[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGICAL CHEMISTRY, HARVARD MEDICAL SCHOOL, AND THE DEPARTMENT OF MEDICINE, PETER BENT BRIGHAM HOSPITAL (DFHW) AND THE DEPARTMENT OF BIOLOGICAL CHEMISTRY, NORTHWESTERN UNIVERSITY SCHOOL OF MEDICINE (JEG)]

Filter Paper Electrophoresis of Lipids in Mixed Solvent Systems[1]

BY DONALD F. HOELZL WALLACH AND JAMES E. GARVIN

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The electrophoretic behavior on paper of a number of naturally occurring and synthetic lipids has been investigated. By control of apparent *p*H, apparent ionic strength, solvent composition and electroendosmosis, true electrophoretic migration of lipids in water-poor media has been achieved. Migration has been shown to be proportional to applied potential gradient and its duration. Apparent *p*H has been found to influence migration of various species in the manner expected from inferences related to their functional groups. For a number of phosphatides these data are the first demonstration that individual ionic species actually exist in solution. The solvent system in which most of the reported results were obtained consisted of trichlorethylene/methanol/water, 47.5/47.5/5, v./v./v. The most useful buffer system was diethylbarbituric acid/potassium diethylbarbiturate 0.0025 m*M* providing an apparent *p*H of 9.8. The migration rates of phosphatidyl serine, phosphatidyl ethanolamine and lecithin in a natural mixture were sufficiently different to provide satisfactory separation of the 3 species in 40 minutes. An acetal phosphatide preparation was separated into three fractions, a platelet lipid extract into four fractions and a proteolipid preparation into five fractions. Low-aqueous filter paper electrophoresis appears to be a general method for the characterization and separation of materials having dissociable groups but which are appreciably soluble only in lipid solvents.

Introduction

Although a number of authors[2-6] have suggested the presence of charged ionic species of lipids in water-free or water-poor solutions, unambiguous evidence of the presence of charged species in such systems has been lacking. Electrophoretic migration of lipids under conditions which rule out aggregate formation would provide proof that they exist in solutions in forms inferred from the known behavior of their dissociable groups. With one exception,[7] previous attempts to demonstrate electrophoresis in water-poor media have met with questionable success.[8-10]

By control of *p*H, ionic strength, solvent composition, heat dissipation, mass flow of solvent and electroendosmosis, true electrophoresis of lipids has been attained in organic solvent systems containing 3–5% v./v. water. Using this technique it has been shown that a number of lipids exist in organic solution as charged species and that they migrate in an electric field with a dependence on potential gradient, *p*H and ionic strength in every way analogous to aqueous systems. In addition a number of useful separations have been demonstrated. Low aqueous filter paper electrophoresis is a general method for separation and characterization of materials which have dissociable groups but which are soluble only in organic solvents.

Experimental

Preliminary experiments conducted with free-hanging paper strips indicated that systematic work would require an apparatus in which escape of solvent vapor from the surface of the paper would be minimal.

Apparatus.—The apparatus in which all the results reported in this paper were carried out is shown in Fig. 1.

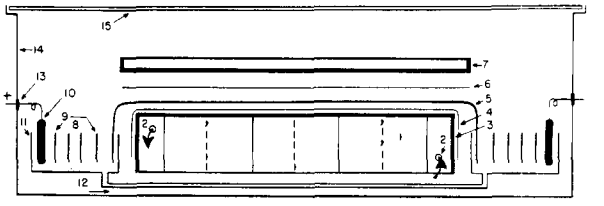


Fig. 1.—Diagram of apparatus: 1, cooling chamber of brass with baffles; 2, inlet and outlet for circulating coolant; 3, Durafilm (polyfluorocarbon) coating on cooling chamber; 4, Saran Wrap film over Durafilm; 5, paper strip for electrophoresis; 6, Saran film over paper strip; 7, Durafilm-coated brass covering block; 8, baffle of Pyrex wool between two glass slides; 9, glass slide baffles; 10, carbon electrodes; 11, glass reservoirs; 12, polyethylene tube; 13, entry port for power lead; 14, stainless steel enclosure; 15, glass lid.

