A CHEMICAL AND HISTOCHEMICAL STUDY OF THE LIPIDES OF THE PYLORIC CECUM OF THE STARFISH, ASTERIAS FORBESI*

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PLATE 48

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In studies on the fatty alcohol ethers of glycerol, the diverticula of the pyloric cecum from the starfish, Asterias forbesi, have been found to be of particular interest, and respiring fragments of these diverticula have been used in metabolic experiments on these ethers (1). It seemed desirable to augment such studies with histochemical and biochemical studies on the lipides of the diverticula. The present paper offers data on the chemical nature of these lipides, which have a rather unusual composition, and attempts to correlate the histochemical and biochemical findings, especially with respect to the limits of sensitivity of some histochemical methods and the differences in the nature of the lipides found in various parts of the cell.

Materials and Methods

Materials.—Starfish about 6 to 8 inches in diameter, shipped alive in lots of 12 from the Marine Biological Laboratory at Woods Hole, were used within 24 hours after arrival in Boston. The animals were killed by dismembering, and the paired diverticula of the pyloric cecum were carefully removed from the arms.

Histological Methods.—From 2 starfish, short segments of one diverticulum were fixed in Bouin's fluid, in Baker's (2) formol-calcium, in weak Bouin's fluid, and in 10 per cent formalin buffered with phosphate to pH 7. Another segment was quickly frozen in a container chilled by alcohol and CO₂ ice; it was then preserved in a deep-freeze at -20°C. The 9 diverticula remaining from each of these animals were used for comparative chemical examination of the lipides.

The blocks fixed in Bouin's fluid were embedded in paraffin, sectioned at 5 µ, and stained with hematoxylin and eosin and with the Masson connective tissue mixture. The blocks fixed in formol-calcium were prepared according to the Baker (2) acid hematein method for demonstrating phosphatides, and those fixed in weak Bouin's fluid were used for the pyridine controls.

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The blocks fixed in formalin were sectioned after 1 week on the freezing microtome at 20 μ. At this time the unfixed frozen blocks were sectioned at 20 μ in the Linderström-Lang cryostat, mounted, and fixed for 15 minutes in 10 per cent buffered formalin (3). Thoroughly washed sections of both types were stained by the following methods: (a) 1 per cent Sudan black B in 70 per cent alcohol, 1 and 7 minutes, for total lipides; (b) the Schiff reagent, 15 minutes, for free aldehydes; (c) quarter-saturated HgCl₂ solution, 15 minutes, and the Schiff reagent, 15 minutes, for plasmals; (d) the Ashbel and Seligman (4) naphthoic acid–hydrazide method, for free carbonyls; (e) quarter-saturated HgCl₂ solution, 15 minutes, and the Ashbel and Seligman method, for plasmals; (f) the Schultz method, for cholesterol; (g) a 2 per cent solution of Nile blue sulfate, for distinguishing acid from neutral lipides. (h) Another section was mounted unstained for study under the polarizing and fluorescence microscopes. Control sections for each of these 8 procedures were first extracted for 30 minutes with acetone at room temperature.

Chemical Methods.—All lipides were extracted by the procedure of Folch et al. (5). The methods for the isolation and determination of unsaponifiable fractions and for the determination of α-glycerol ethers were described by Karnovsky and Brunn (1). Lipide phosphorus was determined by the methods of Fiske and SubbaRow (6) and Gortner (7), slightly modified. Cholesterol was determined by the method of Bloor (8).

