Phospholipids of Pork Muscle Tissues

M. KUCHMAK and L. R. DUGAN, JR., Department of Food Science, College of Agriculture, Michigan State University, East Lansing, Michigan

Abstract
The lipids extracted from four locations on a hog carcass were fractionated into nonphospholipids, phosphatidyl ethanolamine, phosphatidyl serine, lecithins, and sphingomyelins. The identities of the phospholipid fractions were established and their quantitative contents were determined by three methods: gravimetrically, from analysis of the phosphorus content, and from standard curves of infrared absorptions. Variations in content were present in the phospholipid fraction, after removal of solvents under reduced pressure with a rotary evaporator, and completion of evaporation under a stream of nitrogen, was taken up with a small volume of chloroform and put on a silicic acid column.

Chromatography. Silicic acid columns were prepared in chloroform using at least 50 mg of silicic acid per one mg of phospholipids to be separated. The scheme of chromatographic separation on silicic acid is shown in Table I.

Nonphospholipids were eluted with chloroform as fraction I. The effluent was checked by the Salkowski test for cholesterol and, after obtaining a negative test, the volume of effluent was measured; this was referred to as a volume and is used in the context of the third column in Table I. The volume of chloroform was doubled in order to elute the nonphospholipid fraction.

Acetone, referred to as a scavenger by Nelson and Freeman (4), was used to remove pigmented material. It was usually possible to follow this visually as a band and about $\frac{1}{2}$ of a volume of acetone was sufficient.

The third fraction was eluted with one volume of 10% methanol in chloroform, followed with 15% methanol until a ninhydrin positive test was obtained. For this test, equal volumes of sample, ninhydrin, and 2,4-lutidine, were used. A portion giving a ninhydrin positive test was combined with the next fraction.

Cephalins, as fraction IV, were eluted with one volume of 20% methanol followed by 25% methanol in chloroform until disappearance of the ninhydrin test. They were separated into phosphatidyl ethanolamine and phosphatidyl serine on a hydrated silicic acid column as described by Rouser, et al. (5). The elution pattern was followed with the ninhydrin test and phosphatidyl ethanolamine as fraction IVa was eluted with 25% methanol in chloroform followed by phosphatidyl serine as fraction IVb eluted with methanol.

For elution of fraction V, containing lecithins, from a silicic acid column 4 volumes of 35% methanol in chloroform were used, and the sphingomyelins were then eluted as fraction VI with two volumes of methanol.

Solvents were removed from all fractions under reduced pressure in a rotary evaporator and transferred quantitatively to an ultrafine bacteriological filter, and filtered with suction. In this way the escape of silicic acid fines was prevented. Filters were transferred to weighed 25 ml glass stopped Erlenmeyer flasks, and solvents were removed under a stream of nitrogen, and weighing. The phospholipid fraction, after removal of solvents under reduced pressure with a rotary evaporator, and completion of evaporation under a stream of nitrogen, was taken up with a small volume of chloroform and put on a silicic acid column.

TABLE I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Eluent</th>
<th>Volume</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Chloroform</td>
<td>2</td>
<td>Nonphospholipids *</td>
</tr>
<tr>
<td></td>
<td>Acetone</td>
<td>2</td>
<td>Phospholipids</td>
</tr>
<tr>
<td>II</td>
<td>Acetone</td>
<td>1</td>
<td>Nonphospholipids *</td>
</tr>
<tr>
<td>III</td>
<td>10% Methanol b</td>
<td>1</td>
<td>Cerebrosides (1)</td>
</tr>
<tr>
<td>IV</td>
<td>20% Methanol</td>
<td>1</td>
<td>Cephalins</td>
</tr>
<tr>
<td>V</td>
<td>25% Methanol</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>35% Methanol</td>
<td>1</td>
<td>Lecithins</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>1</td>
<td>Sphingomyelins</td>
</tr>
</tbody>
</table>

a Explained in text.
b Listed percentage of methanol is in chloroform (v/v).

