EVALUATION OF THE USE OF SODIUM DODECYL SULFATE FOR THE IDENTIFICATION OF THE STRUCTURAL ANTIGENS OF INFLUENZA A/PR8.

by

Nora Elizabeth MacDonald

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Department of Microbiology and Immunology,
Faculty of Medicine,
University of Ottawa,
Ottawa.

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LIST OF ABBREVIATIONS

ANTIGENS (Ag)

HS	Human immune serum globulins
IgG	Human immune gamma globulin
BJ	Bence Jones protein
C_3 1/10	Influenza virus A/PR8 concentrated
SDG/C_3	Influenza virus A/PR8 concentrated and purified by sucrose density gradient
SDS	Sodium dodecyl sulfate in saline
DOC	Sodium deoxycholate in saline
Tween 20	Tween 20 in saline
β	β mercaptoethanol at 0.1%

ANTISERA AND SERA

ES	Normal horse serum
RS	Normal rabbit serum
GaH	Goat anti human serum
GaHγ	Goat anti human immunoglobulins
GaHγG	Goat anti human IgG (Gamma globulin)
GaHFab	Goat anti human Fab fragment

Ga H	Goat anti human gamma chain
GaHBJ	Goat anti human Bence Jones protein
RaS/C_2	Rabbit anti influenza A/PR8 concentrate
RaS/Ne	Rabbit anti normal egg soluble antigen

ANTIGEN TREATMENTS

-/0.1%, 0.5%,1% 2% SDS	Antigen treated with specific concentration of Sodium dodecyl sulfate
-/1% DOC	Antigen treated with 1% sodium deoxycholate
-/1% Tween 20	Antigen treated with 1% Tween 20
-/P	Antigen treated by cold precipitation
-/A	Antigen treated with acetone
-/K	Antigen treated with saturated potassium chloride
-/d	Antigen treated by dialysis
-/β	Antigen treated with 0.1% β mercaptoethanol

ABSTRACT

An evaluation of the use of sodium dodecyl sulfate (SDS) for the identification of the structural antigenic components of influenza A/PR8 was carried out.

Background for this evaluation was provided by examination of the effects of SDS on the antigens of known antigen-antobody reactions measured by the ability of the treated antigen to participate in the immunodiffusion reaction. Treatment of antigens with SDS resulted in the nonspecific precipitation of serum by SDS in immunodiffusion reactions. SDS treatment also caused some precipitation of the antigens. The concentration of SDS used was important in determining the amount of antigenic activity lost; 1% SDS caused far more loss than 0.1%. Attempts to restore antigens treated with 1% SDS to their original antigenic characteristics by standard methods of SDS removal were not successful. Treatment of antigens with 0.1% SDS compared favorably to results obtained with two other detergent treatments, 1% Tween 20 and 1% sodium deoxycholate (DOC).

Investigations with influenza A/PR8, revealed that SDS was a useful agent for disruption of this virus and characterization of released components by immunodiffusion reactions but only if a concentration of 0.1% SDS

was used, not 1% as is commonly used, and if the possibility of nonspecific precipitation by SDS was kept in mind when analyzing these reactions. However, because of this problem, it is suggested that DOC at 1% may prove to be a more suitable disrupting agent.

I - INTRODUCTION

Influenza virus has been the object of much research. Attention has been particularly focused on the elucidation of the virus structure in relation to its biological properties such as hemagglutinin, neuraminidase and antigenic activities.

In recent years, the anionic detergent sodium dodecyl sulfate (SDS) has been extensively used to disrupt the virus into subviral components which are then closely examined. Attempts to relate these released components back to the intact virion have been hampered by the varied effect of SDS on biological properties. In some experiments the released component will have retained a biological function while in others the same function will have been lost.

The subject of this thesis is the analysis of the effects of SDS on a specific biological property, the ability of an antigen to participate in the immunodiffusion reaction, of a known system. With this background, an evaluation of the use of SDS for identification of the structural antigenic components of influenza A/PR8 is carried

out. It is hoped that the results presented here may provide at least part of the reason for the conflicting results obtained when SDS has been used.

A REVIEW OF THE LITERATURE

II - A REVIEW OF THE LITERATURE

1. Influenza virus

1.1 Introduction

Influenza virus belongs to the myxovirus group which includes those RNA, lipid-containing, viruses which have a specific affinity for certain mucoproteins. Andrewes et al (1955) originally defined the group as viruses 60-200 nm in diameter, which agglutinate red blood cells and enzymatically elute from them, are sensitive to ether and cause respiratory disease.

More than any other viruses that infect man, influenza viruses, especially type A, show great genetic variability. Antigenic changes appear to be continually occurring within the influenza A type. Two kinds of variation in surface antigens have been distinguished. The first, called immunological or antigenic drift (Burnet, 1955) involves relatively minor changes in the surface antigens giving rise to a succession of immunologically related progeny. The second kind of variation involves a major antigenic shift in which the

new virus population has surface antigens completely unrelated to the old.

The capacity to participate in genetic interactions has been suggested as a possible basis for these continually occurring antigenic changes. The type specific antigen, located in the nucleocapsid, does not appear to undergo variation and antibody induced by this antigen does not neutralize infectivity of the virus. However, genetic recombination studies have revealed that the two virus-coded surface antigens, hemagglutinin and neuraminidase, are associated with the antigenic variation. These strain specific antigens do induce neutralizing antibody formation. Thus, because of the important role played by hemagglutinin and neuraminidase in antigenic variation and immunity to infection, much work has been done on the examination of the components of influenza virus. A total of four components have been characterized according to biological activity; hemagglutinin, neuraminidase, ribonucliоprotein (RNP) and host component, while at least six antigenic components have been detected (Johnson and Westwood 1971).

1.2 Morphological Studies

Electron microscope studies, by the negative straining technique, of intact particles showed the virus

to be pleomorphic ranging from nearly spherical to rod-shaped. (Hoyle et al, 1961). Predominantly, the particles were spherical with a diameter of 800-1000 Å. Recently, freeze-etching and freeze-drying techniques have shown that the particles may be more uniform than has been originally believed since the particles seen were icosahedral with a diameter of 1080 Å. It was suggested that the observed pleomorphism may be to a great extent a preparation artifact (Nermut and Frank, 1971).

The most distinctive morphological feature described by Hoyle et al (1961) was the 100 Å fringe of projections present on the surface. Archetti et al (1967), Almeida and Waterson (1967), and Nermut and Frank (1971), have shown that the spikes were regularly spaced and that three spikes formed an equilateral triangle. Almeida and Waterson (1967) observed that the envelope appeared to be composed of subunits surrounded by either five or six subunits similar to viruses showing cubic symmetry. However, unlike the fixed distribution of "fives" and "sixes" for the adenovirus capsid, the arrangement showed a random distribution. Furthermore, it must be remembered that it is uncertain whether the observed "spikes" do in fact project from the virus envelope because the phosphotungstic acid (PTA), used for staining binds tightly to proteins but not to carbohydrates or lipids. Thus,

non-staining lipid or carbohydrate units, alternating with PTA binding material could result in a spike-like appearance (Scholtissek et al, 1969). In fact, Nermut and Frank (1971), on the basis of their freeze-etching results suggested that the space between the spikes may indeed be filled with some amorphous material. However, in general, they concluded that their freeze-etching observations confirmed the results obtained by negative straining as far as the surface structure was concerned.

Disintegration of the virus into components has been attempted with a number of agents, most commonly, ether (Hoyle, 1952), sodium deoxycholate (DOC) (Laver, 1961) and sodium dodecylsulfate (SDS) (Laver, 1964). In morphological studies on the effects of ether, SDS and DOC, Nermut (1970) reported that ether, 0.1% SDS and 0.1% DOC completely removed the spikes and after prolonged action the membrane usually disintegrated. The released nucleoprotein did not appear to be affected by either DOC or ether whereas SDS apparently disrupted its morphological integrity.

More detailed E/M studies have been carried out by several groups of workers in attempts to elucidate the morphological structure of the released viral subunits (Laver and Valentine, 1969, Drzeniek et al, 1968, and Webster and Darlington, 1969). On the basis of these

studies structures for hemagglutinin and neuraminidase have been proposed. Hemagglutinin subunits, in the presence of SDS, appeared to be rods approximately 40 Å wide and 140 Å long. (Laver and Valentine, 1969) See Figure 1a.

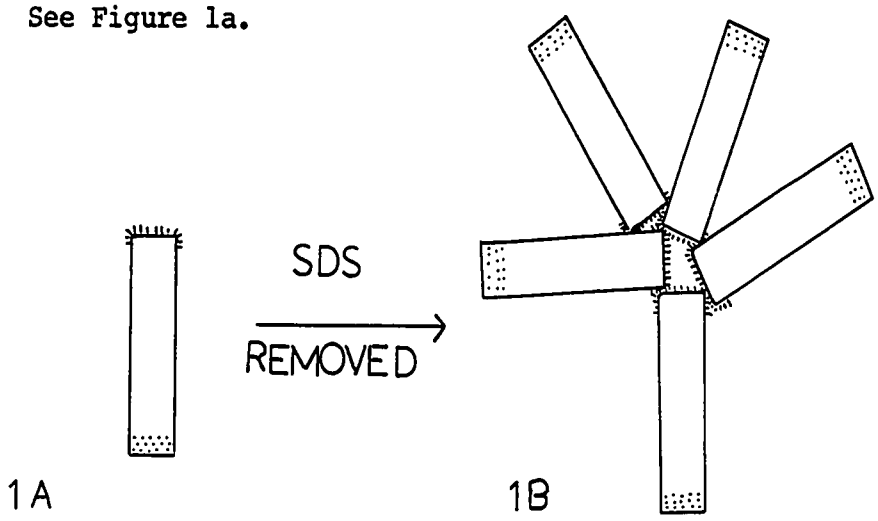


Fig 1A, B Model for structures of isolated hemagglutinin subunits in the presence of SDS and after SDS removal.
(After Laver and Valentine, 1969).

It was reported that when the SDS was removed by cold precipitation followed by either washing with cold acetone or treatment with saturated potassium chloride, aggregates formed which consisted of radiating rods. See Figure 1b. In the presence of SDS these isolated subunits were found to adsorb to the stroma of red blood cells. After SDS removal, hemagglutinin activity was recovered. Webster and Darlington (1968) observed morphologically similar structures for their aggregated hemagglutinin preparations, as well as for their

nonaggregated subunit preparations.

Isolated neuraminidase subunits, also in the presence of SDS, appeared as oblong structures, 85 Å by 50 Å, with a centrally attached fibre 100 Å long with a knob about 40 Å in diameter at its end (Laver and Valentine, 1969). See Figure 2a.

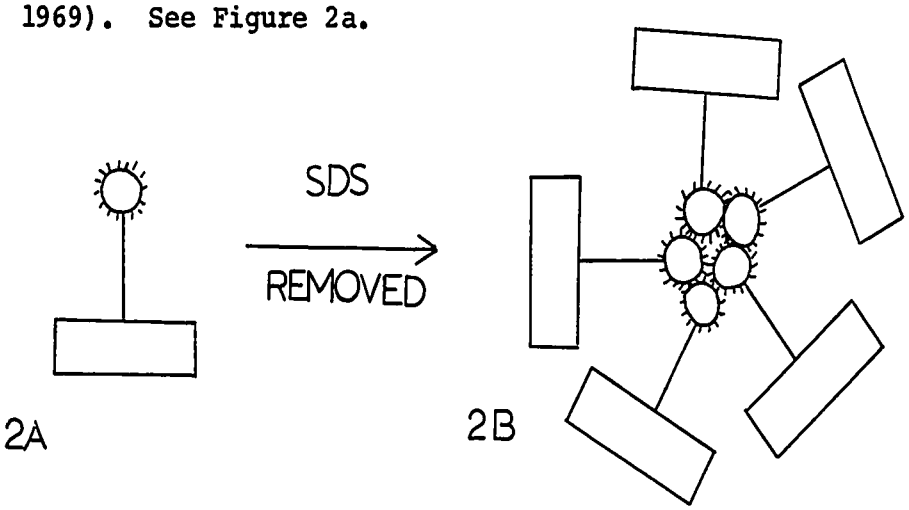


Fig 2A, B. Model for structures of isolated neuraminidase subunits in the presence of SDS and after SDS removal.
(After Laver and Valentine, 1969).

When SDS was removed, the subunits aggregated to form dandelion-shaped groups. See Figure 2b. The isolated subunits were thought to maintain their neuraminidase activity even in the presence of SDS; however, the level and specificity of enzyme activity was not reported. Webster and Darlington (1969) and Drzeniek et al (1968) observed similar structures for aggregated preparations, however, only Webster and Darlington observed similar nonaggregated subunits.

Another component which has been visualized by electron microscopy is the ribonucleoprotein (RNP). Hoyle et al. (1961), Almeida and Waterson (1969) and others, have shown it to be a coiled helical structure. A large discrepancy between the structures visualized within spontaneously ruptured particles and those seen with ether extraction has been reported (Almeida and Waterson, 1969). Enveloped RNP was 60 Å in diameter while the ether extracted component was 90 Å or more. Moreover, while the average length of enveloped RNP was calculated to be 10,000 Å to 50,000 Å and included some up to 80,000 Å, the average length of extracted RNP was 600 Å to 800 Å with the greatest length seen being 2,500 Å.

These observed differences between enveloped and extracted RNP illustrate some of the difficulties in relating chemically isolated components back to morphological structures seen in the intact virus. In fact, no morphological structures have been seen in intact virions which resemble the morphology of isolated hemagglutinin and neuraminidase as observed by Laver and Valentine (1969).

1.3 Biochemical Studies

Many attempts have been made to correlate the polypeptides of influenza isolated by biochemical techniques, with the known structural proteins of the virion. The

basic biochemical approach has been the isolation of sub-viral components with recognizable biological activity, and subsequent dissociation into their component polypeptides for further analysis. Several problems are inherent to this approach. For example, the smaller the structure isolated the greater the probability that it may consist of a single protein species but the less chance that it retains biological activity. Furthermore, the actual choice of technique limits the information obtained. For example, many protein separation techniques often destroy or do not detect lipids and or carbohydrates, therefore lipoproteins and glycoproteins may not be identified. Since the lipid and carbohydrate moieties are possibly essential for the biological activity of the subunits the correlation of biological activity with chemical and morphological structure may become impossible. However, biochemical studies of this nature have provided a great deal of information concerning the relationship between the mechanisms of virus replication and the intact virion.

There has been a great deal of conflicting evidence concerning the role of host proteins in influenza virus. It has now been shown, using this biochemical approach, that all the major polypeptides of the virion are newly synthesized, virus directed, proteins and not incorporated preformed host proteins. (Haslam et al, 1970a). However,

varying numbers of polypeptides have been reported by different groups: White et al (1970) found three major polypeptides although they observed that the number varied according to experimental conditions; Content and Duesberg (1970) reported the separation of viral components into three major electrophoretic zones consisting of probably five or more polypeptides, three of which were glycoproteins; Schulze (1970) and Compans et al (1970) both reported seven polypeptides, four of which were glycoproteins. These variations in number and type no doubt reflect virus strain differences, differences in host cell contributions and most importantly the differences in preparation techniques.

Correlation of these isolated polypeptides with a biological function has not been particularly easy. The polypeptide associated with hemagglutinin activity is thought to be a glycoprotein with a host derived carbohydrate moiety (Schulze, 1970; Haslam et al, 1970b; and Compans et al, 1970). The molecular weight of this component determined by various workers has ranged from 47,000 to 77,000, possibly due again to different host systems and different techniques of isolation. Furthermore, it has been suggested by Haslam et al, (1970b) that components may be biologically active at several stages of degradation leading to different determined molecular

weights. Neuraminidase function has also been tentatively associated with one or more glycoproteins. (Compans et al, 1970; Schulze, 1970; and Haslam et al, 1970b).

Three carbohydrate-free polypeptides have been identified by Schulze (1970) in the core of the virus. One was associated with the viral RNA, the second was suggested to be a membrane layer about the RNP, while the function of the third was unknown. Compans et al (1970) have also confirmed the existence of this last polypeptide.

The viral RNA is another structural component which has been intensively examined. Studies of sedimentation coefficients (Pons, 1967; Barry and Davies, 1968; and others) and electrophoretic characteristics (Pons and Hirst, 1968 a and b; and Etchison et al, 1971) have all shown that the isolated RNA occurs as fragments. However, none of these pieces have been shown to possess biological functions such as the ability to cause infection or the ability to be genetically rescued (Etchison et al, 1971). A direct correlation of these findings of fragmentation with the morphological results described earlier by Almeida and Waterson (1969) has not yet been made and the conformation of the RNA within the intact virion is still subject to debate.

1.4 Immunological Studies

Immunological analysis of influenza virus and its

components have revealed much useful information. Several studies have been carried out on the biologically active components of influenza viruses. Antibodies directed against neuraminidase did not greatly inhibit hemagglutinin (Meier-Ewert and Dimmock, 1970). However, antihemagglutinin did inhibit neuraminidase activity and steric hindrance has been suggested to explain this phenomenon (Webster and Darlington, 1969). This suggestion was supported by their observation that treatment with Tween 20, a nonionic detergent, removed this inhibition. Therefore these two biological properties are considered to be antigenically distinct but spatially related.

Neuraminidase and ribonucleoprotein antigens from SDS disrupted viral concentrates have been identified in immunoprecipetin tests (Schild and Perira, 1969). Examination of a recombinait virus along similar lines revealed that it contained subtype specific antigens derived from each parent virus strain (Schild et al, 1970; and others). These findings add support to the theory that genetic recombination is the basis for antigenic variation. However, closer examination by Schild et al (1970) of the recombinant hemagglutinin and neuraminidase revealed that the hemagglutinin resembled its parent in all properties tested but the neuraminidase did not. Although the neuraminidases were related antigenically and had similar

Michaelis constants (Km) they differed in electrophoretic mobility and stability following SDS treatment. Therefore antigenic variation appears to be more complicated than just recombination; some modification in the surface antigens also seems to occur. It is possible that the host may exert selective pressures or that the host moiety of the neuraminidase or hemagglutinin may provide some necessary modification to them.

This importance of host contribution has been supported by immunodiffusions, carried out in this laboratory, which have shown that of a total of six structural antigens, one was classified as having host properties, two were viral and all the remainder showed both host and viral relationships (Johnson and Westwood; 1971).

1.5 Conclusions

Although much work has been done on influenza virus and its components, the nature of the virus morphologically, biochemically and immunologically is still uncertain. Much of the data from the experiments reviewed here is conflicting and difficult to interpret due, in part, to a lack of understanding of the effects of the disrupting agents. For example, SDS treatment destroyed neuraminidase activity for some viruses but not hemagglutinin while

with others the situation was reversed. Similarly, a comparison of antigenic components released by DOC and SDS procedures revealed that they were not only different in number but also had different identities (Johnson and Westwood, 1971). Analysis of how SDS acts as well as an evaluation of its effects on protein can only help in interpreting studies involving virus disruption.

2. Theory of Sodium Dodecyl Sulfate Action

Sodium dodecyl sulfate (SDS) is a protein denaturant. Joly (1965) defines denaturation as any modification of the secondary, tertiary or quaternary structure of the protein molecule, excluding any breaking of covalent bonds. Thus, any change in the original native configuration which does not alter the amino acid sequence is denaturation.

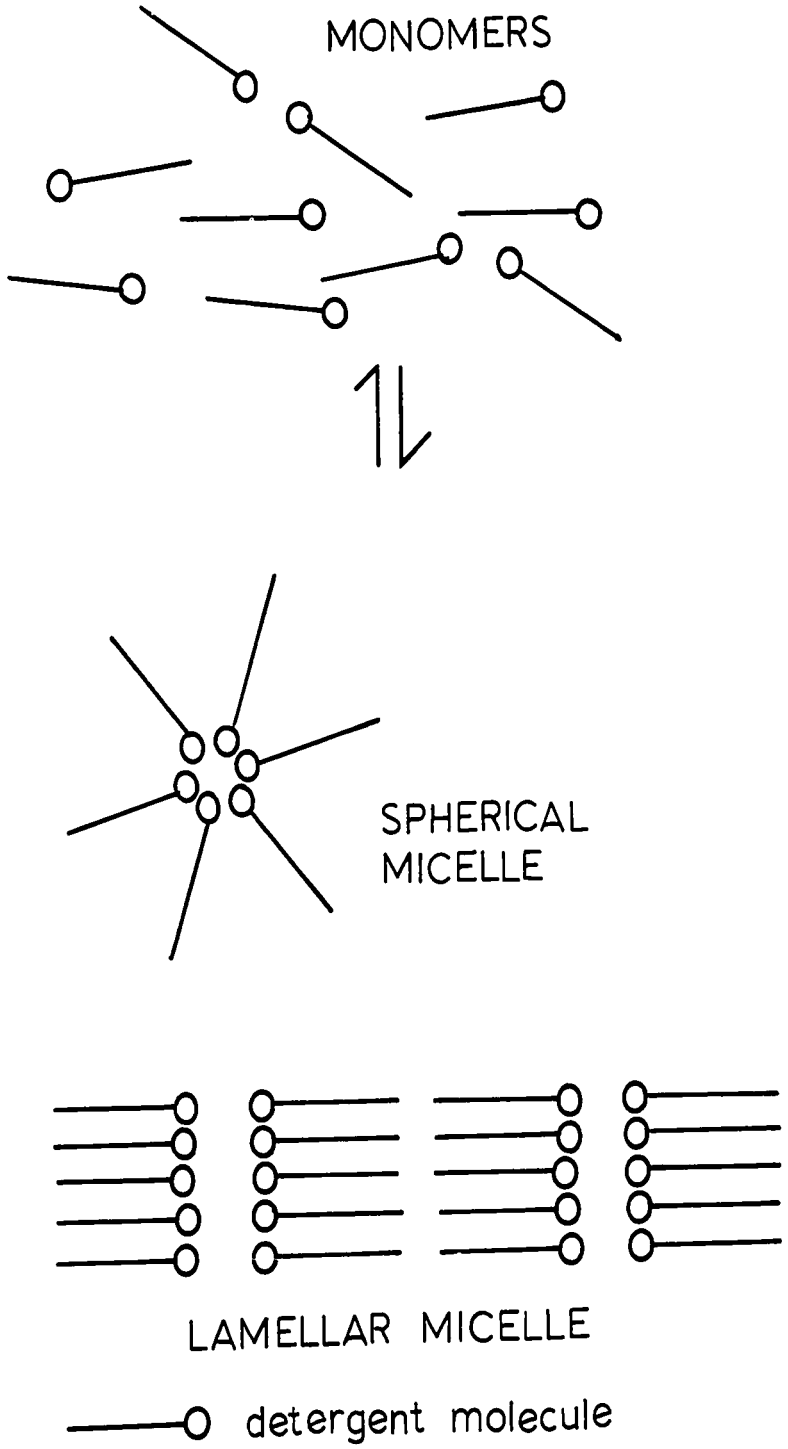
SDS belongs to the class of protein denaturants called detergents, and is anionic in nature. Detergents in this context are surface active agents which occupy a unique position among protein denaturants because they can produce drastic conformational changes at about 1/100 of the concentration required by other denaturants such as urea. (Tanford, 1968). This is a result of the higher binding forces between detergents and proteins than

between proteins and other denaturants. Detergent protein interactions are restricted to the side chains of the protein while urea, quanidine hydrocloride and other denaturants compete for hydrogen bonds. Thus the physical changes a detergent induces on a protein are to a large extent due to modifications in tertiary structure (Joly, 1965). However, detergents may disrupt hydrogen bonds indirectly as a result of intramolecular electrostatic repulsion or perturbation in the stabilization of the secondary structure by the non-polar side chains.

