CHROMATOGRAPHIC PURIFICATION AND SEROLOGIC
STUDIES OF A BEEF HEART LECITHIN*

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The flocculation tests currently used for the serodiagnosis of syphilis in-
volve the use of an aqueous emulsion of cholesterol, beef heart lecithin,
and cardiolipin which undergoes aggregation when treated with Wassermann antibody (1). The role of cholesterol in this reaction has been de-
picted as providing a particulate surface which enhances the visibility of
the aggregates formed by the antigen-antibody reaction (2). However,
the function of the two phospholipides has not yet been clearly defined.

Before undertaking further immunological studies on the rôle of lecithin
and cardiolipin, an attempt was made to ascertain the purity of these two
phospholipides, as prepared customarily for serologic use. Chromatograms
of several preparations of beef heart lecithin on Magnesol-Celite indicated
the presence of more than one component. The present report describes
the isolation of a chromatographically homogeneous, immunologically ac-
tive lecithin from beef heart lecithin prepared commercially for serologic
tests.

EXPERIMENTAL

Source of Materials—Chromatographic analyses and serologic assays
were carried out with the following preparations of beef heart lecithin:
(a) lecithin prepared from dehydrated beef heart (Difco) according to
Pangborn (3), (b) several lots of the crude CdCl₂ complex supplied through
the courtesy of Dr. H. D. Piersma of the Lederle Laboratories Division,
American Cyanamid Company, and further purified in this laboratory by
Pangborn’s procedure (3), (c) commercially prepared (LaMotte and Le-
derle) beef heart lecithin used in the serologic diagnosis of syphilis, (d) a
preparation procured through the courtesy of Dr. Mary C. Pangborn.

Except for small variations in the weight proportions eluted from indi-
vidual zones, all the preparations yielded similar chromatograms. The

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serologic activity was always associated with the material eluted from one zone. The chemical analyses reported below were obtained on samples prepared from a lot of the crude CdCl₂ complex. The iodine numbers determined before chromatography by Niemierko's (4) modification of the method of Rosenmund and Kuhnenn (5) varied between 66 to 77 on different preparations.

**Chromatographic Procedure**

Magnesol¹ was used as the adsorbent for the chromatographic studies and was treated as follows: A thick slurry was prepared by suspending the dry powder in about 0.9 per cent aqueous HCl. The suspension was passed through a Büchner filter and the filter cake washed with distilled water until the filtrate gave negative tests for chloride ion. The Magnesol was then washed with 95 per cent ethanol and dried at room temperature overnight and in an electric oven at 100-120° for 24 hours. The thoroughly dried material was subsequently powdered in a ball mill and mixed with Celite² in proportions of 5 parts (by weight) of Magnesol to 1 of Celite. As indicated below, a weaker adsorbent was used in some instances. This was prepared by further heating a sample of the above mixture in an electric furnace for 12 hours at 800° (6).

In a typical chromatographic study, the adsorbent was packed in a glass column (50 x 250 mm.) (7). After impregnation of the adsorbent with benzene (Merck, c.p.), 500 mg. of lecithin in 15 ml. of benzene were added at the top of the column, which was then developed with 500 ml. (about two and one-half column lengths) of 2 per cent (by volume) tert-butyl alcohol in benzene. The column was then extruded and streaked with an aqueous solution of 1 per cent potassium permanganate in 2.5 N sodium hydroxide (8). Two zones appeared on the column. Zone I extended for 2 cm. from the top of the column. Zone II originated 10 cm. from the lower face of Zone I and was 2 cm. long. The permanganate streak was carefully scraped away, the zones were cut, and the adsorbed material in each zone was eluted with 500 ml. of absolute ethanol. The concentration of the developer that had passed through the column yielded additional material (Zone III). The following material was recovered: for Zone I, 166 mg., Zone II, 160 mg., and Zone III, 170 mg. Serologic activity was confined to the material eluted from Zone I for each of the beef heart lecithins. Since the present investigation was concerned with the isolation of the beef heart lecithin component associated with serologic activity, as estimated by the flocculation tests used in the serodiagnosis

¹ A synthetic, hydrated magnesium acid silicate manufactured by the Westvaco Chlorine Products Company, South Charleston, West Virginia.

² No. 535 obtained from Johns-Manville, New York.
of syphilis, further attempts at purification were confined to the eluate of Zone I.

