Nonsteroidal Components of the Unsaponifiable Fraction of Human Liver*

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In 1952 Penn (21) described the preparation of an unsaponifiable fraction of liver from cancer-bearing patients which gave a serological reaction with sera of cancerous individuals, but usually not with normal serum or serum from persons with noncancerous diseases (12). The difficulty of obtaining sufficient stable test material for extensive cancer screening tests led to the development of a substitute for the human lipide fraction (23). This substitute, ethyl choladienate, was compared in a clinical screening program (13) with the results of the previous testing with the human liver fraction. It was believed that the human fraction gave fewer positive reactions with normal sera and sera from noncancerous disease than did ethyl choladienate. Subsequently, three reports appeared (4, 20, 31) disputing Penn's claims for ethyl choladienate, which in turn were countered by another publication from Penn's laboratory recommending a change in serological procedure (22). No results of further examination of the liver fraction have been reported, although, from the method of preparation, it obviously does not contain ethyl choladienate.

The authors felt that a logical extension of Penn's earlier work entailed: (a) the characterization of the naturally occurring serum flocculants in human liver, (b) identification of the reactant(s) in serum of cancer to ethyl choladienate and the characterized liver substance(s), and (c) clarification of certain aspects of the chemistry of ethyl choladienate itself on which so much work was being based. This approach appeared to offer a faster, less laborious solution to questions of specificity, basic significance, and final acceptance or rejection than extensive clinical screening.

This report is concerned with the first of the three objectives stated above: the characterization of the naturally occurring serum flocculants in cancerous human liver.

Lipide constituents from cancerous liver and other tissues have been studied many times by different groups of investigators who have had as their objective the evaluation of the carcinogenicity of such materials (33) and the isolation of carcinogens from such sources (19, 32). While agreement has been reached that carcinogenic activity is associated with the crude unsaponifiable fraction, the responsible agent has not been identified.

With the exception of the ubiquitous steroids, particularly cholesterol, both saturated and unsaturated hydrocarbons, and small amounts of carotenoid pigments, little else has been identified in the unsaponifiable fraction of normal or cancerous human liver (32). None of these substances is responsible for the flocculation reaction.

MATERIALS AND METHODS

Reagents.—Solvents were of reagent grade; those received in glass bottles were used without additional purification. Pentane and hexane, pure grade, obtained from the Phillips Petroleum Company in metal drums, were redistilled before use. The silicic acid employed was Baker's, lot No. 8814 (best) and No. 2351. The alumina was a catalyst grade, A1-0109P, obtained from Harshaw Scientific Company and was ground to 200 mesh, redried, and mixed with 20 per cent of its weight with oven-dried Celite before use.

Preparation of crude active unsaponifiable fraction from human liver.—A total of 20 kg. of liver, obtained at autopsy, was ground, autolysed under toluene, and the lipides were isolated and saponified, following in general the procedure employed by Penn (21). The alkaline alcoholic hydrolysate of the liver lipides was diluted with water to contain 20 per cent alcohol, extracted with hexane-ether (1:1) and finally with ether. The solvent extracts, after being washed thoroughly with water, were concentrated under reduced pressure to contain 20 per cent alcohol, extracted with hexane-ether (1:1) and finally with ether. The solvent extracts, after being washed thoroughly with water, were concentrated under reduced pressure to an oily residue which was partially freed of cholesterol by crystallisation from acetone. The solute remaining (40.5 gm.) was treated with an equal amount of betaine hydrazide to remove ketonic material (11) and finally with digitonin to remove residual cholesterol. The remaining 25.3 gm. of dark red oil was transferred to 250 cc. of hexane for chromatographic separation.

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Chromatograms on silicic acid.—The silicic acid chromatograms were all run in a comparable fashion following the general procedure described by Fillerup and Mead (9). Fractions were usually collected by volumes equivalent to column retention volume of solvent.

Partition on Celite-supported mineral oil.—Reversed phase partition chromatograms were run according to the procedure described by Howard and Martin and others (5, 17, 30). The stationary phase was Celite-supported heavy, domestic white paraffin oil (Fisher Scientific Co., viscosity 885/880), and the mobile phase was various percentages of acetone in water (v/v). Columns were run at 35°C, and flow rates varied from 0.57 to 0.88 cc/sec/cm/min for columns of 3.36 X 30 cm. The retention volume was usually about 175 cc., and fractions of 25 or 50 cc. were collected.