In aqueous solutions, detergents exist as monomers and as micellar aggregations. See Figure 3. The relative concentrations of each component depend upon the total detergent concentration, the ionic strength and the temperature. At a given ionic strength, increasing the total detergent concentration will NOT result in an increase in monomer concentration above a specific value called the critical micelle concentration (CMC). Above the CMC each new molecule of detergent added to the system becomes incorporated into a micelle. The CMC decreases with increasing ionic strength at constant temperature.

Based on this tendency of detergents to form micelles, a mechanism for the formation of a complex was proposed by Lundgren (1945). He suggested that several

FIGURE 3.
CONFIGURATIONS OF DETERGENT MOLECULES IN AQUEOUS SOLUTIONS.(AFTER BERNFIELD,1963)

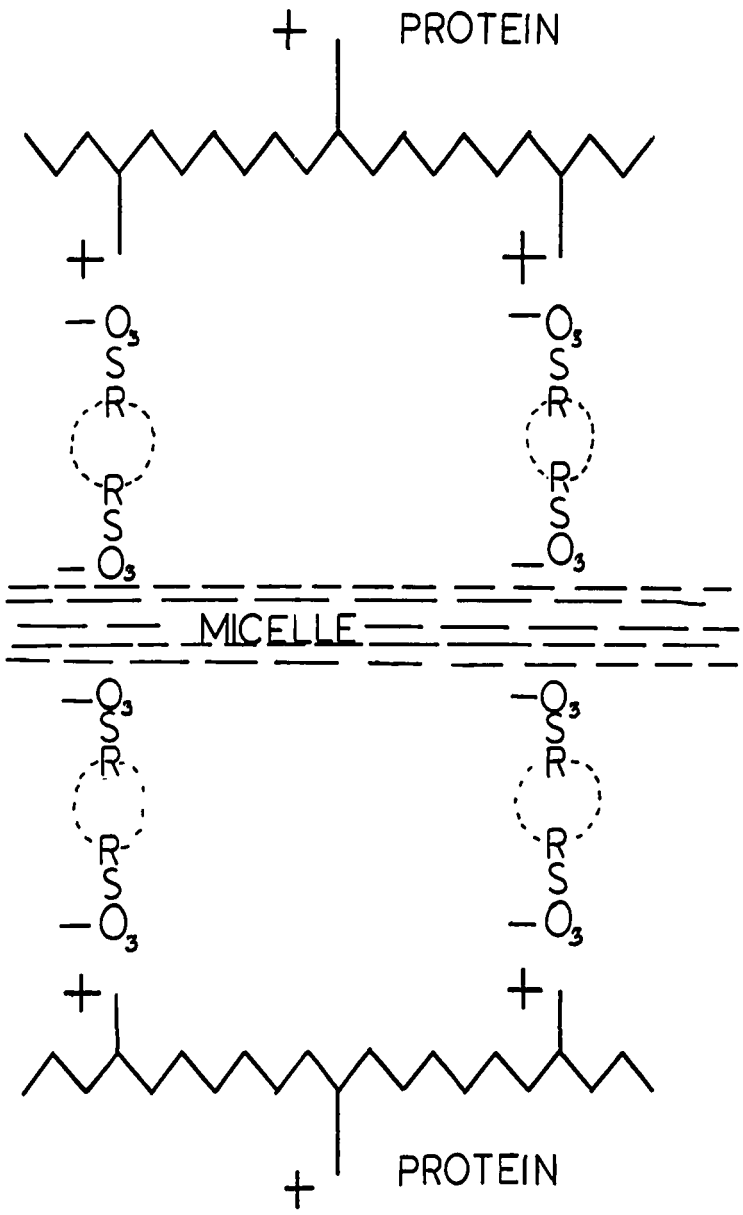


polypeptide chains are held together by a detergent micelle through electrostatic forces while the micelle itself is held together by nonpolar bonds. See Figure 4. Bernfield (1963) using this theory postulated that the formation of such a configuration would strain the bonds necessary for quaternary and tertiary structure as well as secondary structure, thus causing them to break and denature the protein.

More recent studies have elucidated a number of points about SDS-protein interaction (Pitt-Rivers and Impiombato, 1968; Weber and Osborn, 1969; Reynolds and Tanford, 1970 a and b; and Fish et al, 1970). Reynolds and Tanford (1970a) found that only the monomeric form of SDS bound to protein, the micellar form was not involved. Since the ionic strength of the solution is important in determining the concentration of the total SDS in the monomeric form and at low ionic strengths, the monomeric forms of SDS is favored; more detergent was bound to proteins in low ionic strength solutions than in high.

Furthermore, the reaction between SDS and protein was nonspecific. All reduced proteins ie. those treated with β mercaptoethanol or other agents which break disulfide bonds, bound identical amounts of SDS at the same equilibrium monomer concentration (Reynolds and Tanford, 1970a). The proteins used in these studies

FIGURE 4.
PROPOSED DETERGENT MICELLE-PROTEIN COMPLEX. (AFTER LUNDGREN, 1945)



included the very basic histones, the large helical myosin molecules and the complex proteins of ghost red blood cells. Two binding ratio levels of SDS to protein were observed and each depended upon the equilibrium monomer concentration. The binding ratio values were:

0.4g SDS/g protein at Cf 5-8 x 10^{-4}M SDS monomer

and

1.4g SDS/g protein at Cf 7 8 x 10^{-4}M SDS monomer (Reynolds and Tanford, 1970a).

Pretreatment of protein with guanidine hydrochloride, another denaturant, and β mercaptoethanol followed by dialysis against water (to remove guanidine hydrochloride), β mercaptoethanol and SDS gave the same result as treatment with SDS and β mercaptoethanol alone. Thus the structural conformation of the protein did not appear to alter the SDS binding capacity as long as the disulfide bonds had been reduced. (Reynolds and Tanford, 1970a). If the protein had not been reduced, up to fifty percent less SDS was bound (Pitt-Rivers and Impiombato, 1968). It was suggested that the SDS complex protected the sulfhydryl groups in the polypeptide chain not only from the surrounding medium but from each other.

A gross conformation of the SDS-protein complex has been tentatively postulated by Reynolds and Tanford (1970b) from studies of the intrinsic velocity of the

complex. The protein-SDS complexes assumed rod-like shapes and preliminary E/M studies have confirmed this observation. Values have been calculated for the dimensions of this rod-like particle based on its resemblance to an elongated ellipsoid. At the SDS binding level of 0.4 these have been calculated to be

Major axis 0.61 Å per amino acid residue

Minor axis 14 Å and was constant

while at the SDS binding level of 1.4

Major axis 0.74 Å per amino acid residue

Minor axis 18 Å and was constant

(Reynolds and Tanford, 1970b).

The rod lengths obtained for SDS-protein complexes were compared with the lengths of native tropomyosin and paramyosin. These two proteins consist of two α helices side by side twisted slightly about one another. The lengths correspond to 0.66 Å per residue for tropomyosin and 0.72 Å per residue for paramyosin. Therefore, Reynolds and Tanford (1970b) suggested that a possible model consistent with this data would be a helical polypeptide chain folded back on itself near its middle to give a double helical rod with SDS forming a shell about the rod. Other data, such as ORD spectra, have shown that while the polypeptide chain may have been primarily in the form of a helix it was not 100% helical.

3. Discussion

The data from experiments examining the components from influenza virus tends to be confusing. Part of this can be attributed to artifacts added by the treatments employed.

Some problems when SDS has been used have been discussed in the literature. Gould et al (1964) in examining various physicochemical effects, including that of nonaqueous solvents on antibody activity observed that dialysis for ten days against sodium chloride-phosphate buffer changed daily followed by several passages through a Dowex 1 column was insufficient to remove all the SDS from an SDS-antibody solution. SDS at a concentration of 6% was found to completely destroy the precipitate formed by an antigen and its antibody. However, 2.2% SDS only caused a 40% drop in the precipitate formed in an antibody-antigen precipitin reaction. When antigen was added to normal γ globulin treated with SDS, approximately 10% nonspecific precipitation resulted.

Another problem with SDS is that unless proteins complexed with SDS are in their reduced form, the estimates of molecular weight determined by either gel chromatography or electrophoresis are invalid (Weber and Osborn, 1969; and Fish et al, 1970). Very recently, Palmer et al (1971), have described certain nonspecific precipitations effects

of SDS in a agar diffusion and immunoelectrophoresis. SDS has also been shown to inactivate some enzymes (Rogers and Yusko, 1969); however, in addition, it can protect other enzymes from further inactivation by other denaturants (Groves et al, 1951).

Treatment of membranes of Mycoplasma laidlawi with SDS resulted in only some lipid-protein bonds being disrupted (Rodwell et al, 1967) indicating that SDS is only partially effective as a lipid solvent. Nevertheless, the ability of SDS to solubilize lipids has been a large factor in its selection as an influenza virus disrupting agent.

Thus, as more has become known about the mechanism of protein denaturation by SDS, the problems with interpretation of experimental results have increased. Therefore with the theory of SDS action in mind, an examination of the effects of SDS treatment on a specific biological property, antigen combining ability as measured by immunodiffusion, was carried out.

PURPOSE

III - PURPOSE

The purpose of these investigations was to evaluate the use of SDS for the identification of structural antigenic components of influenza A/PR8. Two basic questions needed to be answered before the influenza system could be examined :

1. Does SDS treatment of the antigen modify or abolish its reactivity in the immunodiffusion test?
2. If the antigen has been modified can these changes be reversed by removing SDS by standard techniques such as cold precipitation, precipitation with saturated potassium chloride or washing with cold acetone?

A series of experiments testing the effects of SDS on known antigens measured by their ability to participate in immunodiffusion reactions were carried out first. Then the effect of SDS treatment of influenza A/PR8 on its antigenic components was examined.

MATERIALS AND METHODS

IV - MATERIALS AND METHODS

1. Antigens

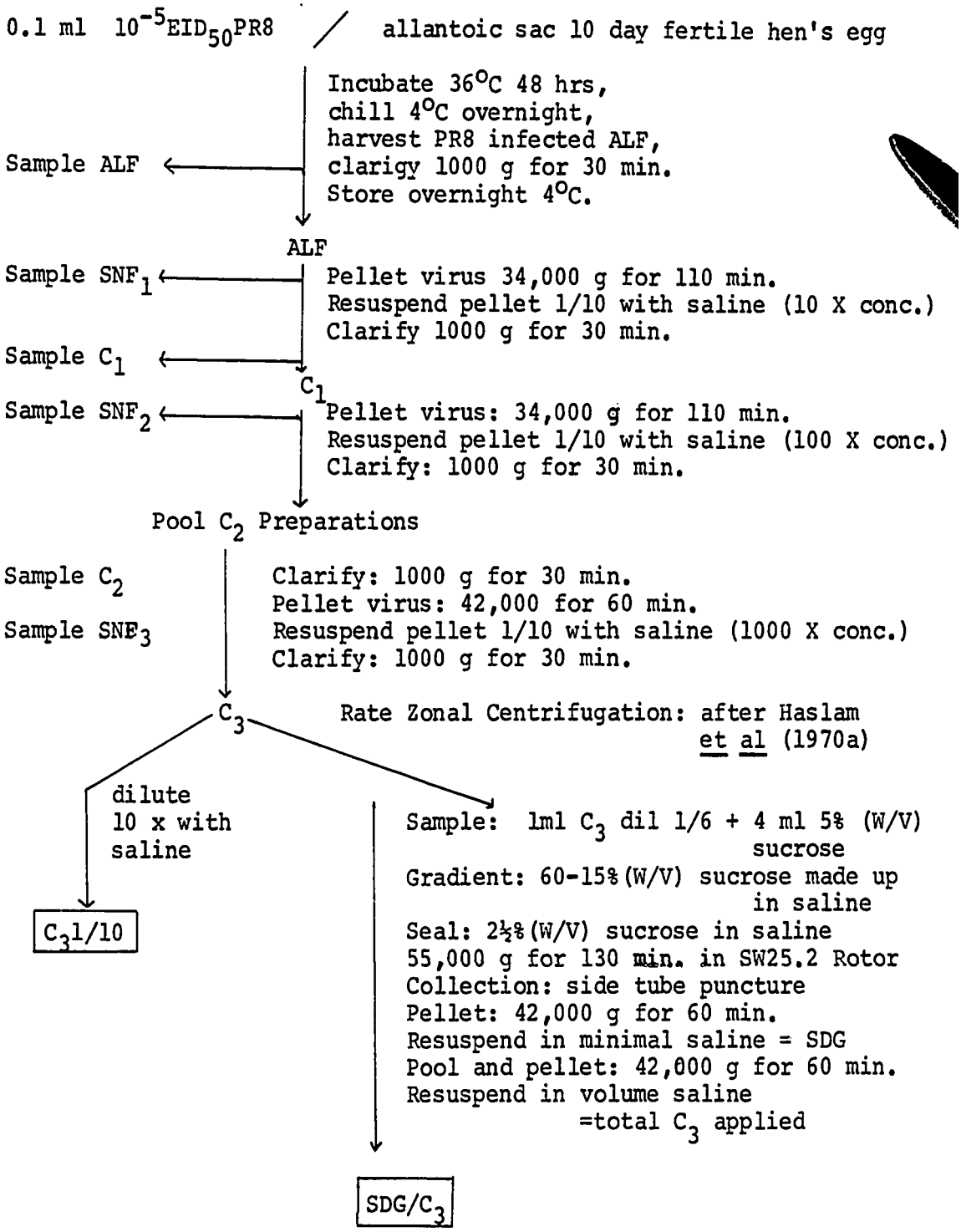
1.1 Virus

0.1 ml of influenza A/PR8($10^{-5}EID_{50}$), containing penicillin (100u/ml) and streptomycin (100 mg/ml) was injected into the allantoic cavity of 10-day-old embryonated hens' eggs which were incubated for 48 hours at 36° C and then chilled overnight at 4°C. The allantoic fluid (ALF) was harvested and clarified. Virus was prepared by differential centrifugation, and partially purified by rate zonal centrifugation, in a sucrose gradient made up in saline, as shown in Figure 5. The gradiented virus, SDG C_3, the concentrates, C_2 and C_3; samples of ALF, C_1, C_2 and C_3 and their respective supernatant fluids designated SNF_1, SNF_2 and SNF_3 were stored at -80°C.

1.2 Human Serum Proteins

Human Immune Serum Globulin (HS), (Lederle, 254-764) containing 16.5 $\pm$ 1.5% gammaglobulin was obtained from the local pharmacy. Using the Lowry method, it was

Figure 5. Influenza A/PR8 Virus Preparation



found to contain 213 mg. protein/ml. (Leggett Bailey, 1962).

Human Immune Gamma Globulin (IgG) (IgG-5) containing 20 mg. protein/ml.was a gift through the courtesy of Hyland, California.

Bence Jones Protein (BJ), (BJK-2), kappa chain protein, containing 5 mg. protein/ml. was also obtained as a gift through the courtesy of Hyland, California.

2. Sera

2.1 Normal Sera

Normal rabbit sera (RS), normal human sera (HS), normal horse sera (ES) and normal goat sera (GS) were all available in the laboratory.

2.2 Antisera

Rabbit sera against normal egg soluble antigen (RaS/NE) was prepared by intramuscular injections of 1 ml. samples of antigen (20.7 mg. protein/ml) once a week for four weeks followed by booster shots every other week for six weeks. Freund's complete adjuvant was incorporated into the second injection. The rabbits were bled by heart puncture, again given booster shots once a week for two weeks and finally bled out by heart puncture. All sera were tested by immunodiffusion, pooled and stored at -20^{o}C.

Rabbit serum against influenza A/PR8 virus concentrate (RaS/C_2) was supplied by Dr. C. M. Johnson (Johnson and Westwood, 1971).

Commercial antihuman sera were purchased from Hyland, California :

Goat Antihuman Serum for Immunoelectrophonesis (GaH) ($3802C_2$)

Goat Antihuman Immunoglobulins (GaHɣ) (S202C1)

Goat Antihuman IgG (GaHɣG); H and L chain specific (8201M001A1)

Goat Antihuman Fab fragment (GaHFab) (8258E005A1)

Goat Antihuman ɣ chain (GaɣH) (8257H001A1)

Goat Antihuman free kappa L-chain (GaHBJ) (8255M001A1)

3. Detergents:

The following disrupting agents were purchased from Fisher Scientific: Tween 20; Sodium desoxycholate, (DOC), powdered, purified; and Sodium dodecyl sulfate, (SDS).

4. Purification of Sodium Dodecyl Sulfate

Sodium dodecyl sulfate (SDS) was purified according to a method developed by Roeppnack (1965). After extraction of SDS with 1 part butanol, a further extraction with 5 parts N,N dimethylformamide was carried

out. The solution was filtered to remove the inorganic salts. The filtrate was evaporated nearly to dryness until the SDS crystalized and was then treated with diethyl ether to complete the precipitation. The crystals were filtered, washed with diethyl ether and dried in a dessicator jar for 48 hours. The percent recovery was approximately 60%. This procedure narrowed the melting point range from 201°C - 210°C to 198° - 200°C indicating that a higher degree of purity had been achieved (Fieser, 1957).

5. Viral Assay Methods

5.1 Virus Hemagglutination Titrations

Two fold serial dilutions were made in Perspex trays using 0.2 ml. volumes in saline. An equal amount, 0.2 ml, of 1% rooster red blood cells were added and the results were read after incubation at room temperature for 60 minutes. The 50% end point was estimated by interpolation between complete agglutination and the absence of agglutination.

5.2 Virus Infectivity Titrations

Serial tenfold dilution series were made in saline containing penicillin and streptomysin. 0.1 ml. of each

dilution was inoculated into the allantoic sac of each of four 10-day-old chick embryos. After incubation for 48 hours at 35°C, the eggs were chilled overnight at 4°C, the allantoic fluids were individually harvested and tested for hemagglutination with 1% rooster red blood cells. The 50% infectious dose was calculated by the method of Kärber. (Jawetz et al, 1968).

6. Detergent Treatments

6.1 Sodium Deoxycholate (DOC)

DOC treatment was carried out by the addition of 1% DOC (1 part 10% DOC in saline to 9 parts sample), to the sample followed by incubation for 30 minutes at room temperature. The samples treated were Hs, $Hs_{\frac{1}{2}}$, IgG, BJ and influenza virus A/PR8 as C3 1/10 and SDG/C3. Immunodiffusion tests were run on the day these samples were treated. All samples were then stored at 4°C until needed. If a sample was used again, it was warmed to room temperature. This method was adapted from Styk et al (1970).

6.2 Tween 20

Tween 20 treatment was carried out by the addition of 1% Tween 20 (1 part 10% Tween 20 in saline to 9 parts sample) to the sample followed by incubation for 30 minutes

at room temperature. The samples treated were HS, IgG, and influenza virus A/PR8 C3 1/10 and SDG/C3. Immunodiffusion tests were run on the day these samples were treated. All samples were then stored at 4°C until needed.

6.3 Sodium Dodecyl Sulfate (SDS)

1% SDS samples were prepared by adding 1 part 10% SDS in saline to 9 parts of sample. The samples were incubated at room temperature for 30 minutes to 1 hour. The samples treated were HS, $HS_{\frac{1}{2}}$, IgG, BJ and influenza virus A/PR8:C3 1/10 and SDG/C3. Immunodiffusion tests were run on the day of preparation. All samples were stored at 4°C. If a sample was used again it was warmed to room temperature.

0.1%, 2% and 0.5% SDS samples were similarly prepared.

Some samples were pretreated with 0.1% β mercaptoethanol before treatment with SDS. The samples were allowed to incubate for 10 minutes before SDS was added.

7. Removal of SDS

7.1 Cold Precipitation

Samples were stored at least overnight at 4°C followed by centrifugation at 400 g for 15 minutes at 4°C.

The pellet was discarded and the supernatant fluid was tested for SDS content (See Experimental Results Section 2) and by immunodiffusion. This method was adapted from Laver and Valentine (1969).

7.2 Potassium Chloride Precipitation

A few drops of saturated KCl were added to samples which had been pretreated by cold precipitation and cooled to 0°C. The mixture was allowed to stand 2-3 hours at 0°C and then was centrifuged at 400 g for 15 minutes to remove precipitated SDS. The pellet was discarded and the supernatant fluid was tested for SDS content and by immunodiffusion. This method was also adapted from Laver and Valentine (1969).

7.3 Cold Acetone Precipitation

Cold acetone (-20°C) was added to the samples (4 parts acetone to 1 part sample) which had been pretreated by cold precipitation and cooled to 0°C, by stirring slowly. The mixture was stored overnight in an ice bath and then centrifuged at 1500 g for 30 minutes. The supernatant fluid was poured off and discarded. The pellet was resuspended in cold saline to about 1/10 of the original sample volume. The solution was then tested for SDS content and by immunodiffusion. This method was adapted from Laver and Valentine (1969).

8. Immunodiffusion

Immunodiffusion tests were carried out according to the continuous flow micro double diffusion technique described by Johnson et al (1964) using stainless steel chromatography clips to hold the templates in place. The 0.1% thiazine red R staining procedure was used throughout and all reactions were routinely recorded by direct photographic enlargement using a Beseler enlarger.

9. Protein Estimations

The protein content of all samples was estimated by the method of Lowry et al (1951) using the sodium citrate modification of Leggett Bailey (1962).

EXPERIMENTAL RESULTS

V - EXPERIMENTAL RESULTS

1. Introduction

Components, with associated biological functions, released by influenza virus disruption are difficult to correlate with known viral structures. However, one biological property easily checked is antigenicity which can be assayed by immunodiffusion. Since immunological reactivity is determined by the conformation of the surface molecules and SDS has been shown to modify protein conformation, treatment with SDS may change the antigenicity of the component as measured by its ability to participate in immunodiffusion precipitation reactions. In order to provide a background for evaluating immunodiffusion reactions with SDS disrupted virus, experiments with SDS treated antigens were carried out.

