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Characterization of Lipids from Canine Adrenal Glands*

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Received December 31, 1962

Neutral lipids, which represented approximately 90% of total adrenal lipids, were isolated by silicic acid chromatography. Methyl esters obtained from cholesterol esters and triglycerides by methanolysis were determined by gas-liquid chromatography. The cholesterol esters were mainly unsaturated, containing high proportions of 8,11,14-eicosatrienoic acid, arachidonic acid, and adrenic acid (7,10,13,16-docosatetraenoic acid) in addition to oleic acid, which was the major component. Over 90% of the cholesterol in canine adrenal gland existed in the esterified form. Adrenal phosphatides were partially purified by silicic acid chromatography. The methyl esters liberated from individual phosphatides by mild alkaline hydrolysis were studied by gas-liquid chromatography. Arachidonic and adrenic acids were distributed in all of the fractions, with relatively higher concentrations in phosphatidylethanolamine. Phosphatidylethanolamine and phosphatidylcholine showed a marked specificity for stearic and palmitic acids, respectively. Lysophosphatidylcholine was surprisingly rich in unsaturated fatty acids. More complete characterization of the phosphatides was provided by anion-exchange chromatography of the water-soluble products of mild alkaline hydrolysis. Under the conditions of Hawthorne and Hübscher (1959), modified for microanalyses and with an extended range of eluting solvents, phosphorus-containing products related to cardiolipin, phosphatidic acid, phosphatidylserine (and lysophosphatidylserine), phosphatidylcholine (and lysophosphatidylcholine), phosphatidylinositol, diphosphoinositide, and triphosphoinositide were observed. The major components were glycerylphosphorylethanolamine and glycerylphosphorylcholine, which accounted for more than 60% of the phosphorus. In addition, the mixture of phosphatides contained traces of sphingomyelin and plasmalogens. Further characterization of cardiolipin, the polyphosphoinositides, and the serine-containing phosphatides will be required before exact structures can be assigned.

It has been recognized for a number of years that adrenal tissue contains high concentrations of polyenoic acids (Sinclair, 1958; Klenk and Eberhagen, 1960), but systematic investigations of the nature of these acids were not available until Dailey *et al.* (1960) reported the fatty acid composition of adrenal cholesterol esters from a variety of species and a number of pure polyenoic acids isolated from bovine adrenal phosphatides were fully characterized (Klenk and Eberhagen, 1960). In confirmation of the structural identifications made by Klenk and Eberhagen, we recently established the structures of three polyenoic acids, isolated from canine adrenal lipids, as 8,11,14-eicosatrienoic acid, 5,8,11,14-eicosatetraenoic (arachidonic) acid, and 7,10,13,16-docosatetraenoic acid (Chang and Sweeley, 1962).

* This work was supported in part by a grant-in-aid (A-4307) from the U. S. Public Health Service.

The occurrence of relatively high proportions of 7,10,13,16-docosatetraenoic acid, for which we wish to propose the name "adrenic acid,"[1] in canine adrenal lipids was surprising, since extensive analyses of other mammalian tissues and body fluids by a number of investigators have indicated little if any of this component. Medium to high levels of adrenic acid[2] were found in the cholesterol esters from adrenal glands of rat, dog, and man, but the cholesterol esters from blood of these

[1] We were reluctant to increase the body of trivial nomenclature but wished to avoid repetitive use of the more awkward generic name. The choice of adrenic acid rested on the proportions of this polyenoic acid in adrenal glands from rat, dog, and man, in which it occurs in much higher concentration than any other mammalian tissue.

[2] Identification by these authors was based on retention times observed on gas chromatography, which indicated a C_{22}-tetraenoic acid. The component which they so designated was almost certainly adrenic acid.

species contained no more than traces of this polyenoic acid (Dailey *et al.*, 1960). Adrenic acid was also reported in a mixture of glycerophosphatides isolated from brain (Klenk and Lindlar, 1955) and bovine liver (Klenk and Tomuschat, 1957), but in the case of lipids from liver it was observed only after fractional distillation of methyl esters yielded a C_{22}-polyene fraction which represented a small percentage of the total fatty acids. Lipids from mouse liver contain traces of a C_{22}-tetraenoic acid (Nelson, 1962), and this component has been observed at trace levels in the fatty acids of fish liver and heart (Richardson *et al.*, 1962).

Sinclair (1958) has raised the interesting but entirely speculative suggestion, based on the high levels of polyenoic acids in adrenal tissue, that essential fatty acids may play a role in the biosynthesis of steroidal hormones. Indeed, little attention has been directed to the fate of these acids, esterified with cholesterol, when the cholesterol moiety is utilized for the synthesis of corticosteroids. Whatever the role of polyenoic acids in adrenal metabolism may be, a further investigation of their occurrence and distribution in various simple and polar lipids seemed justified.

In this study of the lipids of canine adrenal gland, methods are presented for the characterization of neutral lipids by silicic acid chromatography and of phosphatides by a combination of silicic acid chromatography of intact lipids and ion-exchange chromatography of the water-soluble products of mild alkaline hydrolysis of phosphatides. Analyses of fatty acids isolated from purified lipids were carried out by gas-liquid chromatography.