A hollow brass box 20 cm. wide, 20 cm. long and 5 cm. deep, with alternating 18 × 5 cm. internal baffles acted as cooling block. Forced circulation of cold 70% alcohol through the box maintained the surface at −5°.

The top and sides of this box, as well as of the brass blocks used to compress the electrophoretic strips, were coated with an inert film "Durafilm L"[11] tested to withstand 3,000 v. However, since this coat did not provide perfect electrical insulation under our working conditions, and after some weeks of use, allowed some arcing at the corners, a single layer of Saran Wrap[12] was applied to the top and sides of the block. This produced a smooth and electrically impermeable cooling support for the electrophoretic paper.

Minimum solvent loss was assured by covering the paper strip with a wider sheet of Saran. The overlapping edges of the plastic film would adhere tightly to the top of the cooling chamber, thus sealing the edges of the paper.

Neither polyethylene nor Teflon films were effective. The Saran-covered electrophoretic strips were compressed with Durafilm-coated brass blocks 20 cm. long, 1.5 cm. thick and the same width as the strip. These weights were necessary to prevent pooling of solvent on the paper.

The reservoirs were histological staining dishes; 2 pairs could be accommodated in the present apparatus, 3″ × 1″ glass slides with glass wool between them acted as baffles. Electrodes were 2″ × 1″ carbon brushes. Hydrostatic equilibrium was maintained by connecting the reservoirs with bridges consisting of 1 mm. bore polyethylene tubing.

(1) This work was supported by a fellowship and grant from the American Cancer Society to (D.F.H.W.), and by Research grant #4734 from the U.S.P.H.S., National Institutes of Health.

(2) H. Fishgold and E. Chain, *Proc. Royal Soc.* (*London*), **B117**, 239 (1935).

(3) E. Chain and I. Kemp, *Biochem. J.* (*London*), **28**, 2052 (1934).

(4) R. Kuhn, I. Hauser and W. Bryndowne, *Chem. Ber.*, **68**, 2386 (1935).

(5) D. R. Howton, *Science*, **119**, 420 (1954).

(6) J. E. Garvin and M. L. Karnovsky, *J. Biol. Chem.*, **221**, 211 (1956).

(7) J. E. Garvin, *Federation Proc.*, **15**, 552 (1956).

(8) W. Kannigiesser, *Biochem. Z.*, **325**, 12 (1953).

(9) A. J. C. Barnett, H. Lees and D. K. Smith, *Biochem. J.* (*London*), **53**, XXXIII (1953).

(10) M. H. Paul and E. L. Durrum, THIS JOURNAL, **74**, 4721 (1952).

(11) American Durafilm Co., 2300 Washington St., Newton, Mass.

(12) Dow Chemical Co., Midland, Mich.

These were left open at all times and did not affect current flow.

The entire assembly was enclosed in a vapor tight stainless steel box with a removable glass lid. Since the solvent vapor concentration in this container is high, some form of ventilation is desirable when the lid is removed.

The power supply was regulated to 0.01% and could deliver up to 1,500 v. at 20 ma.

Solvents.—A large number of solvent systems, all containing water, were examined, including various proportions of chloroform, trichloroethylene, tetrachloroethylene and diisobutyl ketone as non-polar components and methanol, ethanol, 1,2-propanediol, glycerol, formamide, dimethylformamide, dimethylsulfoxide, diethylene glycol monomethyl ether and diethyleneglycol monoethyl ether as solvents of intermediate polarity. Most of these systems were either not effective solvents for phospholipids or did not permit sufficient current flow. In our experience the mixture 47.5/47.5/5 v./v./v. trichloroethylene/methanol/water was most successful and was used in all the experiments reported here. Trichloroethylene was substituted for chloroform to reduce evaporation without resorting to the very toxic saturated halogenated hydrocarbons.