The material of Zone I was rechromatographed to establish further its chromatographic homogeneity. A chromatographic column (30 × 250 mm.) was packed with the Magnesol-Celite which had been heated at 800° for 12 hours (6). After wetting the column with benzene, 150 mg. of the eluate of Zone I dissolved in 5 ml. of benzene were added at the top of the column, which was then developed with 200 ml. of 25 per cent (by volume)
absolutely ethanol in benzene. The column was washed with 200 ml. of benzene, extruded, and streaked with permanganate. Two zones were visible at distances of 1 cm. (Zone IA) and 5 cm. (Zone IB) from the top of the column. These were cut and the materials contained therein were eluted, each with 250 ml. of absolute ethanol. 50 mg. of serologically inactive material were recovered from Zone IA. Zone IB yielded 100 mg. of a serologically active substance (see Table I). The eluate from Zone IB could be rechromatographed on heated Magnesol-Celite without further chromatographic separations, even with a 3-fold increase in the volume of developer.

Isolation and Characterization of Serologically Active Component in Beef Heart Lecithin

It was noted during the course of this work that the eluate of Zone IB formed a complex with CdCl₂ which was only slightly soluble in ethanol. The serologically inactive material of Zone IA did not form this insoluble complex. Subsequently, CdCl₂ was used to effect the separation of material from Zone I into its active and inactive components without resort to the second chromatographic procedure. The ethanolic eluate of Zone I was concentrated under reduced pressure at 30-40° in an atmosphere of CO₂ to approximately 10 ml. A saturated solution of CdCl₂ in ethanol was then added dropwise until maximal turbidity developed, and the precipitate was removed by centrifugation.

The supernatant fluid, when concentrated to dryness in vacuo at 30-40° under a stream of CO₂, yielded a solid material which was serologically inactive and corresponded chromatographically to the material from Zone IA. The precipitate, prepared with the eluate of either Zone I or Zone IB, was dissolved in a small volume of benzene (about 4 ml. of solvent for 100 mg. of the CdCl₂ precipitate) and absolute ethanol added until a slight turbidity developed. Microscopic crystals separated on chilling the solution in the ice box overnight. The compound melted with decomposition between 160° and 180°, depending on the rate of heating. The compound was analyzed for C₄₂H₅₅O₃NP(CdCl₂)₄.

Calculated. C 44.76, H 7.34, N 1.24, P 2.75, Cl 12.5, iodine No. 22.6

Found. C 45.36, H 7.35, N 1.25, P 2.74, Cl 11.9, iodine No. 21.2

A 10 per cent solution in benzene showed no optical activity.

Further analyses of the eluate of Zone IB were made after the removal of the CdCl₂ from the lecithin molecule by a modification of Levene's

*The combustion analyses were performed by the Clark Microanalytical Laboratory, Urbana, Illinois.

4 Rosenmund-Kuhnhenn method (4, 5).
method (9). 100 mg. of the complex were dissolved in 15 ml. of benzene. 1 ml. of 80 per cent alcohol was added and ammonia gas passed through the solution. The precipitate which formed was removed by centrifugation and nitrogen was bubbled through the supernatant fluid to remove the ammonia. 50 ml. of absolute ethanol were added and the solution was concentrated to about 10 ml. The weight of the lecithin, as estimated by drying and weighing an aliquot, was 75 mg. This compound crystallized from ethanol-acetone (1:1) and melted at 140–150° (decomposition, depending on the rate of heating). The compound was unstable and on standing turned into a viscous brown sirup. A carbon-hydrogen analysis was not obtained. The nitrogen and phosphorus contents of a freshly prepared sample agreed with the formula C_{32}H_{52}O_{5}NP.

Calculated. N 1.84, P 4.08, iodine No. 33.4
Found. “ 1.82, “ 4.06, “ 37.4–40.3

SeroLogic Activity of Preparations of Beef Heart Lecithin and Their Chromatographically Separated Components

As shown in Table I, serologic assays were performed with the preparations of beef heart lecithin before and after chromatographic separation. Cholesterol-lecithin-cardiolipin emulsions were prepared as for the V. D. R. L. microfloculation test (1) by mixing 0.9 per cent NaCl solution with an alcoholic solution containing 0.9 per cent cholesterol and 0.03 per cent cardiolipin, in addition to the beef heart lecithin or the designated fraction. Several concentrations of each fraction were tested with sera of known reactivity, as well as with saline and negative serum controls.

