CHROM. 4512

SEPARATION OF GANGLIOSIDES, CORTICOSTEROIDS AND WATER-SOLUBLE NON-LIPIDS FROM LIPID EXTRACTS BY SEPHADEX COLUMNS*

CHARLES TERNER, ELEK I. SZABO** AND NORTON L. SMITH***
Department of Biology, Biological Science Center, Boston University, Boston, Mass. 02215 (U.S.A.)
(Received November 10th, 1969)

SUMMARY

Two column chromatographic systems (benzene–Sephadex and hexane–Sephadex) are described which effectively separate water-soluble intermediates from lipid extracts of tissues incubated with radioactive precursors.

Biphasic solvent systems were prepared by equilibrating either benzene or hexane with aqueous ethanol. Sephadex G-25 was allowed to swell in the aqueous ethanol (stationary phase). From crude lipid extracts the mobile phase (benzene or hexane) eluted neutral and phospholipids; polar lipids and non-lipids were retained, but were eluted by aqueous ethanol. The hexane–Sephadex column was more effective in separating gangliosides from glycerolipids and separated corticosteroids from sterols, ketosteroids and progestins.

INTRODUCTION

The necessity to remove water-soluble contaminants from lipids extracted from tissues has led to the development of a number of methods of purification. Of the procedures in which lipids in solution in organic solvents are washed with water, the best known and most effective is that of FOLCH *et al.*[1], in which the lipids are first extracted with chloroform–methanol (2:1), and then partitioned between chloroform and aqueous methanol containing salt. This technique is effective in removing the bulk of water-soluble contaminants, but when these are radioactive, high counting traces remaining in the chloroform fraction can be removed only after repeated washing with aqueous salt solution until the concentrate of the final wash (in our experience the 7th or 8th) is free from measurable radioactivity. The paper by WELLS AND DITTMER[2], in which the use of Sephadex columns for the removal of non-lipid contaminants was described, demonstrated the possibility of using Sephadex columns for the single step removal of water-soluble radioactive precursors and metabolites.

* Supported by grants CA-08444 and DE-00153 from the National Institutes of Health, United States Public Health Service.
** Present address: V. A. Research Hospital, Chicago, Illinois.
*** Participant in summer training program for dental students supported by the National Institute of Dental Research.

Since the solvent system described was not performing adequately in our hands, another system based on benzene was developed in our laboratory and described in a thesis[3]. In the meantime, an elaborate system was reported by SIAKOTOS AND ROUSER[4] and later simplified procedures were described by WUTHIER[5] and by MAXWELL AND WILLIAMS[6], all employing modifications of the original solvent system: chloroform–methanol–water, recommended by WELLS AND DITTMER[2]. After comparing our solvent system over a period of time with those previously published, we found it to be superior in separating power, speed and ease of operation. Whereas this and the other methods cited do not share the advantage offered by the system of SIAKOTOS AND ROUSER[4] of separating gangliosides from phospholipids, we describe in this paper a second solvent system based on hexane which purifies crude lipid extracts and separates efficiently not only gangliosides, but also corticosteroids.

EXPERIMENTAL

Preparation and operation of Sephadex columns

A mixture of either (a) benzene or (b) hexane with ethanol (95 %) and water (100:80:20) was shaken in a separating funnel and allowed to stand until two phases had formed. The lower phase (LP) consisted of the aqueous ethanolic component, the upper phase (UP) of the benzene or the hexane phase. The systems will be referred to as benzene column or hexane column, and the upper phases as UP (benzene) or UP (hexane). Since thorough equilibration of the two phases was found to be essential, the solvents were left in the funnel for storage and separated only before use.

Sephadex G-25 (fine grade, bead form) was stirred into the aqueous ethanolic phase (LP) and allowed to swell for several hours in a stoppered vessel. The slurry was poured into a column plugged with glass wool and packed under a few pounds of pressure using nitrogen. Small chromatographic glass columns, with a bulb and side arm, but without a tap, were found the most practical*.

