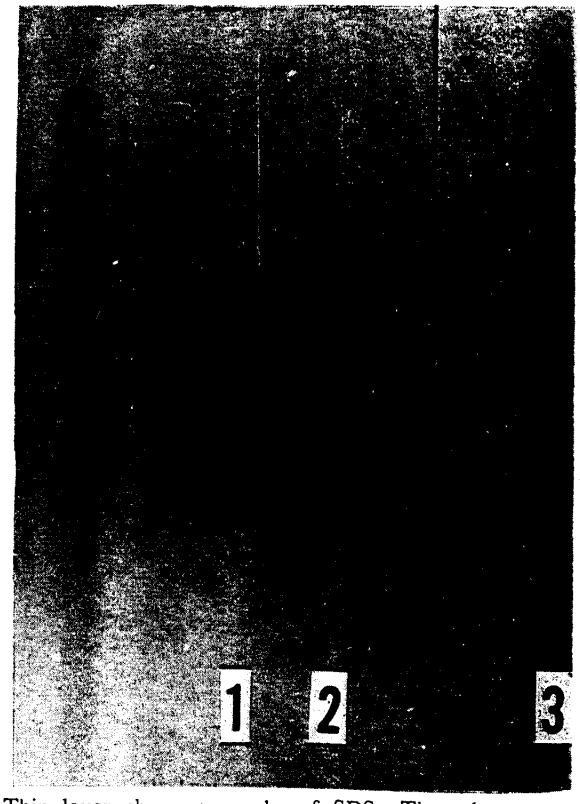

Fig. 1. Thin layer chromatography of SDS. The solvent was composed of chloroform, methanol and 0.1N sulfuric acid in a 80 : 19 : 1 volume. The solution of SDS was developed for 40 minutes at 20–25°C. Under U. V. light irradiation after spraying with dichlorofluorescein, SDS was detected as a yellow spot at Rf 0.35 (No. 1 and 2). Under distilled water spraying, SDS was detected as a red spot at the the same Rf (No. 3).

RESULTS

The sensitivity of SDS was compared by the method of U. V. light irradiation and the method of water spray between 10^{-1}M to 10^{-5}M con-

centrations (pH 6.1) by TLC. Under U. V. light irradiation after spraying with dichlorofluorescein, SDS was detected at 10^{-1}M to 2×10^{-3}M (2.3 μg) on TLC as a yellow spot at Rf 0.35. Under distilled water spraying, SDS was detected at 2.75×10^{-5}M (0.31 μg) as a red spot at the same Rf (Table 1, Fig. 1).

TABLE 1 SENSITIVITY OF THIN-LAYER CHROMATOGRAPHY

SDS (M)	SDS spot	
	Under U. V. light	Under water spray
10^{-1}	+	+
10^{-2}	+	+
2×10^{-3}	+	+
10^{-3}	−	+
10^{-4}	−	+
2.75×10^{-5}	−	+
10^{-5}	−	−

SDS, sodium dodecyl sulfate

The absorption curve of SDS-rosaniline hydrochloride complex showed a smooth shoulder at approximately 500 nm and maximum absorption at 550 nm (Fig. 2). A linear correlation was present between the SDS level (0.1 to 1.2 μg/ml) and absorbance at 550 nm (Fig. 3).

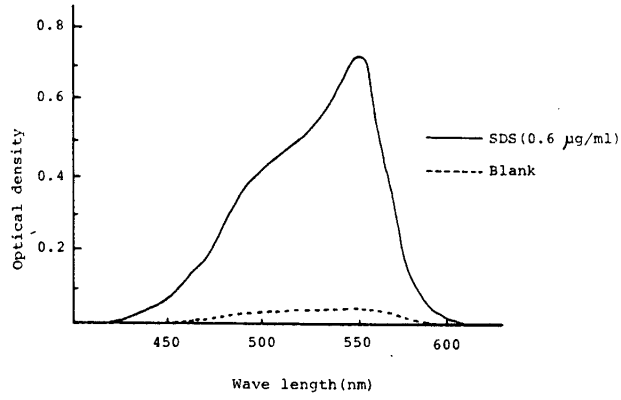

Fig. 2. Absorption spectrum of SDS-rosaniline HCl complex. SDS was extracted from Kieselgel H with methanol and coloured by rosaniline hydrochloride. A blank was obtained at both steps.

To examine SDS recovery after TLC, the SDS was mixed with human serum and developed on TLC. SDS was extracted from TLC with alcohol

and measured by the colorimetric method using rosaniline hydrochloride (Table 2). The recovery rate of SDS from SDS-containing serum ranged from 85% to 92% depending upon the SDS concentration.

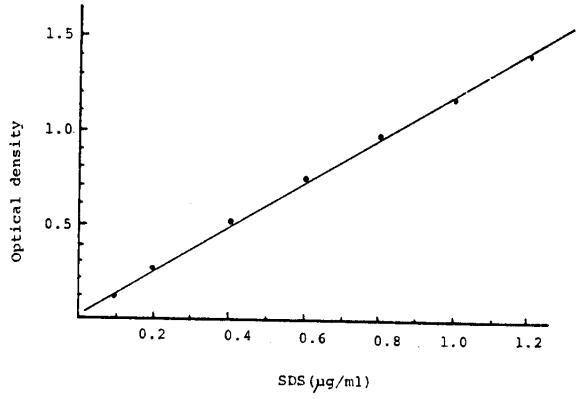

Fig. 3. Correlation of absorbance of SDS-rosaniline HCl complex with SDS concentration.