Experimental Procedures

Material.—Freshly frozen adrenal glands from healthy mongrel male dogs were the source of lipids. Sodium α-glycerophosphate was purchased from Fisher Scientific Co., Pittsburgh, Pa. Glycerylphosphoryl-*myo*-inositol cyclohexylamine salt, glycerylphosphoryl-*myo*-inositol phosphate cyclohexylamine salt, and glycerylphosphoryl-*myo*-inositol diphosphate barium salt were kindly donated by Dr. Clinton E. Ballou, University of California, Berkeley. Synthetic sphingomyelin, used as a chromatographic standard for thin-layer chromatography, was a gift from Dr. D. Shapiro, Weizmann Institute of Science, Rehovoth, Israel. Phospholipase A, in *Crotalus adamanteus* venom, was supplied by Ross Allen's Reptile Institute, Silver Springs, Fla. Silica Gel G, used for thin-layer chromatography, was supplied by Brinkmann Instruments, Inc., Great Neck, N. Y. Unisil, a special heat-activated, size-graded silicic acid, was obtained from Clarkson Chemical Co., Williamsport, Pa. Column packings for gas-liquid chromatography were supplied by Applied Science Laboratories, State College, Pa., and Analabs, Hamden, Conn. Chromatographic standards for gas-liquid chromatography were kindly made available by the Division of Research Grants, National Institutes of Health, Bethesda, Md. All other chemicals and solvents were of reagent grade and were used as supplied unless specially noted. Water used for ion-exchange chromatography was doubly distilled in an all-glass apparatus.

Routine Analytical Methods.—Esters were determined colorimetrically by the method of Rapport and Alonzo (1955), cholesterol analyses were done by the procedure of Zlatkis *et al.* (1953), and phosphorus determinations were based on Bartlett's method (1959). Thin-layer chromatography, used to monitor all purifications of lipids by silicic acid chromatography, was carried out by standard procedures (Mangold, 1961) except that the plates were coated by dipping them into a tank containing a well-mixed suspension of Silica Gel G (1 part) in chloroform (2 parts) and methyl alcohol (1 part), a procedure (Peifer, 1962) which gave uniform and highly reproducible thin films. Spots were developed by exposure of the plate to iodine vapor.

Isolation of Lipid from Adrenal Glands.—The procedure of Folch *et al.* (1957) was adapted for extraction and washing of total crude lipids. The weighed frozen glands were cut into small pieces and extracted three times with approximately 35 ml of chloroform and methyl alcohol (2:1, v/v). These extractions were carried out in a mortar by grinding with a small quantity of fat-free sand. Combined extracts were filtered through fluted paper and the clear, yellowish filtrates were mixed thoroughly with 0.2 volume of distilled water. After standing overnight at 2°, the upper aqueous layers were removed by aspiration, the lower layers were mixed with a small portion of absolute ethyl alcohol, and the solutions were evaporated to dryness *in vacuo* in a rotating evaporator. Crude lipids were weighed immediately and dissolved in chloroform.

Separation of Crude Total Lipid into Neutral and Polar Fractions.—According to the procedure originally described by Borgström (1952), the mixture of total lipids, dissolved in chloroform, was percolated onto a column (2.0 cm i.d.) containing 20 g of Unisil (100–200 mesh) suspended in chloroform. Neutral lipids were eluted with chloroform (150 to 250 ml) until negative tests for lipid were obtained by the technique (Lands and Deans, 1962) of spotting 5 to 10 μl of eluate on a ferrotype plate. Phosphatides were eluted from the column with methyl alcohol (100 to 150 ml). Weights of the fractions were recorded, the lipids were dissolved immediately in chloroform, and the solutions were stored in a deep freeze for further analyses.

Chromatographic Separation of Neutral Lipids.—It was possible to separate mixed neutral lipids into four classes, hydrocarbons, cholesterol esters, triglycerides, and sterol plus partial glycerides, by chromatography on silicic acid according to the procedure described by Horning *et al.* (1960). The weighed lipid (200–300 mg) was dissolved in 25 ml redistilled hexane and percolated onto a column (2.0 cm i.d.) containing 15 g of Unisil (100–200 mesh) suspended in hexane. Lipids were eluted from the column with (*A*) 6% benzene in redistilled hexane, 75 ml; (*B*) 20% benzene in hexane, 225 ml; (*C*) 65% benzene in hexane, 225 ml; and (*D*) pure chloroform, 120 ml. Fractions (15 ml) were collected in test tubes, dry weights of lipid in each tube were recorded, and the chromatographic separations were observed graphically by a plot of weight *versus* tube number (Fig. 1). The first pooled fraction, eluted by (*A*), containing hydrocarbons, was usually very small and was not further investigated. Pooled fractions eluted by (*B*), (*C*), and (*D*) were evaporated to dryness *in vacuo*, weights were recorded, and the samples were dissolved in hexane in volumetric flasks. Colorimetric determinations of ester and cholesterol were carried out on small aliquots and free fatty acid was determined in (*D*) by titration with 0.001 N sodium hydroxide (only traces were found).

