The Use of Sephadex for the Removal of Nonlipid Contaminants from Lipid Extracts*

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When lipid extracts are passed through columns prepared of Sephadex in chloroform-methanol-water the extracts are freed of nonlipid contaminants. The removal of amino acids, carbohydrates, nucleotides, HCl, and inorganic phosphate from lipid extracts by this procedure is of the order of 99%. Lipid recoveries of 98–100%, as determined by the recovery of total fatty acids and of C^{14}-cholesterol, are obtained with most extracts.

The contamination of lipid extracts with nonlipid compounds has been long recognized as a problem in the chemical characterization of the lipids in these extracts (MacLean, 1915; Folch and Van Slyke, 1939; Christensen, 1939). The procedure which recently has been most commonly used to remove these contaminants is based on partitioning chloroform-methanol solutions with water or salt solutions (Folch *et al.*, 1951, 1957). Other methods, used to a more limited extent, include adsorption on cellulose (Lea and Rhodes, 1953; Smith, 1954) and Hyflo Super-Cel (Westley *et al.*, 1957) columns; dialysis (Sinclair, 1948; Folch, 1949); paper electrophoresis and chromatography (Westley *et al.*, 1957); and chromatography on silicic acid–impregnated paper (Biezenski, 1962).

Except for the Folch procedure and the use of silicic acid–impregnated paper, none of these methods has been checked for its effectiveness to remove anything more than amino acids or for recovery of lipid. All these procedures suffer from one defect or another. With some extracts, the Folch procedure produces emulsions which can be broken only by centrifugation or standing for very long times. The Folch procedure also fails to remove inorganic phosphate. Chromatography on cellulose fails to remove inorganic phosphate and loss of phosphoinositides has been reported (Garcia *et al.*, 1956). The other methods mentioned above have extremely limited capacity or take an excessively long time, or both. This paper describes a rapid, highly effective method for the removal of nonlipid contaminants with no serious loss of lipid.

MATERIALS

Amino acids, carbohydrates, cholesterol, and urea labeled with C^{14} were obtained from California Corporation for Biochemical Research, Los Angeles. Reagent grade chloroform and methanol were used without further purification. All solvents were mixed in proportion by volume. Reagent grade anthrone was recrystallized from ethyl acetate. Sephadex is a product of Pharmacia Fine Chemicals, Uppsala.

ANALYTICAL PROCEDURES

Inorganic Phosphorous.—Determination was by the method of Fiske and Subbarow (1925). Total phosphorous was determined after prior digestion with perchloric acid.

Carbohydrate.—Assay of carbohydrate was by a modification of the procedure of Radin *et al.* (1956). Aliquots containing 20–125 μg of galactose or 10–60 μg of glucose were taken to dryness in 18 × 150–mm screw-cap tubes at 80° in a vacuum oven. The samples were hydrolyzed with 0.5 ml of 3 N H_2SO_4 at 100° for 2 hours. The tubes were cooled to −18° in a freezer and 5 ml of ice cold anthrone reagent (500 mg recrystallized anthrone in 25 ml of concentrated H_2SO_4, allowed to stand 4 hours, and diluted 1:15 with 27 N H_2SO_4) was added. The reaction mixture was heated for 10 minutes at 100° and, after the mixture was cooled to room temperature, absorbancies were measured at 625 mμ. Galactose or glucose standards were run with each assay.

Total Fatty Acids.—Determination was made by saponification followed by extraction, methylation, and quantitative assay of the methyl esters. Lipid samples containing between 4 and 6 μeq of fatty acid were saponified with 2 ml of 3 N KOH in 50% ethanol for 2 hours at 100°. The hydrolysate was acidified with HCl and the fatty acids were extracted into petroleum ether. The petroleum ether extract was taken to dryness under a stream of nitrogen at room temperature and the fatty acids were methylated with BF_3-methanol (Metcalfe and Schmitz, 1961). The fatty acid methyl esters were extracted into petroleum ether and the extract was dried at room temperature under a stream of nitrogen. The residue was dissolved in 3 ml of petroleum ether. One-ml aliquots were assayed for fatty acid methyl esters by the method of Rapport and Alonzo (1955) with the exception that the tubes, instead of being handled individually, were treated simultaneously in a rack using a 65° water bath for the hydroxylamine reaction, and that a vacuum oven at 65° was used to take the reaction mixture to dryness.