TABLE 2 RECOVERY OF SODIUM DODECYL SULFATE (SDS) IN SERUM COMBINING THIN-LAYER CHROMATOGRAPHY AND ROSANILINE HYDROCHLORIDE COLORIMETRY

SDS mixed in serum (μg/ml)	No. of experiments	Recovered SDS (M±SD) (μg/ml)	(%)
0.400	5	0.338±0.010	84.60±2.62
1.00	5	0.919±0.038	91.98±3.83

Dialyzed erythrocyte membrane solution was filtered through a Sephadex G-25 column. The chromatogram had two peaks under O. D. 280 nm, and one peak by the Lowry-Folin method (Fig. 4). SDS was determined as 10^{-3}M in fractions from No. 16 to 20, and 10^{-5}M from fractions No. 29 to 44. The fractions from No. 15 to No. 22 were collected and re-chromatographed through the same Sephadex G-25 column. The protein content in the filtered fractions was the same as the first filtration. SDS was not present in any fraction under either U.V. light irradiation or distilled water spray procedures on TLC. The SDS content in this refiltered fraction was examined by rosaniline hydrochloride colorimetry and was revealed as being under 10^{-6}M (Table 3). Thus, fraction No. 17, 18 and 19 on Sephadex G-25 column removed SDS at the level of 10^{-6}M.

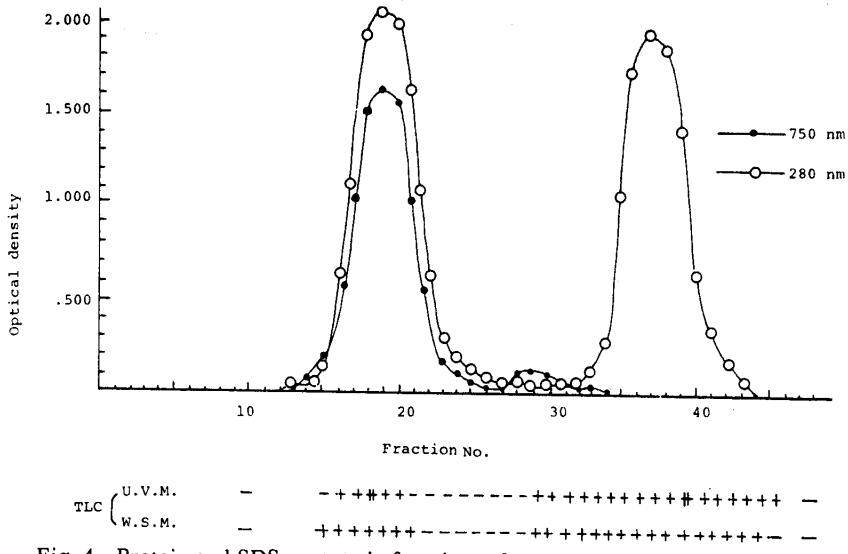

Fig. 4. Protein and SDS content in fractions of solubilized erythrocyte membrane through Sephadex G-25 column. Protein concentration was revealed by absorption of O. D. 280nm and by the Lowry-Folin method. SDS was estimated with TLC by methods of U. V. light irradiation (U. V. M.) and water spray (W. S. M.). These SDS spots on TLC were classified into three reaction patterns: intensely positive (#), positive (+) and negative (−).

TABLE 3 SODIUM DODECYL SULFATE (SDS) AND PROTEIN CONCENTRATION IN SEPHADEX G-25 FRACTIONS OF GEL REFILTRATION

Fraction No.	SDS [M]	Protein concentration μg/ml
14	8.3×10^{-7}	85
15	1.5×10^{-6}	325
16	1.9×10^{-6}	830
17	2.3×10^{-6}	1020
18	2.4×10^{-6}	1020
19	2.0×10^{-6}	925
20	7.5×10^{-7}	770

Antigenicity was still positive in these fractions in immunoelectrosyneresis against sera of patients with SLE in contrast to the absence of precipitates in the sera of healthy subjects (Fig. 5). Therefore, all precipitates by immunoelectrosyneresis were genuine antigen-antibody aggregates and were not nonspecific interactions in the gels.

