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A simple chromatographic technique for removal of non-lipid contaminants from lipid extracts*

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SUMMARY

A simple procedure for the removal of non-lipid contaminants from lipid extracts is described. The lipids are applied in a line to a silicated paper and eluted in a descending manner into a beaker with 20% methanol in chloroform. The impurities remain on the paper. The procedure, which is carried out in an atmosphere as anhydrous as is practical, is best conducted at 30° and takes 16 to 24 hours. The amount of lipid material that can be purified on one paper is equivalent to that extracted from 1 g of tissue. The recovery of all classes of lipids is close to 100% without selective loss or obvious degradation. Chromatographic proof is provided for the removal of all ninhydrin and ammoniacal silver nitrate-staining contaminants. Inorganic phosphate present in the lipid extract is also completely removed but other salts may persist in very small quantities. The procedure was applied to lipids extracted from rat liver, kidney, and heart. Quantitative analyses of all the nitrogen-containing residues were carried out and the sum agrees well with the total nitrogen. Chromatography of the acid hydrolysates revealed only two ninhydrin-staining compounds, identified as serine and ethanolamine.

Lipid extracts of biological material invariably contain non-lipid contaminants (1, 2), the removal of which presents a difficult and often a time-consuming problem. This contamination is only partly caused by the solubility of non-lipid substances in lipid solvents. The main factor responsible for the persistence of contaminants is the strong "solubilizing" property of certain lipids, particularly phospholipids (1), which, because of their polar characteristics, are able to form micelles around non-lipids in non-polar solvents (3). Consequently, simple methods of purification, such as repeated extractions, pressure filtration, or even selective precipitation (3) are inadequate. Lecithin in particular displays this "solubilizing" property, further enhanced by the presence of even small amounts of water in the extract (4). For this reason extracts of fresh animal tissue, and especially of plasma, are particularly liable to contamination. The nature of the contaminants differs to a certain degree depending on the type of the original material; e.g., plasma extracts contain an appreciable amount of urea, which is found in only negligible amounts in extracts from other tissues. Three main classes of contaminants have been found: free amino acids such as serine, valine, leucine, etc. (5, 6), and possibly peptides (7); sugars, particularly glucose, but also fructose or galactose (8); and inorganic salts which may include small amounts of inorganic phosphate (9). In an effort to remove the contaminants, a number of techniques have been evolved including passage through cellulose columns (6, 10), dialysis (11), electrodialysis (12), countercurrent distribution (1), electrophoresis, proteolytic enzymes (13), paper chromatography (7), and a series of methods employing water-washing or water-solvent partition under varying conditions (14 to 20). Unfortunately, the techniques are inefficient, or involve loss or degradation of material, or can be applied on microscale only, or are too tedious and time-consuming.

The technique to be described was developed in the course of studies on the distribution of phospholipids in subcellular fractions of rat organs (21). The adequacy of the procedure has been tested mainly with regard to tissue phospholipids, but it has been applied equally well to other lipid mixtures such as plasma extracts. The technique consists of a single chromatographic passage of a lipid extract down a silicic acid-impregnated paper, with 20% methanol in chloroform as the eluent. The lipids are eluted directly into a beaker, whereas the non-lipid contaminants remain on the paper. The procedure takes 16 to 24 hours and, by use of one paper strip, purifies lipids extracted from 1 g of tissue with nearly 100% recovery of all varieties of lipids and without obvious degradation. The puri-

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fied lipids are chromatographically free of non-lipid ninhydrin-staining material and of reducing substances detectable with ammoniacal silver nitrate and are almost completely free of inorganic salts.

MATERIALS

All reagents were of analytical grade. Redistillation was found unnecessary.

Silicated Paper. Chromatographic paper Whatman No. 40 (double acid washed) was cut into strips 14 in. long and $4^1/_2$ in. wide. Each strip was lightly impregnated with silicic acid; heavy impregnation resulted in incomplete recovery of phospholipids. The method was based on that described by Kirchner and Keller (22): each strip was run once through a solution of sodium silicate and water, 1:1 (v/v), and was stroked down once on each side with a glass rod to remove excess silicate. It was then immersed in 6 N HCl for 30 minutes, and the excess acid was washed off in running water until the washings produced no precipitate with 1% $AgNO_3$ (about 10 minutes). The paper was then dried in air, placed in anhydrous diethyl ether for 30 minutes, dried in a chromatographic oven for 30 minutes at 45°, and pressed between two glass plates for at least 24 hours. Immediately before use, the paper was hung in the chromatographic oven for a few minutes at 110° and cut to shape suitable for elution. This was accomplished by producing sloping edges of the lower third of the strip and by fashioning a tongue at the bottom.

