THE CHEMICAL INVESTIGATIONS OF CORPUS LUTEUM.

IV. THE ACETONE-SOLUBLE FAT.

BY GEORGE F. CARTLAND* AND MERRILL C. HART.

(From the Research Laboratories, The Upjohn Company, Kalamazoo.)

(Received for publication, October 15, 1925.)

Most modern investigators agree that the acetone extract of the ovary contains the hormone which governs the phenomena of estrus. Evidence seems to be quite conclusive also that this growth-stimulating and regulating factor is contained in the unsaponifiable fraction. This dramatic search for the so called active principle has more or less overshadowed other important questions as to the physiological action of the ovary. For instance, what is the chemical nature of the neutral fat of corpus luteum? Are the fatty acids of this fat similar in nature to the fatty acids incorporated in the molecules of the different phosphatides? Are these phosphatides specific in their chemical structure or are they closely related to the phosphatides elaborated in other glandular structures, indicating a common function?

Our experimental investigation of the fatty acids in the neutral fat of corpus luteum shows that there are present the same fatty acids that have been shown by Levene and others to be present in lecithin and cephalin isolated from other tissues. This would indicate that the neutral fat functions more or less as a menstruum for the production of these non-specific lipoids. We would expect therefore that the hydrolysis of the lecithin and cephalin from corpus luteum would give fatty acids similar to those obtained from the phosphatides isolated from other tissues. This would remove the possibility that there was anything specific in the physiology of these phosphatides and focus more clearly attention as to just

* Holder of the Upjohn Cooperative Fellowship at Kalamazoo College (1924-25). This paper is based upon the thesis presented by Mr. Cartland to the Faculty of Kalamazoo College, in partial fulfillment of the requirements for the degree of Master of Science.
what is the exact function of these phosphatides in the metabolic processes.

In the neutral fat of corpus luteum we have shown the presence of approximately 8 per cent of arachidonic acid. Wesson\textsuperscript{1} has suggested the possibility that arachidonic acid is an intermediate product in the metabolism of at least part of the fatty acids which contain fewer than 20 carbon atoms. Of great importance in this question of the function of arachidonic acid in metabolism, is our finding of relatively large quantities (4.8 per cent) of a hexa-unsaturated acid of the C\textsubscript{20} series. This also brings up the question as to the relation of the phosphatides, such as lecithin and cephalin, to the utilization by the body of this arachidonic acid. It is quite possible that the production of arachidonic acid regulates more or less the chemical physical properties of these phosphatides and thus plays a very important part in the metabolic processes. This question will be attacked later by carrying out this same sort of chemical investigation on corpora lutea collected at different known stages of pregnancy and attempting to correlate this chemical information with the known physiological conditions represented. Before this was done, however, we considered it of importance to carry out this preliminary study on the commercially important, easily procurable, pharmaceutical corpus luteum.

**EXPERIMENTAL.**

The sample of corpus luteum used in the following experiments represented carefully collected, hand-dissected material, dried immediately after collection in a high vacuum and below 40\degree C. in the commercial way. The dried corpus luteum represents approximately 6 parts of the fresh corpus luteum substance. It was representative of the material found on the pharmaceutical market in that it had been taken from such a large number of cattle as to represent a good average sample. It contained 4.5 per cent moisture and 5.4 per cent ash.

The material was ground to pass through a No. 20 sieve. 5.5 kilos of this dried ground tissue were exhaustively extracted with five 5 liter portions of acetone.

The combined acetone extracts were concentrated to about 1

\textsuperscript{1} Wesson, L. G., *J. Biol. Chem.*, 1925, lxv, 235.
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liter. This solution upon standing deposited some crystalline material which was shown to consist of cholesteryl palmitate, potassium stearate, fatty acids, phosphatides, and a mixture of the amino acids leucine, isoleucine, and valine.

The acetone filtrate and washings from the above described crystalline material were evaporated to dryness and the residue dissolved in 1 liter of pure acetone. On standing in the cold for some time material separated which when dried in vacuo weighed 9.025 gm. Examination of this material indicated that it consisted of cholesteryl palmitate, a potassium soap, possibly sphingomyelin, and an alcohol-soluble phosphatide similar to lecithin.

The acetone filtrate and washings from the above described material were concentrated and the oily residue dissolved in 2 liters of absolute alcohol. Upon standing in the cold this solution gradually deposited some white material. This was filtered off, washed, and dried in vacuo. This material weighed 5.66 gm. and investigation showed it to consist of cholesteryl palmitate mixed with the fat, tristearin.

The filtrate from this fatty material gave nothing more of a crystalline nature when held in the cold at various concentrations. The entire alcohol solution measuring 2 liters was then precipitated completely with alcoholic cadmium chloride solution. A white flocculent precipitate of the lecithin cadmium chloride compound was obtained. The alcoholic mother liquor from this precipitation was taken to dryness and the oily residue dissolved in ether, filtered from the insoluble cadmium chloride, and the ether solution concentrated to a volume of 2 liters.

The ether solution of the fat was extracted with water and 1 per cent sulfuric acid to remove water-soluble nitrogen bases and inorganic salts. Water extracted 108 gm. of solid material containing 2.27 per cent N, 0.32 per cent P, and 29 per cent cadmium (48 per cent cadmium chloride). The 1 per cent sulfuric acid extract was small and contained a total of 0.11 gm. of nitrogen and 0.11 gm. of phosphorus.