Alternatively, fatty acid methyl esters were determined quantitatively by gas-liquid chromatography on a 183 × 0.4–cm column of 12% ethylene glycol succinate on 60/70 mesh Anakrom A. Chromatograms were run at 180° in a Barber-Coleman Model 10 chromatograph equipped with an ionization detector.

Radioactivity.—Radioactivity was measured by drying aliquots on 2-cm planchets and counting the radioactivity on a Nuclear-Chicago D47 Micromil flow counter.

METHODS

Lipid Preparations and Extracts.—Except as indicated, chloroform-methanol extracts of ox brain and liver (Folch *et al.*, 1957) were used without partitioning with water or salt solutions. Chloroform-methanol extracts of yeast (Bligh and Dyer, 1959) were prepared after grinding the yeast with glass beads in a colloid mill. The cells were centrifuged from the chloroform-methanol-water extraction mixture and, except when indicated, were used without washing by the addition

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of chloroform and water. The unwashed extracts were taken to dryness and dissolved in chloroform-methanol-water, 60:30:4.5. Acidified chloroformmethanol extracts of the residue left from brain after chloroform-methanol extraction (Dittmer and Dawson, 1961) were used without partitioning with water. Petroleum ether extracts of ox brain (Folch, 1949) were taken to dryness and redissolved in chloroform-methanol-water, 60:30:4.5. Phosphatidic acid was prepared from egg lecithin and carrot phospholipase D (Einset and Clark, 1958).

Preparation of Sephadex Columns.—Fines were removed from Sephadex by suspending 1 g of dry Sephadex in 30 ml distilled water, allowing the larger particles to settle for 15 minutes, and decanting the supernatant. This washing procedure was repeated at least five times. Water was removed by washing twice with acetone. The Sephadex was dried in a Buchner funnel and then in air overnight. Severe mechanical manipulation of the washed Sephadex to eliminate large aggregates or excessive stirring in preparing the columns was avoided in order to minimize the production of fines.

One g of the washed and dried Sephadex was suspended in 5 ml of chloroform-methanol-water, 60:30:4.5 and the suspension was poured into a 0.6 × 40–cm column equipped with a Teflon stopcock and plugged with glass wool. The trapping of bubbles in the column was prevented by pouring the Sephadex suspension into 5 ml of the chloroform-methanol-water solvent already in the column and by gentle agitation of the suspension with a glass rod. The Sephadex was allowed to settle, the solvent was drained to the top of the Sephadex, and then the column was washed with an additional 5 ml of solvent. For preparation of 5-g columns, a 1.0 × 62–cm column was used and quantities of materials were increased 5-fold. In general, it was found desirable to use a column which gave a diameter-to-height-of-packed-Sephadex ratio of no more than 1:15.

Treatment of Lipid Extracts on Sephadex Columns.—In the procedure finally developed, the lipid is dissolved in a mixture of chloroform-methanol-water, 60:30:4.5, at any concentration short of saturation. Up to 15 ml of this solution is passed through a 1-g column at a rate of no more than 0.1 ml/min and the effluent is collected in bulk. The column is then washed at the same flow rate with 5 ml of chloroform-methanol, 2:1, containing no water, and this wash is combined with the original effluent. The lipid of the extract, freed of nonlipid contaminates, is recovered in this combined fraction. Nonlipid material held on the column can be removed by washing the column with 8 ml of methanol-water 1:1. Larger columns are run with proportionately larger volumes of lipid extract and eluting solvent and the flow rate is also increased proportionately. The Sephadex may be reused by repeating the washing procedure to remove fines. If the column is not eluted with methanol-water, rewashing the Sephadex should be preceded by a methanol wash to remove chloroform.

