Horiguchi, M., and Kandatsu, M. (1960), *Bull. Agr. Chem. Soc. Japan 24*, 565.
Hudson, R. F. (1964), *Pure Appl. Chem. 9*, 371.
Kittredge, J. S. (1965), Ph.D. Thesis, University of California, La Jolla, Calif.
Kittredge, J. S., and Hughes, R. R. (1964), *Biochemistry 3*, 991.
Kittredge, J. S., Roberts, E., and Simonsen, D. G. (1962), *Biochemistry 1*, 624.
Kosolapoff, G. M. (1947), *J. Am. Chem. Soc. 69*, 2112.
Liang, C. R., and Rosenberg, H. (1966), *Biochim. Biophys. Acta 125*, 548.
Myers, T. C., and Jibril, A. O. (1957), *J. Org. Chem. 22*, 180.
Quin, L. D. (1964), *Science 144*, 1133.
Quin, L. D. (1965), *Biochemistry 4*, 324.
Rosenberg, H. (1959), *J. Chromatog. 2*, 487.
Rosenberg, H. (1964), *Nature 203*, 299.
Rosenthal, A. F., and Geyer, R. P. (1958), *J. Am. Chem. Soc. 80*, 5240.
Rouser, G., Kritchevsky, G., Heller, D., and Lieber, E. (1963), *J. Am. Oil Chemists' Soc. 40*, 425.
Segal, W. (1965), *Nature 208*, 1284.
Smith, I., Ed. (1960), in Chromatographic and Electrophoretic Techniques, New York, N. Y., Interscience, p 84.
Troll, W., and Cannon, R. K. (1953), *J. Biol. Chem. 200*, 803.
Zeleznick, L. D., Meyers, T. C., and Titchener, E. B. (1963), *Biochim. Biophys. Acta 78*, 546.

The Reactions of Sulfatide with Metallic Cations in Aqueous Systems*

Morris B. Abramson, Robert Katzman, Robert Curci, and Clarence E. Wilson

ABSTRACT: Sulfatide when converted to the sodium salt could be dispersed in water by ultrasonic radiation to form stable systems. Titrations and reactions with metallic cations showed the strongly acidic behavior of this lipid. Addition of univalent and divalent metal chlorides produced increases in turbidity and coagulation showing the relative affinity of the sulfate group for the cations was Ca > Mg > K > Na > Li. Analysis of the lipid coagulated by adding salts or mixtures of salts showed a stoichiometric relation of lipid to cation where each Ca^{2+} and Mg^{2+} bridges two lipid molecules. Selective binding of K > Na and Ca > Mg is found by turbidimetric measurements and by analysis of coagula.

Mixed dispersions of sulfatide with either lecithin or cerebroside show cation association by sulfatide with inclusion of neutral lipid in the coagula. Similarities are shown by acidic lipids and cation-exchange resins with analogous acid groups in their reactions with cations.

Theories of ion transport across biologic membranes as well as the establishment of membrane potentials assume specific binding of metallic cations at negative-charge sites on the membrane surface. Investigations in this laboratory directed toward the determination of such specificities have already shown the relative order of binding in aqueous systems of Ca^{2+}, Mg^{2+}, Li^+, Na^+, K^+, and TMA^+[1] by the acidic phospholipids, phosphatidic acid, and phosphatidylserine. The binding of cations by acidic phospholipids was postulated to be an intermediate step in membrane ion transport by Hokin and Hokin (1960), although now the relationship of the lipid to transport is considered to be more complex (Hokin, 1966). However, Tobias *et al.* (1962) have implicated the binding of cations by phospholipids in the physical changes occurring in the membrane in transport and potential phenomena.

Lipids with ionized sulfate groups constitute an important component of membranes in the central nervous system. These sulfate groups are more strongly acidic than the lipid phosphate or carboxyl groups. In the present paper, we give data on the ionic characteristics of sulfatide and measurements of the interaction of the ionized sulfate group of this lipid with alkali and alkaline earth cations in aqueous systems. Whereas the phospholipids have been shown to bind Na^+ more strongly than K^+, we have found that aqueous dispersions of sulfatide react more strongly with K^+ than Na^+. This result was anticipated by the finding that sulfatide can be purified by precipitation as the potassium salt (Blix, 1933; Lees *et al.*, 1959). An

* From the Saul R. Korey Department of Neurology, Albert Einstein College of Medicine, New York, New York. *Received August 23, 1966.* Supported by the Office of Saline Water, U. S. Department of the Interior, and Research Grant NB-03356 from the National Institutes of Health, U. S. Public Health Service.

[1] Abbreviation used: TMA^+, tetramethylammonium ion.

interesting general agreement is found with the results published by Breyer (1965), who studied ion binding in a two-phase chloroform–methanol–water system. Green *et al.* (1961) reported that the binding of K^+ by brain mitochondria was due to the sulfatide present.

For the study of the reactions of aqueous systems of phospholipids with cations, two experimental procedures were found to be effective. These are a measurement of the hydrogen ion released by the lipid (Abramson *et al.*, 1966) and the changes in the turbidity of dispersed systems (Abramson *et al.*, 1965) on addition of the cation. Since the first procedure cannot be applied to the studies of sulfatide because of its essentially complete acid ionization, we have used turbidimetric titrations. This procedure clearly demonstrates that the surface groups of this lipid react differently with different cations. To show that these differences are related to varying amounts of the cations associated with the lipid, we have employed a procedure for separating the lipid from its aqueous medium and analyzing the cation present. These results give actual measures of the cations associated with the lipid and may be related to the changes in charge and structure that are indicated by turbidity changes.

Since biologic membranes contain a mixture of lipid components with a variety of polar groups neighboring each other, we are presently investigating the effects of such interactions of different charged groups by studying the properties of mixed-lipid dispersions. We present here the properties of some aqueous systems containing sulfatide mixed with cerebroside or lecithin. We will report some properties of mixed acidic lipids in a later paper.

