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The Chemical, Physical, and Immunological Characterization of Soluble Components Extracted from Group A, Type 12 Streptococcal Cell Membrane

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THE CHEMICAL, PHYSICAL, AND IMMUNOLOGICAL CHARACTERIZATION OF SOLUBLE COMPONENTS EXTRACTED FROM GROUP A, TYPE 12 STREPTOCOCCAL CELL MEMBRANE

by

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VITA

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LIST OF ABBREVIATIONS

1.	BUT-LIS-TE-CM-Ppt.	Insoluble residue resultant from successive extraction of SCM with TE-buffer, LIS, and butanol
2.	BUT-TE-CM	Aqueous phase resultant from butanol extraction of the insoluble residue resultant from treatment of SCM with TE-buffer
3.	$CaCl_2$	Calcium chloride
4.	cm	Centimeter(s)
5.	d	Day(s)
6.	DEAE cellulose	Diethylaminoethyl cellulose
7.	^oC	Degrees centigrade
8.	EDTA	Ethylenediaminetetraacetate
9.	GBM	Glomerular basement membrane
10.	Genetron	Trifluorotrichloroethane
11.	GLC	Gas-liquid chromatography
12.	GLCA	Aqueous phase resultant from Genetron extraction of GLCM
13.	GLCM	Aqueous phase from Genetron extraction of LIS solubilized SCM material
14.	gm	Gram(s)
15.	h	Hour(s)
16.	ID	Immunodiffusion
17.	IEP	Immunoelectrophoresis
18.	l	Liter(s)

19.	LIS-BUT-TE-CM	LIS solubilized material from the insoluble residue resultant from successive extraction of SCM with TE buffer and butanol
20.	LIS-BUT-TE-CM-Ppt.	Insoluble residue resultant from successive extraction of SCM with TE-buffer, butanol, and LIS
21.	LIS-CM	LIS solubilized SCM material
22.	LIS-CM-Ppt.	Insoluble material resultant from LIS extraction of SCM
23.	LIS-TE-CM	LIS solubilized material from the insoluble residue resultant from TE-buffer treatment of SCM
24.	M	Molar
25.	mA	Milliamperes
26.	meq	Milliequivalent(s)
27.	mg	Milligram(s)
28.	min	Minute(s)
29.	ml	Milliliter(s)
30.	MRBC	Mouse red blood cells
31.	NaCl	Sodium chloride
32.	NaOH	Sodium Hydroxide
33.	O. D.	Optical Density
34.	PAGE	Polyacrylamide gel electrophoresis
35.	PBS	Phosphate buffered saline
36.	PGT-CM	Aqueous phase resultant from Genetron extraction of material solubilized by trypsin digestion of streptococcal cell membrane
37.	rpm	Revolutions per minute

38.	SCM	Streptococcal cell membrane
39.	SDS	Sodium dodecylsulfate
40.	sec	Second(s)
41.	SLS-CM	Soluble component from sodium lauryl sulfate extraction of SCM
42.	TCA	Trichloroacetic acid
43.	TE-buffer	0.01 M Tris-EDTA buffer, pH 8.9
44.	TE-CM	Material solubilized by treatment of SCM with TE-buffer
45.	TE-GLCA	GLCA following dialysis vs. TE-buffer
46.	TE-GLCA-CAP	Precipitate resultant from centrifugation of TE-GLCA after dialysis vs. isotonic $CaCl_2$
47.	TE-GLCA-CAS	Supernatant resultant from centrifugation of TE-GLCA after dialysis vs. isotonic $CaCl_2$
48.	TEMED	Tetramethylethylenediamine
49.	TH broth	Todd Hewitt broth
50.	Tris-HCl	Tris-(hydroxymethyl)-amino-methane hydrochloride
51.	TLC	Thin layer chromatography
52.	u	Micron(s)
53.	ul	Microliter(s)
54.	uM	Micromole(s)
55.	v/v	Volume per unit volume
56.	w/v	Weight per unit volume
57.	w/w	Weight per unit weight
58.	X	Times

CHAPTER I

INTRODUCTION

Historically, the immunological cross reactivity of antibody prepared against group A, type 12 streptococcal cell membrane (SCM) for specific antigens found in or on human glomerular basement membrane (GBM) has been primarily established by the use of immunodiffusion analysis (8, 46), passive hemagglutination (37, 46) and indirect fluorescent antibody tests (6, 7, 8). The observation of the immunological relatedness of host and parasite structures utilizing serological methods is common and has been well documented. The protein nature of cross reactive antigens between SCM and GBM also has been established (7). The antigenicity of proteins has been attributed to the various structural aspects of proteins (2, 4), which have been classified as either primary (the amino acid sequence of the polypeptide chain as determined by peptide bonds), secondary (those parts of the polypeptide chain stabilized into helically coiled conformations by hydrogen bonding and hydrophobic interactions), tertiary (the three dimensional conformation of the polypeptide chain stabilized by covalent and non-covalent forces), or quaternary (the interaction of protein subunits to form multichain protein complexes) (3, 10, 39). Thus, the immunological cross reaction of GBM and SCM may have been due to the sharing of either conformational or

sequential determinants or combinations of both. Therefore, the determination of the structural basis for the immunological relatedness of GBM and SCM is fundamental to the study of the characteristics which govern the antigenicity of proteins.

Previous investigations have revealed that antisera prepared against soluble components obtained from SCM had never displayed the strong cross reactivity with GBM as had the whole SCM. Two possibilities may explain this observation. One explanation would be that the cross reactions which had been observed between SCM and GBM were due to conformational determinants, and the extraction methods used to obtain soluble components destroyed or modified these conformational determinants. The other possibility would be that the cross reactivity between GBM and SCM had been due to sequential determinants, and that extraction methods either had failed to isolate soluble components containing the sequential determinants or again the sequential determinants had been destroyed during the extractions (for example by enzymatic cleavage).

The complexity of the problem was further compounded by the elucidation of the fluid structure of bilayered biological membranes and the constant turnover of the constituents of the membranes (59). In addition, it has been well established that only certain M protein type strains of

group A streptococci are nephritogenic (31). Thus the nephritogenicity of the cytoplasmic membranes of these strains of group A streptococci, though unrelated to, was associated with the production of a protein (M protein). The M protein antigens had not been found to be involved in any of the cross reactions between GBM and SCM. Therefore, in order to understand the observed immunochemical cross reactivity between SCM and GBM, further investigation into the chemical and structural similarities of SCM and GBM was necessary.

A. Chemical composition and physical characterization of SCM

SCM has been isolated from whole streptococcal cells with the use of a phage associated muralysin to digest the cell wall and yield protoplasts, or by mechanical methods which disrupt the cell, followed by differential centrifugation to separate walls from membranes (23). Purity of SCM was usually determined by the concentration of rhamnose, a component of the group specific carbohydrate of the group A streptococcus. A concentration of rhamnose constituting less than 1% w/w of the dry weight of the membrane preparation was considered to be characteristic of a cell wall free material. In general, SCM was composed of 25% lipid, 70% protein, 3% phosphorous and ribonucleic acid (RNA), and 2% carbohydrate, predominantly in the form of glucose (22, 30). Protoplasts contained lower levels of rhamnose and hexosamine

(another constituent of the group specific carbohydrate), and slightly higher levels of phosphorous and RNA than membranes isolated from mechanically disrupted cells (22). In addition, observation under electron microscopy revealed protoplasts to be less fragmented than membranes isolated by mechanical methods (22). A lipoprotein complex was found to compose 85% of the cell membrane (22). Fatty acids which have been found in the lipid fraction included lauric, myristic, palmitic, palmitoleic, stearic, oleic, linoleic, and arachidonic. Branched fatty acids were absent, and the presence of phosphatidyl choline and phosphatidyl serine was tentatively established (22).

Panos et al. (50) solubilized the protoplast membrane of a non-typable group A streptococcus by heating the membranes in a solution of sodium dodecyl sulfate (SDS) and mercaptoethanol for 0.5 h. After polyacrylamide gel electrophoresis (PAGE) at alkaline pH (pH 8.6) of the solubilized membrane, a single, fast moving band was observed. Alkaline PAGE of the solubilized membrane in the presence of 8.0 M urea yielded several slower migrating bands, none of which had the mobility of the single fast moving band obtained in the absence of 8.0 M urea.

B. Characterization of components isolated from SCM

Chemical cleavage and digestion with proteolytic enzymes have comprised the majority of methods utilized to

obtain soluble components from SCM.

1.) Components isolated by chemical cleavage and proteolytic digestion of SCM. After extracting membranes of group A, type 25 streptococci with a mixture of ethyl ether and ethanol, Freimer (22) digested the delipidated membranes with trypsin. After the digestion, roughly half of the membrane protein was solubilized without release of further lipid. Immunoelectrophoresis in agar of the trypsin solubilized type 25 SCM versus antiserum to whole SCM yielded two overlapping precipitin arcs migrating to the cathode. Similar treatment of type 12 SCM produce essentially the same results (22).

Markowitz and Lange (46) digested SCM with trypsin. A soluble fraction (PGT-CM) was obtained from the aqueous phase of extraction of the trypsin digest with the fluorocarbon trifluorotrichloroethane (Genetron). PGT-CM was composed of 80% protein, 7% carbohydrate, 3.7% phosphorous, and 0.2% hexosamine. PGT-CM was water soluble and sparingly acid soluble. The chemical composition of PGT-CM was consistent with that of a glycoprotein. Glucose comprised 75% of the carbohydrate moiety along with traces of rhamnose, ribose, galactose, glucosamine, and galactosamine. Aspartic, glutamic, alanine, and glycine were amino acids present in highest concentrations while histidine, methionine, arginine, and tyrosine were the amino acids in lowest concentration. Ultracentrifugal data and the behavior of PGT-CM on Sephadex

indicated the fraction had a m.w. of 8,500 daltons. Electrophoretically, PGT-CM had a component which migrated to the cathode. Employing anti-whole SCM sera, PGT-CM displayed cross reactivity with soluble GBM antigens in immunodiffusion analysis, passive cutaneous anaphylaxis, and passive hemagglutination assays.

After digestion of PGT-CM with papain, peptide maps were obtained by high voltage electrophoresis (37). The fingerprint maps obtained were almost superimposable with the maps of a similarily obtained soluble GBM fraction.

Lange (36) reported the use of SDS, cyanogen bromide, and deoxycholate to obtain soluble fractions from SCM which were non-dialyzable and immunologically cross reactive with soluble GBM components. Treatment of SCM with trichloroacetic acid (TCA), pancreatic lipase, Triton X-100, 2-chloroethanol, pepsin, and collagenase failed to produce the same results. Separation of soluble components via DEAE-cellulose chromatography yielded a number of constituents. The first fraction obtained from each soluble preparation was eluted with 0.01 M phosphate buffer, pH 7.75. Without exception, Fraction 1 obtained from each soluble preparation produced a single cross reactive line with a soluble GBM component in agar gel analysis, while other eluted fractions were non-cross reactive. Soluble components of SCM, which consistently displayed cross reactivity with soluble GBM components, were those with the highest carbohydrate content of

the soluble SCM preparations studied (37).

2.) Components obtained from SCM by other extraction methods. Treatment of the residue remaining after ethanol extraction of type 6 streptococcal cell membrane with Emulphogene BC 720 yielded a soluble fraction (62). Elution on DEAE-cellulose chromatography of this soluble fraction with a linear gradient of ammonium bicarbonate (0.01 - 0.025 M, pH 8.3) yielded five distinguishable peaks.

Lacane and Panos (34) isolated a lipoprotein from the cell membrane of a non-typable group A streptococcus by extraction with a combination of chloroform-methanol and phenol. The lipoprotein migrated with the tracking dye in PAGE and had a m.w. of less than 10,000 daltons. Treser, et. al. (61) isolated a lipoprotein component in the supernatant following centrifugation of a suspension of SCM in phosphate buffered saline (PBS). The lipoprotein had a m.w. of 120,000 daltons and displayed three bands on cellulose acetate electrophoresis with a mobility in the gamma globulin region. The lipoprotein was composed of 85% protein, 10.95% lipid, and 1.5% hexose. This lipoprotein absorbed the activity of a fluorescein isothiocyanate labelled antisera (obtained from patients with post-streptococcal glomerulonephritis), vs. autologous kidney sections.

C. Purpose of the present work

The short review presented above should serve to

demonstrate the fragmentary knowledge of the streptococcal cell membrane. Also, the information has involved several different M protein types of streptococci and is not restricted to the "nephritogenic" strains of group A streptococci (31). Unique structural characteristics might very well exist which differentiate membranes of nephritogenic streptococci from non-nephritogenic streptococci.

The chemical basis for the observed cross reactivity between GBM and SCM, though previously evaluated (6, 7, 37, 38, 46), remains to be elucidated. The isolation of soluble components from either SCM or GBM is necessary if conventional chromatographic and immunological characterization procedures are to be utilized in the elucidation of the chemical and structural basis for the immunological cross reactivity of the two membrane preparations. The majority of the information presently known concerning the immunological cross reactivity of soluble fractions from SCM and GBM has been reported mainly due to the efforts of Markowitz and Lange (46).

However, the strength of the cross reactivity of the soluble SCM fractions has always been of a magnitude lower than that of the whole, insoluble SCM (personal observation of C. F. Lange and A. S. Markowitz). The loss of immunogenicity as well as antigenicity in the soluble SCM fractions may have been due to the fact that the fractions were largely obtained by methods involving proteolytic and

chemical cleavage of the parent membrane preparation.

The report of a possible cellular component in the immunopathogenesis of post-streptococcal glomerulonephritis (66, 67, 68) and acute rheumatic fever (51, 66) provides additional impetus for the isolation of soluble SCM components. A particulate SCM fraction inhibited migration of lymphocytes and caused lymphoblastogenesis in lymphocytes obtained from patients with post-streptococcal glomerulonephritis (68). Soluble components isolated by trypsin digestion of SCM failed to stimulate cellular immunity (51, 67). In order to further investigate or understand the role of soluble SCM antigens in the cellular immune response during post-streptococcal glomerulonephritis and acute rheumatic fever, both alternate methods for extraction of SCM may need to be developed as well as chemical elucidation of the immunogenic components. There has been good evidence (2) which supports the distinction between cellular versus humoral immune stimulators.

The ultimate goal of the present research, therefore, is to develop an extraction procedure, utilizing methods other than chemical or proteolytic cleavage, which yields soluble antigenic components of type 12 SCM detectable by rabbit antisera. Further requirements include the chemical and immunological characterization of the isolated components and their behavior in various chromatographic systems with the goal of obtaining a homogeneous material displaying

most if not all of the humoral immunological activity displayed by the parent SCM.

Various methods were utilized to extract soluble components from SCM. The methods involved treatments of SCM which disrupt the weak cohesive forces that hold the bilayers of biological membranes together. Examples of these weak cohesive interactions are ionic interactions, hydrogen bonding, Van der Waal's forces, and hydrophobic bonding (56). Changes in temperature, ionic strength, and pH all can disrupt the weak cohesive interactions. Alternatively, sequestering of divalent cations or treatment of the membrane with detergents will also cause their disruption. Thus, the combination of treatments utilized, and the order in which they are applied can markedly affec membrane stability (56) as well as the composition of the final product.

Surfactive reagents are believed to exert their effects on membranes by the disruption of lipid from protein yielding a material composed of association between lipid, protein, and detergent (53, 57). Ionic and non-ionic detergents, and bile salts are such surfactants commonly used. SDS (sodium dodecyl sulfate or commonly sodium lauryl sulfate), an ionic detergent, apparently binds to all membrane components uniformly and is capable of forming protein-SDS complexes saturated with the detergent (1.5-3.3 gm SDS/gm protein) (33). The major biological function of deoxycho-

late and other bile salts is to solubilize phospholipid by incorporation into mixed micelles resulting in a release of membrane protein (33). However, deoxycholate also binds to proteins. Triton X-100 (Triton), a non-ionic detergent, gradually depletes membrane lipoproteins of their lipid content and causes little or no conformational changes in the released protein (33).

Triton and deoxycholate are considered to be less denaturing than SDS because they do not form the "saturated complexes" with membrane proteins as found in protein-SDS interactions (43). In addition, SDS and other ionic detergents bind to both hydrophilic and hydrophobic regions of proteins, causing unfolding of the peptide chain and consequent loss of function and antigenicity (33). In contrast, employment of non-ionic detergents permits the majority of membrane proteins to retain their biological characteristics in aqueous solution, because hydrophilic proteins do not bind these detergents (12, 27, 29, 32, 58). However, it should be noted and emphasized that extraction methods involving any of the three detergents suffer due to the lack of an adequate method to remove all traces of the detergent after extraction. The inability to completely remove SDS becomes especially critical for immunological characterization of membrane proteins, since SDS alone is known to form non-immune precipitates in gel immunodiffusion analysis (26). However, these precipitates are readily distinguishable from

immunoprecipitates in the saline solubility of the former but not the latter. Acknowledging these limitations, a component from SCM extracted with SDS treatment can still be utilized in immunologic studies, especially as an immunogen. For the most part, the extraction methods have avoided the use of detergents to solubilize components.

The disruption of hydrophobic interactions between lipid and protein using organic solvents has proved to be an effective method for the solubilization of membrane proteins. Genetron has been successfully applied to extraction of SCM in the past (46) and was utilized in the present study. In addition, the effect of n-butanol on SCM was investigated. The usefulness of n-butanol for the extraction of biological membranes was originally reported by Morton (48).

Extraction of membranes with n-butanol usually results in four phases: an upper phase, consisting of butanol and membrane lipids; a middle phase consisting of lipoprotein at the interface between the butanol and aqueous phases; a lower (aqueous) phase, consisting of solubilized membrane protein; and finally a precipitate consisting of aggregated and/or denatured proteins in the lower phase. However, denaturation of protein is minimal due to the low solubility in water and lipophilicity of butanol.

The contribution of divalent cations to the structure of biological membranes has been demonstrated by the

release of peripheral membrane proteins after treatment with chelating reagents (59). Recently, Archer et al. (1) reported the extraction of membrane proteins from Acholeplasma laidlawii with ethylenediaminetetracetate (EDTA). In the present study, the effect of EDTA on SCM has also been investigated.

Previous data on PGT-CM, a soluble component extracted from SCM displaying immunologic cross reactivity with a soluble component from GBM employing specific antisera, attributed the chemical composition of a glycoprotein to PGT-CM (46). The glycoprotein nature of PGT-CM led to the use of the extraction procedure of Marchesi and Andrews (45) which employed lithium diiodosalicylate (LIS) to isolate the blood group glycoprotein antigens from erythrocytes, producing a new soluble component from the SCM (LIS-E-CM) (unpublished results of A. S. Markowitz). To date reports on the extraction of microbial materials with LIS have been limited in number. Goel and Lemcke (24) extracted Mycoplasma gallisepticum membranes with LIS. Material solubilized by LIS was further extracted with butanol. The resultant material contained 43% of the total membrane protein. Following centrifugation of the LIS extracted material on a cesium chloride gradient, three fractions were obtained, one of which was glycoprotein in nature. However a lipoprotein fraction was also isolated, suggesting that LIS did not selectively extract glycoproteins. After extraction of

avian tumor viruses (ATV) with LIS, Maldow et al. (44) obtained a material which antagonized the binding of ATV to chicken embryo fibroblast cells and reduced the transforming capacity of the virus for the cells. No mention was made of the chemical nature of the material extracted by LIS.

In the present study it was proposed to isolate soluble components from SCM by treatment of SCM with SDS, N-butanol, Genetron, LIS, and EDTA. Chromatography of soluble components was accomplished utilizing diethylamino ethyl (DEAE) cellulose, Sephadex (Pharmacia Fine Chemicals, Co., Uppsala, Sweden), and PAGE. Chemical characterization of soluble components included amino acid analysis (AAA), thin layer chromatography (TLC) of lipids, and gas-liquid chromatography (GLC) of lipids. Although logistics and sample quantity did not allow the application of all of these methods to all of the components, a serious attempt was made to completely characterize chemically one or more of the components.

Antisera were prepared in rabbits against most of the soluble components. Immunological characterization of the soluble components depended on the nature of the components isolated, as discussed above in the case of the SDS extractions. Methods included immunoelectrophoresis (IEP), immunodiffusion (ID), precipitin analysis, and indirect fluorescent antibody tests on mouse kidney sections.

The present studies were designed to establish extrac-

tion methods capable of producing a variety of soluble, immunologically significant components of SCM. Information based on the physical properties and obtained from the chemical structural data of the compounds will serve a dual purpose. First, a new base can be established for the investigation of the structural basis of an immunological cross reaction between material of bacterial and mammalian origin. And, secondly, a better understanding of the forces contributing to the structure of a bacterial membrane will be gained by observing the effect of various extraction reagents on SCM.

CHAPTER II

MATERIALS AND METHODS

A. Isolation of SCM

Cell membranes of group A, type 12 streptococci were isolated by methods described previously (46). The strain of streptococcus was a gift from Dr. A. Markowitz and was originally isolated from an acute glomerulonephritic patient. Streptococci were grown in 10 l batches of Todd-Hewitt (TH) broth (Lot #A9DE1C, BBL, Cockeysville, MA) in a Biokulture Fermentor (Fermentation Design Inc., Allentown, PA). Initially, an inoculation flask which contained 1 l of TH broth was inoculated with streptococci and grown overnight in standing culture at 37o C. The 14 l fermentor vessel, containing 7 l of sterile TH broth, was then inoculated with the starter culture. The culture was aerated with filtered, compressed air at 2,000 ml/min and agitated with a gyration speed of 200 rpm. After 5 h, 1 l each of sterile 10.0% w/v glucose (Anhydrous dextrose, Mallinckrodt Chemical Works, St. Louis, MO) and 8.0% w/v sodium bicarbonate (Mallinckrodt) were added to the culture. The aeration was then discontinued, and after a total of 18 h of growth, the bacteria were heat killed at 56° C for 1 h. The bacteria were harvested in a Sharples centrifuge at full speed (a setting of 120 on the

rheostat), washed 3X in normal saline, weighed and stored frozen. The yield in wet weight of cells was on the average 3-4 gm/l of TH broth. Purity of culture was checked by gram staining and by streaking of samples on sheep blood agar (BBL) from: the inoculum of the starter culture; the starter culture before inoculation into the fermentor vessel; and the vessel culture before heat killing.

For isolation of SCM, approximately 75 gm wet weight of whole streptococcal cells were suspended in 1 l of distilled water. The cell suspension was poured into an Eppenbach Homo-Mixer (Gifford-Wood Co., Hudson, NY). Approximately 600 ml of Superbrite (R) glass beads, type 120-5005 (3M Company, St. Paul, MN) were added to the cell suspension. Cell breakage proceeded for 35 min with an aperature of 3·2 on the Homo-Mixer and a setting of 80 on the Powerstat (R) (Superior Electric Co., Bristol, CN). The resultant slurry was filtered by suction through a large course sintered glass filter. A preservative solution consisting of 1.0% w/v Thimerosal (commonly, merthiolate; Sigma Chemical Co., St. Louis, MO) and 1.4% w/v sodium borate (Fisher Scientific Co., Fairlawn, NJ) was added to the slurry to make a final concentration of approximately 1.0% w/v in preservative.

The slurry was passed through a Sharples centrifuge at top speed. The supernatant of the Sharples spin was passed at 6,000 rpm through a Sorvall KSB "Szent-Gyorgyi and Blum" Continuous Flow System (DuPont Co., Newton, CN), which

had been assembled on a Sorvall RC2-B centrifuge. The supernatant thus obtained was passed through the continuous flow system at 17,000 rpm. The pellets from the 17,000 rpm spin represented SCM. SCM was washed 3X with distilled water and lyophilized. The concentration of rhamnose was determined on the lyophilized material by the method of Dische and Shettles (17). Only preparations with a rhamnose concentration of less than 1.0% w/v were considered to be SCM free of significant wall contamination.

B. Extraction of SCM

1.) Extraction of SCM by treatment with Genetron and LIS. A modification of the LIS extraction procedure originally reported by Marchesi and Andrews (45) was utilized to extract soluble components from SCM. An outline of the procedure is displayed in Fig. 1. A 0.3 M solution of LIS (Eastman Kodak Co., Rochester, NY) in 0.05 M Tris (hydroxymethyl aminomethane) (Fisher)-HCl buffer, pH 7.5 was utilized for the extractions. SCM was extracted in 200 mg batches by suspending 25.0 mg dry SCM/1.0 ml LIS-Tris solution. The suspension was homogenized in a Sorvall Omni-Mixer at 60° C for 10 min with ten 30 sec intervals. Following the homogenization the slurry was diluted with two volumes of distilled water, then mixed overnight with a magnetic stirring bar at 4° C. The slurry was centrifuged at 15,000 rpm for 20 min. The precipitate was washed 3X by suspension

in distilled water followed by centrifugation at 15,000 rpm. The washed precipitate was lyophilized and labelled LIS-CM-P (Precipitate from LIS extraction of SCM).

The supernatant from the LIS extraction of SCM (LIS-CM) was extracted with two volumes of trichlorotrifluoroethane (Genetron), by mixing with a magnetic stirring bar at top speed for 5 min. The phases were allowed to separate by standing, or by centrifugation at 2,000 rpm in an International Equipment Co. model HN-S table top centrifuge. Three phases were obtained. The upper, aqueous phase was carefully suctioned off with a Pasteur pipet, without disturbing the thin particulate layer at the interphase between the lower Genetron phase and the upper aqueous phase. The upper aqueous phase was poured into Spectrapor (Spectrum Medical Industries, Terminal Annex, Los Angeles, CA) dialysis tubing having a molecular exclusion of 3,500 daltons, and dialyzed vs. distilled water for 3 d. The dialyzed material, designated GLCM (aqueous phase from Genetron extraction of the supernatant from LIS extraction of SCM), was extracted with Genetron, as described below.