Since only small amounts of SDS need to be present to cause protein denaturation (Reynolds and Tanford, 1970a), it seemed critical to know how much SDS was present in test antigen solutions after treatment with SDS and after attempted removal of SDS by various means. Two systems for assay of SDS concentration were used.

2. Standardization of SDS Assay Systems

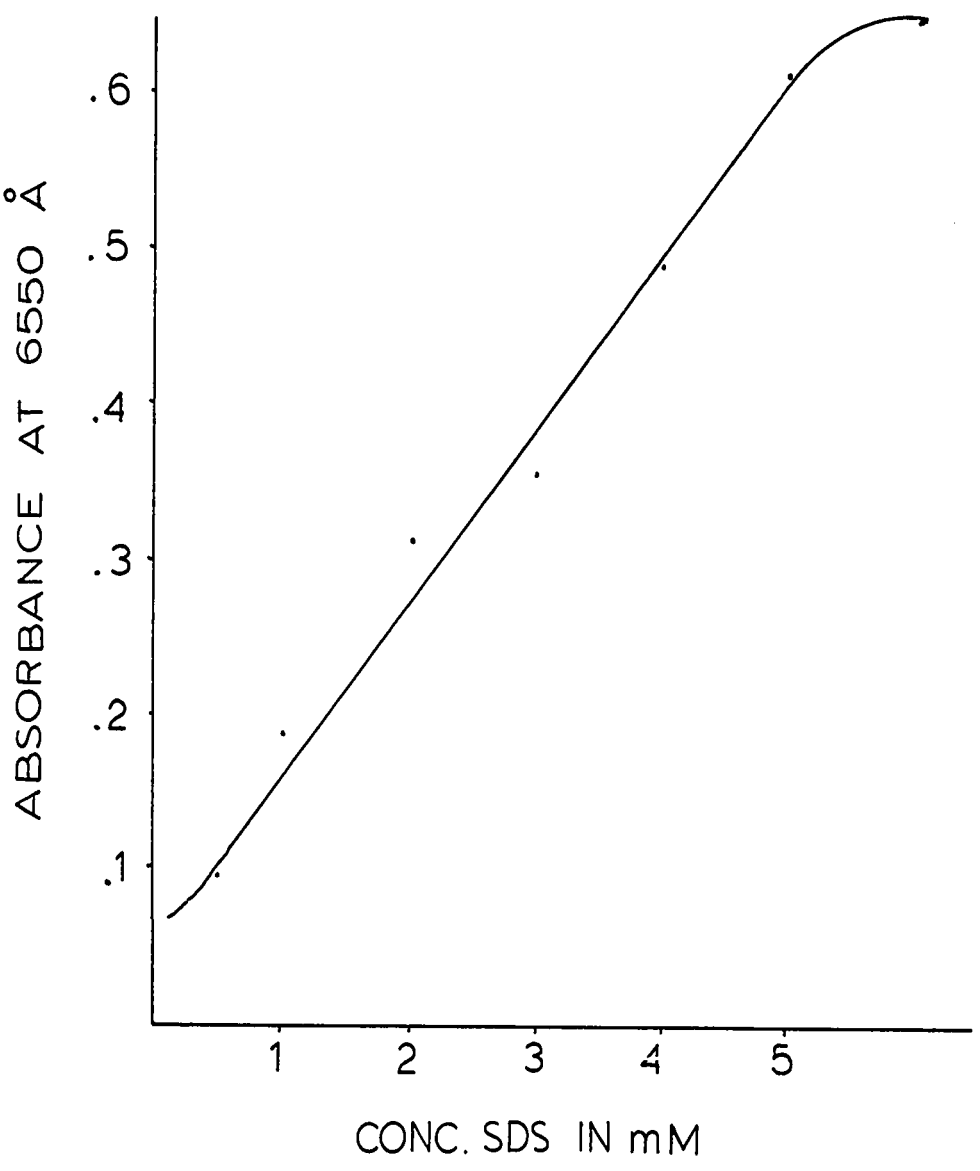
2.1 Methylene Blue - Chloroform Technique

SDS, an alkyl sulfate, forms a complex with methylene blue which can be extracted with chloroform and then detected by reading the optical density of the colored solution at 6550 Å (Mukerjee, 1956). Reynolds and Tanford (1970a) used these observations to develop a method for the quantitative determination of SDS. Based on their results the following technique was used: Five ml. of methylene blue (21 mg./litre) were added to 0.05 ml. of sample. The sample was shaken and then extracted with 20 ml. of chloroform. The optical density of the extract was read at 6550 Å on a Beckman DB spectrophotometer and standard curves were prepared. (Figure 6). As judged by linearity the limits of sensitivity of this technique were between 0.5 mM to 5 mM SDS. Standard curves were also prepared for SDS in the presence of known quantities of protein and β mercaptoethanol. There was no significant difference between the curves for SDS in saline and SDS with protein and or β mercaptoethanol. (Appendix)

2.2 Hemolysis Technique

The hemolysis technique was based on the observation of Rideal and Taylor (1957) and others that red blood cells lyse in the presence of SDS.

FIGURE 6.
CONCENTRATION OF SDS IN SALINE AS MEASURED BY ABSORBANCE OF METHYLENE BLUE - SDS CHLOROFORM EXTRACTED SOL. AT 6550 Å.

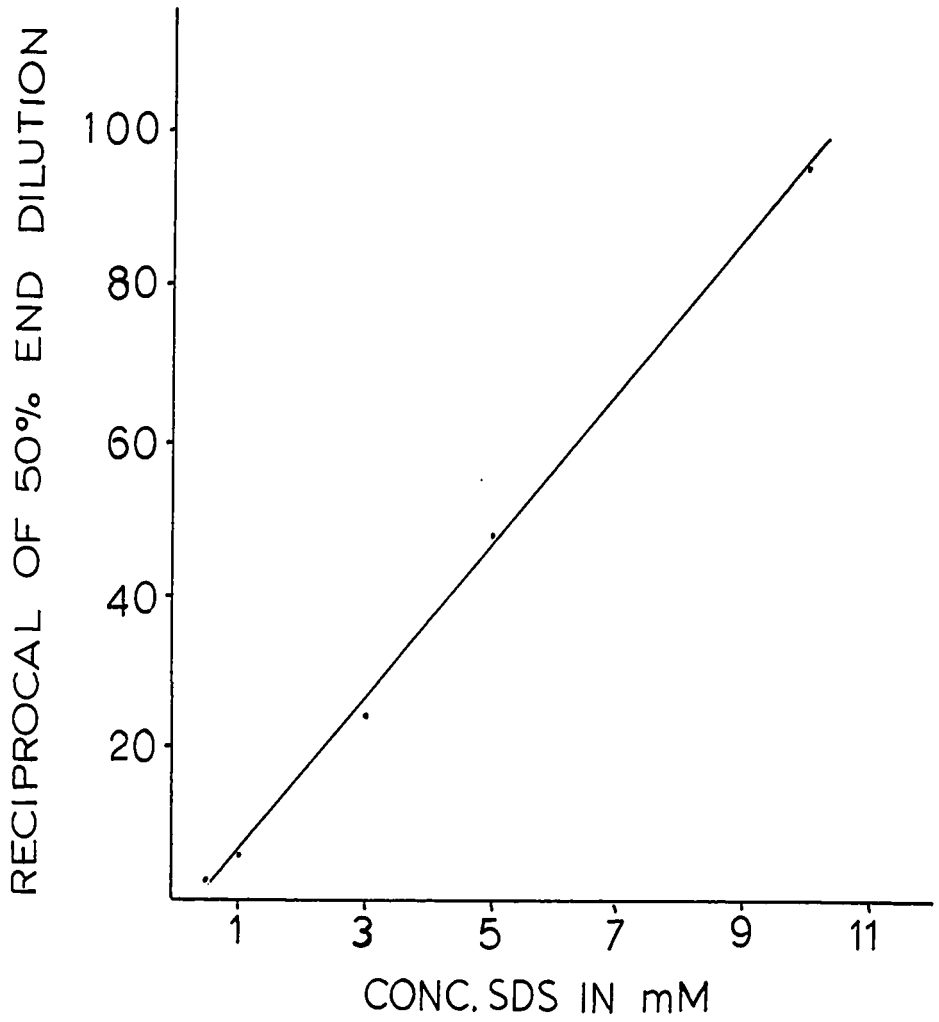


Two-fold serial dilutions of SDS solutions were made in Perspex trays using 0.2 ml. volumes in saline. An equal amount, 0.2 ml, of washed red blood cells (1% rooster, 1% human '0', 1% sheep or 0.5% rooster red blood cells) were added. The trays were read for hemolysis (absence of a button) or nonhemolysis (presence of a button) after incubation for 30 minutes at room temperature. The 50% end point was interpolated between complete lysis and no lysis. Standard curves were prepared for SDS concentration variations for all the erythrocytes listed. (Figure 7.). The relationship between hemolysis and SDS concentration appeared to be linear. No significant difference was found between the different red blood cells. (Appendix). Standard curves were also prepared for SDS in the presence of protein and β/mercaptoethanol. No significant difference was found between the presence and absence of protein.

2.3 Evaluation of the Two Techniques

In comparing these two procedures for determining SDS concentration, the hemolysis technique seemed to be simpler and less prone to technical problems than the methylene blue method. Due to the properties of this dye, even trace amounts of basic material on glassware changed the optical density of the chloroform-extracted SDS complex. For this reason, all glassware had to be

FIGURE 7.
SDS CONC. DETERMINATION USING
HEMOLYSIS TECHNIQUE WITH 1% ROOSTER RBC.



scrupulously clean. The hemolysis technique appeared to be as sensitive as the methylene blue method since as little as 0.5 mM SDS could be detected by both procedures. The methylene blue method did have one great advantage in that samples of only 1/10 of the size used for the hemolysis technique were required. However, this difference could be reduced, if sample quantity was limited, by developing a micro instead of macro hemolysis technique. The hemolysis procedure was the method of choice for most SDS determinations reported in these studies.

3. Effects of SDS on Protein

3.1 Introduction

The effect of SDS treatment of antigens was demonstrated by immunodiffusion reactions. The first system chosen for study was the reaction between the antigens present in human immune serum globulin (HS), 170 mg. protein/ml., and their corresponding antibodies in goat antihuman serum (GaH). All preparations which were treated with SDS were assayed for SDS content at the time of testing in the immunodiffusion reactions.

The normal reaction between GaH and HS is shown in Plate 1 Figure 1. A total of eight precipitin lines have developed which are clearly defined and easily resolved. The haze about the central antiserum well was attributed mainly to the binding of excess protein to the cellulose

PLATE 1

Immunodiffusion Reactions Between Untreated and SDS Treated Human Immune Serum Globulins (HS) and Goat Antihuman Serum (GaH).

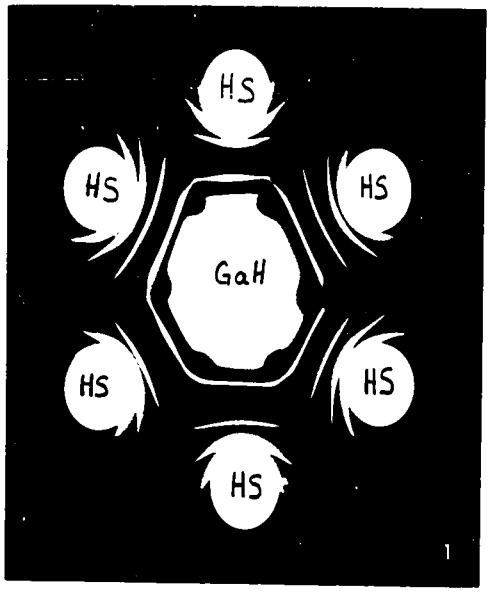
1. The reaction between untreated Human Immune Serum Globulins (HS) and Goat Antihuman Serum (GaH) is shown. A total of eight precipitin lines have developed.

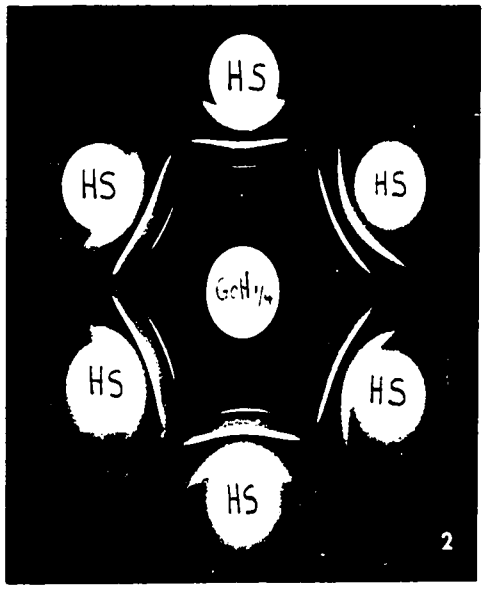
2. The reaction between HS and GaH diluted ¼ is shown. Only four lines have developed with this serum dilution.

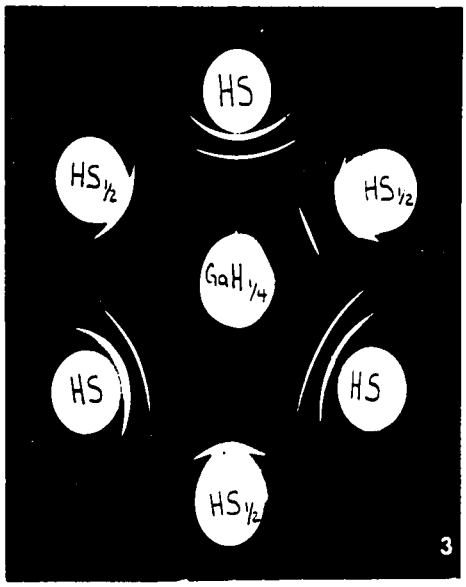
3. The reaction between HS and HS diluted ½ with the antiserum $GaH_{\frac{1}{4}}$ is shown. Five lines have developed with $HS_{\frac{1}{2}}$ but only four with HS undiluted.

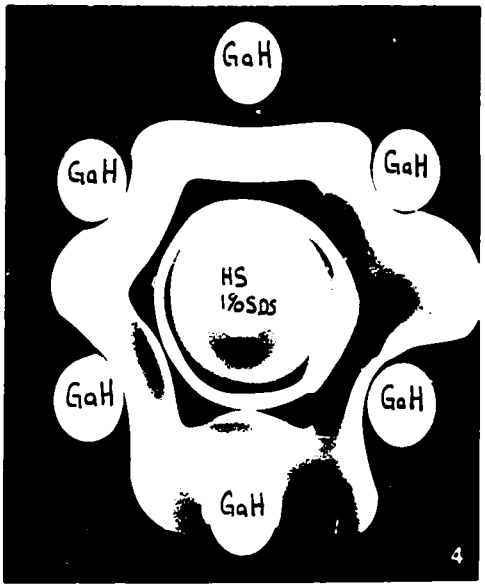
4. The effect of treatment of HS with 1% SDS is shown. Two relatively sharp lines have developed between HS/1% SDS and GaH. Wide bands of precipitation can be seen near the antiserum and angigen wells.

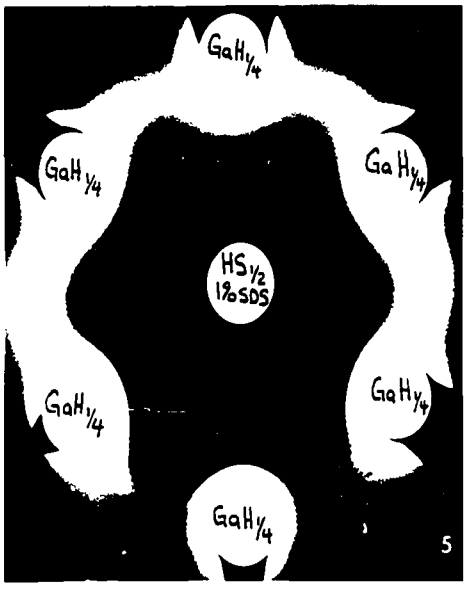
5. The effect of treatment of HS diluted ½ with 1% SDS is shown. No distinct precipitin lines have formed. Only a hazy band has developed about the antiserum wells ($GaH_{\frac{1}{4}}$)

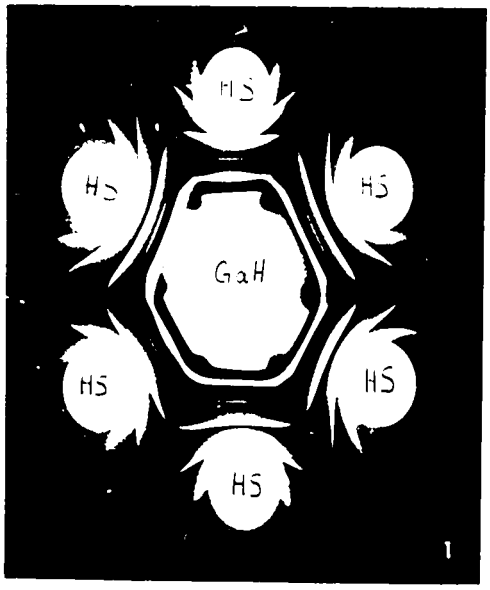

HS
HS
HS
GaH
HS
HS
1

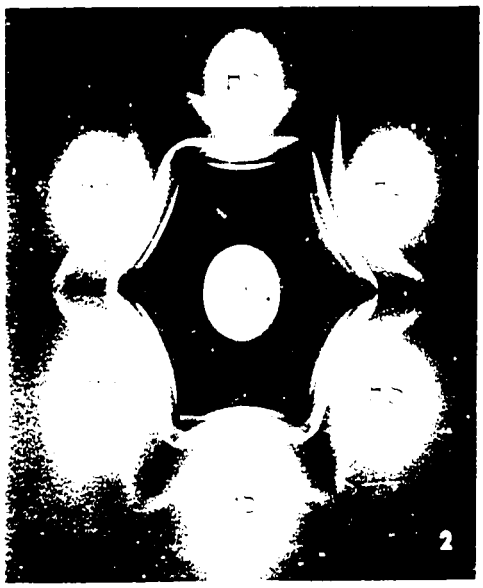

HS
HS
HS
GaH 1/4
HS
HS
2

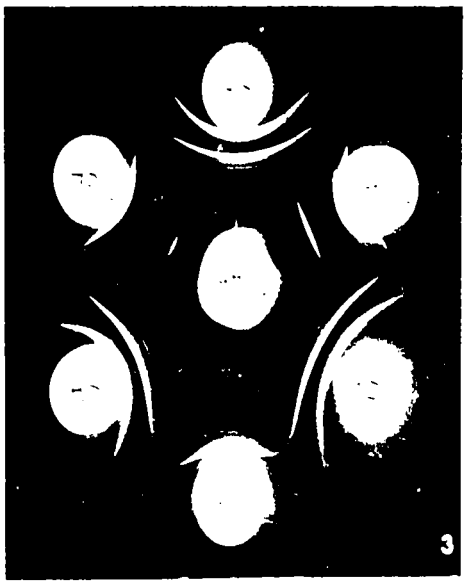

HS
HS 1/2
HS 1/2
GaH 1/4
HS
HS
HS 1/2
3


GaH
GaH
GaH
HS
1% SDS
GaH
GaH
GaH
4


GaH 1/4
GaH 1/4
GaH 1/4
HS 1/2
1% SDS
GaH 1/4
GaH 1/4
GaH 1/4
5


HS
HS
HS
GaH
HS
HS
HS
1


2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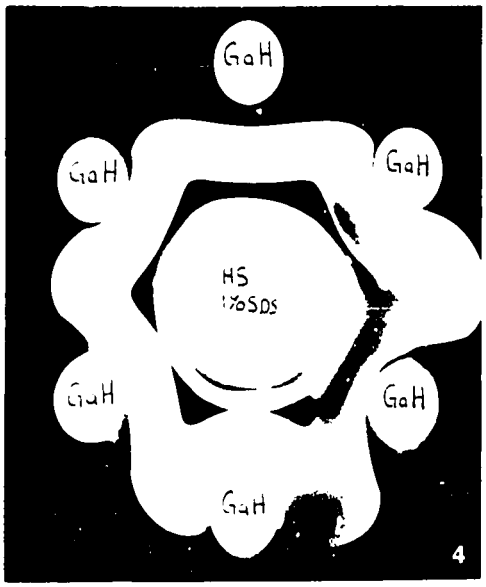
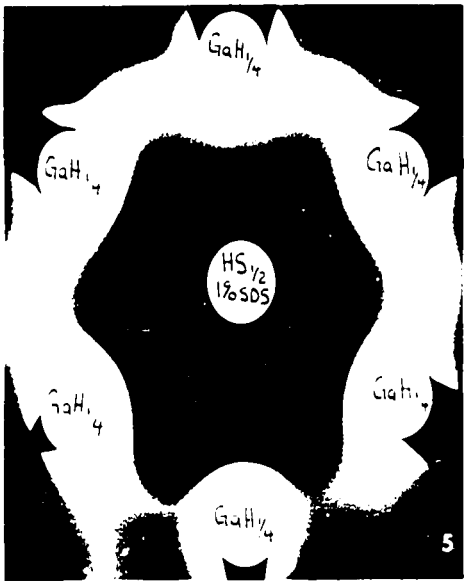

GaH
GaH
GaH
HS
1%SDS
GaH
GaH
GaH
4


GaH1/4
GaH1/4
GaH1/4
HS1/2
1%SDS
GaH1/4
GaH1/4
GaH1/4
5

acetate, combined with some nonspecific antibody precipitation. Figures 2 and 3 show the effect of varying the concentration of the antigens, HS, and the antiserum, GaH. It can be seen that fewer lines (4) have developed with GaH diluted ¼,(Figure 2), than with GaH (Figure 1).However, at least five precipitin lines have developed when a dilution of HS (½) was used (Figure 3).Thus at these dilutions the ratio of antigen to antibody appeared to be closer to the optimal as judged by an increase in number and quality of the developed lines than for HS with GaH ¼. The reagents were used at both these dilutions throughout these experiments unless specified otherwise.

The problems involved when the antigen was treated with SDS are well illustrated in Figures 4 and 5 of Plate 1. Figure 4 shows the results of testing HS treated with 1% SDS, the concentration most commonly used to disrupt influenza, with GaH. At least two relatively sharp lines have developed just out from the HS/1% SDS well. A wide band of precipitation could be seen near the antiserum, GaH, wells. Similarly a wide band was also seen around the antigen well, HS/1% SDS. Interpretation of this type of reaction is extremely difficult. Do the wide bands represent non-specific precipitation of antigen-antibody complexes or perhaps non-specific precipitation of antigen or antibody? Are the two sharp lines midway between the

wells true antibody-antigen precipitin lines? If the antigen and the antiserum were both diluted, ½ and ¼ respectively, but the antigen concentration of SDS remained at 1%, the results of the immunodiffusion reaction were quite different (Figure 5). No distinct precipitin lines were visible, only a hazy band about the antiserum, GaH ¼, wells. Does this loss of distinct lines, as compared to Figure 4, represent a total loss in recognizable antigen due to the increased ratio of SDS to antigen or is it simply a dilution factor?