Cutting water content below 3% (by volume) restricted lipid migration sharply but with this in mind other systems could be tailored for special uses.

Supporting Phase.—Schleicher and Schuell No. 589 green ribbon filter paper was the supporting phase in all experiments reported here. It was cut into strips 30 cm. long and 2.5–7.5 cm. wide, taking care that the machine direction was in the axis of electrophoretic migration. This paper appeared to have the least electroendosmosis of any papers tested. A number of other untreated filter papers were tested as well as acetylated paper, siliconed paper, silicic acid impregnated paper, phosphate-buffer impregnated paper, $CaCl_2$ impregnated paper, $MgCl_2$ impregnated paper, glass fiber sheets and siliconed glass fiber sheets. Each supporting phase was found to have its own individual characteristics. For example, a greatly increased rate of electroendosmosis toward the anode was observed with the glass fiber sheets and there were striking changes in the mobilities of both phosphatidyl serine and phosphatidylethanolamine. The variables introduced by each modification require systematic exploration.

Buffers.—Regulation of the apparent *p*H of the solvent system was achieved by the use of proton donor–proton acceptor pairs readily soluble in the mixture. Three such pairs were employed in the experiments reported here. For each pair two parameters, descriptive of the acidity imparted to their solvent system, were noted, (1) the *p*H calculated for the pair if dissolved in aqueous solution and (2) the apparent *p*H measured with the glass electrode and calomel half-cell reference electrode immersed in the solvent mixture containing the pair in question. Representative data for our experiments were:

proton-acceptor proton-donor	Calcd	Measured
potassium barbiturate barbituric acid	7.4	9.8
potassium benzoate benzoic acid	4.2	7.2
potassium acetate acetic acid	4.0	6.2

The exact significance of the apparent *p*H values was not entirely clear, but they were readily reproducible and provide an index to the dissociation of functional groups in a particular solvent mixture. The usefulness of apparent *p*H was greatly enhanced by the clear relationship of electrophoretic migration to apparent *p*H shown later (Fig. 3).

The apparent ionic strength of the buffers was most commonly calculated at 0.005 or below. These values were obtained by assuming that the concentrations of the proton-acceptor and proton-donor present in the water-poor medium were as calculated for an aqueous system. Although this assumption was probably not exactly true, it provided a useful index by which conductivity of the buffers could be compared and controlled. At apparent ionic strength 0.005 the current flow in the paper at 54 volts/cm. of length was in the vicinity of 20 microamperes/cm. of paper width (Schleicher and Schuell No. 589 green ribbon), although the exact value depended upon the wetness of the paper strip. At this level of current flow, gross evidence of solvent evaporation was not seen. At current flow ten times this value, visible condensation upon the glass lid near the anode and distortion of the electrophoretic pattern were regularly observed.

Temperature.—All experiments reported in this paper were carried out in a −5° cold room. In addition, forced circulation through the cooling chamber of 70% alcohol at −5° was maintained constantly during operation of the apparatus. Similar experiments at +5° showed excessive evaporation of solvent.

Potential Gradient.—Direct measurements were made of the voltage drop across the length of a 7.5 cm. wide strip of S and S No. 589 green ribbon paper with 1,500 v. applied. The strip was moistened with barbital buffer at apparent *p*H 9.8 and apparent ionic strength 0.0025 in trichloroethylene/methanol/water (47.5/47.5/5 v./v./v.). Two mm. wide strips of aluminum foil were placed across the paper under the Saran cover at 2 cm. intervals and the potential gradient between these strips was measured with a high impedance voltmeter (20,000 ohms/volt).