The lower Genetron phase was pooled with Genetron from other extractions. The pooled Genetron phases were redistilled at 47.6° C and the residue left in the flask dissolved in petroleum ether (redistilled, Mallinckrodt). The material dissolved in petroleum ether was dried by flash evaporation, washed by the Folch procedure (21) dried under

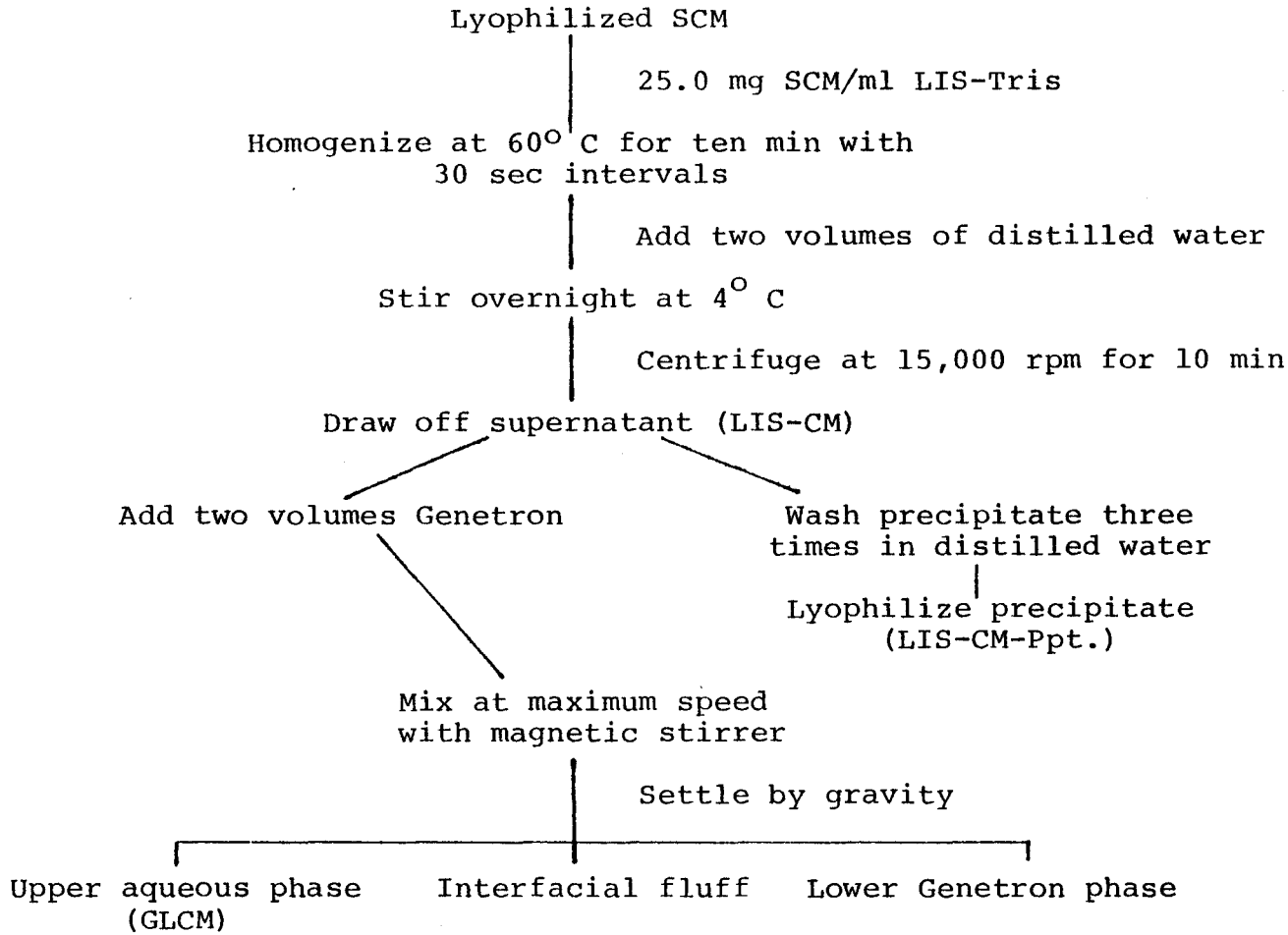

Fig. 1. Procedure for extraction of SCM with LIS and Genetron

a stream of nitrogen, resuspended in petroleum ether, and stored at 0° C pending further analysis.

The interfacial fluff, which had adhered to the sides of the test tube after removing the Genetron phase with a Pasteur pipet, was rinsed out of the tube with distilled water. The material was flash evaporated to remove traces of Genetron and stored frozen.

As outlined in Fig. 2, GLCM was extracted with an equal volume of Genetron. After addition of Genetron to GLCM, the two phases were homogenized in the Omni-Mixer for 6 min with three 1 min intervals. The homogenized material was stored frozen overnight. After thawing, the six min homogenization was repeated. The homogenized material was centrifuged at 2,000 rpm for 10 min. Three phases were obtained. The upper aqueous phase was placed in 3,500 dalton exclusion Spectrapor dialysis tubing and dialyzed 3 d vs. distilled water. The dialyzed material, termed GLCA (aqueous phase from Genetron extraction of GLCM), was stored frozen.

The lower Genetron phase was removed from the tube with a Pasteur pipet and stored frozen. The interfacial fluff, which adhered to the side of the tube, was rinsed out of the tube with distilled water. An equal volume of ethyl ether (Mallinckrodt) was added to the suspension, and the two phases mixed overnight at 4° C with a magnetic stirring bar. The two phases were allowed to separate upon standing. After isolation, the ethyl ether phase was flash evaporated,

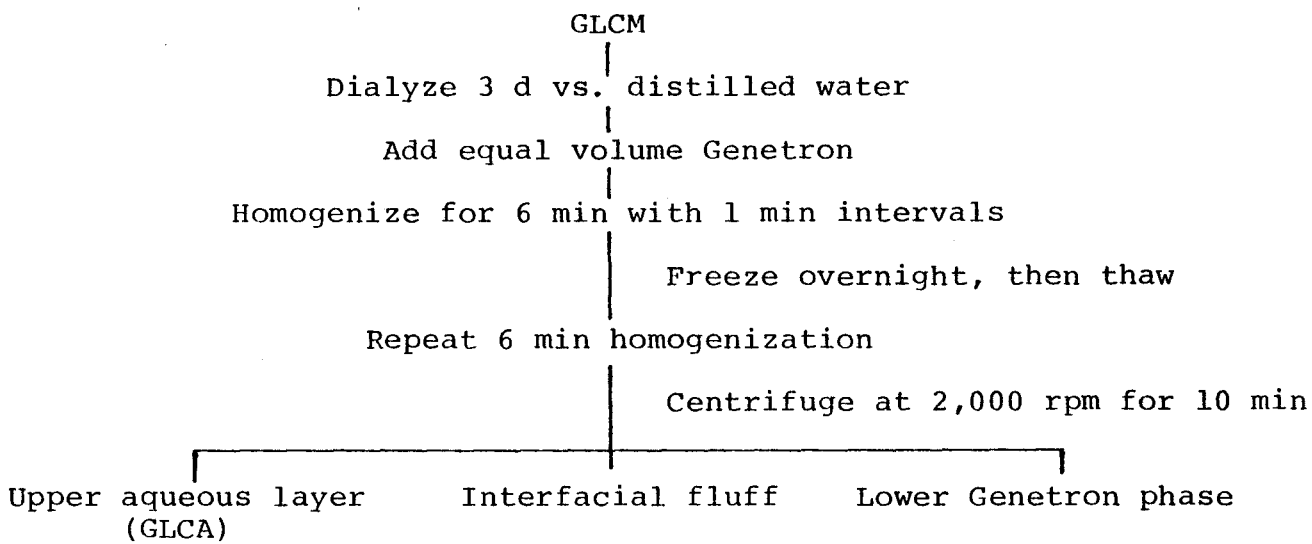

Fig. 2. Procedure for extraction of GLCM with Genetron.

washed by the Folch procedure, dried under a stream of nitrogen, dissolved in petroleum ether, and stored at 0° C pending further analysis. The aqueous phase was flash evaporated to remove traces of ether, then lyophilized and stored. The lower Genetron phase from Genetron extraction was stored at 4° C.

All components extracted with LIS in this study were dialyzed vs. three changes of TE buffer followed by dialysis vs. three changes of distilled water. Therefore, in this text the designation of TE preceding an abbreviation for the title of any SCM material implies that the material was dialyzed vs. TE buffer before dialysis vs. distilled water.

2.) Treatment of SCM with EDTA. Suspension of SCM in 0.01 M Tris-EDTA (TE) buffer, pH 8.9 caused the release of membrane components. Displayed in Fig. 3, the procedure consisted of suspending 25 mg of SCM/ml of TE buffer, followed by mixing with a magnetic stirring bar for 1 h at 4° C. The suspension was centrifuged for 20 min at 15,000 rpm. After carefully drawing off the supernatant, the precipitate was resuspended in the same volume of fresh TE buffer, and the suspension was poured into 10,000 dalton molecular exclusion dialysis tubing. The material was dialyzed overnight at 4° C vs. TE buffer. The suspension was centrifuged at 15,000 rpm for 20 min, the supernatant was drawn off, and the precipitate was resuspended in the same volume of fresh TE buffer. The material was again dialyzed vs. TE buffer at

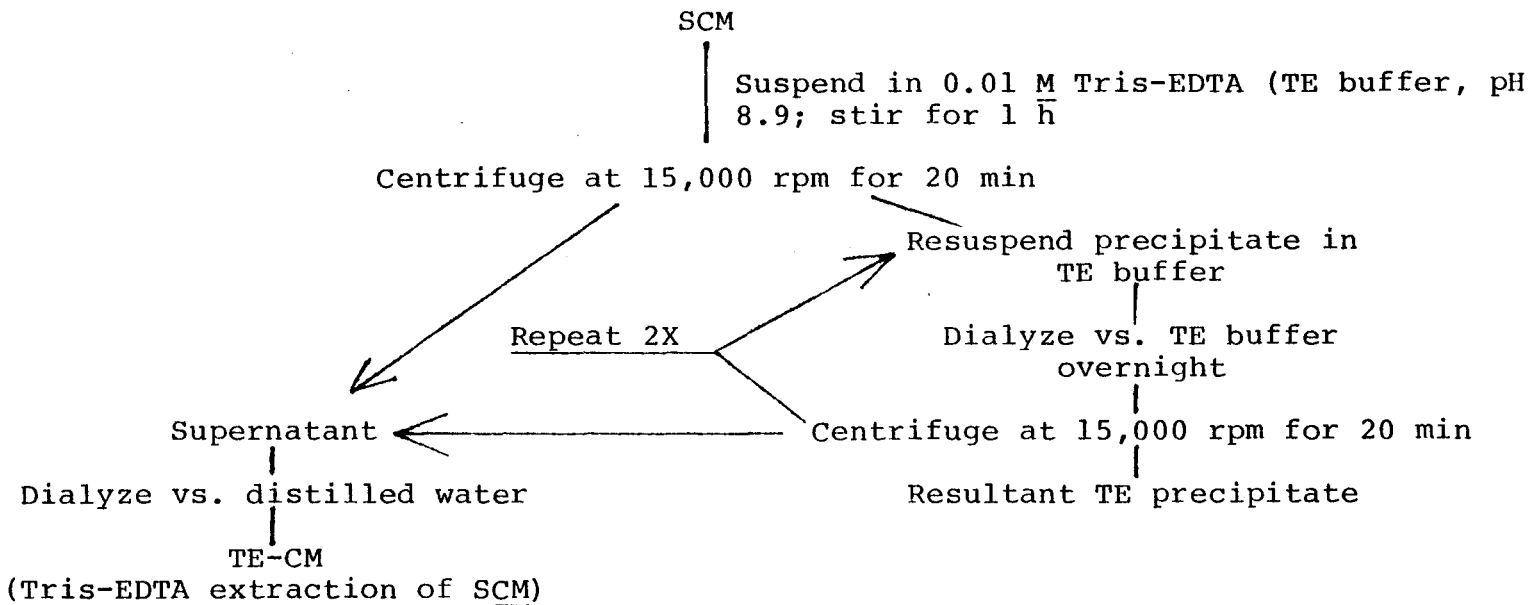

Fig. 3. Extraction of SCM with 0.01 M Tris-EDTA (TE) buffer, pH 8.9.

4° C overnight. The suspension was centrifuged at 15,000 rpm for 20 min. Supernatants from all three TE extractions were pooled, dialyzed vs. distilled water and lyophilized. The lyophilized material was designated TE-CM (Tris-EDTA extraction of SCM). The insoluble material was resuspended in TE buffer to a volume corresponding to that of the original TE extraction, and extracted either with butanol or LIS as described below.

3.) Extraction of the insoluble residue from extraction of SCM by TE buffer with butanol and LIS. As depicted in Fig. 4 and Fig. 5, the precipitate resultant from extraction of SCM with TE buffer was extracted in succession with either butanol and LIS, or with LIS and butanol.

For the purpose of the LIS extractions, the assumption was made that the material to be extracted weighed the same as the original amount of SCM before extraction with TE-buffer, or before extraction with TE buffer and butanol. On that basis, the suspension of material to be extracted was centrifuged, and the resultant precipitate resuspended in a volume of LIS-Tris solution corresponding to the weight/volume ratio described above for the Genetron-LIS extraction (25 mg SCM/ml LIS-Tris). The suspension was mixed overnight with a magnetic stirring bar at 4° C, followed by centrifugation at 15,000 rpm for 2 min. The supernatant was drawn off, dialyzed vs. three changes of TE

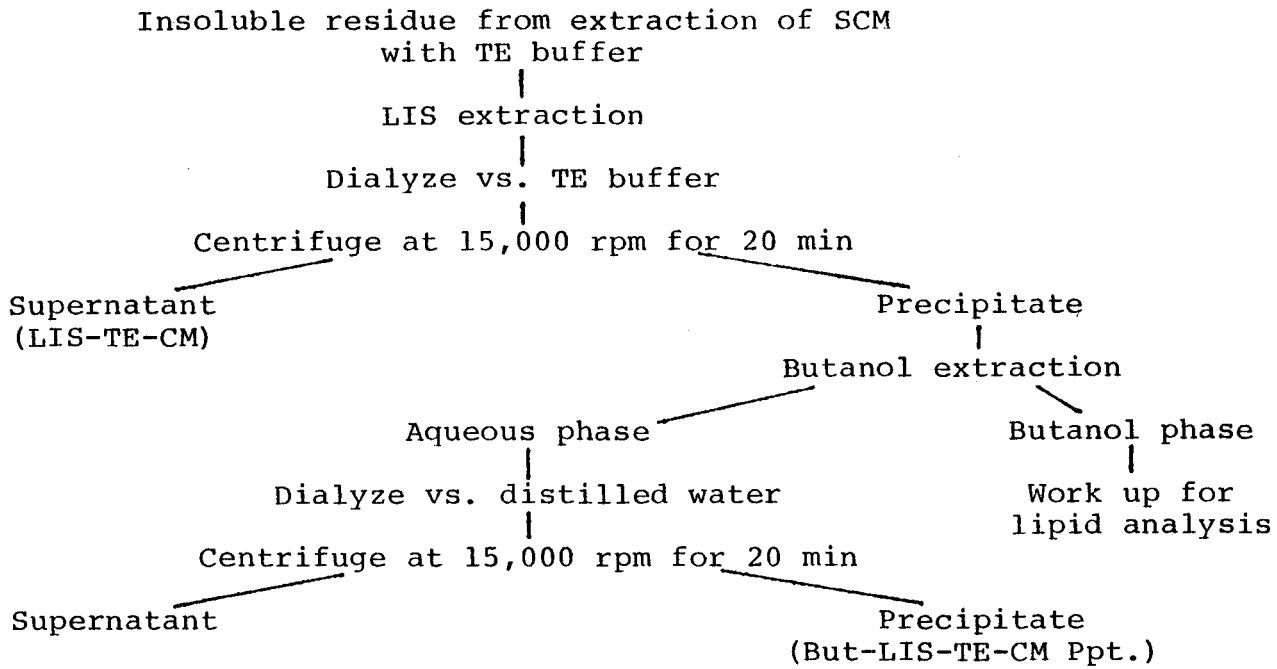

Fig. 4. Extraction of the insoluble residue from extraction of SCM with TE buffer by successive extraction with LIS and butanol.

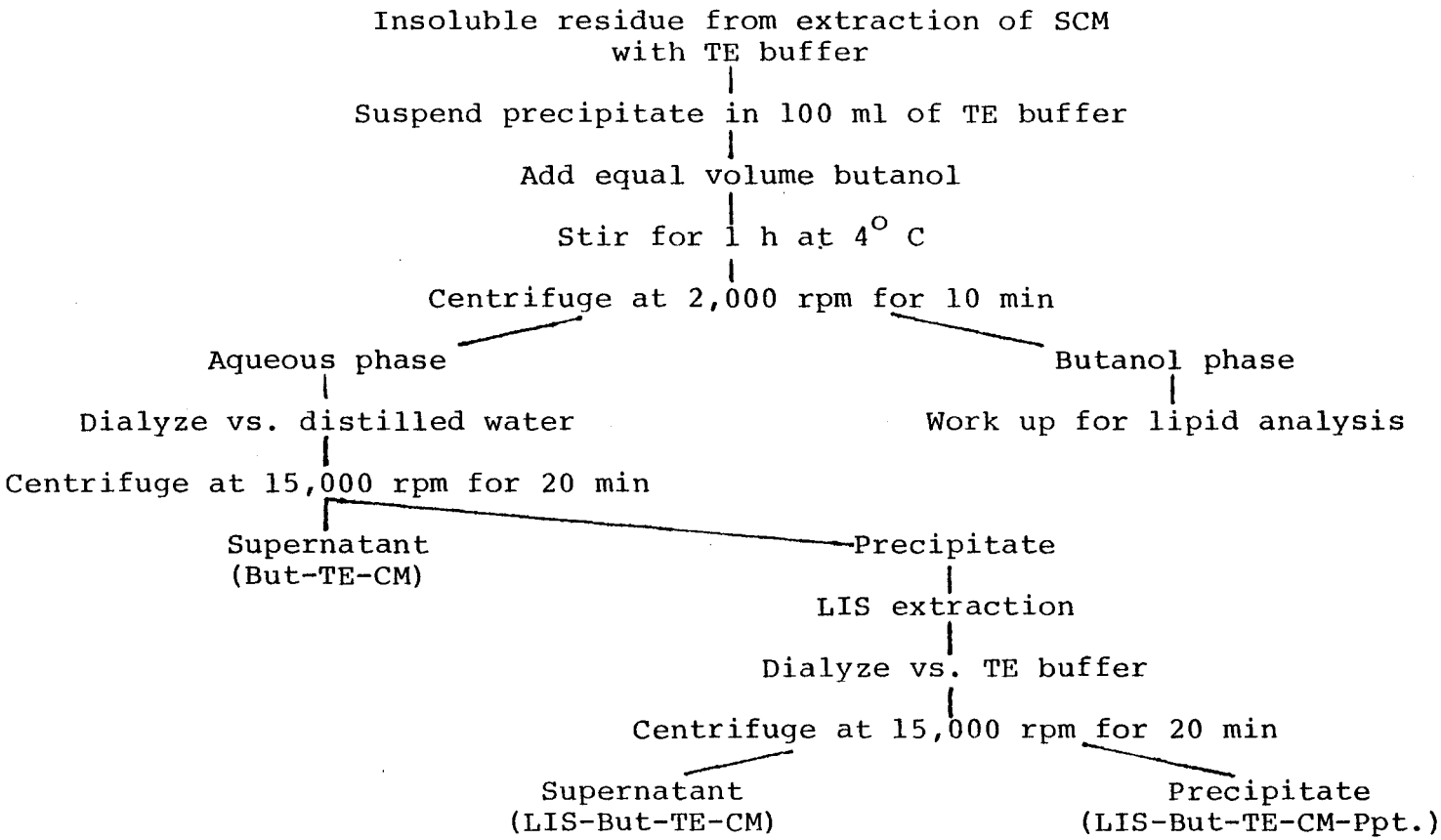

Fig. 5. Extraction of the insoluble residue from extraction of SCM with TE buffer by successive extraction with butanol and LIS

buffer, followed by dialysis vs. three changes of distilled water and lyophilized. LIS extraction of the precipitate resultant from TE extraction of SCM was designated LIS-TE-CM (the supernatant from LIS extraction of the precipitate from Tris-EDTA extraction of SCM), and the LIS extraction of the precipitate resultant from extraction of SCM successively with TE buffer and butanol was designated LIS-But-TE-CM (the supernatant from LIS extraction of the precipitate from butanol extraction of the precipitate from TE extraction of SCM).

Precipitates resultant from TE extraction of SCM or LIS extraction of the precipitate from TE extraction of SCM were resuspended in 100 ml of TE buffer. An equal volume of redistilled n-butanol (Mallinckrodt) was added to each fraction. The two phases were mixed with a magnetic stirring bar for 1 h at 4° C. Separation of phases was accomplished by centrifugation at 2,000 rpm. The upper aqueous phase and interfacial fluff were carefully drawn off, poured into 10,000 dalton m.w. exclusion tubing, and dialyzed 3 d vs. distilled water. The dialyzed material was centrifuged at 15,000 rpm for 20 min. The supernatant was drawn off and lyophilized, and designated either But-TE-CM (butanol extraction of the precipitate from TE extraction of SCM) or But-LIS-TE-CM (butanol extraction of the precipitate from LIS extraction of the precipitate from TE extraction of SCM), depending on the material which was originally extracted.

The insoluble residues remaining after the two extraction sequences were designated LIS-But-TE-CM-Ppt. and But-LIS-TE-CM-Ppt. (Ppt. = Precipitate).

The pool of butanol extractions was dryed on a flash evaporator, resuspended in chloroform/methanol (2/1, both solvents from Mallinckrodt and redistilled) and washed by the Folch procedure. The washed lipids were dried under a stream of nitrogen, dissolved in petroleum ether, and stored at 0° C pending further analysis.

The interaction of TE-GLCA with divalent cations was investigated by the following method. TE-GLCA was dissolved in 0.01 M Tris-HCl buffer, pH 8.9, and poured into 3,500 m.w. exclusion Spectrapor dialysis tubing. The solution was dialyzed vs. three changes of isotonic (1.7% w/v) aqueous calcium chloride ($CaCl_2 \cdot H_2O$) (Mallinckrodt). The suspension which formed was centrifuged at 15,000 rpm for 20 min. The supernatant was poured into 3,500 dalton exclusion Spectrapor tubing and dialyzed vs. three changes of distilled water. The supernatant fraction was designated TE-GLCA-CAS. The precipitate was dissolved in TE-buffer, then dialyzed vs. three changes of TE buffer followed by three changes of distilled water. The precipitated fraction was designated TE-GLCA-CAP.

C. Trypsin digestion of SCM

Samples of the insoluble residue from LIS extraction

of SCM (LIS-CM-Ppt.) and the interfacial fluff from Genetron extraction of GLCM were digested with bovine pancreas trypsin (Sigma). A weight ratio of 100/1 (mg membrane preparation/mg trypsin) was used for the digestion. The membrane preparation and trypsin were mixed together in 0.07 M sodium phosphate buffer, pH 8.1, to a volume corresponding to 1.0 ml of buffer/10 mg of membrane digested. The solution was stirred intermittently for 6 h at 37° C, followed by stirring overnight at 4° C with a magnetic stirring bar. The suspension was dialyzed vs. distilled water for 3 d in 10,000 dalton exclusion dialysis tubing, the distilled water being changed each day. The suspension was then centrifuged at 15,000 rpm for 30 min. The supernatant was carefully drawn off with a Pasteur pipet and lyophilized. The precipitate was washed out of the tube with distilled water and lyophilized. Soluble components thus obtained were designated either Tryp.-LIS-CM-Ppt. or Tryp.-GLCM-Ppt., depending on the insoluble residue from which they had originated.

D. Antisera

Young adult (2.5 kg) New Zealand White rabbits were used for all antisera production. These animals were housed in the AAALAC approved Animal Research Facility of Loyola University Medical Center. Complete Freund's adjuvant was composed of Marcol 52 (Humble Oil and Refining Co.) containing lyophilized Mycobacterium tuberculosis strain H37Ra

cells (2.5-5.0 mg/20 ml complete Freund's adjuvant); Falba (Pfaltz and Bauer, Inc., Flushing, NY); and phosphate buffered saline (PBS), containing the particular antigen, in a ratio of 2:1:1. Initially, each rabbit received 2.0 mg of antigen in complete Freund's adjuvant distributed in the foot pads and subcutaneously in the neck. Fourteen days later, each animal was injected intramuscularly with 1.0 mg of antigen in incomplete Freund's adjuvant. One week after the last inoculation, the animals were exsanguinated.

Antisera were prepared in individual rabbits against whole SCM and the following components obtained from SCM: SLS-CM (soluble component from sodium lauryl sulfate extraction of SCM), LIS-CM-Ppt. (precipitate from LIS extraction of SCM), GLCM (aqueous phase material from Genetron extraction of the soluble component from LIS extraction of SCM), GLCA (aqueous phase from Genetron extraction of GLCM), GLCA Frac. I (fraction I from DEAE cellulose chromatography of GLCA), GLCM-Ppt. (interfacial fluff from Genetron extraction of GLCM), TE-GLCA (GLCA dialyzed vs. TE buffer), TE-CM (soluble component from extraction of SCM with TE buffer), LIS-But-TE-CM (soluble component from successive Lis and butanol extraction of insoluble material from TE extraction of SCM), LIS-TE-CM (soluble component from LIS extraction of insoluble material from TE extraction of SCM), But-TE-CM (soluble component from butanol extraction of insoluble material from TE extraction of SCM), But-LIS-TE-CM-Ppt.

(precipitate from successive extraction of SCM with TE buffer, LIS and butanol), LIS-But-TE-CM-Ppt. (precipitate from successive extraction of SCM with TE buffer, butanol, and LIS), and finally Tryp.-LIS-CM-Ppt. (soluble material obtained from Trypsin digestion of insoluble material from LIS extraction of SCM). The abbreviations and descriptions of composition for the SCM components utilized in this study as immunogens are summarized in Table 1.

E. Adsorption of Antisera

Anti-SCM (AG-3), anti-SLS-CM (#22), and anti-GLCM (AG-10) were adsorbed on human glomerular basement membrane (GBM). One ml of antisera was adsorbed/10 mg. GBM. The insoluble GBM was suspended in glycine-HCl buffer, pH 3.0 and stirred for 1 h at room temperature. The suspension was centrifuged at 2,500 rpm for 20 min. The pellet was washed 3X by resuspending in saline and centrifuging at 2,500 rpm for 20 min. Following the saline wash, the pellet was suspended in the appropriate antiserum, incubated 2 h at 37° C with rotation, and then rotated overnight at 4° C. The suspension was centrifuged at 2,500 rpm for 20 min. The adsorbed antiserum was removed carefully with a Pasteur pipet. Antibody was eluted from the GBM with glycine-HCl as described above for treatment of GBM prior to adsorption.

F. Agarose Gel Analysis of Soluble SCM Components

Immunodiffusion and immunoelectrophoresis in agarose

gel of soluble SCM components were accomplished by the method of Demus and Mehl (14) for membrane proteins. A 1.7% w/v solution of each soluble component was made up in 0.1 M Tris-HCl buffer, pH 8.7, containing 1.0 M urea (Analytical reagent, Mallinckrodt) and 1.0% w/v Triton X100 (Scintillation Grade, Eastman).

Immunodiffusion was accomplished in gels composed of 1.0% w/v agarose (Type IV, Lot #87C-0288, Sigma) in 0.1 M Tris-HCl buffer, pH 8.7, containing 1.0 M urea and 1.0% v/v Triton. Samples were diffused for 48 h against antisera at room temperature in a moist chamber. Plates were then washed a total of 3 d vs. three changes of normal saline followed by three changes of distilled water. Gels were dried before staining as described below.

Immunoelectrophoresis was accomplished in gels composed of 2.0% w/v agarose in 0.1 M Tris-HCl buffer, pH 8.7 containing 1.5 M urea and 2.0% v/v Triton. Electrophoresis under water cooling was run for 10 h at 3 mA and 90 volts per slide in a Gelman (Gelman Instrument Co., Ann Arbor, MI) immunoelectrophoresis apparatus utilizing a Vokam-Shandon type 2541 power supply (Consolidated Laboratories, Inc., Chicago Heights, IL). The electrode buffer was 0.1 M Tris-HCl, pH 8.7 containing 1.5 M urea and 2.0% v/v Triton. After electrophoresis, antisera were added to troughs and immunodiffusion carried out for 48 h in a moist chamber at room temperature. Gels were washed and dried as described above

Table 1. Summary of SCM components utilized to immunize rabbits.

