Immunochemical Studies of Organ and Tumor Lipids

XI. A SIMPLIFIED PREPARATION OF CYTOLIPIN H*

MAURICE M. RAPPORT,† HERBERT SCHNEIDER, AND LISELOTTE GRAF

From the Department of Biochemistry, Albert Einstein College of Medicine, Yeshiva University, and the Division of Experimental Pathology, Sloan-Kettering Institute for Cancer Research, New York 61, New York

(Received for publication, November 6, 1961)

Cytolipin H is a glycosphingolipid whose haptenic function was first demonstrated as a result of studies of the antigenic components of a human epidermoid carcinoma (1, 2). Analysis of the isolated molecule showed that it contained fatty acid, a lipid base, glucose, and galactose in equimolar proportions and thus had a composition similar to that of a substance isolated from ox spleen (3). Subsequently it was shown that cytolipin H isolated from ox spleen was immunochemically indistinguishable from that isolated from human tumor, despite small differences in degree of unsaturation and specific rotation (4). Further studies have now revealed other structural features, namely, that the two monosaccharide residues are bound as a disaccharide with the configuration of lactose (5), that the fatty acid residue is principally sphingosine (6). Further study of this molecule is necessary in order to develop more fully the relation between structure and immunological activity, but the supply of sphingolipid has been extremely limited. On one hand, large amounts of human carcinoma tissue are not available, and on the other, the method of isolation from ox spleen described by Klenk and Rennkamp (3) is very indirect, since their primary objective was the isolation of gangliosides rather than cytolipin H. We have therefore restudied the method of isolation with a view to developing an efficient procedure that would yield a pure product in gram quantities. The method developed involves the following steps: (a) dehydration of the tissue, (b) extraction of lipids and removal of water-soluble impurities from them, (c) preparation of an ether-acetone-insoluble fraction, (d) preparation of an acetic acid-acetone-insoluble fraction, (e) chromatography on Florisil, and (f) chromatography on silicic acid. In this way it was possible to isolate over 1.5 g of cytolipin II from 40 kg of ox spleen, or approximately 75% of that estimated to be present in this tissue.

EXPERIMENTAL PROCEDURE

Analytical procedures for carbohydrate based on color reactions produced directly with anthrone (7) and, after hydrolysis, with o-anisidinephenyl (8) were indicated in an earlier paper (4). Determination of iodine number and serological analysis by complement fixation have also been described (4, 9). Silicic acid for chromatography was prepared from J. T. Baker's Silicic Acid (10). Florisil (Florisil Company), 100 to 200 mesh, 649° activated, was used directly.

Extraction of Lipids from Dehydrated Ox Spleen—Ox spleen (10 kg) that had been stored at −20° after being chopped in an electric meat grinder was thawed and then dehydrated with acetone as follows. The tissue was suspended in 15 liters of acetone, stirred occasionally, and left overnight at room temperature. After suction filtration, the residue was again treated with 15 liters of acetone in the same way. The residue obtained on filtration was air-dried in a shallow tray for several hours. The resulting light brown powder was extracted with 12 liters of chloroform-methanol (2:1) to which 600 ml of water had been added, with mechanical stirring for 6 hours. After suction filtration, the amber brown filtrate was shaken with 0.7 its volume of water and placed at 5° overnight. The residue re-extracted with chloroform-methanol filtered, and this filtrate was then used to extract the next batch of spleen. The clear yellow upper phase and white interfacial layer were removed by suction, and the reddish brown chloroform layer was evaporated to dryness in a vacuum. From 10 kg of tissue, 199 g of crude lipid were obtained.

Preparation of Ether-Acetone-insoluble Fraction—A solution of 199 g of water-washed crude lipid in 1300 ml of ethyl ether containing 30 ml of water was placed in a 2-liter glass-stoppered cylinder, and 400 ml of acetone were added. After these components were mixed and allowed to stand at 5° for 1 to 3 days, a compact precipitate, light tan to white in color, settled to the bottom of the cylinder. The clear brown supernatant solution was removed. The precipitate was centrifuged bottles with the aid of ether-acetone (4:1) and washed three times by centrifugation at 5°. The precipitate was collected by dissolving it in chloroform-methanol (2:1), and the solvent was then completely removed. From 199 g of crude lipid, 35.3 g of a colorless ether-acetone-insoluble fraction were obtained.