As noted by Horning *et al.* (1960) the moisture content of the silicic acid is extremely important in the successful execution of this chromatographic separation. The moisture content of Unisil, as obtained from the manufacturer, was usually adequate for these separations with mixed solvents as given, but it is advisable to monitor the results by thin-layer chromatography. Slight overlaps may be avoided in subsequent analyses by minor adjustments of the compositions of the eluting solvents.

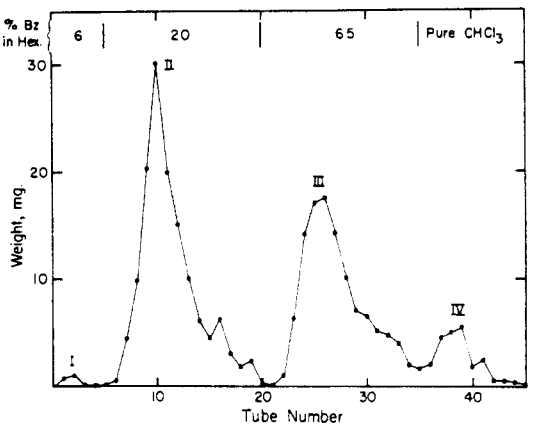


FIG. 1.—Silicic acid chromatography of neutral lipids from sample 92; hydrocarbons (I), cholesterol esters (II), triglycerides (III), and free sterol plus partial glycerides (IV) were obtained. Analyses of these fractions are summarized in Table II.

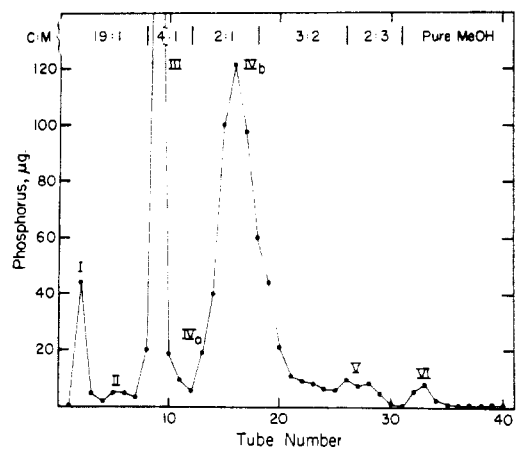


FIG. 2.—Silicic acid chromatography of phosphatides from sample 92. Identifications and yields of fractions I–VI are given in Table IV.

Fatty acids from cholesterol esters and triglycerides were determined as methyl esters by gas-liquid chromatography. The lipids (20 to 70 mg) were mixed with 90 ml of methyl alcohol containing 3% sulfuric acid and 10 ml of benzene. Complete methanolysis of the lipids was achieved by refluxing the mixture for 4 hours. After cooling, methyl esters were extracted with three portions (30 ml) of redistilled hexane. The combined extracts were washed with water, dried over anhydrous sodium sulfate, and taken to dryness *in vacuo*. Solutions of methyl esters in hexane, containing approximately 10 mg per ml, were analyzed by gas-liquid chromatography. The column, 6 ft. by 1/4 in. i.d., contained 15% ethylene glycol-succinate polyester coated on 80–100 mesh Chromosorb W, and was operated at 170° with an argon inlet pressure of 15 psi. A Barber-Colman Model 10 instrument, equipped with 2.5-cm argon ionization detectors containing 56 µc of Ra^{226}, was used for the analyses. Peaks were identified by comparisons of retention times with those of standards, and individual fatty acids were expressed in terms of area per cent of observed peaks on the chromatogram.

Chromatographic Separation of Phosphatides.—The procedure was based on established methods for silicic acid chromatography of phosphatides (Lea *et al.*, 1955; Hanahan, *et al.*, 1957; Zilversmit *et al.*, 1961). Four grams of Mallinckrodt silicic acid (100 mesh) or 7 g of Unisil (200–325 mesh) was dried in an oven at 135° for at least 18 hours just before use. The activated silicic acid, slurried in chloroform, was packed into a column (1 cm i.d.) under gravity-flow (or 2 to 5 psi in the case of Mallinckrodt silicic acid). A sample of mixed phosphatides, containing approximately 1 mg P, was dissolved in 50 ml of chloroform and percolated onto the column after an aliquot (0.2 ml) was removed for P assay. The order of elution and volumes of solvents were as follows: chloroform–methyl alcohol (CM) (19:1, v/v), 90 ml; CM (4:1), 75 ml; CM (2:1), 80 ml; CM (3:2), 110 ml; CM (2:3), 65 ml; and pure methyl alcohol, 70 ml. Fractions of 12 to 15 ml were collected in test tubes and measured aliquots from each tube were assayed for total P. Separations were presented graphically as total P *versus* tube number (Fig. 2).