Title	Description
SCM	Whole, untreated SCM
SLS-CM	Soluble component from sodium lauryl sulfate extraction of SCM
LIS-CM-Ppt.	Precipitate from LIS extraction of SCM
GLCM	Aqueous phase obtained from Genetron extraction of the soluble component from LIS extraction of SCM
GLCM-Ppt.	Interfacial fluff from Genetron extraction of GLCM
GLCA	Aqueous phase from Genetron extraction of GLCM
GLCA Frac. I	Fraction I from DEAE cellulose chromatography of GLCA
TE-GLCA	GLCA dialyzed vs. 0.05 M Tris-EDTA buffer, pH 8.9
TE-CM	Soluble component obtained from extraction of SCM with TE buffer
LIS-But-TE-CM	Soluble component obtained from successive extraction with butanol and LIS of the insoluble residue from TE extraction of SCM
LIS-TE-CM	Soluble component resultant from extraction with LIS of the insoluble residue from TE extraction of SCM
But-TE-CM	Soluble component resultant from butanol extraction of the insoluble residue from TE extraction of SCM
But-LIS-TE-CM-Ppt.	Precipitate from successive extraction of SCM with TE buffer, LIS, and butanol
LIS-But-TE-CM-Ppt.	Precipitate resultant from successive extraction of SCM with TE buffer, butanol and LIS
Tryp.-LIS-CM-Ppt.	Soluble material obtained from digestion with trypsin of the insoluble residue from LIS extraction of SCM

for immunodiffusion gels.

After drying, the gels were stained with 0.1% w/v Napthalene Black 12B (Allied Chemical and Dye Corporation, New York, NY) in methanol, glacial acetic acid, and water (5/2/5), and then destained in a solution of methanol, glacial acetic acid, and water (7/1/2).

G. Analysis of Soluble SCM Components in Cellulose Acetate Membrane

Cellulose acetate electrophoresis using Beckman electrophoresis membranes (Beckman Instruments, Inc., Fullerton, CA) of soluble components obtained from SCM was accomplished in 0.075 M barbital buffer, pH 8.6, using a Beckman Model R-101 Microzone Electrophoresis Cell with the type 2541 Vokam-Shandon Power Supply. Electrophoresis was run at 100 volts for 45 min. Cellulose acetate membranes were stained in 0.2% w/v Ponceau S (3.0% w/v trichloroacetic acid) stain, decolorized with 5.0% v/v acetic acid and dried.

Cellulose acetate immunodiffusion of soluble SCM components was accomplished utilizing Beckman cellulose acetate electrophoresis membranes. The membranes were prepared using a Beckman membrane embosser, and antisera and antigen solutions applied using a 0.5 ul Beckman Radial Applicator. After the antisera and antigen solutions were applied the strips were incubated overnight in Squibb Mineral Oil (E. R. Squibb and Sons, New York, NY), washed two times in petro-

leum ether (1 min/wash), washed 3X in normal saline, stained with Ponceau S, decolorized with 5.0% v/v acetic acid, and dried.

H. Fluorescent Antibody Tests

Indirect fluorescent antibody tests were done on C3H mouse kidney, heart, and lung sections utilizing antisera prepared against various components extracted from SCM. Tissue from adult, newborn, 3 d and 5 d old mice were utilized in the study. Tissue sections were sliced 2 microns (u) thick and fixed onto glass slides by immersion in acetone.

Prior to use, sera were first inactivated at 56° C for 30 min. Antisera and respective pre-immunization sera were then tested for their ability to agglutinate mouse red blood cells (MRBC) by mixing a drop of sera with a drop of a 3% suspension of fresh MRBC in saline. Sera displaying no agglutination after 1 min were considered negative. For adsorption of positive sera, 1.0 ml of a 10% solution of MRBC in saline was centrifuged at 1,500 rpm. After removal of the saline supernatant, 1.0 ml of serum was added to the packed cells. Following an incubation period of 10 min, the suspension was centrifuged at 1,500 rpm. The adsorbed serum was decanted into a clean test tube, and retested for agglutination of MRBC as described above. Sera which displayed agglutinating activity were readsorbed, as described above with a fresh batch of MRBC.

Aliquots of adsorbed antisera, adsorbed preimmunization sera, and normal saline were overlayed on tissue sections and incubated at room temperature for 1 h. Slides were washed 3 times in phosphate buffered saline (PBS), for 10 min/wash. The slides were dried by pressing between bibulous paper and then overlayed with a 1/15 dilution of fluorescein conjugated sheep anti-rabbit gamma globulin. The slides were incubated for 30 min at room temperature in a moist chamber and then washed with PBS and dried as described above. Sections were mounted with 90% glycerol, 10% PBS cover slipped and observed with a Leitz Wetzlar (E. Leitz, Inc., Rockleigh, NJ) fluorescence microscope for fluorescent staining.

I. Amino Acid Analysis

Quantitative amino acid analyses of whole SCM and extracted SCM components were performed with either a Beckman model 120 C amino acid analyzer (Palo Alto, CA) or JOEL model JLC-5AH amino acid analyzer (Cranston, NJ) using methods previously described (37, 46). Approximately 1.0 mg amounts of samples were hydrolyzed in evacuated test tubes containing 1.0 ml of 5.7 M constant boiling HCl, at 110° C for 22 h. After hydrolysis, the tubes were opened and the contents dried over sodium hydroxide in a vacuum dessicator. Samples were solubilized with 0.2 M citrate buffer, pH 2.2 and duplicate 1.0 ml samples were placed on the short and long column respectively.

J. Carbohydrate Analysis

Total hexoses were determined on SCM preparations by the orcinol procedure of Rosewear and Smith (55) using a glucose standard. Methyl pentose determinations were by the method of Dische and Shettles (17) using a rhamnose standard.

K. Lithium Determination

Concentrations of lithium in various soluble components extracted from SCM were kindly determined by atomic absorption-emission spectrophotometry by the Department of Clinical Chemistry of Loyola McGraw Hospital. A lithium standard of 1.56 meq, LIS and soluble SCM components were dissolved in 0.05 M Tris-HCl buffer, pH 7.5. Concentrations of lithium were determined on an Instrumentation Laboratory, Inc. Atomic Absorption-Emission Spectrophotometer model IL 253.

L. Phosphorous Determination

Total phosphorous determinations on TE-GLCA were accomplished by the method of Chen et al. (9). Disodium phosphate (anhydrous, Mallinckrodt) dissolved in water was utilized to establish standard curves. Optical densities were determined on a Coleman Jr. Spectrophotometer (Coleman Instruments, Maywood, IL).

M. Nitrogen Determination

Total nitrogen content in soluble SCM components was determined as ammonia by the method of Lanni et al. (39).

Standard curves were established using a Brook (R) ammonium sulfate standard (0.1 mg nitrogen/ml, ALOE Scientific Co., St. Louis, MO). Optical densities were determined on the Coleman Junior Spectrophotometer.

N. Water Determination

Water concentrations in various soluble components extracted from SCM were determined by Galbraith Laboratories, Inc., Knoxville, TN, utilizing Karl Fischer water analysis.

O. Ashing Procedure

Prior to the ashing procedure, the porcelain crucible was heated over a Meeker bunsen burner repeatedly until a constant dry weight was obtained. The sample to be ashed was then placed into the crucible and heated red hot for 10 min. The crucible was allowed to cool, and the inner wall and lid were rinsed with 30% v/v hydrogen peroxide (Fisher) while carefully collecting the rinse in the crucible. The crucible was again heated over the bunsen burner for 10 min, allowed to cool and weighed to determine the ash content of the sample.

P. Polyacrylamide Gel Electrophoresis of Soluble SCM Components

Various methods were used to analyze soluble SCM preparations by polyacrylamide gel electrophoresis (PAGE). Initially, the disc method of Davis and Ornstein (13) was utilized with a 7.0% w/v acrylamide running gel in 0.375 M

Tris-HCl buffer, pH 8.9, and a 3.0% w/v acrylamide stacking gel in 0.125 M Tris-HCl buffer, pH 6.7. Gels were polymerized (by the addition of 0.025% by volume of tetramethylethylenediamine (Eastman) and ammonium persulfate (Fisher)) in glass tubes with an inner diameter of 5.0 mm and length of 10 cm. The electrode buffer contained 0.025 M Tris and 0.192 M glycine, with a pH of 8.3. Approximately 200-400 ug of membrane protein in solution of 20% w/v sucrose (Mallinckrodt) were layered on top of the stacking gel with a 50 ul Hamilton syringe (Hamilton Co., Whittier, CA). Gels were run at 100 volts, 30 mA (or about 5 mA/gel) until the bromphenol blue tracking dye reached a level of about 1 cm above the bottom of the gel.

A modification of the procedure of Dewald et al. (16) was used to run alkaline 5.0% w/v polyacrylamide gels in the presence of 0.1% v/v Triton X-100. Running gels were 5.0% w/v acrylamide and 0.1% v/v Triton in 0.375 M Tris-HCl buffer, pH 8.9. The stacking gel was 3.0% w/v acrylamide and 0.1% v/v Triton in Tris-phosphoric acid (5.7 gm Tris, 25.6 ml 1.0 M phosphoric acid to 100 ml) buffer, pH 7.2.

Gels were either poured into glass tubes as described above or into 1 mm thick slabs using the 15.9 cm X 14 cm glass plates of an Aquebogue vertical gel apparatus (Aquebogue Machine and Repair Shop, Aquebogue, NY). Gels were polymerized by addition of TEMED and ammonium persulfate, as described above. The upper tank buffer contained

0.1% v/v Triton in Tris-Glycine buffer, pH 8.7. The lower tank buffer was 0.5 M Tris-HCl, pH 8.1. Samples were dissolved in 0.1 M Tris-HCl buffer, pH 8.5, which was 1.0% v/v in Triton and 10.0% w/v in sucrose. Using a 50 ul Hamilton syringe, 100-200 ug of membrane protein were applied onto tube gels; on slab gels, between 80-100 ug were applied. Gels were electrophoresed at 30 mA/slab gel and 5 mA/tube gel until the bromphenol blue tracking dye reached a point 1.0 cm above the bottom of the gel.

PAGE of soluble SCM components were run in slab gel in the presence of SDS essentially by the procedure of Laemmli (35). The running gel contained 10.0% w/v acrylamide and 0.1% w/v SDS in 0.375 M Tris-HCl buffer, pH 8.8. The stacking gel contained 3.0% w/v acrylamide and 0.1% w/v SDS in 0.125 M Tris-HCl buffer, pH 6.8. In addition, stacking and running gels were 8 M in urea (Mallinckrodt) for certain analyses. Membrane preparations were boiled for 1.5 min in a solution which consisted of 2.0% w/v SDS, 10.0% v/v glycerol (Fisher), 5.0% v/v 2-mercaptoethanol (Eastman), and 0.001% w/v bromphenol blue in 0.0625 M Tris-HCl buffer, pH 6.8 (and 8 M urea for SDS-urea PAGE). Gels were polymerized by addition of TEMED and ammonium persulfate, as described above. The electrode buffer, pH 8.3, contained 0.025 M Tris, 0.192 M glycine, and 0.1% SDS. After application of between 80-100 ug of membrane protein, electrophoresis was carried out at 30 mA/gel for 2 h. Although m.w. standards

(Schwarz-Mann, Orangeburg, NY) of human gamma globulin, horse apoferitin, and BSA were also run, no attempt was made at determining molecular weights of unknowns due to the numerous bands which were obtained after staining (see Results).

After removal from tubes or from between glass plates, gels were fixed and stained by a variety of procedures. Gels run by the procedure of Davis and Ornstein were fixed and stained in a solution of 1.0% w/v buffalo black (Allied Chemical, Morristown, NJ) in 7.0% acetic acid (destaining with 65% distilled water, 25% ethanol, and 10% glacial acetic acid). Gels containing either Triton or SDS were fixed by the method reported in Application Note #306 of the LKB company. Gels were fixed in a solution of 11.4% trichloroacetic acid (Mallinckrodt) and 3.4% w/v sulphosalicylic acid (Mallinckrodt) in methanol/water (3/7). Gels were stained in a solution of 0.1% w/v Coomassie Brilliant Blue R-250 (Biorad Laboratories, Richmond, CA) in 7.0% v/v glacial acetic acid. Gels were destained in a solution of distilled water, ethanol, and glacial acetic acid (65/25/10).

Q. Ion Exchange Chromatography of Soluble SCM Components

Fractionation of GLCA and TE-GLCA on DEAE cellulose (Eastman) was accomplished using a continuous pH and ionic strength gradient composed of the following sodium phosphate buffers: 0.005 M, pH 7.8; 0.005 M, pH 7.0; 0.01 M, pH 6.1; 0.04 M, pH 5.0; 0.1 M, pH 5.0; 0.3 M, pH 4.0; and a 0.2 M

phosphoric acid solution made in 0.5 M NaCl, pH 1.3. Also, a continuous gradient composed of the following Tris-HCl buffers was utilized: 0.01 M -- pH 8.9, 8.5, 7.8, 7.0, and 6.0; and 0.3 M, pH 6.0.

The material to be chromatographed was dissolved either in 0.005 M sodium phosphate buffer, pH 7.8 or 0.01 M Tris-HCl buffer, pH 8.9 depending on whether a phosphate buffer gradient or Tris-HCl buffer gradient, respectively, was utilized for elution. The solution was then applied onto the DEAE-cellulose bed. All fractions were collected in 10.0 ml test tube amounts with a Fractomat (Buchler Co., Fort Lee, NJ) automatic fraction collector. Fractions were scanned at 280 nm and recorded on a Gilson tricorder. Appropriate tubes comprising each fraction were pooled.

Aliquots of each fraction were concentrated to smaller volumes using a Millipore Immersible Separator Kit (Millipore, Bedford, MA), which had a Pellicon (R) molecular filtration membrane that excluded solute greater than 10,000 daltons. Optical densities of the filtrate and concentrated solution were determined using a Perkin Elmer Model 139 UV-Visible Spectrophotometer (Tokyo, Japan). If the optical densities of respective solutions comprising a fraction were approximately equal, the material absorbing at 280 nm in the fraction was considered to be less than 10,000 daltons, and desalted on Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden). If the O. D. of the concentrated solution was

obviously greater than the filtrate, the fraction was placed into 10,000 dalton exclusion dialysis tubing (Union Carbide, Chicago, IL) and dialyzed in distilled water for 3 d with daily water changes. After desalting, all samples were lyophilized and stored.

Following saponification of TE-GLCA and extraction of lipids (described below), the resultant aqueous phase material was neutralized with 1.0 M NaOH. The neutralized material was passed over an Amberlite MB-3 mixed ion exchange resin (Mallinckrodt).

Prior to use, the MB-3 resin was washed with 3 bed volumes of distilled water. After application of the entire neutralized sample, the color change in the resin penetrated down no further than 2/3 of the resin bed in the column. The neutral fraction was then eluted with 3 bed volumes of water. The eluate was concentrated on a flash evaporator at 40° C to yield the neutral fraction.

The resin was then rinsed with 3 bed volumes of 3.0 M HCl, and the eluate concentrated on the flash evaporator at 40° C. The concentrated sample was hydrolyzed at 80° C in 3.0 M HCl for 3 h, and then passed over a fresh bed of MB-3 resin. The resin was then washed with 3 bed volumes of distilled water to yield the acid hydrolyzed neutral fraction.

R. Molecular Exclusion Chromatography

GLCM was fractionated on Sephadex (Pharmacia) G-

50. Approximately 10 mg of membrane protein in solution of 0.05 M Tris-HCl buffer, pH 7.5 were applied onto a gel bed of length 75 cm and diameter of 2.5 cm with a void volume of 430 ml. Fractions were eluted with 0.05 M Tris-HCl buffer, pH 7.5, at a flow rate of 0.75 ml/min. All fractions were collected in 10.0 ml test tube amounts with a Fractomat automatic fraction collector. Fractions were scanned at 280 nm and recorded on a Gilson tricorder. Appropriate tubes were pooled, dialyzed against deionized water at 4° C for 3 d, lyophilized and stored.

Desalting of appropriate fractions from DEAE cellulose chromatography of GLCA, described in section Q of Materials and Methods above, was accomplished on Sephadex G-25. Appropriate fractions were lyophilized, resuspended in 30.0 ml of distilled water, and applied to a column containing a 30 X 2.5 cm Sephadex gel bed with a void volume of 45.0 ml. Fractions were collected in 10.0 ml test tube amounts with the Fractomat automatic fraction collector, scanned at 280 nm and recorded on the Gilson tricorder. Aliquots from tubes comprising fractions were tested for the presence of free ion using a 1.0% w/v ammoniacal silver nitrate solution. Fractions which displayed no precipitation with silver nitrate were lyophilized and stored.

S. Thin Layer Chromatography of Lipids

Thin layer chromatography of lipids obtained from Genetron and butanol extraction of SCM was accomplished on

Uniplate (R) glass plates coated with Silica gel G, layer thickness of 250 u (Analtech Inc., Newark, DE). All lipid fractions were washed by the Folch procedure (21) prior to chromatography to remove non-lipid material. Thin layer chromatography of the following lipid fractions was accomplished: chloroform/methanol (2/1) extraction of G-LIS-CM; the residue remaining after distillation of the Genetron used for extraction of LIS-CM; a petroleum ether extraction of the interfacial fluff from Genetron extraction of G-LIS-CM; and lipid obtained from butanol extraction of SCM. Standards (all purchased from Sigma Chemical) included lecithin, cholesterol palmitate, cholesterol, tripalmitin, palmitoleic acid, palmitic acid, myristic acid, stearic acid, and oleic acid. All solvents were purchased from Mallinckrodt and were redistilled before use. Sample application points were marked with a lead pencil on a line 15 mm above the bottom of the plate, with adjoining points spaced 15 mm apart. Plates were dried in a 110^{o} C oven for 1 h before use.

Ten ul of a 1% w/v solution of the respective standard of unknown lipid solution were spotted on the application points with either a calibrated platinum loop or a 10 ul Hamilton syringe (Hamilton Co., Reno, NE). Chromatography was accomplished in Shandon Panglas T. L. C. Chromatanks, lined with Whatman #3 filter paper (W. & R. Balston, Ltd., England) to effect solvent saturation. Plates were dipped

in the developing solvent to a level approximately 5 mm from the bottom edge of the plate. Solvent was run to a level 100 mm (previously marked with pencil) above the sample application line. Plates were then dried, and sprayed with the appropriate reagents described below. R_f values were determined utilizing the equation:

$$R_f = \frac{\text{Distance of lipid spot from start}}{\text{Distance of solvent front from start}}$$

All solvent systems described below were prepared on a volume to volume basis. Chromatographs of neutral lipids were developed with a solvent consisting of petroleum ether/ethyl ether/acetic acid (90/10/1). Samples were dissolved in petroleum ether for application. After application of samples dissolved in chloroform, plates for chromatography of phospholipids were prewashed with a solvent consisting of acetone/petroleum ether (1/3) to the upper edge of the plate, thus removing neutral lipids. Plates were dried for 10-15 min, and then developed with a solvent consisting of diisobutyl ketone/ethyl ether/acetic acid (40/15/2).

A 10% w/v solution of phosphomolybdic acid (Mallinckrodt) in absolute ethanol was used as a general lipid spray.

Phospholipids were specifically stained with molybdenum blue utilizing the method of Dittmer and Lester (18). Plates specifically developed for phospholipids were run in duplicate and stained, respectively, with each reagent.

T. Gas-Liquid Chromatography of Fatty Acids

Identification of fatty acids in butanol and Genetron extracts of SCM, and TE-GLCA was accomplished by gas-liquid chromatography. Saponification of samples was accomplished by the method of Weppelman et al. (65). One ml of methanol (Mallinckrodt, redistilled) and 1.0 ml of 10.0 M potassium hydroxide (Mallinckrodt) were added to 5.0 mg of lipid material or membrane protein. The mixture was heated under refluxing conditions at 90° C for 2 h. After cooling, non-saponifiable lipids were extracted 3 times with equal volumes of petroleum ether (Mallinckrodt, redistilled). The resultant aqueous phase was acidified with 6.0 M hydrochloric acid. Saponifiable lipids were then extracted 3 times with equal volumes of petroleum ether as described above for non-saponifiable lipids. Petroleum ether extracts of non-saponifiable and saponifiable lipids were washed three times with water and dried under a stream of nitrogen prior to esterification for gas-liquid chromatographic analysis.

Methyl esters of fatty acids were produced with a 2.5% w/v solution of methanolic-hydrogen chloride (Instant Methanolic-HCl Kit (R), Applied Science Laboratories, State College, PA). After the addition of excess methylating reagent (approximately 150 ul methanolic-HCl/mg sample) the mixture was allowed to stand at room temperature for 30 min prior to injection for chromatography.

Analyses were accomplished utilizing a Beckman model GC 45 gas-liquid chromatograph equipped with a flame ionization detector. The glass column (6 feet in length, 2 mm inner diameter) was packed with 10% Silar-10C on 100 mesh Gas Chrom Q (Applied Science Laboratories). The temperature of the injection port was 258° C. Initially, the column temperature was isothermal at 120° C for 2 min; then temperature programmed to increase at a rate of 5° C/min up to 200° C. The temperature was maintained at 200° C until completion of the run. The flow rate of helium, the carrier gas, was 40 ml/min.

Peak areas were determined by manual calculation, and concentrations of individual fatty acids were reported relative to palmitic acid, the concentration of which was designated as 1 unit. The methyl ester of arachidonic acid was included in all runs as an internal standard. The following standards were run to get various peak identifications: the methyl esters of palmitic, palmitoleic, oleic, linoleic, linolenic, myristic, stearic, and lauric acids (Applied Science Laboratories).

RESULTS

A. Properties of components extracted from SCM with LIS due to the presence of LIS in the extracts. The color of lyophilized proteinaceous material often offers a clue to its state of purity. Thus lyophilized SCM was greyish-white in appearance. The color of the LIS-TRIS solution used for extracting SCM was greenish-brown. Following dialysis vs. 3 changes of distilled water, LIS extracts of SCM retained a reddish-brown tinge. Lyophilized insoluble residues and soluble components from LIS extraction were all pinkish-red in color. The color was suspected to be due to the presence of LIS, which was apparently bound to the extracted SCM components in a manner which rendered the LIS resistant to dialysis.

Initially, spectrophotometric methods were used to quantitate the amount of LIS in solutions of SCM components extracted with LIS. The molar extinction coefficient of LIS, as reported by Marchesi and Andrews (45), was 4×10^3 at 323 nm.

Extraction of SCM with LIS and Genetron, as described above in section B of Materials and Methods, involved a final concentration of approximately 40 mg LIS/ml of extract (8.0 ml of LIS-TRIS solution containing 953.6 mg of LIS diluted to a final volume of 24 ml). GLCA represented

the aqueous phase from two successive extractions with Genetron of the initial 24.0 ml volume. After dialysis vs. three changes of distilled water, the concentration of LIS in GLCA was 0.58 mg LIS/ml of extract, as determined spectrophotometrically.

Several observations attributed to the presence of LIS in GLCA necessitated the development of methods for further removal of LIS. During the course of spectrophotometric determinations of LIS in extracts, it was observed that LIS also absorbed significantly at 280 nm (a 1.0 mg/ml solution of LIS in 0.05 M Tris-HCl buffer, pH 8.9 had an O.D. of .75 at 280 nm). Thus spectrophotometric methods for the quantitation of protein in LIS extracts were rendered useless by the presence of LIS.

Solutions of LIS-Tris formed precipitates in agarose gel vs. various antisera. Displayed in Fig. 6 is the reaction of two LIS-TRIS solutions (0.3 M and 0.15 M in LIS) with a pre-immunization rabbit serum. Not all antisera displayed precipitation with LIS-TRIS controls. Additionally, antisera which did display non-immune precipitation with LIS varied with respect to the concentration of LIS necessary to produce the reaction. A goat serum (Kallestad Laboratories, Minneapolis, MN) precipitated with a 10 mg/ml solution of LIS-TRIS, while a rabbit anti-LIS-CM serum precipitated with a 20 mg/ml solution of LIS-TRIS but not with the 10 mg/ml solution of LIS-TRIS (data not shown).

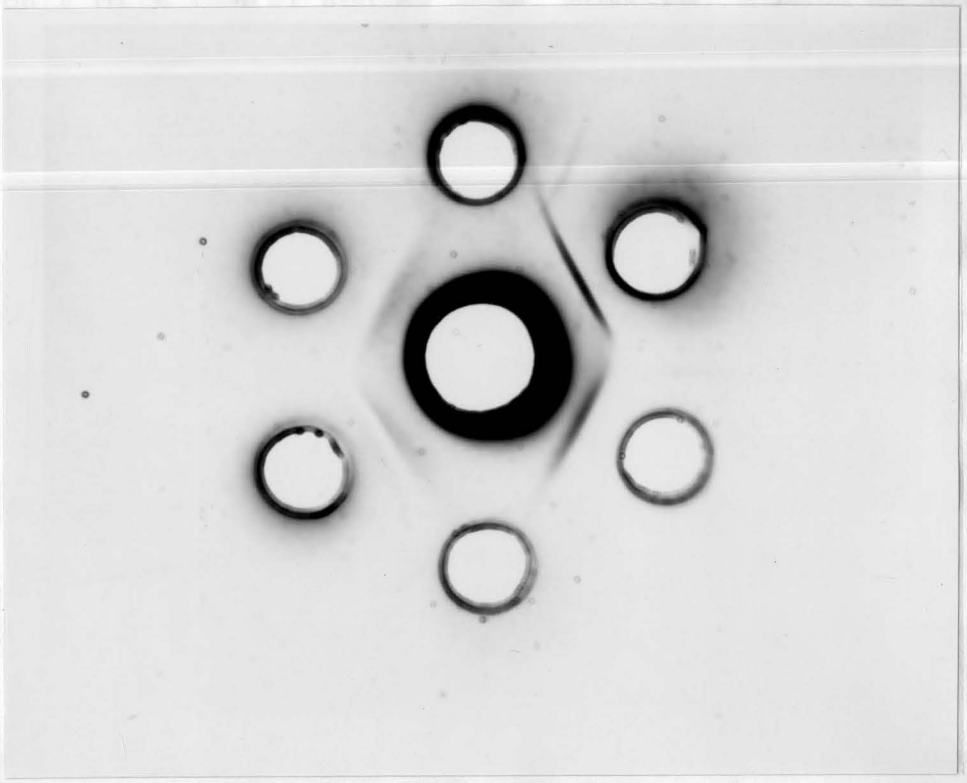

Fig. 6. Non-immune precipitation of LIS-TRIS solutions with a rabbit pre-immunization serum. Wells: 1 and 4, 0.05 M Tris-HCl buffer, pH 7.5; 2 and 3, 0.15 M LIS in 0.05 M Tris-HCl buffer, pH 7.5; and 5 and 6, 0.3 M LIS in 0.05 M Tris-HCL buffer, pH 7.5.

The precipitated material was not washed out by the saline and distilled water washings of the gel described above in section F of Material and Methods. However, a halo of precipitated material, which consistently surrounded wells containing LIS in agarose gel, was washed out.