As is pointed out below, elution with a combined volume of lipid extract and eluting solvent of more than 20 ml will cause the elution of nonlipid contaminants. However, eluting the column with at least 5 ml of chloroform-methanol, 2:1, is necessary to quantitatively remove lipid from the column. Accordingly, a maximum of 15 ml of lipid extract can be used with each gram of Sephadex. It is also important to note that the work reported here shows that nonlipid contaminants are not effectively removed by this method from lipid solutions which have been previously partitioned in a system of chloroform-methanol-water or salt solution. However, lipid solutions which have been previously washed may be used if they are made 0.05 N with respect to HCl or $MgCl_2$. Finally, it should be noted that there is no limitation on the concentration of the lipid in the solution as long as it is in true solution.

Results

Investigation of Optimum Conditions.—The selection of chloroform-methanol-water, 60:30:4.5, as a solvent was based on the fact that chloroform-methanol extracts (Folch *et al.*, 1951) of most tissues have approximately these proportions. Further, columns prepared with mixtures of chloroform and methanol with either larger or smaller concentrations of water were found to be no more effective and also required more attention because of fluctuations in flow rates during the elution of the lipid.

When Sephadex was used without washing to remove fines, a chloroform-insoluble residue was eluted. Assays on hydrolysates of this material indicated that it is carbohydrate and was probably Sephadex.

Columns prepared from three grades of Sephadex, G-25 fine, G-25 medium, and G-75 medium were tested for their ability to remove C^{14}-glucose from a chloroform-methanol extract of liver. C^{14}-glucose was added to the extract to a concentration of 5 mμmoles/ml of extract. This extract had a count rate of 130,000 cpm/ml. Glucose was used because preliminary experiments indicated that it was the most difficult to remove of those compounds tested. The columns prepared with G-25 medium and G-25 fine Sephadex removed 99.8% of the radioactivity of the first 5 ml of extract passed through the column, while the G-75 retained only 72.5%. Recovery of the lipid from the column with subsequent elution of chloroform-methanol, 2:1, required 8 ml with the G-25 medium and 5 ml with the G-25 fine. This further elution also eluted 1.5% of the radioactivity placed on the G-25 medium column and no detectable counts from the G-25 fine column. On the basis of this experiment, it was decided that G-25 fine Sephadex would be the most satisfactory for this application and all of the subsequent work reported was done with G-25 fine Sephadex.

Efficiency of Removal of Nonlipid Contaminants.—Columns were checked for their ability to remove trace amounts of labeled 1-C^{14}-glutamic acid, 1-C^{14}-lysine, 1-C^{14}-serine, 1-C^{14}-tyrosine, and 1-C^{14}-galactose. When each of these was added individually or all together to liver chloroform-methanol extracts in a concentration of 5 mμmoles/ml, the columns removed from 99.8 to 100% of the radioactivity. Elution of the columns with up to 15 ml of chloroform-methanol, 2:1, to remove the lipid eluted no more than an additional 0.5% of the total activity. A minimum of 99.3% of labeled urea was removed from liver and brain extracts.

The removal of inorganic phosphate was tested using two different solutions. A phosphatidic acid preparation containing a total of 7.65 mg of phosphorous of which 41.2% was present as inorganic phosphate and, 3 ml of a petroleum ether extract of brain (Folch, 1949) containing 1.2 mg of total phosphorous of which 8% was present as inorganic phosphate were each dissolved in 5 ml of chloroform-methanol-water, 60:30:4.5. Ninety-nine per cent recovery of organic phosphate was obtained with both of these solutions when they were passed through 1-g columns and eluted with 5 ml of chloroform-methanol, 2:1. No detectable inorganic phosphate could be assayed in the eluate of the phosphatidic acid, and only 0.3% of the inorganic phosphate was not retained from the brain extract.