The purified fat weighed 720 gm. and contained 0.36 per cent N and 0.24 per cent P. For preliminary analysis, 35 gm. of the fat

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\(^3\) Hart, M. C., and Heyl, F. W., *J. Biol. Chem.*, 1925, lxvi, 639.
were dried to constant weight and the following determinations made. Where possible official methods as given in Bulletin 107, United States Department of Agriculture, were used, otherwise Lewkowitsch\(^6\) was followed:

\[
\begin{align*}
\text{Specific gravity} & \quad \frac{100^\circ}{100^\circ} \quad 0.9031 \\
\text{Iodine No. (Hanus)} & \quad 66 \\
\text{Acid value} & \quad 78 \\
\text{Saponification value} & \quad 154 \\
\text{Unsaponifiable} & \quad 15.6 \text{ per cent} \\
\text{Reichert Wollny value} & \quad 0.76 \\
\text{Cholesterol} & \quad 10.2 \text{ per cent} \\
\text{Acetyl value} & \quad 36
\end{align*}
\]

700 gm. of the fat in ether solution were extracted with 10 per cent potassium hydroxide to remove the free fatty acids. The ether solution of neutral fat was then taken to dryness and saponified by boiling for 8 hours with 20 per cent alcoholic potash. Most of the alcohol was removed by distillation,\(^7\) and the residual soap dissolved in water. The alkaline soap solution was then extracted repeatedly with ether to remove the unsaponifiable material. The fatty acids from the saponification of the neutral fat were obtained by decomposing the soap solutions with 20 per cent hydrochloric acid and extracting the liberated fatty acids with ether.

By this procedure, the 700 gm. of fat were separated into the following three fractions:

1. 254 gm. free fatty acids.
2. 270 " fatty acids from neutral fat.
3. 105 " unsaponifiable material.

The unsaponifiable material was crystallized from alcohol to remove cholesterol. 58 gm. of cholesterol were obtained melting at 143–144°C. The alcoholic mother liquor from the cholesterol

\(^6\) Lewkowitsch, J., Chemical technology and analysis of oils, fats and waxes, London, 6th edition, 1921, i.

\(^6\) Method of MacArthur (MacArthur, C. G., \textit{J. Am. Chem. Soc.}, 1919, xii, 1232) gave 66.3 per cent cholesterol in the unsaponifiable fraction, or 10.2 per cent in the total fat.

\(^7\) All distillations were conducted at reduced pressure in an atmosphere of nitrogen.
was ampouled in an atmosphere of nitrogen and reserved for future physiological and chemical examination.

The Free Fatty Acids.

The 254 gm. of free fatty acids in ether solution were extracted with varying strengths of alkaline solutions. The fatty acids were obtained from the alkaline extracts by acidifying with hydrochloric acid and extracting the liberated fatty acids with ether. The following fractions were obtained:

- 10 per cent ammonium carbonate extract, 16 gm. acids.
- 10 " " sodium " " 236 " "
- 10 " " potassium hydroxide " 2 " "

The Ammonium Carbonate Extract of the Free Fatty Acids.

This fraction consists of 16 gm. of a dark brown oil having a strong odor of volatile acids. Nothing crystalline was obtained upon allowing the alcoholic solution to stand at various concentrations in the ice box.

The oil was mixed with 10 per cent sulfuric acid and steam distilled. The total volatile acids in the 700 cc. distillate were equivalent to 3.3 cc. of normal acid. The solution was neutralized and concentrated to 100 cc. The soap solution was acidified, the volatile acids extracted with ether, and dried over anhydrous sodium sulfate. The ether was removed in vacuo, the residual acid dissolved in 5 per cent ammonia solution, and the excess ammonia boiled off. The solution was precipitated with silver nitrate, the precipitate filtered, washed, and dried. The silver salt was analyzed for silver.

Analysis. 0.0669 gm. substance: Ag 0.0267.

Calculated for $C_4H_9O_2Ag$. Ag 42.8.

" " $C_{10}H_{16}O_2Ag$. " 38.5.

Found. Ag 39.9 per cent.

These results suggest that the volatile acids consist of a mixture of caprylic and capric acids.

The non-volatile acids from the steam distillation were extracted with ether, washed free from sulfuric acid, and dried over anhydrous sodium sulfate. The ether was removed and the residual acids distilled in vacuo. The distillate, consisting of 6.8 gm. of a
Corpus Luteum. IV

partially solid oil, distilled at 170-200°C. at a pressure of 3 to 4 mm. of mercury. The tarry residue in the distilling flask was not further examined. The distilled acids crystallized from 40 cc. of 95 per cent alcohol yielded 0.65 gm. of palmitic acid melting at 58-61°C.

*Analysis.* 0.1561 gm. substance: CO₂ 0.4292, H₂O 0.1782.
Calculated for C₁₆H₃₂O₂. C 75.0, H 12.5.
Found. C 75.0, H 12.8.

The mother liquor from these crystals was separated into the saturated and unsaturated acids by the lead salt-ether process. 1.8 gm. of saturated acids and 3.5 gm. of unsaturated acids were obtained.

The saturated acids were crystallized from alcohol and two fractions obtained.

Fraction 1. (0.39 gm.) m.p. 55-56°C.
*Analysis.* 0.1490 gm. substance: CO₂ 0.4099, H₂O 0.1693.
Calculated for C₁₆H₃₂O₂. C 75.0, H 12.5.
Found. C 75.0, H 12.7.