The release from acetal linkage of α-glycols with a free primary carbinal group in unsaponifiable and other fractions was measured by treating the lipide with alcoholic HCl, and, after adding excess pyridine, oxidizing the α-glycols with periodic acid. The formaldehyde, representing total α-glycol primary alcohol groups, was determined as described elsewhere (1). The procedure for releasing α-glycols in acetal linkage and oxidizing these with periodic acid was as follows: An aliquot of lipide (2 to 5 mg.), usually in chloroform-methanol, was evaporated to dryness under N₂, and taken up in 2 ml. absolute alcohol. Ten drops of 6 N HCl (aqueous) were added, and the tube rotated to mix thoroughly. Controls were run with distilled water replacing the 6 N HCl. Further tests were carried out on butyl alcohol, with and without acid, as standards. Blanks were run with and without acid. All samples were allowed to stand overnight at room temperature. One-half ml. redistilled pyridine and 1 ml. alcoholic H₂O₂ (1 per cent; 0.044 μ) were added, and the tubes again mixed. After 1 hour, 0.5 ml. 1.2 μ sodium arsenite was added, and the mixture carefully titrated with 6 N aqueous HCl until no further iodine was freed and discharged by the arsenite. The volume was then made to 10 ml., celite added, and the mixture filtered through a No. 42 filter paper. Formaldehyde was then determined (1).

Lipide aldehydes were determined by the method of Feulgen and Grünberg (9) somewhat modified.¹

Lipides from Cellular Fractions.—An attempt was made to obtain information about the location of the various lipide fractions by subjecting homogenized tissue to differential centrifugation prior to the extraction of lipide.

Cellular fractions were obtained as follows: The tissue was thoroughly homogenized in the cold room (4°C.) with a Potter-Elvehjem homogenizer (10), in a 1 M sucrose solution. The latter was used instead of 0.88 μ sucrose (11) since it more nearly approaches the tonicity of sea water. The homogenate was centrifuged at 2,000 r.p.m. (International horizontal rotor, No. 240, 50 ml. centrifuge tubes) for 30 minutes so that the “free” lipide ("droplets") rose to the surface. This was carefully skimmed off and blended in a Waring blender with petroleum ether (b.p. 30–60°C.) for 2 minutes. The mixture was then centrifuged, and the

¹ The authors are indebted to Dr. G. Schmidt for details of a modification of the method of Feulgen and Grünberg (9), for a determination of acetal phosphatides depending on the release of lipide phosphorus, as water-soluble phosphorus, and for a sample of pure acetal phosphatide, which has been used as a standard throughout this work.
petroleum ether layer separated, filtered, and evaporated to dryness at 60°C. under N₂. The residue was taken up in chloroform and the extract filtered and made to volume. This material is referred to as the “free” lipid, and is designated fraction I.

The homogenate, after removal of the “free” lipid, was mixed thoroughly, chilled to 4°C. for 30 minutes, and the following further fractions made, using a servall SS-34 centrifuge:

(II) A fraction was spun down at 1,200 × g for 20 minutes. This was the “nuclear” fraction (11).

(III) A fraction was recovered from the supernatant of (II) after spinning down at 20,000 × g for 30 minutes. This corresponded to the “mitochondrial” fraction, and was bright yellow in color.

(IV) The supernatant from (III) was diluted to 0.25 M sucrose, centrifuged at 20,000 × g for 90 minutes, and a fraction corresponding to the “microsome” fraction obtained. The fraction was colorless. The supernatant was discarded.

All sedimented fractions were washed twice by resuspending in sucrose solution and re-sedimenting. The washings were added to the original supernatant fractions. The lipides of the solid fractions obtained were extracted by the chloroform-methanol method of Folch et al. (5).

Microscopic examination was made of fractions II, III, and IV by (a) staining with 0.1 per cent methylene blue, pH 6, (b) staining with a dilute solution of Janus green B in 1 M sucrose, (c) staining with 1 per cent Sudan black B, and (d) viewing under the darkfield microscope.