Operating Procedure.—The apparatus was always kept in the cold room. A sheet of Saran Wrap was first spread over the top and sides of the cooling chamber. The clinging tendency of the Saran film caused it to form a smooth, tightly adherent skin over the top and sides of the cooling chamber. Chilled buffer was added to the reservoirs and a free-flowing connection between reservoirs was established through the bridge. The ensemble was then allowed to come to hydrostatic equilibrium. Next, suitably marked paper strips were placed with their positive and negative ends dipping into the liquid of the appropriate reservoirs. After alignment, they were rapidly moistened with buffer using a syringe and immediately covered with another sheet of Saran wide enough to provide a 1 cm. margin on each side of the paper strip. If the paper was sufficiently wet the Saran formed a smooth, snug cover which adhered to the lower Saran layer on each side of the paper strip. This sealed the paper strip along its entire length except for the gap between the ends of the cooling block and the solvent surface in the reservoir. The strips were loaded by applying the lipid in question at the exact mid-point between the ends of the cooling block. In doing this, it was important to avoid drying of the strip which would lead to local changes in solvent composition and ionic strength. This was minimized by rolling back the top Saran coating to expose the loading site and quickly applying the 20–30 microgram sample in 4–6 microliters of chloroform/methanol 2/1, v./v., and smoothly but rapidly replacing the film. A Durafilm coated block of appropriate width was put on the strip, the current turned on and timing commenced. After the required time had elapsed the power supply was turned off, the upper covering layers lifted off and the paper strip removed for staining.

Most consistent results were obtained by loading the wet strip exactly mid-way between the two reservoir surfaces. Dry loading was not satisfactory.

Experiments with uncharged molecules such as triglycerides showed that there were two factors leading to mass flow of solvent across the paper strip; the first, evaporation from the paper surface, produced liquid movement up from the reservoirs and toward the center of the paper. It was independent of current flow and was greatest at the ends of the strip. The second factor was electroendosmosis leading to liquid flow toward the anode. These flow patterns were additive at the cathode end of the strip and opposed each other at the anode, possibly leading to the slowing of migration near the anode.

Staining Techniques.—Amino-nitrogen containing compounds were located by a ninhydrin staining solution consisting of 2.5 g. of ninhydrin, 2.5 ml. of lactic acid (85%), 50 ml. of methyl cellosolve, 50 ml. of acetic acid, 50 ml. of water and 350 ml. of acetone.

Choline containing lipids were localized by the method of Levine and Chargaff[13] but substituting a 30-minute wash in running water for the butanol–water wash.

Triglycerides were located with Sudan Black D in 50% ethanol followed by 3 washes in 30% ethanol.

A 0.04% solution of rhodamine B in 50% ethanol was useful for a large number of materials. The Schiff reagent

(13) C. Levine and E. Chargaff, *J. Biol. Chem.*, **192**, 465 (1951).

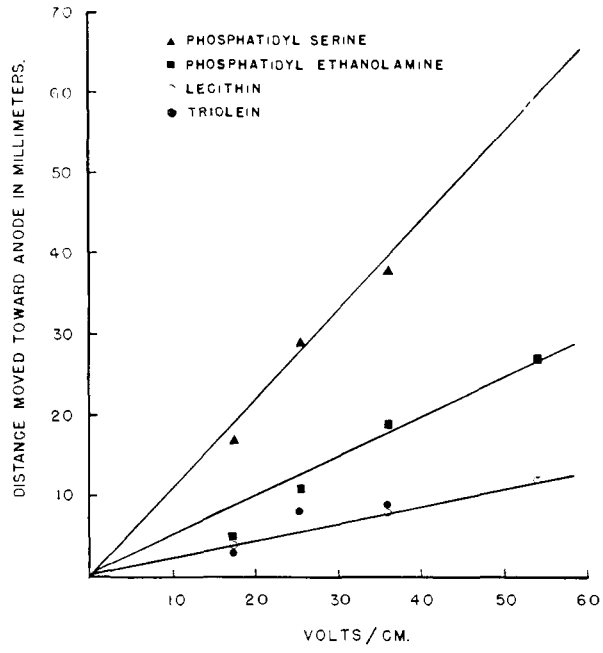


Fig. 2.—Effect of the potential gradient upon the electrophoretic migration of some lipids: phosphatidyl serine from beef brain; phosphatidyl ethanolamine and lecithin from a natural mixture present in egg yolk phosphatide; triolein. Buffer: potassium barbiturate/barbituric acid, apparent ionic strength 0.005, apparent *p*H 9.8. Spotting reagents: ninhydrin for phosphatidyl serine and phosphatidyl ethanolamine, phosphomolybdic acid–stannous chloride method for lecithin, Sudan Black B for triolein. Duration of experiments: 40 min.

according to Lillie[14] was used to locate aldehyde containing compounds.