Fraction 2. (0.92 gm.) m.p. 52-54°C.
*Analysis.* 0.1601 gm. substance: CO₂ 0.4388, H₂O 0.1804.
Calculated for C₁₆H₃₂O₂. C 75.0, H 12.5.
Found. C 74.8, H 12.6.

Both of these fractions analyze for palmitic acid indicating that the saturated acids of the ammonium carbonate extract consist entirely of palmitic acid. Myristic acid is not present in this fraction.

The unsaturated acids (3.5 gm.) from the lead salt separation consisted of a brown oil having an iodine number of 151 and a neutralization value of 174. As the neutralization value is abnormally low probably due to the condensation of the unsaturated acids, this fraction was reduced with hydrogen and colloidal palladium by the method of Paal. The saturated acids so obtained were crystallized from alcohol. 2.0 gm. of unsaturated acids yielded 1.27 gm. of crystalline acids melting at 59-60°C.

Analysis. 0.1495 gm. substance: CO₂ 0.4179, H₂O 0.1751.
Calculated for C₁₈H₃₆O₂. C 76.0, H 12.7.
" " C₂₀H₄₀O₂. " 76.9, " 12.8.

These analytical results indicate a mixture of stearic and arachidic acids. The unsaturated acids, therefore, consist of a mixture of C₁₈ and C₂₀ acids. The iodine number of the unsaturated acids (151) and the analysis of the reduced acid indicate that these acids consist of a mixture of 70 per cent oleic (C₁₈H₃₄O₂) and 30 per cent arachidonic (C₂₀H₃₂O₂) acids. Arachidonic acid was later isolated from the unsaturated acids of the sodium carbonate extracts.

The Sodium Carbonate Extract of the Free Fatty Acids.

The 236 gm. of acids extracted by 10 per cent sodium carbonate were dissolved in 95 per cent alcohol and cooled in the ice box. The crystalline acids which separated were recrystallized from 95 per cent alcohol. 40 gm. of crystals melting at 53–55°C. were obtained. As these acids were similar to the fatty acids from the neutral fat, they were joined with these and studied together.

The alcoholic mother liquor from the above crystallization yielded 182 gm. of fatty acids. These acids were distilled in vacuo. The distillate consisted of 155 gm. of a clear yellow oil which partially solidified upon cooling. 22 gm. of these acids were separated into the saturated and unsaturated acids by the lead salt-ether process. This separation yielded 12.1 gm. of liquid acids having an iodine number of 165, and 8 gm. of solid acids having an iodine number of 80. This method of separation was unsatisfactory because of the large amount of unsaturated acids contaminating the saturated fraction. The remainder of the acids (133 gm.) were separated by the barium-lead salt process.⁹

This separation is based upon the fact that the barium salts of oleic acid and also of the saturated acids are insoluble in a mixture of benzene and ether. The barium salts of the more unsaturated acids are soluble in this mixture. Oleic acid is separated from the saturated acids by the solubility of its lead salt in ether, the lead salts of the saturated acids being insoluble in ether.

By this means the acids were separated into the following three fractions.

1. 90 gm. unsaturated acids, iodine No. 165.
2. 11 " intermediate fraction, iodine No. 123.
3. 30 " saturated acids, iodine No. 21.

These fractions were later joined for analysis to like fractions of acids from the neutral fat.

The Potassium Hydroxide Extract.

These acids (2 gm.) were crystallized from alcohol. A small amount of crystalline acids was obtained which melted at 55°C. This appeared to be a similar mixture to that obtained from the sodium carbonate extract of free acids, and was not further examined.

The Fatty Acids of the Neutral Fat.

These acids weighing 270 gm. were dissolved in ether and extracted with varying strengths of alkaline solutions. The fatty acids were recovered from the alkaline extracts in the usual manner. In this case, 10 per cent ammonium carbonate and 10 per cent potassium hydroxide extracted only small amounts of acids from which nothing crystalline could be obtained. Most of these acids (267 gm.) were extracted by 10 per cent sodium carbonate.

The acids of the sodium carbonate extract were crystallized from 95 per cent alcohol. The crystalline acids which separated, after recrystallization from alcohol, weighed 34 gm. and melted fairly sharply at 54°C. A mixed melting point with pure myristic acid indicated that this was not myristic acid. These solid acids were later examined together with the other saturated acids of the fat.

The alcoholic mother liquor from the above crystallization yielded 233 gm. of acids. These were distilled in vacuo. The distillate consisted of 213 gm. of a yellow oil which partially solidified at room temperature, and had an iodine number of 127. These acids were separated into three fractions by the barium-lead salt process. The following fractions were obtained.
1. 146 gm. unsaturated acids, iodine No. 169.
2. 20 " intermediate fraction, iodine No. 120.
3. 45 " saturated acids, iodine No. 21.

These fractions had similar properties to like fractions obtained from the free fatty acids. Before further analytical work, like fractions of the free acids and those from the neutral fat were joined. The saturated acids obtained by the barium-lead salt separation were recrystallized from alcohol and joined to the crystalline acids which separated from the mixed acids before distillation.