Thus, in answer to the first question posed at the onset of these experiments, it was apparent from Figures 4 and 5, that 1% SDS treatment of the antigen (HS) did modify the reactivity of the antigen in the immunodiffusion test.

3.2 Effects of SDS on Immunodiffusion Reactions

a) Nonspecific Precipitation

When HS½/1% SDS was diffused against normal rabbit serum (RS) in the standard manner of the immunodiffusion test, precipitin "lines", developed (Plate 2, Figure 1). Since no lines could be demonstrated using untreated HS½, in control wells, the "lines", both narrow and diffuse between HS½/1% SDS and RS were attributed to nonspecific precipitation of serum proteins by the SDS diffusing from the antigen wells. This effect was also noted using sera

PLATE 2

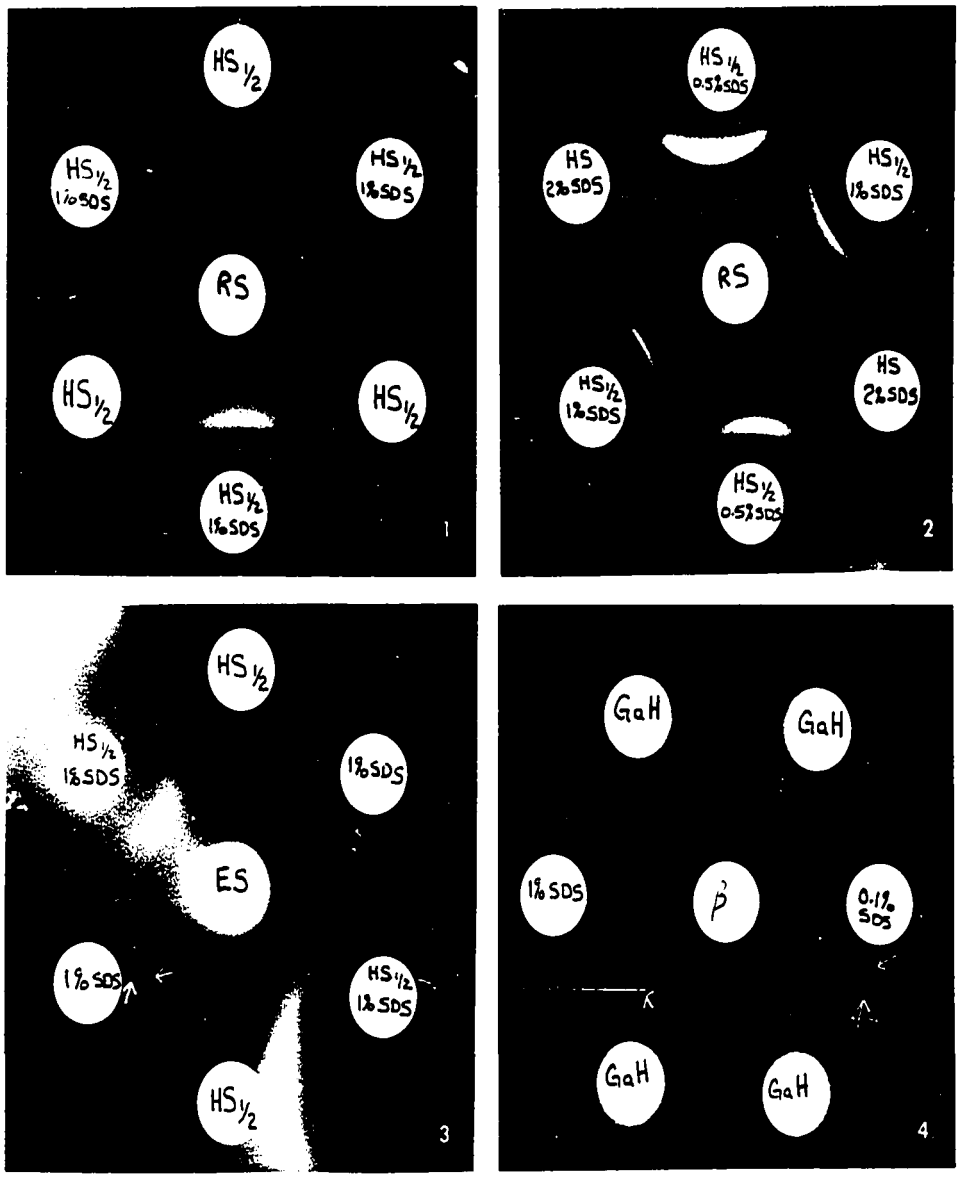
Immunodiffusion Reactions Demonstrating the Nonspecific Precipitation of Serum by SDS Either Alone or with Antigens.

1. Nonspecific precipitation of normal rabbit serum (RS) by SDS present in $HS_{\frac{1}{2}}$/1% SDS samples is shown. No reaction has developed with untreated $HS_{\frac{1}{2}}$ but lines can be seen between $HS_{\frac{1}{2}}$/1% SDS and RS.

2. The effect of varying the concentration of SDS (0.5% to 2%) with HS or $HS_{\frac{1}{2}}$ on the ability of SDS to cause nonspecific precipitation of normal rabbit serum (RS) is shown. Lines have developed between all SDS treated samples and RS.

3. The reaction between SDS at 1% either in saline or with HS and normal horse serum (ES) is shown. Lines (arrowed) have developed between both SDS samples with or without $HS_{\frac{1}{2}}$ and ES. No lines have formed with non-SDS containing $HS_{\frac{1}{2}}$.

4. The reaction between SDS at 1% and 0.1% in saline and β mercaptoethanol at 0.1% in saline and Goat Antihuman serum (GaH) is shown. No reaction was detected with β mercaptoethanol but lines (arrowed) developed with both SDS samples and GaH.


HS ½
HS ½ 1% SDS
HS ½ 1% SDS
RS
HS ½
HS ½
HS ½ 1% SDS
1
HS ½ 0.5% SDS
HS 2% SDS
HS ½ 1% SDS
RS
HS ½ 1% SDS
HS 2% SDS
HS ½ 0.5% SDS
2
HS ½
HS ½ 1% SDS
1% SDS
ES
1% SDS
HS ½ 1% SDS
HS ½
3
GaH
GaH
1% SDS
P
0-1% SDS
GaH
GaH
4

RS
1
RS
GaH
GaH
3
4

from humans, goats and horses.

Plate 2 Figure 2 shows the results of reacting antigens, treated with concentrations of SDS ranging from 0.5 to 2%, with normal rabbit serum. "Lines" developed with all the SDS treated antigens. In fact, two very narrow "lines", very similar to antigen-antibody specific precipitin lines were seen with HS/2% SDS (Figure 2). Further experiments revealed that the presence of even 0.1% SDS in the antigen could cause nonspecific precipitation of serum proteins in immunodiffusion reactions. Lines also developed when SDS alone was tested against sera in the immunodiffusion reaction (Figures 3 and 4). Even at 0.1% SDS, the nonspecific precipitation occurred, (Figure 4). The intensity and number of "lines" which developed did not appear to be definitely related to the concentration of SDS. In some instances several sharp lines developed if 2% SDS was present while only a fuzzy line if the concentration was 0.1%. In other cases, the results were reversed. However, for all concentrations of SDS tested, the addition of 0.1% β mercaptoethanol did not alter the development of "lines". Not surprisingly, 0.1% β mercaptoethanol alone did not seem to have any effect when tested against serum (Figure 4).

Therefore on the basis of these results, the haze

which developed about the wells containing antigen, HS or HS½, and SDS can now be interpreted as nonspecific SDS precipitation of antigen (Plate 2, Figure 1, 2 and 3).

In summary, then, SDS whether alone or with the antigen preparation at concentrations of 0.1% or greater, reacts with antiserum in cellulose acetate immunodiffusion reactions to form precipitin "lines" which are artifacts. This conclusion has recently been confirmed by published observations of Palmer et al (1971) that SDS reacts with whole serum in agar diffusion and immunoelectrophoresis to form artifactual precipitin lines. Thus, due to the ability of SDS to cause reaction artifacts, immunodiffusion reactions in which participating reactants have been exposed to SDS must be cautiously analysed. These "lines" which SDS causes have been named SDS precipitin lines.

b) Effect of SDS on Specific Ag-Ab Reactions

The effect of treatment of HS with 1% SDS, the concentration commonly used to disrupt influenza virus, is shown in the immunodiffusion reaction in Plate 3 Figure 1. At maximum of seven precipitin lines have developed in the control reaction between HS and GaH¼ (Figure 1). Two lines, possibly three, were visible in the HS/1% SDS reaction, only one of which definitely linked to show a reaction of identity between HS and HS/1% SDS. However,

PLATE 3

Immunodiffusion Reactions showing the Effects of Treatment of Human Immune Serum Globulins (HS) with various concentrations of SDS and with 0.1% β mercaptoethanol.

1. HS treated with 1% SDS is compared with untreated HS in this reaction. The antiserum is Goat Antihuman Serum ($GaH_{\frac{1}{4}}$). A maximum of seven lines are seen in the control reaction, HS with $GaH_{\frac{1}{4}}$, but only three lines are seen with HS/1% SDS. One of these lines shows a reaction of identity with the control, HS.

2. A comparison of the effects of HS treated with 1% SDS and 0.1% SDS with untreated HS is shown. The antiserum is $GaH_{\frac{1}{4}}$. Six lines are seen in the control reaction, HS and $GaH_{\frac{1}{4}}$. Five or six are seen with HS/0.1% SDS but only two of these link to show reactions of identity with the control. One of these lines links around to show identity with one of the three lines formed with HS/1% SDS.

3. In this reaction, HS pretreated with 0.1% β mercaptoethanol (HS/β) and then treated with 1% SDS (HS/β 1% SDS) is compared with the controls, HS and HS/β. The antisera are GaH and Goat Antihuman Serum Globulins (GaHγ). Five lines which form reactions of identity are seen between HS and HS/β with GaH and two with GaHγ. An abrupt change in lines (arrowed) is seen near HS/1% SDS β, with which new lines have formed.

4. A reaction between HS containing varying amounts of protein with different concentrations of SDS is compared with untreated HS (170 mg protein/ml). The antiserum is $GaH_{\frac{1}{4}}$. At least four lines are seen with the control, HS, while with all SDS preparations fewer were formed.

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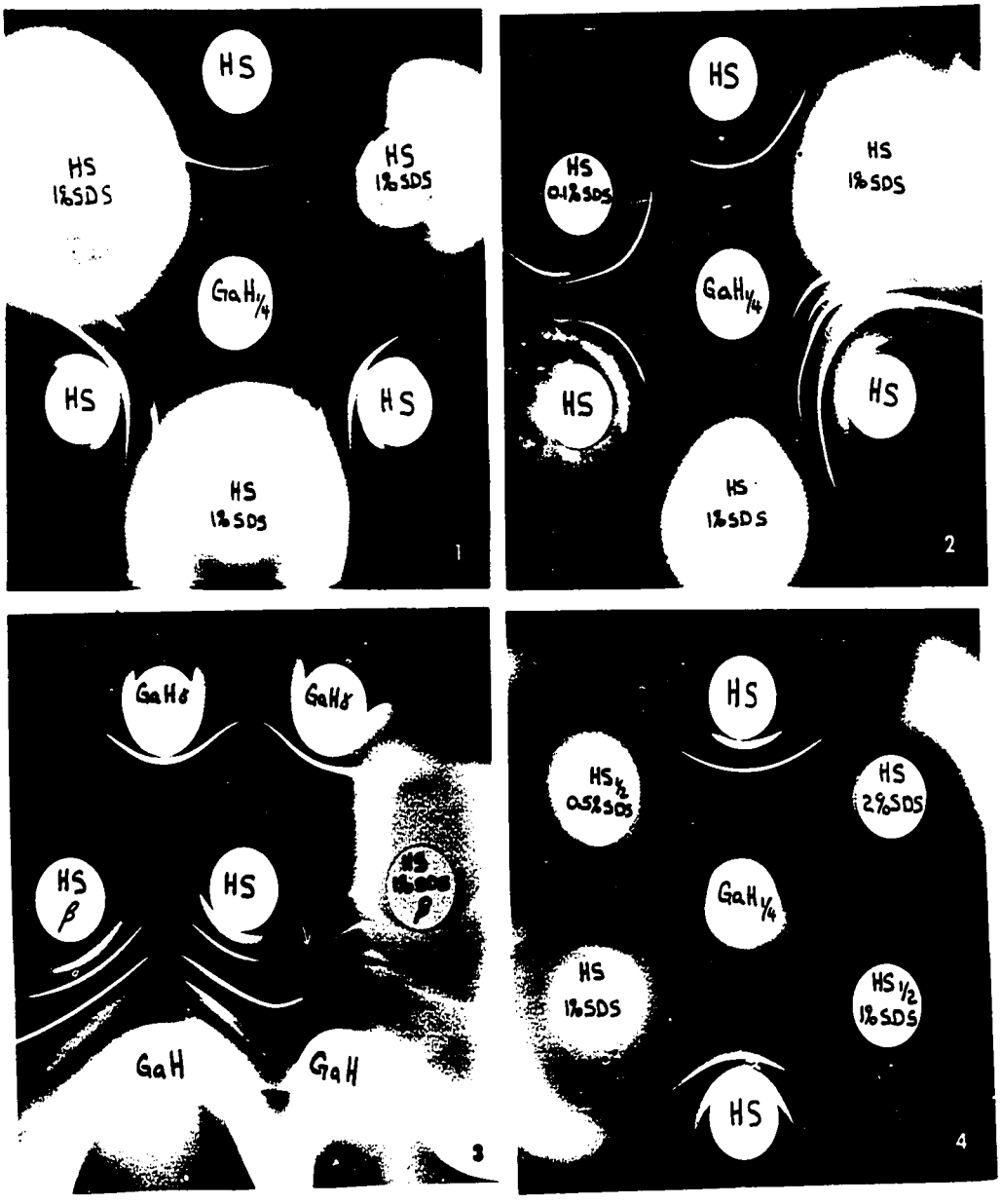

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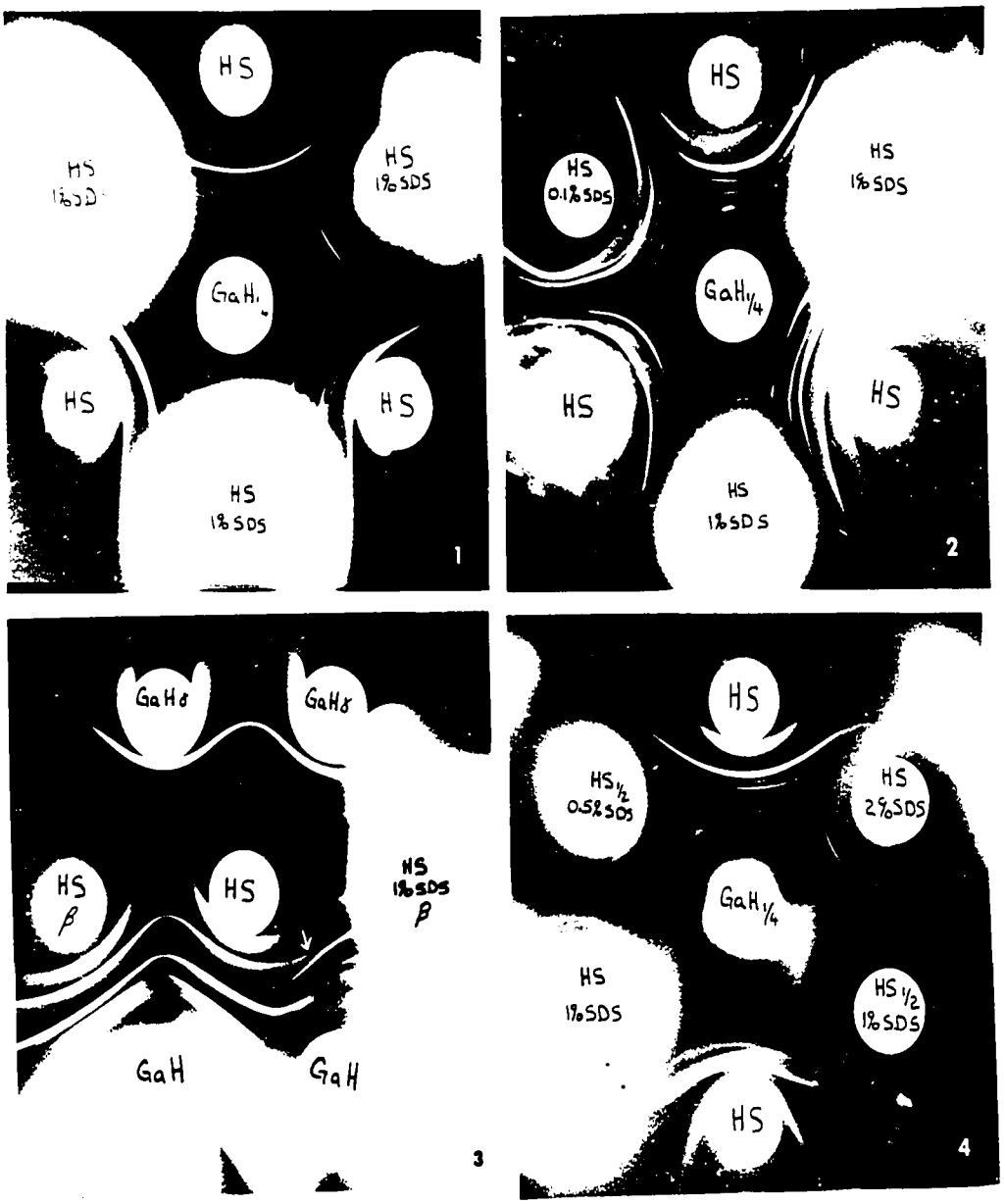

this reaction was obscured by the presence of the haze associated with the SDS precipitation of HS and it was not possible to determine any relationships between the remaining antigens, nor could it be determined whether those components present in the control preparation were lost by SDS treatment.

If HS was treated with only 0.1% SDS the results were quite different (Plate 3, Figure 2). At least six precipitin lines have developed in the control reaction between HS and GaH½ and one of these showed a reaction of identity with a line from the HS/1% SDS reaction. With the 0.1% SDS treated HS, at least five and possibly six lines can be counted. Reactions of identity between HS/0.1% SDS and HS control developed between two of these lines. However, one of these lines of identity also links around to show identity with a line from HS/1% SDS. The low number of reactions of identity developing between the antigens of HS/0.1% SDS and HS with GaH½ was seen in all samples tested. A possible explanation might be that at this low concentration SDS may only partially alter the structure of the antigens, allowing them to be recognized by the antibodies but altering their diffusion rates so linkages are difficult to form.

When HS was treated with 0.1% β mercaptoethanol prior to testing by immunodiffusion, as Plate 3 Figure 3 shows, at least five linked precipitin lines developed between HS/β and HS with GaH. Similarly, at least two linked precipitin lines formed between these two samples and GaH½. However, if HS/β was treated with 1% SDS, the result was similar to that seen with HS/1% SDS. (Figure3). Only one precipitin line formed a reaction of identity between HS and HS/1% SDS β with GaH. The rest of the reaction lines formed with HS were abruptly changed. New lines developed between HS/1% SDS β and both GaH and GaH½.

Since Reynolds and Tanford (1970a) observed that two binding levels of SDS to protein existed following equilibrium dialysis, both of which were dependent on the equilibrium monomer concentration, the effect of varying the SDS content with protein content on immunodiffusion reactions was examined. As Plate 3 Figure 4 shows, if the SDS concentration was increased to 2%, only one line developed compared to the three for HS/1% SDS. One of these lines linkedto form a reaction of identity with one of the four lines developed between HS and GaH¼. If both HS and 1% SDS were diluted by a factor of two i.e. HS½/0.5% SDS, although only one line developed in the reaction shown in Figure 4, in Plate 5, two lines formed

and one linked with a line from HS½ to form a reaction of identity. Thus similar results were obtained When the ratio of SDS to protein was maintained at a constant; ie HS/1% SDS, HS½/0.5% SDS with GaH¼.

In summary, the following conclusions regarding the effect of SDS on immunodiffusion reactions can be drawn. The concentration of SDS present in the antigen solution appeared to be critical: 0.1% seemed to cause only slight modifications while 1% seemed to abolish nearly all reactivity. The amount of SDS present with respect to protein content also was important in determining the degree of modification. Finally, the presence of β mercaptoethanol at 0.1% did not appear to have much effect on the antigens of HS nor did it moderate the effect of SDS on the antigen.

3.3 Removal of SDS

As illustrated in Plate 3, Figure 2, the presence of 1% SDS modified the ability of HS to participate in the immunodiffusion reaction, while 0.1% SDS appeared to cause little change. Therefore, a series of experiments were carried out to determine if by removing the SDS, the samples could be restored to their original reacting characteristics or at least to an improved reacting level. Three procedures for removal were used. All samples were

TABLE 1. Percentage SDS Removed from HS-SDS Samples by Cold Precipitation, Acetone Treatment, and Saturated Potassium Chloride Treatment.

Removal Technique	SDS Content* Before	After	% Removed
Cold Precipitation	1%	~0.8%	20%
	0.5%	~0.45%	10%
Cold Precipitation + KCL Treatment	1%	~0.15%	85%
	0.09%	0%	100%
Cold Precipitation + Acetone Treatment	1%	0%	100%
	0.09%	0%	100%

* measured by Hemolysis Technique

treated by cold precipitation (P), followed by either acetone (A) or potassium chloride (K) treatment. The percentage of SDS removed by these procedures is presented in Table 1.

a) Cold Precipitation

Using the cold precipitation technique, up to 20% of the SDS could be removed from the samples tested (Table 1). These cold precipitation preparations (P) were tested by immunodiffusion. The effect of cold precipitation on the reactivity of HS½/0.5% SDS is shown in Plate 4. Four lines have developed between HS½ and GaH½. With HS½/0.5% SDS, two possibly three lines have formed, one of which clearly showed a reaction of identity with HS½ while one definitely did not. Following cold precipitation, $HS_{\frac{1}{2}}$/0.5% SDS/P, no marked improvement was detected. However, although a faint new line has developed (arrowed) it could not be ascertained whether this represented a renatured antigen or a SDS precipitin line. Further tests with cold precipitation samples revealed that while native HS and $HS_{\frac{1}{2}}$ were unaffected by this procedure, SDS-treated samples (1% or 0.5%) were not restored to their original reacting capacity.

b) Acetone Treatment

Using the acetone technique 100% of the SDS could

PLATE 4

Immunodiffusion Reaction showing the Effect of SDS Removal From Human Immune Serum Globulins Samples (HS) by Cold Precipitation.