Experimental Section

Preparation of the Sodium Form of Sulfatide. The method of Lees *et al.* (1959) was used to extract sulfatide from beef brain. The purest fraction, sulfatide A, is obtained as the potassium salt. Two procedures were employed to convert this product to the sodium salt form. In one, a coarse aqueous suspension of the sulfatide A was made in 0.1 M NaCl and shaken 12 hr at 2°. The supernatant was removed after centrifuging, and the solid was retreated with 0.1 M NaCl. The solid was washed with chloroform–methanol–water (1:1.5:0.2, v/v) and brought to −20°, thereby reducing the solubility of the lipid. The liquid was removed after centrifuging, the washing procedure was repeated three times at −20°, and the solid was dried under vacuum.

In a second procedure, the sulfatide A was dissolved in chloroform–methanol (2:1, v/v) and one-fifth its volume of 0.1 M NaCl was added. The two-phase system was agitated for several hours at 2°, and the aqueous layer was removed. An equal volume of 0.1 M NaCl was added, and the partitioning was then repeated. The lower phase was removed and washed three times with small volumes of water. This procedure gave a more complete conversion to the sodium sulfatide salt than the first procedure; however there were greater losses to the aqueous layer on washing. The sulfatide was analyzed for galactose by a procedure used by Svennerholm (1956) which showed 18.1% galactose. This value is similar to that reported by Lees *et al.* (1959), although 5% less than the theoretical value. Phosphorus analyzed by a modified procedure based upon that of Chen *et al.* (1956) showed negligible phosphorus (0.005 μmole/mg).

Thin layer chromatography was carried out with plates coated with silica gel HR (E. Merck A. G., Darmstadt). Lipid (100 μg) was deposited. The solvent system used was chloroform–methanol–water (70:30:4, v/v), and the plates were developed with I_2 vapors and in some instances charred with concentrated sulfuric acid. Both sulfatide and cerebroside standards were run on each plate. All preparations of sulfatide A showed one spot with no detectable cerebroside. Relying upon the absence of any significant amount of phospholipid, the chromatographic purity of the preparations, and the complete agreement of the ionic behavior of our material with that anticipated for a monoester of sulfuric acid, we have employed the structure given for sulfatide by Thannhauser *et al.* (1955), and have used a molecular weight of 925.

Sodium and potassium were determined following nitric acid digestion by means of a Baird Atomic flame photometer using an internal standard with an accuracy of 3%. Calcium and magnesium were analyzed with a Perkin-Elmer atomic absorption spectrophotometer. Calcium samples were prepared to contain 1% lanthanum chloride in hydrochloric acid. Magnesium samples contained 0.1% lanthanum chloride. Accuracies for calcium and magnesium were 5 and 3%, respectively.

Cation Content of Lipid. In the course of the preparation of sulfatide we analyzed the product at various stages to ascertain the cation content. Although repeated preparations were not identical, Table I gives the typical content of cations in the phosphorous-free sulfatide A and the sulfatide B before and after passage through the Florisil column. The cations of one preparation of sulfatide A converted to the sodium form are also given. They are typical of the lipid used in the experiments reported here.

Turbidimetric Measurements. A weighed amount of the sodium form of sulfatide was dissolved in chloroform–methanol (2:1) in a 20-ml glass tube, and the solvent was evaporated, to leave a solid deposit. Water (5 ml) was added, and the lipid was dispersed by ultrasonic radiation by the procedure described in earlier publications. It was then diluted with redistilled water to a concentration of approximately 2×10^{-4} M and was filtered through a 0.8-μ Millipore filter. The dispersions were stored at 2° and from five- to eight-aliquot samples were used for turbidimetric titrations within a 5-day period. Aliquots of 9 ml were brought to 10 ml with 0.5 M Tris, pH 7.3. Turbidity measurements were made in a semioctagonal cell in a Brice-Phoenix photometer. Light intensities were measured at 0, 90, 45, and 135°. Except for one series of experiments at 38–40°, all others were performed at 24

TABLE I: Cation Content of Sulfatide Preparations.

Sample of Sulfatide	Cation Content of Lipid (μmole/μmole) Na	K	Ca	Mg	Total Equiv of Cations/ Mole of Lipid
		Sulfatide A			
As extracted	0.030	0.80	0.016	0.010	0.87
After conversion to Na salt	0.81	0.11	0.011	0.005	0.95
		Sulfatide B			
Before Florisil column	0.069	0.59	0.015	0	0.69
After Florisil column	0.32	0	0.026	0.31	0.99

± 1°. Small additions of 3 M KCl or 4 M NaCl and LiCl or 0.1 M $CaCl_2$ and $MgCl_2$ were made by means of a microsyringe, and dilution effects were small. These reagents were also filtered through a 0.8-μ Millipore filter. Addition of salt caused rapid changes in turbidity, and readings of light intensity were stable after 5 min except when heavy coagulation occurred. After coagulation, the precipitate was separated by centrifugation and dissolved in chloroform–methanol. Thin layer chromatography of the precipitate showed only sulfatide; there was no evidence of cerebroside or other degradation products.

Determination of Cations in Coagulated Sulfatide. In these experiments 5-ml aliquots of dispersions containing 1 mg/ml of the sodium sulfatide salt were brought to the concentration of cation which had been found by turbidimetric studies to produce coagulation (unnecessary excess of cation was avoided). Where two or more cations were added, a solution of the cations was prepared in the desired concentration ratio, and this was added to the lipid dispersion to reach the final concentration. All systems were kept for 12 hr so that coagulation would be completed. The systems were centrifuged to compact the solid, and most of the supernatant was removed, leaving approximately 0.2 ml. This was used to aid the transfer of the coagulate to a preweighed 0.8-μ Millipore filter. Filtration was carried out under a gentle vacuum. The solid was kept in a small area of the filter to reduce the volume of the wetting liquid. The Millipore filter with the wet solid was quickly weighed, dried under vacuum overnight, and reweighed. A series of control experiments in which water was passed through a Millipore filter showed negligible changes in weight after drying. The loss in weight of the filter containing the wet coagulate on drying gave the weight of the water wetting the solid and filter. In subsequent calculations this loss in weight is assumed to be equal to the volume of supernatant solution present in the filter.