The size of the column was selected according to the amount of lipid to be purified; *e.g.* a column of O.D. 1 cm was packed with the gel to a height of 9 to 10 cm. To ensure saturation with LP, at least 7 ml LP were passed through the column under sufficient nitrogen pressure for discrete drops to emerge. This was followed by 7 ml of UP to displace the preceding LP. Incomplete extrusion of LP was shown by the emergence from the tip of the column of opaque drops indicating separation of water from the organic solvent and more UP was passed through until the effluent drops were clear.

The lipid, dissolved in 2–3 ml of UP, was then applied to the top of the column and allowed to seep into the gel. Elution was started immediately by adding UP and applying sufficient nitrogen pressure to move the mobile phase through the column. Up to 500 mg of crude liver lipid could be purified on a 1 × 10 cm column, with virtually 100 % recovery of the total lipid in 5 ml of eluate. The volume of eluate was therefore routinely restricted to 7 ml, or 2 ml more than needed to force the lipid material through the column.

Polar substances were recovered by continuing the elution with 7 to 10 ml LP (aqueous ethanol). In the hexane column, the breakthrough of LP was easily re-

* Metaloglass Inc., Boston, Mass.

cognized when the first drop of aqueous ethanol fell to the bottom of the collecting tube filled with hexane (starburst effect). After stripping by passing a larger volume of LP, the columns could be re-equilibrated with UP and used again.

Material

The lipids were extracted from the various tissues by homogenizing in chloroform–methanol (2:1). To test the efficiency of the columns in removing polar material, ^{14}C-labeled substrates were added to rat liver lipids in chloroform–methanol (2:1) solution. In order to prepare ^{14}C-labeled lipids, intact cell suspensions (trout eggs, mammalian and fish spermatozoa), slices, minces and homogenates of rat and guinea pig mammary gland were incubated with the radioactive substrates; these were 0.04–0.1 *M* solutions of 1-[^{14}C]acetate (specific activity 2.5 mCi/mmole), [^{14}C]glucose (r.l.), 1,3-[^{14}C]glycerol, 2-[^{14}C]pyruvate and 1-[^{14}C]glucosamine (0.1–1 mCi/mmole).

The lipid extracts were concentrated in a rotary flash evaporator with repeated addition of benzene.

Gangliosides were isolated from lipid extracts of hamster brain by partitioning between chloroform and aqueous methanol according to FOLCH *et al.*[1] and recovered from the aqueous methanolic phase.

Steroids were purchased from Mann Research Laboratories, New York. They were added in 1 mg amounts to 500 mg crude lipids.

The radioactivity of lipids was determined either by counting thin layers on planchets in a gas flow counter with a thin window, or by counting in a liquid scintillation counter.

Thin-layer chromatography

Fractions containing steroids were analyzed by thin-layer chromatography using pre-coated silica gel plates with fluorescent indicator (S/G, Q1-F, Quantum Industries, Hanover, N.J.) in the solvent system: chloroform–methanol–water (90:10:1). Ketosteroids were detected by spraying with Zimmerman reagent, corticosteroids with Tetrazolium Blue.

Gangliosides were chromatographed on pre-coated silica gel plates (Brinkman Instruments Co., Westbury, N.Y.) in the solvent system: *n*-propanol–water (7:3). The zones were detected by spraying with resorcinol reagent[7]. Exposure of the plates to iodine vapor aided in the visualization of other material.

RESULTS

Retention of non-lipid material

Water-soluble inorganic and organic substances dissolved in UP and applied to either the benzene– or the hexane–Sephadex column were retained and could not be displaced even by large volumes of UP. They were, however, recovered by elution with LP.

When ^{14}C-labeled substrates, commonly used in tissue incubation, (acetate, glucose, glucosamine, glycerol) were added to a lipid extract and the mixture, dissolved in UP, was passed through the column, the radioactivity was retained, the emerging lipids being virtually free from radioactivity.

At first the column appeared to be less effective in removing [^{14}C]pyruvate,

since it allowed some of its radioactivity to be eluted with UP. This was found to be due to the presence of less polar impurities in the radioactive pyruvate. Pre-treatment of stock solutions of [^{14}C]pyruvate by passage through a Sephadex column, elution of the impurity with UP and recovery of the pyruvate with LP was therefore necessary to prepare pure pyruvate solutions.

Lipids which had been labeled by incubation of surviving tissue with a radioactive precursor retained their radioactivity on passing through the column. Their specific activity, determined after the first passage through the Sephadex column, remained constant on repeated re-running through fresh columns.