Cold precipitation treated $HS_{\frac{1}{2}}$ 0.5% SDS is compared with untreated $HS_{\frac{1}{2}}$ and Goat Antihuman Serum ($GaH_{\frac{1}{2}}$). Four lines are seen in the control $HS_{\frac{1}{2}}$ and $GaH_{\frac{1}{2}}$ but only two or possibly three are seen with $HS_{\frac{1}{2}}$/0.5% SDS. With $HS_{\frac{1}{2}}$/0.5% SDS/P (ie following cold precipitation) a new line may have formed arrowed.

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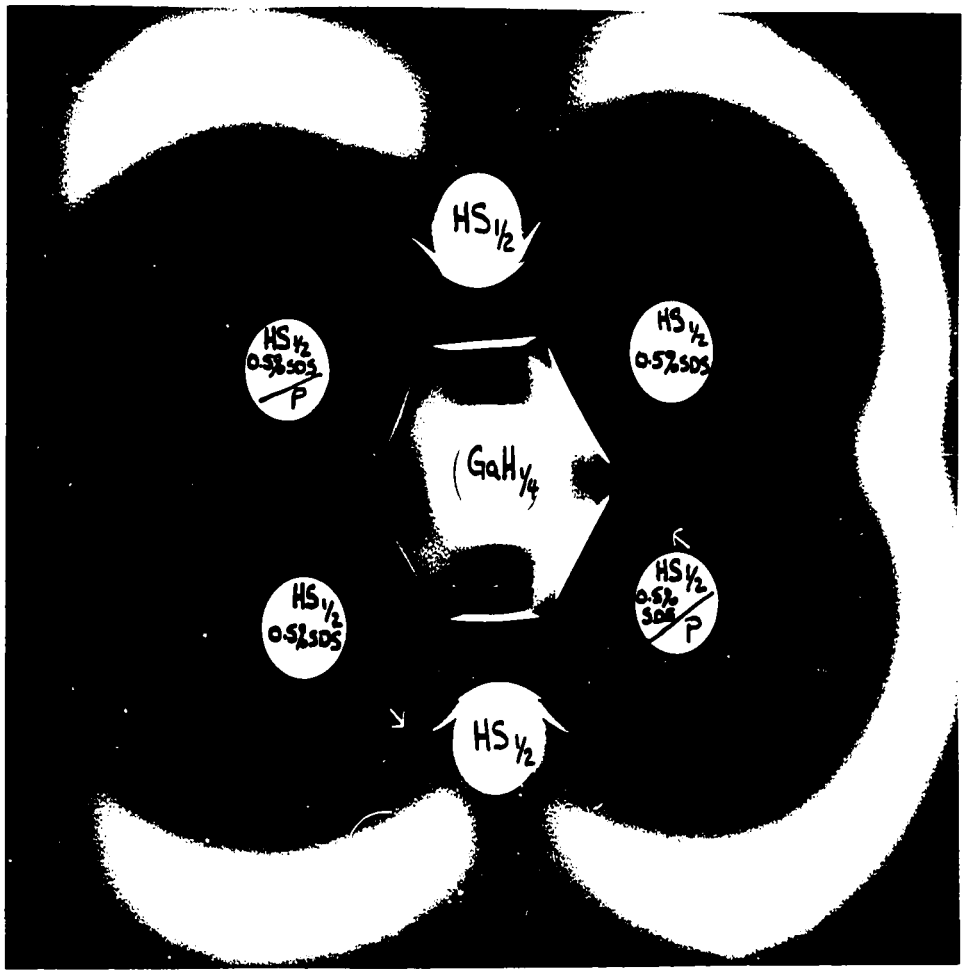
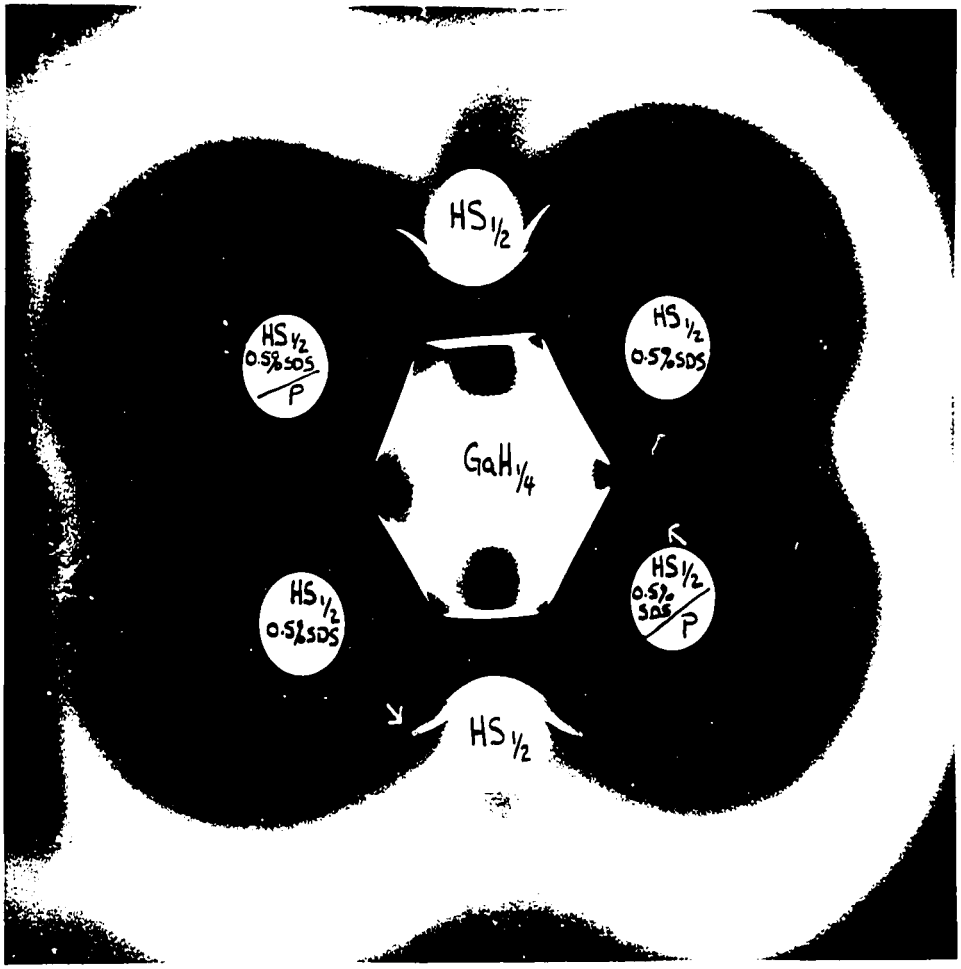
cold
d.




HS 1/2
HS 1/2 0.5% SDS / P
HS 1/2 0.5% SDS
GaH 1/4
HS 1/2 0.5% SDS
HS 1/2 0.5% SDS / P
HS 1/2

be removed from samples containing 1% SDS and 0.1% SDS. (Table 1). Immunodiffusion results with acetone-treated samples are presented in Plate 5.

In control reactions (Plate 5, Figure 1), between four and five precipitin lines have developed between HS and $GaH_{\frac{1}{4}}$. However, after treatment of HS with acetone (HS/A) all but one of these lines were lost. This remaining line did show a reaction of identity with a line from HS. In Figure 2, with HS/0.1% SDS treated with acetone (HS/0.1% SDS/A), only one precipitin line formed. However, this line did link with one of the three or four lines formed with HS/0.1% SDS and HS. When HS/1% SDS was treated with acetone, (Figure 3), only a faint line (arrow) developed between HS/1% SDS/A and $GaH_{\frac{1}{4}}$. This was in contrast to the three between HS and HS/0.1% SDS, and $GaH_{\frac{1}{4}}$. Thus the removal of SDS by acetone treatment did not restore the antigenic characteristics of HS/1% SDS back to the level of even HS/0.1% SDS.

Figure 4 illustrates the effects of acetone treatment on HS/1% SDS β. With HS, at least six and possibly seven precipitin lines have developed with GaH, one of which may have formed a reaction of identity with a line developed near HS/1% SDS β. However, the HS precipitin lines appeared to travel through the HS/1%

PLATE 5

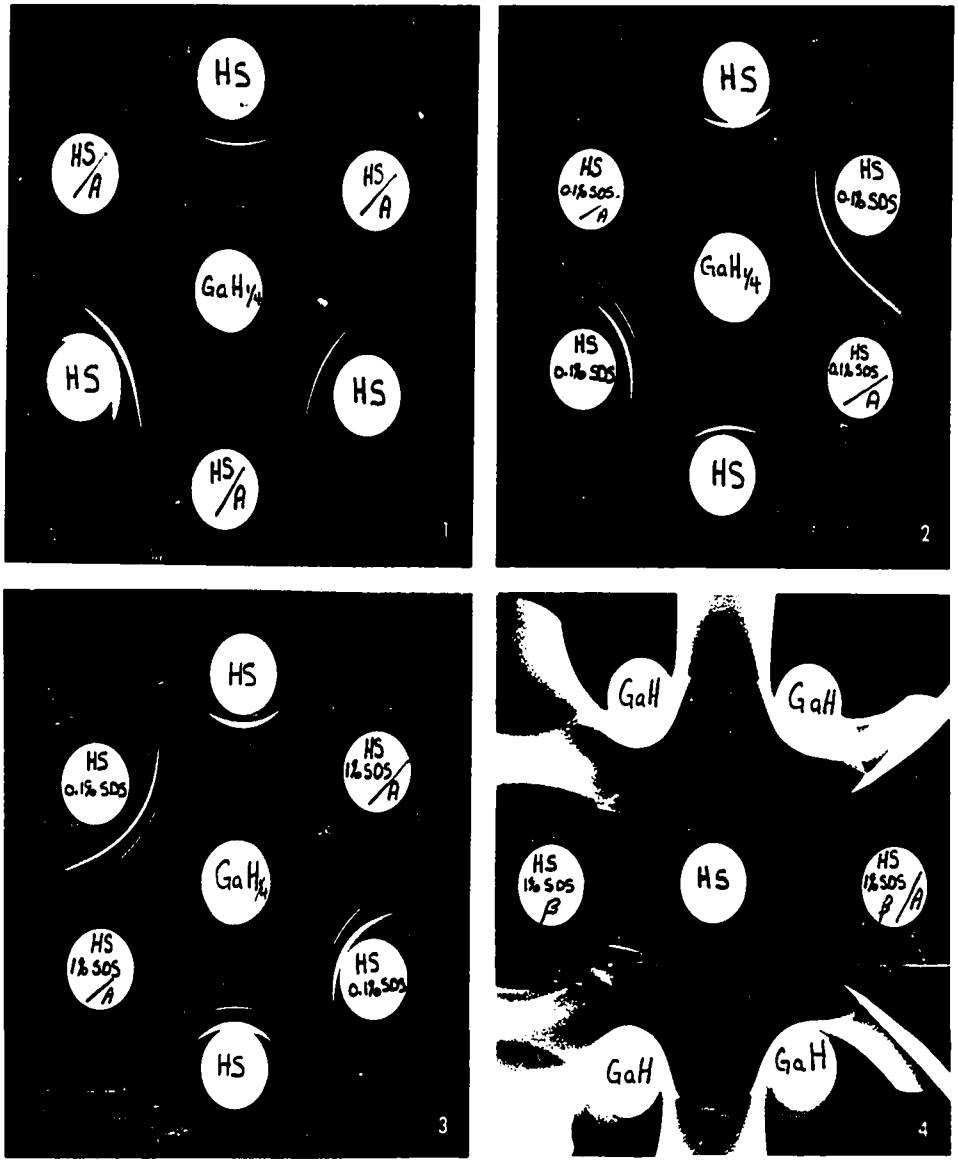
Immunodiffusion Reactions Demonstrating the Effect of Acetone Treatment for SDS Removal on Human Immune Serum Globulins Samples (HS) both with and without SDS.

1. In this reaction acetone treated HS (HS/A) is compared with native HS. The antiserum Goat antihuman serum ($GaH_{\frac{1}{4}}$). Four or five lines are seen with the control (HS) but only one (arrowed) is seen with HS/A.

2. In this reaction acetone treated HS samples pretreated with 0.1% SDS (HS/0.1% SDS/A) is compared with HS/0.1% SDS and native HS. The antiserum is $GaH_{\frac{1}{4}}$. Three or four lines have developed with HS and HS/0.1% SDS/A.

3. In this reaction acetone treated HS pretreated with 1% SDS (HS/1% SDS/A) is compared with HS/0.1% SDS and native HS. Only a very faint line (arrowed) is seen with HS/1% SDS/A compared to at least three lines seen with HS and HS/0.1% SDS and the antiserum $GaH_{\frac{1}{4}}$.

4. In this reaction acetone treated HS pretreated with 0.1% β mercaptoethanol and 1% SDS (HS/1% SDS β /A) is compared to native HS, and HS/1% SDS β. The antiserum is GaH. At least six lines are seen between HS and GaH one of which may have formed a reaction of identity with a line near HS/1% SDS β. In contrast, the HS precipitin lines appear to travel through the HS/1% SDS β/A sample for reactions of nonidentity (arrowed). One line is seen between HS/1% SDS β/A and GaH.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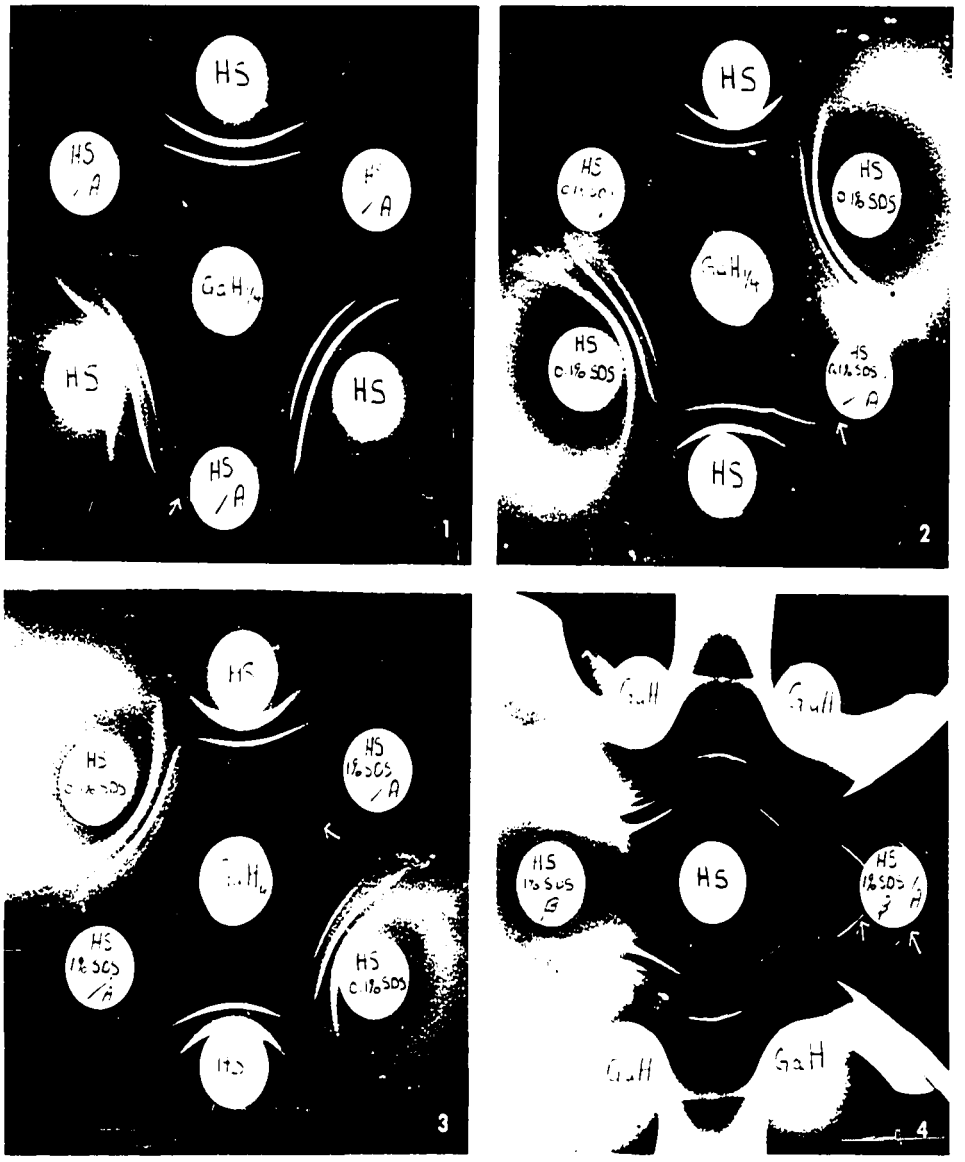
HS/A
HS/A
GaH4
HS
HS
HS/A
1
HS
HS 0.1% SDS/A
HS 0.1% SDS
GaH4
HS 0.1% SDS
HS 0.1% SDS/A
HS
2
HS
HS 0.1% SDS
HS 1% SDS/A
GaH4
HS 1% SDS/A
HS 0.1% SDS
HS
3
GaH
GaH
HS 1% SDS β
HS
HS 1% SDS β/A
GaH
GaH
4


HS
HS / A
HS / A
GaH
HS
HS
HS / A
1
HS
HS 0.1% SDS
HS 0.1% SDS
GaH
HS 0.1% SDS
HS 0.1% SDS / A
HS
2
HS
HS 1% SDS / A
HS 1% SDS / A
HS 0.1% SDS
3
GaH
GaH
HS 1% SDS B
HS
HS 1% SDS / A
GaH
GaH
4

SDS β/A sample as if it was not there (reaction of nonidentity). Only one line appeared to have developed between HS/1% SDS β/A and GaH and this was attributed to precipitation of GaH.

Thus the acetone treatment appeared to be efficient at removing SDS but caused great reduction in antigenic capacity even in non-SDS treated samples.

c) Saturated Potassium Chloride Treatment

Using the saturated potassium chloride (K) technique, up to 85% of the SDS could be removed from samples containing 1% SDS (Table 1). Immunodiffusion results with potassium chloride treated samples are presented in Plate 6.

The effect of potassium chloride treatment on HS alone is demonstrated by the reaction shown in Figure 1. Six precipitin lines have developed between HS and GaH. If HS was treated with potassium chloride (HS/K), at least five lines formed indicating that potassium chloride treatment had only a slight effect on the antigenic properties of HS as measured by immunodiffusion. Similar results were seen when HS/K was dialysed to remove potassium chloride (HS/Kd). Plate 5, Figures 2 and 3 show the reactions with potassium chloride treated HS/1% SDS. In Figure 2, although five

PLATE 6

Immunodiffusion Reactions Demonstrating the Effect of Saturated Potassium Chloride Treatment for SDS Removal on Human Immune Serum Globulin (HS) Samples both with and without SDS.

1. The effect of KCL treatment on HS alone is shown in this reaction. The antiserum is GaH. Six lines are seen with HS while five are seen with HS/K (ie treated with KCL).

2. The effect of KCL treatment on HS/1% SDS (Hs/1% SDS/K) is shown in this reaction. The controls are HS and HS/1% SDS while the antiserum is $GaH_{\frac{1}{4}}$. Five lines have developed with HS but only one or possibly two link to show reaction of identity with HS/1% SDS/K. Other lines (arrowed) have formed between HS/1% SDS, HS/1% SDS/K and $GaH_{\frac{1}{4}}$.

3. The effect of KCL treatment on HS/1% SDS (HS/1% SDS/K) is shown in this reaction. The antiserum is $GaH_{\frac{1}{4}}$. In the control reactions, five lines have formed between HS and $GaH_{\frac{1}{4}}$, but no reactions of identity are seen with HS/1% SDS or HS/1% SDS/K. However, a reaction of identity is seen between HS/1% SDS and HS/1% SDS/K with $GaH_{\frac{1}{4}}$. Also a line has developed between the wells containing the two SDS samples (arrowed).

4. The effect of KCL treatment on HS/0.1% SDS (HS/0.1% SDS/K) is shown in this reaction. The antiserum is $GaH_{\frac{1}{4}}$. While at least five lines have formed between one control, HS and $GaH_{\frac{1}{4}}$, and four or five between the second control, HS/0.1% SDS and $GaH_{\frac{1}{4}}$ only three are seen with HS/0.1% SDS/K and $GaH_{\frac{1}{4}}$.