Nucleotide removal was checked by adding 0.1 ml of a 0.1 M solution of either ATP or AMP to 5 ml of liver lipid extract and passing it through a 1-g column. Five ml of chloroform-methanol, 2:1, was used to quantitatively elute the lipid. The optical density at 260 mμ of the combined eluate was compared with that of the lipid extract to which no AMP or ATP had been added and was found to be identical.

To obtain comparable data for the procedure most commonly used, the liver and phosphatidic acid solutions described were partitioned with 0.1 M KCl (Folch *et al.*, 1957). This effectively removed all the glutamic acid, serine, tyrosine, AMP, and ATP, and 99.5% of the lysine, 98% of the glucose, and 96% of the urea. In this respect the use of Sephadex is comparable to or better than the Folch procedure. The Folch procedure removed only 83% of the inorganic phosphate from the phosphatidic acid solution and there was a loss of 7% of the organic phosphate into the water phase. The superiority of a Sephadex column for this purpose is also demonstrated in its ability to remove inorganic phosphate from chloroform-methanol solutions of petroleum ether extracts of brain (Folch, 1949). Partitioning with neither salt solutions nor cellulose columns (Lea and Rhodes, 1953) quantitatively removes inorganic phosphate from this fraction although the rather anomalous use of cation-exchange resins removes up to 95% of the inorganic phosphate (Dittmer and Dawson, 1961).

The use of acidified chloroform-methanol in lipid extraction procedures makes it desirable to have a method for the removal of HCl. Five ml of an acidified chloroform-methanol extract of brain residue which had been previously extracted with chloroform-methanol (Dittmer and Dawson, 1961) was applied to a 1-g Sephadex column and washed through with 5 ml of chloroform-methanol, 2:1. The total eluate was extracted with methanol-water, 1:1, and the extract was titrated with standard base. The titratable acid was equivalent to that obtained with a chloroform-methanol blank not run through Sephadex. The amount of titratable acid determined by this procedure when applied to acidified extracts agreed satisfactorily with the amount of acid added.

Labeled amino acids were not effectively removed by Sephadex from extracts which had been previously partitioned with water or salt solutions, taken to dryness and redissolved in chloroform-methanol-water, 60:30:4.5. Chloroform-methanol extracts of brain and liver partitioned with water or 0.1 M KCl (Folch *et al.*, 1957) and extracts of yeast partitioned with water (Bligh and Dyer, 1959) were taken to dryness and dissolved in chloroform-methanol-water, 60:30:4.5 at a concentration of 30 μmoles of lipid phosphorous per ml. When labeled amino acids were added to these extracts and the extracts were chromatographed on Sephadex, only 96% of the radioactivity was removed from the brain extract, 86% from the liver, and 88% from the yeast. The removal of glucose was not affected. When yeast lipids which had been partitioned with water were separated into neutral and phospholipid fractions by chromatography on silicic acid (Borgstrom, 1952) it was found that the binding of amino acids was associated entirely with the phospholipid fraction.

The addition of HCl to these lipid solutions to give a final concentration of 0.01 M, or of $MgCl_2$ to a concentration of 0.005 M, improved the removal of nonlipid contaminants. Of the added amino acids, 99.5–99.7% is removed by Sephadex. As is indicated, lipid extracts which have not been partitioned with water may be taken to dryness, dissolved in chloroform-methanol-water, 60:30:4.5, and chromatographed on Sephadex without affecting the efficiency with which nonlipid contaminants are removed.