Effects of LIS on electrophoresis of LIS containing components are displayed in Fig. 7 and Fig. 8. Fig. 7 displays the result of electrophoresis of GLCA in cellulose acetate membrane. Apparently, LIS caused diffusion off line of neighboring samples toward the sample containing LIS, as observed in the diffusion pattern of normal rabbit serum (NRS) toward GLCA. The effects of LIS on neighboring samples in slab gel PAGE is depicted in Fig. 8. The tendency for samples to spread out perpendicular to the direction of electrophoresis and the wavy line composed of the fused fastest migrating component in each fraction depict effects of LIS observed in slab gel PAGE.

B. Removal of LIS from LIS Extracted Components of SCM. Following dialysis of GLCA vs. 0.01 M Tris-EDTA (TE) buffer, pH 8.9, LIS was not detectable in GLCA by absorption at 323 nm. The apparently LIS free material was designated TE-GLCA, to signify the dialysis of GLCA vs. TE buffer. Lyophilized TE-GLCA was white in appearance, in contrast to the pinkish-red appearance of GLCA (see section A above of this chapter).

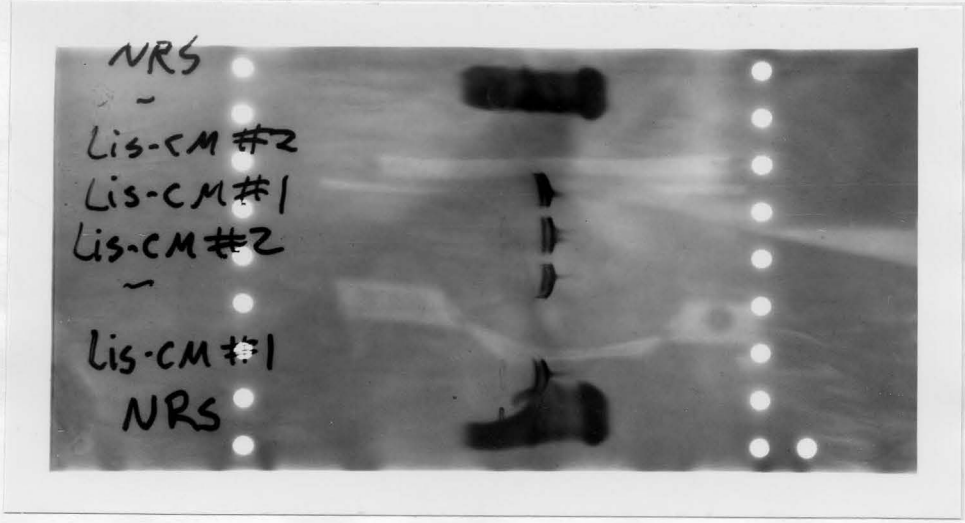

Fig. 7. Effect of LIS on migration of components in cellulose acetate electrophoresis. Samples consisted of, from top to bottom: 1) Normal rabbit serum (NRS); 2), 3), and 4) GLCA; 5) GLCA; and 6) NRS. Separation of NRS in sample 1 from GLCA in sample 2 prevented diffusion of NRS toward GLCA as observed between samples 5 and 6.

Fig. 8. Slab PAGE of various SCM components displaying the effect of LIS on the electrophoretic migration of neighboring samples which contained LIS. Samples included, from left to right: 1) Bovine serum albumin; 2) SLS extract of SCM; 3) Trypsin digest of SCM; 4) Fraction I from DEAE cellulose chromatography of GLCA; 5), 6), 7) and 8) various GLCA extracts. Samples 6 and 7, and 7 and 8 were separated by empty application wells. The fused wavy line composed of the fastest migrating component was believed to be caused by the presence of LIS in GLCA.

The more sensitive method of atomic absorption emission spectrophotometry was utilized to quantitate the concentration of LIS in lyophilized samples. Concentrations of LIS were determined on the basis of the quantity of lithium in each sample. Determinations on several samples are reported in Table 2. GLCA was 47.2% by weight in LIS; TE-GLCA was 0.41% by weight in LIS. Therefore, TE-GLCA was contaminated with less than 0.5% by weight with LIS.

DEAE cellulose chromatography of TE-GLCA yielded two fractions, as described below. Fraction II had a LIS concentration similar to the parent TE-GLCA (0.44% w/w). Fraction I contained a higher concentration of LIS than the parent TE-GLCA (21.8% w/w vs. 0.41% w/w).

Following dialysis of TE-GLCA vs. three changes of distilled water, precipitation occurred in the contents of the dialysis tubing. Occasionally, before lyophilization, the dialyzed material was centrifuged at 20,000 rpm for 20 min. The resultant precipitate was termed PTE-GLCA; the supernatant STE-GLCA. As recorded in Table 2, PTE-GLCA was 0.64% w/w in LIS.

In order to remove LIS from insoluble residues resultant from LIS extraction of SCM, the precipitates were suspended in TE buffer and stirred at room temperature until the pinkish-red color disappeared. The slurry was then poured into Spectrapor tubing, and dialyzed vs. 3 changes of TE buffer and 3 changes of distilled water before lyophil-

Table 2. Concentration of LIS in various samples as determined by atomic absorption emmission spectrophotometry. Samples were dissolved in 1.0 ml of 0.05 M Tris-HCl buffer, pH 7.5. Concentrations of lithium are reported in milliequivalents (meq)/L, where 1 meq of lithium = 6.94 mg.

Sample Identification	Dry weight of sample (mg)	Lithium (meq/L)	ug LIS/ml	% LIS w/w of sample
PTE-GLCA	0.68	0.011	4.33	0.64
TE-GLCA	0.78	0.008	3.19	0.41
GLCA	0.65	0.775	306.87	47.2
Fraction I from DEAE cellulose chromatography of TE-GLCA	0.85	0.469	185.4	21.8
Fraction II from DEAE cellulose chromatography of TE-GLCA	0.80	0.009	3.54	0.44

NOTE: The concentrations of % LIS w/w of sample recorded above in Table 2 were determined prior to the discovery of the hygroscopic nature (described below in section E of RESULTS) of the soluble components extracted from SCM. Therefore, the values for % LIS w/w of sample must be considered as minimum values, and may actually be as low as half the actual value.

ization.

Complete removal of LIS from the soluble extracts eliminated the deleterious effects due to the LIS as observed in slab PAGE and cellulose acetate electrophoresis. Further it safeguarded against the possibility of nonimmune precipitation in agarose gel.

C. DEAE Cellulose Chromatography of Soluble SCM Components.

Two fractions were obtained from DEAE cellulose chromatography of TE-GLCA, as displayed in Fig. 9. Fraction I appeared in the equilibration buffer and Fraction II was eluted in the range pH 5.0-5.5. Dry weight recovery of TE-GLCA in the two fractions totaled less than 10% w/w (data not shown). The loss was attributed to the insolubility of TE-GLCA below pH 6.0 (personal observation of the author). Utilizing the 10,000 dalton exclusion molecular separator, Fraction I contained material of m.w. greater than 10,000 daltons; Fraction II contained material of m.w. less than 10,000 daltons. Sephadex G-25 chromatography (employing water) of Fraction I produced two peaks. The first fraction appeared in the void volume; the second fraction appeared in the eluted volume.

Elution with the Tris buffer gradient produced a single fraction, which appeared in the equilibration buffer (data not shown).

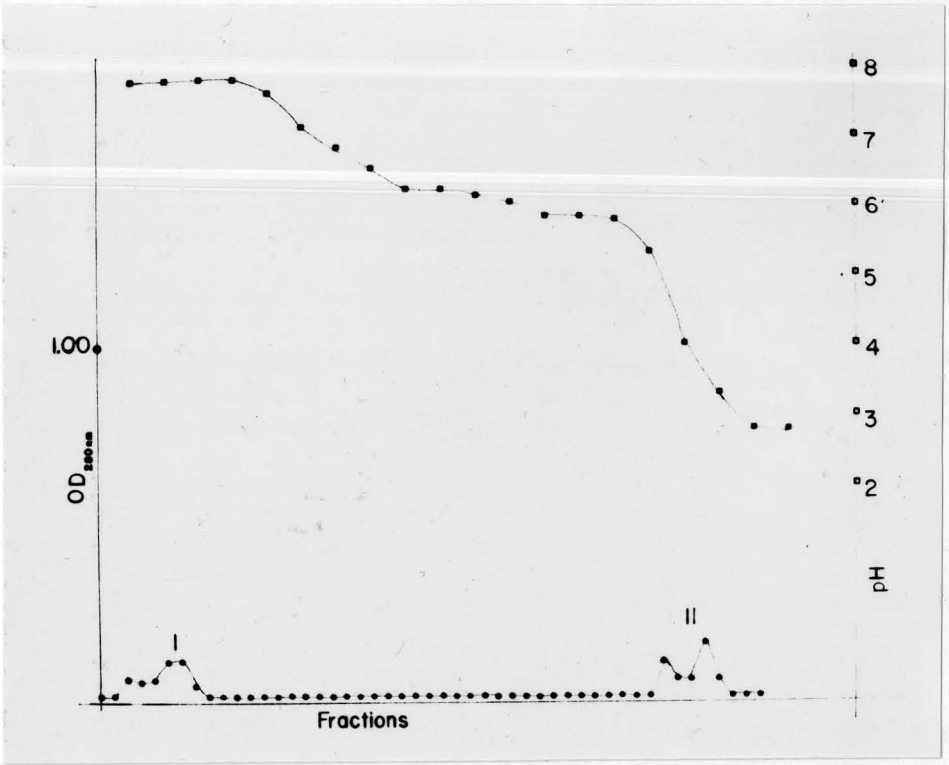

Fig. 9. DEAE cellulose chromatography of TE-GLCA. Elution was accomplished with a continuous pH and ionic strength gradient composed of the following sodium phosphate buffers: 0.005 M, pH 7.8; 0.01 M pH 7.0; 0.01 M, pH 6.1; 0.04 M, pH 5.0; 0.1 M pH 5.0; 0.3 M, pH 4.0; and a 0.2 M phosphoric acid solution in 0.5 M NaCl, pH 1.3. Fraction I appeared in the equilibration buffer; Fraction II was eluted in the range pH 5.0-5.5.

D. Sephadex Chromatography of GLCM

Chromatography of GLCM on Sephadex G-50 consistently yielded a major fraction in the void volume and a minor fraction in an included volume, as displayed in Fig. 10. The same result was obtained after chromatography of GLCM on Sephadex G-200 (data not shown). The exact recovery of GLCM in the two fractions was not determined due to: 1) the absorption of LIS at 280 nm, which made spectrophotometric determination of protein in the fractions impossible, and 2) GLCM was applied to the Sephadex gel bed as a solution, on which only a Kjeldahl nitrogen had been determined. Thus although the significance of dry weight recoveries on the two fractions was questionable, approximately 90% of the protein as determined by Kjeldahl nitrogen was recovered in Fraction I. Lyophilization of Fraction II yielded a yellow film on the sides of the lyophilization flask, and therefore could not be accurately weighed.

E. Recovery of the Initial Dry Weight of SCM in Soluble and Insoluble components Resultant from Extraction of SCM.

Recoveries of the initial dry weights of SCM (utilized for extractions) in components isolated from SCM are reported in Table 3. Methods I and II involved extraction procedures utilizing equal quantities of the same starting material, TE-CM-Ppt., which was the insoluble resi-

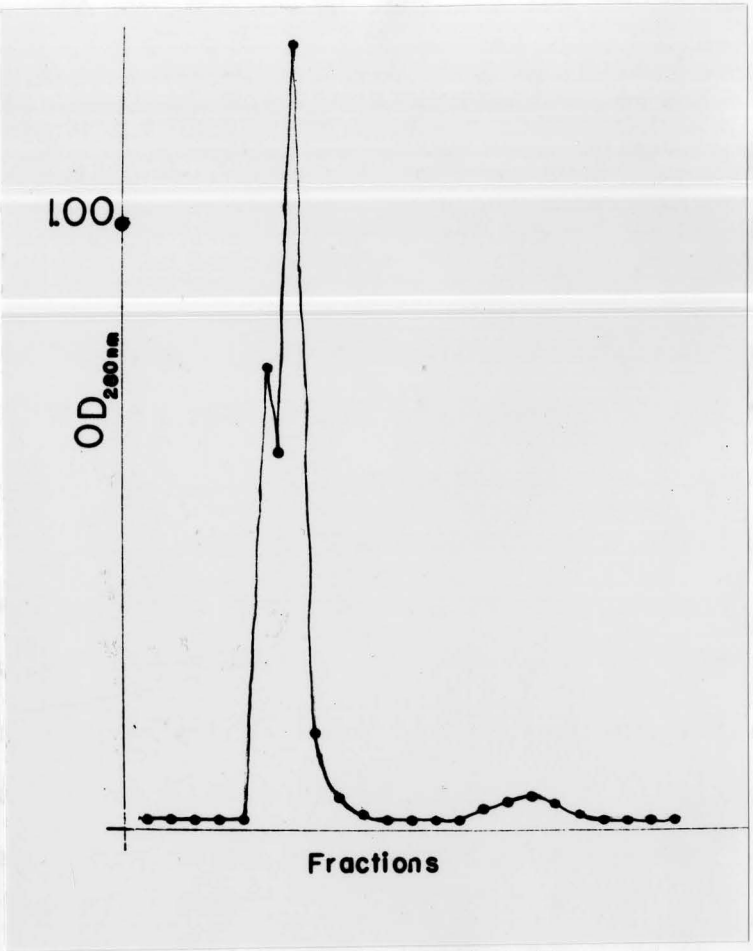

Fig. 10. Sephadex G-50 chromatography of GLCM. Approximately 10.0 mg of GLCM was layered on top a gel bed of 75 cm X 2.5 cm with a void volume of 430 ml. Elution was with 0.05 M Tris-HCl buffer, pH 7.5 at a flow rate of 0.75 ml/min. Fraction I appeared in the void volume; Fraction II in the included volume.

due remaining after extraction of SCM with TE buffer. The two methods differed only by the order in which the insoluble residue was extracted by butanol or LIS. Method III involved extraction of SCM with LIS followed by extraction of the supernatant (LIS-CM) with Genetron.

As recorded in Table 3, the dry weight recovery of LIS-BUT-TE-CM-Ppt. (554.9 mg) was greater than BUT-LIS-TE-CM-Ppt. (298.9 mg). Thus extraction of TE-CM-Ppt. with LIS prior to treatment with butanol apparently served to solubilize the insoluble residue to a greater extent than extraction with butanol prior to treatment with LIS. However, extraction of TE-CM-Ppt. with LIS before extraction with butanol caused an almost two-fold loss in total dry weight recovery as compared to extraction with butanol before LIS (48.1% vs. 81.7%). In addition, extraction of the insoluble residue resultant from LIS extraction of TE-CM-Ppt. did not serve to isolate a soluble component (see Table 3, Method II). The protein composition of the two insoluble residues resultant from extraction methods I and II were equivalent; LIS-BUT-TE-CM-Ppt. was 59.8% protein w/w, and BUT-LIS-TE-CM-Ppt. was 59.45% protein w/w.

Data recorded in Table 3 on protein concentrations in LIS-BUT-TE-CM (24.3%, w/w) and LIS-TE-CM (27.9%, w/w) suggested that protein recoveries of TE-CM-Ppt. were equal in the two soluble components (37.4 mg).

Extraction of SCM by Method III yielded the high-

Table 3. Dry weight and protein recoveries of SCM in fractions obtained by two extraction methods. Results of methods I and II represent the average recovery from two extractions; of method III the average recovery from the first three of the twenty extractions accomplished by that method.

Method I Starting material: 900 mg TE-CM-Ppt. (631.8 mg protein)
(Insoluble residue from extraction of SCM with 0.01 M Tris-EDTA buffer, pH 8.9)

Fraction	Dry Weight (mg)	% Protein (w/w)	Protein (mg)
BUT-TE-CM	26.3	57.8	15.2
LIS-BUT-TE-CM	153.8	24.3	37.4
LIS-BUT-TE-CM-Ppt.	554.9	59.8	331.7
Total	735.0		384.3
% Recovery	81.7		60.8

Method II Starting material: 900 mg TE-CM-Ppt. (631.8 mg protein)

LIS-TE-CM	134.05	27.9	37.4
BUT-LIS-TE-CM	0.0	0.0	0.0
BUT-LIS-TE-CM-Ppt.	298.9	59.45	177.7
Total	432.95		215.1
% Recovery	48.1		34.0

Method III Starting Material: 200 mg SCM (141 mg protein)

GLCM	37.75	57.0	21.5
LIS-CM-Ppt.	127.05	63.9	81.2
Total	164.80		102.7
% Recovery	82.4		72.8

est recoveries of protein and dry weight in the isolated components (see Table 3). GLCM was the soluble component obtained from the aqueous phase of Genetron extraction of LIS-CM; LIS-CM-Ppt. was the insoluble residue remaining after extraction of SCM with LIS. Total recovery of the initial dry weight of SCM in GLCM and LIS-CM-Ppt. was 82.4%; per cent protein recovery (w/w) of SCM in GLCM and LIS-CM-Ppt. was 72.8%.

F. Amino Acid Analyses

Amino acid analyses of soluble components extracted from SCM and the insoluble residues of SCM resultant from various extraction procedures are reported in Table 3 and Table 4, respectively. Amino acids were reported in micro moles (uM)/100 uM, and hexosamines as uM/100 ug protein. The percentage of protein composing the dry weight of each sample was reported for all samples.

Soluble components extracted from SCM were hygroscopic; the water content of samples stored in a vacuum dessicator over phosphorous pentoxide varied, on a dry weight basis, from 15-20% for soluble components (the dry weight of each sample was determined after heating a vacuum dessicator, containing the sample and phosphorous pentoxide, in a 110^{o} C oven for 1 h). Thus determinations on percent protein content in soluble components were considered to be based on an approximation of the dry weight for each sample, and

Table 4. Amino acid analyses of insoluble components resultant from extraction of SCM by various methods. Concentrations of amino acids are reported as uM/100 uM amino acids. Each analysis represents the average of at least three separate analyses, unless indicated otherwise (*), in which case analyses were run in duplicate. Range of average uM values are recorded.

Sample:	TE-LIS-CM-Ppt.	TE-GLCM-Ppt.	LIS-BUT-TE-CM-Ppt.	BUT-LIS-TE-CM-Ppt.*
Amino Acid				
Lysine	9.00 (8.53- 9.14)	8.48 (8.27- 9.52)	8.17 (7.74- 8.49)	8.05 (6.86- 9.29)
Histidine	0.88 (0.83- 1.19)	1.79 (1.48- 2.11)	1.38 (1.34- 1.40)	1.42 (1.24- 1.61)
Ammonia	11.9 (11.4 -12.4)	11.2 (10.5 -14.5)	13.7 (13.5 -13.7)	15.4 (14.6 -16.0)
Arginine	3.97 (3.77- 4.08)	3.98 (3.69- 4.00)	3.64 (3.42- 3.81)	3.48 (3.40- 3.56)
Aspartic Acid	7.76 (7.57- 7.90)	7.70 (7.54- 7.77)	7.55 (7.22- 7.77)	6.05 (5.08- 7.08)
Threonine	4.50 (4.04- 4.69)	4.20 (3.89- 4.54)	4.32 (4.17- 4.50)	3.39 (2.86- 3.94)
Serine	4.44 (4.32- 4.54)	4.55 (4.21- 4.81)	4.04 (3.50- 4.17)	3.39 (3.29- 3.49)
Glutamic Acid	10.7 (10.4 -10.9)	10.9 (10.6 -10.9)	10.3 (10.0 -10.4)	11.0 (11.0 -11.1)
Proline	1.91 (1.77- 2.42)	2.33 (2.27- 2.35)	1.91 (1.79- 2.01)	1.84 (1.63- 2.05)
Glycine	5.47 (5.04- 5.79)	5.18 (4.93- 5.46)	5.48 (4.99- 5.90)	5.97 (4.60- 7.29)
Alanine	16.1 (15.3 -16.5)	15.8 (15.8 -16.0)	15.8 (15.3 -16.0)	19.1 (18.5 -19.6)
Cysteic Acid	0.18 (0.10- 0.30)	0.19 (0.11- 0.27)	0.11 (0.07- 0.15)	0.0
Valine	5.88 (5.71- 5.9)	4.85 (3.52- 6.02)	5.95 (5.82- 6.03)	4.37 (4.05- 4.72)
Methionine	1.06 (1.04- 1.22)	1.14 (0.93- 1.35)	1.03 (1.01- 1.04)	1.15 (1.12- 1.19)
Isoleucine	4.82 (4.76- 4.83)	5.07 (5.06- 5.08)	4.23 (3.31- 5.17)	4.60 (4.50- 4.70)
Leucine	6.65 (6.34- 6.76)	7.10 (7.09- 7.11)	6.97 (6.92- 6.98)	6.21 (6.15- 6.26)
Tyrosine	1.94 (1.66- 2.23)	2.38 (2.35- 2.42)	2.24 (2.16- 2.30)	1.83 (1.67- 2.00)
Phenylalanine	2.82 (2.71- 2.86)	3.14 (3.10- 3.17)	3.24 (3.13- 3.31)	2.77 (2.73- 2.81)
% Protein	67.4	62.3	59.6	59.45
uM Hexosamine/ 100 ug protein	0.028 (.027-.028)	0.025 (.022-.027)	0.018 (.014-.022)	0.027 (.023-.031)

therefore were minimal values, especially since the laboratory relative humidity varied daily and all samples were not weighed under identical conditions. Insoluble residues were at a maximum 5% water by weight.

Alanine, glutamic acid, lysine, and aspartic acid were the four amino acids of highest concentration in SCM and the other 4 insoluble residues analyzed (TE-LIS-CM-Ppt., TE-GLCM-Ppt., LIS-BUT-TE-CM-Ppt., and BUT-LIS-TE-CM-Ppt.), and composed an average 43% of the total amino acid content of the insoluble residues. Aspartic acid, glutamic acid, leucine, and alanine composed 45% of the total amino acid residues of the soluble components extracted from SCM. Allisoleucine was detected in SCM, TE-CM, and BUT-TE-CM. Insoluble components were higher in protein content than soluble fractions, and ranged from 67.4% for TE-LIS-CM-Ppt. to 22.8% for LIS-BUT-TE-CM. Hexosamine was present in every fraction. Concentrations of hexosamine were highest in insoluble residues, and ranged from 0.028 uM hexosamine/100 ug protein in TE-LIS-CM-Ppt. to 0.002 uM hexosamine/100 ug protein in LIS-TE-CM.

As reported in Table 5, SCM was 70.5% protein by weight. Amino acids present in highest concentration in SCM were alanine (11.3 uM/100 uM), glutamic acid (10.4), aspartic acid (9.82), and lysine (8.11). Amino acids present in lowest concentration were methionine (0.19 uM/100 uM), cysteic acid (0.46), histidine (1.91), proline (2.24), and

Table 5. Amino acid analyses of SCM and of soluble components extracted from SCM. Concentrations of amino acids are reported as uM/100 uM amino acids. Each analyses represents the average of at least three separate analyses.

Sample:	SCM	TE-CM	BUT-TE-CM
Lysine	8.11 (8.10- 8.12)	5.24 (4.90- 5.70)	4.65 (4.34- 4.91)
Histidine	1.91 (1.49- 2.29)	1.22 (1.05- 1.38)	1.05 (1.02- 1.08)
Ammonia	9.52 (8.89-10.5)	10.2 (9.91-10.9)	7.60 (7.10- 7.77)
Arginine	4.23 (4.19- 4.69)	3.29 (3.02- 3.59)	3.24 (2.87- 3.42)
Aspartic Acid	9.82 (9.44-10.2)	12.3 (12.05-13.1)	12.4 (12.01-12.6)
Threonine	5.31 (5.19- 5.43)	6.05 (5.96- 6.33)	5.60 (5.42- 5.68)
Serine	4.15 (4.02- 4.28)	4.42 (4.15- 4.82)	4.56 (4.01- 5.31)
Glutamic Acid	10.4 (10.1 -11.6)	11.4 (11.1 -12.2)	12.1 (11.7 -12.2)
Proline	2.24 (1.81- 3.31)	2.78 (2.54- 3.02)	2.41 (1.98- 2.86)
Glycine	6.86 (6.48- 7.24)	6.185 (6.05- 6.54)	7.08 (6.58- 7.51)
Alanine	11.3 (11.1 -11.6)	7.69 (7.30- 8.49)	8.94 (7.96-10.0)
Cysteic Acid	0.46 (0.36- 0.55)	0.40 (0.21- 0.61)	0.17 (0.15- 0.19)
Valine	7.03 (7.02- 7.04)	7.40 (7.30- 7.72)	9.22 (8.61- 9.60)
Methionine	0.19 (0.17- 0.30)	-- Trace --	1.54 (1.37- 1.78)
Isoleucine	5.71 (5.60- 5.87)	6.73 (6.23- 6.79)	6.25 (6.22- 6.28)
Leucine	7.50 (7.16- 7.60)	8.83 (8.25- 9.72)	7.59 (7.51- 7.60)
Tyrosine	2.27 (2.01- 2.47)	2.22 (2.19- 2.22)	1.92 (1.84- 1.97)
Phenylalanine	2.85 (2.29- 3.13)	3.57 (3.52- 3.57)	3.47 (3.27- 3.57)
Allisoleucine	0.11 (0.08- 0.15)	-- Trace --	0.10 (0.06- 0.13)
% Protein	70.5	54.2	57.8
uM Hexosamine/ 100 ug Protein	0.0085 (0.0084-0.0086)	0.007 (0.005-0.0085)	0.0032 (0.0032)

Table 5. (Continued) Amino acid analyses of SCM and of soluble components extracted from SCM. Concentrations of amino acids are reported as uM/100 uM amino acids. Each analyses represents the average of at least three separate analyses.

Sample:	TE-GLCA	LIS-TE-CM	LIS-BUT-TE-CM
Lysine	6.07 (5.92- 6.15)	7.46 (6.81- 7.88)	5.37 (5.15- 5.59)
Histidine	1.38 (1.06- 1.66)	1.99 (1.84- 2.08)	2.145 (1.96- 2.33)
Ammonia	8.09 (7.39- 8.39)	12.9 (11.9 -13.4)	10.7 (9.73-11.66)
Arginine	4.09 (3.86- 4.12)	4.24 (4.15- 4.66)	4.29 (4.20- 4.38)
Aspartic Acid	10.9 (10.2 -11.3)	9.45 (9.04- 9.65)	10.1 (9.87-10.2)
Threonine	5.61 (5.40- 5.75)	4.88 (4.56- 5.19)	5.39 (5.22- 5.59)
Serine	4.65 (4.50- 4.75)	4.48 (4.15- 4.67)	4.73 (4.35- 5.11)
Glutamic Acid	11.2 (10.0 -12.0)	9.38 (8.99- 9.54)	10.8 (9.85-11.8)
Proline	3.00 (2.78- 3.08)	2.87 (2.65- 3.01)	2.69 (2.68- 2.70)
Glycine	6.52 (5.75- 6.69)	6.34 (6.25- 6.36)	7.31 (6.74- 7.88)
Alanine	7.58 (7.36- 7.64)	7.57 (7.19- 7.72)	7.125 (7.05- 7.20)
Cysteic Acid	0.20 (0.20- 0.25)	0.13 (0.09- 0.14)	0.045 (0.01- 0.09)
Valine	6.88 (5.92- 7.46)	5.64 (5.12- 6.00)	6.54 (6.08- 6.99)
Methionine	2.21 (2.19- 2.25)	1.24 (1.11- 1.56)	1.57 (1.36- 1.90)
Isoleucine	6.38 (6.15- 6.42)	6.40 (6.16- 6.56)	6.285 (6.25- 6.32)
Leucine	9.05 (8.94- 9.23)	8.99 (8.72- 9.28)	8.51 (8.11- 8.75)
Tyrosine	2.26 (2.14- 2.57)	2.15 (2.08- 2.22)	2.39 (2.18- 2.65)
Phenylalanine	3.98 (3.73- 4.07)	3.87 (3.67- 3.945)	4.04 (3.70- 4.38)
Allisoleucine	0.0	0.0	0.0
% Protein	24.8	32.4	22.8
uM Hexosamine/ 100 ug Protein	0.0025 (0.0025)	0.002 (0.00145-0.00255)	0.005 (0.0038-0.0065)

tyrosine (2.27). Allisoleucine was detected in SCM (0.11 uM/100 uM).