RESULTS

Histology and Histochemistry of the Starfish Diverticulum.—Two diverticula of the pyloric cecum extend from the stomach into each of the 5 arms, where they are suspended by mesenteries. In cross-section, each diverticulum appears subdivided into 2 or 3 tubes, the walls of the tubes being greatly folded (Fig. 1). The high epithelium of the wall rests on a basement membrane, outside of which there is a serosal layer (Fig. 2). The epithelial cells are extremely tall and narrow; their nuclei lie for the most part in a broad band in the middle third of the layer. Many of the epithelial cells bear long flagella (12). The apical surfaces of the cells display a conspicuous striated border, underneath which is a rather broad fibrillar zone in which there are no granules. The remaining supranuclear portion of the cytoplasm contains many granules, most of which are acidophilic.

Frozen sections from the formalin-fixed blocks and from the unfixed blocks reacted alike in the histochemical tests for lipides. In unstained preparations, the droplets appeared yellow. Sections stained with Sudan black B demonstrated crowded lipide droplets in the epithelial cells occupying the entire length of the cells except for the brush border and the immediately underlying fibrillar zone (Fig. 3). The sizes of the droplets varied in different parts of the sections, but the larger ones appeared to have arisen by confluence of smaller ones during the histological manipulation. All of the stainable droplets were removed by extracting the sections for 30 minutes with acetone. After extraction, only diffuse staining of the cytoplasm occurred.

The lipide droplets stained pink with Nile blue sulfate, indicating their
neutral nature (13). They failed to exhibit a Schultz cholesterol reaction, birefringence, or fluorescence. They reacted neither with the Schiff reagent for aldehydes nor with the Ashbel-Seligman (4) hydrazide reagent for carbonyl groups, whether or not they were pretreated with mercuric chloride. On the other hand, the cytoplasm surrounding the droplets, which appeared virtually colorless when the sections were exposed directly to the Schiff or the Ashbel-Seligman reagent, became lightly stained if the sections were first treated with mercuric chloride. By definition, this is the true plasmal reaction (3). This cytoplasmic staining was diminished but not entirely prevented by prior extraction of the sections with acetone. Neither the droplets nor any other structures in the cells stained by the Baker (2) acid hematein test for phosphatides.

**TABLE I**

*Chemical Values for Starfish Diverticulum Total Lipide*

<table>
<thead>
<tr>
<th>Lipide</th>
<th>Starfish 1</th>
<th>Starfish 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total lipide percentage of fresh weight of diverticula.</td>
<td>14.2</td>
<td>7.8</td>
</tr>
<tr>
<td>Phosphorus percentage of total lipide...</td>
<td>1.02</td>
<td>0.94</td>
</tr>
<tr>
<td>Acetal lipides*</td>
<td>&quot; &quot; &quot; &quot;</td>
<td>4.0</td>
</tr>
<tr>
<td>Unasaponifiable fraction</td>
<td>&quot; &quot; &quot; &quot;</td>
<td>12.6</td>
</tr>
<tr>
<td>Sterols‡</td>
<td>&quot; &quot; &quot; &quot;</td>
<td>4.2</td>
</tr>
<tr>
<td>Glycerol ethers§</td>
<td>&quot; &quot; &quot; &quot;</td>
<td>3.9</td>
</tr>
</tbody>
</table>

* Calculated as acetal phosphate of Thanhhauser et al. (14).
‡ Calculated as cholesterol.
§ Calculated as butyl alcohol.

*Chemical Values on Starfish Diverticulum Lipides.*—A chemical analysis of the diverticulum lipide of the 2 starfish used for histological study yielded the results reported in Table I.

The absorption spectrum of the total lipide was determined, with the results reported in Text-fig. 1. The substance absorbing strongly at 470 μm would appear to be carotenoid, but insufficient data are available to make a more specific identification possible. Carotene and xanthophyll have been reported to occur in species of *Asterias* (15), but the compound does not appear to be either of these. It is unstable, the characteristic absorption disappearing after a few days' standing.