**TABLE I.**

**Distillation of Unsaturated Acids.**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Weight (gm)</th>
<th>Boiling point (°C)</th>
<th>Pressure (mm Hg)</th>
<th>Iodine No.</th>
<th>Neutralization value</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.1</td>
<td>To 180</td>
<td>1.2-1.4</td>
<td>106</td>
<td>191.5</td>
<td>293</td>
</tr>
<tr>
<td>2</td>
<td>20.2</td>
<td>180-185</td>
<td>1.5</td>
<td>117</td>
<td>193.0</td>
<td>291</td>
</tr>
<tr>
<td>3</td>
<td>32.2</td>
<td>185-190</td>
<td>1.4-1.5</td>
<td>140</td>
<td>193.7</td>
<td>290</td>
</tr>
<tr>
<td>4</td>
<td>36.4</td>
<td>190-195</td>
<td>1.5</td>
<td>159</td>
<td>188.6</td>
<td>297</td>
</tr>
<tr>
<td>5</td>
<td>34.9</td>
<td>195-200</td>
<td>1.5</td>
<td>189</td>
<td>185.4</td>
<td>302</td>
</tr>
<tr>
<td>6</td>
<td>22.3</td>
<td>200-205</td>
<td>1.4-1.5</td>
<td>224</td>
<td>178.0</td>
<td>315</td>
</tr>
<tr>
<td>7</td>
<td>11.4</td>
<td>205-207</td>
<td>1.5</td>
<td>251</td>
<td>171.4</td>
<td>327</td>
</tr>
<tr>
<td>8</td>
<td>7.0</td>
<td>207-210</td>
<td>1.5</td>
<td>274</td>
<td>166.9</td>
<td>336</td>
</tr>
<tr>
<td>Residue</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>199</td>
<td></td>
</tr>
</tbody>
</table>

**Examination of the Highly Unsaturated Acids.**

These acids consisted of 236 gm. having an iodine number of 169. The iodine number indicated that highly unsaturated acids are present.

209 gm. of these acids were fractionally distilled at a pressure of 1.5 mm. of mercury. Five fractions and a residue were obtained. These were refractionated into eight fractions. Neutralization values and iodine numbers of the fractions were determined and their mean molecular weights calculated. These results are summarized in Table I.

From fractions 1 and 2, there separated a small amount of solid fatty acids which had the characteristics of the saturated acids of the fat and was not further examined.

Each of the eight fractions from the above distillation was
dissolved in dry ether, cooled to 0°C., and saturated with bromine. After standing overnight in the ice box, the ether-insoluble bromides were separated by centrifuging, washed free from bromine with cold ether, dried, and weighed. Excess of bromine was removed from the ether-soluble bromides by emulsification with sodium thiosulfate solution.

The ether-soluble bromides were then dried, dissolved in dry ethyl alcohol, and debrominated by boiling for 3 hours during which a stream of dry hydrogen chloride was passed through the solution, and zinc dust added in small quantities. The alcoholic solution was diluted with a large volume of water and the acid extracted with ether. The acid so obtained (which also contains

### Table II.

**Bromination of Unsaturated Acids.**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Acid taken (gm.)</th>
<th>Ether-insoluble bromides (gm.)</th>
<th>Br in bromides (Carius) (per cent)</th>
<th>Tetra-bromide (gm.)</th>
<th>Dibromide (gm.)</th>
<th>Acid from dibromide (gm.)</th>
<th>Iodine No. (Acid from dibromide)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.0</td>
<td>0.78</td>
<td>63.5</td>
<td>0.39</td>
<td>10.7</td>
<td>6.15</td>
<td>96</td>
</tr>
<tr>
<td>2</td>
<td>10.3</td>
<td>1.15</td>
<td>61.9</td>
<td>0.69</td>
<td>14.3</td>
<td>8.07</td>
<td>103</td>
</tr>
<tr>
<td>3</td>
<td>11.4</td>
<td>2.50</td>
<td>65.4</td>
<td>0.06</td>
<td>18.0</td>
<td>9.53</td>
<td>116</td>
</tr>
<tr>
<td>4</td>
<td>13.7</td>
<td>4.06</td>
<td>64.7</td>
<td>0.06</td>
<td>22.0</td>
<td>11.3</td>
<td>128</td>
</tr>
<tr>
<td>5</td>
<td>13.9</td>
<td>8.0</td>
<td>64.5</td>
<td>0.21</td>
<td>17.1</td>
<td>8.30</td>
<td>135</td>
</tr>
<tr>
<td>6</td>
<td>14.0</td>
<td>13.0</td>
<td>64.1</td>
<td>0.14</td>
<td>18.3</td>
<td>8.50</td>
<td>151</td>
</tr>
<tr>
<td>7</td>
<td>9.9</td>
<td>11.3</td>
<td>65.0</td>
<td>0.20</td>
<td>12.4</td>
<td>5.32</td>
<td>161</td>
</tr>
<tr>
<td>8</td>
<td>5.7</td>
<td>6.55</td>
<td>66.4</td>
<td>0.35</td>
<td>7.8</td>
<td>2.78</td>
<td>173</td>
</tr>
</tbody>
</table>

ethyl ester) was again brominated. In this way a further quantity of ether-insoluble bromides was obtained. These bromides were weighed but were not joined to the first crop because they contained the bromides of the ethyl esters which were formed during the debromination.

The ethereal filtrate from the second crop of insoluble bromides was emulsified with sodium thiosulfate to remove excess bromine, dried over anhydrous sodium sulfate, and the ether removed. The bromides were then taken up in petroleum ether and the insoluble tetrabromide fraction separated by centrifuging. The bromides soluble in petroleum ether were dried and weighed. This dibromide fraction was then debrominated with alcoholic
hydrogen chloride and zinc. The ethyl ester so obtained was saponified, the soap decomposed with dilute sulfuric acid, and the fatty acid extracted with ether, the ether solution dried, and the ether removed. The iodine number was determined for each fraction. The results of the bromination are given in Table II.