Ion-Exchange Chromatography of Phosphorus-Containing Products of Mild Alkaline Hydrolysis of Phosphatides.—The procedure for mild alkaline hydrolysis of lipids, as developed by Dawson (1954) and modified by Hübscher *et al.* (1960), was used. A sample of mixed phosphatides, containing approximately 3 mg of P, was dissolved in 0.5 ml of chloroform–methyl alcohol (2:1, v/v) in a 50-ml centrifuge tube with glass stopper. The solution of lipid was mixed with 0.3 ml of 0.5 N sodium hydroxide in anhydrous methyl alcohol and the reaction mixture was allowed to stand at room temperature for about 15 minutes. After addition of 20 ml of distilled water and 20 ml of diethyl ether, the biphasic mixture was shaken and centrifuged. The ether layer was transferred to a flask and the aqueous layer was reextracted once with 15 ml of ether. The combined ether layers were washed with water, dried over anhydrous sodium sulfate, and saved for analyses of methyl esters and aldehydes (from plasmalogens).

The aqueous layer, containing water-soluble glyceryl-phosphoryl products from the methanolysis of alkali-labile lipids, was immediately neutralized with 1 ml of 0.1 M boric acid. The solution was transferred quantitatively to a 50-ml volumetric flask and diluted to the mark with redistilled water. Aliquots (0.5 ml) were removed for P analysis, and 45 ml of solution was mixed with 4 ml of 0.1 M sodium tetraborate and then diluted with redistilled water to 100 ml. This solution was the charge for ion-exchange chromatography.

A published procedure (Hawthorne and Hübscher, 1959) for ion-exchange chromatography of glyceryl-phosphoryl products of mild alkaline hydrolysis was scaled down for accurate microanalyses of mixtures containing from 1 to 4 mg of P. It was also necessary to extend the range of eluting buffers used by these authors in order to obtain complete resolution of the components.

The column consisted of a piece of 6-mm glass tubing, 15 in. long, to which were fused a Teflon stopcock and a 50-ml centrifuge tube with glass stopper. A small plug of glass wool was pushed into the bottom of the column, close to the stopcock, as a support for the resin. About 4 g of resin, Dowex 1 (chloride) (50–100 mesh), was washed twice in a beaker with 6 N hydrochloric acid, then with water until free of chloride ion. A slurry of the resin in redistilled water was poured into the column to a settled height of 22 cm. The resin was converted to the formate form by continuous washing with 3 M ammonium formate until negative tests for chloride ion were obtained in the eluate. Finally, the column was washed free of ammonium ion with 5 mM sodium tetraborate. Owing to shrinking and swelling of the resin after washing with buffers of different concentrations, the final bed varied in height from 18 to 20 cm.

The solution of products of mild alkaline hydrolysis,

n approximately 5 mM sodium tetraborate and containing no ammonium formate, was pipetted onto the column. Glycerylphosphorylcholine was not retained by the resin and was eluted during this initial charging of the column (see Fig. 3). The column was then eluted step-wise with the following mixtures of buffers: (*A*) 5 mM sodium tetraborate (STB)–10 mM ammonium formate (AF), 500 ml; (*B*) 5 mM STB–40 mM AF, 225 ml; (*C*) 5 mM STB–60 mM AF, 150 ml; (*D*) 5 mM STB–90 mM AF, 200 ml; (*E*) 5 mM STB–150 mM AF, 150 ml; (*F*) 5 mM STB–300 mM AF, 200 ml; and (*G*) 5 mM STB–600 mM AF, 200 ml. Fractions of 20 ml were collected for elutions with (*A*) through (*E*), and 25-ml fractions were collected with the last two buffers. Aliquots of 2 to 10 ml were withdrawn from each tube for P assay, the volume depending on the phosphorus content in each area as determined in a trial run. The analysis required from 40 to 60 hours and was carried out over a 5- to 7-day period with interruptions at night. A typical elution curve is shown in Figure 3.

The purified fractions, pooled on the basis of observed peaks as shown in Figure 3, were deionized, for paper chromatographic studies, in the following manner. With the exception of glycerylphosphorylcholine (I, Fig. 3), fractions were diluted with an equal volume of distilled water and percolated onto a Dowex 1 (formate) column (1 × 20 cm). The column was washed with about 100 ml of distilled water to remove all sodium tetraborate while leaving the glycerylphosphoryl product. The absorbed product was then eluted from the resin with 50 ml of ammonium formate solution of twice the original molarity required to elute the peak. For example, to recover the product initially eluted by (*D*), 5 mM sodium tetraborate–90 mM ammonium formate, 50 ml of 180 mM ammonium formate solution was used. Most of the water was removed *in vacuo* at 40°, after which the solution was frozen and lyophilized under high vacuum. During the process of lyophilization ammonium formate was sublimed from the mixture. It was necessary to add a small volume of water and repeat lyophilization several times to remove all of the ammonium formate. The recovered product was dissolved in 0.5 ml of 50% ethyl alcohol for paper chromatographic identification. In extremely small samples traces of ammonium formate tended to interfere during paper chromatography.

The initial fraction could not be purified in the same way, since glycerylphosphorylcholine was not retained by Dowex 1 (formate) columns. This solution was percolated through a column of IRC-50 Amberlite cation resin (H form) to remove sodium ions. One drop of 3 N ammonium hydroxide was added to the eluate and the solution was taken to dryness *in vacuo*. The residue was dissolved in 0.5 ml of 50% ethyl alcohol for paper chromatography.