Samples were introduced onto the column dissolved in paraffin oil contained on siliconed Celite slurried in aqueous acetone. The height of the zone of Celite containing the admitted sample was usually about 10 per cent of the over-all column length.

<table>
<thead>
<tr>
<th>Table 1</th>
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<tbody>
<tr>
<td><strong>SOLUTE PARTITION IN THE FIRST CHROMATOGRAM</strong></td>
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<tr>
<td>The total fraction of 25.3 gm. was chromatographed on a 5.5 X 23 cm. column of silicic acid (600 gm.). The column retention volume of solvent was 675 cc. The total recovery of solute was 87 per cent.</td>
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<tr>
<td></td>
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<tr>
<td>Fraction</td>
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<tr>
<td>II</td>
</tr>
<tr>
<td>III</td>
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<tr>
<td>IV</td>
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<tr>
<td>V</td>
</tr>
<tr>
<td>VI</td>
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</table>

Eluted solids were recovered by distillation of acetone from the eluates, extraction of the aqueous residue with ether, and evaporation of the ether. Losses due to steam volatility and poor extraction occurred in some instances, but attempts to monitor solute concentrations in the eluate by a sensitive device used to detect changes in capacitance were unsuccessful.¹

The concentration of mineral oil in eluates containing less than 65 per cent acetone was negligible, and these fractions were purified directly by crystallization or were tested serologically without removal of traces of mineral oil. Samples for infrared examination and samples from eluates containing more than 65 per cent acetone were dissolved in pentane or cyclohexane, absorbed on silicic acid to wash out small amounts of hydrocarbons, and finally eluted with absolute ether or acetone.

Urea fractionation.—Urea fractionation was carried out by a slight modification of the method described by Fetterly (8).

Examination of spectra.—Infrared spectra were obtained with a Baird Model B double-beam infrared spectrophotometer equipped with a sodium chloride prism. Solid samples were examined in potassium bromide pellets and both solid and liquid fractions in solutions in appropriate solvents. Ultraviolet spectra were obtained with a Cary recording spectrophotometer. The mass analysis and computation were carried out on equipment in general use in the petroleum industry for analyzing hydrocarbon samples and monitoring the cracking pattern for the mass range of 65-477.

Testing of fractions.—The method employed in testing fractions is detailed in the following paper (87).

RESULTS

Fractionation of Crude Liver Unsaponifiable

The total unsaponifiable fraction, isolated from 26 kg. of liver, amounted to 0.92 per cent of the wet liver weight. Of this, 25.3 gm. remained after removal of cholesterol and ketonic materials. This total was absorbed onto 600 gm. of silicic acid (23 X 5.5 cm. column) from hexane and eluted in six fractions by successive washing with pentane; 5, 10, and 25 per cent ether in pentane; 100 per cent ether; and 100 per cent acetone. The results of the partition afforded by the initial separation are detailed in Table 1. Aliquots of the six fractions from which the total weights were determined were oils and were all relatively pigmented.

Finer separation of the constituents of fractions II through VI was achieved on 20 X 4 cm. columns of silicic acid by slow development of the chromatogram with the use of smaller changes in solvent polarity and greater development volumes than were employed in the initial separation. Aliquots of the eluates were taken serially, tested for their reaction to trichloroacetic acid, antimony trichloride, and acetic anhydride-sulfuric acid, and those adjacent fractions giving comparable color reactions were pooled. Aliquots were again taken for weight and serological testing. A summary of the refractionation is given in Table 2, together with the serological findings on the various fractions.

Examination of Individual Fractions

In the belief that serological activity of these fractions might deteriorate with time, we turned our attention first to fractions D, E, and L, which provided the largest amount of active material and which, on an admittedly subjective basis, were thought to possess the most satisfactory serological properties. Subsequently, the composition of several of the other fractions was also examined.

Fractions I, A-1, A-2, B-1, and B-2.—These fractions, while serologically unsatisfactory, were given cursory examination because of their appreciable size. All were essentially free of urea-complexing substances, and aliquots of fractions I and B-1 gave, respectively, 0.67 per cent and 113 per cent of their weight of HCl adducts. Fraction B-1 corresponds apparently to the squalene-like substance, hepene, of Dimter (7) and

¹ We are indebted to Dr. Gunther Steinberg for calling the method to our attention and for assistance with the first runs.