**The Ether-Insoluble Bromides.**

This substance consisted of material which in fractions 1 to 6 was a pure white, easily powdered substance. The bromides from fractions 7 and 8 became gray when dried *in vacuo* at 60°C. These bromides blackened and decomposed at 225–230°C. The bromine content (Table II) indicates that these fractions consist of a mixture of arachidonic octobromide \((C_{20}H_{32}O_2Br_8)\) and a hexabromide.

18.7 gm. of the ether-insoluble bromides from fractions 4, 5, and 6 were extracted in a Soxhlet with benzene. The benzene-insoluble bromide (6.1 gm.) was washed with large quantities of cold dry ether. The resulting product (5.7 gm.) blackened and decomposed at 237–238°C. This was analyzed for bromine.

*Analysis.* 0.1465 gm. substance: AgBr 0.2321.
Calculated for \(C_{20}H_{32}O_2Br_8\). Br 67.8.
Found. Br 67.4.

These results indicate that the product is nearly pure arachidonic octobromide.

5.5 gm. of the purified octobromide were debrominated with alcoholic hydrogen chloride and zinc dust by the method of Wesson. The resulting ethyl ester was saponified and the acid obtained in the usual manner. 1.1 gm. of unsaturated acid were obtained.

0.0920 gm. acid absorbed 0.2846 gm. iodine. Iodine No. 309.
Calculated for \(C_{20}H_{32}O_2\). Iodine No. 335.

0.78 gm. of this acid was reduced in alcoholic solution with hydrogen and colloidal palladium. 0.68 gm. of a crystalline acid was obtained melting at 65–67°C. After two crystallizations from 95 per cent alcohol, 0.43 gm. of a pure white crystalline acid

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was obtained which melted at 68–69°C. Further crystallization failed to raise the melting point. This acid mixed with stearic (m.p. 69) melted at 61–62°C., indicating that it is not stearic acid.

Analysis. 0.1443 gm. substance: CO₂ 0.4067, H₂O 0.1670.
Calculated for C₂₀H₄₂O₂.  C 76.9, H 12.9.
Found.  C 76.9, H 12.9.

The reduced acid is pure arachidic acid, thus proving that the unsaturated acid in the fat is arachidonic acid, C₂₀H₃₂O₂.

The benzene extract upon cooling deposited 7.5 gm. of a bromide which blackened at 220–225°C. This material was crystallized from benzene into two fractions, both of which blackened at 218–222°C.

Fraction 1. 5.38 gm.
Analysis. 0.1479 gm. substance: AgBr 0.2242.
Found.  Br 64.5.
Fraction 2. 0.63 gm.
Analysis. 0.1435 gm. substance: AgBr 0.2168.
Found.  Br 64.3.
Calculated for C₂₀H₃₂O₂Br₆.  Br 67.8.
“  “  C₁₈H₂₉O₂Br₆.  “ 63.3.

These analytical results indicated a hexabromide, but the blackening of these bromides at 220°C. instead of melting at 181°C. indicated that it is not linolenic hexabromide (C₁₈H₂₉O₂Br₆).

Fractions 1 and 2 were joined, suspended in absolute ethyl alcohol, and debrominated with zinc dust and dry hydrogen chloride. The ethyl ester so obtained was saponified, the soap decomposed with dilute sulfuric acid, and the liberated acid extracted with ether. 5.7 gm. of bromides yielded 1.95 gm. of acid having an iodine number of 270. (0.1058 gm. absorbed 0.2857 gm. iodine.)

1.8 gm. of this acid were reduced with hydrogen and colloidal palladium. After one crystallization, 1.5 gm. of acid were obtained melting at 67–68°C. This was recrystallized from alcohol into two fractions and analyzed.

Analysis.
Fraction 1. 1.2 gm., m. p. 68–69°C.
0.1544 gm. substance: CO₂ 0.4359, H₂O 0.1818.
Found.  C 77.0, H 13.2.
Fraction 2. 0.2 gm., m. p. 66-67°C.
0.1537 gm. substance: CO₂ 0.4308, H₂O 0.1781.
Found.  C 76.5, H 13.0.
Calculated for C₁₈H₃₆O₂.  C 76.9, H 12.9.

These analytical results indicate that fraction 1 is pure arachidic acid and fraction 2 is impure arachidic acid. Since most of the acid is represented in fraction 1, we conclude that practically all of the reduced acid is arachidic, proving that the unsaturated acid in the fat is of the C₂₀ series. The iodine number of the unsaturated acid (270) and also the bromine content (64.5 per cent) of the benzene-soluble bromides from which this acid was obtained, indicate that it is a mixture of about equal parts of arachidonic acid (C₂₀H₃₄O₂) and a C₂₀ acid with three double bonds having the formula C₂₀H₃₄O₂. If this unsaturated acid were a mixture of arachidonic acid and linolenic acid (C₁₈H₃₀O₂), the reduction product would have been 73 per cent stearic acid and 27 per cent arachidic, instead of nearly all arachidic, as we found. These analytical results prove the presence of an unsaturated acid in the fat having the formula C₂₀H₃₄O₂.

The Petroleum Ether-Insoluble Bromides.

The amounts of petroleum ether-insoluble bromides (Table II) were very small in all of the fractions. Those from fractions 1 and 2, consisting of 1.08 gm., were taken up in a small amount of cold dry ether and filtered from the insoluble higher bromides. The ether solution was taken to dryness and the residue repeatedly extracted with petroleum ether. The insoluble residue (0.7 gm.) was crystallized in two fractions from a minimum amount of dry ether. Both fractions melted at 110-112°C. Linolic tetrabromide melts at 113-114°C.