The procedure for mild alkaline hydrolysis and ion-exchange chromatography, described above, was also applied to purified phosphatides obtained by silicic acid chromatography. In these cases some of the eluting buffers were omitted, depending on the nature of the phosphatide under investigation. Typical curves, showing the products obtained from phosphatidic acid, phosphatidylethanolamine, and phosphatidylcholine fractions, are shown in Figure 4.

Authentic samples of glycerophosphoric acid (GPA), glycerylphosphorylinositol (GPI), glycerylphosphorylinositol phosphate (GP_2I), and glycerylphosphorylinositol diphosphate (GP_3I) were individually carried through the elution scheme on ion-exchange columns to establish their positions of elution for purposes of identification (Fig. 5).

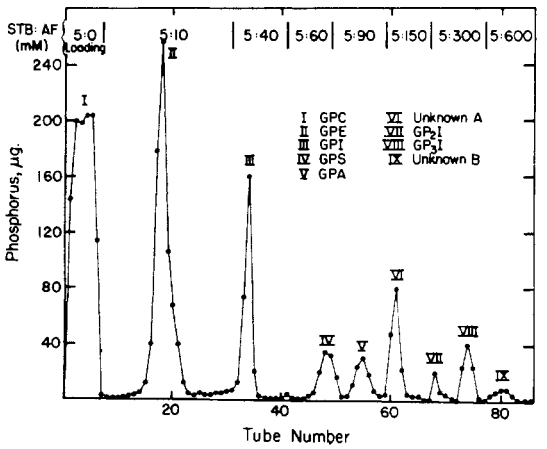


FIG. 3.—Dowex 1 (formate) chromatography of mild alkaline hydrolysate from total phosphatides of sample 92. STB and AF refer to sodium tetraborate and ammonium formate. Identifications and yields of the fractions are given in Table VI.

Paper Chromatography of Glycerylphosphoryl Products of Mild Alkaline Hydrolysis.—Individual fractions from ion-exchange columns, deionized as described previously, total hydrolysates, and authentic samples were chromatographed on Whatman No. 1 paper, prewashed with acetic acid, in three solvent systems (Dawson, 1960; Wheeldon, 1960). These systems were (I) phenol saturated with water–NH_3 (100:0.1, v/w), (II) phenol saturated with water–acetic acid–ethyl alcohol (100:10:12, v/v), and (III) methyl alcohol–98% formic acid–water (80:13:7, v/v). The papers were developed by ascending chromatography for 17 to 18 hours (systems I and II) or for 7 to 8 hours (system III) at room temperature. Chromatograms were dried in a hood for 12 to 24 hours. With the phenol-containing systems, air-dried chromatograms were washed once with dry diethyl ether. Ninhydrin was used to locate products containing an amino group, and an ammonium molybdate spray was used to locate phosphorus-containing components (Dawson, 1960). Chromatograms once sprayed with ninhydrin could not be sprayed a second time with the molybdate reagent because the background turned blue so rapidly that trace spots could not be detected.

Analysis of Aldehydes Derived from Plasmalogens.—The procedure described by Dawson (1954) was followed for these analyses, but they were not carried out routinely since the level of plasmalogens in canine adrenal phosphatides was low. A sample of total adrenal phosphatides was subjected to mild alkaline hydrolysis and the alkali-stable lipids were extracted along with methyl esters into diethyl ether. The lysoplasmalogens were purified by silicic acid chromatography, and aldehydes were liberated by mild acid hydrolysis. Alternatively, aldehydes were liberated from total phosphatides by mild acid hydrolysis (Dawson, 1954). Aldehydes were analyzed by gas-liquid chromatography before and after hydrogenation on the same columns that were used for methyl esters.

Positional Analysis of Methyl Esters of Phosphatidylcholine with Phospholipase A.—A solution of 20 mg of lyophilized venom from *Crotalus adamanteus* in 0.1 ml of 0.1 M calcium chloride and 0.2 ml of isotonic saline solution was adjusted to *p*H 7.2 to 7.6 with 0.1 N sodium hydroxide[3] (Hanahan *et al.*, 1954). A solution of phosphatidylcholine (up to 50 mg) in 10 ml

[3] These conditions were described in a personal communication from M. Rodbell.