Eluent. Twenty per cent methanol in chloroform was used.

Chromatographic Apparatus. Standard glass jars and troughs suitable for descending chromatography were employed. The size of the jar depends on the number of papers used at one time. The large rectangular jar, 12 × 12 × 24 in., accommodates six papers.

PROCEDURE

Lipid Extraction. Any solvent system can be employed in extracting lipids. The final extract, however, should be evaporated to dryness and taken up in an appropriate solvent at the concentration of about 5 mg of lipid P per ml. Phospholipids constitute about one-half to two-thirds of total lipids in most biological materials of animal origin (23) and can serve as a convenient indicator for approximate total lipid concentration. Furthermore, any loss of material from incomplete elution will involve phospholipids rather than the non-polar lipids, which move with the solvent front. Moreover, in view of the ease and accuracy of phosphorus determination (24), amounts of lipid mixture taken for various subsequent procedures are expressed in terms of their lipid P content.

Purification. Approximately 0.2 ml of the concentrated extract (about 1 mg lipid P) is applied to the paper along a transverse line 25 cm from the bottom, falling just short of the edge. Best results are obtained when the line of application is about 2 cm wide. When the solvent has evaporated, the paper is placed in the jar in the manner usual for descending chromatography. A small beaker is placed on the bottom of the jar so that the tip of the paper fits loosely into it. The trough is filled with the solution of 20% methanol in chloroform and the chromatogram is allowed to run for 16 to 24 hours. Adequate jar saturation is important and is best accomplished at 30°. Under these circumstances the solvent will reach the bottom of the paper in under 4 hours and will drip freely into the beaker. At room temperature the descent is slower but, providing the jar saturation is adequate, no difficulties are encountered. When the run is over, the beaker contains purified lipids in a volume of 10 to 30 ml, depending on the conditions of the run. Impurities remain on the paper. The amount of lipid thus purified corresponds roughly to the amount extracted from 1 g of rat liver or other lipid-rich tissue. When lipid extracts containing 2 mg of lipid P were run, the recovery of phospholipids was at least 90% without loss of other lipids. For larger quantities, therefore, it is better to run more papers rather than to overload the paper. The time of the run is important in that shorter runs may result in loss of material (95% lipid P recovered after 12 hours), whereas impurities may elute during longer runs. It was also found in experiments with non-silicated papers that, although recovery was excellent, non-lipid ninhydrin-staining material was present in the eluate. This indicates that cellulose alone is not sufficient to adsorb all the non-lipid material.

RECOVERY

The technique was applied to lipids of adult rat liver, kidney, and heart extracted in the postabsorptive state with a chloroform-methanol-ethanol mixture, 5:4:1 (v/v). Table 1 shows quantitative recoveries of lipid P, total cholesterol (25), and triglyceride (26) of close to 100%. Blank silicated papers run under identical conditions produced an eluate containing 2.0 to 3.0 μg of phosphorus per paper—a negligible amount. In all purification procedures, some form of denaturation or degradation (by hydrolysis, oxidation, etc.) is a distinct possibility. For that reason, a neutral developing mixture was chosen and the run con-

TABLE 1. QUANTITATIVE RECOVERY OF LIPID CLASSES IN RAT ORGAN EXTRACTS SUBJECTED TO PURIFICATION

Tissue	Amount of Lipid P Applied to Paper	Amount of Lipid P in Eluate	Recovery of Lipid P
	μg	μg	%
Liver	1,148	1,117	97.5
	"	1,102	96.3
Kidney	1,141	1,182	103.5
	"	1,157	101.5
Heart	751	732	97.7
	"	722	96.3
Tissue	Amount of Cholesterol Applied to Paper	Amount of Cholesterol in Eluate	Recovery of Cholesterol
	μg	μg	%
Liver	338	318	94.0
	"	328	97.2
Kidney	800	823	102.8
	"	837	104.7
Heart	187	197	104.4
	"	169	90.4
Tissue	Amount of Triglyceride Applied to Paper	Amount of Triglyceride in Eluate	Recovery of Triglyceride
	μg	μg	%
Liver	5,916	6,140	103.8
Kidney	3,390	3,338	98.4
Heart	4,012	4,135	103.0