* Separated into phosphatidyl ethanolamine (I Vb) and phosphatidyl serine (I Vb) on a hydrated silicic acid silicate column.
stream of nitrogen. Flask contents were dried overnight in a vacuum desiccator over calcium chloride and weighed. Flasks were transferred with a suitable spectro grade solvent to volumetric flasks and used for infrared (IR) spectra and other analyses.

Preparation of Standards. Isolated phospholipid standards of desirable purity are usually not available. Synthetic compounds of rigorous purity, if available, have limited solubility for use for IR spectra in solutions, and they may have different extinction coefficient values in IR absorption spectra than naturally occurring phospholipids because of different fatty acid composition and location (6). It was desirable, therefore, to prepare our own standards from hog ham muscle. The phosphorus content, qualitative thin-layer chromatography, and IR spectra were used as criteria of purity of the fractions isolated for standards. Phosphorus was determined by the method of Beveridge and Johnson (7). Thin-layer chromatography was conducted on silica gel G, according to Stahl, with solvent of chloroform-methanol-water, 65/25/4, v/v/v, according to Wagner (8). Chromatograms were developed with 0.2% ninhydrin in n-butanol-aqueous acetic acid 10%, 95/5, and aqueous sulfuric acid, 1/1, v/v (9). Both phosphatidyl ethanolamine and phosphatidyl serine were hydrolyzed in 3N HCl in sealed tubes at 100°C for 16 hr (5). After hydrolysis, the free fatty acids were extracted into petroleum ether, and the water-acid layer was evaporated to dryness in order to remove hydrochloric acid. The residue was dissolved in water and chromatographed with corresponding reference compounds (ethanolamine and serine) on the plate coated with silicie acid (TLC). For developing, a ninhydrin spray was used. The cerebroside fraction after acid hydrolysis was tested with anthrone reagent (10) for possible presence of galactose.

IR measurements were made with a Beckman IR5 double beam recording spectrophotometer, equipped with a sodium chloride prism.

Results and Discussion

Evaluation of Standards. Standards were quite pure on a basis of phosphorus content. The cerebroside fraction had no phosphorus and the phospholipids assayed 97–103% of theoretical phosphorus content.

Thin-layer chromatography of the cerebroside fraction revealed no component which reacted with ninhydrin, but gave a single spot when developed with sulfuric acid, with an Rf value of 0.85. Phosphatidyl ethanolamine and phosphatidyl serine each gave single spots with ninhydrin and sulfuric acid and with Rf values of 0.61 and 0.37, respectively. Acid hydrolysis products of both aminophosphatides gave identical ninhydrin positive spots with their appropriate reference compounds ethanolamine and serine. Lecithins gave a negative ninhydrin reaction only after rechromatographing on silicie acid and gave a spot (developed with sulfuric acid) with an Rf of 0.40. Sphingomyelins gave a ninhydrin negative spot with Rf of 0.31 and another faint spot corresponding to lecithin, even after rechromatographing on silicie acid.

Acid hydrolysis products of the fraction considered to be cerebrosides gave inconclusive results with anthrone reagent. IR spectra of this fraction did not confirm the presence of cerebrosides, and identity was not established. This fraction is not included in further quantitative presentation of phospholipids, because of the small quantities involved. IR spectra of isolated phospholipid standards are shown in Figure 1.

Since the absorption cells were not entirely matched, we have discontinued regions on the spectra corresponding to the regions of absorption by solvents. Spectrum A represents phosphatidyl ethanolamine, and spectrum B phosphatidyl serine. Both spectra exhibit band characteristics of cephalins at 9.8 μ, which are poorly developed in isolated cephalins as compared with those of the synthetic, and appear rather as inflections. There is no trace of a lecithin band at 10.3 μ and, common to both cephalins and lecithins, the ester carbonyl band at 5.8 μ is strongly developed. Since there is no ester carbonyl band at 5.8 μ in the siphingomyelin spectrum, and the 10.3 μ band is not present in either isolated cephalin, both bands at 5.8 and 9.8 μ might be used for calibration curves.

Spectrum C represents lecithins with strong bands