A number of phosphorus containing compounds were localized by the method of Bandurski and Axelrod.[15]

Lipids.—Lipids used in these experiments were: L-a-(dimyristoyl)-lecithin and L-a-(dimyristoyl)-glycerophosphoric acid, synthetic materials kindly provided by Dr. Erich Baer.

Pure, crystalline potassium diphosphoinositide[16] was kindly supplied by Dr. J. Folch. It was converted into the calcium salt by precipitation from aqueous solution with calcium chloride.

Sulfatide A (cerebron sulfonic acid containing 3% sulfur), "-10 sulfatide" a sulfaphosphatide fraction obtained in the preparation of sulfatide A and an acetone insoluble, ether soluble acetal phosphatide fraction from beef brain obtained during the preparation of proteolipid C were all generously provided by Drs. J. Folch and M. Lees.

Sphingomyelin (95% of N present as choline), sphingomyelinic acid (the sphingomyelin analog of phosphatidic acid) and ethanolamine phosphatide (Folch Fraction V) were kindly supplied by Dr. Gerhardt Schmidt.

Phosphatidyl serine was prepared by the method of Folch.[17] The crude egg phospholipid was prepared by twice precipitating from total egg yolk lipid with cold acetone.

The total platelet lipid was prepared according to Folch.[18] Brain proteolipid A and B were prepared according to Folch.[19]

The synthetic lipopeptide was prepared by Dr. Johannes van Ormondt[20]; stearic acid, Eastman Kodak No. 402; triolein, molecular distillation products, Emery Industries, Inc.

(14) R. D. Lillie, *Stain Technology*, **26**, 163 (1951).
(15) R. S. Bandurski and B. Axelrod, *J. Biol. Chem.*, **193**, 405 (1951).
(16) J. Folch, *ibid.*, **177**, 505 (1949).
(17) J. Folch, *ibid.*, **177**, 497 (1949).
(18) J. Folch, M. Lees and G. H. Sloan-Stanley, *ibid.*, **266**, 497 (1957).
(19) J. Folch and M. Lees, *ibid.*, **191**, 807 (1951).
(20) J. Van Ormondt, *ibid.*, **114**, lxxvii (1936).

Results and Discussion

(1) **Potential Gradient.**—The rate of movement of a component was directly proportional to the magnitude of the imposed potential gradient as shown in Fig. 2. The slow movement of triolein, which is uncharged, and lecithin, which has no net charge at this *p*H, is due to electroendosmosis.

(2) ***p*H.**—As shown in Fig. 3, of the three phospholipids tested, only the migration rate of phosphatidyl ethanolamine was affected by alteration of apparent *p*H from 7.2 to 10.9. This lipid