The Millipore filter and solid were digested in 0.5 ml of concentrated nitric acid at 90° for 30 min and dissolved except for a small residue. The solution was diluted to an appropriate volume, and aliquot samples were taken for analysis. Similar aliquots of the supernatant obtained after centrifugation of the system were also analyzed for cations. Periodically, blank Millipore filters were digested and analyzed for cations. These contained negligible potassium, small amounts of magnesium and calcium, and approximately 0.24 μmole of sodium (range 0.19–0.27) per filter. Control experiments showed no loss of cation when water was passed through the filter.

From the volume of the supernatant estimated to be on the filter and the analysis of the cation concentrations of the supernatant, a correction could be made for the cations present on the filter due to the wetting supernatant. This correction and a correction for the cation found in the blank Millipore filter when subtracted from the total cation content of the solid gave the cations present in the lipid coagulum. In a similar manner, the dry weight of the solid was corrected for the weight of dried salts from the supernatant solution.

Results

Properties of Aqueous Dispersions. Preparations of sulfatide containing sodium as the predominant cation dispersed readily in water forming relatively clear systems on exposure to ultrasonic radiation for short periods (2–3 min). Sulfatide in which potassium was predominant required longer periods of radiation at higher intensities and produced more turbid systems. A preparation containing a ratio of potassium:sodium of 3:1 could not be dispersed, but when this ratio was changed to 1:2, relatively clear dispersions readily formed. The pH of the dispersions of either the sodium or potassium form ranged from 4.7 to 5.3.

Titrations of these dispersions with acid and base showed no buffer capacity from pH 2.75 to 10. This indicated the strong acid character of this lipid. After titration the lipid was extracted from the aqueous medium, and thin layer chromatograms showed no change from the original material. This indicated its stability within the range of pH used. The addition of $CaCl_2$ to a concentration of 0.01 M in systems at pH 4.7 containing 3.38 μmoles of sulfatide in 5 ml released only 0.033 μmole of H^+. The addition of alkali metal chlorides produced negligible H^+ ion

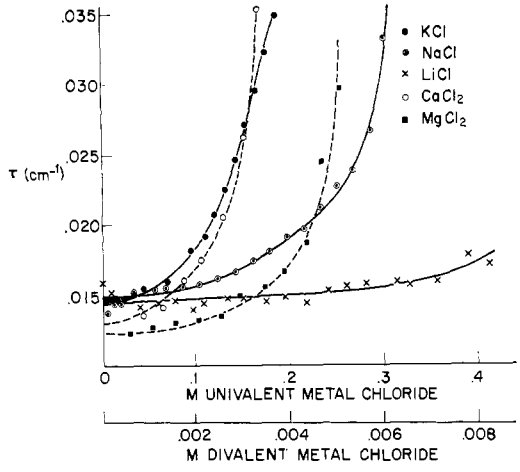

FIGURE 1: Changes in turbidity of aqueous systems of sulfatide on addition of univalent and divalent metal chlorides. Systems contained approximately 2×10^{-4} M sulfatide in 0.05 M Tris at pH 7.3; λ 546 mμ.

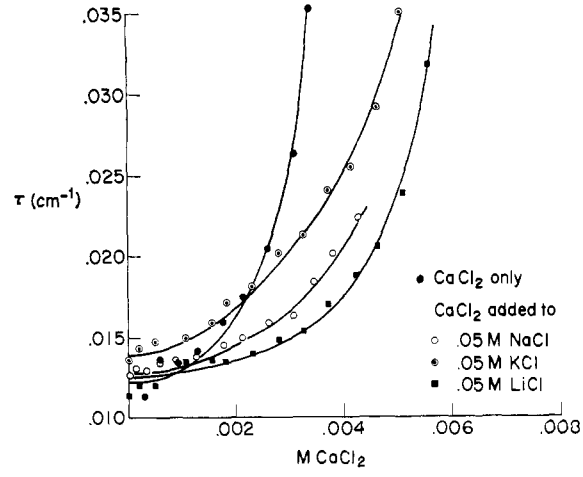

FIGURE 2: Changes in turbidity of aqueous systems of sulfatide containing 0.05 M alkali metal chlorides on addition of $CaCl_2$. Systems contained approximately 2×10^{-4} M sulfatide in 0.05 M Tris at pH 7.3; λ 546 mμ.

release, in keeping with the highly ionized nature of the sulfate groups.

Turbidimetric Measurements. Dispersed systems of the potassium salt of sulfatide with a concentration of approximately 2×10^{-4} M had turbidities of the order of 0.030 at 546 mμ. When converted to the sodium sulfatide, dispersions were formed with lower turbidities (0.013). Similar low turbidities were measured for sulfatide B preparations which also had a high sodium content.

The addition of Tris buffer to a concentration of 0.01 M produced little change in the turbidity of the systems. On adding solutions of univalent and divalent metal chlorides, turbidity changes occurred at concentrations of cation which were different for the various metals (Figure 1). For the univalent chlorides, KCl produced an increase in turbidity beginning at 0.07 M KCl, and heavy coagulation or flocculation occurred at 0.15 M KCl. Parallel experiments with NaCl showed no increase in turbidity until a concentration of 0.12 M was reached, and coagulation was heavy at 0.30 M. Confirmation of these effects was observed in experiments in which systems of dispersed sulfatide were brought to a desired cation concentration and permitted to coagulate on standing 24 hr. Very little coagulate formed in 0.1 M KCl, but complete coagulation resulted in 0.15 M KCl. Contrary to this, similar systems in 0.20 M NaCl formed no precipitate, and only part of the sulfatide was coagulated in 24 hr in 0.33 M NaCl. The addition of LiCl or TMACl produced no increase in turbidity until 0.5 M concentration was reached; above this, small increases in turbidity occurred with increasing concentrations.