In all batches of starfish diverticulum lipide so far examined, the phosphorus content of the phosphatide fraction, isolated by acetone precipitation in the presence of MgCl₂ (16) and reprecipitation with acetone after taking up in chloroform, has never exceeded 2.6 per cent. The mean figure was 2.43
per cent, with an average deviation from the mean of 0.09. Thus, in order to convert the percentage of phosphorus in the fat to a percentage of phosphatide, a factor of 40 would be appropriate rather than the conventional 25. The phosphatide content of the two fats examined above (Table I) would then become 40.8 and 37.6 per cent, respectively. Repeated reprecipitation of the isolated phosphatide, without the use of MgCl₂, failed to raise the phosphorus content.

Lipide aldehydes were detected not only in the acetone-precipitated phosphatide fraction (acetal phosphatides or plasmalogens) but also in the acetone-soluble neutral fat, even though the latter contained only traces of phosphorus (0.02 ± 0.008 per cent). Repeated isolation and retreatment of the neutral fat did not eliminate these aldehydes, presumably present in the form of acetals. Examination of unsaponifiable fractions prepared from whole lipide or neutral fat indicated the presence of aldehydes in these fractions also. Fatty acids extracted after acidification of aqueous saponification mixtures gave, in all cases, negative or negligible aldehyde titers. Aldehyde content of some starfish fat fractions is reported in Table II. In the case of phosphatide fractions measurements were made of the water-soluble phosphorus released by saponification at 37°C and of that released after treating the saponification mixture with acid and mercuric chloride. The difference represented acetal phosphatide phosphorus (17) and compared reasonably well with the results obtained by the Schiff reaction (9).

The proportion of α-glycerol ethers in the unsaponifiable fractions of star-
fish diverticulum lipide increased with age (1). As can be seen in Text-fig. 2, lipide aldehydes showed a corresponding increase. That the positive aldehyde test obtained on unsaponifiable fractions was probably due to glycol acetals and not to aldehydes formed by epoxidation and splitting of fatty acid double bonds is suggested by the fact that mineral acid treatment, at room temperature, of the unsaponifiable fraction led to an increase in α-glycol titer, as may also be seen in Text-fig. 2. This release of α-glycols, yielding formaldehyde with periodate, has been confirmed on several unsaponifiable fractions. Tests with a sample of pure crystalline acetal phosphatide by the method employed for the fractions above indicated, however, only about one-third to one-half of the theoretical yield of formaldehyde. Consequently, the release of α-glycol groups in the case of the unsaponifiable fractions is probably not complete. Purified α-glycerol ether samples, described elsewhere (1), showed neither a positive Schiff aldehyde test nor a release of α-glycol groups by gentle acid treatment.

**TABLE II**

Aldehyde Content of Starfish Lipide Fractions

(Expressed as per cent acetal in each fraction calculated as acetal phosphatide.)

<table>
<thead>
<tr>
<th>Starfish</th>
<th>Total lipide</th>
<th>Phosphatide</th>
<th>Neutral fat</th>
<th>Unsaponifiable fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.2</td>
<td>2.4</td>
<td>1.4</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>4.0</td>
<td>4.9</td>
<td>0.2</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>—</td>
<td>5.6</td>
<td>1.6</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>—</td>
<td>10.0</td>
<td>—</td>
<td>9.2</td>
</tr>
<tr>
<td>5</td>
<td>6.2</td>
<td>—</td>
<td>—</td>
<td>5.7</td>
</tr>
</tbody>
</table>

Cellular Fractions of the Starfish Diverticula.—On histological examination, little free lipide was present in any of the “nuclear,” “mitochondrial,” or “microsome” fractions. The “nuclear” fraction (II) consisted of numerous basophilic nuclei together with considerable amounts of cellular debris and large numbers of granules. Of the latter, only a few were colored with Janus green B. Presumably the rest were secretory granules (12). The “mitochondrial” fraction (III) consisted almost entirely of discrete granules, about one-half of which stained a pale green with Janus green B. The “microsomal” fraction (IV) consisted principally of clumps of highly basophilic particles together with some granules. The particulate nature of the clumps was especially evident under the darkfield microscope.