Preparation of the Acetic Acid-Acetone-insoluble Fraction—To a suspension of 35 g of ether-acetone-insoluble lipid in 160 ml of warm (50°) glacial acetic acid, 240 ml of acetone were added. After centrifugation at 20° for 20 minutes at 3000 r.p.m. in an International No. 1 centrifuge, the pale yellow supernatant solution was decanted; the precipitate was washed three times with 200-ml portions of ether-acetone (4:1) at 5°. The lipid was then dried by dissolving it in chloroform-methanol (2:1) and evaporating the solvent in a vacuum. From 35 g of ether-acetone-insoluble fraction, 15.7 g of acetic acid-acetone-insoluble fraction were obtained. Material from four runs was combined.
April 1962
M. M. Rapport, H. Schneider, and L. Graf

(63 g of lipid) and dissolved in 400 ml of chloroform-methanol (2:1). The hazy solution was filtered through Florisil: each 100-ml portion of solution was filtered through a 20-g column (2.5 x 10 cm) and washed through with 350 ml of warm solvent. If the column became clogged, the upper surface was stirred up. The lipid recovered after this procedure weighed 49.4 g (representing 40 kg of spleen). This material was then fractionated on large Florisil columns.

Chromatography on Florisil—A column of adsorbent measuring 60 x 3.5 cm was prepared from 250 g of Florisil suspended in 600 ml of chloroform-methanol (4:1). The column was washed with 600 ml of the same solvent, and a solution of 10 g of the acetic acid-acetone-insoluble fraction in 20 ml of chloroform-methanol (4:1) was placed on it. Five bulk fractions were collected, as follows: I, 1000 ml of chloroform-methanol (4:1); II, 250 ml of chloroform-methanol (4:1); III, 200 ml of chloroform-methanol (4:1); IV, 3000 ml of chloroform-methanol (2:1); and V, 2500 ml of chloroform-methanol (2:1). The weight and anthrone color value of each fraction were determined. Those with a value in excess of 55% of that expected for the pure material (usually fractions II, III, and IV) were pooled and subjected to chromatography on silicic acid. The combined yield from 49.4 g of the acetic acid-acetone-insoluble fraction was 12.6 g. This step serves to separate cerebrosides from cytoside, the former being eluted more rapidly from Florisil. The two small volume fractions, II and III, are collected in order to insure a sharp separation.

Chromatography on Silicic Acid—A column of adsorbent measuring 85 x 3.5 cm was prepared from a suspension of 500 g of silicic acid and 50 g of Celite No. 535 in 1 liter of acetone. The column was washed with 1 liter each of methanol, acetone, and chloroform, in that order. A solution of 8.6 g of Florisil-purified lipid in 25 ml of chloroform-methanol (95:5) was put on the column with the aid of 25 ml of chloroform. Five fractions were collected by discontinuous gradient elution, as shown in Table I. The remaining 4 g of Florisil-purified lipid were fractionated in the same way with a 250-g column of silicic acid and one-half the solvent volumes. Fractions with an anthrone color value in excess of 80% of that of pure cytolipin H (eluted with chloroform-methanol, 92:8) were combined to give 2.5 g of almost colorless material. The behavior of this product on recrystallization from pyridine-acetone indicated the presence of impurities (rapid precipitation, failure to form spheres). It was therefore reprocessed on a 100-g Florisil column with chloroform-methanol (2:1), and the collection of 41 fractions of 40 ml each. Anthrone analysis showed a major peak in Fractions 8 to 20 (Fig. 1). The material in these fractions was combined and recrystallized by dissolving it in 30 ml of pyridine and adding 180 ml of acetone at 70° followed by slow cooling (2). The yield was 1.64 g of colorless cytolipin H with a hexose content of 38.2% (o-aminodiphenyl) and an anthrone color value 102% of that of the standard. The iodine number was 27.1. Thin layer chromatography on silicic acid of 40 µg with chloroform-methanol (7:3) containing 4% water showed the presence of only one spot migrating with a velocity relative to that of phrenosine (Rfphren) of 0.65. The Rp of phrenosine was 0.77. Immunochemical characterization is shown in Fig. 2A, in which the isofixation curve (II) for the preparation is compared with that of a pure sample of cytolipin H.