The greater effect of divalent cations was shown by an increase in turbidity that took place at 2×10^{-3} M $CaCl_2$ and heavy coagulation at 4×10^{-3}. A system brought to a concentration of 3×10^{-3} M $CaCl_2$ coagulated completely on standing overnight. $MgCl_2$ required higher concentrations, producing an increase in turbidity at 4.5×10^{-3} M and coagulation at 1×10^{-2} M.

Experiments carried out with the dispersed systems maintained at 38–40° showed the same relative behavior to cations as at 24°. At the higher temperature the initial turbidity of a comparable system was lower than at 24°, and the concentrations of NaCl, KCl, or $CaCl_2$ required to increase turbidities were from 15 to 25% greater than at the lower temperature.

Antagonistic effects of univalent and divalent cations are shown in Figure 2. The addition of higher concentrations of $CaCl_2$ was required to produce an increase in turbidity in the presence of 0.05 M concentration of univalent cations than in their absence. The order of effectiveness as indicated by the increased concentration of $CaCl_2$ required was $Li^+ > Na^+ > K^+$. Similar effects were found for the addition of $MgCl_2$ in the presence of 0.05 M concentration of univalent ions, except that the comparable concentrations of $MgCl_2$ were approximately twice those of $CaCl_2$. In a like manner, the presence of NaCl or KCl required the addition of higher concentrations of $CaCl_2$ to cause coagulation on standing.

The converse influence of divalent cations on the effectiveness of univalent cations was also followed by turbidimetry. Dispersions of sulfatide A were brought to a concentration of $CaCl_2$ equal to 1.5×10^{-3} M. This resulted in a moderate increase in turbidity. Additions of NaCl to one such system produced at low concentrations (<0.1 M) a decrease in turbidity followed by an increase in turbidity at concentrations >0.2 M. LiCl produced only a decrease in turbidity at low concentrations with no further increase. KCl produced no change at low concentrations but increased the turbidity above 0.06 M. Similar effects of the univalent cations were observed when added to systems contain-

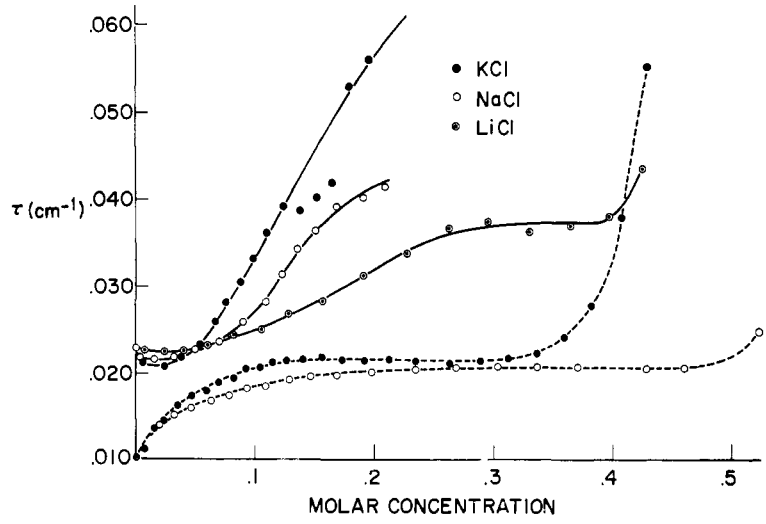

FIGURE 3: Changes in turbidity of aqueous systems of sulfatide codispersed with either cerebroside or lecithin on addition of alkali metal chlorides in 0.05 M Tris at pH 7.3; λ 546 mμ. ———, 0.114 mg/ml of sulfatide (1.2×10^{-4} M) + 0.089 mg/ml of cerebroside (1.1×10^{-4} M). ---------, 0.18 mg/ml of sulfatide (2×10^{-4} M) + 0.175 mg/ml of lecithin (2×10^{-4} M).

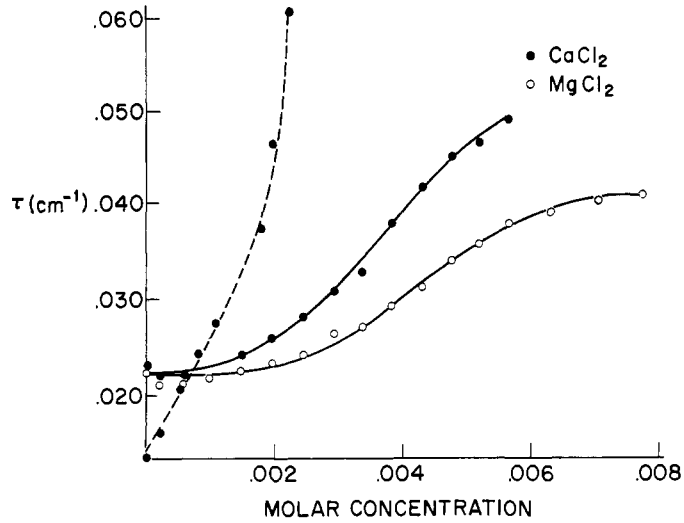

FIGURE 4: Changes in turbidity of aqueous dispersions of sulfatide codispersed with either cerebroside or lecithin on addition of alkaline earth chlorides in 0.05 M Tris at pH 7.3; λ 546 mμ. ———, 0.114 mg/ml of sulfatide (1.2×10^{-4} M) + 0.089 mg/ml of cerebroside (1.1×10^{-4} M). ---------, 0.18 mg/ml of sulfatide (2×10^{-4} M) + 0.175 mg/ml of lecithin (2×10^{-4} M).

ing initially 2.8×10^{-3} M $MgCl_2$.