ducted in an atmosphere as anhydrous as possible. As phospholipids were of particular interest, they were studied in more detail. Chromatography was used to show that no individual compound was selectively lost. Comparison of chromatograms of the phospholipids before and after purification (27) showed the presence of the same compounds in both cases. Their R_f values were also identical. The chromatographic spots detected with Rhodamine 6G (27), ninhydrin, and phosphomolybdic acid (28) were more compact, with little trailing, after removal of contaminants. Preservation of plasmalogens was verified with 2,4-dinitrophenylhydrazine (27). Lysolecithin, a slow-moving polar phospholipid, is present in only small amounts in the tissues examined. Therefore, plasma lipid extracts—much richer in lysolecithin—were used. Although quantitative comparisons were not made, the appearance of chromatographic spots did not suggest any loss. The state of the esterified fatty acids was not determined, but the preservation of fatty acid unsaturation of the "cephalins" was tested by using them as platelet substitutes in the thromboplastin generation test (29). Brain "cephalin" subjected to the purification procedure gave clotting times equal to controls. Saturation or peroxidation of "cephalin" fatty acids would have resulted in complete loss of activity (30). The lipids were further subjected to chemical analyses for total nitrogen (31), serine (32), ethanolamine (32), choline (33), sphingomyelin (34), and inositol (35).[1] The results (Table 2) show good quantitative as well as qualitative recoveries of individual phospholipids. The possibility of isomerization, such as may occur after passage through a silicic acid column (36), was not investigated.

TABLE 2. CHEMICAL ANALYSIS OF RAT ORGAN PHOSPHOLIPIDS IN EXTRACTS SUBJECTED TO PURIFICATION*

Tissue	Serine	Ethanolamine	Total Choline	Sphingomyelin	Inositol	Nitrogen
Liver	0.06	0.24	0.54	0.09	0.07	0.96
Kidney	0.06	0.20	0.59	0.16	0.10	1.11
Heart	0.22	0.29	0.37	0.08	0.05	0.96

* Results, which are averages of several determinations, are expressed in moles per mole lipid P.

TABLE 3. REMOVAL OF INORGANIC SALTS FROM RAT ORGAN EXTRACTS BY THE PURIFICATION PROCEDURE*

	Liver	Purified Liver	Kidney	Purified Kidney	Heart	Purified Heart
Sodium	11.2	4.5	22.2	6.5	15.9	6.9
Potassium	10.5	0.5	7.9	0.8	6.5	0.6
Chloride	12.4	trace	25.5	4.8	14.1	9.7
Inorganic phosphorus	27.1	0.0	20.0	0.0	18.0	0.0

* Aliquots of lipid mixtures before and after purification were washed with water (17), and the water extracts were analyzed. All results expressed in μg per mg lipid P.

EFFECTIVENESS OF PROCEDURE

The main contaminants—inorganic salts, amino acids, urea, sugars—were studied for completeness of removal. Chromatographic comparisons were used for amino acids, sugars, and urea. This was not possible in the case of inorganic salts; for them a water-washing technique was employed as follows: aliquots of the impure and purified lipids (about 0.5 mg lipid P) were washed once with water by the method of Folch *et al.* (17). Sodium and potassium (determined by flame photometry), chloride[2] (37), and inorganic phosphorus contents of the washings are shown in Table 3. Assuming that the water technique is adequate for removal of inorganic salts, about 2% of total phosphorus is in-

[1] Inositol determinations were kindly performed by Dr. V. Skipski of the Sloan-Kettering Institute, New York, New York.

[2] Sodium, potassium, and chloride were determined through the courtesy of Mr. G. Ross, of Montefiore Hospital, New York, New York.

organic phosphate, which is completely removed by the chromatographic passage. The removal of other salts was considerable but not complete.