TE-LIS-CM-Ppt. (the precipitate resultant from LIS extraction of SCM, dialyzed vs. TE buffer) and TE-GLCM-Ppt. (the interfacial fluff resultant from Genetron extraction of GLCM, dialyzed vs. TE buffer) were similar in protein content (67.4% and 62.3% by weight, respectively), hexosamine content (0.028 uM hexosamine/100 ug protein and 0.025 uM hexosamine/100 ug protein, respectively), and amino acid content. Alanine, glutamic acid, lysine, and aspartic acid were the amino acids of highest concentration in both insoluble residues; and cysteic acid, methionine, histidine, proline, and tyrosine were amino acids of lowest concentration in both insoluble residues. Average concentrations of eight (arginine, aspartic acid, serine, glutamic acid, alanine, cysteic acid, methionine, and isoleucine) out of the seventeen amino acid residues determined on analysis were within 5% agreement.

Close agreement between respective concentrations of individual amino acids was not found between LIS-BUT-TE-CM-Ppt. (the insoluble residue from successive extraction of SCM with TE buffer, butanol, and LIS) and BUT-LIS-TE-CM-Ppt. However, the two insoluble residues were similar in protein content (59.6% and 59.45% by weight, respectively). In addition, the amino acids of highest concentration (alanine, glutamic acid, and lysine) and of lowest concentration (cys-

teic acid, methionine, histidine, proline, and tyrosine) were the same in both insoluble residues.

TE-GLCA was composed of 24.8% protein by weight. Glutamic acid (11.2 uM/100 uM), aspartic acid (10.9), leucine (9.05), and alanine (7.58) were amino acids present in highest concentration (see Table 5). Cysteic acid (0.20 uM/100 uM), histidine (1.38), methionine (2.21), tyrosine (2.26), and proline (3.00) were amino acids present in lowest concentration.

TE-CM was composed of 54.2% protein by weight. Aspartic acid (12.3 uM/100 uM), glutamic acid (11.4), leucine (8.83), alanine (7.69), and valine (7.40) were amino acids present in highest concentration (see Table 5). Methionine (trace), cysteic acid (0.40 uM/100 uM), histidine (1.22), tyrosine (2.22), and proline (2.78) were amino acids present in lowest concentration.

BUT-TE-CM was composed of 57.8% protein by weight. Aspartic acid (12.4 uM/100 uM), glutamic acid (12.1), valine (9.22), alanine (8.94), and leucine (7.59) were amino acids in highest concentration (see Table 5). Cysteic acid (0.17 uM/100 uM), histidine (1.05), methionine (1.54), and tyrosine (1.92) were the amino acids in lowest concentration.

LIS-TE-CM was 32.4% protein by weight. Aspartic acid (9.45 uM/100 uM), glutamic acid (9.38), leucine (8.99), alanine (7.57), and lysine (7.46) were amino acids present in highest concentration (see Table 5). Methionine (1.24

uM/100 uM), cysteic acid (0.13), histidine (1.99), and tyrosine (2.15) were amino acids present in lowest concentration. LIS-TE-CM possessed the lowest hexosamine content (0.002 uM/100 ug protein) of the soluble components extracted from SCM.

LIS-BUT-TE-CM was composed of 22.8% protein by weight. Glutamic acid (10.8 uM/100 uM), Aspartic acid (10.1), leucine (8.51), and alanine (7.125) were amino acids present in highest concentration (see Table 5). Cysteic acid (0.045 uM/100 uM), methionine (1.57), histidine (2.145), tyrosine (2.39), and proline (2.69) were amino acids present in lowest concentration in LIS-BUT-TE-CM.

G. Hexose Concentration in Soluble Components Extracted from SCM.

The hexose concentration in TE-GLCA, TE-CM, LIS-TE-CM, and LIS-BUT-TE-CM was (w/w): 0.4%, 1.4%, 0.8%, and 0.9%, respectively.

H. Immunodiffusion Analysis of Soluble SCM Components in Triton-Agarose Gel.

Soluble components extracted from SCM precipitated with antisera prepared against both homologous and heterologous SCM preparations. In general, the soluble components displayed a maximum of two lines of precipitation with a given antiserum. Antigens either failed to react with a given antiserum, or formed lines of identity with antigens

in neighboring wells. Lines of non-identity or partial identity were not observed between the soluble SCM components utilized in this study. None of the soluble SCM components displayed lines of precipitation with any of the anti-glomerular basement membrane sera tested. Soluble SCM components were tested against a number of antisera prepared against the same immunogen. In the discussion that follows, reference to a positive reaction of a particular antiserum with a given component does not imply that the component reacted with all antisera of that specificity. Contrariwise, reference to a negative reaction of a component with a given antiserum specificity implies that all antisera of that specificity failed to react with the component unless stated otherwise. The following is a summary of the reactions of each soluble component with various antisera.

TE-GLCA precipitated with antisera prepared against the following components, forming one line of precipitation; SLS-CM, LIS-CM, G-LIS-CM, TE-GLCA, and BUT-TE-CM. TE-GLCA formed two lines of precipitation with four different anti-whole SCM sera, as displayed in Fig. 11. In general, anti-sera forming one line of precipitation with TE-GLCA displayed identity with one of the lines of precipitation formed by reaction of TE-GLCA with anti-whole SCM sera. TE-GLCA did not precipitate with antisera prepared against the following insoluble SCM components; LIS-CM-Ppt. (see Fig. 11),

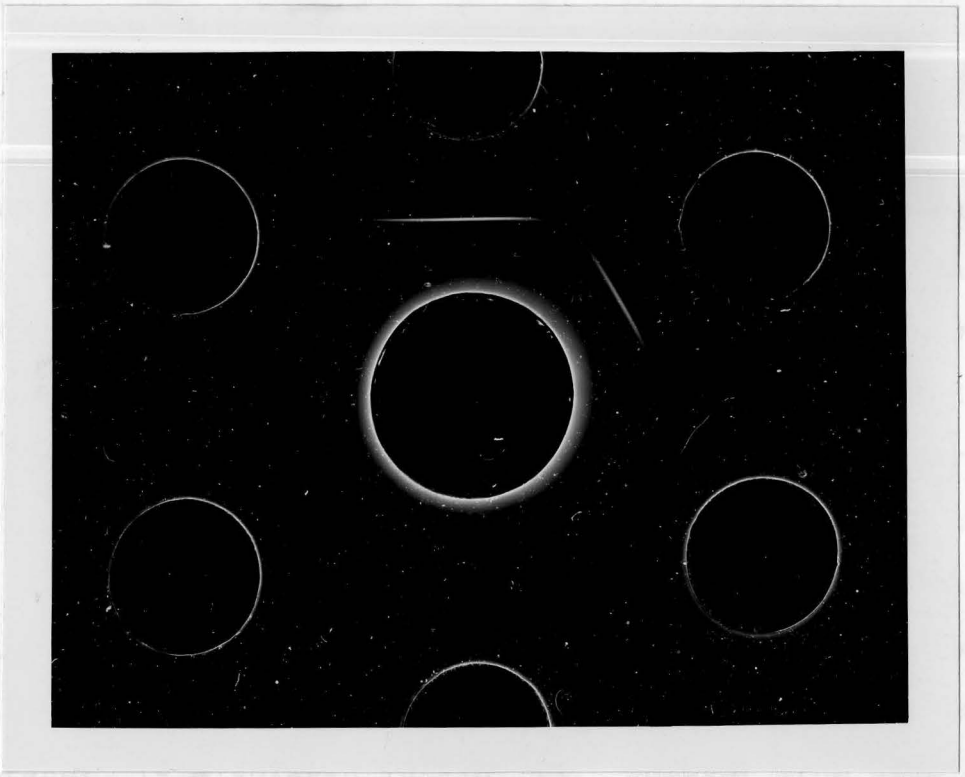

Fig. 11. Reaction of TE-GLCA (center well) with anti-whole SCM sera, forming two distinct lines of identity. Contents of outer wells: 1) AG-1 anti-SCM, 2) AG-2 anti-SCM, 3) AG-3 anti-SCM, 4) AG-4 anti-SCM, 5) anti-LIS-CM-Ppt., and 6) anti-GLCM. TE-GLCA did not react with anti-LIS-CM-Ppt. and formed one line of precipitation with anti-GLCM.

G-LIS-CM-P, trypsin solubilized TE-LIS-CM-Ppt., and the insoluble residues left from extraction of SCM with TE buffer, LIS, and butanol.

Fraction II, eluted at pH 5.0-5.5 from DEAE cellulose chromatography of TE-GLCA, reacted with anti-LIS-CM sera forming one line of identity between 3 anti-LIS-CM sera, a line of identity between an anti-LIS-CM serum and an anti-SLS-CM serum, and two distinct lines of precipitation with two anti-SLS-CM sera (see Fig. 12). Fraction I, eluted in the equilibration buffer from DEAE cellulose chromatography of TE-GLCA was not antigenic with any of the antisera tested. The reaction of an antiserum, prepared against Fraction II, with PGT-GBM (a soluble component obtained from Genetron extraction of a trypsin digest of human glomerular basement membrane), is displayed in Fig. 13. However, the same anti-Fraction II serum displayed no reactivity with either the parent immunogen or any other soluble SCM component.

The first fraction from Sephadex G-50 chromatography of GLCM displayed reactivity with anti-whole SCM sera and anti-GLCM sera. Fraction II from Sephadex chromatography of GLCM was not antigenic with any of the sera tested.

TE-CM reacted with antisera against the following SCM components, forming 2 lines of precipitation: SCM, SLS-CM, a soluble component from deoxycholate extraction of SCM (DOC-CM), LIS-CM, TE-GLCA, and BUT-TE-CM. Antisera prepared against the following SCM components formed a single

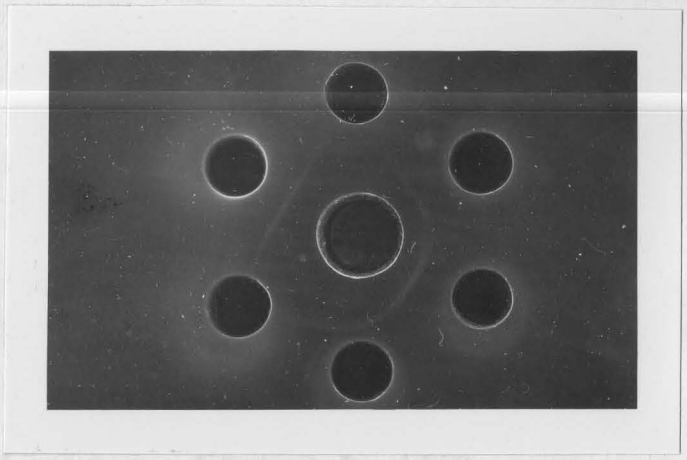

Fig. 12. Reaction of Fraction II from DEAE cellulose chromatography of TE-GLCA (center well) with various antisera. Contents of outer wells: 1) anti-Fraction II, 2) #1169 anti-PNG-LIS-CM (Genetron extract of a pronase digestion of LIS-CM), 3) anti-LIS-CM, 4) #1168 anti-PNG-LIS-CM, 5) #22 anti-SLS-CM, and 6) A-38 anti-SLS-CM. A line of identity was formed between wells: 2, 3, and 4; and wells 4 and 5 (not visible in print). The line of precipitation between the antiserum and well #6 was distinct from the line of identity between wells 4 and 5.

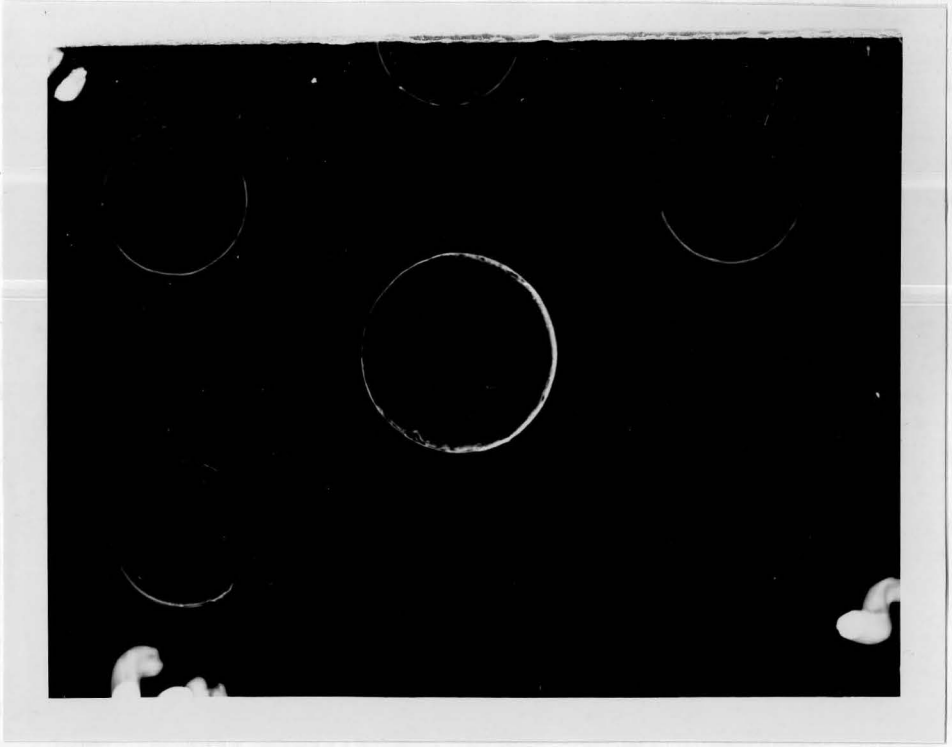

Fig. 13. Reaction of a soluble component of human glomerular basement membrane (GBM), PGT-GBM (obtained by digestion of GBM with trypsin followed by extraction with Genetron), with various antisera. Numbered wells contained antisera prepared against the following components: 1) Fraction II from DEAE cellulose chromatography of TE-GLCA, 2) #1169 PNG-LIS-CM, 3) LIS-CM, 4) #1168 PNG-LIS-CM, 5) #22 SLS-CM, and 6) A-38 SLS-CM. Anti-Fraction II produced three lines of precipitation with PGT-GBM; there was no reaction between PGT-GBM and the other antisera tested.

line of precipitation with TE-CM: GLCM, TE-CM, LIS-BUT-TE-CM, LIS-TE-CM, and LIS-BUT-TE-CM-Ppt. #16. TE-CM did not precipitate with antisera prepared against: trypsin solubilized TE-LIS-CM-Ppt., BUT-LIS-TE-CM-Ppt., or LIS-BUT-TE-CM #17 and #18.

BUT-TE-CM displayed two lines of precipitation with antisera prepared against the following components; SCM, GLCM, and SLS-CM. Immunological testing of BUT-TE-CM was restricted by the limited quantity of available antigen.

LIS-BUT-TE-CM displayed a single line of precipitation with antisera prepared against the following SCM components: SCM, GLCM, LIS-CM, TE-CM, LIS-BUT-TE-CM, and LIS-BUT-TE-CM-Ppt. #16. LIS-BUT-TE-CM displayed two lines of precipitation with two antisera prepared against BUT-TE-CM. LIS-BUT-TE-CM did not display reactivity with antisera prepared against: trypsin solubilized TE-LIS-CM-Ppt., BUT-LIS-TE-CM-Ppt., LIS-BUT-TE-CM-Ppt. #17 and #18, DOC-CM, SLS-CM, and LIS-TE-CM.

LIS-TE-CM displayed two lines of precipitation with antisera prepared against GLCM, TE-CM, and LIS-BUT-TE-CM. LIS-TE-CM displayed one line of precipitation with antisera prepared against LIS-CM, SLS-CM, LIS-TE-CM, and LIS-BUT-TE-CM-Ppt. #16. LIS-TE-CM displayed no reactivity with antisera prepared against LIS-BUT-TE-CM-Ppt. #17 and #18, and DOC-CM.

Examples of reactions of identity between soluble components are displayed in Fig. 14 and Fig. 15. Fig. 14

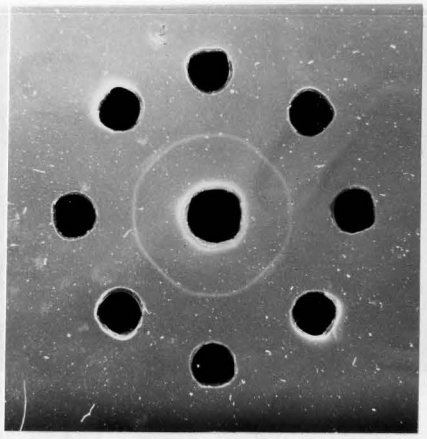

Fig. 14. Reaction of soluble components extracted from SCM with an antiserum prepared against GLCM. Well: 1) BUT-TE-CM, 2) LIS-BUT-TE-CM, 3) LIS-TE-CM, 4) TE-CM, 5) LIS-TE-CM, 6) BUT-TE-CM, 7) TE-GLCA, and 8) TE-CM. A line of identity was formed between all the components; TE-CM displayed an additional line of precipitation with the antisera.

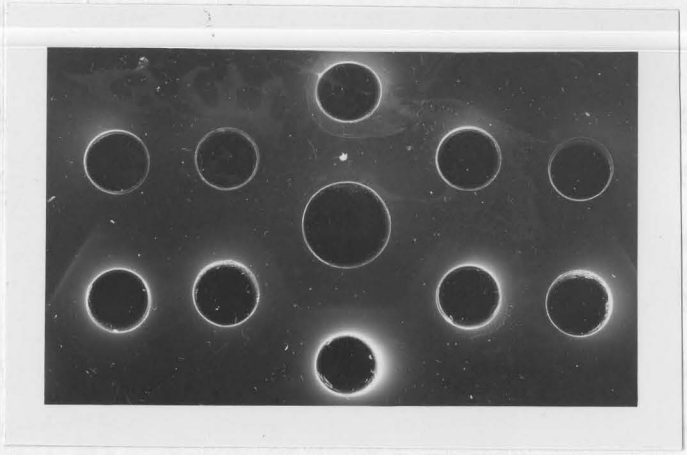

Fig. 15. Demonstration of the immunological relatedness of LIS-TE-CM, LIS-BUT-TE-CM, and SLS-CM. Precipitation with an anti-LIS-TE-CM serum produced a line of identity between LIS-TE-CM (well 3), LIS-BUT-TE-CM (well 4) and SLS-CM (well 5). TE-CM (well 1), TE-GLCA (well 2), and trypsin solubilized GBM (well 6) gave no reaction with the antiserum.

displays a line of identity resultant from precipitation of an anti-GLCM serum and TE-CM, TE-GLCA, LIS-TE-CM, BUT-TE-CM, and LIS-BUT-TE-CM. Fig. 15 displays the line of identity formed by precipitation of anti-LIS-TE-CM serum with LIS-TE-CM, LIS-BUT-TE-CM, and SLS-CM.

I. Immunoelectrophoretic Analysis in Agarose Gel of Soluble Components Extracted from SCM. Fig. 16 displays results of a typical immunoelectrophoretic analysis of various soluble components extracted from SCM. Following electrophoresis of samples, immunodiffusion of the components was accomplished vs. an anti-whole SCM serum (AG-3) and an anti-GLCM serum (AG-10). The soluble components examined demonstrated the presence of one or two constituents. One constituent had no apparent electrophoretic mobility under the conditions of the analysis; the other constituent had an anodal mobility slower than human serum albumin. Identity between the two constituents was not apparent in those soluble components which contained both constituents.

As displayed in Fig. 16, TE-CM and BUT-TE-CM displayed both components vs. the AG-10 serum, and displayed only the component with anodal mobility vs. the AG-3 serum. LIS-BUT-TE-CM and LIS-TE-CM displayed only the non-mobile component vs. both antisera. TE-GLCA displayed only the component with anodal mobility vs. both antisera. SLS-CM displayed the non-motile component vs. the AG-10 serum, and was non-reactive with the AG-3 serum.

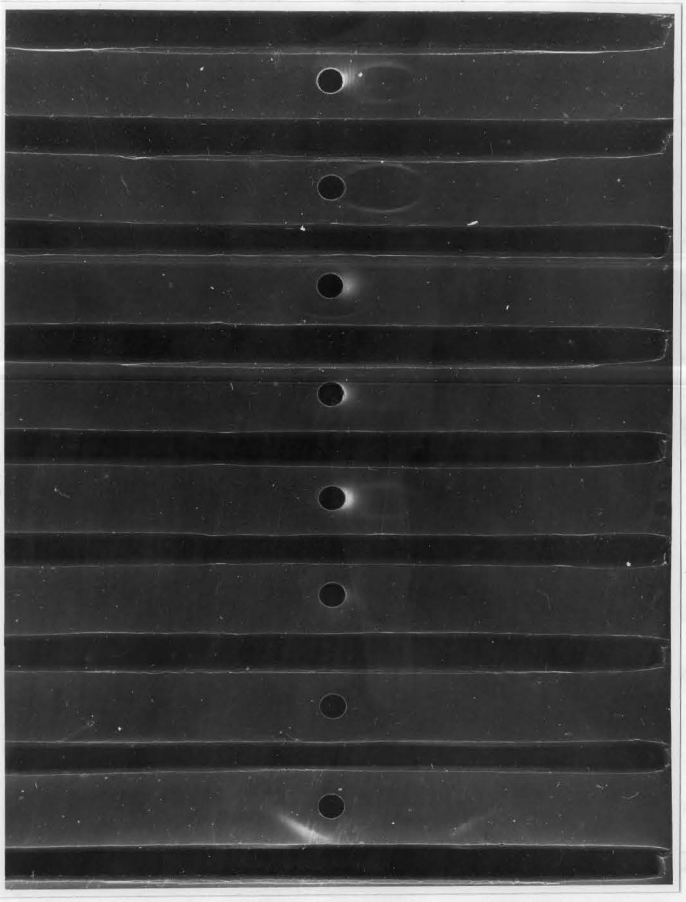

Fig. 16. Immunoelectrophoretic analysis in agarose gel of soluble components extracted from SCM. Gels were composed of 2.0% agarose w/v in 0.01 M Tris-HCl buffer, pH 8.7 with 1.0 M urea and 2.0% v/v Triton X-100. Electrophoresis was run for 5 h at 90 volts, 3 mA/gel, and was followed by immunodiffusion vs. antisera for 48 h in a moist chamber at room temperature. The anode was to the right of the figure, the cathode to the left. Wells, from top to bottom, contained: TE-CM, BUT-TE-CM, LIS-BUT-TE-CM, LIS-TE-CM, TE-GLCA, SLS-CM, human gamma globulin (HGG), and human serum albumin (HSA). Troughs, from top to bottom, contained: anti-SCM (AG-3), anti-GLCM (AG-10), AG-3, AG-10, AG-3, AG-10, AG-3, anti-HGG, and anti-HSA. Up to two of the same, identical constituents were observed in each soluble component. One constituent was non-mobile, the other had an anodal mobility slower than albumin. BUT-TE-CM and TE-CM displayed both constituents vs. AG-10, but displayed only the constituent with anodal mobility vs. AG-3. LIS-BUT-TE-CM and LIS-TE-CM displayed only the non-mobile constituent vs. both antisera. TE-GLCA displayed only the constituent with anodal mobility vs. both antisera. SLS-CM was not reactive with either antiserum.

J. Polyacrylamide Gel Electrophoresis of Soluble Components Extracted from SCM. Initially, PAGE of soluble components extracted from SCM was accomplished in 7.0% w/v acrylamide gels by the method of Davis and Ornstein (13). Displayed in Fig. 17 are results of PAGE on LIS-CM (LIS solubilized material of SCM), GLCM (soluble aqueous phase material from Genetron extraction of SCM), GLCA (soluble aqueous phase material from Genetron extraction of GLCM), and TE-GLCA (soluble material resultant from dialysis of GLCA vs. TE buffer). Essentially the same pattern of staining was observed for each extract; a band which migrated slightly ahead of the bromphenol blue dye front, and a band at the interface of the stacking and running gels. Although the band at the interface appeared to be in the running gel, 5.0% w/v acrylamide gels were run to determine if the material was actually excluded from the running gel due to the molecular size of the material. PAGE of the soluble components on 5% acrylamide gels produced the same staining pattern as that obtained on 7% acrylamide gels (data not shown). However, the exclusion of high m.w. components was still suspected (see below). PAGE of SLS-CM (soluble component obtained from sodium lauryl sulfate extraction of SCM), TE-CM (soluble component obtained by treatment of SCM with TE buffer), LIS-TE-CM (soluble component extracted with LIS from TE treated SCM), BUT-TE-CM (soluble component extracted with butanol from TE treated SCM), and LIS-BUT-TE-CM (soluble

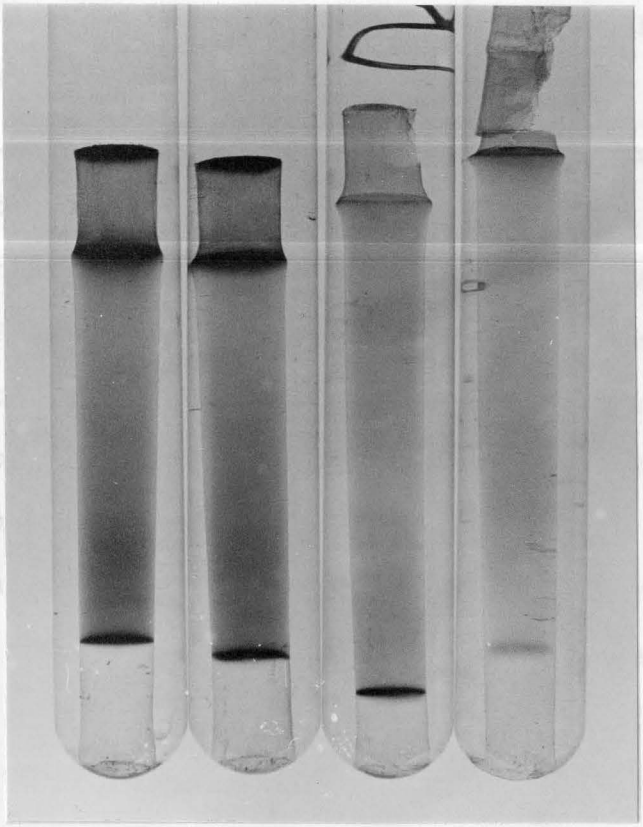

Fig. 17. PAGE in 7% w/v acrylamide gels of TE-GLCA and soluble components produced during the isolation of TE-GLCA. From left to right: 1) LIS-CM, 2) GLCM, 3) GLCA, and 4) TE-GLCA. A component at the interface of the stacking and running gels and a component migrating slightly ahead of the tracking dye were observed in all four fractions. Gels were stained with Napthalene Black.

component extracted with LIS from butanol and TE buffer treated SCM) on 5% acrylamide gels produced the identical staining pattern of 1 band migrating slightly ahead of the bromphenol blue dye front and a band at the interface of the stacking and running gels (data not shown).