TABLE I
THE RETENTION OF RADIOACTIVE NONLIPID COMPOUNDS ON SEPHADEX COLUMNS

Column	Total cpm/ Fraction	Per Cent of Applied cpm in Fraction	Total Per Cent of cpm Applied not Retained
I[a]			
Fraction 1	50	0.25	0.25
2	200	0.50	0.75
3	150	0.25	1.00
4	500	0.63	1.63
5	1350	1.35	1.98
6	1500	1.25	3.23
7	1750	1.25	4.78
8	2600	1.63	6.41
II[b]	4400	1.10	
III[c]	225	0.06	
IV[d]	4000	1.00	
V[e]	600	0.20	

[a] A chloroform-methanol extract of brain was taken to dryness and dissolved in chloroform-methanol, 60:30, and 4.5 parts of an aqueous solution was added which was 0.1 M with respect to each of C^{14}-labeled galactose, glutamic acid, lysine, and serine. The final solution contained 1000 cpm/ml of each of the amino acids and galactose added. Forty ml of this solution was passed through a 1-g Sephadex column and 5-ml fractions of the eluate were collected. [b] Twenty ml of chloroform-methanol extract of brain containing only trace amounts of C^{14}-labeled galactose, glutamic acid, lysine, and serine was run on a 1-g column. The column was eluted with 5 ml of chloroform-methanol 2:1. [c] Same as II except only 15 ml of lipid extract was used. [d] Same as II except a chloroform-methanol extract of liver containing trace amounts of C^{14}-labeled galactose, glutamic acid, lysine, and serine was used. [e] Same as III except a chloroform-methanol extract of liver containing 300,000 cpm of C^{14}-labeled glucose alone was used.

Recovery of Lipids.—Aliquots of 5 ml of chloroform-methanol-water, 60:30:4.5, solutions of chloroform-methanol extracts of liver and brain, and an acidified chloroform-methanol extract of brain residue left after chloroform-methanol extraction contained, respectively, 8.35, 5.10, and 3.84 μeq of fatty acid as determined by acyl ester assay of the methylated fatty acids prepared from petroleum ether extracts of acidified alkaline hydrolysates. After treatment on 1-g Sephadex columns, 8.30, 5.05, and 3.55 μeq of fatty acid was recovered. This represented recoveries of 99.4, 99.1, and 92.5% for the respective lipid preparations. Gas-liquid chromatography of the liver lipid fatty acid methyl esters showed recoveries of the order of 100% of each of the six major fatty acids present.

A specific check on the recovery of free palmitic acid and of cholesterol was made by treating 5 ml chloroform-methanol-water, 60:30:4.5, solutions of 12.5 μmoles of palmitic acid, and 245 mg of cholesterol containing 94,500 cpm of 4-C^{14}-cholesterol. After treatment, 12.3 μmoles of palmitic acid and 243 mg of cholesterol containing 93,000 cpm were recovered in the eluent. This represents 98.8% recovery of palmitic acid and 98.9% by weight of cholesterol or 98.5% of the radioactivity.

Capacity of Sephadex Columns.—The capacity of the columns was checked for both the removal of nonlipid material and also for the total amount of lipid and

TABLE II
EFFECT OF ELUTION OF SEPHADEX COLUMNS ON RETENTION OF NONLIPID CONTAMINANTS[a]

Fraction of Eluate	Solvent I[b] Total cpm/ Fraction	Solvent I[b] Per Cent of Applied cpm in Fraction	Solvent II[c] Total cpm/ Fraction	Solvent II[c] Per Cent of Applied cpm in Fraction
1	50	0.16	50	0.06
2	200	0.65	200	0.65
3	2500	8.0	175	0.55
4	5000	16.0	525	1.68
5	4750	15.2	475	1.52

[a] Five ml of a chloroform-methanol extract of liver which contained 62,500 cpm of C^{14}-labeled glucose was run on a 1-g Sephadex column and then eluted with 25 ml of solvent. The eluate was collected in 5-ml fractions. [b] Chloroform-methanol-water, 60:30:4.5. [c] Chloroform-methanol, 2:1.

lipid solution which could be run through without causing elution of nonlipid contaminants. Forty ml of a chloroform-methanol-water solution of brain lipid to which nonendogenous C^{14}-labeled amino acids and carbohydrates had been added was passed through a Sephadex column and the eluate was collected in 5-ml fractions. These fractions were assayed for radioactivity (Table I).