The lipides obtained from the various fractions were analyzed, with the results reported in Table III. The lipides of the final supernatant fraction were not obtained, owing to the large amount of sucrose present and the
Text-Fig. 2. Correlation of the content of $\alpha$-glycerol ethers, aldehydes, and $\alpha$-glycols with terminal primary carbinol groups released from acetals, in the unsaponifiable fractions of the lipides obtained from starfish of different sizes. In all cases except that of the 2 inch starfish the lipide was extracted from the diverticula. In the case of the 2 inch starfish it was necessary to extract the total lipide of the whole animal (1).

**TABLE III**

*Analysis of Lipide Extracted from Various Cellular Fractions*

(The values are percentages of the total lipide of each cellular fraction.)

<table>
<thead>
<tr>
<th>Lipide</th>
<th>I &quot;Droplets&quot;</th>
<th>II &quot;Nuclei&quot;</th>
<th>III &quot;Mitochondria&quot;</th>
<th>IV &quot;Microsomes&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphorus</td>
<td>0.06</td>
<td>1.02</td>
<td>0.99</td>
<td>0.75</td>
</tr>
<tr>
<td>Acetal lipides*</td>
<td>2.2</td>
<td>7.7</td>
<td>6.6</td>
<td>5.6</td>
</tr>
<tr>
<td>Unsaponifiable fraction</td>
<td>13.8</td>
<td>30.5</td>
<td>17.5</td>
<td>44.7</td>
</tr>
<tr>
<td>Sterols†</td>
<td>1.1</td>
<td>6.1</td>
<td>4.5</td>
<td>4.0</td>
</tr>
<tr>
<td>Glycerol ethers§</td>
<td>4.2</td>
<td>2.2</td>
<td>2.5</td>
<td>3.0</td>
</tr>
</tbody>
</table>

* Calculated as the acetal phosphatide of Thannhauser et al. (14).
† Calculated as cholesterol.
§ Calculated as batyl alcohol.
considerable volume of the fraction. The main features revealed in Table III are the low phosphatide, acetal, and steroid content of the "free" or droplet lipides and the high unsaponifiable fraction of the "microsome" fraction (IV). All lipide fractions exhibited a broad absorption band at about 470 m\(\mu\) with \(E_{1\%}^{1\text{cm}}\) values ranging from 0.4 to 0.7 at this wave-length.

**DISCUSSION**

Several points may be made with respect to the comparison of histochemical and chemical results. Histochemically the lipide droplets do not contain detectable amounts of sterol or of acetal aldehyde, and chemically the values for these constituents in the "free" lipide are low. The minimum concentration of sterol in lipide necessary for a detectable Schultz reaction is between 6 and 12 per cent (18), whereas the concentration in the "free" lipide is 1.1 per cent. It may also be assumed that the 2.2 per cent listed in Table III for the acetal content of the droplets is somewhat high, since it was calculated in terms of the pure acetal phosphatide of Thannhauser et al. (14); in terms of a mixture of equal amounts of palmitaldehyde and stearaldehyde it would be 1.3 per cent.

A further correlation might be made between the failure by both Anderson (12) and ourselves to obtain a positive Baker (2) test for phosphatides, in either the droplets or in the cytoplasm, and the low phosphorus content of the isolated phosphatides. A considerable proportion of the total lipide of the diverticulum is phosphatide, as has been shown by chemical isolation. It should be noted that the actual recovery of neutral fat and phosphatides after precipitation and reprecipitation of the latter, and two treatments of the former with acetone, was consistently over 90 per cent, and the actual yield of phosphatide isolated was about 30 per cent of the total lipide. The failure of the Baker test is thus in all probability not due to low concentrations of phosphatide but may be due to the low phosphorus content of the phosphatide fraction. In the histochemical test, the hematein is presumed to react with the phosphate radical. The sensitivity of this test has never been determined, although its specificity seems established (19).