The assumption was made that the electrophoretic pattern on PAGE of the soluble materials was not a true reflection of their composition, but may rather have been the result of the formation of large molecular weight aggregates in aqueous solution from the individual components comprising a given soluble material. Therefore, PAGE of the soluble components was run in the presence of Triton X-100 in 5% acrylamide gels. The inclusion of Triton was designed to disrupt the aggregates, thus allowing the individual components to enter the running gel. Typical results of Triton-PAGE are displayed in Fig. 18A. All soluble components possessed a constituent (marked with metal wire in TE-CM, tube #1) migrating faster than the bromphenol blue tracking dye (marked with wire in SLS-CM, tube #3). All components possessed a constituent at the interface of the running gel and stacking gels; however the constituent was virtually absent in BUT-TE-CM (tube #6).

Due to the diffuse staining in some of the gels, which made visualization of bands in the photographic print difficult, Fig. 18B was included to highlight certain constituents in the gels. LIS-TE-CM (tube #2), SLS-CM (#3),

Fig. 18A. Triton-Page in 5% w/v acrylamide gels of various soluble components extracted from SCM. From left to right, tube: #1 TE-CM, #2, LIS-TE-CM; #3, SLS-CM; #4, TE-GLCA; #5, LIS-BUT-TE-CM; and #6, BUT-TE-CM. All components possessed a constituent migrating slightly ahead of the tracking dye (positioned by the wire in tube #3). A band at the interface between the stacking and running gels was virtually absent in BUT-TE-CM, but present in all five of the other components. Gels were stained with Coomassie Blue.

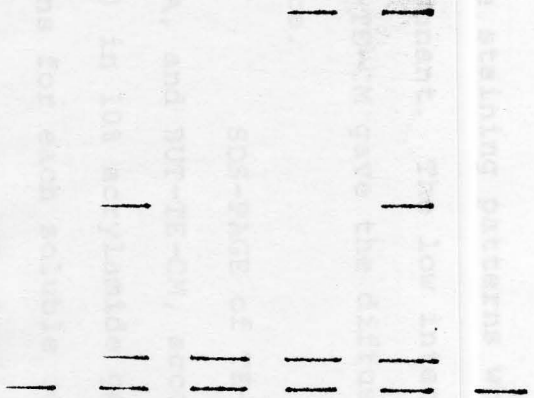

Fig. 18B. Diagram illustrating major constituents shared between the six soluble components.

TE-GLCA (#4), and LIS-BUT-TE-CM (#5) shared a band in the alpha region and a band in the gamma region. Both constituents were absent in TE-CM (#1) and BUT-TE-CM (#6). TE-CM, LIS-TE-CM, LIS-BUT-TE-CM, TE-GLCA, and SLS-CM displayed diffuse staining patterns which were characteristic for each component. The low intensity of the diffuse staining in BUT-TE-CM gave the diffuse staining a questionable significance.

SDS-PAGE of TE-CM, LIS-TE-CM, LIS-BUT-TE-CM, TE-GLCA, and BUT-TE-CM, accomplished by the method of Laemmli (35) in 10% acrylamide gels, produced multiple banding patterns for each soluble component (see Fig. 19). The number of bands made calculation of molecular weights of individual constituents an impossible task. The intense staining at the interface of the stacking and running gels suggested the possibility that LIS-TE-CM was apparently not completely disaggregated by treatment with mercaptoethanol and SDS. LIS-TE-CM, TE-GLCA, LIS-BUT-TE-CM, and TE-CM contained constituents of at least 67,000 daltons as evidenced by comparison of the position of bands in the soluble components with the position of BSA in Fig. 19. Comparison of the position of the heavy chain of HGG with banding pattern of BUT-TE-CM revealed the absence of constituents in BUT-TE-CM with m.w. greater than 50,000 daltons.

All five soluble components had constituents which migrated with the tracking dye. In order to determine if a

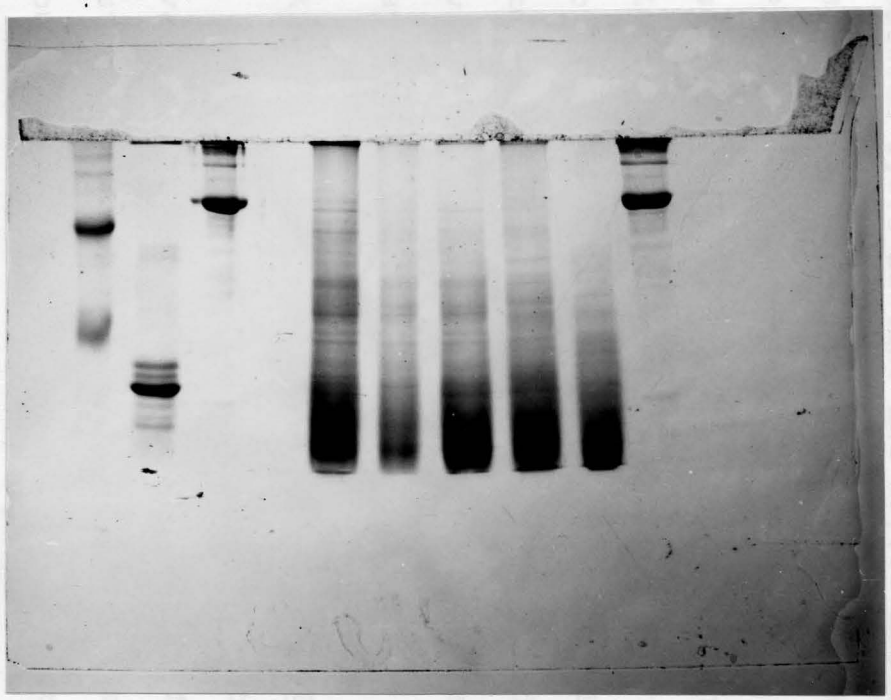

Fig. 19. SDS-PAGE of soluble components extracted from SCM in 10% w/v acrylamide gels by the procedure of Laemmli (35). Samples were, from left to right: human gamma globulin (HGG) (light chain m.w. 25,000 daltons, heavy chain 50,000 daltons), horse heart cytochrome C (12,400 daltons), bovine serum albumin (BSA) (67,000 daltons), LIS-TE-CM, TE-GLCA, LIS-BUT-TE-CM, TE-CM, BUT-TE-CM, and BSA. LIS-TE-CM, TE-GLCA, LIS-BUT-TE-CM, and TE-CM contained constituents of at least 67,000 daltons. No constituent was present in BUT-TE-CM with m.w. greater than 50,000 daltons. The fastest migrating constituent in each soluble component migrated with the tracking dye. The gel was stained with Coomassie Blue.

low molecular weight constituent was present in the components, the SDS-PAGE system of Swank and Munkres (60) was utilized. The incorporation of 8.0 M urea into 12.5% acrylamide gels was designed to decrease the pore size of the gels and thus increase the separation of peptides with m.w. between 1,000 daltons and 10,000 daltons. A low m.w. constituent was observed utilizing the Swank and Munkres SDS-PAGE system. However, the resolution of higher m.w. constituents was poor, as evidenced by the smearing behind the leading low m.w. constituent observed in Fig. 20. The m.w. of the low m.w. constituent was calculated to be 4,000 daltons. Measurements of m.w. for peptides below 10,000 daltons with the Swank and Munkres system were considered to have a standard deviation of 18%.

K. Thin Layer Chromatography of Lipid Extracts

Direct comparison of individual constituents in various lipid extracts of SCM was difficult. R_f values of standards varied between chromatograms and all three (the butanol extract of SCM, and the Genetron extracts of LIS-CM and GLCM) lipid extracts analyzed were never chromatographed on the same plate since they were obtained at different periods of the study. Therefore, although identity between constituents of individual extracts could not be established, several similarities between the three extracts were observed. Table 6 contains average R_f values for standards,

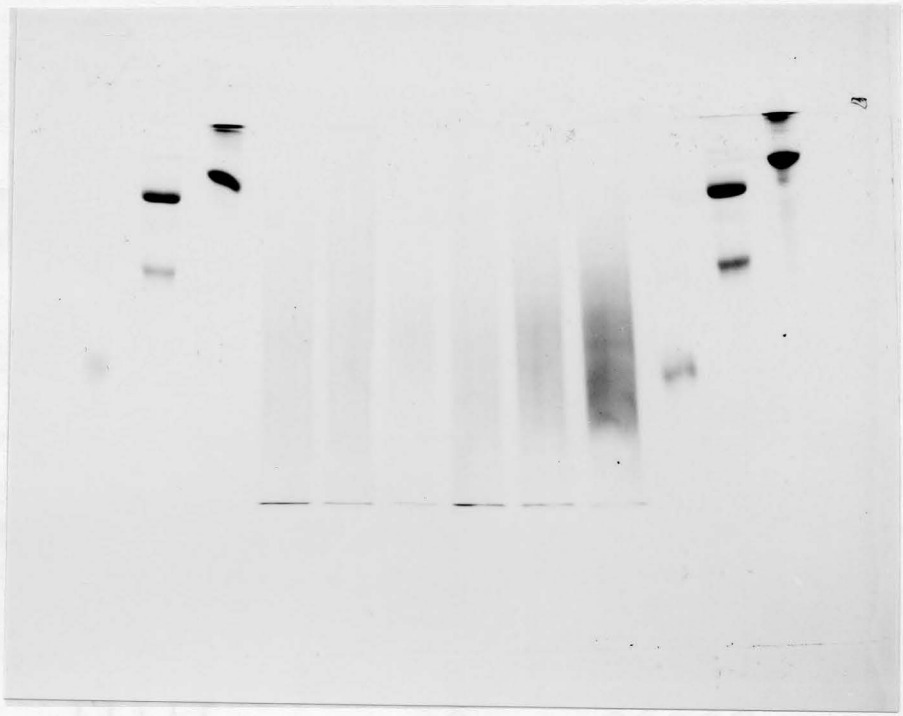

Fig. 20. SDS-PAGE, in 12.5% w/v acrylamide gel containing 8.0 M urea, of soluble components extracted from SCM. Samples were, from left to right, cytochrome C, HGG, BSA, LIS-BUT-TE-CM, LIS-TE-CM, BUT-TE-CM, TE-CM, TE-GLCA, SLS-CM, cytochrome C, HGG, and BSA. The fastest moving constituent present in every soluble component was calculated to have a m.w. of 4,000 daltons. Considerable smearing of higher m.w. constituents occurred in the system. Arrow designates tracking dye front. Staining was with Coomassie Blue.

Table 6. R_f values for standards, and lipid constituents in the Genetron extract of GLCM. Thin layer chromatography was accomplished on glass plates coated with Silica Gel G, utilizing a developing solvent consisting of petroleum ether/ether/acetic acid (90/10/1). Range of R_f values for each lipid are in parenthesis.

Standards:	R_f
Lecithin	Origin
Cholesterol	0.062 (0.049-0.072)
Palmitic Acid	0.194 (0.192-0.195)
Palmitoleic Acid	0.218 (0.216-0.219)
Tripalmitin	0.238 (0.223-0.246)
Cholesterol Palmitate	0.615 (0.608-0.631)

Lipid constituents in the Genetron extract of GLCM:

Constituent #	R_f
1	Origin
2	0.031 (0.027-0.033)
3	0.046 (0.044-0.051)
4	0.146 (0.132-0.153)
5	0.238 (0.216-0.245)
6	0.638 (0.638-0.639)

and unknown lipid components in the Genetron extract of GLCM. Data on the Genetron extract of GLCM typified the data obtained for all three extracts; therefore statements on the lipid composition of the Genetron extract of GLCM generally held true for the other two extracts.

Mobilities of the general lipid classes in the solvent utilized (petroleum ether/ether/acetic acid, 90/10/1) were, in order of increasing R_f value: phospholipids (at the origin), 1- and 2-monoglycerides, cholesterol, 1,2- and 1,3-diglycerides, fatty acids, triglycerides, and cholesterol esters. All three lipid extracts contained six distinguishable components. A component at the origin, which stained specifically for phospholipid with molybdenum blue, was present in all three extracts. Comparison of R_f values recorded in Table 6 for lipid components in the Genetron extract of GLCM with R_f values for standards, suggested the presence of the following lipid classes in the extract: two monoglycerides (R_f values 0.031 and 0.046), a diglyceride (R_f 0.146), and a component which was either a triglyceride or an unsaturated fatty acid (R_f 0.238). A definite identification of the latter component could not be made due to the wide range of R_f values for the unknown (R_f 0.216-0.245) and standards (palmitoleic acid, R_f 0.216-0.219, tripalmitin, R_f 0.223-0.246). A sixth component with an R_f value (0.638) higher than cholesterol palmitate (0.615), suggested the presence of hydrocarbons in the extract. A similar compo-

nent with an R_f value higher than cholesterol palmitate was present in the butanol extract of SCM and the Genetron extract of LIS-CM.

Although the resolution of individual phospholipid standards was poor in the solvent utilized (diisobutyl ketone/formic acid/water, 40/15/2), three individual phospholipid components were distinguishable in the Genetron extract of GLCM (and in the other two lipid extracts). R_f values for standards were: 0.192 (phosphatidyl choline, commonly lecithin), 0.208 (L-distearoyl-α-glycerol phosphoryl N, N-dimethyl thanolamine), and 0.217 (L-dipalmitoyl-α-glycerol phosphoryl N, N-dimethylethanolamine). R_f values for the three unknown phospholipid components in the Genetron extract of GLCM were 0.167, 0.233, and 0.375. All standards and unknown phospholipid components stained specifically for phospholipid with molybdenum blue.

L. Gas-Liquid Chromatography of Fatty Acids in Saponfied Lipid Extracts. Following saponification, the fatty acid compositions of various lipid extracts of SCM (the butanol extract of SCM, and the Genetron extracts of LIS-CM and GLCM) and of TE-GLCA were determined by gas-liquid chromatography. Concentrations of individual fatty acids were determined relative to a concentration of 1 unit for palmitic acid, which was the fatty acid of highest concentration in all the lipid extracts. Fatty acid compositions of the individual extracts are recorded in Table 7. A typical chroma-

Table 7. Fatty acid composition of various saponified lipid extracts of SCM and of the lipid fraction obtained from saponification of TE-GLCA. Values for individual fatty acids are reported relative to a concentration of 1 for palmitic acid, which was the fatty acid of highest concentration in every fraction.

Fatty Acid	Butanol Extract of SCM	Genetron Extract of LIS-CM	Genetron Extract of GLCM	Lipid Fraction from Saponified TE-GLCA
Lauric	0.01	0.10	0.006	0.08
Myristic	0.055	0.25	0.13	0.07
Palmitic	1	1	1	1
Palmitoleic	0.195	0.125	0	0.185
Stearic	0.15	0.38	0.26	0.13
Oleic	0.32	0.50	0.02	0.24
Linoleic	0	0.125	0.06	0

to gram, obtained from chromatography of the saponifiable lipids of TE-GLCA, is displayed in Fig. 21.

Lauric acid, myristic acid, palmitic acid, palmitoleic acid, stearic acid, and oleic acid were fatty acids presend in all four of the extracts examined. Linoleic acid was present in the Genetron extracts of LIS-CM and GLCM, but was absent in the butanol extract of SCM and the saponified lipid fraction from TE-GLCA. In general, with the exception of palmitic acid, unsaturated fatty acids comprised the majority of the fatty acid constituents of the extracts. The fatty acid composition of the butanol extract of SCM and saponified lipid fraction of TE-GLCA were in close agreement with the exception of lauric acid.

M. Chemical Analysis of TE-GLCA

The complete chemical characterization of one of the soluble components extracted from SCM was undertaken utilizing TE-GLCA. A problem which hampered the effort was the hygroscopicity of, in addition to TE-GLCA, the various weighing materials (weighing paper, aluminum "boats", porcelain crucibles) utilized. The rapid rate of water regain by the weighing materials presented a problem which could not be surmounted. Therefore, all data on percent compositions of various constituents in TE-GLCA must be considered to be minimum estimates.

Following dessication of TE-GLCA in a vaccuum des-

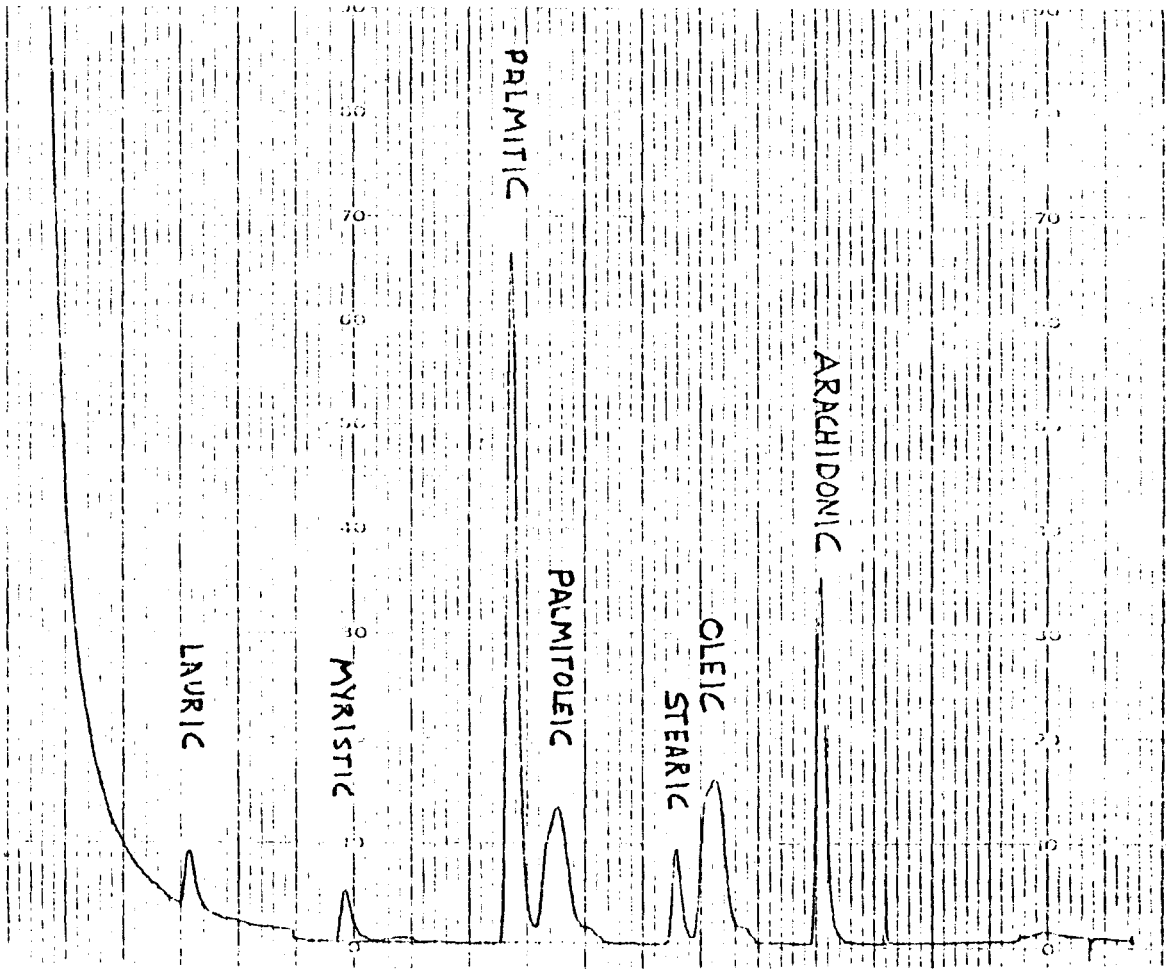

Fig. 21. Typical chromatograph resultant from gas-liquid chromatography of a saponified lipid fraction from TE-GLCA. Standards and unknown samples were analyzed as the methyl esters of their fatty acids on a Beckman Model GC 45 gas chromatograph. Identifiable fatty acids, from left to right, were: lauric, myristic, palmitic, palmitoleic, stearic, oleic, and arachidonic (included as an internal standard). The arachidonic acid peak represents the approximate (±0.5 ug) peak height for the 2.0 ug of the fatty acid.

sicator containing phosphorous pentoxide for 1 h at 110° C, the water content by weight in TE-GLCA was determined to be 19.6%. Table 8 contains data on the percent composition of various constituents in TE-GLCA based on the wet weight of TE-GLCA, which was the weight of TE-GLCA after exposure to the laboratory atmosphere overnight. TE-GLCA was found to contain (w/w) 24.8% protein (as determined by amino acid analysis), 0.4% hexose, 0.01% phosphorous, 4.1% lipid, 3.73% nitrogen, 11.76% water (as determined by Karl Fischer analysis), and 0.0% ash. Thus, of the apparent dry weight of TE-GLCA, the total analyses accounted for 41.06% of the starting weight of TE-GLCA. After neutralization, saponified TE-GLCA was passed over an Amberlite MB-3 ion exchange resin. Elution with water yielded no material in the neutral fraction. Likewise, no material was eluted in the acid hydrolyzed neutral fraction. Based on the percent nitrogen content, the percent protein composition of TE-GLCA was 23.3% w/w (ug nitrogen X 6.25 = ug protein), in close agreement with the figure obtained by amino acid analysis. Thus the possibility of a contribution by other nitrogenous substances, not detected by the methods utilized to chemically characterize TE-GLCA, was eliminated. The absence of either a significant neutral fraction or ash substance led to the conclusion that TE-GLCA was binding water resistant to dessication at 110° C, and was therefore an extremely hygroscopic material. This conclusion was supported by the significant water

Table 8. Chemical composition of TE-GLCA. Values for concentrations of constituents are reported on the basis of percent w/w and ug/100 ug protein.

Constituent	%w/w	ug/100 ug protein
Protein	24.8	100
Hexose (Hexosamine)	0.4 (0.089)	1.6 (0.358)
Phosphorous	0.01	0.04
Lipid	4.10	16.5
Nitrogen	3.73	15.04
Water	11.76	47.6
Ash	0.0	0.0

content of TE-GLCA, as determined by Karl Fischer analysis (11.76% w/w). Table 8 also contains precent compositions of various constituents in TE-GLCA based on 100 ug of protein, and ignoring the contribution of water. Based on 100 ug of protein, TE-GLCA contained 1.6% hexose (0.358% hexosamine), 0.04% phosphorous, 16.5% lipid, 15.04% nitrogen, and 47.6% water.

N. Fractionation of TE-GLCA by Precipitation with Isotonic Calcium Chloride. Amino acid analyses, reported in Table 9, suggested that fractionation of TE-GLCA by dialysis vs. isotonic $CaCl_2$ resulted in a protein enriched (CAS, 69% protein by weight) component in the supernatant, and a component in the precipitate of approximately the same protein content (CAP, 27.8% protein by weight) as the parent TE-GLCA (24.8% protein). The hygroscopicity of the components prevented the determination of quantitative recoveries of dry weight and protein of TE-GLCA in CAS and CAP. The data which was obtained indicated recoveries of over 100% of the initial quantities of TE-GLCA treated with $CaCl_2$ (data not shown). Approximately 20% of the starting dry weight of TE-GLCA was recovered in the supernatant after treatment with $CaCl_2$; the other 80% was recovered in CAP.

Major differences detected by amino acid analysis included an increase in concentration of acidic amino acid residues and proline in CAS (aspartic-12.3%, glutamic-14.0% as compared to CAP (aspartic-9.14%, glutamic-9.52%).

Table 9. Amino acid analyses of TE-GLCA, the supernatant from precipitation of TE-GLCA with $CaCl_2$ (CAS), and material from TE-GLCA precipitated with $CaCl_2$ (CAP). Concentration of individual amino acids are reported in uMol/100 uMol amino acids. All analyses were run at least in triplicate, except for CAS, which was run in duplicate.

Amino Acid	TE-GLCA	CAS	CAP
Lysine	6.08 (5.92- 6.15)	5.78 (5.65- 5.92)	6.01 (5.83- 6.05)
Histidine	1.38 (1.06- 1.66)	0.43 (0.38- 0.43)	0.32 (0.31- 0.32)
Ammonia	8.09 (7.39- 8.39)	7.55 (7.49- 7.59)	13.6 (12.4 -14.9)
Arginine	4.09 (3.86- 4.12)	3.42 (3.27- 3.56)	4.05 (3.75- 4.22)
Cysteic Acid	0.22 (0.20- 0.25)	0.19 (0.13- 0.25)	0.26 (0.23- 0.29)
Aspartic	10.9 (10.2 -11.3)	12.3 (11.9 -12.7)	9.14 (8.90- 9.67)
Threonine	5.61 (5.40- 5.75)	5.58 (5.16- 5.99)	5.11 (4.51- 5.62)
Serine	4.65 (4.50- 4.75)	4.96 (4.86- 5.26)	5.24 (5.00- 5.35)
Glutamic	11.2 (10.0 -12.0)	14.0 (14.0 -14.1)	9.52 (8.92-10.1)
Proline	3.00 (2.78- 3.08)	2.76 (2.65- 2.87)	3.26 (3.21- 3.28)
Glycine	6.52 (5.75- 6.69)	6.68 (6.34- 7.01)	6.81 (6.76- 6.85)
Alanine	7.58 (7.36- 7.64)	8.22 (8.19- 8.24)	7.71 (7.19- 8.06)
Valine	6.88 (5.92- 7.46)	5.37 (4.32- 6.42)	5.27 (4.94- 5.48)
Methionine	2.21 (2.19- 2.25)	1.90 (1.86- 1.93)	2.085 (2.00- 2.12)
Isoleucine	6.38 (6.15- 6.42)	6.17 (5.85- 6.49)	5.87 (5.74- 5.89)
Leucine	9.05 (8.94- 9.23)	8.49 (7.87- 8.49)	9.04 (8.84- 9.55)
Tyrosine	2.26 (2.14- 2.57)	2.60 (2.48- 2.71)	2.59 (2.33- 2.67)
Phenylalanine	3.98 (3.73- 4.07)	3.47 (3.40- 3.54)	4.08 (3.81- 4.22)
% Protein	24.8	69.0	27.8
Hexosamine (uM/100 ug protein)	0.0025 (0.0025)	---	0.009 (0.009)

A large increase in ammonia content was observed in CAP (13.6%) over CAS (7.55%) and TE-GLCA (8.09%). Hexosamine was not detected in CAS, suggesting its total recovery in CAP.