² II-F Conductometer, Beckman Bulletin 254.
fraction B of Stanger et al. (32). It was not further studied.

Fraction I and A-1, rechromatographed on alumina, gave a large (50 per cent), colorless liquid hydrocarbon fraction, I-a, eluted promptly with pentane, a contiguous slightly slower-moving pale yellow semi-solid fraction (43 per cent), and finally three small fractions containing red and yellow pigments which accounted for the remainder. Of these, fraction I-a was submitted to mass spectroscopic analysis3 and compared with resemblance between the unknown and Nujol, there seems little doubt that the unknown is mineral oil."

Fractions C, D, E, and F.—Fractions C, D, and E in pentane-ether solution all deposited crystalline material on standing in the cold. The crystalline portion of fraction E after recrystallization from pentane-ether was serologically active and melted at 51° C. Its infrared spectrum in CCl₄ was similar to that of hexadecanol, but elemental analysis indicated that it most probably consisted of a mixture of aliphatic alcohols. Since the non-crystalline portions of fractions C, D, E, and F showed stronger absorption at 7.25 μ than expected of unbranched aliphatic compounds, these fractions were partitioned with urea. From a total of 1.10 gm. of material, 428 mg. of a white, waxy complexing solid and 670 mg. of noncomplexing yellow oil were obtained. Both fractions were serologically active. The waxy solid was resolved into its constituents by reverse phase partition between aqueous acetone and Celite-supported mineral oil. A partition chromatogram of an aliquot of the urea-complexing material is shown in Chart 1. The peak eluted with 68 per cent acetone, fractions 40–58 inclusive (47.5 mg.), was recrystallized directly from hexane and gave 28 mg. of white plates, m.p. 48.5°–49.5° C.; 1-hexadecanol, 49.3° C. The infrared spectrum in KBr and the
serological activity were identical with those of hexadecanol.

Found: C, 79.25; H, 14.09.

Fractions 71–89 inclusive (61.2 mg.), on recrystallization from hexane, gave 28 mg. of ma-

**TABLE 3**

<table>
<thead>
<tr>
<th>Hydrocarbon type</th>
<th>Fraction I-a (mole per cent*)</th>
<th>Nujol (mole per cent*)</th>
<th>Heavy mineral oil (mole per cent*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paraffins</td>
<td>14</td>
<td>13</td>
<td>8</td>
</tr>
<tr>
<td>Noncondensed naphthenes</td>
<td>34</td>
<td>39</td>
<td>28</td>
</tr>
<tr>
<td>Condensed naphthenes (2-ring)</td>
<td>24</td>
<td>17</td>
<td>23</td>
</tr>
<tr>
<td>Condensed naphthenes (3-ring)</td>
<td>15</td>
<td>12</td>
<td>19</td>
</tr>
<tr>
<td>Condensed naphthenes (4-ring)</td>
<td>11</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Condensed naphthenes (5-ring)</td>
<td>3</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Condensed naphthenes (6-ring)</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

* Values are rounded off to the nearest whole number.

From the amounts of these substances which were isolated, it is estimated that these livers contained a minimum of 11 and 14 mg/kg, respectively, for hexadecanol and octadecanol. The residue appearing in the 72.5 per cent acetone fraction amounted to 2.3 mg/kg of liver. The actual amounts present were probably considerably greater than those found because of losses during isolation.

The combined portion of fractions C, D, E, and F which did not complex with urea had an iodine number of 159. Prior to finding the facility with which these fractions could be investigated with partition methods, several abortive attempts were made to resolve the noncomplexed fraction via bromo derivatives and by chromatography of various esters. The small amount remaining was finally submitted to partition chromatography. In contrast to the saturated fraction, this material gave many small active fractions eluted with acetone concentrations of 65, 67.5, 70, 72.5, and 75 per
cent. Adequate amounts of pure components for serological testing and identification were not obtained. Infrared absorption spectra of these fractions indicated the presence of unsaturated normal and branched aliphatic or acyclic alcohols. It might be expected that at least traces of phytol, vitamin A alcohol, and oleoyl, linoleoyl, and linolenoyl alcohols would appear in the noncomplexed fraction, though the first three named are inactive (27).