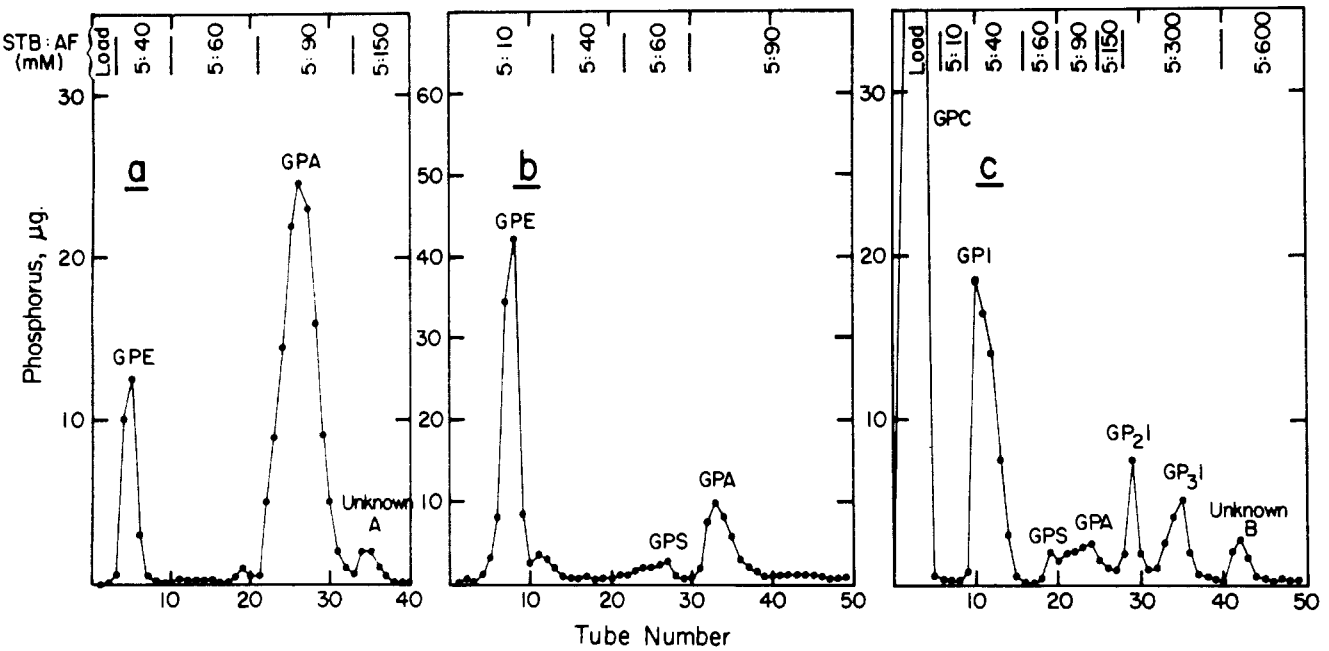


FIG. 4.—Dowex 1 (formate) chromatography of mild alkaline hydrolysates from fractions 134-I (*a*), 134-III (*b*), and 134-IV (*c*) obtained by silicic acid chromatography of adrenal phosphatides (see Table IV). The results for the phosphatidylcholine fraction (*c*) are presented on an expanded scale for clarity; the GPC peak accounted for 80 per cent of total phosphorus.

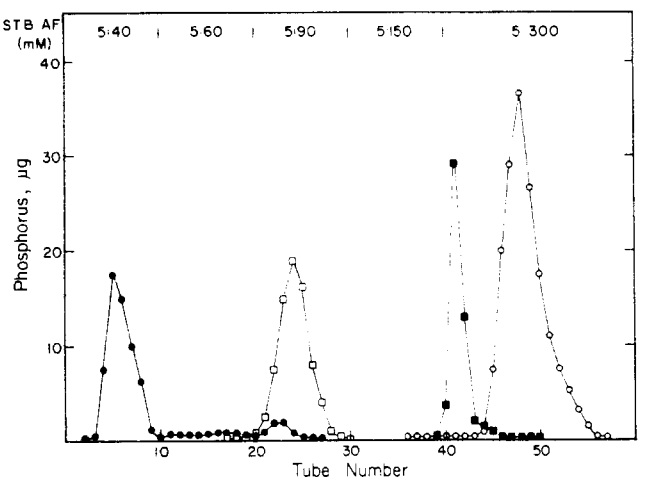


FIG. 5.—Dowex 1 (formate) chromatography of authentic glycerylphosphorylinositol (●), α-glycerophosphoric acid (□), glycerylphosphorylinositol phosphate (■), and glycerylphosphorylinositol diphosphate (○).

of diethyl ether was added, and the mixture was shaken vigorously and allowed to stand for 18 hours at room temperature. The solvent was removed under a stream of nitrogen, and the residue was extracted twice with 10 ml of chloroform–methyl alcohol (2:1, v/v). The combined extracts were evaporated to dryness *in vacuo* and the residue, dissolved in chloroform, was percolated onto a column containing 5 g of Unisil (100–200 mesh). Free fatty acids, liberated from phosphatidylcholine by the phospholipase A, were eluted with 50 ml of chloroform. The residue of fatty acids after evaporation of solvent was converted to methyl esters by the procedure described by Radin *et al.* (1960), and the methyl esters were analyzed by gas-liquid chromatography.

RESULTS

Yields of total lipids, neutral lipids, and phosphatides from several analyses of canine adrenal glands are given in Table I. In general the wet weight of a pair of glands ranged from 1.5 to 3.0 g and yields of total lipids varied from 0.3 to 0.8 g. It was observed that, regardless of the size of the adrenal gland, the average yield of total phosphatides was approximately 10% of the total lipid.