Fig. 22 displays the result of Triton-PAGE in 5% acrylamide gels of TE-GLCA, CAS and CAP. The striking total absence of bands in the gamma region was immediately obvious in CAS, while the banding pattern of CAP was similar to the parent TE-GLCA. SDS-PAGE of the components in 12.5% acrylamide gels containing 8.0 M urea is displayed in Fig. 23. The low m.w. 4,000 dalton component of the parent TE-GLCA was conserved in both the supernatant and precipitate fraction. Comparison of the diffuse staining pattern of each component suggested the concentration in CAS of constituents in the m.w. range of 12,400 - 25,000 daltons.

Antigenic differences were also observed between the three components. Fig. 24 displays the increase in antigenicity observed in CAS as compared to CAP and TE-GLCA. Reaction of CAS with: anti-SCM (AG-3), anti-GLCA (AG-10), and anti-SLS-CM (#22) produced lines of precipitation not observed in reactions of CAP and TE-GLCA with the same antisera. In the case of AG-3, one line of identity was formed between all three components, one line of identity was formed between CAS and TE-GLCA alone, and CAS formed a distinct precipitin line of its own with AG-3. Reaction of the three components with AG-10 produced a line of identity between

Fig. 22. Triton-PAGE in 5% acrylamide gels of TE-GLCA (Tube #1), CAP (#2), and CAS (#3). Staining of CAP revealed a banding pattern similar to that of TE-GLCA; there was a total absence of staining in the gamma region of the CAS gel. Staining was with Coomassie Blue.

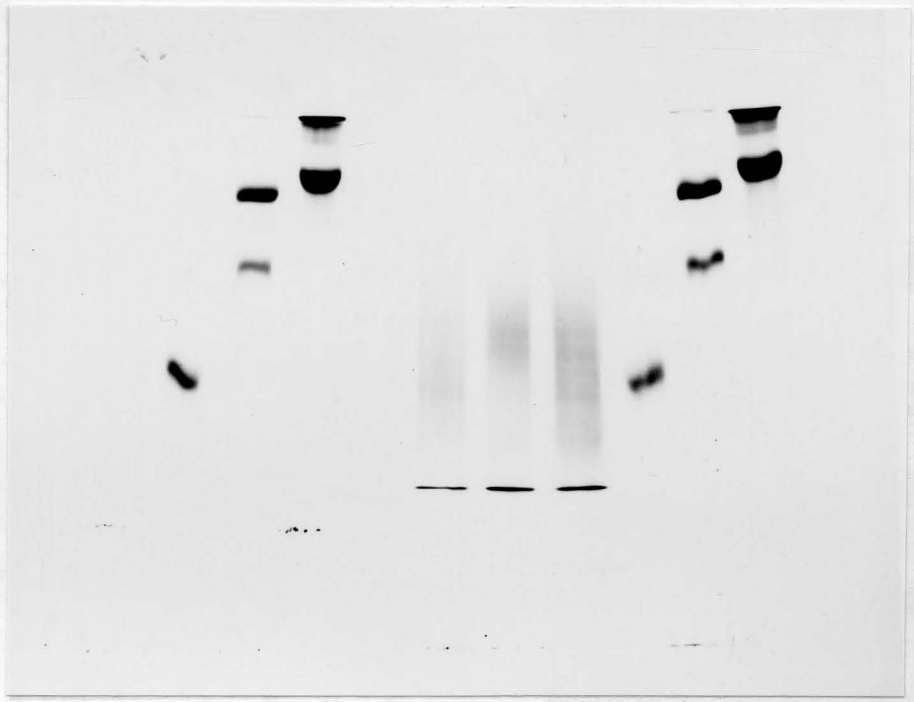

Fig. 23. SDS-PAGE, in 12.5% acrylamide gels containing 8.0 M urea, of (from left to right): cytochrome C, HGG, BSA, CAS, CAP, TE-GLCA, cytochrome C, HGG, and BSA. TE-GLCA, CAS and CAP all contained a 4,000 dalton component. CAP had a concentration of constituents in the m.w. range of 12,400 - 25,000 daltons. Arrow indicates position of tracking dye front. Staining of the gel was with Coomassie Blue.

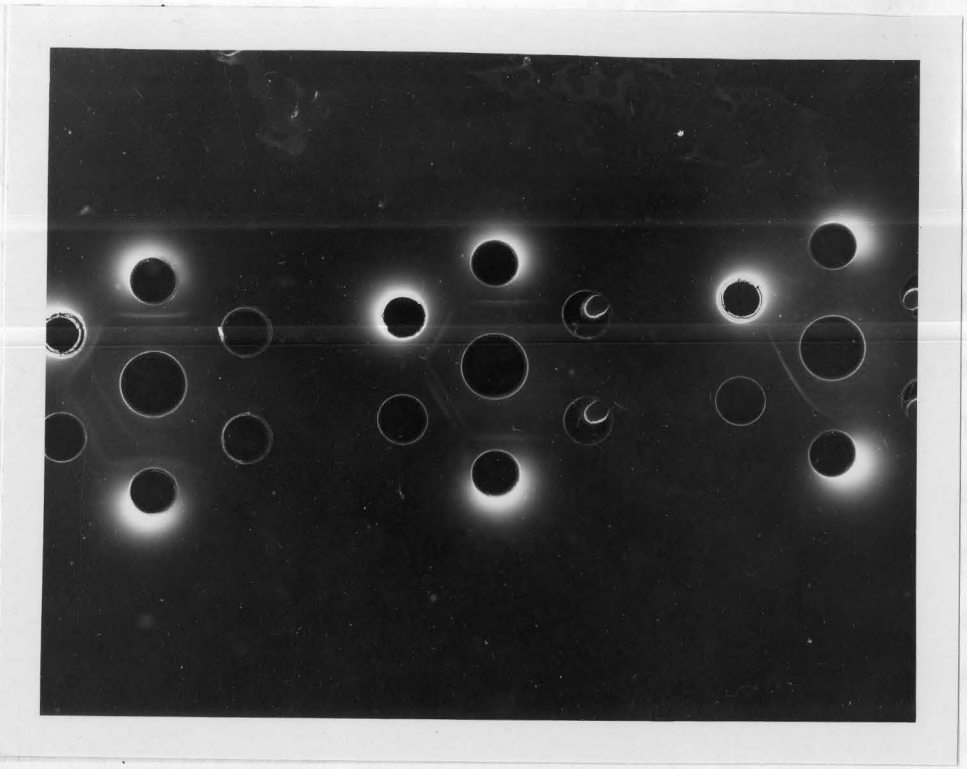

Fig. 24. Immunodiffusion analysis of TE-GLCA, CAP, and CAS in Triton-urea agarose gel. Wells contained, for all three patterns: #1, TE-GLCA, #2, CAP; #3, CAS; and #4, TE-GLCA. Wells #5 and #6 were empty. From left to right, antisera in center wells were: anti-SCM (AG-3), anti-GLCM (AG-10), and anti-SLS-CM (#22). Reaction of the antigen with AG-3 produced: a line of identity between TE-GLCA, CAP and CAS; a line of identity between CAS and TE-GLCA; and a line of precipitation unique to CAS. Reactions of the antigens with AG-10 produced: a line of identity between TE-GLCA, CAP, and CAS; and a line of precipitation unique to CAS. Reaction of the antigens with #22 produced a single line of precipitation between CAS and the antiserum, and no reaction with the other antigens.

all three components, and a distinct individual line between CAS and AG-10. CAS produced a single line of precipitation with #22; CAP and TE-GLCA failed to display reaction with #22.

Other antigenic differences were observed between the three components vs. the same antisera which had been adsorbed on human glomerular basement membrane. Examples are displayed in Fig. 25. Reaction of the three components with GBM adsorbed AG-3 serum revealed the absence of the distinct individual line of precipitation formed between non-adsorbed AG-3 and CAS. Reaction of the three components with GBM adsorbed AG-10 revealed the absence of the distinct, individual line of precipitation formed between non-adsorbed AG-10 and CAS; also the line of precipitation which displayed identity between TE-GLCA, CAS, and CAP was absent in CAP. The reaction of CAS with GBM adsorbed #22 was unchanged.

Immunoelectrophoresis of the three components further substantiated the increase in antigenicity of CAS over TE-GLCA and CAP (see Fig. 26). Reaction of CAS with AG-10 revealed a component with no electrophoretic mobility in addition to the component with anodal mobility. The immunoelectrophoretic pattern of CAS was apparently identical to BUT-TE-CM (butanol extract of TE buffer treated SCM). The constituent in CAS, reactive with #22 anti-SLS-CM serum, which was absent in TE-GLCA and CAP, was the component with no electrophoretic mobility (see Fig. 26). TE-GLCA and CAP

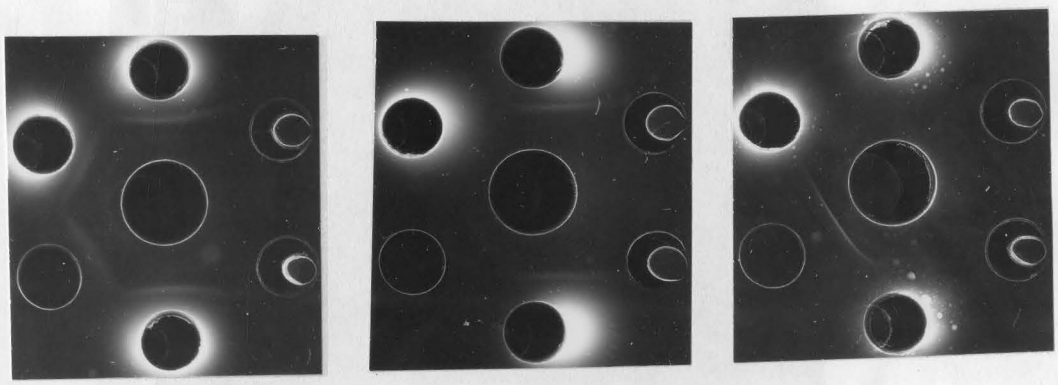

Fig. 25. Immunodiffusion analysis of TE-GLCA, CAS, and CAP vs. antisera adsorbed on human glomerular basement membrane (GBM). Outer wells contained in every pattern: #1, TE-GLCA; #2, CAP; #3, CAS; and #4, TE-GLCA. Well #5 and #6 were empty. Center wells, from left to right, contained the following: AG-3, AG-10, and #22 adsorbed antisera. Comparison with reactions with unadsorbed sera in equivalent patterns in Fig. 24 revealed: the loss of the line of precipitation unique to reaction of CAS with antiserum AG-3; loss of reactivity of CAP with AG-10; and loss of the line of precipitation unique to reaction of CAS with AG-10.

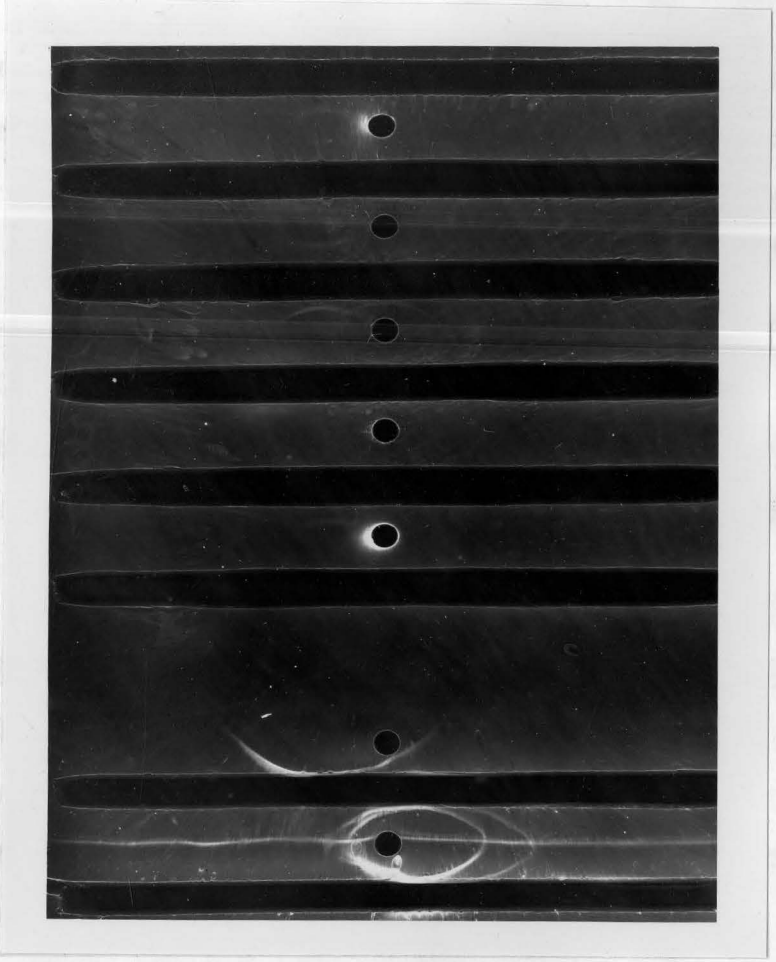

Fig. 26. Immunoelectrophoretic analysis of TE-GLCA, CAP, CAS, BUT-TE-CM, and SLS-CM. The anode was to the left of the figure, the cathode to the right. Troughs contained, from top to bottom, anti-GLCA (AG-10), anti-SLS-CM (#22), AG-10, #22, AG-10, #22, anti-whole human serum, anti-human gamma globulin. Wells contained, from top to bottom: TE-GLCA, CAS, BUT-TE-CM, SLS-CM, CAP, human serum albumin, and human gamma globulin. TE-GLCA and CAP each had a single, identical component with anodal mobility and a non-mobile component. CAS also produced a non-mobile component with antiserum #22, while SLS-CM was not reactive with its homologous serum.

displayed no reaction with antiserum #22 in immunoelectrophoresis, consistent with the results of immunodiffusion.

In order to rule out non-specific reactions of the antigen with antisera due to the presence of Ca^{++}, TE-GLCA was dissolved in 0.01 M Tris-HCl buffer, pH 8.9 containing 1.7 mM $CaCl_2$ (which was a concentration at which no visible precipitation of TE-GLCA occurred). The antigenicity of TE-GLCA was unaffected by the treatment (data not shown).

O. Indirect Fluorescent Antibody Tests Utilizing Antisera Prepared Against Components Extracted from SCM.

Recorded in Table 10 are the results of indirect fluorescent antibody tests on various mouse tissues utilizing antisera prepared against SCM and components extracted from SCM. Intensities were graded on a basis relative to the 3+ intensity of a mouse anti-GBM serum.

In general, the antisera failed to stain the alveolar basement membrane of C3H mouse lung tissues (22/22 sera), or the sarcolemmal membrane of C3H mouse heart tissue (19/22 sera). The intensity of staining with antisera on glomerular basement membrane (GBM) of 5 d old C3H mouse glomeruli was as strong as (8/22 antisera) or better than (6/22) the GBM of adult C3H mouse glomeruli. However, the intensity of staining with antisera on GBM of adult Swiss mouse glomeruli was greater than (12/22) or equal to (7/22) the intensity of staining on GBM of 3 d old Swiss mouse glomeruli. The most

Table 10. Indirect fluorescent antibody tests on mouse tissues utilizing antisera prepared against SCM and various components extracted from SCM. Intensities were graded on a basis relative to the 3+ intensity of an anti-mouse GBM serum on mouse glomerular basement membrane.

Antiserum Specificity	C3H Mouse Kidney			Adult C3H Mouse			Swiss White Mouse Kidney		
	0 d	3 d	5 d	Kidney	Lung	Heart	0 d	3 d	Adult
SCM (27-Ia)	+	±	±	±	–	±		±	±
SLS-CM (27-IIa)	–		±	±	–	–		±	+
SLS-CM (27-IIb)	–	–	+	+	–	–		±	1+
SLS-CM (27-IIc)	–	–		+	–	–		±	1+-2+
TE-CM (29-4)			±	–	–	–		–	±
TE-CM (29-5)			±	±	–	–		–	+
LIS-BUT-TE-CM (29-6)			+	+	–	±		–	1+
LIS-BUT-TE-CM (29-7)			±	±	–	–		±	1+
LIS-TE-CM (29-8)			+	+	–	±		+	+
LIS-TE-CM (29-9)			±	–	–	–		±	±
BUT-TE-CM (29-10)			+	±	–	–		+	+
BUT-TE-CM (29-11)			–	±	–	–		–	±
LIS-BUT-TE-CM-Ppt. (29-16)			+	±	–	–		±	±
Frac. II from DEAE Cellulose Chrom. of TE-GLCA (24-M)			±	–	–	–		–	1+
SCM (AG-1)			±	±	–	–	–	+	+
SCM (AG-2)			–	±	–	–	±	+	1+
SCM (AG-3)			–	±	–	–	±	–	±
SCM (AG-4)			±	+	–	–	–	–	+
GLCM (AG-9)			–	+	–	–	±	+	±
GLCM (AG-10)			±	±	–	–	+	–	–
GLCM (AG-11)			±	+	–	–	±	+	+
GLCM (AG-12)			±	±	–	–	±	+	+

potent antisera tested were: SLS-CM (27 III-b, 1+), SLS-CM (27 IIIc, 1+-2+), LIS-BUT-TE-CM (29-6, 29-7, 1+), Frac. II from DEAE cellulose chromatography of TE-GLCA (24-M, 1+), and anti-SCM (AG-2, 1+). All preimmunization sera tested were uniformly negative on the tissues.

CHAPTER IV

DISCUSSION

The physical and immunological properties of a biological substance have as their basis the chemical composition of the given biological substance. The soluble components studied in this investigation were all of SCM origin. Therefore, it was assumed that the complete chemical analysis of one of the components, TE-GLCA, would reflect the general chemical composition of all the soluble components. Knowledge of the chemical composition of one of the major soluble components would then aid in the interpretation of physical and immunological data on the components.

Chemical analysis of TE-GLCA, reported in Table 8, resulted in the inability to account for more than 40% of the apparent dry weight of the component. Protein (24.8%), hexose (0.4%), phosphorous (0.01%), lipid (4.1%), and water (11.76%) accounted for only 41.1% of the total apparent dry weight. The nitrogen content substantiated the percent protein composition of the component as determined by amino acid analysis, thus eliminating the contribution of other nitrogenous substances such as purines and pyrimidines. The absence of a significant neutral fraction (thus eliminating the presence of glycerol and therefore membrane teichoic

acids) or ash substance (eliminating the contribution of inorganic ions) led to the recognition of the possibility that TE-GLCA was extremely hygroscopic and was binding more than twice its weight in water.

Karl Fischer analysis determined the water content of TE-GLCA to be 11.76% by weight, thus supporting the hypothesized hygroscopic nature of TE-GLCA. The 11.76% figure may actually represent a minimal value, depending on the solubility of TE-GLCA in the Karl Fischer reagent (unfortunately, technical information regarding the solubility of TE-GLCA in the Karl Fischer reagent was not available). Assuming that TE-GLCA was not totally soluble in the Karl Fischer reagent, water bound in the constituents of TE-GLCA insoluble in the reagent would conceivably not be detectable.

The mode of binding of water by TE-GLCA in relation to its chemical composition and physical properties, was beyond the scope of this investigation. The other two soluble components obtained from LIS extraction of SCM, LIS-BUT-TE-CM and LIS-TE-CM, also had protein (Table 5) and hexose compositions similar to TE-GLCA. Although lipid concentrations were not determined on LIS-BUT-TE-CM and LIS-TE-CM due to limited quantities of material, these LIS extracted components were presumed to share the extreme hygroscopic properties of TE-GLCA.

An obvious effect of the hygroscopic nature of the membrane components would be observed in any procedure in-

volving the weighing of the components, in which the contribution of water to the final weight would be enormous. The effect on the physical behavior of the membrane components of their hygroscopicity, in procedures such as PAGE and molecular sieve chromatography, remained unknown.

The hygroscopic nature of the soluble components was only recognized and confirmed after all various chemical tests were run. Recalculation of the contribution of various constituents to the chemical composition of TE-GLCA on the basis of 100 ug protein resulted in a 16.5% lipid content. This rather significant lipid content was in contrast to the 4.1% figure determined on an apparent dry weight basis, and displayed the difficulty of determining the chemical composition of a hygroscopic substance.

Due to the recognition of the contribution of a significant lipid fraction to the composition of TE-GLCA, aliquots of TE-GLCA were saponified, and the fatty acids in the saponified lipid extracts were identified and quantitated by gas-liquid chromatography. Palmitic acid was the fatty acid in highest concentration, followed by a predominance in concentration of unsaturated fatty acids (Table 6). The concentration of the individual fatty acids in the saponified lipid extracts of TE-GLCA (lauric, 0.08; myristic, 0.07; palmitoleic, 0.185; stearic, 0.13; oleic, 0.24), expressed relative to the concentration of palmitic, were surprisingly similar to the fatty acid concentrations in a butanol extract

of whole SCM (lauric, 0.01; myristic, 0.055; palmitoleic, 0.195; stearic, 0.15; oleic, 0.32). This was in contrast to the differences in fatty acid concentrations between the butanol extraction of SCM, and the Genetron extractions of LIS-CM and GLCM, all three of which appeared to be similar, based on the results of thin layer chromatography of the unsaponified extracts (Table 5). The importance of this observation, and the extent to which, if any, it reflected the properties of SCM itself or possible differential extraction properties of butanol and Genetron (which was utilized in the extraction of TE-GLCA), was not determined.

The fatty acids in the saponified lipid fraction of TE-GLCA were believed to be "bound" or "non-extractable" lipids for three reasons. First, lipid was not detected by gravimetric analysis in a chloroform-methanol extract of GLCA. Second, TE-GLCA was essentially material resultant from two Genetron extractions of LIS-CM, and one would expect that all extractable lipid in TE-GLCA would have been removed by the two Genetron extractions. Finally, the relative concentrations of several fatty acids, recorded in Table 6, were significantly higher in the saponified lipid fraction of TE-GLCA than in the Genetron extraction of GLCM, the latter of which resulted in GLCA (which after dialysis vs. TE buffer resulted in TE-GLCA). Examples were oleic (0.24 to 0.02), palmitoleic (0.185 to 0), and lauric (0.08 to .006).

Based on the lipid and the carbohydrate (16.5 and

1.6 ug/100 ug protein, respectively) content of TE-GLCA, TE-GLCA may be classified as a glycolipoprotein component. The nature of the individual constituents comprising the component is presently unknown. These may vary from that of the parent component and were not detectable due to concentration. Neither identity of the residues comprising the carbohydrate component, nor the modes of binding of carbohydrate to TE-GLCA were determined. Here, too, their low concentration made these determinations difficult.

The soluble components extracted from SCM were similar in amino acid composition, but not identical (Table 5). Thus, on the basis of amino acid composition alone, the soluble components would be expected to have similar physical properties. LIS-TE-CM and LIS-BUT-TE-CM had the most closely related amino acid compositions of the soluble components. All of the soluble components were acidic proteins, as evidenced by their high aspartic (TE-GLCA, 10.9; TE-CM, 12.3; BUT-TE-CM, 12.4; LIS-TE-CM, 9.45; LIS-BUT-TE-CM, 10.1) and glutamic (TE-GLCA, 11.2; TE-CM, 11.4; BUT-TE-CM, 12.1; LIS-TE-CM, 9.38; LIS-BUT-TE-CM, 10.8) acid content. The amino acid compositions of the soluble components were also similar to SCM, with the exception of two amino acids. As compared to SCM, a marked decrease in alanine content (SCM, 11.3; TE-GLCA, 7.58; BUT-TE-CM, 7.69; LIS-TE-CM, 7.57; LIS-BUT-TE-CM, 7.125) in most of the soluble components was observed, as was a marked increase in the content of methio-

nine (SCM, 0.19; TE-GLCA, 2.21; BUT-TE-CM, 1.54; LIS-TE-CM, 1.24; LIS-BUT-TE-CM, 1.57) components (with the exception of TE-CM).

The soluble components could logically be split into two groups for comparison. One group, consisting of those components resultant from LIS extraction of SCM, would include TE-GLCA, LIS-TE-CM, and LIS-BUT-TE-CM. All three components were very similar in amino acid composition. However, the significantly higher ammonia content of LIS-TE-CM (12.9) and LIS-BUT-TE-CM (10.7), as compared to TE-GLCA (8.09), suggested a higher content of glutamine and asparagine in LIS-BUT-TE-CM and LIS-TE-CM. The per cent protein compositions (TE-GLCA, 24.8; LIS-TE-CM, 32.4; and LIS-BUT-TE-CM, 22.8) of the three soluble components were also similar. The low concentrations of protein in the LIS extracted components, and perhaps of TE-CM and BUT-TE-CM as well, were believed to be a reflection of the assumed hygroscopic nature of the soluble components. Based on the chemical composition of TE-GLCA (see data determined as ug of individual constituents/100 ug protein, recorded in Table 8), the soluble components were all believed to be predominantly protein in composition.

The other group of soluble components would consist of BUT-TE-CM and TE-CM, based upon their identical appearance in immunoelectrophoresis (Fig. 16). Striking differences in content of individual amino acids were not apparent between

the two components (Table 5). The percent protein compositions of the two components were also similar.

The most striking difference between the insoluble residues resultant from the extractions of SCM, and the soluble components and parent SCM, was the very high content of alanine in the insoluble residues (Table 4). The content of alanine in the insoluble residues was nearly twice that in the soluble components. The insoluble residues also had higher hexosamine content than the parent SCM and the soluble components. Both hexosamine and alanine are known constituents of the cell wall peptidoglycan of group A streptococci (22). Thus, the higher concentration of these two constituents in the insoluble residues suggested that the insoluble residues were enriched for the residual cell wall contaminants of the parent SCM utilized for extraction.

Unfortunately, the hygroscopicity of the soluble components was not recognized before determining data on the dry weight recoveries of SCM in extracted components recorded in Table 3. Obviously, the recovery of "dry weights" in the soluble components was actually due to a significant amount of water. Therefore, recoveries of total protein in the soluble components may represent the most significant data.

Based on dry weight recoveries in extracted components of the initial dry weight of SCM utilized for extraction, the most striking observation was the difference in total recoveries resulting from the extraction of SCM with

butanol. Extraction of the insoluble material resultant from TE extraction of SCM (TE-CM-Ppt) with n-butanol prior to extraction with LIS resulted in a higher total recovery of initial dry weight (81.7%), as compared with the total recovery observed when TE-CM-Ppt was extracted with LIS prior to extraction with butanol (48.1%). Since the recovery of protein was the same in both LIS extracted components (LIS-BUT-TE-CM and LIS-TE-CM, 37.4%), the loss of material may have occurred as a result of a more complete disruption of TE-CM-Ppt, due to a more efficient extraction of lipid by butanol following extraction of TE-CM-Ppt with LIS. Thus, BUT-LIS-TE-CM-Ppt would be predicted to have a lower lipid content than LIS-BUT-TE-CM-Ppt, a hypothesis made in retrospect and therefore not tested. Additionally, a high percentage of low m.w. components, which would be lost during dialysis, may have resulted due to extraction of TE-CM-Ppt with LIS followed by extraction with butanol.

Extraction of TE-CM-Ppt with butanol prior to LIS extraction resulted in a soluble component (BUT-TE-CM). A soluble component was not isolated following extraction of LIS extracted TE-CM-Ppt with butanol. Electrophoresis revealed the presence of two constituents in BUT-TE-CM (Fig. 16); both LIS extracts (LIS-TE-CM and LIS-BUT-TE-CM) possessed a single, identical constituent. Possibly, LIS was disrupting interactions between membrane constituents which were interactions stable to butanol treatment, thus resulting

in the loss of a constituent in the LIS extracts.