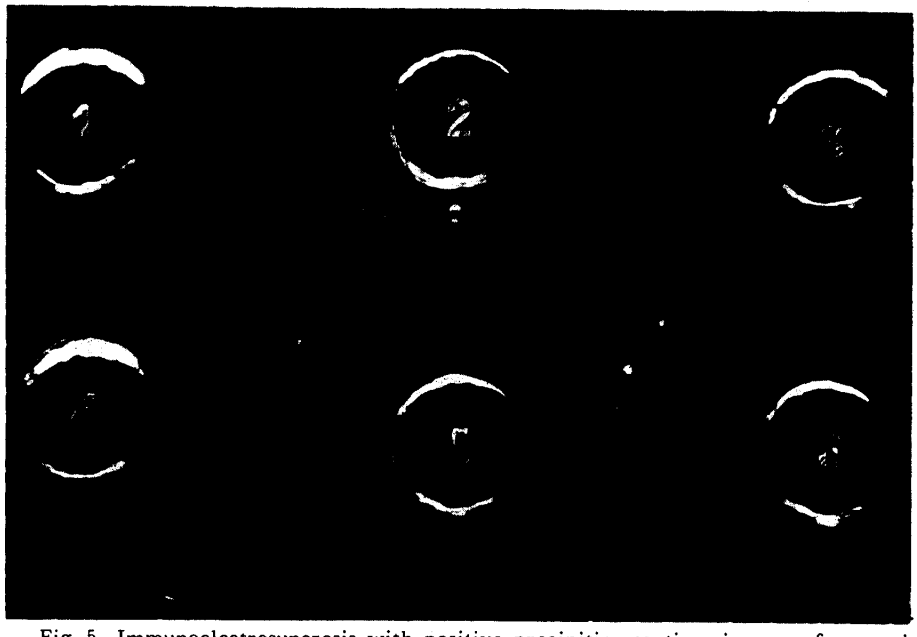

Fig. 5. Immunoelectrosyneresis with positive precipitin reactions in sera of systemic lupus erythematosus (well No. 4 and 5), against membrane solution after reducing SDS to the level of 10^{-6}M (well No. 1, 2 and 3). The serum of a healthy individual (well No. 6) showed no reaction. The anode is on the upper side.

DISCUSSION

Mangold and Kammereck (6) reported a method for detecting fatty acids with TLC. These investigators used chloroform-methanol-sulfuric acid as solvent and dichlorofluorescein as the indicator. The present study demonstrated that the lower limit of this method of detecting SDS was at a concentration of 10^{-3}M, and the procedure was successful for detection of SDS in serum containing fatty acids, proteins, glycoproteins and phospholipids. This method was considered effective for detecting SDS in solubilized erythrocyte membrane. Proteins, amino acids and glycoproteins remained on the TLC at Rf 0, while phospholipids, cholesterol and free fatty acids were detected respectively at Rf 0.1–0.2, 1 and 1.

SDS was unexpectedly detected on TLC chromatoplate as a red spot within 30 min. of spraying with 0.2% alcoholic solution of 2′, 7′-dichlorofluorescein and distilled water. By this improved method, SDS was detected at a level of 10^{-5}M compared with the limit of 10^{-1}M by the method of Mangold and Kammereck (6).

SDS has also been detected by the simple colorimetric method (7), paper chromatography (10) and gas chromatography (11). The colorimetric method

with rosaniline hydrochloride is known to have higher sensitivity at SDS levels from 10^{-7}M to 10^{-8}M.

In the present study, SDS was detected in solubilized erythrocyte membrane using the rosaniline hydrochloride colorimetric method with TLC. The Kieselgel H corresponding Rf region of SDS was collected and extracted with methanol, and finally colored by rosaniline hydrochloride. SDS was quantitated according to the prescribed calibration curve. SDS was recovered at more than 90% of the original SDS content in human serum. When petroleum ether or chloroform was used instead of methanol as the extraction solvent, the recovery of SDS was reduced to less than 30% of the original SDS content. On the other hand, the use of boiling water resulted in the recovery of 90% of SDS, but we preferred methanol to boiling water as solvent, because of the ease of evaporation.

When human erythrocyte membrane was solubilized with SDS and 2-mercaptoethanol, the membrane proteins disaggregated and conformed to the SDS-protein complex (12, 13). Therefore, if SDS is removed from the disaggregated membrane solubilized with SDS, reconstruction of the membrane might be induced (14).

The removal of SDS from the disaggregated membrane-SDS complex was performed by procedures, such as dialysis (15), gel filtration with Sephadex G-25 (8) and Bio-Rad AG-2$\times$10 (16), ion-exchange with Dowex 1, 2, 50 (17) and Dowex 2$\times$8 (15). Quantitative estimation of the removal of SDS was performed by measuring the ^{35}S-SDS cpm, but the concentration of SDS remaining in the membrane protein was not successfully estimated. In the present study, we also applied the combined quantitative method to eluted fractions of SDS-solubilized erythrocyte membrane by Sephadex G-25 column. The maximum SDS content in the first filtered fraction with Sephadex G-25 column was 10^{-3}M and in the refiltered fraction, 10^{-6}M.

The recovery of membrane proteins through these filtrations was demonstrated to be 100%. The SDS concentration of 10^{-6}M is negligible in comparison with the membrane protein concentration. Nevertheless, membrane protein containing SDS at concentrations of 10^{-6}M was shown to act as an antigen against the anti-membrane antibody by immunoelectrosyneresis, and therefore, all precipitates in the gels were in fact antigen-antibody aggregates and not non-specific interactions in the gel. The precipitin reaction in gels, thus, could be considered aggregates of the antigen-antibody reactions.

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