This experiment suggested that the total capacity of the column is limited by either the total volume of solution passed through the column and/or the total amount of nonlipid material in the lipid solution. To further elucidate this, columns through which 5 ml of the lipid solution had been passed were eluted with 25 ml of either chloroform-methanol-water, 60:30:4.5, or chloroform-methanol, 2:1. Five ml fractions were collected and assayed for radioactivity (Table II). These experiments show that the ability of Sephadex columns to remove and hold nonlipid compounds increases when subsequent elution is carried out with less polar solvents and also supports the conclusion that the limit of the capacity of the column depends mainly on the volume of solvent eluted through. If this is true, then the concentration of lipid and contaminants in the lipid solution should not greatly affect the performance of the column. This was checked by preparing 15-ml solutions with different concentrations of both lipid and contaminants and passing these through a 1-g column which was subsequently eluted with 5 ml of chloroform-methanol, 2:1. Both the retention of nonlipid contaminants and the recovery of fatty acid was checked (Table III). The retention of nonlipid material was excellent for all of the solutions; however, the fatty acid recovery for the most concentrated solution was only 88%. As indicated in Table III, this material was placed on the column as a suspension rather than as a true solution and the poor recovery of fatty acid was probably because of this fact. The only limitation on the concentration of lipid, then, appears to be the saturation point of the solvent.

The expedient of increasing the capacity of the column by increasing the size of the column, the volume of lipid extract, and the volume of eluting solvent used was also tried. A 5-g column was used and the retention of radioactivity and recovery of fatty acid from 75 ml of lipid extract eluted with 25 ml of chloroform-methanol, 2:1, was compared with that obtained with a 1-g column on which 15 ml of lipid extract was eluted with 5 ml of chloroform-methanol. This data has been included in Table III. The retention of nonlipid amino acids and carbohydrates and recovery of lipid fatty acid was good, and not significantly different in the two columns.

Because it would be desirable to carry out the procedure at low temperatures in working with the more labile lipids, the procedure was also checked at 4°. It was found that at this temperature an additional 5 ml of chloroform-methanol, 2:1, was required to give quantitative recovery of the total fatty acid. As seen in the data given in Table III, this did not appreciably increase the elution of nonlipid contaminants.

DISCUSSION

Although not all grades of Sephadex were tried for this application, the simple experiment testing the retention of glucose served to compare Sephadex of different mesh sizes but with the same pore size, and also Sephadex of the same mesh size but different pore sizes. The results indicate that for this application the smaller the pore size and particle size of the Sephadex the greater its ability to remove nonlipid contaminants and retain these contaminants during subsequent elution.

Since most of the known nonlipid contaminants of lipid extracts fall into the categories of the compounds which were tested in the experiments described, it appears that within the limitations discussed below this procedure is as satisfactory as any of those now

TABLE III
CAPACITY OF SEPHADEX COLUMNS FOR THE REMOVAL OF NONLIPID CONTAMINANTS FROM BRAIN EXTRACTS AND THE RECOVERY OF TOTAL FATTY ACID[a]

Column	Volume of Brain Extract (ml)	Fatty Acids in Extract (μeq)	Total cpm Original Extract	Per Cent Fatty Acids in Eluate	Per Cent of cpm in Eluate
I[b]	5	26.7	7,750	101.0	0
II[b]	50[c]	1335	77,500	98.0	0.25
III[b]	200[c]	5340	310,000	88.0	0.28
IV[b]	15	66.6	60,000	99.1	0.58
V[d]	75	336.0	300,000	99.0	0.48
VI[b,e]	15	66.6	60,000	97.0	0.50
VII[f]	15	50.6	60,000	102.0	0.75