Originally, the LIS-Genetron extraction procedure, which ultimately resulted in TE-GLCA, was considered to extract soluble components which displayed strong cross reactivity with GBM antisera in immunodiffusion. However, the observation of non-immune precipitation between LIS-Tris solutions and various antisera questioned the significance of the earlier observations. The absorption of LIS at 280 nm, and the adverse effects of LIS on electrophoresis of extracted components in cellulose acetate membrane and acrylamide gel, added further impetus for the development of methods for the removal of LIS from the LIS extracted components. Initially, LIS had been used predominantly to extract glycoprotein constituents from mammalian cell membranes (45). Applications of LIS to extraction of microbial membranes were limited (24,44). Supposedly, LIS had been easily removed from extracts of red blood cell ghosts (45). Since the extraction of mammalian cell membranes with LIS had consistently been preceded by treatment of the cells with EDTA, the effects of EDTA on LIS extracted components from SCM was investigated. Dialysis of LIS extracted SCM components vs. TE buffer proved effective in the removal of LIS from the extracts. The observation that LIS was removed by dialysis vs. TE buffer may have special significance due to the recent observation that extraction of the integral membrane protein glycophorin from human erythrocyte

ghosts with LIS resulted in a preparation heavily contaminated with LIS (54). Up to 10 moles of LIS was bound/mole of glycophorin. The bound LIS could not be removed by washings with a variety of polar organic solvents or by treatment with sodium deoxycholate.

The removal of LIS by dialysis vs. EDTA may serve to indicate the mode of binding of LIS to the soluble components. The fact that LIS was detectable by spectrophotometric methods, and since lithium was quantitatable by atomic absorption spectrophotometry, suggested the possibility that the LIS molecule was bound to the extracted components. LIS may have been bound through lithium to the free carboxyl groups of the acidic amino acid residues in the extracted components. EDTA possibly would disrupt the interaction by binding competitively to LIS through lithium, thus removing LIS from the extracts after dialysis vs. water.

The behavior of GLCM on Sephadex G-50 (Fig. 10) may actually have reflected the high content of LIS in GLCM. Although the actual concentration of LIS in GLCM was not determined, it could not logically be less than that of GLCA, which was the soluble component resultant from Genetron extraction of GLCM. The concentration of LIS in GLCA, as reported in Table 2, was 47.2% w/w. Fraction I appeared in the void volume and Fraction II was in the included volume. The results suggested that GLCM was predominantly a high molecular weight component. Whether the results were due to

the presence of high concentrations of LIS was never investigated. Immunodiffusion analysis of Fraction I and Fraction II vs. anti-SCM and anti-GLCM sera revealed that antigenic reactivity resided in Fraction I alone. Therefore, Fraction II possibly represented the presence of free residual LIS in GLCM. Also, the absence of reactivity of Fraction II exemplified the variability in non-immune precipitation of LIS with different sera.

Results of DEAE-cellulose chromatography of TE-GLCA were generally inconclusive. Poor recoveries of initial apparent dry weights (which may in part have been a reflection of the hygroscopicity of the component), of aliquots of TE-GLCA utilized for chromatography, were consistently observed. Another property of TE-GLCA which frustrated these investigations was demonstrated by the tendency for TE-GLCA to precipitate below pH 6.0 and therefore was considered as a major cause of the poor recoveries. However, Fraction II was eluted at an acidic pH (5.0-5.5) and proved to be of considerable antigenic significance. Regardless, the low recoveries (less than 10% w/w) of the starting material in the eluted fractions were of a magnitude prohibitive of further experimentation with DEAE cellulose.

According to recent advances in the study of biological membranes (59), the proteins comprising the soluble components extracted from SCM are either "integral" or "extrinsic" membrane proteins. Integral membrane proteins

have distinct hydrophobic and hydrophilic domains on the same molecule, and are thus referred to as being amphipathic molecules (27). Integral membrane proteins are only released from membranes by treatment of membranes with disruptive agents such as detergents or organic solvents (25). Within the membrane, integral membrane proteins were considered to be closely associated with phospholipids through hydrophobic bonding with the hydrocarbon "tails" of the phospholipids (25). When liberated from the membrane, integral membrane proteins were generally water insoluble (64).

The majority of membrane proteins are considered to be "extrinsic" to the bilayered membrane "continuum" (63). Extrinsic membrane proteins are removed by relatively mild methods without disrupting the phospholipid matrix of the membrane. Thus proteins are released through the treatment of membranes with chelating reagents, extremes of pH, or high salt concentration (27). Extrinsic membrane proteins were water soluble, and were bound through polar interactions to integral membrane proteins and the polar "heads" of phospholipids (25). The association of extrinsic proteins with the polar heads of phospholipids may be mediated via divalent cations (47, 52).

Extractions of membranes with high detergent concentrations or organic solvents tend to dissociate lipid from protein in membranes and in lipoproteins (28). Once delipidated and in the absence of detergent, membrane pro-

teins tend to form aggregates in aqueous solution (19, 20).

Various methods involving EDTA, LIS, butanol, and Genetron were used in the present study to extract membrane components from SCM. Investigations into the physical properties of the soluble components extracted from SCM, led to the realization that difficulties were being encountered which could only be attributed to the membrane origin of the components. PAGE of the extracted components, in the presence or absence of detergent, may have revealed characteristics of the membrane components attributable to their membrane origin. As described below, the results of SDS-PAGE on the components gave support to recent criticism (42) of the procedure as it applies to membrane proteins.

LIS is believed to disrupt membranes in a manner similar to the action of SDS (45). Assuming that LIS acted as a detergent, the following is a possible explanation for the appearance of the extracted components on PAGE in the presence and absence of detergents. The problem to be resolved was the explanation for the apparent heterogeneity of the extracted components on detergent-PAGE, as compared to the homogeneity (1-2 constituents) of the extracted components in immunodiffusion and immunoelectrophoretic analysis in Triton-agarose gel, keeping in mind that the latter was the critical criterion for the biological signifiance of these constituents.

Immunological data from immunoelectrophoresis did

not support the appearance of the extracted components on PAGE in the absence of detergent. All of the extracted components displayed two constituents on PAGE (data not shown), yet not all of the components displayed two constituents in immunoelectrophoresis (Fig. 16). A logical assumption was that the component at the interface of the stacking and running gels actually represented delipidated integral membrane proteins, which formed large molecular weight aggregates in the aqueous environment, and therefore could not migrate through the gel. Since the supposed complexes did migrate through the 3% acrylamide stacking gel but not the 5% running gel, this placed a possible m.w. of the complexes at greater than 500,000 daltons (13). The presence of the same fast moving constituent in each soluble component suggested the presence of water soluble extrinsic membrane protein constituents. A release of extrinsic membrane protein constituents by the extraction procedures utilized to obtain each soluble component could be expected to occur.

However, the fast moving component was probably not antigenic. Two facts supported this hypothesis. First HSA migrated slower than the fast moving component (which migrated ahead of the tracking dye) in both Triton-PAGE and in PAGE without Triton (data not shown). However, HSA migrated slightly faster than the constituent with anodal mobility observed in BUT-TE-CM, TE-GLCA, and TE-CM after immunoelectrophoresis (Fig. 16). Secondly, all of the soluble compo-

nents displayed the fast moving constituent in both PAGE and Triton-PAGE; however SLS-CM, LIS-TE-CM and LIS-BUT-TE-CM did not display a constituent with anodal mobility.

Therefore, the antigenic constituents were apparently present in the proposed large molecular weight aggregates, which were excluded from the PAGE running gel. In an attempt to disrupt the aggregates, PAGE was run in the presence of Triton X-100. The interaction of Triton X-100 with delipidated integral membrane proteins is known to reflect the interactions with lipid of the integral membrane proteins (11, 27). Triton was bound to the hydrophobic regions of the amphipathic proteins, thus replacing the lipid (11, 27). An important distinction was the failure of extrinsic membrane proteins to bind Triton (11, 27).

However, PAGE of the soluble components in the presence of Triton yielded multiple banding patterns with diffuse staining throughout regions of the gels (Fig. 18). Due to the presence of components at the interface of the stacking and running gels, the possibility existed that complete dissociation of the aggregates was not effected by Triton. Therefore, disruption of the large molecular weight aggregates in Triton may have caused the formation of a wide range of lower m.w. protein-detergent micelles, capable of migrating into the running gel and leading to the possibility that the banding patterns were no reflection of the true composition of the components. However, the presence of

bands shared between the soluble components suggested the possibility that those bands were not the result of chance aggregation of individual constituents, but rather reflected actual constituents in the soluble components.

SDS-PAGE results on the soluble components extracted from SCM also suggested a heterogeneity in composition not supported by immunoelectrophoresis results. However, the results of SDS-PAGE may have not reflected the true number of different m.w. constituents in the components, for the following reasons.

The theoretical background of SDS-PAGE is still unclear, especially as it applies to membrane proteins (40, 42). In order for the results of SDS-PAGE to reflect the m.w. of the true number of individual polypeptides comprising a given component, the following assumptions must be made for each individual polypeptide (42): i) The binding of SDS to a polypeptide must disrupt all but covalent interactions within the polypeptide chain and between polypeptide chains. ii) All interactions between lipids and proteins must be destroyed by binding of SDS. iii) The binding of SDS must be constant/unit polypeptide chain length, and all charge differences between polypeptide chains must be abolished by the polysulphate structures formed. iv) Conformational differences between the SDS saturated polypeptides must not exist. Thus, differences in the frictional drag between SDS-polypeptide complexes, which is a property reflecting the

molecular dimensions of the polypeptide and the viscosity of the medium in which it is suspended, are due only to variations in molecular weights of the complexes. Unfortunately, the assumptions have almost exclusively been verified by water soluble proteins of known quaternary structures, and the release of membrane proteins from their intimate association with lipid may cause anomalous behavior of membrane proteins in SDS-PAGE (42).

Utilizing TE-GLCA as an example, the multiplicity of m.w. constituents in the soluble components may be explained on the basis of amino acid composition and the hygroscopic nature of the soluble components (assuming the other soluble components shared the hygroscopic property of TE-GLCA). The high percentage of acidic amino acid residues in the soluble components may have caused poor binding of SDS by the soluble components (42). Thus, complete disruption by SDS of the high molecular weight aggregates in the soluble components may not have occurred, and it could not be assumed that all of the constituents possessed the same conformation.

The extreme affinity of the soluble components for water may have caused preferential binding of SDS to hydrophobic regions of the polypeptides comprising the soluble components. This preferential binding of SDS to hydrophobic regions in the case of leghaemoglobin was theorized to have caused a decrease in the frictional drag of the molecule,

thus leading to a lower than actual apparent m.w. as determined on SDS-PAGE (40). Thus, the m.w. of the constituents stained in gels following SDS-PAGE of the soluble components (Fig. 19) and the presence of a constituent in each soluble component migrating with the tracking dye front, may have been artifactual results.

By analogy, therefore, due to the high acidic amino acid content and hygroscopicity of the soluble components, the results of SDS-PAGE on the soluble components may have actually represented the migration of aggregates of various conformations. Also, the low apparent m.w. of the aggregates, may actually have been due to an artificially high mobility due to decreased frictional drag caused by anomalous SDS binding.

The decreased resolution of constituents in SDS-PAGE in the presence of 8 M urea supported the criticisms of Nielsen and Reynolds (49), who stated that use of urea in SDS-PAGE was unsound. SDS and urea caused different types of conformational changes in proteins (49). Therefore, it could not be assumed that all proteins possessed the identical conformation in the presence of a mixture of urea and SDS. The multitude of conformations induced by the mixture of SDS and urea may have caused the smearing observed on SDS-PAGE in the presence of 8 M urea.

In contrast to data obtained by PAGE on the composition of the soluble components, data obtained from immuno-

electrophoresis of the components indicated the presence of up to two antigenic constituents in the soluble components (Fig. 16). One component had an anodal mobility slower than albumin; the other was not mobile. TE-CM and BUT-TE-CM contained both constituents. The significance of the absence of the non-motile constituent in TE-GLCA and absence of the motile constituent in LIS-BUT-TE-CM and LIS-TE-CM is presently unknown. The absence of at least one of the constituents, present in TE-CM and BUT-TE-CM, in all of the LIS extracts may have been attributable to unknown properties of LIS which affected its extraction of membrane proteins. Furthermore, the fact that Genetron was utilized in the extraction of TE-GLCA, but not in the extraction of LIS-BUT-TE-CM and LIS-TE-CM, may have caused the difference in immunoelectrophoretic composition between TE-GLCA and the other two LIS extracted components. The fact that differences were present between the soluble components suggested that the various extraction procedures did yield the isolation of components of different immunological compositions.

The possibility existed that the results of immunoelectrophoresis reflected the immune response of the rabbits to the proposed aggregates of the constituents comprising the components. Antigenic determinants may have been masked, or formed due to the incorporation of the constituents into the aggregates. Thus, based on the assumption that the delipidated integral membrane proteins formed aggregates,

comparison of the results of immunoelectrophoresis with results of PAGE could not be made with any significance of correlation.

With the exception of the adsorption studies involving CAS and CAP (Section N of Results), immunological cross reaction with GBM by soluble SCM components was not observed by immunodiffusion tests in agarose gel. Anti-GBM sera did not react with soluble SCM antigens and anti-SCM sera did not react with soluble GBM antigens. The results may have been due to a concentration effect; due to the hygroscopicity of TE-GLCA (and presumably for the other soluble components extracted from SCM) the actual protein concentrations in the antigen solutions may have been too low. Since the cross reactive antigens are believed to be protein in composition (7) the low concentration of protein in the antigen solutions may have been the cause of the negative results.

With the exception of a single antiserum LIS-BUT-TE-CM-Ppt #16, the soluble components failed to precipitate with antisera prepared against the insoluble residues resultant from the extractions of SCM. Thus, the soluble components possessed little if any antigenic homology with the insoluble residues. Either all the antigens constituting the soluble components were extracted exhaustively from SCM by the methods utilized, or the immunogenicity of the insoluble residues was markedly altered by treatment with the reagents utilized for extraction.

Cross reactivity with mouse GBM of antisera prepared against the soluble components extracted from SCM was established by indirect fluorescent antibody tests on mouse kidney sections (Table 10). Antisera prepared against SCM, SLS-CM, TE-CM, LIS-BUT-TE-CM, LIS-TE-CM, BUT-TE-CM, Fraction II from DEAE cellulose chromatography of TE-GLCA (Frac II), and GLCM were shown to possess cross reactivity with mouse GBM. The antisera failed to react with mouse lung and heart tissue, thus demonstrating the specificity for GBM of the antisera.

Antisera prepared against SCM, SLS-CM, LIS-BUT-TE-CM, and Frac II possessed a comparable strength of reactivity with antisera prepared against the three soluble components displaying reactions of generally higher potencies than the antisera prepared against SCM. The reaction of anti-Frac II with mouse GBM in indirect fluorescent antibody tests confirmed reaction of the identical antiserum with PGT-HUGL III (third fraction from DEAE cellulose chromatography of soluble material resultant from Genetron extraction of a trypsin digest of GBM) observed in immunodiffusion in agarose gel.

The problems encountered with DEAE cellulose fractionation of TE-GLCA in this study are not unique; difficulties in obtaining a suitable method for fractionation of membrane proteins have been recognized (19). Therefore, the observation that TE-GLCA could be separated into two fractions by dialysis vs. isotonic calcium chloride ($CaCl_2$) was

investigated as a possible method for further characterization of the extract. Upon dialysis vs. $CaCl_2$, a precipitate was formed in the contents of the dialysis tubing. The precipitate fraction (CAP) was separated from the supernatant fraction (CAS) by centrifugation. Dialysis of TE-GLCA vs. $CaCl_2$ was originally undertaken after the recognition of the possible role of divalent cations in the structure of SCM, due to the observation that suspensions of SCM in TE buffer released significant quantities of soluble material. An unknown relationship may exist between the ability of LIS to extract membrane components and the role of divalent cations in the structure of SCM. LIS was removed from GLCA by apparently being bound by EDTA following dialysis vs. TE buffer (to yield TE-GLCA), thus behaving as a divalent cation. Secondly, constituents of TE-GLCA could bind calcium ion, thus suggesting a possible analogy between the binding of LIS to the constituents and the binding of calcium to the constituents. Since TE-GLCA was a component extracted from whole SCM, the suggestion that components precipitated by calcium in TE-GLCA would not be present, had the SCM been treated with TE buffer prior to LIS extraction, may seem warranted. However, the fact that LIS-TE-CM (a component extracted with LIS from TE treated SCM) displayed properties similar to TE-GLCA upon dialysis vs. isotonic $CaCl_2$ (data not shown), suggested a role of calcium ion in the SCM structure greater than just that of binding extrinsic membrane proteins.

The assessment of the use of $CaCl_2$ on the extracts lead to many new evaluations and observations. Comparison of chemical, physical, and immunological characteristics of CAP and CAS suggested several "concentration" effects in CAS. CAS contained over twice the protein concentration of CAP (Table 9). On Triton-PAGE, CAP had a banding pattern similar to the parent TE-GLCA, while CAS displayed a total absence of banding in the gamma region, but had a similar banding pattern to TE-GLCA and CAP in higher mobility regions (Fig. 22). SDS-PAGE revealed the presence of several constituents in CAS in the m.w. range of 12,400 - 25,000 daltons which were absent in TE-GLCA and CAP (Fig. 23), keeping in mind all previous reservations made in regard to estimation of these substances m.w.

The most striking differences between CAS and CAP were observed immunologically by immunodiffusion and immunoelectrophoresis in agarose gel. The antigenicity of CAS vs. anti-GLCM (AG-10), anti-SCM (AG-3), and anti-SLS-CM (#22) sera was increased as compared to TE-GLCA and CAP (Fig. 24). New, distinct lines of precipitation, absent in TE-GLCA and CAP, were observed in the reaction of CAS with the three antisera. The absence of the new and distinct lines in reaction of CAS with two of the antisera (AG-10 and AG-3), which had been previously adsorbed on whole human GBM, suggested that antibody specific for the "new" antigenic determinants in CAS had been adsorbed on human GBM (Fig. 25). Thus, dialy-

sis of TE-GLCA vs. $CaCl_2$ may have served to either i) concentrate SCM antigens present in TE-GLCA, or ii) held the proteins into a critical conformation necessary for antigenicity and related more to their native structure, thus allowing for cross reaction with GBM.

Immunoelectrophoresis revealed that CAP and TE-GLCA were identical in appearance; having only a single constituend with anodal mobility (Fig. 26). CAS had an immunoelectrophoretic composition identical in appearance to BUT-TE-CM, due to the presence of a non-motile constituent, in addition to the constituent with anodal mobility observed in TE-GLCA and CAP.

Further investigation into the chemical, physical and immunological properties of CAS may serve to explain the observation of "concentration effects" vs. conformational changes in CAS over CAP and TE-GLCA. For example, it is presently unknown if the difference in protein concentration between the fractions was due to the relative hygroscopicity of the fractions, and if so to what does one attribute these differences in hygroscopicity. The solubility characteristics of CAS would have to be investigated in order to determine if the application of ion exchange chromatography would prove to be feasible for further fractionation of CAS.

Immunologically, the apparent increase in the antigenicity of CAS over CAP and TE-CLCA posed intriguing possibilities worthy of speculation and discussion. An antiserum

specific for CAS has not been produced at the present time, since the recognition of the binding of calcium ion to constituents of TE-GLCA was a relatively recent development. Therefore, the observed immunological reactions of CAS were only between CAS and heterologous anti-streptococcal sera. The apparent enhancement in CAS of streptococcal antigens cross reactive with GBM may have been due totally to the concentration effects, as previously mentioned. These "concentration effects" can be restated to suggest two phenomena: i) a more accurate protein concentration based on weight; and ii) the selection of protein structures from the milieu possessing the ability to complex with calcium ion, and structural relatedness to GBM antigens. Thus, the streptococcal antigens cross reactive with GBM may fortuitously have the property of binding calcium. Alternatively, the "release" of CAS from TE-GLCA may have resulted in a change of conformation in CAS, that is an intrachain reaction, thus resulting in a more antigenic form of CAS. Perhaps due to the interaction of CAS and CAP in TE-GLCA, important sequential determinants were "hidden" or "masked". The possibility exists that divalent cations are fundamental to the antigenicity of these constituents through the creation of a lattice structure in solution (implying incomplete removal of calcium from CAS after dialysis vs. EDTA), thus optimizing precipitation with antibody. Regardless, it is apparent that the absence of treatment of TE-GLCA with calcium minimized immunologic

activity, whereas its presence enhanced it.

One can only speculate on the role of calcium (or magnesium, a possibility which was not investigated) ion in the native SCM and GBM structures. The ability of constituents in TE-GLCA to bind calcium may signify a role for calcium (or magnesium) in the incorporation of these constituents in native SCM. Whatever the case, elucidation of the structural basis for the cross reactivity of SCM with GBM will have to entail further investigation into the physical properties of the membrane components themselves. Only with a better understanding of the interactions between membrane components, once isolated from the membrane structure, will further fractionation or isolation procedures become apparent.

CHAPTER V

SUMMARY

The immunological cross reactivity of antibody specific for human glomerular basement membrane (GBM) and group A, type 12 streptococcal cell membrane (SCM) has been established (6, 7, 8, 36, 37, 46). Previous attempts at isolating soluble cross reactive components from either GBM or SCM had resulted in the loss of immunological cross reactivity in the soluble components as compared to the parent membrane preparation. However, the isolation of soluble components from either SCM or GBM was necessary if conventional chromatographic and immunological characterization procedures were to be utilized in the elucidation of the chemical and structural basis for the immunological cross reactivity of the two membrane preparations.

A modification of the lithium diiodosalicylate (LIS) extraction procedure of Marchesi and Andrews (45) was utilized to extract soluble components from streptococcal cell membrane in an attempt to isolate soluble SCM components displaying immunological cross reactivity with GBM. Initially, the LIS extracts were observed to display strong cross reactivity with anti-GBM sera in agarose gel immunodiffusion analysis. However, the presence of non-dialyzable LIS in the extracts was found to cause non-immune precipitation with

various unrelated antisera. Also, anomalous behavior of the LIS extracts in PAGE and electrophoresis in cellulose acetate membrane was attributed to the presence of residual LIS in the extracts. Dialysis of the LIS extracts vs. 0.01 M Tris-EDTA buffer, pH 8.9 (TE buffer) was observed to remove all but trace amounts of LIS from the extracts, as measured by atomic absorption-emission spectrophotometry. Removal of LIS from the extracts eliminated the effects attributed to LIS in immunodiffusion, PAGE, and electrophoresis in cellulose acetate membrane.

The soluble component resultant from LIS extraction of SCM, followed by two successive Genetron extractions of aqueous phase material, was termed GLCA. Dialysis of GLCA vs. TE buffer to remove LIS from the fraction yielded the soluble component termed TE-GLCA. Chemical analysis of TE-GLCA revealed a composition compatible to that of an acidic glycolipoprotein. TE-GLCA was hygroscopic, binding up to approximately 12% of its weight in water. DEAE cellulose chromatography of TE-GLCA yielded two fractions. The first fraction appeared in the equilibration buffer, and was not antigenic as determined by immunodiffusion in agarose gel. The second fraction, eluted at pH 5.0-5.5, was antigenic with several heterologous SCM antisera in immunodiffusion analysis. Poor recoveries of starting material after DEAE cellulose chromatography of TE-GLCA (less than 10%) were attributed to the property of constituents in TE-GLCA to precipitate below

pH 6.0.

Other soluble components were extracted from SCM with TE buffer, LIS, butanol, and combinations thereof. With respect to TE-GLCA, all had similar chemical compositions and physical properties.

The soluble components all displayed anomalous behavior on PAGE and detergent-PAGE. The properties were attributed to the membrane origin of the components and the effects of their subsequent dissociation from membrane lipid when extracted into aqueous solution. Thus, the lack of migration of constituents in the components in PAGE in the absence of detergent was attributed to the tendency for delipidated membrane proteins to aggregate in aqueous solution, forming large molecular weight aggregates incapable of migrating into the gels due to their size. The formation of protein-detergent micelles in the presence of Triton X-100 was believed to have caused complex banding patterns on Triton-PAGE and smearing in various regions of the gels. The chemical compositions and hygroscopic nature of the soluble components was proposed to have caused anomalous SDS binding, resulting in artifactual banding patterns and molecular weight values. Thus, the acquisition of significant information from PAGE analysis of soluble components was severely hampered by the physical properties of the components themselves.

Immunoelectrophoretic analysis of the soluble com-

ponents revealed the presence of up to two constituents in each of the components. One component was not mobile under the conditions of electrophoresis, the other had an anodal mobility slower than albumin. Immunodiffusion analysis revealed that all of the soluble components were antigenically related to each other, but did not share antigenic determinants with the insoluble residues resultant from the extraction procedures. All soluble SCM components failed to display reaction with anti-GBM sera. However, an antiserum prepared against the second fraction from DEAE cellulose chromatography of TE-GLCA precipitated with a GBM extract, but failed to react with the homologous antigen.

Indirect fluorescent antibody test on mouse kidney, heart, and lung sections, utilizing rabbit antisera prepared against SCM and soluble components extracted from SCM displayed: i) the specificity of the antisera for kidney tissue, and ii) that antisera prepared against SLS (sodium lauryl sulfate) and LIS extracted SCM soluble components produced fluorescent staining of GBM on par with or stronger than antisera prepared against the parent SCM.

Finally, it was discovered that dialysis of TE-GLCA vs. isotonic calcium chloride ($CaCl_2$) resulted in the isolation of two fractions, CAS and CAP. CAP was material in TE-GLCA precipitated by $CaCl_2$, while CAS remained in solution. CAS was subsequently shown to i) be distinct from CAP and TE-GLCA on Triton-PAGE analysis, ii) be higher in protein con-

tent than CAP and TE-GLCA, iii) display greater antigenicity in agarose gel immunodiffusion analysis than CAP and TE-GLCA, and iv) to be an enrichment of streptococcal antigens cross reactive with GBM. The increase in antigenicity of CAS over CAP and TE-GLCA was attributed to effects of calcium ion. i) The formation of CAP following dialysis of TE-GLCA vs. TE buffer released antigenic determinants (CAS) which were "hidden" or "masked" in TE-GLCA, due to blocking of sequential determinants or unfavorable conformations of the constituents. ii) A concentration effect, since CAS was significantly higher in protein content than CAP or TE-GLCA. iii) Incomplete removal of calcium ion from CAS by dialysis vs. TE buffer. Thus lattice structures formed by calcium bridges between constituents in CAS led to more efficient precipitation with antibody, or binding of calcium to constituents in CAS led to intrachain conformational changes antigenically more favorable.

Due to the interaction of calcium with soluble SCM components and the effects due to the presence of calcium on the antigenicity of the soluble components, it was proposed that divalent cations i) possessed an obligatory role in the structural integrity of SCM, and ii) were either involved directly with the antigenicity of streptococcal structures cross reactive with GBM or could be exploited in the isolation of the cross reactive antigens.

CHAPTER VI

BIBLIOGRAPHY

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