When chromatograms of unpurified tissue lipid extracts are run on silicic acid-impregnated papers (27), impurities remain at the origin and can be detected with appropriate stains. Amino acids stain very strongly with Rhodamine 6G, fluoresce purple in the ultraviolet light, and may be easily mistaken for a part of the phospholipid sample. A number of such chromatograms, to which 20 μg lipid P was applied, were developed and stained with ninhydrin for α-amino acids and with ammoniacal silver nitrate (38) for reducing substances. Unless very large amounts were applied, the purified lipids did not leave any stainable material at the origin.

The impure and purified lipids were subsequently subjected to unidimensional descending chromatography on Whatman No. 1 paper, with *n*-butanol-acetic acid-water mixture (39). Under these conditions lipids migrate in bulk with the solvent front whereas amino acids, sugars, urea, and other compounds undergo chromatographic separation. The amount of lipid applied was 20 μg lipid P. Staining with ammoniacal silver nitrate revealed glucose and a faint unidentified spot on the chromatogram of the impure lipids but absence of spots after purification.

Urea was detected as follows: the paper, after drying, was sprayed with a solution of urease in a phosphate buffer pH 7.5 and left at room temperature for 30 minutes. It was then sprayed carefully with Nessler's reagent. A yellow spot appeared, with R_f value of about 0.5. The method permitted detection of 10 μg of urea, which was absent in the purified extracts.

The results of ninhydrin staining of liver and kidney lipids are shown in Figure 1. In both cases the impure extracts contained a large number of free amino acids (not identified further), which were absent from the purified preparations. The bulk of lipids traveled with the solvent front. They were identified with Oil Red O but they also stained with ninhydrin because of their phosphatidyl serine and phosphatidyl ethanolamine contents. There was one other ninhydrin-staining spot detectable on chromatograms to which pure lipids were applied. It had a high R_f value and was almost continuous with the main lipid fraction. Although unlikely to be a free amino acid, the spot was subjected to a series of investigations to determine whether it was an impurity or a ninhydrin-staining lipid component that for some reason did not move with the rest of the lipids. The spot did not take up the Oil Red O, but the dye is not as sensitive as ninhydrin. It showed purple fluorescence under ultraviolet light following Rhodamine 6G, but so do free amino acids. Attempts at identification subsequent to elution with water or chloroform-methanol were unsuccessful. The chromatographic mobility of the spot was compared with amino acids that would display high R_f values under the conditions of the run (leucine, isoleucine, phenylalanine) (39), and with a mixture of phosphatidy