Mixed Lipids. Weighed portions of sulfatide and cerebroside or lecithin were dissolved together by warming in chloroform–methanol (2:1).[2] After thorough mixing, the solvent was evaporated. The solid residue, a mixture of lipids, was dispersed in water by ultrasonic radiation. The dispersions were stable and did not show any change over periods of several days. The presence of cerebroside increased the turbidity. Figure 3 shows the effect of added univalent cations on dispersions containing 1.14 mg of sulfatide and 0.89 mg of cerebroside/10-ml aliquot. The effect of Ca^{2+} and Mg^{2+} (Figure 4) is less pronounced than that observed for sulfatide alone. Higher concentrations of both these cations are required to produce coagulation. The order of effectiveness, $Ca^{2+} > Mg^{2+} > Na^{+} > Li^{+}$, is the same as for sulfatide alone, except that the differences between the univalent cations are

[2] Both the cerebroside (phrenosin–kerasin, 2:1) obtained from beef brain white matter by column separation and the egg lecithin were chromatographically pure as determined on thin layer.

TABLE II: Cation Content of Sulfatide Coagulated from Aqueous Systems.

Initial System (M)	Concentration of Supernatant Solution (M) Na	K	Ca	Mg	Cation Content of Lipid (μmole/μmole) Na	K	Ca	Mg	Total Equiv of Cations/ Mole of Lipid
I KCl (0.15)		0.15			0	0.97			0.97
II NaCl (0.10)–KCl (0.10)	0.096	0.096			0.39	0.81			1.20
III $CaCl_2$ (0.033)			0.033		0.12[a]	0	0.43[a]		0.98[a]
					0.14	0	0.44		1.02
IV $MgCl_2$ (7.5×10^{-3})				7.4×10^{-3}	0.013	0.024		0.49	1.01
V $CaCl_2$ (3×10^{-3})–$MgCl_2$ (3×10^{-3})	0.61×10^{-3}		2.7×10^{-3}	2.8×10^{-3}	0.017		0.29	0.10	0.80
VI $CaCl_2$ (4.7×10^{-3})–$MgCl_2$ (4.7×10^{-3})	Not analyzed	0.01	4.7×10^{-3}	4.7×10^{-3}	0.12	0.29	0.15	0.079	0.87
VII NaCl (0.075)–KCl (0.055)–$CaCl_2$ (4.5×10^{-3})–$MgCl_2$ (7.5×10^{-3})	0.0743	0.054	4.5×10^{-3}	7.6×10^{-3}	0.26	0.39	0.13	0.073	1.07
VIII NaCl (0.046)–KCl (0.125)–$CaCl_2$ (2.52×10^{-3})–$MgCl_2$ (7.50×10^{-3})	0.046	0.12	2.5×10^{-3}	7.1×10^{-3}	0.18	0.72	0.008	0.026	0.97

[a] Coagulate washed free of supernatant solution.

decreased.

Several systems containing sulfatide and lecithin in different ratios were investigated. The turbidities of the lecithin-containing dispersion were lower than for sulfatide alone. Figures 3 and 4 show the changes in turbidity of a system containing 1.8 mg of sulfatide and 1.75 mg of lecithin/10-ml aliquot. The presence of lecithin appears to stabilize the dispersions toward univalent cations. An initial increase in turbidity occurs at concentrations of univalent cations <0.1 M, but then greater concentrations are required for coagulation than for sulfatide alone. $CaCl_2$, however, showed the same effectiveness in coagulating the sulfatide–lecithin dispersion as it did for sulfatide alone. Pure lecithin dispersions were unaffected by $CaCl_2$ to concentrations of 0.006 M.

The coagulated systems of mixed lipids were centrifuged, and the coagulated solid was dissolved in chloroform–methanol (2:1). Thin layer chromatograms showed the presence of each of the lipids dispersed with relative intensities of spots comparable with that of the original mixture of lipids. This indicates the simultaneous coagulation of both lipids as components of the same dispersed particle.

Cations in Coagulated Sulfatide. The cations present in the coagulated specimens were analyzed to see whether they might measure the relative extent of association of the different metal ions with the dispersed polyanion. In all cases, the total cation content approached the stoichiometric amount for the lipid present and indicated complete availability of the sulfate groups for reaction (Table II).

Coagula containing only univalent cations or univalent cations plus small amounts of calcium when washed free of the adhering supernatant liquid were redispersed and could not be analyzed. System III is an example of one containing high calcium which was washed with small portions of water four times without redispersion. The analysis of the cation content in this washed precipitate and an identical sample, which was not washed but which was corrected for the adhering supernatant liquid, show reasonable agreement. This finding increases confidence in the corrected analysis of unwashed, coagulated lipids and permits the study of systems with univalent cations.

The analysis of system I shows that the sodium sulfatide is converted to the potassium salt in 0.15 M KCl. In the presence of solutions of mixed NaCl and KCl in equimolar concentrations as in system II, the sulfatide coagulates with a ratio of potassium: sodium = 2:1. A similar system using sulfatide B coagulated in 0.1 M KCl and 0.1 M NaCl and had a ratio K:Na = 1.89. The systems containing either $CaCl_2$, $MgCl_2$, or both present clear indications of the association of one divalent cation with two sulfatide ions, thus necessitating the formation of a bridging unit. The systems to which only divalent cations were added showed that the sodium present as the original

TABLE III: Comparative Values of Cations in Forming Associations with Sulfatide Anions.

	Analytical Data (Cation Equiv/Mole of Lipid in Coagulate) (Cation Concn (normal) in Supernatant)			Turbidimetric Data Molar Concn of Cations in Equiv Systems at Equal Turbidities (τ = 0.020)		
	Na	K	K/Na	Na	K	1/(K/Na)
System II	4.0	8.4	2.1			
System VII	3.5	7.2	2.1	0.216	0.107	2.0
Sulfatide B	2.4	4.6	1.9			
	Ca	Mg	Ca/Mg	Ca	Mg	1/(Ca/Mg)
System V	106	37	2.9	2.48×10^{-3}	4.55×10^{-3}	1.84
System VI	32	17	1.9			
System VII	62	19	3.3			

TABLE IV: Cation Content of Coagulated Mixed Dispersions of Sulfatide and Other Lipids.