Analyses.
Fraction 1. 0.1124 gm. substance: AgBr 0.1410.
Found.  Br 53.4.
Fraction 2. 0.1709 gm. substance: AgBr 0.2180.
Found.  Br 54.3.
Calculated for C₁₃H₁₇O₂Br₄.  Br 53.3.

These analytical results indicate that the petroleum ether-insoluble bromide is linolic tetrabromide.
The Acids from the Petroleum Ether-Soluble Bromides.

These acids obtained by debrominating the liquid bromides (Table II) represent the largest part of the unsaturated acids. The iodine values, varying from 96 in fraction 1 to 173 in fraction 8, indicate that acids more unsaturated than oleic are present. Rollett has shown that when linolic acid is treated with bromine, only about 50 per cent of it is converted into a crystalline, petroleum ether-insoluble bromide, the other 50 per cent appearing as an oily petroleum ether-soluble bromide, which when debrominated yields linolic acid. Therefore, it seemed likely that linolic acid might be present in these fractions.

These acids were reduced with hydrogen and colloidal palladium and the resulting saturated acids crystallized three times from 95 per cent alcohol. These crystalline acids were analyzed. The results are given in Table III.

These analytical results show that fractions 1 to 5 consist of

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Unsaturated acid taken</th>
<th>Iodine value</th>
<th>Saturated acid after crystallization</th>
<th>Melting point</th>
<th>Substance</th>
<th>CO₂</th>
<th>H₂O</th>
<th>O</th>
<th>H</th>
<th>Neutralization value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gm.</td>
<td>gm.</td>
<td>°C.</td>
<td>gm.</td>
<td>gm.</td>
<td>gm.</td>
<td>gm.</td>
<td>per cent</td>
<td>per cent</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.0</td>
<td>96</td>
<td>1.38</td>
<td>65</td>
<td>0.1434</td>
<td>0.3978</td>
<td>0.1643</td>
<td>75.7</td>
<td>12.8</td>
<td>197</td>
</tr>
<tr>
<td>2</td>
<td>3.0</td>
<td>103</td>
<td>1.75</td>
<td>66-67</td>
<td>0.1374</td>
<td>0.3828</td>
<td>0.1589</td>
<td>76.0</td>
<td>12.9</td>
<td>197</td>
</tr>
<tr>
<td>3</td>
<td>3.0</td>
<td>116</td>
<td>1.77</td>
<td>66-67</td>
<td>0.1508</td>
<td>0.4197</td>
<td>0.1721</td>
<td>75.9</td>
<td>12.8</td>
<td>196</td>
</tr>
<tr>
<td>4</td>
<td>3.0</td>
<td>128</td>
<td>1.86</td>
<td>64-65</td>
<td>0.1540</td>
<td>0.4293</td>
<td>0.1760</td>
<td>75.8</td>
<td>12.7</td>
<td>195</td>
</tr>
<tr>
<td>5</td>
<td>3.0</td>
<td>135</td>
<td>1.72</td>
<td>63-64</td>
<td>0.1585</td>
<td>0.4414</td>
<td>0.1821</td>
<td>76.0</td>
<td>12.9</td>
<td>191</td>
</tr>
<tr>
<td>6</td>
<td>3.0</td>
<td>151</td>
<td>1.86</td>
<td>62</td>
<td>0.1528</td>
<td>0.4276</td>
<td>0.1750</td>
<td>76.3</td>
<td>12.8</td>
<td>186</td>
</tr>
<tr>
<td>7</td>
<td>3.0</td>
<td>161</td>
<td>1.17</td>
<td>65-66</td>
<td>0.1438</td>
<td>0.4043</td>
<td>0.1672</td>
<td>76.7</td>
<td>13.0</td>
<td>181</td>
</tr>
<tr>
<td>8</td>
<td>2.6</td>
<td>173</td>
<td>0.73</td>
<td>68-69</td>
<td>0.1587</td>
<td>0.4310</td>
<td>0.1772</td>
<td>76.5</td>
<td>12.9</td>
<td>169</td>
</tr>
</tbody>
</table>

Calculated for

\[ \text{C}_{18}\text{H}_{30}\text{O}_2 \] ........................ 69 | 76.0 | 12.7 | 197.5
\[ \text{C}_{20}\text{H}_{40}\text{O}_2 \] ........................ 77 | 76.9 | 12.9 | 179.8

unsaturated acids of the C18 series. Fractions 6, 7, and 8 contain increasing amounts of acids of the C20 series. The iodine values of fractions 6, 7, and 8 also indicate that some arachidonic acid is present which did not separate as an ether-insoluble bromo-derivative.

To get further information about these unsaturated acids from the dibromide fractions, the oxidation products were studied. 6 gm. of acid obtained from the reduction of the dibromide of fraction 3 (iodine No. 116) were oxidized in alkaline solution with potassium permanganate.\(^\text{12}\) 1.8 gm. of water-insoluble oxidation products were obtained. Of this material, ether dissolved 1.6 gm. Upon concentrating and cooling the ether solution, the dihydroxystearic acid crystallized out. This was recrystallized from ether and boiled three times with 200 cc. of water. The water-insoluble residue was crystallized from 95 per cent alcohol. 0.568 gm. of a white crystalline acid was obtained which melted at 129–130°C.