Fraction G.—Fraction G consisted essentially of cholesteryl, probably contaminated with traces of activity from fraction F.

Fractions H and J.—Recovery of solute from partition chromatograms of aliquots of fractions H and J was very poor (30 per cent). Recovered solute was partitioned fairly uniformly among the serial fractions containing 65—77 per cent acetone, and activity was centered in the 70 per cent acetone eluates. Fractions H and J had remained standing for some months prior to partitioning, and their subfractions were not examined further.

Fraction K.—An aliquot of 216 mg. of fraction K was partitioned on Celite-supported mineral oil as described above; 17 mg. eluted in the first seven retention volumes with 60 per cent acetone. The subsequent two retention volumes of 65 per cent acetone eluate contained 10.7 mg. of a white crystalline solid, melting at 85°-86° C. The remainder of the material on the column was eluted irregularly with continued development.

The crystalline solid in the 65 per cent acetone eluate from the remainder of fraction K gave a total of 26.4 mg. after repeated recrystallization from methanol, m.p. 86.5° C. From the ultraviolet absorption spectrum of this material, the following E1%_1 cm. values were calculated: 2420 A: 4.7; 2470: 5.2; 2530: 6.1; 2580: 6.7; 2610: 6.3; 2640: 5.3; and 2690: 4.8. The infrared spectrum in KBr showed strong bands at 3520, 1640, 1550 cm.⁻¹ and a weak band at 3093 cm⁻¹ characteristic of mono-substituted amides. Strong bands at 1502, 751, and 699 cm.⁻¹ characteristic of uninterrupted methylene chains were present. The aromatic band near 1600 cm⁻¹ was largely obscured by the amide band in that region, and C-H stretching frequencies associated with aromatic rings were present at 3040 cm⁻¹.


Hydrogenation of 15.7 mg. over platinum in absolute ethanol gave 12 mg. of white powder from methanol softening at 68° C. and melting at 69°-70° C. The characteristic ultraviolet absorption around 2580 A was eliminated; the infrared showed loss of all four bands assigned to the aromatic structure and revealed the polymethylene doublet in clearer detail.

The hydrogenation product was only partially hydrolyzed after being heated 5 hours in 0.5 cc. of 85 per cent H₃PO₄ at 150° C. The acidic hydrolysis product, isolated conventionally and sublimed at 180°/1 mm., was identified (18) with good probability from the infrared spectrum in KBr as palmitic acid.

The amine was recovered as a gummy HCl salt; however, no structural assignment could be made from its surprisingly sharp infrared spectrum in KBr. Absorption bands characteristic of amine hydrochlorides were present and a strong band at 1657 cm⁻¹ which we would assign to the cyclohexyl group.

The small amount of material available precluded further study of this interesting serologically inactive compound.

Fraction L.—One-third of fraction L (300 mg.) was partitioned between mineral oil on Celite and aqueous acetone as described above. Active fractions interspersed with inactive fractions eluted with 60, 65, 72.5, and 75 per cent acetone. The combined 60 per cent acetone eluates from this column and similar fractions obtained from the remainder of fraction L were pooled to give 450 mg. of solute, the bulk of the activity present in fraction L. This was repartitioned starting with eluting solvent containing less acetone to give the chromatogram shown in Chart 2.

Fractions 27-42, inclusive, of the second peak were recrystallized twice from acetone to give 29.2 mg. of white plates, m.p. 60.5°-62.0° C.; d-a-hexadecylglyceryl ether, 62.5°-63.5° C. A mixed m.p. with the synthetic compound was 62.5°-63.5°.


The fractions 53-64, inclusive, of the third peak, Chart 2, were recrystallized twice from acetone to give 15.9 mg. of white plates melting at 71.3° C.; d-octadecyglyceryl ether, 71.2° C. A mixed melting point with an authentic sample was 70.8°-71.2° C.

Anal. Caled. for C₂₁H₄₄O₃: C, 73.20; H, 12.87. Found: C, 71.84; H, 12.56.

The infrared spectra of both alcohols from fraction L compared fairly well with authentic samples of the corresponding glyceryl ethers. A sample of the hydrogenated unsaponifiable fraction of ratfish liver oil, partitioned under the same conditions

We are indebted to Dr. E. Baer, University of Toronto, for a gift of d-batyl and d-chimyl alcohol which he had prepared (3).
given for the chromatogram in Chart 2, gave crystalline fractions of chimyl and batyl alcohols, m.p. 62°–63° C. and 71°–71.5° C., emerging, respectively, in the same positions as the compounds from the human liver. The serological behavior of the synthetic compounds was indistinguishable from that found for compounds isolated from human and ratfish liver.