Initial System	Concentration of Supernatant Solution (M) Na	Ca	Mg	Cation Content of Lipid μmole/μmole of Sulfatide[a] Na	Ca	Mg	Total Equiv of Cations/ Mole of Sulfatide
IX, 2.85 mg of sulfatide–2.83 mg of lecithin in 3.03×10^{-3} M $CaCl_2$–3.03×10^{-3} M $MgCl_2$	0.58×10^{-3}	2.8×10^{-3}	2.8×10^{-3}	0.039	0.27	0.12	0.82
X, 2.75 mg of sulfatide–2.75 mg of cerebroside in 2.87×10^{-3} M $CaCl_2$–2.87×10^{-3} M $MgCl_2$	0.50×10^{-3}	2.7×10^{-3}	2.6×10^{-3}	0	0.24	0.15	0.78

[a] Assuming the same mole fraction of sulfatide in the coagulate as in the dispersion.

counterion appeared in the final supernatant. This resulted from the exchange of divalent for univalent cations. The concentration of sodium in the supernatant agreed within the experimental limits with the equivalent of divalent metal that presumably was associated with the entire lipid sample used. This calculation was based upon the cation content found in the coagulated portion, which in most instances was within 80% of the theoretical weight of lipid.

System VI with equimolar concentrations of calcium and magnesium shows relative amounts of calcium: magnesium in the coagulate of 1.90 while system V is somewhat higher, 2.8. System VII was designed to obtain a measure of the relative affinities of the four cations of interest in a mixed system. The concentrations of the salts used were such that on the basis of earlier experiments the amounts of any of the cations in the coagulated material would not be either excessively high or low. Again the order of selectivity is as predicted, but the ratio of calcium:magnesium was 3.2:1, which takes into account the supernatant concentrations. The variability of the calcium:magnesium concentrations between 1.86:1 and 3.2:1 as shown in Table III indicates the limitations of this methodology.

Cations in Mixed-Lipid Systems. A dried mixture of equal weight sulfatide and lecithin was dispersed in water and brought to a concentration of 3.03×10^{-3} M $CaCl_2$ and 3.03×10^{-3} M $MgCl_2$. This resulted in coagulation. The analysis of the supernatant and coagulum (Table IV) shows the order of selectivity of calcium:magnesium to be the same as in sulfatide alone, with an equivalent release of sodium from the sulfatide. From the weight of the coagulated lipid mixture and the mole fraction of sulfatide in the original material, the total cation found agrees with the sulfatide present. This indicates that the lecithin does not associate with cations under these conditions. A similar mixture of sulfatide and lecithin coagulated in 0.1 M

NaCl and 0.1 M KCl could not be filtered in the usual manner. This coagulum, in the absence of divalent cations, possibly undergoes micellar changes which permit its passage through the filter.

A dispersion of mixed sulfatide and cerebroside coagulated in a solution of equimolar $CaCl_2$ and $MgCl_2$ showed the same order of selectivity of these two cations and the total equivalence as for sulfatide alone. Thin layer chromatographs of portions of the coagulated sample showed the presence of each lipid used in what appeared to be the same relative amounts as in the original materials. Furthermore, where a coagulum was formed which was filterable, the supernatant when extracted for lipid showed either the complete absence of lipids or only faint spots on thin layer chromatographs.

A sulfatide dispersion, system VIII, was brought to a concentration of univalent and divalent cations similar to that present in intracellular fluid. The coagulated lipid and the supernatant solution were analyzed in the manner described. The relatively high concentration of KCl in the supernatant resulted in the inclusion of potassium as the major cation of the lipid and a smaller than anticipated content of divalent cations. At these low values for cation concentration, relative binding cannot be estimated accurately. It is significant, however, that in this mixture of cations, sulfatide exists predominantly as the potassium salt, and the low content of divalent cations precludes any significant degree of bridging between these lipid molecules.

Discussion

The relative amounts of the different cations associated with the lipid are estimated here by the analytical results of the coagulated samples and by the concentrations of cations that produce a sharp increase in turbidity in comparable systems (Table III). Our analytical data show stoichiometry between the total number of sulfate groups present and the equivalents of cations in accord with the presumed orientation of the ionic groups in the aqueous media. The lipid can be converted in practically its entirety from one cationic form to another by changes in the cationic content of the media. However, in solutions of a single salt or in a solution of mixed salts the concentration of cation that is required for converting the sulfatide to that cationic form varies with the cation. The relative values for the cation required for this conversion found by the turbidimetric and the analytic methods are in agreement. This leads to the view that the change in turbidity follows the formation of a close association of the lipid with a cation. A comparison of the effectiveness of like-charged cations in producing increased turbidities then measures the relative effectiveness of formation of an association with the lipid. In reaching these comparative values for like-charge types, it is not necessary to predicate any mechanism or model for the interaction.

In comparing univalent and divalent cations, recognition must be given to the observed ratio of sulfatide: cation found in the analytical data of 1:1 for univalent cations and 2:1 for divalent cations. It is then desirable to compare these cations utilizing a selectivity coefficient which includes the charges of the ions. Treating the lipid, with its ionogenic groups on the surface of the solid particle, in a manner similar to the resin phase of a cation-exchange resin, a selectivity coefficient can be set up comparing both univalent and divalent cations with a reference ion, sodium. Using $\bar{X}_B$ for the ion fraction of sodium and $\bar{X}_A$ for the cation A in the lipid phase and X_B and X_A for their ion fractions in solution, the rational selectivity coefficient is given by the equation ${}^{N}K_B{}^{A} \equiv \bar{X}_A{}^{|Z_B|}X_B{}^{|Z_A|}/\bar{X}_B{}^{|Z_A|}X_A{}^{|Z_B|}$ (Helfferich, 1962). It must be pointed out that these coefficients give only relative values for closely related systems. The selectivity coefficients, in terms of sodium = 1, are found from the data of system VII (Table II) to be calcium, 34.8, magnesium, 12.6, and potassium, 2.05.