With respect to the histochemical test for plasmals, it is also noteworthy that acetone extraction of the sections diminished but did not abolish the carbonyl reaction in the cytoplasm. This diminution could be due to the removal of the acetals found by chemical means in the neutral fat and its unsaponifiable fraction. The existence of such non-phosphatide acetals is of considerable interest when considered in the light of the suggestion of Baer, Rubin, and Fischer (20) regarding the origin of \(\alpha\)-glycerol ethers from acetal phosphatides. Neutral fat acetals may represent an intermediate state in such a metabolic process. The unsaponifiable fractions of all the cellular fractions obtained by centrifugation were found to contain aldehydes, pre-
sumably acetals—the titer ranging from 2.5 to 7.0 per cent. Hack (21) reported similarly the finding of aldehyde-containing lipides which were acetone-soluble and hence probably not phosphatides.

A further point of considerable importance is the fact that no pseudoplasmal reaction (3) developed in the droplets during fixation. This fact would strengthen the contention that the chemically determined aldehydes were characteristic of the lipides rather than artefacts formed during the isolation and handling of the fats. Further, since the pseudoplasmal reaction has been shown to be the result of the oxidation of fatty acid double bonds (22), more detailed studies on the composition of starfish fats and their environment would be of interest.

SUMMARY

The lipides of the diverticula of Asterias forbesi have been studied by histochemical and biochemical means.

Correlations between results obtained by histochemical examination of sections, and chemical analysis of isolated lipide have been made, particularly with respect to phosphatides, steroids, and aldehyde lipides.

The results of the histochemical study were in good agreement with the chemical data as to the nature of the phosphatide fraction, the presence of acetone-soluble aldehyde lipides, and the composition of the free droplet fat. Homogenized diverticula were differentially centrifuged in order to establish the distribution of types of lipides in the various cellular components.

In addition, data have been presented which demonstrate a direct correlation between the titer of α-glycerol ethers and that of acetone-soluble lipide acetals in the unsaponifiable fraction.

BIBLIOGRAPHY

LIPIDES IN STARFISH PYLORIC CECUM


EXPLANATION OF PLATE 48

Fig. 1. Low-power photomicrograph of a cross-section of a diverticulum of a starfish pyloric cecum. Fixed in Bouin's fluid, sectioned at 5 μ, stained by the Masson trichrome method, photographed with an orange filter. The point of the mesenteric attachment is indicated by the arrow. At this level, the diverticulum consists of 2 large lateral tubes and a smaller median one. The walls of the lateral tubes are extensively folded and convoluted. X 12.

Fig. 2. A high-power photomicrograph of a segment of the wall of the diverticulum. Fixed in Bouin's fluid, sectioned at 5 μ, stained with Harris' hematoxylin and eosin, photographed with green and orange filters. The epithelium consists of tall (ca. 200 μ), slender columnar cells, whose nuclei (n) lie approximately in the central region. The apical borders of the cells (above) display a conspicuous brush border (bb). The cells rest on a broad basement membrane ( bm), outside of which lies a serosal layer (s). The supranuclear portions of the epithelial cells are distinctly granular except for a fibrillar region (f) underlying the brush borders. X 400.

Fig. 3. A high-power photomicrograph of a segment of the wall of a diverticulum. Fixed in buffered formalin, sectioned on the freezing microtome at 20 μ, stained with Sudan black B for 7 minutes, photographed with a blue filter. The epithelial cells are crowded with lipide droplets except in the brush border and the underlying fibrillar region. The droplets are generally small, although in some instances they appear to have coalesced into larger globules. The serosal cells contain minute sudanophilic granules. All lipide droplets are removed by a half-hour extraction with acetone, leaving only a weak and diffuse staining of the cytoplasm. X 400.
(Karnovsky et al.: Lipides in starfish pyloric cecum)