[a] Brain extract in chloroform-methanol-water, 60:30:4.5, which was 1.1 mM with respect to added C^{14}-labeled galactose, glutamic acid, serine, tyrosine, and lysine was used in all the experiments. The extract contained 4.3 mg of total solid per ml. [b] A 1-g column was used and was eluted with 5 ml of chloroform-methanol, 2:1, after the lipid solution was passed through. [c] This volume of extract was taken to dryness and dissolved or, with the 200-ml sample, suspended in 5 ml of chloroform-methanol-water, 60:30:4.5. [d] A 5-g column was used which was eluted with 25 ml of chloroform-methanol, 2:1, to recover the lipid. [e] Column run at 4°. [f] Column the same as VI except eluted with 10 ml of chloroform-methanol, 2:1.

used and is superior for the removal of inorganic phosphate.

The determination of fatty acid in lipid extracts before and after treatment on Sephadex columns is a reliable criterion for the recovery of all lipids except hydrocarbons, steroids, nonesterified ethers, and various other lipid components that occur in tissues in relatively small amounts. The recoveries of the order of 99% obtained probably indicate that the loss of lipid on Sephadex is negligible. It is particularly noteworthy that brain extracts containing sulfolipids and gangliosides and the fraction containing highly polar phosphatidic acid showed no significant loss of fatty acid. That sterols are not lost in the procedure is indicated by the good recovery of cholesterol obtained.

The data presented in Tables I, II, and III indicate that the limiting factor on the capacity of the columns is in the total volume of lipid extract and eluting solvent passed through the column and is not greatly affected by the concentration of the lipid. Because of the convenience of using small columns the use of as concentrated solutions as possible is desirable. However, the relatively dilute extracts obtained from most tissues can be treated without concentration by simply scaling up the size of the columns. This is particularly desirable with extracts of labile lipids which might be degraded during concentration. Regardless, optimum performance is obtained when up to 15 ml of a chloroform-methanol-water, 60:30:4.5, solution of lipid is eluted through the column with 5 ml of chloroform-methanol, 2:1, for every gram of Sephadex. At 4°, an additional 5 ml of chloroform-methanol, 2:1, is required to elute the lipid quantitatively. It should be emphasized that the actual concentration of lipid or contaminant is not critical but the combined volume of lipid solution and eluting solvent should not exceed 20 ml/g of Sephadex. Elution with excessive volumes will cause the elution of nonlipid material. Also, prior partitioning of extracts with water or salt solution results in poor retention of nonlipid contaminants, although this can be corrected by adding HCl or $MgCl_2$ to the lipid solution.

Finally, it may be pointed out that the use of Sephadex for this application has the advantage of being rapid and easily adapted to either a micro or macro scale. With lipid preparations subject to oxidation, it can be used in the cold or, with minor modifications, in an inert atmosphere.

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Studies of Chemically Reacting Systems on Sephadex. I. Chromatographic Demonstration of the Gilbert Theory*

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The presence of rapid reversible association in proteins has previously been demonstrated by either electrophoretic or sedimentation velocity experiments. In the present study, a chromatographic procedure employing Sephadex G-100 is described whereby similar qualitative infomation may be obtained. Patterns comparable with the schlieren peaks observed in the ultracentrifuge have been derived from the elution profiles of α-chymotrypsin in 0.01 M sodium phosphate, *p*H 7.9. All the features of the transport behavior of such systems, theoretically predicted by Gilbert, have been observed experimentally in a chromatographic procedure for the first time.

The possible effects of chemical interconversion of solute species during transport experiments has been the subject of several theoretical studies. For a substance existing in two isomeric forms, or in association equilibrium, as many as three peaks or spots can result when the rate of reaction is slow relative to the time of migration (Keller and Giddings, 1960; Cann and Bailey, 1961; Scholten, 1961; Mysels and Scholten, 1962). This type of behavior has been observed, e.g., in the chromatography of glucuronic acid and its lactone

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