TABLE I

DISTRIBUTION OF NEUTRAL LIPID AND PHOSPHATIDES IN CANINE ADRENAL GLAND

Sample	Pairs of Glands Used	Total Lipid (mg)	Neutral Lipid (mg)	Phosphatide (mg)	Phosphorus (µg per mg Phosphatide)
641	1	800	710	82	27
633	1	550	470	60	31
92	5	2150	1900	210	36
134	4	1550	1300	155	29
574	1	307	279	29	—
136	2	739	704	56	—

Neutral Lipids.—Table II summarizes the results from silicic acid chromatography of three samples of neutral lipids; a typical pattern of elution is shown in Figure 1. Hydrocarbons constituted a very small percentage of the total lipid, and no further studies were made of this material. The fraction (IV) containing sterol, partial glycerides, and traces of free fatty acids has not been characterized except for colorimetric analyses of ester and cholesterol and titrations for free fatty acids. Since this fraction probably contained both mono- and diglycerides, gas-liquid chromatography analyses of methyl esters were not done.

Cholesterol esters and triglycerides together represented 90 to 95% of neutral lipids, and these fractions were examined in some detail. Colorimetric determinations of cholesterol and ester, as given in Table II, indicated that the products were nearly pure. In later experiments proof of purity also rested on exami-

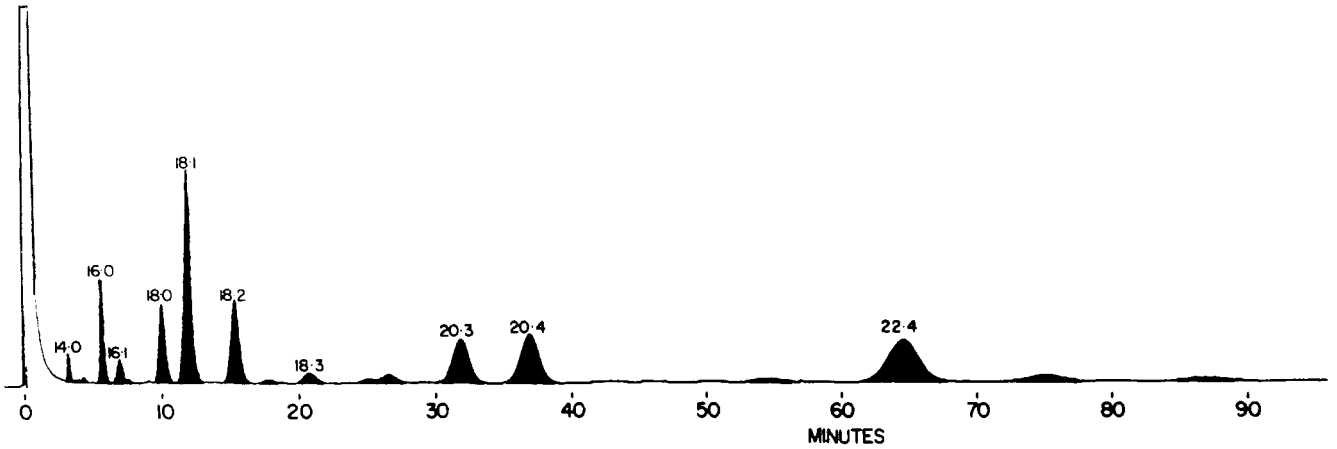


FIG. 6.—Gas-liquid chromatographic analysis of methyl esters from cholesterol esters (sample 574).

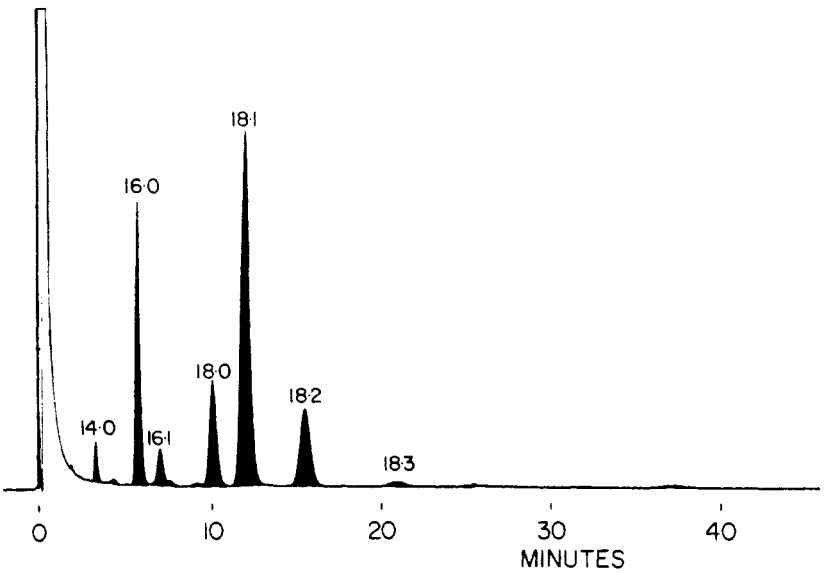


FIG. 7.—Gas-liquid chromatographic analysis of methyl esters from triglycerides (sample 574).

nations of the products by thin-layer chromatography in petroleum ether–diethyl ether–acetic acid (80:20:1, v/v).[4]

Results of fatty acid analyses of the cholesterol esters and triglycerides, by gas-liquid chromatography of methyl esters isolated after methanolysis of the lipids, are given in Table III. The fatty acids esterified with cholesterol were mostly unsaturated; of these the dominant component was always oleic acid, with lower proportions of linoleic, arachidonic, adrenic, and eicosatrienoic acids in that order. The triglyceride fraction contained mainly oleic acid (about 50 per cent) and palmitic acid (about 20 per cent). Typical chromatographic recordings of the methyl esters from cholesterol esters and triglycerides are presented in Figures 6 and 7.