Dihydrocytolipin H—A solution of 100 mg of cytolipin H in 100 ml of methanol at room temperature was prepared by adding 10 mg of pure sphingomyelin, heating to the boiling point, and cooling. Hydrogenation under 40 pounds of excess pressure of hydrogen was carried out for 1 hour at room temperature in a Parr hydrogenator in the presence of 80 mg of platinum oxide catalyst. The precipitate which formed was redissolved by warming, and the catalyst was removed by filtration. After removal of the solvent under reduced pressure, the lipid residue was taken up in 5 ml of chloroform-methanol (4:1) and fractionated on a 10-g Florisil column. Fractions of 10-ml volume were collected, six with chloroform-methanol (4:1) and 15 with chloroform-methanol (2:1). On the basis of anthrone color value, Fractions 8 through 15 were combined and evaporated to dryness to give 80 mg of product. This was recrystallized twice from pyridine-acetone at 70° (2.5 ml plus 5 ml) to give 36 mg of dihydrocytolipin H with an anthrone value 99% of that of the standard, an iodine number of 0, and a phosphorus content below 0.01%. Thin layer chromatography of 40 µg of this material as described above showed the presence of only one spot,
for effective separation. The knowledge that cytolipin H was a glycosphingolipid and contained no phosphorus permitted these substances from other tissues. A number of isolation experiments applying the simplified method to human epidermoid carcinoma tissue on a 2- to 4-kg scale did not give a pure product in yield comparable with that obtained by the method first developed (2). Other methods of extraction, such as that proposed by Bligh and Dyer (12), were rejected because of difficulties encountered in later phases of purification.

Although the anthrone color value is a useful indicator in following the course of isolation and establishing the purity of the final product, it must be used cautiously because spleen contains a number of glycolipids with high sugar content. Some fractions were obtained whose anthrone value was very similar to that of cytolipin H, but they were unreactive with anticytolipin H. These fractions were also readily distinguished from cytolipin H by their solubility. It has so far been possible to place a very high degree of reliance on the combination of recrystallization behavior and anthrone color value in establishing the purity of preparations of cytolipin H. Pure preparations are readily soluble in small volumes of pyridine at ordinary temperature. On addition to a pyridine solution at 70° of 6 volumes of acetone at 70°, no precipitate forms (precipitate at this point indicates the presence of impurity). Allowing the solution to cool slowly to room temperature results in the formation of fairly large, individual spheres adhering to the wall of the glass vessel. When these spherules have the correct anthrone color value, and the supernatant solution, which usually contains less than 5% of the initial material, has a comparable value (13), the preparation is pure. Cerebrosides show a similar crystallization behavior, but the anthrone color value is much lower.

In one case when the described method was applied to 100 pounds of spleen, it was found possible to complete the purification by recrystallization immediately after chromatography on Florisil, thus eliminating chromatography on silicic acid. The yield in that run, however, was just under half of that found in the experiment presented. Elimination of chromatography on silicic acid was suggested by two properties of the material obtained from the Florisil column: a high anthrone color value and insolubility in chloroform-methanol (95:5).

The characterization of dihydrocytolipin H does not establish that the allylic hydroxyl group is intact, since hydrogenolysis may have occurred during hydrogenation. Further chemical study of the product will be required. However, immunological reactivity may be conservatively assessed in relation simply to the loss of the double bond in the sphingosine residue. Complete retention of reactivity by dihydrocytolipin H shows that the double bond in the sphingosine residue is not a structural feature of significance for the immunochromatographic properties of cytolipin H. Thus, an entirely satisfactory product should be available by synthetic methods used to prepare dihydrocerebrosides (14).

**SUMMARY**

A method has been developed for isolating cytolipin H from ox spleen with a high degree of efficiency. The steps involve dehydration of the tissue with acetone, extraction of lipids with chloroform-methanol, removal of water-soluble impurities, preparation of a sphingolipid fraction by removing ether-soluble and acetic acid-soluble lipids, chromatography on Florisil, and chromatography on silicic acid. From 40 kg of spleen, over 1.5 g of...
pure cytolipin H were obtained. Dihydrocytolipin H was prepared by catalytic reduction of cytolipin H and found to retain completely the immunochemical properties of the parent molecule.

Acknowledgments—We wish to thank Mr. Daniel H. Fackre, Mr. Nicholas F. Alonzo, Miss Loretta Giuffra, and Mrs. Suzanne Kerr for invaluable assistance. We are also grateful to Dr. K. Folkers and Dr. T. A. Jacob, Merck, Sharp and Dohme, and Mr. S. Rosenberg, Sylvana Chemical Company, for carrying out early steps in two of the large scale preparations.

REFERENCES
Maurice M. Rapport, Herbert Schneider and Liselotte Graf

PREPARATION OF CYTOLIPIN H
Immunochemical Studies of Organ and Tumor Lipids: XI. A SIMPLIFIED