The benzene–Sephadex column has been used routinely in this laboratory in the purification of crude lipids labeled by *in vitro* incorporation of ^{14}C-labeled precursors. From the purified lipids, eluted with UP (benzene), glycerides, phosphatides, cholesterol[8] and cerebrosides[9] were isolated by column or thin-layer chromatography. Gangliosides were not easily separated on benzene–Sephadex columns since they began to emerge in the last 2 ml of UP (benzene) and therefore tended to be distributed between UP and LP. On the hexane–Sephadex column, however, the gangliosides were completely retained during elution of other lipids by UP (hexane), and could then be eluted with LP. Thin-layer chromatography of brain gangliosides recovered from LP of hexane columns yielded the same number of resorcinol-positive zones as recovered from the aqueous methanol portion of the same brain lipid extract, which had been separated by the method of FOLCH *et al.*[1].

Separation of ketosteroids and corticosteroids

The hexane column also proved useful in the separation of sterols and steroids. Cholesterol, testosterone and progesterone were eluted quantitatively (hexane) together with the glycerolipids from which they could then be separated by thin-layer chromatography. The corticosteroids, cortisone, hydrocortisone and deoxycorticosterone were retained on the column and could subsequently be eluted with LP. Thin-layer chromatography of UP and LP showed that a trace amount of deoxycorticosterone had escaped into UP, although the major portion was in the fraction eluted by LP. Careful fractionation by reducing the volume of UP, or use of longer columns may result in more complete retention of less polar corticosteroids.

DISCUSSION

Compared with other Sephadex column systems, based on chloroform, the two new solvent systems, based on the less polar benzene and hexane, offer distinct advantages. Sharp separations of the important classes of less polar lipids from more polar lipids and non-lipids can be accomplished with ease by one passage of mobile phase (UP), followed by one stripping with stationary phase (LP), without the need for fractional collection of either eluate. Because of the great holding capacity of Sephadex columns and the small volumes of both phases needed for complete elution, the whole procedure, including evaporation of the solvents, can be completed within 30 min. No special technical skill is required and several columns can be run concurrently. The only precaution needed is to maintain the equilibrium between the stationary and the mobile phases during the passage of the latter through the column. Persistent cloudiness of the emerging drops of mobile phase is an indication of partial

extrusion of stationary phase. When this is observed, the lipid sample, if already on the column, is best recovered by stripping with LP and rerun on a new column. If there should be reason to doubt the completeness of removal of radioactive non-lipid contaminants, the samples can be rerun on new columns, repeatedly if desired, without loss of lipid material until constant specific activity is attained.

The benzene–Sephadex column is preferred in the routine isolation and purification of neutral and phospholipids; for the separation of gangliosides and corticosteroids, the hexane column is more suitable, but greater care is required to maintain the equilibrium between the two phases. Extrusion of stationary phase can be avoided by slowing the rate of elution of UP. The hexane column offers an effective and unusually rapid method of separation of groups of steroids, *e.g.* it easily separates progesterone (eluted by UP) from corticosteroids which are eluted by LP, free from glycerolipids and phospholipids.

REFERENCES

1 J. FOLCH, M. LEES AND G. H. SLOANE STANLEY, *J. Biol. Chem.*, 226 (1957) 497.
2 M. A. WELLS AND J. C. DITTMER, *Biochemistry*, 2 (1963) 1259.
3 E. I. SZABO, *Ph. D. Thesis*, Boston University, 1966.
4 A. N. SIAKOTOS AND G. ROUSER, *J. Am. Oil Chemists' Soc.*, 42 (1965) 913.
5 R. E. WUTHIER, *J. Lipid Res.*, 7 (1966) 558.
6 M. A. B. MAXWELL AND J. P. WILLIAMS, *J. Chromatog.*, 31 (1967) 62.
7 J. R. WHERRETT AND J. N. CUMINGS, *Biochem. J.*, 86 (1963) 378.
8 A. A. GIANELLY AND C. TERNER, *Endocrinology*, 83 (1968) 1311.
9 C. TERNER, L. A. KUMAR AND T. S. CHOE, *Comp. Biochem. Physiol.*, 24 (1968) 941.