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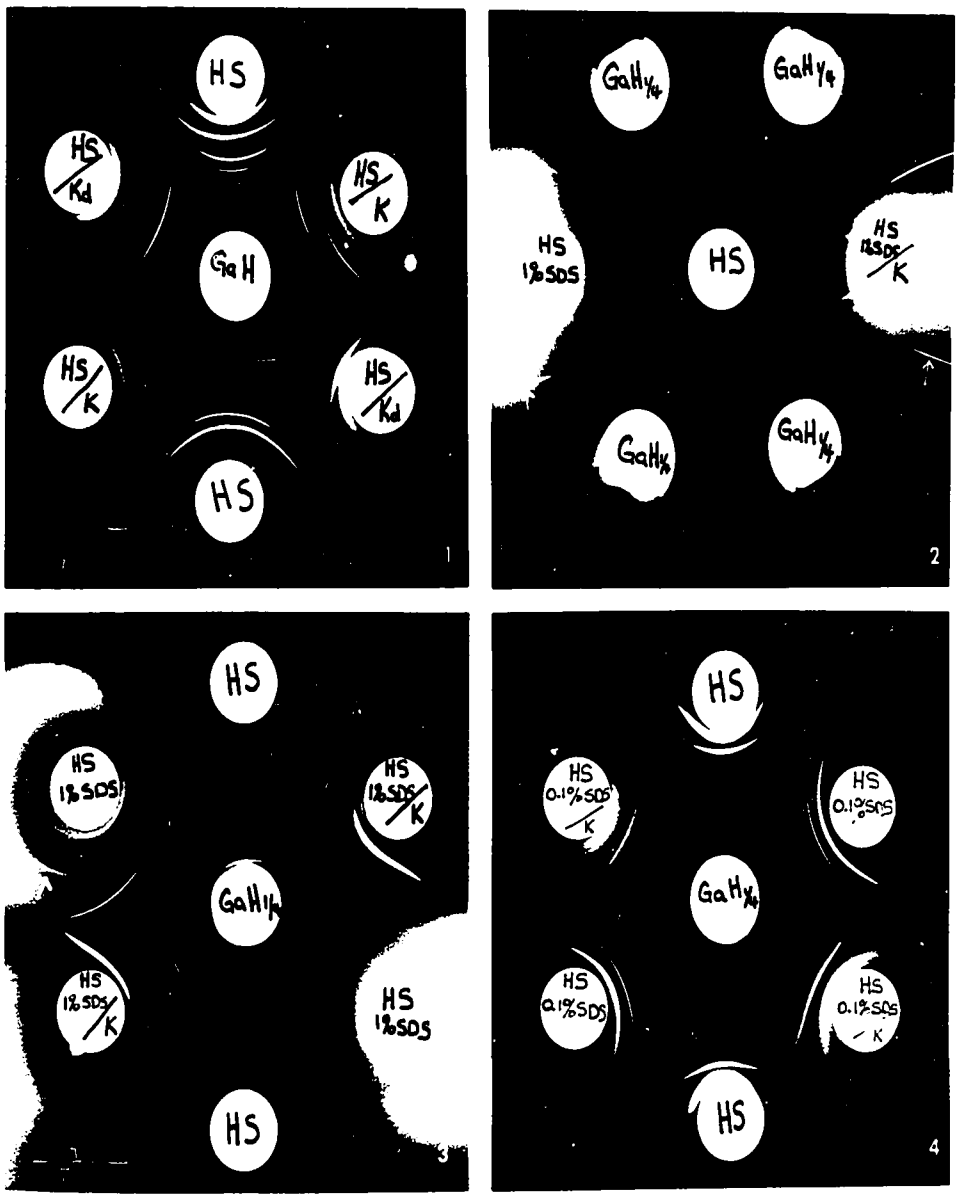
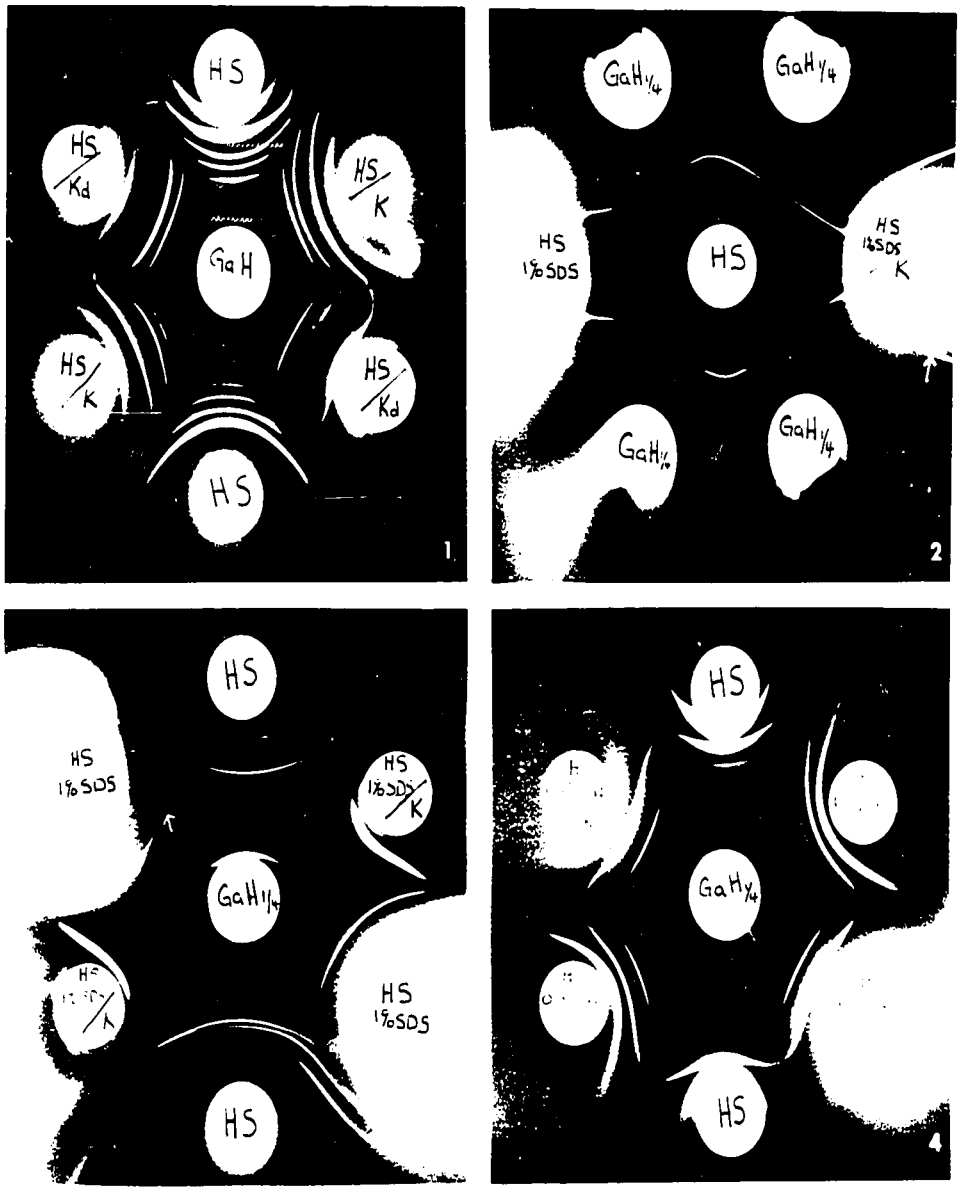



HS
HS/Kd
HS/K
GaH
HS/K
HS/Kd
HS
1
GaH1/4
GaH1/4
HS
1%SDS
HS
HS
1%SDS/K
GaH1/4
GaH1/4
2
HS
HS
1%SDS
HS
1%SDS/K
GaH1/4
HS
1%SDS
HS
HS
GaH1/4
HS
3
4

precipitin lines have developed between HS and $GaH_{\frac{1}{4}}$, only one, possibly two, of these has linked to show a reaction of identity with HS/1% SDS/K. However, although this was a slight improvement compared to HS 1% SDS with which it was impossible to see any linkages because of the haze, in both cases, HS/1% SDS and HS/1% SDS/K, new "lines" have developed. These may have represented uncovered antigens but more probably were SDS precipitin lines.

Figure 3 also shows the results of reactions with HS/1% SDS potassium chloride treated samples. Five precipitin lines have developed between HS and $GaH_{\frac{1}{4}}$, however, unlike the reaction shown in Figure 2, none of these lines showed reactions of identity with either HS/1% SDS or HS/1% SDS/K. In this example a reaction of identity was seen between HS/1% SDS and HS/1% SDS/K with $GaH_{\frac{1}{4}}$. It was interesting to note that a line developed between HS/1% SDS and HS/1% SDS/K (arrowed). This line was attributed to precipitation of SDS from the HS/1% SDS sample caused by residual potassium chloride.

In Figure 4, a reaction with potassium chloride treated HS/0.1% SDS is shown. While at least five precipitin lines have formed between HS and $GaH_{\frac{1}{4}}$ and four, possibly five between HS/0.1% SDS and $GaH_{\frac{1}{4}}$, following potassium chloride treatment of the SDS sample (HS/0.1% SDS/K) only

three have developed. Thus, although potassium chloride treatment removed all the SDS from the 0.1% SDS sample (Table 1), it also decreased the ability of HS/0.1% SDS to participate in the immunodiffusion reaction.

Therefore, while the potassium chloride treatment was capable of reducing the SDS concentration of samples, it also caused some loss in antigens for low (0.1%) SDS containing HS and only slight improvement for high (1%) SDS samples.

d) Conclusions on Removal of SDS

From the experiments carried out to remove SDS, the acetone technique appeared to be the most efficient at removing SDS but also caused almost a total loss in the antigenic character of HS, even non-SDS treated. Cold precipitation, while being the mildest treatment, removed only a limited amount of SDS and did not lead to improved reactions. Potassium chloride treatment, while not as efficient as acetone did remove most of the SDS and did not markedly affect untreated HS; however, little improvement in HS/1% SDS samples was seen.

Thus the answer to the second question posed at the onset of these experiments appears to be no. The removal of SDS by acetone, potassium chloride or cold precipitation treatment could not reverse modifications in the antigen

caused by SDS treatment, especially 1% SDS. In fact, HS appeared to be irreversibly denatured by treatment with 1% SDS.

3.4 Comparison of SDS with other Denaturing Agents

In order to assess the effects of SDS, several experiments were done using Tween 20, a neutral detergent, and DOC, an anionic detergent, to treat HS. The results of immunodiffusion reactions with these samples are seen in Plate 7.

Since SDS had been found to cause nonspecific precipitation of both antiserum, GaH, $GaH_{\frac{1}{4}}$ and antigens, HS, $HS_{\frac{1}{2}}$ (Section 3(a)) several experiments were carried out to see if Tween 20 or DOC could also cause similar results. In Plate 7, Figure 1, no precipitin lines have developed between HS, HS/1% Tween 20 and RS. However, a haze did form about HS/1% DOC samples which gave the appearance of lines between these samples and RS. This haze was attributed to nonspecific precipitation of HS by DOC since dialysis of the HS 1% DOC samples against saline overnight decreased the haze formation but did not obliterate it. (Figure 2). Thus Tween 20 at a concentration of 1% apparently did not cause non-specific precipitation of either reactant whereas DOC at 1% tended to cause slight precipitation of its reaction mixture.

PLATE 7

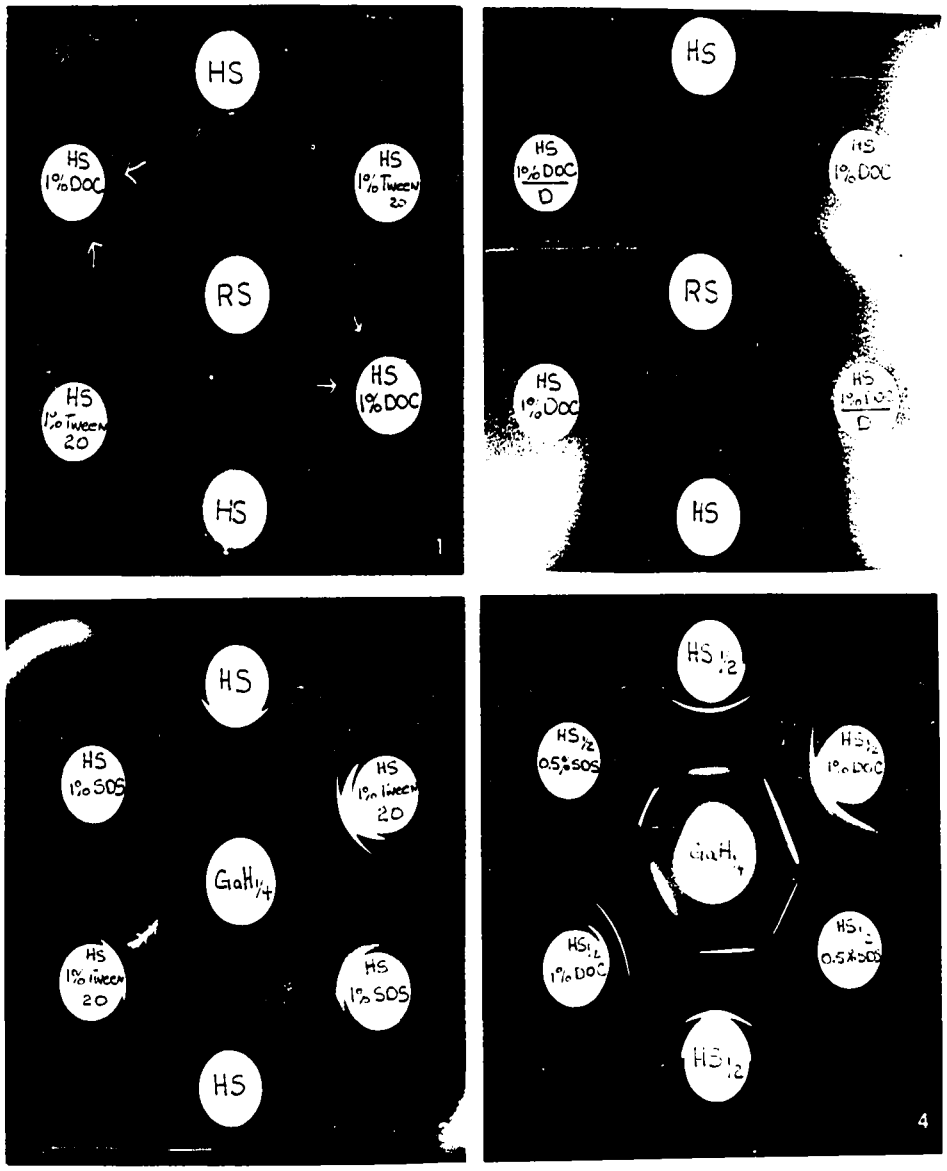
Immunodiffusion Reactions Comparing SDS Treatment of Human Immune Serum Globulins (HS) with 1% Tween 20 and 1% DOC Treatments.

1. The reaction between normal rabbit serum (RS) and HS, HS treated with 1% DOC (HS/1% DOC) and HS treated with 1% Tween 20 (HS/1% Tween 20) is shown. No reaction lines are seen between HS, HS/1% Tween 20 and RS but a haze and faint lines (arrowed) are seen with HS/1% DOC.

2. The reaction between normal rabbit serum (RS) and HS, HS/1% DOC and HS/1% DOC dislysed overnight against saline (HS/1% DOC/d) is shown. The haze about the HS/1% DOC wells is much larger than about the HS/1% DOC/d wells. No reaction is seen with HS alone.

3. This reaction shows the effect of 1% Tween 20 treatment of HS (HS/1% Tween 20) as compared to the effect of 1% SDS treatment (HS/1% SDS) or no treatment (HS) with the antiserum $GaH_{\frac{1}{4}}$. While only three lines have formed with HS/1% SDS, at least four have developed with HS and HS/1% Tween 20.

4. This reaction shows the effect of 1% DOC treatment of $HS_{\frac{1}{2}}$ ($HS_{\frac{1}{2}}$/1% DOC) as compared to the effect of 0.5% SDS treatment ($HS_{\frac{1}{2}}$/0.5% SDS) or no treatment ($HS_{\frac{1}{2}}$) with the antiserum $GaH_{\frac{1}{4}}$. Six lines are seen with $HS_{\frac{1}{2}}$, two or three with $HS_{\frac{1}{2}}$/0.5% SDS and four with $HS_{\frac{1}{2}}$/1% DOC.


HS
HS 1% DOC
HS 1% Tween 20
RS
HS 1% DOC
HS 1% Tween 20
HS
1
HS
HS 1% DOC D
HS 1% DOC
RS
HS 1% DOC
HS
HS
HS 1% SDS
HS 1% Tween 20
GaH1/4
HS 1% Tween 20
HS 1% SDS
HS
HS1/2
HS1/2 0.5% SDS
HS1/2 1% DOC
GaH1/4
HS1/2 1% DOC
HS1/2 0.5% SDS
HS1/2
4

1
4

In Figure 3, the reaction showing the effects of 0.5% SDS on $HS_{\frac{1}{2}}$ and 1% DOC on $HS_{\frac{1}{2}}$ are seen. Six lines have developed between the control wells of $HS_{\frac{1}{2}}$ and $GaH_{\frac{1}{4}}$ while only two or possibly three developed between $HS_{\frac{1}{2}}$/0.5% SDS and $GaH_{\frac{1}{4}}$. In contrast, at least four lines have formed between $HS_{\frac{1}{2}}$/1% DOC and $GaH_{\frac{1}{4}}$. Thus DOC at 1% appeared to cause far less damage to the antigens of HS than SDS at 0.5% did; however, treatment with 1% DOC did result in some loss.

The effects of 1% SDS on HS and 1% Tween 20 on HS are seen in the reaction shown in Figure 4. Only two lines developed with HS/1% SDS while at least four have formed with HS/1% Tween 20 and HS untreated with $GaH_{\frac{1}{4}}$. Thus treatment with Tween 20 at 1% seemed to result in little if any loss in antigens.

In conclusion, SDS treatment resulted in the greatest loss of antigenic properties of HS as compared to DOC and Tween 20, DOC, caused slight loss, while Tween 20 treatment seemed to cause minimal if any change.

3.5 The IgG System

The second system chosen to determine the effect of SDS was the reactions between human IgG and the goat antisera to human IgG (GaHγG), to Fab fragment (GaHFab) and to human γ chain (GaγH). This system was chosen because IgG has been well characterized. Due to the

limited quantity available however only a few experiments could be carried out.

The normal reaction between IgG and the antisera GaHγG, GaγH and GaHFab and 1:2 dilutions of each is shown in Plate 8 Figure 1. Although no lines developed between the antigen IgG and antisera GaHγG or GaHγG$_{\frac{1}{2}}$, at least four lines developed between IgG and GaHFab and GaHFab$_{\frac{1}{2}}$. Two of these appeared to link to form reactions of identity with the two lines developed between IgG and GaγH and GaγH$_{\frac{1}{2}}$. Although the reason behind the lack of precipitin reaction between IgG and GaHγG is unknown, it is suggested that since GaHγG was heavy and light chain specific and since light chains in some IgG molecules may be masked (Weir, 1967), the concentrations of these reactants may have been in the wrong proportions.

In Plate 8, Figure 2, the reaction between 1% SDS treated IgG and the antisera GaHγG, GaHFab and GaγH is shown. One linked precipitin line has formed between IgG/1% SDS and GaHFab and GaγH. At least two lines have developed between IgG 1% SDS and GaHγG. These two lines have linked to form reactions of identity with two other lines developed with GaγH, GaHFab. Whether these two lines represented two specific antigenic components revealed by SDS treatment or SDS precipitin lines was uncertain. However, due to the fuzziness of these two lines

PLATE 8

Immunodiffusion Reactions showing the Effects of SDS Treatment of Human IgG and Human Bence Jones Protein.

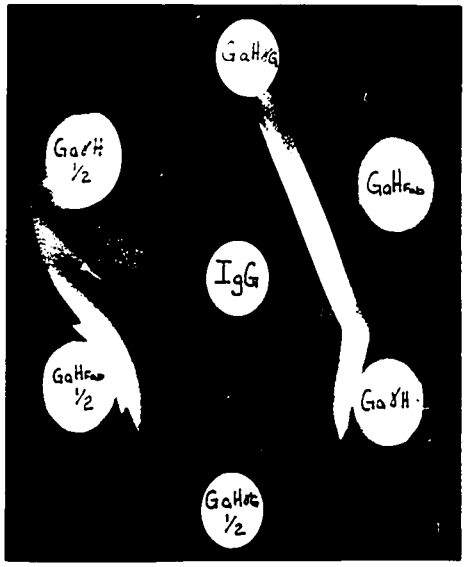
1. The reaction between IgG and the antisera Goat Antihuman Gamma G Globulin (GaHγG), Goat Antihuman Fab Fragment (GaHFab) and Goat Antihuman Gamma Chain (Gaγ H) is shown. Although no reaction is seen with GaHγG or GaHγ$G_{\frac{1}{2}}$, four lines have formed with GaHFab, GaHFab$_{\frac{1}{2}}$, two of which form reactions of identity with the two lines developed with Gaγ H, Gaγ$H_{\frac{1}{2}}$.

2. The effect of treatment with 1% SDS on IgG is shown. The antisera are GaHγG, GaHFab and Gaγ H. Three lines are seen between IgG/1% SDS and GaHFab, Gaγ H. Two of these link to form reactions of identity with the two lines formed with GaHγG. (arrowed).

3. The reaction between the pellet from cold precipitation of IgG 1% SDS (ie Pellet IgG/1% SDS/P) and the antisera GaHFab, Gaγ H and GaHγG is shown. A fuzzy line is seen with GaHγG while a wide hazy band is formed between GaHFab, Gaγ H and the pellet.

4. The reaction between IgG/1% SDS treated with KCL and GaHFab, Gaγ H and GaHγG is shown. No lines are seen with GaHγG but three are seen with Gaγ H and two with GaHFab one of which formed a reaction of identity with a line formed with Gaγ H.

5. A reaction comparing the treatments of 0.1% SDS and 1% DOC on Bence Jones Protein Kappa chain specific (BJ) with native BJ using the antiserum,Goat Antihuman free K L-chain (GaHBJ) is shown. No reaction is seen with BJ but two or three lines are seen with both BJ/1% DOC, BJ/0.1% SDS (arrowed).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GaHFab
IgG
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Ga&H
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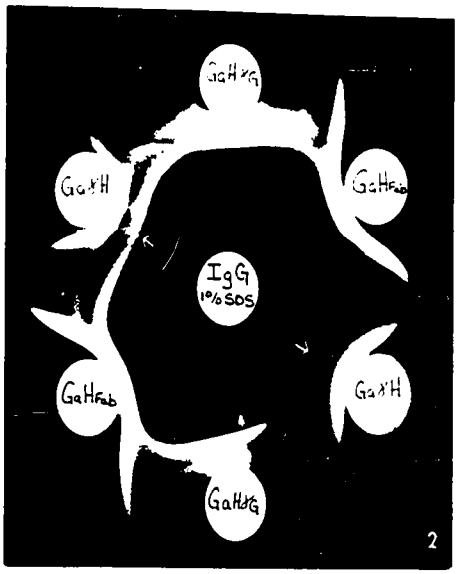
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GaH&G
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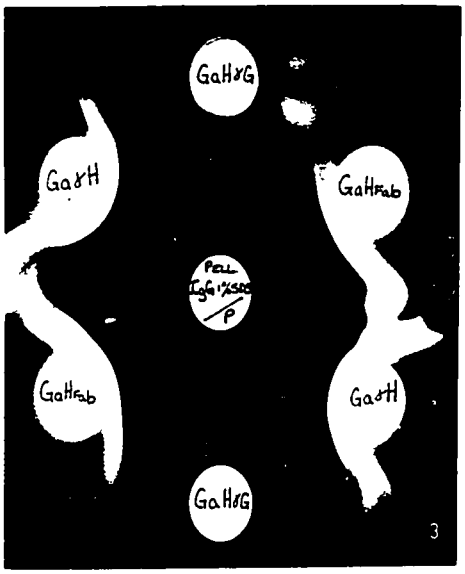
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GaHFab
Pell IgG 1% SDS / P
GaHFab
Ga&H
GaH&G
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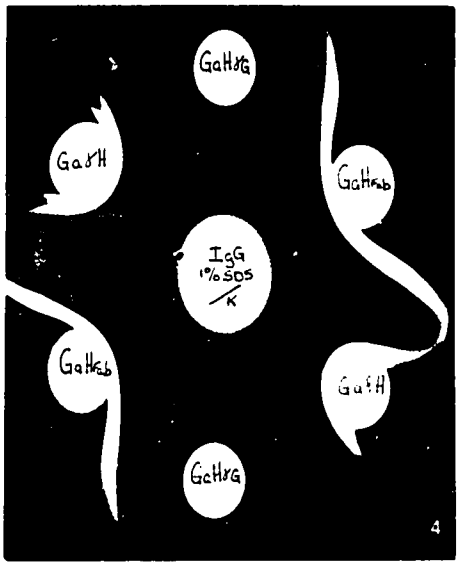
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Ga&H
GaHFab
IgG 1% SDS / K
GaHFab
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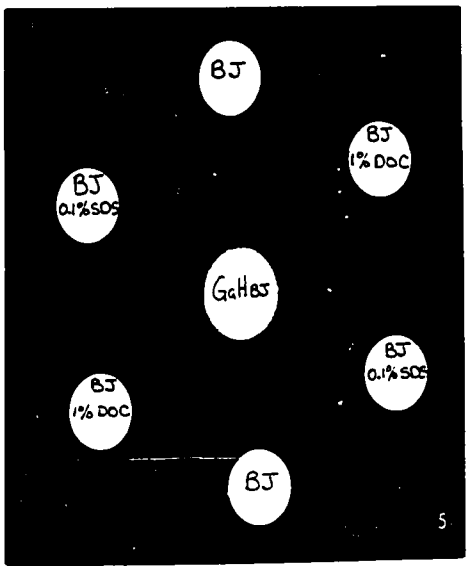
GaH&G
4


BJ
BJ 1% DOC
BJ 0.1% SDS
GaHBJ
BJ 0.1% SDS
BJ 1% DOC
BJ
5

1
2
3
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5

they were attributed to nonspecific SDS precipitation.