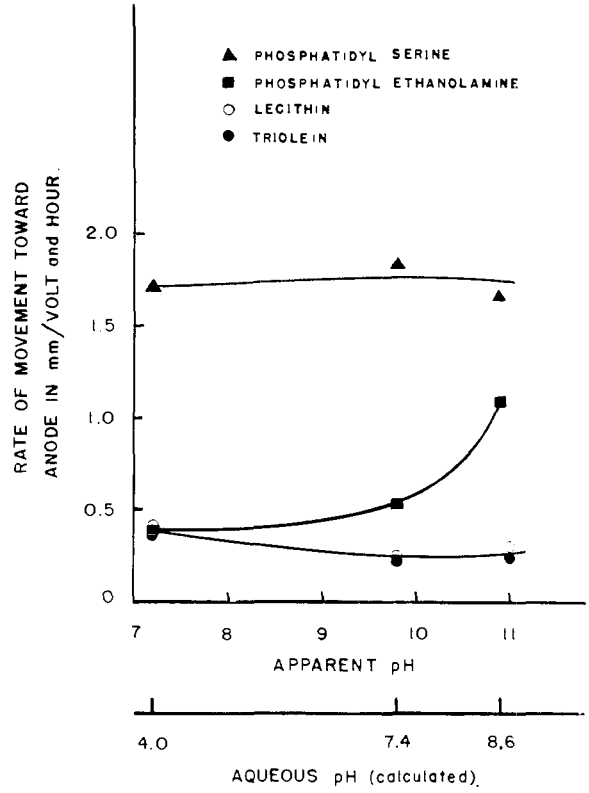


Fig. 3.—Effect of the apparent *p*H on the electrophoretic migration of some lipids: phosphatidyl serine from beef brain; phosphatidyl ethanolamine and lecithin from a natural mixture present in egg yolk phosphatide; triolein. Buffers: all were 0.005 in apparent ionic strength; the buffer at apparent *p*H 7.2 was potassium benzoate/benzoic acid, the buffers at apparent *p*H 9.8 and apparent *p*H 10.9 were potassium barbiturate/barbituric acid. Potential gradient: 54 volts/cm. Current: (av.) 13 μamp./cm. of paper width. Spotting reagents: ninhydrin for phosphatidyl serine and phosphatidyl ethanolamine, phosphomolybdic acid–stannous chloride method for lecithin, Sudan Black B for triolein. Duration of experiments: 30 or 40 minutes.

behaved as though uncharged at apparent *p*H 7.2, showed some electrophoretic migration at apparent *p*H 9.8 and moved rapidly at apparent *p*H 10.9. These data support the contention that the dissociable groups of ethanolamine phosphatide are indeed dissociated at the higher *p*H values and that ionic species actually exist in the solvent system used.

Titration data in 99% 2-ethoxyethanol and 1%

TABLE I

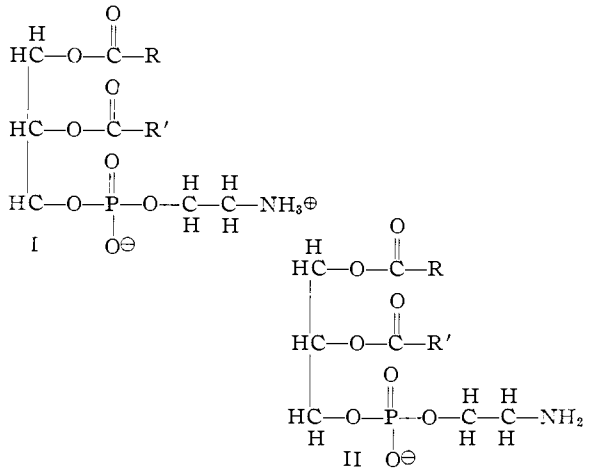
MIGRATION OF SOME LIPIDS DURING FILTER PAPER ELECTROPHORESIS IN WATER-POOR SOLVENTS

Lipid species or preparation	Mm./hr.[b]	Stain	Color
Sphingomyelinic acid[a]	2.52 ± 0.08	Rhodamine B	Red
Phosphatidyl serine	1.89 ± .04	Ninhydrin	Violet
Acetal phosphatide (most rapidly moving)	1.70 ± .1	Ninhydrin	Violet
Sulfatide A (brain)	1.59 ± 0	Rhodamine B	Red
Stearic acid	1.22 ± 0.07	Rhodamine B	Red
Phosphatidic acid (synthetic)	1.11 ± .0	Rhodamine B acidified	Red
Lipopeptide-a-aminocaprylic	0.89 ± .07	Rhodamine B	Pink
Acetal phosphatide (second component)	.85 ± .08	Ninhydrin	Violet
Phosphatidyl ethanolamine (Folch Fr. V)	.67 ± .07	Ninhydrin	Violet
Phosphatidyl ethanolamine (egg yolk)	.56 ± .07	Ninhydrin	Violet
Calcium diphosphoinositide	.48 ± .08	Periodic acid + Schiff reagent	Transient violet
L-a-(dimyristoyl)-lecithin (synthetic)	.33 ± .04	Phosphomolybic acid method	Blue
		Rhodamine B	Pink
Acetal phosphatide (slowest component)	.26 ± .04	Phosphomolybdic acid method	Blue
Lecithin (crude, from egg yolk)	.26 ± .04	Phosphomolybdic acid method	Blue
Triolein	.22 ± 0	Sudan Black B	Black
Sphingomyelin	.19 ± 0	Phosphomolybdic acid method	Blue