Analysis. 0.1403 gm. substance: \(\text{CO}_2 \ 0.3499, \ H_2O \ 0.1443.\)
Calculated for \(\text{C}_{18}\text{H}_{36}\text{O}_4\). \(\text{C} \ 68.3, \ H \ 11.4.\)
Found. \(\text{C} \ 68.0, \ H \ 11.5.\)

This is dihydroxystearic acid indicating the presence of oleic acid in this fraction.

The ether-insoluble, sativic fraction (0.2 gm.) was crystallized from boiling water, extracted with ether, and recrystallized from 95 per cent alcohol. 0.09 gm. of acid melting at 155–162°C. was obtained. In order to obtain more of this material, 8 gm. of acid from the reduction of the dibromide of fraction 4 (iodine No. 128) were oxidized and the satvic fraction obtained. 0.22 gm. of an acid melting at 155–160°C. was obtained. This material was repeatedly extracted with ether and the residue recrystallized from 70 per cent alcohol. 0.15 gm. of satvic acid melting at 163–165°C. was obtained.

Analysis. 0.1371 gm. substance: \(\text{CO}_2 \ 0.3101, \ H_2O \ 0.1306.\)
Calculated for \(\text{C}_{18}\text{H}_{36}\text{O}_4\). \(\text{C} \ 62.1, \ H \ 10.3.\)
Found. \(\text{C} \ 61.7, \ H \ 10.6.\)

\(^{12}\) Lewkowitsch, J., Chemical technology and analysis of oils, fats and waxes, London, 6th edition, 1921, i, 575.
The isolation of sativic acid from the oxidation products indicates the presence of linolic acid (C_{18}H_{32}O_{2}) in this fraction.

The aqueous mother liquors from the tetrahydroxystearic and dihydroxystearic acids were examined for linusic acid (C_{18}H_{36}O_{8}) but only a small amount of acid melting at 135–140°C. was obtained. Linolenic acid is not present in this fraction.

**Examination of the Intermediate Fraction of Unsaturated Acids.**

The 30 gm. of acids whose barium salts were insoluble in the benzene-ether mixture and whose lead salts were ether-soluble, were again converted into their barium salts and extracted with the benzene-ether mixture. Only a small amount of the barium salts was now insoluble in this solvent.

The insoluble barium salt was decomposed with hydrochloric acid and the fatty acid extracted with ether. 0.9 gm. of acid was obtained having an iodine number of 83. 0.6 gm. of this acid was reduced with hydrogen and colloidal palladium by the method of Paal. 0.58 gm. of crystalline acids was obtained which after recrystallization from alcohol melted at 56–61°C. Mixed with palmitic acid, it melted at 52–62°C.

**Analysis.** 0.2147 gm. substance: CO_{2} 0.5878, H_{2}O 0.2380.
Calculated for C_{16}H_{30}O_{2}. C 75.0, H 12.5.

These analytical results indicate that the reduced acid is palmitic and that the unsaturated acid in the fat is a C_{18} acid of the oleic series.

The barium salts soluble in the benzene-ether mixture, when decomposed with hydrochloric acid and extracted with ether, yielded 26.8 gm. of acid having an iodine number of 125. 16 gm. of this acid were dissolved in dry ether, cooled to 0°C., and saturated with bromine. 0.8 gm. of ether-insoluble bromides was obtained which blackened at 230°C.

**Analysis.** 0.1532 gm. substance: AgBr 0.2354.
Calculated for C_{20}H_{32}O_{2}Br_{8}. Br 67.8.
“ C_{18}H_{30}O_{2}Br_{8}. “ 63.3.
Found. Br 65.4.

These results suggest a mixture of arachidonic octobromide and a hexabromide. There was not sufficient material to separate these by extraction with hot benzene.
0.5 gm. of an ether-soluble, petroleum ether-insoluble tetrabromide was obtained which after repeated extraction with petroleum ether melted and blackened at 135–140°C.

Analysis. 0.1891 gm. substance: AgBr 0.2436.
Calculated for C_{18}H_{30}O_{2}Br_{4}. Br 53.3.
“ ” C_{18}H_{30}O_{2}Br_{4}. “ 63.3.
Found. Br 54.8.
The analysis indicates impure linolic tetrabromide.

25.7 gm. of liquid bromides soluble in petroleum ether were obtained. This dibromide fraction was debrominated by boiling with alcoholic hydrogen chloride and zinc dust. The resulting ethyl esters formed during this debromination were saponified and the acids obtained by decomposing the soap and extracting with ether. 14 gm. of acids having an iodine number of 107 were obtained. 3 gm. of this acid were reduced with hydrogen and colloidal palladium and the resulting solid acid crystallized twice from 95 per cent alcohol. 1.33 gm. of crystals melting at 62–65°C. were obtained. The filtrate yielded 0.3 gm. of acid melting at 61–62°C.

Analyses.
Fraction 1. 0.1518 gm. substance: CO_{2} 0.4211, H_{2}O 0.1732.
Fraction 2. 0.1498 gm. substance: CO_{2} 0.4146, H_{2}O 0.1715.
Found. C 75.5, H 12.8.
Calculated for C_{18}H_{36}O_{2}. C 76.0, H 12.7.
“ ” C_{18}H_{36}O_{2}. “ 75.0, “ 12.5.

These analytical results indicate a mixture of palmitic and stearic acids. The unsaturated acids of the dibromide fraction are, therefore, a mixture of oleic acid and a C_{26} acid of the oleic series. The high iodine number (107) indicates the presence of a small amount of more unsaturated acids which did not separate as crystalline bromides during the bromination.