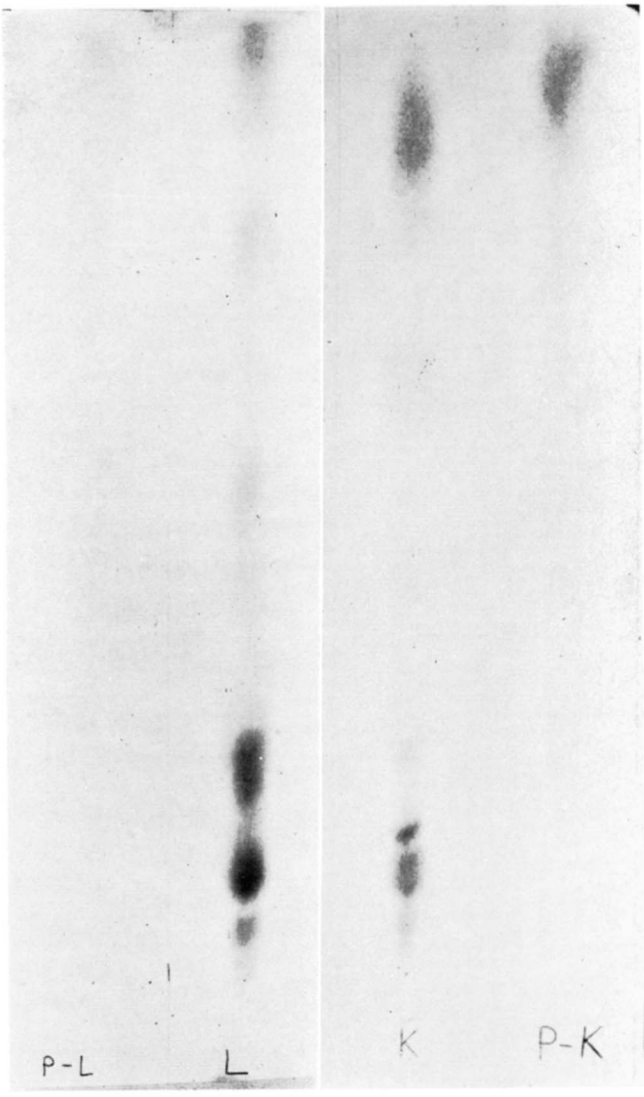

FIG. 1. Descending chromatograms of rat liver and kidney lipids developed with *n*-butanol-acetic acid-water (38) on Whatman No. 1 paper and stained with ninhydrin. L: liver; K: kidney lipids before purification; P-L and P-K: after purification. Lipid extract corresponding to 20 μg lipid P was applied to each paper. A large number of spots representing amino acid contamination are present on L and K but not on P-L and P-K. The heavy spot near the solvent front represents the lipids. A faint spot just behind it is the only other spot detectable with ninhydrin in purified material; it was not identified (see text) but was shown not to be a free amino acid.

serine and phosphatidyl ethanolamine prepared from rat brain by silicic acid column chromatography (40). The spot showed a considerably higher mobility than any amino acid tested; it was slower than ethanolamine phosphatide, which traveled with the solvent front, but was only slightly behind serine phosphatide. The supposition that the spot was not a free amino acid was confirmed by chromatography of the products of lipid acid hydrolysis. The purified lipid mixtures, containing approximately 200 μg lipid P, were hydrolyzed for 3 hours with 2 ml of 6 N HCl at 100°, dried, filtered, dried again, and finally taken up in 10% isopropanol (39). Chromatography with *n*-butanol-acetic acid-water as before displayed only two ninhydrin-staining spots, identified by means of pure compounds as ethanolamine and serine. Quantitative determinations of the freed amino groups were not done, but large amounts of hydrolysates were applied to the paper so that even a small amount of another amino acid would have been detected. This was taken as further evidence that the spot was not a contaminant, as it would have persisted after hydrolysis. The chromatography of the hydrolyzed lipids also showed that serine was the only amino acid (ninhydrin-stainable) that could not be removed from the extracts of the three organs by the purification procedure.

There are doubtless other contaminants in lipid extracts of tissues besides those mentioned; their amounts are probably negligible and they were not investigated.

DISCUSSION

The procedure described offers several advantages over existing methods. It is very simple, demanding little technical skill or special training, and requires little time on the part of the operator. The apparatus needed is standard equipment in any biochemical laboratory. Since the eluent is composed of the same solvents as those used in the original extractions, the possibility of denaturation of lipids is *a priori* small. Avoidance of water further adds to this feature. The loss of material is negligible and certainly not selective. This cannot be said with assurance about water-washing techniques because lysophosphatides are soluble in water. The most important feature of the procedure is the high degree of purification achieved. This is particularly obvious in the case of free amino acids, as they are detectable chromatographically with ninhydrin in most instances in quantities below 1 μg (39). Although the amount of lipid applied to the chromatograms (Fig. 1) contained 0.5 mg of phospholipid as well as other lipids, no trace of a free amino acid was detected. In addition, nitrogen analyses (Table 2) show "proper" N:P ratios. Nevertheless, it must be recognized that absolute purification of lipids is difficult and traces of free amino acids or some other nitrogen-containing compounds may persist.

The removal of amino acid contamination is essential in studies concerned with the establishment of lipid structure or synthesis. The existence of lipids containing various amino acids has been postulated by a number of authors (7), but phosphatidyl serine is the only one whose occurrence in animal tissues has been definitely established. As pointed out by Wren (13), it is difficult to be sure that an amino acid recovered from an apparently pure lipid extract is not a contaminant in spite of several different methods of purification. The procedure described may help in the elucidation of the problem of lipid-amino acid composition or incorporation.