[a] Based on experiments of 30 minutes duration. [b] Potential gradient 1 volt/cm.

water[6] suggested the following charge distribution for ethanolamine phosphatide:

(I) at apparent *p*H 7.8 and (II) at apparent *p*H 10.9



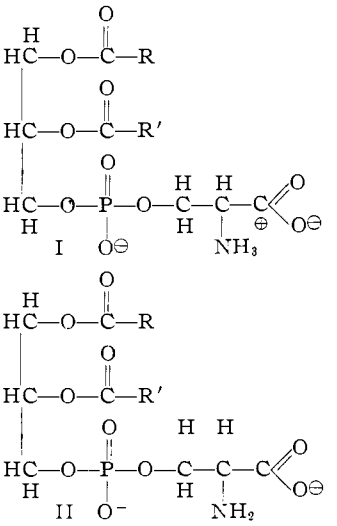
The migration data obtained here are in good agreement with these findings.

In the case of phosphatidyl serine, the titration data suggest charge distribution (I) at apparent *p*H 7.2 and 9.8 and (II) at apparent *p*H 10.9.

The rapid migration of phosphatidyl serine at apparent *p*H 7.2 and 9.8 agrees with the titration values but the lack of any increase in migration rate of phosphatidyl serine at apparent *p*H 10.9 is not readily explained. On the basis of Garvin's titration data, one would expect a net charge of −2 with a subsequent increase in electrophoretic mobility. However, the solvent system used in Garvin's titrations is decidedly different from the buffered solvent systems used here and further studies will be necessary before strict comparisons are possible.

As expected from the zwitterion structure of lecithin with both a strong acid and a strong base,[5] there is no net charge and hence no electrophoretic movement. The small movement that does occur is due to electroendosmosis.

(3) **Time.**—In general the distance of migration toward the anode was proportional to the duration of the imposed potential gradient. However, as shown in Fig. 4, movement slowed down sharply as the end of cooling block was approached. As discussed above, this slowing is probably due to mass flow of solvent up from the reservoir.



Here again the slow movement of triolein and lecithin is presumably endosmotic.

Variability.—In these studies where the distance migrated was measured from the starting point to the measured mid-point of a staining area, as much as 20% variation between duplicates occurred in the cases of slowly moving components. Rapidly moving species showed only about 5% variation. Meticulous attention to rapid and precise application of materials and proper alignment of strips parallel to the electrical axis assured duplication of migration rates within these limits.

Migration of Single Species.—A survey of migration rates toward the anode for a number of lipid species is shown in Table I.

Of particular interest is the lack of migration of calcium diphosphoinositide which although soluble