Fractions M through Q.—Inadequate amounts of fraction M and N remained after test to permit further study. Fractions O, P, and Q were deemed of insufficient interest to warrant further examination. Various fatty alcohols have been reported previously as constituents of mammalian sources. Eicosanol has been found to be a constituent of dermoid cysts (1, 35), while hexadecanol and octadecanol have been found in feces of various species including man (9, 10, 28). A series of aliphatic alcohols including C14, C16, C18, C20, C22, and C24 saturated normal alcohols, docos-12-en-1-ol, tetracos-14-en-1-ol, and the iso-C20, C22, and C24 alcohols were isolated from sebum of the Bantu.

DISCUSSION

Incidental to the primary purpose of this investigation was the examination of the hydrocarbon fraction and the identification of a spectrum of hydrocarbons identical with those in medicinal mineral oil. Unfortunately, records were not kept which would permit identification of each source of liver and, thus, an examination of the patient’s record of medication. Consequently, no quantitative idea can be gained with respect to the absorption and retention of such material. It is of interest that there was no indication of selective absorption of hydrocarbon types. Such a finding is consistent with the metabolic inertness of such compounds and their absorption in macro-molecular dispersion.

Various fatty alcohols have been reported in the past as constituents of mammalian sources. Eicosanol has been found to be a constituent of dermoid cysts (1, 35), while hexadecanol and octadecanol have been found in feces of various species including man (9, 10, 28). A series of aliphatic alcohols including C14, C16, C18, C20, C22, and C24 saturated normal alcohols, docos-12-en-1-ol, tetracos-14-en-1-ol, and the iso-C20, C22, and C24 alcohols were isolated from sebum of the Bantu.

All these fractions were more or less highly pigmented oils or tacky semisolids.
acid not displayed by the synthetic glyceryl ethers.

Batyl and/or chimyl alcohol has been isolated on several occasions from mammalian tissues (14, 24-26), though they are more frequently encountered in the unsaponifiable fraction of certain fish oils (6). Apparently neither alcohol has been reported as a constituent of human liver. It is interesting that the relative proportions of chimyl and batyl alcohol reported here are the inverse of the amounts of the derivative alcohols which were isolated. Chimyl alcohol appears least frequently and in the smallest amounts in fish liver unsaponifiable matter (6).

A disappointingly large percentage of the neutral material in the fractions studied remains unidentified. Particular fractions grouped by their polar properties on silicic acid were by and large still relatively complex mixtures, as revealed by the partition system employed. An examination of a considerable number of infrared patterns obtained on serially spaced fractions from partition columns revealed only minor differences in absorption frequencies or relative transmittancy; the most consistent pattern throughout was the variable large contribution at 1380 cm.⁻¹, compared with that at 1460 cm.⁻¹. The presence of small quantities of a number of branched alcohols corresponding to the branched acids, now recognized as minor constituents of mammalian tissues (29), seems consistent with this finding, though the presence of traces of steroids is not excluded. Some branching of aliphatic alcohols is not inconsistent with possession of serological reactivity (27).

**SUMMARY**

The unsaponifiable fraction of liver from individuals dying of cancer has been fractionated to identify the components responsible for the serological reaction of this fraction with serum from cancerous individuals. Three major active constituents, hexadecanol and batyl and chimyl alcohols, were identified. Among the serologically inactive constituents, octadecanol and a hydrocarbon fraction essentially identical with medicinal mineral oil were also identified. A crystalline amide of palmitic acid was isolated and partially characterized, and evidence was obtained for the presence of small quantities of an extensive number of methyl substituted alcohols.

**ACKNOWLEDGMENTS**

The authors wish to thank Dr. George Hall, who obtained for us serum samples of normal and cancerous individuals which were required for following the progress of fractionation. We are indebted to Mrs. Barbara Crocken and Miss Mary Rotheram for technical assistance, and to Dr. Andrew Dowdy for his expressed interest and encouragement during the progress of the work.

**REFERENCES**


20. PEACOCK, A. C.; and Williams, G. Z. A Study of the


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