Substitution of the individual, serologically inactive chromatographic fractions for the commercial product yielded coarse, partially aggregated antigen suspensions which were not further clumped in the presence of sera containing Wassermann antibody. The eluates from Zones I and IB, in contrast to those from Zones IA, II, and III, formed antigen suspensions which were visually and serologically indistinguishable from the original commercial preparation (see Table I). The data given in Table I are representative of the results obtained with the various preparations of beef heart lecithin listed above and their chromatographic components.

Recombination of the individual chromatographic fractions (Table I) in the proportions isolated yielded a reconstituted beef heart lecithin which exhibited optimal activity at a concentration of 0.20 per cent (total), closely similar to that of the non-chromatographed material (0.26 per cent).

The serologic behavior of the active lecithin fraction (Zone IB eluate) obtained from two commercial preparations of beef heart lecithin was further studied in tests with 605 sera submitted for routine serodiagnosis.
Each of these sera was subjected to parallel tests with the standard V. D. R. L. antigen (1), the Eagle flocculation test antigen (1), and an antigen containing the eluate from Zone IB in optimal concentration. Complete agreement in all three tests was observed in 97 per cent of the 605 sera. These sera included 132 specimens that yielded positive reactions with the Eagle and V. D. R. L. test antigens. The discrepancies observed with the antigens prepared from the eluates of Zone IB were no more numerous than those which occurred with the two standard serologic test antigens.

**DISCUSSION**

Investigations of the mechanism of the flocculation tests used for the serodiagnosis of syphilis have been hampered by the impurity of the reagents employed. The isolation of a chromatographically pure lecithin, whose cadmium chloride complex has the probable empirical formula C_{42}H_{83}O_{8}NP(CdCl₂)₂, makes available one of these reagents in purified form. Since the eluate of Zone IB, which contains the purified lecithin, constitutes approximately 20 to 30 per cent of the weight of the commercial lecithin used as starting material, it would be expected to possess a proportionately greater activity on a weight basis of the starting material. However, this was not the case and it is therefore necessary to assume either that a loss of active material occurred as a result of deterioration during chromatographic separation, or that the other fractions separated by chromatography exerted some accessory function. The experimental data seem to be in accord with the latter alternative since, on recombination of Zones I, II, and III in the proportions in which they were isolated, optimal activity was achieved with approximately material of the same weight as the original beef heart lecithin. Thus it would seem that the other fractions, i.e. the eluates of Zones II and III, although devoid of activity when used alone, contribute in some manner towards producing a satisfactory emulsion and thus enhance the activity of the eluate of Zone IB. Further exploration of this finding, possibly in terms of the surface-active properties of the several components, might be considered.

The possibility of chemical degradation also appears unlikely since the material of Zone IB can be isolated from the eluate of Zone I by either rechromatography or the formation of a cadmium chloride addition compound. Both products possess similar activity and are comparable in terms of their iodine numbers on a molar basis. Moreover, on rechromatography with a relatively large volume of developer, Zone IB was not further fractionated. These findings would seem to indicate that the purified lecithin in Zone IB is stable under the manipulative procedures employed in its isolation from commercial beef heart lecithin.

It is of immunological interest to note that synthetic α-lecithins (dipalmitoyl and dimyristoyl) have been successfully substituted for the nat-
urally occurring beef heart lecithin in flocculation tests for the laboratory
diagnosis of syphilis (10, 11). Determinations of the iodine number for
the purified lecithin in the eluate of Zone IB indicate the presence of one
double bond in the molecule, thus differentiating this substance from the
synthetic lecithins, which contain only saturated fatty acids. It would
therefore appear that the nature of the fatty acids present in the lecithin
molecule may not be critical in terms of its serologic activity. Additional
information on this question may emerge from further studies now in prog-
ress on the chemical and immunological characterization of the chroma-
tographically purified lecithin.

SUMMARY

1. Commercial beef heart lecithin has been fractionated chromatograp-
ically and a lecithin cadmium chloride addition compound of the probable
molecular formula C_{42}H_{82}O_{18}N_{P}(CdCl_{2})_{2} has been isolated from one of the
fractions.

2. In conjunction with cardiolipin and cholesterol, this lecithin forms a
suitable emulsion in the Venereal Disease Research Laboratory flocculation
test for syphilis. The other fractions isolated chromatographically from
beef heart lecithin were individually inactive in serologic tests.

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