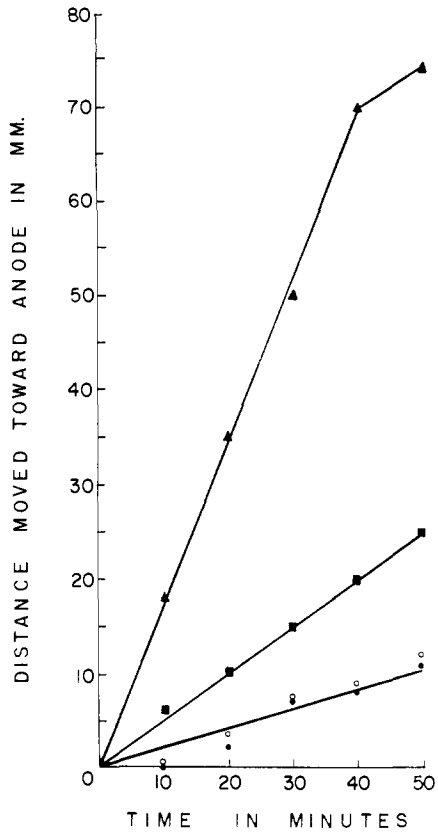


Fig. 4.—Effect of duration of the experiment upon the electrophoretic migration of some lipids: phosphatidyl serine from beef brain; phosphatidyl ethanolamine and lecithin from a natural mixture present in egg yolk phosphatide; triolein. Buffer: potassium barbiturate/barbituric acid, apparent ionic strength 0.005, apparent *p*H 9.8. Potential gradient: 54 volts/cm. Current: 26 microamperes/cm. of paper width. Spotting reagents: ninhydrin for phosphatidyl serine and phosphatidyl ethanolamine, phosphomolybdic acid–stannous chloride method for lecithin, Sudan Black B for triolein.

in the solvent system evidently is undissociated at apparent *p*H 9.8 whereas the water-soluble potassium salt migrates rapidly toward the anode in aqueous electrophoresis.[18]

Separation of Mixtures.—Complete separation of phosphatidyl serine from phosphatidyl ethanolamine and lecithin was obtained readily in both natural and artificial mixtures.

Whereas the phosphatidyl ethanolamine present in natural mixture separates well from lecithin at apparent *p*H 9.8, there is considerable overlapping of these species in artificial mixtures. At apparent *p*H of 10.9, however, there was marked separation of ethanolamine phosphatide from lecithin and it was evident that the negatively charged species of phosphatidyl ethanolamine predominated at this *p*H more than at apparent *p*H 9.8, while lecithin retained its zero net charge.

Lipid mixtures from brain and platelet extracts have been electrophoretically resolved into multiple components. The separation of phosphatidyl serine, phosphatidyl ethanolamine, acetal phosphatides and chlorine containing phosphatides was readily demonstrable.

Electrophoresis in water-poor solvent systems appears well suited to fractionation of proteolipid mixtures.[17] Tracings of an electrophoretogram of mixed brain proteolipids A and B are shown in Fig. 5. There appear to be five components with

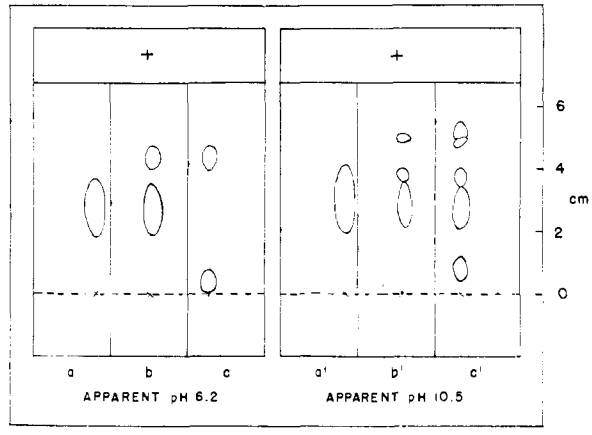


Fig. 5.—Low-aqueous filter paper electrophoresis of proteolipid from brain. Lipid: proteolipids A + B from beef brain prepared according to Folch.[18] Buffer of apparent *p*H 6.2: potassium acetate/acetic acid, apparent ionic strength 0.025. Buffer of apparent *p*H 10.5: potassium barbiturate/barbituric acid, apparent ionic strength 0.025. Potential gradient: 15 volts/cm. of paper length. Current: 7 μamp./cm. of paper width. Duration of experiments: 4 hr. Spotting reagents: (a and a') phosphorus method of Bandurski and Axelrod,[15] and (b and b') Schiff reagent, (c and c') ninhydrin.

greater complexity shown at apparent *p*H 10.6 than at 6.2.

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BOSTON 15, MASS.