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REFERENCES

1. Hanahan, D. J. *Lipide Chemistry*. New York, John Wiley & Sons, 1960.
2. Dawson, R. M. C. *Biol. Revs.* **32**: 188, 1957.
3. Wren, J. J. *J. Chromatog.* **4**: 173, 1960.
4. Baer, E., D. Buchnea, and A. G. Newcombe. *J. Am. Chem. Soc.* **78**: 232, 1956.
5. Wren, J. J., and H. K. Mitchell. *Proc. Soc. Exptl. Biol. Med.* **99**: 431, 1958.
6. Smith, R. H. *Biochem. J.* **57**: 130, 1954.
7. Westley, J., J. J. Wren, and H. K. Mitchell. *J. Biol. Chem.* **229**: 131, 1957.
8. Smith, R. H. *Biochem. J.* **57**: 140, 1954.
9. Marinetti, G. V., M. Albrecht, T. Ford, and E Stotz. *Biochim. et Biophys. Acta* **36**: 4, 1959.
10. Lea, C. H., D. N. Rhodes, and R. D. Stoll. *Biochem. J.* **60**: 353, 1955.
11. Sinclair, R. G. *J. Biol. Chem.* **174**: 343, 1948.
12. Polonovski, J., and L. Douste-Blazy. In *Biochemical Problems of Lipids*, edited by G. Popják and E. LeBreton. London, Butterworth's Scientific Pub., 1956, p. 64.
13. Wren, J. J. *Nature* **185**: 295, 1960.
14. Folch, J., and D. D. van Slyke. *J. Biol. Chem.* **129**: 539, 1939.
15. Christensen, H. N. *J. Biol. Chem.*, **129**: 531, 1939.
16. Folch, J., I. Ascoli, M. Lees, J. A. Meath, and F. N. LeBaron. *J. Biol. Chem.* **191**: 833, 1951.
17. Folch, J., M. Lees, and G. H. Sloane-Stanley. *J. Biol. Chem.* **226**: 497, 1957.
18. Bligh, E. G., and W. J. Dyer. *Can. J. Biochem. Physiol.* **37**: 911, 1959.
19. Blankenhorn, D. H., and E. H. Ahrens, Jr. *J. Biol. Chem.* **212**: 69, 1955.
20. Shorland, F. B., L. W. Bruce, and A. S. Jessop. *Biochem. J.* **52**: 400, 1952.

21. Biezenski, J. J., and T. H. Spaet. *Biochim. et Biophys. Acta.* **51**: 221, 1961.
22. Kirchner, J. G., and G. J. Keller. *J. Am. Chem. Soc.* **72**: 1867, 1950.
23. Deuel, H. J., Jr. *The Lipids.* New York, Interscience Publishers, Inc., 1955, vol. 3.
24. King, E. J. *Biochem. J.* **26**: 292, 1932.
25. Bloor, W. R. *J. Biol. Chem.* **24**: 227, 1916.
26. van Handel, E., and D. B. Zilversmit. *J. Lab. Clin. Med.* **50**: 152, 1957.
27. Marinetti, G. V., J. Erbland, and J. Kochen. *Federation Proc.* **16**: 837, 1957.
28. Chargaff, E., C. Levine, and C. Green. *J. Biol. Chem.* **175**: 67, 1948.
29. Spaet, T. H. *Amer. Practit.* **7**: 403, 1956.
30. Troup, S. B., C. F. Reed, G. V. Marinetti, and S. N. Swisher. *J. Clin. Invest.* **39**: 342, 1960.
31. Conway, E. J. *Microdiffusion Analysis and Volumetric Error*, 4th ed., London, Crosby Lockwood & Son, Ltd., 1957.
32. Nojima, S., and N. Utsugi. *J. Biochem.* (Japan) **44**: 565, 1957.
33. Appleton, H. D., B. N. La Du, Jr., B. B. Levy, J. M. Steele, and B. B. Brodie. *J. Biol. Chem.* **205**: 803, 1953.
34. Schmidt, G., J. Benotti, B. Hershman, and S. J. Thannhauser. *J. Biol. Chem.* **166**: 505, 1946.
35. Campling, J. D., and D. A. Nixon. *J. Physiol.* **126**: 71, 1954.
36. Hirsch, J., and E. H. Ahrens, Jr. *J. Biol. Chem.* **233**: 311, 1958.
37. Cotlove, E., H. V. Trantham, and R. L. Bowman. *J. Lab. Clin. Med.* **51**: 461, 1958.
38. Partridge, S. M. *Biochem. J.* **42**: 238, 1948.
39. Block, R. J., E. L. Durrum, and G. Zweig. *A Manual of Paper Chromatography and Paper Electrophoresis.* New York, Academic Press, Inc., 1955, p. 75–83.
40. Spaet, T. H., H. I. Horowitz, D. Zucker-Franklin, J. Clintron, and J. J. Biezenski. *Blood* **17**: 196, 1961.