Noteworthy points of similarity can be observed in the properties of acidic lipids and cation-exchange resins. In both categories, we are dealing with essentially insoluble high molecular weight polyacids. For the lipid dispersions which are in the form of bilayers or micellar units, we can visualize an oriented hydrocarbon structure with the ionogenic groups directed to the aqueous phase. Several investigators have shown a similarity between the action of cation exchange resins and biologic systems such as erythrocyte ghosts or microsomes (Carvalho *et al.*, 1963; Kavanau, 1965). The reactions of acidic lipids implicate these compounds as ion exchangers of biologic membranes.

Titration studies of resins with sulfonic acid, phosphonic acid, and carboxylic acid groups show characteristics paralleling those of the lipids with similar acid groups. It should be noted the lipids are esters of sulfuric or orthophosphoric acids while the related resins are not esters. Titrations of aqueous dispersions of phosphatidylserine and phosphatidic acid show buffering at neutral pH due to the incomplete ionization of the acid groups. This property has been used in the study of the ion-exchange properties of these lipids in this laboratory and by others (Christensen and Hastings, 1940; Dervichian, 1955; Hendrickson and Fullerton, 1965). Phosphoric acid resins show similar buffering (Bregman and Murata, 1952). In contrast, we find that sulfatide dispersions show no buffer capacities in the pH range studied, sulfatide being completely ionized in the form of a salt in our aqueous systems. Sulfonic acid resin is also completely ionized above pH 2 (Bregman, 1953).

The selective uptake of cations by sulfatide parallels the sulfonic acid resins with an order of selectivity of K > Na > Li. The selectivities of different resin types containing different acid groups are in the same order as those of lipids containing related acid groups. These selectivities for alkali ions referred to sodium are given in Table V.

These selectivities do not of themselves prove specific chemical binding, but they do indicate an ionic associa-

TABLE V: Selectivities for Alkali Ions.

	Resins			Lipids	
	Sulfonic[a]	Carboxylic[a]	Phosphonic[a]	Sulfatide	Phosphatidic Acid
Li	0.51	1.39	1.54	<1	1.1
Na	1.00	1.00	1.00	1.00	1.00
K	1.25	0.88	0.66	2.05	0.56

[a] Adapted from Bregman (1953).

tion. This association of cations with polyanions depends upon the charge, radius, and hydration of the cation and the polarizability of the anion in comparison with that of water. These polarizabilities are of the order $PO_4^{3-} > COO^- > H_2O > SO_4^{2-}$ (Bregman, 1953). We may view the association of the cation with the anionic group as dependent upon a competitive balance between the attractions of the cation for water and for the anion. The small cations which produce greater polarization of phosphates than of water bind more strongly to this anion than the large cations, and the selectivity is found to be Li > Na > K. Since sulfate is polarized less than water, the small cations retain their large hydration shell in preference to the anion site. The larger cations with decreased hydration are attracted more strongly to the anion, and the selectivity is K > Na > Li. It is also not surprising to find similar trends apparent in the solubilities of the inorganic metallic salts of these anions. The molar solubilities of the sulfates are in the order of Mg > Ca; Li > Na > K; while for the acid phosphates, it is K > Na > Mg > Ca.

The studies reported here provide an interesting basis for comparison with those of Breyer (1965) and permit some general observations on the nature of the binding of cations by acidic lipids. Employing a procedure similar to that used by Folch *et al.* (1957), Breyer partitioned sulfatide salts between upper phase (chloroform–methanol–water, 3:48:47, v/v) and lower phase (chloroform–methanol–water, 86:14:1, v/v) in which mixtures of metallic chlorides were dissolved in the upper phase. It may be pointed out in connection with Breyer's data that the unequal binding of the two cations will alter the composition of the upper phase differently so that at equilibrium the cation content of the upper phase will no longer be equal for both metals. Furthermore, the release of sodium from the sodium sulfatide used as starting material will also alter the ion content of the upper phase. These changes will be most pronounced in the experiments at low salt concentrations. The procedure we employ gives the concentrations of cations present in the aqueous system in equilibrium with the sulfatide salt whose total cation is analyzed.

Breyer found, using equinormal mixtures of NaCl and KCl with total concentration of salt ranging from 0.005 to 0.2, that the ratio of K:Na in the sulfatide present in the lower phase was relatively constant at 1.67. A similar series in which the upper phase contained equinormal $CaCl_2$ and $MgCl_2$ in a like range of concentrations gave a ratio of Ca:Mg content of the sulfatide in the lower phase of 1.92. The values for K:Na are somewhat lower than the ratios we obtained from the turbidimetric and analytic data (approximately 2). The ratio of Ca:Mg is similar.

The agreement of our findings with those of Breyer leads to the view that in both types of experiments the ratio of the cations bound is determined by the reaction in the aqueous (or upper) phase. To explain this agreement we must assume that either the solubilities of the various sulfatide salts in the lower phase vary in the same manner as the ionic specificities of sulfatide anion in the aqueous systems of our experiments, or that the various salt forms of sulfatide are equally soluble in the lower phase solvent. It is reasonable to conceive that the sulfatide salts are equally soluble in the lower phase solvent and possibly other nonpolar solvents including the hydrocarbon barrier of a lipid membrane. Studies of some phospholipids in nonpolar solvents showed the formation of small micelles which presumably have their hydrocarbon chains directed to the solvent (Legault-Demare and Faure, 1959). The salt forms of sulfatide, if micellar in the lower phase, would have the polar groups and the metallic element directed away from the solvent. They would thus show very little difference in their attractions for the nonpolar components of the solvent.