The immunodiffusion reaction with a pellet from cold precipitation of IgG/1% SDS is shown in Figure 3. The pellet had been allowed to warm to room temperature before testing, following preparation by the cold precipitation method. A wide hazy band formed between the "pellet" and GaŸH and GaHFab, however, this band did not sweep around to include GaHŸG. A less intense hazy band formed between GaHŸG and the "pellet". This latter band was probably an SDS precipitin line. In contrast, since the wide hazy band did not bend near GaHŸG, it may have been due both to nonspecific SDS precipitation and also to specific antibody-antigen precipitation. Thus, the pellet appeared to contain not only SDS but also some IgG. This loss of antigen by the cold precipitation process could be very important if only small amounts of component were present in the test solutions.

In Figure 4, the reaction between potassium chloride treated IgG/1% SDS and GaHŸG, GaŸH and GaHFab is shown. At least three lines appeared to have formed between IgG/1% SDS/K and GaŸH although none have formed with GaHŸG. At least two, although one was not well resolved, have developed with GaHFab, one of which linked to form a reaction of identity with GaŸH. The actual identity of these lines could not be ascertained from this reaction although it appeared that potassium chloride

treatment could definitely remove some SDS since the fuzzy SDS precipitin lines seen with IgG/1% SDS in Figure 2 did not developed in this reaction.

3.6 The Bence-Jones Protein System

Another system which was briefly examined (again due to limited reagents) was the reaction between Bence Jones Protein (BJ), kappa chain specific and goat anti-human free kappa L chain (GaHBJ). The reactions between GaHBJ and the antigen solutions of BJ, BJ/0.1% SDS and BJ/1% DOC are seen in Plate 8, Figure 5. No lines formed between BJ and GaHBJ. This may have been due to the limited protein concentration of the BJ solution (5 mg/ml). In contrast, two possibly three lines have developed with both BJ/0.1% SDS and BJ/1% DOC. These lines did not link and were close to the antigen wells. Since both SDS and DOC, but to a lesser extent, have been shown to cause HS, a serum preparation, to precipitate nonspecifically these lines may have represented nonspecific precipitation of the serum like proteins in BJ. Analysis of this reaction clearly illustrates the difficulty of identifying specific and nonspecific precipitin lines in weak antigen-antibody systems.

3.7 Conclusions about the Effects of SDS on Protein

SDS, whether alone or with antigen, at concentrations of 0.1% or greater, reacted with serum in cellulose acetate immunodiffusion to form SDS precipitin lines which were artifiacts and did not represent true antigen-antibody precipitin lines. SDS at concentrations as low as 0.1% also reacted with antigen to cause nonspecific precipitation.

In specific antigen-antibody reactions, SDS at 0.1% caused less change in antigenic activity than at 1%, at which concentration SDS appeared to destroy nearly all antigenic properties. The presence of 0.1% βmercaptoethanol appeared to have no effect.

None of the removal procedures, acetone, potassium chloride or cold precipitation, were capable of reversing the modifications in the antigen caused by SDS treatment, and acetone treatment appeared to cause further modifications to the antigen, even untreated antigen.

When compared with the two other detergents, 1% Tween 20 and 1% DOC, SDS at the same concentration was far more destructive. However, SDS at 0.1% was a more useful agent than the more commonly used 1% because fewer antigenic properties were lost. But even at this concentration, interpretation of the results was hampered because of the ability of SDS to cause nonspecific

precipitation of both the antiserum and the antigens.

4. Influenza Virus A/PR8

With the theory of SDS action postulated by Tanford and his associates and the results of the experiments with HS, IgG and BJ in mind, an investigation of the effect of SDS on antigenic components obtained by disruption of influenza A/PR8 by SDS was carried out.

Two virus preparations were used: a one in ten dilution of concentrated virus pool ($C_3$1/10) and a more purified virus preparation (SDG/C_3). Before treatment, virus infectivity, hemagglutination activity and protein content were determined. These values are presented in Table 2.

The reactions between untreated $C_3$1/10 and SDG/C_3 and rabbit antiserum to influenza virus concentrate (RaS/C_2) are shown in Plate 9. In Figure 1, three precipitin lines have developed between $C_3$1/10 and both RaS/C_2 and RaS/$C_2$1/2. However, in Figure 2, only one possibly two precipitin line have developed between SDG/C_3 and RaSC_2. Since the protein content of these two antigens was almost the same ($C_3$1/10:0.77mg/ml; SDG/C_3:0.66mg/ml) a comparison between these two was valid.

In Plate 10, the ability of SDS to release antigenic components as tested by immunodiffusion is shown.

TABLE 2. Characterization of Influenza A/PR8 Virus Preparations, C_3 and SDG/C_3, According to Infectivity, Hemagglutinin Activity and Protein Content.

Virus Preparation	Infectivity EID_{50}/0.1 ml**	Hemagglutinin Activity* HAU/ml	Protein Content mg./ml.	Ratio $\frac{\text{mg protein}}{10^5 \text{ HAU}}$
C_3	$10^{10.75}$	20,000	7.1	7.1
SDG/C_3	$10^{8.75}$	3,200	0.66	3.88

* Reciprocal of 50% End Point.

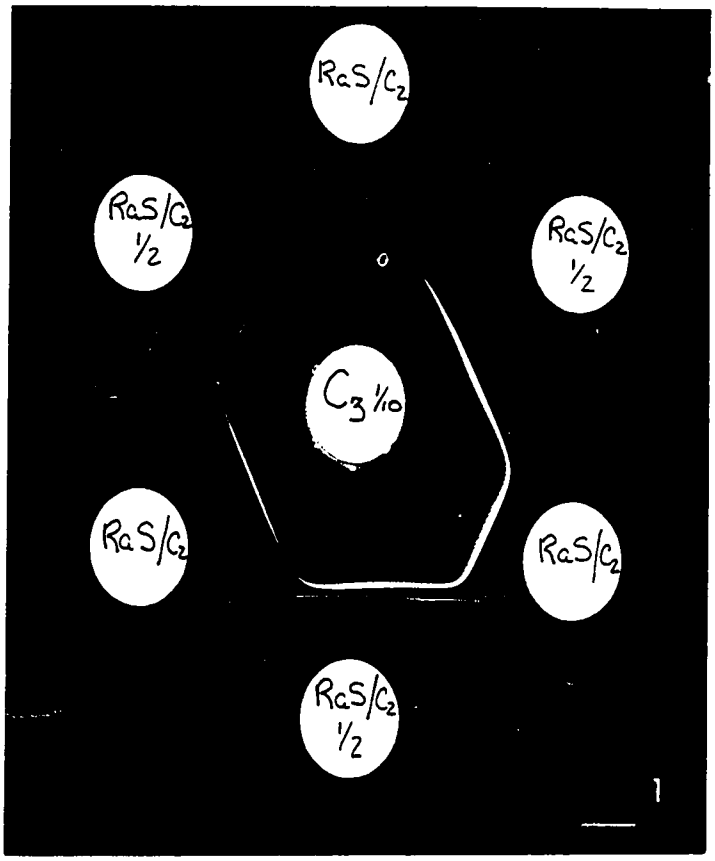
** Kärber,s method for EID_{50} calculation.

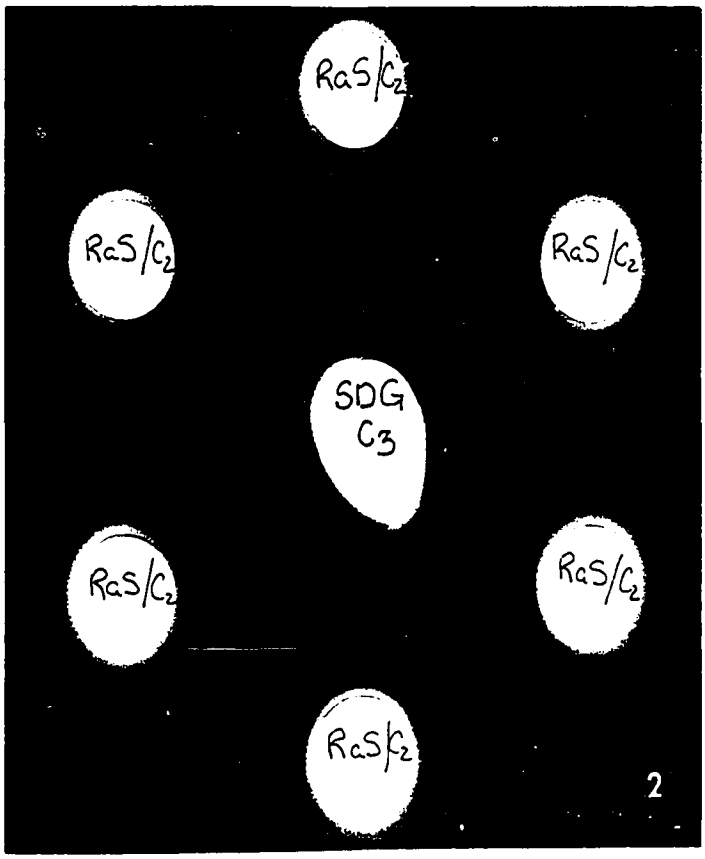
PLATE 9

Immunodiffusion Reactions Demonstrating the Detection of Antigens of Influenza A/PR8 Viral $C_3$1/10 and SDG/C_3 Preparations with Rabbit Anti Influenza Virus Concentrate (RaS/C_2)

1. The reaction between influenza virus A/PR8 $C_3$1/10 preparation and RaS/C_2 and RaS/C_2½ is shown. Three precipitin lines have formed.

2. The reaction between influenza virus A/PR8 SDG/C_3 preparation and RaS/C_2 is shown. Only one or possibly two lines have formed.


RaS/C2
RaS/C2 1/2
RaS/C2 1/2
C3 1/10
RaS/C2
RaS/C2
RaS/C2 1/2
1


RaS/C2
RaS/C2
RaS/C2
SDG C3
RaS/C2
RaS/C2
RaS/C2
2

1

PLATE 10

Immunodiffusion Reactions showing the Effect of SDS Treatment on SDG/C_3 Influenza A/PR8 Virus Preparations with Rabbit Anti Influenza Virus Concentrate (RaS/C_2).

1. The reaction between SDG/C_3 1% SDS and RaS/C_2 is shown. Five or six lines have developed but one is rather wide and fuzzy (arrowed).

2. The reaction between SDG/C_3 0.1% SDS and RaS/C_2 is shown. Five lines have formed.

3. A reaction to compare the SDS treatments of SDG/C_3 (ie SDG/C_3 1% SDS and SDG/C_3 0.1% SDS) with untreated virus is shown. The antiserum is RaS/C_2. Two lines are seen with SDG/C_3 untreated. Four or five are seen with SDG/C_3 0.1% SDS, one of which links to form a reaction of identity (arrowed) with a line developed between untreated SDG/C_3 and RaS/C_2. Only two or three lines are seen with SDG/C_3 1% SDS, one of which (closest to the RaS/C_2 well) is fuzzy.

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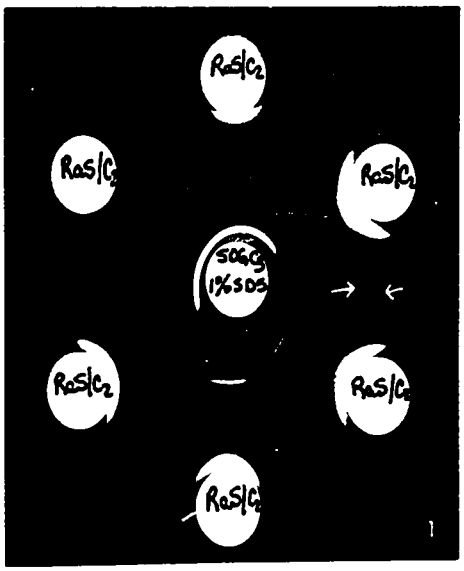
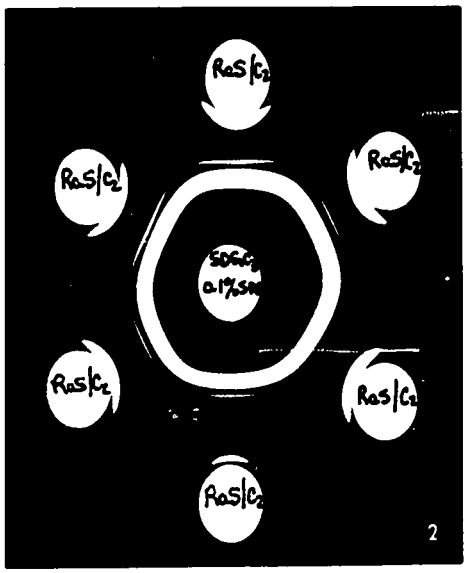
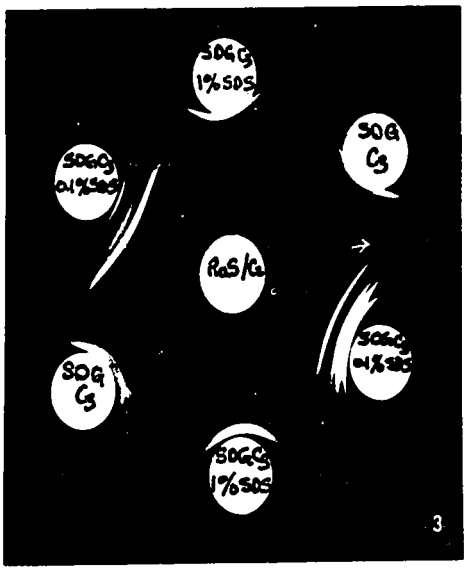
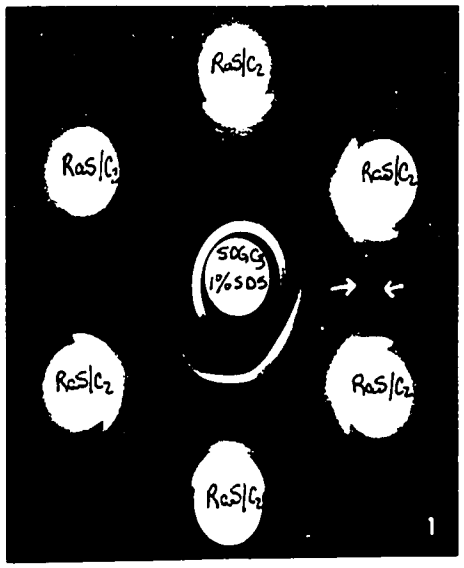
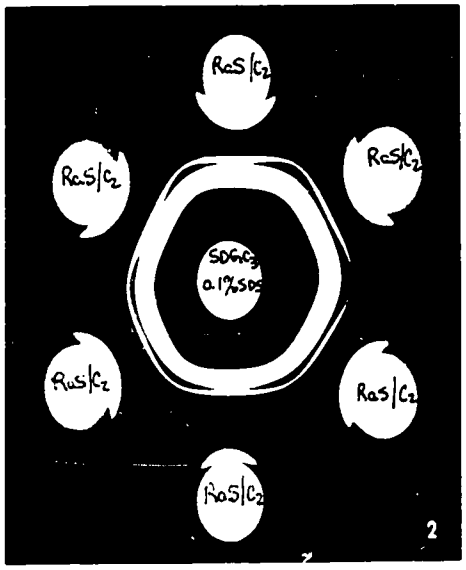
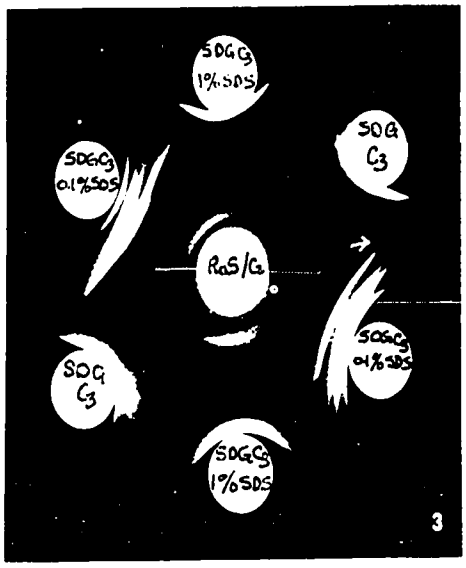







RaS/C2
RaS/C2
RaS/C2
SDGC3
1%SDS
RaS/C2
RaS/C2
RaS/C2
1


RaS/C2
RaS/C2
RaS/C2
SDGC3
0.1%SDS
RaS/C2
RaS/C2
RaS/C2
2


SDGC3
1%SDS
SDGC3
0.1%SDS
SDG
C3
RaS/C2
SDGC3
0.1%SDS
SDG
C3
SDGC3
1%SDS
3

With 1% SDS, five possibly six precipitin lines have developed between SDG/C_3 1% SDS and RaS/C_2 (Figure 1). However, one of these lines was rather wide and fuzzy and probably represented a SDS precipitin line (arrowed). Thus possibly five but more probably four antigenic components have been released by 1% SDS treatment. However, since true antibody-antigen reactions could not be distinguished from SDS precipitin lines in this pattern, none of these lines can be positively identified as released antigenic components. Similarly, in Figure 2, five precipitin lines were seen between SDG/C_3 0.1% SDS and RaS/C_2. This treatment may have resulted in the release of five detectable antigenic components but due to the presence of SDS in the sample and thus the possibility of SDS precipitin line formation, the number of released antigenic components could not be ascertained.

A reaction to compare these two SDS-containing virus preparations (1% SDS and 0.1% SDS) with untreated virus is shown in Figure 3. The untreated control, SDG/C_3, has reacted with RaS/C_2 to form two precipitin lines. Four, possibly five precipitin lines have developed between SDG/C_3 0.1% SDS and RaS/C_2, one of which linked in a reaction of indentity with a precipitin line formed between untreated SDG/C_3 and RaS/C_2 (arrow). In contrast, only two, possibly three precipitin lines

have developed between SDG/C_3 1% SDS and RaS/C_2 and none of these appeared to form a reaction of identity with lines from SDG/C_3. One of the precipitin lines formed between SDG/C_3 1% SDS and RaS/C_2, the one closest to the RaS/C_2 well, appeared to be fuzzy and most probably was a SDS precipitin line.

Therefore, on the basis of these reactions with SDS-treated virus, it would appear that there is a marked difference between the effect of 1% and of 0.1% SDS with respect to both the number and the identity of the antigens released from the virion. After 1% SDS treatment, as shown in Figure 3, only two or three lines formed while following 0.1% SDS treatment at least four developed. However, only one of the lines from the 0.1% SDS sample showed a reaction of identity with untreated virus. Some or all of the other lines may have been SDS precipitin lines.

The results of these experiments were confirmed by repeated tests using $C_3$1/10 preparations with SDS.

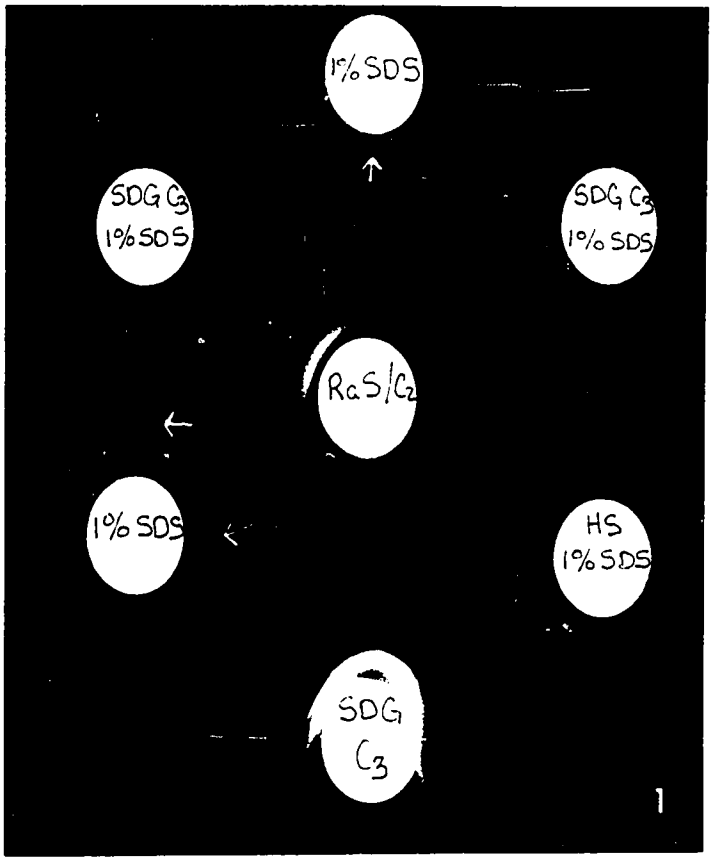
To aid in the interpretation of the results of reactions involving SDS as seen in Plate 11, several experiments involving SDG/C_3, SDG/C_3 1% SDS and RaS/C_2 were carried out. As shown in Plate 11 Figure 1, two precipitin lines developed between SDG/C_3 and RaS/C_2. Three lines have formed between RaS/C_2 and SDG/C_3 1% SDS,

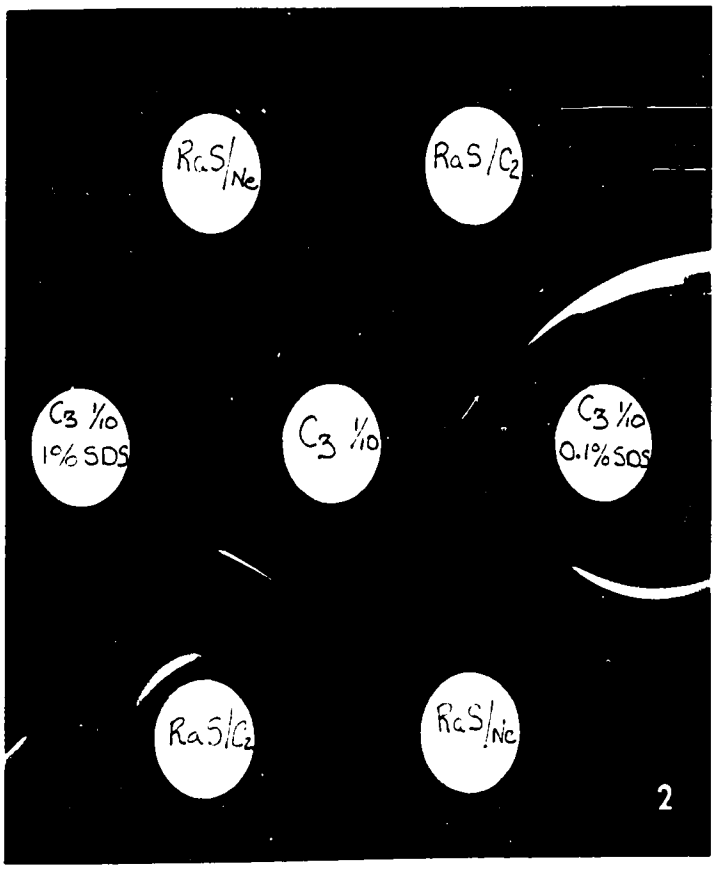
PLATE 11

Immunodiffusion Reactions Demonstrating the Possible Presence of Nonspecific SDS Precipitin lines with SDS Treated Influenza Virus Preparations.