Examination of the Saturated Acids.

The saturated acids from the lead salt separations and those from crystallization of the crude acid mixtures were joined. These acids weighed 149 gm. After recrystallization from 95 per cent alcohol, 129 gm. of crystalline acids were obtained which melted at 53–55°C. When mixed with pure myristic acid (m.p. 54°C.)
the melting point was lowered, indicating that this was not myristic acid. An attempt was made to crystallize fractionally this mixture from alcohol but a satisfactory separation could not be obtained.

### TABLE IV.
**Distillation of Methyl Esters of Saturated Acids.**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Temperature</th>
<th>Pressure (mm, Hg)</th>
<th>Ester</th>
<th>Acid from ester</th>
<th>Acid after crystallization</th>
<th>Melting point (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Up to 160</td>
<td>1.5-1.6</td>
<td>31.4</td>
<td>29.2</td>
<td>21.1</td>
<td>56-56.5</td>
</tr>
<tr>
<td>2</td>
<td>160-161</td>
<td>1.6-1.7</td>
<td>17.7</td>
<td>16.6</td>
<td>14.5</td>
<td>55-56.5</td>
</tr>
<tr>
<td>3</td>
<td>161-168</td>
<td>1.8</td>
<td>11.4</td>
<td>10.7</td>
<td>9.0</td>
<td>56-58</td>
</tr>
<tr>
<td>4</td>
<td>168-172</td>
<td>1.5-1.8</td>
<td>16.3</td>
<td>15.3</td>
<td>11.8</td>
<td>59-62</td>
</tr>
<tr>
<td>5</td>
<td>172-175</td>
<td>1.5-1.7</td>
<td>10.5</td>
<td>9.6</td>
<td>8.5</td>
<td>64-66</td>
</tr>
</tbody>
</table>

### TABLE V.
**Analysis of Saturated Acids.**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Neutralization value</th>
<th>Analysis</th>
<th>Calculated composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CO₂</td>
<td>H₂O</td>
</tr>
<tr>
<td>1</td>
<td>214</td>
<td>0.1541</td>
<td>0.4232</td>
</tr>
<tr>
<td>2</td>
<td>211</td>
<td>0.1471</td>
<td>0.4052</td>
</tr>
<tr>
<td>3</td>
<td>207</td>
<td>0.1519</td>
<td>0.4198</td>
</tr>
<tr>
<td>4</td>
<td>203</td>
<td>0.1559</td>
<td>0.4324</td>
</tr>
<tr>
<td>5</td>
<td>196</td>
<td>0.1553</td>
<td>0.4318</td>
</tr>
</tbody>
</table>

The acids were converted into the methyl esters by refluxing for 4 hours with 200 cc. of methyl alcohol and 10 cc. of sulfuric acid. 131 gm. of methyl esters were obtained. 90 gm. of the esters were fractionally distilled at a pressure of 1.5 to 1.8 mm. of mercury. Each of the five fractions was saponified, the soap decomposed with dilute sulfuric acid, and the liberated fatty
acids extracted with ether. The ether solutions were washed free from sulfuric acid, dried over anhydrous sodium sulfate, and the ether removed. The resulting acids were weighed, crystallized once from alcohol, and the melting points determined. The results are given in Table IV.

The crystalline acids from the above distillation were analyzed for C and H and the neutralization values determined. From the analysis, the percentage of palmitic and stearic acids was calculated for each fraction. The results are tabulated in Table V.

These analytical results show that the saturated acids consist of a mixture of 68 per cent palmitic acid and 32 per cent stearic acid. The mother liquor from the crystallization of fraction 1 was examined for myristic acid. The alcoholic solution was concentrated and the residue crystallized into two fractions. The top fraction (2.9 gm.) melted at 55–56.5°C. and appeared to be identical with fraction 1. The bottom fraction (0.36 gm.) melted at 53–55°C., but when mixed with myristic acid, lowered its melting point.

**Analysis.** 0.1533 gm. substance: CO₂ 0.4192, H₂O 0.1742.
Calculated for C₁₆H₃₄O₂. C 75.0, H 12.5.
Found. C 74.6, H 12.7.

These results indicate impure palmitic acid. Myristic acid is not present in this fraction.

**SUMMARY.**

700 gm. of the fat of the corpus luteum, freed from acetone- and alcohol-insoluble and cadmium chloride-precipitable phosphatides, yielded 524 gm. of fatty acids and 105 gm. of unsaponifiable material. From the unsaponifiable material, 58 gm. of cholesterol were crystallized. 52 per cent of the fatty acids are present as glycerides, the other 48 per cent existing as free fatty acids. 35 per cent of the total fatty acids are saturated and 65 per cent are unsaturated. The experimental work presented in this paper indicates the following composition of the fatty acid mixture: 25 per cent palmitic (C₁₆H₃₂O₂), 11.2 per cent stearic (C₁₈H₃₆O₂), 1.6 per cent (C₁₆H₃₀O₂), 32.8 per cent oleic (C₁₈H₃₄O₂), 16.6 per cent linolic (C₁₈H₃₄O₂), 8 per cent arachidonic (C₂₀H₃₄O₂), and 4.8 per cent of a new hexa unsaturated acid of the C₂₀ series having the formula C₂₀H₃₄O₂.
THE CHEMICAL INVESTIGATIONS OF
CORPUS LUTEUM: IV. THE
ACETONE-SOLUBLE FAT
George F. Cartland and Merrill C. Hart


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