Breyer also reports distribution coefficients for the alkali and alkaline earth metal salts of sulfatide in the upper and lower phase solvents. She found a greater proportion of sodium and potassium sulfatide in the upper phase as compared to calcium and magnesium. The proportion of lipid in the upper phase is inversely related to the binding of the cations to the lipid anion. In the absence of added salts, the sodium and potassium forms of sulfatide are to a great extent dissociated into free ions in aqueous systems. This form remains dispersed in the polar solvent. The form with bound cations dissolves more readily in the nonpolar phase. The association of calcium and magnesium with sulfatide by the bridging of the two molecules by the divalent cation forms a very stable structure in which the head groups of the lipid lose water and become oriented in a manner permitting the hydrocarbon chains to be exposed to nonpolar solvents. This increases the solubility of these salts in the lower phase.

References

Abramson, M. B., Katzman, R., and Curci, R. (1965), *J. Colloid Sci. 20*, 777.

Abramson, M. B., Katzman, R., Curci, R. and Gregor, H. P. (1966), *Biochemistry 5*, 2207.

Blix, G. (1933), *Z. Physiol. Chem. 219*, 82.

Bregman, J. I. (1953), *Ann. N. Y. Acad. Sci. 57*, 125.

Bregman, J. I., and Murata, Y. (1952), *J. Am. Chem.*

Soc. 74, 1867.
Breyer, U. (1965), *J. Neurochem. 12*, 131.
Carvalho, A. P., Sanui, H., and Pace, N. (1963), *J. Cellular Comp. Physiol. 62*, 311.
Chen, P. S., Jr., Toribara, T. Y., and Huber, W. (1956), *Anal. Chem. 28*, 1756.
Christensen, H. N., and Hastings, A. B. (1940), *J. Biol. Chem. 136*, 387.
Dervichian, D. G. (1955), *in* Biochemical Problems of Lipids, Popjak, G., and Lebreton, E., Ed., New York, N. Y., Interscience, p 3.
Folch, J., Lees, M., and Sloane-Stanley, G. H. (1957), Metabolism of the Nervous System, Richter, D., Ed., Oxford, Pergamon, p 174.
Green, J. P., Robinson, J. D., and Day, M. (1961), *J. Pharmacol. Exptl. Therap. 131*, 12.
Helfferich, F. (1962), Ion Exchange, New York, N. Y., McGraw-Hill, p 154.
Hendrickson, H. S., and Fullerton, J. G. (1965), *Biochemistry 4*, 1599.
Hokin, M. (1966), *Federation Proc. 25*, 656.
Hokin, M. R., and Hokin, L. E. (1960), *Intern. Rev. Neurobiol. 2*, 99.
Kavanau, J. L. (1965), Structure and Function in Biologic Membranes, San Francisco, Calif., Holden-Day, p 328.
Lees, M., Folch, J., Sloane-Stanley, G. H., and Carr, S. (1959), *J. Neurochem. 4*, 9.
Legault-Demare, J., and Faure, M. (1959), *Bull. Soc. Chim. Biol. 33*, 1013.
Svennerholm, L. (1956), *J. Neurochem. 1*, 42.
Thannhauser, S. J., Fellig, J., and Schmidt, G. (1955), *J. Biol. Chem. 215, 211.*
Tobias, J. M., Agin, D. P., and Pawlowski, R. (1962), Symposium on the Plasma Membrane, Fishman, A. P., Ed., New York, New York Heart Association.

The Heterogeneity of Bovine Pancreatic Ribonuclease S*

M. S. Doscher and C. H. W. Hirs

ABSTRACT: The initial proteolysis of native bovine pancreatic ribonuclease A by subtilisin BPN′ (Nagarse) occurs at either the bond between residues 21 and 22 or the previously established position between residues 20 and 21. As a result, the ribonuclease S produced contains a mixture of S-peptide molecules composed of residues 1–20 and 1–21 and of S-protein molecules composed of residues 21–124 and 22–124. Resolution of the S-peptide fraction was achieved by gradient elution from a column of Dowex 50-X2. Resolution of the intact S-protein fraction was not attempted. Instead, the fraction was fragmented by successive reaction with cyanogen bromide and performic acid and the products were fractionated by gel filtration through a column of Sephadex G-25. A zone containing a partially resolved mixture of peptides derived from residues 21–29 and 22–29 was obtained. Amino acid analyses of material along this zone provided an estimate of the yield of each peptide. Resolution of the various species of ribonuclease S has not yet been achieved. Examination of the S-peptide fraction of ribonuclease S prepared by the digestion of ribonuclease A with subtilopeptidase A revealed that the yield of the peptide composed of residues 1–20 is higher with this enzyme, although the peptide is still not the sole chromatographic component in the fraction. Additional components of unknown composition and comprising 16% of the fraction are also present.

The modification of native bovine pancreatic ribonuclease A by proteolytic enzymes from two strains of *Bacillus subtilis* has in both cases converted the protein in high yield to a product, designated ribonuclease S, which retains full enzymatic activity (Richards and Vithayathil, 1959; Gordillo *et al.*, 1962). Subtilisin (Güntelberg and Ottesen, 1954) was the enzyme first used to obtain this conversion (Richards and Vithayathil, 1959). The properties of ribonuclease S were consistent with the hypothesis that it was a homogeneous protein formed by the cleavage of the bond between residues 20 and 21 of ribonuclease A. Subsequently, the strain of *B. subtilis* from which subtilisin had been isolated was lost, and it became necessary to develop a procedure utilizing subtilisin BPN′ (Gordillo *et al.*, 1962), an enzyme isolated by Hagihara (1960) from a different strain of *B. subtilis*, and frequently referred to by its trade name (Nagarse). The course of the digestion and the properties of the ribonuclease S formed were very similar to those observed with sub-

* From the Biology Department, Brookhaven National Laboratory, Upton, New York 11973. *Received October 26, 1966.* Research at Brookhaven National Laboratory was performed under the auspices of the U. S. Atomic Energy Commission. One of us (M. S. D.) was aided by a grant from the American Cancer Society (Grant PF-266). A preliminary report of a portion of this work has appeared (Doscher and Hirs, 1966).