1. The reaction comparing 1% SDS in saline, with HS and with SDG/C_3 (ie SDG/C_3 1% SDS) with untreated virus, SDG/C_3 is shown. The antiserum in RaS/C_2. Two lines have developed between SDG/C_3 and RaS/C_2. Three lines have formed with SDG/C_3 1% SDS, however, two of these link to form reactions of identity with lines formed with 1% SDS. A line is also seen with HS/1% SDS.

2. The reaction comparing 0.1% SDS and 1% SDS treatment of $C_3$1/10 virus preparations with native $C_3$1/10 is shown. The antisera are RaS/C_2 and rabbit antinormal egg soluble Antigen (RaS/Ne). Only one line is seen between $C_3$1/10 and RaS/C_2 whereas five have formed between $C_3$1/10 0.1% SDS and RaS/C_2. One of these partially links to form a reaction of partial identity with $C_3$1/10 (arrowed). With $C_3$1/10 1% SDS, three or four lines are seen. There is a line only for $C_3$1/10 1% SDS and $C_3$1/10 0.1% SDS with RaS/Ne.


1% SDS
SDG C3 1% SDS
SDG C3 1% SDS
RaS/C2
1% SDS
HS 1% SDS
SDG C3
1


RaS/Ne
RaS/C2
C3 1/10 1% SDS
C3 1/10
C3 1/10 0.1% SDS
RaS/C2
RaS/Nc
2

1

2

however, two of these linked to form reactions of identity with the SDS precipitin lines formed between 1% SDS and RaS/C_2. Therefore, these two lines of SDG/C_3 1% SDS were probably artifacts and did not represent released viral components.

The results of reactions between the antigens $C_3$1/10 1% SDS, $C_3$1/10 and $C_3$1/10 0.1% SDS and the antisera RaS/C_2 and RaS/Ne (rabbit anti normal egg soluble antigen) are shown in Plate 11 Figure 2. One precipitin line has developed between the $C_3$1/10 well and antiserum RaS/C_2 whereas, five lines have formed between $C_3$1/10 0.1% SDS and RaS/C_2. There appeared to be a reaction of partial identity between one of the five $C_3$1/10 0.1% SDS components and the control $C_3$1/10 reaction (arrowed); however, no reaction of identity was demonstrated with the 1% SDS components. With $C_3$1/10 1% SDS, three possibly four lines formed with RaS/C_2. The presence of host antigens, detectable by RaS/Ne, was only demonstrated in the SDS-treated preparations. The identity of these lines was uncertain since, even at 0.1%, SDS can cause nonspecific precipitin line formation.

In only one reaction (Plate 10, Figure 3) of all the reactions in Plates 10 and 11, could a precipitin line be definitly attributed to the release of an antigenic component by SDS treatment (0.1%) of influenza A/PR8. All the other lines may have been artifacts.

Since SDS precipitin lines may be either sharp or fuzzy, even the very fine lines that developed could have been artifacts. However, excluding the obvious SDS precipitin lines, the fuzzy ones and the ones that showed identity with known SDS precipitin lines, there appeared to be four, possibly five, antigenic components released by 0.1% SDS treatment of the virus detectable by RaS/C_2 and one detectable by RaS/Ne. In contrast, only one viral component was detected after treatment by 1% SDS. Presumably, the other components must have been lost or destroyed by the SDS treatment.

To complete the evaluation of SDS treatment of influenza virus, a series of experiments were carried out comparing the effects of the disrupting agents Tween 20 and DOC with SDS.

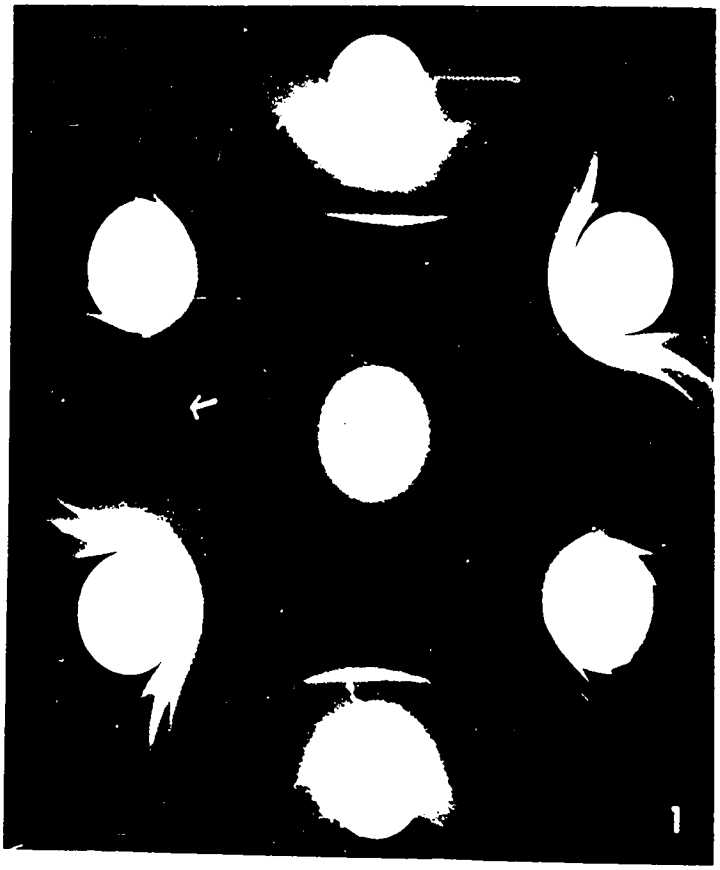
Plate 12 Figure 1 shows the result of an immunodiffusion reaction with 1% DOC treated $C_3$1/10 and 1% Tween 20 treated $C_3$1/10 with RaS/C_2. Three, possibly four lines have developed between the control $C_3$1/10 and RaS/C_2. Two and probably three, of these lines linked to show reactions of identity with two or three of the five lines formed between $C_3$1/10 1% DOC and RaS/C_2. One of the lines formed with the control reactants also linked to show identity with one of the four lines

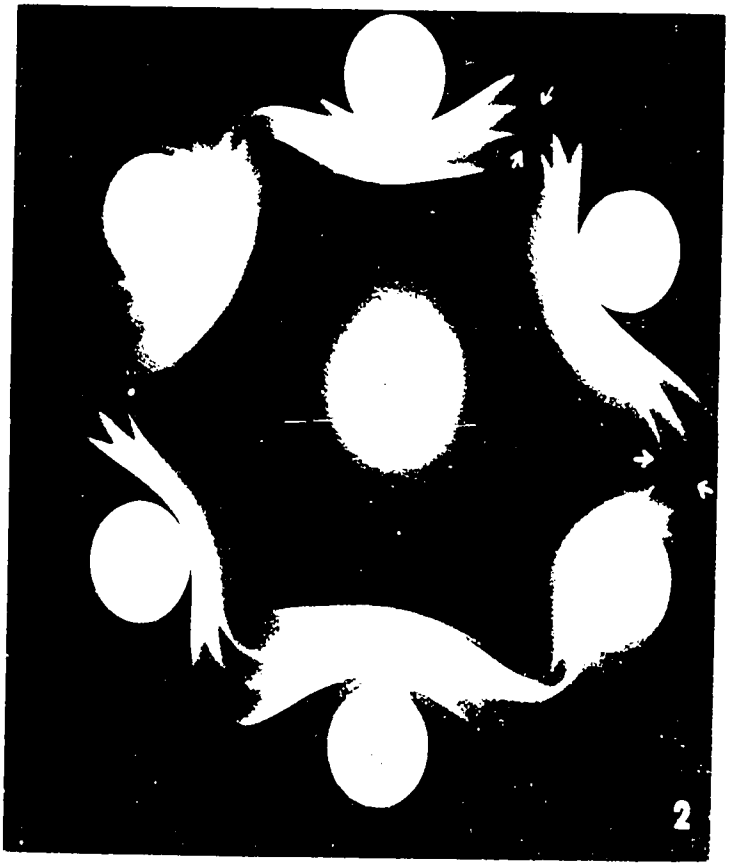
PLATE 12

Immunodiffusion Reactions Comparing 0.1% SDS Treatment of Influenza A/PR8 with Treatment with either 1% Tween 20 or 1% DOC using the Antiserum RaS/C_2.

1. This reaction shows a comparison of 1% Tween 20 and 1% DOC treatment of $C_3$1/10 virus preparations with untreated $C_3$1/10 virus. The antiserum is RaS/C_2. Three or four lines are seen with the control, $C_3$1/10. Two or probably three of these show reactions of identity with two or three of the five lines formed with $C_3$1/10 1% DOC. One of the control lines also showed reaction of identity with one of the four lines developed with $C_3$1/10 1% Tween 20. The DOC and Tween 20 preparations also share a line (arrowed), a reaction of identity.

2. This reaction shows a comparison of 1% Tween 20 and 1% DOC treatments of SDG/C_3 with SDG/C_3 0.1% SDS. The antiserum is RaS/C_2. Four lines have developed with SDG/C_3 0.1% SDS, four or five with SDG/C_3 1% DOC and three or four with SDG/C_3 1% Tween 20. Two lines formed with the DOC preparations show reactions of identity (arrowed) with lines from SDG/C_3 1% Tween 20 and SDG/C_3 0.1% SDS.


1


2

developed with 1% Tween 20 treated virus. The DOC and Tween 20 preparations also appeared to share a line (arrowed), a reaction of identity, which the control did not. Thus treatment with 1% DOC or 1% Tween 20 appeared to release five and four antigenic components respectively.

Plate 12 Figure 2 shows the results of a comparison between SDG/C_3 treated with either 0.1% SDS, 1% DOC or 1% Tween 20. In reaction against RaS/C_2, four lines have developed with SDG/C_3 0.1% SDS, four or five with SDG/C_3 1% DOC and three or four with SDG/C_3 1% Tween 20. Two precipitin lines formed with DOC-treated virus linked to form reactions of identity with two from 0.1% SDS and two from 1% Tween 20 (arrowed) virus samples. Since these two lines were shared by all three preparations and 1% Tween 20 did not seem to cause any type of artifact, at least two viral antigenic components must have been detected after breatment of influenza A/PR8 with 0.1% SDS.

In conclusion then, due to the ability of SDS to cause precipitation artifacts even at the 0.1% level, interpretation of reactions with SDS-disrupted influenza virus A/PR8 was difficult. If SDS alone was tested against SDS virus samples some of the SDS precipitin lines could be identified by reactions of identity. Similarly some

of the true antigen-antibody precipitation reactions could be identified by the developing reactions of identity with untreated virus preparations. However, this latter procedure was not entirely satisfactory since antigenic components may be hidden in the intact virus and therefore would not be available for the formation of reactions of identity. Nevertheless, using these two procedures and also excluding fuzzy lines which were probably SDS precipitin lines, treatment with 0.1% SDS was found to be superior to treatment with 1% SDS since four or five antigenic components not just one were detected by RaS/C_2.

When compared with 1% Tween 20 disrupted virus and 1% DOC disrupted virus, 0.1% SDS disrupted virus appeared to share two or possibly more antigenic components detectable by RaS/C_2. Treatment with either 0.1% SDS or 1% DOC appeared to cause the release of four or five RaS/C_2 detectable components while 1% Tween 20 treatment appeared to release only three or four.

In summary, SDS at 0.1% might be considered a useful agent for disrupting influenza virus A/PR8, causing the release of four or five RaS/C_2 detectable antigenic components. However, interpretation of these immunodiffusion reactions where the reactants have been exposed to SDS must be done with extreme care since SDS even at 0.1% can cause nonspecific precipitation.

DISCUSSION AND CONCLUSIONS

VI - DISCUSSION AND CONCLUSIONS

The purpose of this work was to evaluate the use of SDS in identifying the antigenic components of influenza A/PR8. The basis for this evaluation was to be the effects of SDS on known antigens as measured by their ability to participate in immunodiffusion reactions.

Before investigating the effects of SDS on several known antigens, two methods for assaying SDS content of solutions were standardized; the methylene blue - chloroform technique and the hemolysis technique. Both methods proved to be useful for assaying SDS although the hemolysis technique was simpler and easier to use.

In the investigations of the known systems, SDS was found to be more useful at 0.1% than at the more commonly used 1% since fewer antigenic components were lost. The number and quality of components detectable following 0.1% SDS treatment compared favorably with those detectable following 1% Tween 20 and 1% DOC treatments. However, SDS alone or in the presence of antigen, at concentrations as low as 0.1%, was found to cause nonspecific precipitation of antiserum resulting

in the formation of SDS precipitin lines which closely resembled antigen-antibody specific precipitin lines. SDS also appeared to cause some precipitation of the antigens tested. This nonspecific precipitation made analysis of immunodiffusion reactions more difficult. This problem was not found with 1% Tween 20 treatment. Treatment with 1% DOC however, did appear to cause slight precipitation of its reacting mixture. Removal of SDS from 1% treated samples by any of the standard procedures could not restore their original antigenic properties.

With this background, experiments with SDS disrupted influenza A/PR8 were carried out. When the virus was treated with 0.1% SDS, four or five antigenic components were detected by RaS/C_2; however, if 1% SDS was used only one component was detected. But, both numbers of components can only be an estimate since some of the lines may have resulted from nonspecific SDS precipitation. This problem was partially controlled by showing reactions of identity with lines developed with untreated virus or with SDS alone.

The use of 0.1% SDS for disruption of influenza A/PR8 compared favorably with Tween 20 at 1% since three or four components detected by RaS/C_2, were released by this treatment. 1% DOC treatment of virus appeared to release at least five detectable components.

After treatment with SDS, one host component could be detected by RaS/Ne; however, it was impossible to definitely demonstrate that the precipitin line which had formed was not an SDS precipitin line.

With the theory proposed by Tanford and his associates and these observations in mind a re-examination of the work with SDS disrupted influenza virus may explain some of the inconsistencies in the results of different workers. For example the variation in the molecular weight estimates for hemagglutinin (47,000 to 77,000) may be not only a result of the techniques used, (density gradient and electrophoretic) but also partly due to the binding of different amounts of SDS caused by the different methods of preparation. Similarly, immunodiffusion reactions with SDS-disrupted virus may have been erroneously interpreted due to the ability of SDS to cause nonspecific precipitation. Therefore, when SDS is used to disrupt virus, the concentration of SDS present in the viral solution must be determined. The casual method of adding SDS until the opalescence has disappeared is not a well controlled procedure since the addition of 0.25 ml of either 10% or 1% SDS to 2.25 ml of virus (0.77 mg protein/ml) caused the preparations to instantly clear yet the effect on antigenic properties by these two concentrations of SDS was drastically different.

It is suggested that more studies should be carried out on other antigen-antibody systems. The work presented here has been confined to a study of the effects of SDS on some serum components and no attempt has been made to determine whether only the protein in the samples are affected. More detailed studies using simple haptens should help to provide some of these answers. Furthermore since carbohydrate and possibly lipid moieties also participate in immune reactions, an investigation of the effect of SDS on these with their corresponding antisera should be undertaken.

Another aspect for future study should be concerned with testing for changes in the antigenicity of specific antigens following SDS treatment, especially in view of the fact that SDS might be used to disrupt virus into components for further isolation and immunization. This is particularly important since much current research is directed at the production of influenza subunit vaccines. (Brandon et al, 1969 and Warburton, 1969).

Similarly, work with enzymes and their corresponding antibodies would be particularly interesting since not only antigenic but catalytic properties could also be assayed following SDS treatment. Results from such experiments might shed light on the unpredictability of isolating fully active neuraminidase from influenza virus by SDS treatment.

It follows therefore, that similar studies should be carried out with the other detergents in common use; ie TritonX-100, Nonidet·P·40, the Tweens and DOC. Already, from experiments with DOC-disrupted influenza virus, Styk et al (1970) have suggested that the concentration used for disruption may be very important in determining the number of components detected by immunodiffusion. On the basis of our preliminary experiments, it was suggested that DOC did cause slight precipitation of the reacting mixture. Thus the use of DOC may also result in difficulties in the analysis of immunodiffusion reactions, However, Crumpton (1971) has concluded that antigen-antibody interaction is not grossly affected by 1% concentrations of DOC. Since the immunological reactivity of globular proteins is determined by the conformation of the surface molecules, Crumpton suggests that 1% DOC does not bind to the surface or cause gross conformational changes. Thus the mechanism of DOC protein interaction appears to be quite different from that postulated for SDS. Therefore, correlation of data from experiments using DOC-disrupted virus with that of SDS-disrupted virus becomes an even more delicate operation.

In conclusion, it is suggested that SDS may be considered an acceptable agent for disrupting influenza A/PR8 and characterizing the isolated components by reaction in immunodiffusion tests only; 1) if a concentration of 0.1% is used, not 1% as is commonly

used, and 2) if the probability of nonspecific precipitation by SDS is kept in mind when analyzing these reactions. Furthermore, on the basis of these results, before choosing a detergent for distrupting viral preparations, influenza or others, an intensive examination of the effects of the selected detergent on the experimental system should be thoroughly investigated. Results of these experiments may show that analysis of experiments with other detergent treated virus is as complex and difficult as analysis of immunodiffusion reactions with SDS treated reactants indicating that nondetergent disruptants ie enzymes, may be more useful.

APPENDIX

APPENDIX

Statistical Method

A program was drawn up to statistically compare the standard curves drawn for SDS in saline and in the presence of known amounts of protein with or without β mercaptoethanol based on methods described by Brownlee (1967).

Comparison of Two Regression Lines

$$Y_1 = a_1 + b\ (x - \bar{x}_1)$$

$$Y_2 = a_2 + b\ (x - \bar{x}_2)$$

these lines are estimates of the corresponding true lines :

$$n_1 = \alpha_1 + \beta(x - \bar{x}_1)$$

$$n_2 = \alpha_2 + \beta(x - \bar{x}_2)$$

$a_1 = \bar{Y}_1$

$a_2 = \bar{Y}_2$

The problem is to decide if a single common regression is an adequate fit.

If separate regression lines

$$Y_1 = a_1 + b_1 (x - \bar{x}_1)$$

$$Y_2 = a_2 + b_2 (x - \bar{x}_2)$$

1. find a_i , b_i , S_i^2

if the lines are identical the S_i^2 will be estimates of a common σ^2

$$\frac{S_1^2}{S_2^2} \sim F (n_1 - 2, n_2 - 2)$$

2. if accept this

form a joint estimate of σ^2

$$S^2 = \frac{(n_1 - 2) S_1^2 + (n_2 - 2) S_2^2}{n_1 - 2 + n_2 - 2}$$

3. Ho : $B_1 = B_2$ If the lines are identical their slopes will be identical

if true

then

$$\frac{b_1 - b_2}{S \left\{ \left[\sum_{n}^{n_1} (x_{1n} - \bar{x}_1)^2 \right]^{-1} + \left[\sum_{n}^{n_2} (x_{2n} - \bar{x}_2)^2 \right]^{-1} \right\}^{\frac{1}{2}}}$$

$$\sim t \quad (n_1 + n_2 - 4)$$

if reject $\rightarrow$ lines have different slopes and are not identical

if accept $\rightarrow$ test if lines are coincident

4. Estimate the common slope

$$Y_1 = a_1 + b\ (x - \bar{x}_1)$$

$$Y_2 = a_2 + b\ (x - \bar{x}_2)$$

$$a_1 = \frac{1}{n_1} \sum_{n}^{n_1} y_{1n}$$

$$a_2 = \frac{1}{n_2} \sum_{n}^{n_1} y_{2n}$$

$$b = \frac{\sum_{n}^{n_1} y_{1n} (x_{1n} - \bar{x}_1) + \sum_{n}^{n_2} y_{2n} (x_{2n} - \bar{x}_2)}{\sum_{n}^{n_1} (x_{1n} - \bar{x}_1)^2 + \sum_{n}^{n_2} (x_{2n} - \bar{x}_2)^2}$$

Test if $E\left((a_1 - a_2) - b\ (\bar{x}_1 - \bar{x}_2)\right) = 0$

ie are the lines coincident

If the lines are identical

$$\frac{(a_1 - a_2) - b\ (\bar{x}_1 - \bar{x}_2)}{S\left[\frac{1}{n_1} + \frac{1}{n_2} + \frac{(\bar{x}_1 - \bar{x}_2)^2}{\sum_{j}^{2} \sum_{n}^{n_i} (x_{in} - \bar{x}_i)^2}\right]^{\frac{1}{2}}} \sim t(n, + n_2 - 3)$$

For the curves obtained using the methylene blue-chloroform technique, there was no significant difference between the test curves and the SDS in saline standard curve at the 0.975 level.

Similarly when the curves drawn using the different red blood cell types for the hemolysis technique, no significant difference was determined for

0.5% rooster RBC
1% Human 'O' RBC
1% sheep RBC

as compared to 1% rooster RBC

at the 0.975 level.

Similarly if SDS was in the presence or absence of protein and/or β mercaptoethonol,no significant difference at the 0.975 level was determined when these curves were compared.

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