Phosphatides.—It was necessary to employ several methods to characterize the complex mixture of phosphatides isolated from canine adrenal gland. Exploratory studies of silicic acid columns indicated that the mixture could be separated easily into five fractions classically assigned as phosphatidic acid (or cardiolipin), phosphatidylethanolamine, phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine (see Fig. 2). The yields of various phosphatides obtained from silicic acid chromatography of four samples are summarized in Table IV.

[4] A modification of the solvent system described by Mangold (1961), made by M. Nichaman in this laboratory.

TABLE II
ANALYSIS OF CANINE NEUTRAL LIPIDS BY SILICIC ACID CHROMATOGRAPHY

Sample	641	92	574
Charge, mg	224	285	279
Hydrocarbons, mg	1.4	1.8	—
Cholesterol esters, mg	133	139	100
Total cholesterol, μmoles	169	167	132
Total ester, μmoles	135	173	132
Molar ester–cholesterol	0.8	1.04	1.00
Triglycerides, mg	69	113	132
Total cholesterol, μmoles	0.2	0.2	0.2
Triglyceride (estimated),[a] μmoles	80	132	149
Triglyceride (found), μmoles	85	128	152
Ester, estimated/found	1.06	0.97	1.02
Sterol, partial glycerides, mg	13.5	22.2	—
Total cholesterol, μmoles	11.7	14.8	—
Total ester, μmoles	21.7	25.5	—
Free fatty acid	Trace	Trace	—

[a] Estimated triglyceride was calculated from observed weights and the molecular weight of dioleopalmitin. Triglyceride found was calculated from colorimetric ester analyses.

The results obtained by silicic acid chromatography on columns were duplicated when the mixture was studied by thin-layer chromatography on silica gel. The chromatogram shown in Figure 8 was made with

TABLE III

ANALYSES OF METHYL ESTERS OF FATTY ACIDS DERIVED FROM CHOLESTEROL ESTERS AND TRIGLYCERIDES[a]

Fatty[b] Acid	Sample 641		Sample 92		Sample 574	
	Cholesterol Esters	Triglycerides	Cholesterol Esters	Triglycerides	Cholesterol Esters	Triglycerides
14:0	1.1	2.3	1.5	2.8	0.9	1.9
15:0	Trace	1.7	1.1	Trace	—	—
16:0	7.6	17.9	9.3	19.2	6.4	21.3
16:1	3.5	9.5	3.1	5.4	1.6	3.6
18:0	5.5	8.9	7.2	7.1	7.1	11.2
18:1	31.5	50.1	35.1	50.2	22.9	49.5
(18:2)	16.6	9.0	14.1	13.6	13.2	12.5
(18:3)	3.0	Trace	1.3	Trace	1.1	—
20:3	8.8	—	7.3	—	11.0	—
20:4	11.8	—	13.5	—	13.3	—
22:4	9.1	—	6.5	—	19.0	—
(22:5)	1.1	—	Trace	—	2.6	—
Others	0.4	0.6	—	1.7	0.9	—

[a] Composition is expressed in terms of area per cent. [b] C:X indicates carbon chain length and degree of unsaturation. Acids for which positional analyses of double bonds have not been carried out are indicated by parentheses.

TABLE IV

SILICIC ACID CHROMATOGRAPHY OF CANINE ADRENAL PHOSPHATIDES

Fraction	Solvent (C:M)[a]	Components	Distribution of P (Weight %) 92[b]	134[b]	136	574
I	19:1	Phosphatidic acid Cardiolipin	5.0	5.6	6.7	4.6
II	19:1	Unknown	1.3	0.5	0.8	1.1
III	4:1	Phosphatidylethanolamine Phosphatidylserine (trace)	35.0	33.0	37.8	39.3
IVa	2:1	Phosphatidylinositol (major) Phosphatidylcholine (minor)	6.0	5.7	3.0	3.7
IVb	2:1 3:2	Phosphatidylcholine (major) Phosphatidylinositol (minor) Lysophosphatidylserine (minor)	44.8	45.0	43.6	44.3
V	2:3	Sphingomyelin (major) Phosphatidylcholine (minor)	3.5	2.7	5.7	4.9
VI	Pure MeOH	Lysophosphatidylcholine	1.6	1.0	2.5	1.9

[a] Chloroform–methyl alcohol. [b] All fractions recovered from these samples were individually subjected to mild alkaline hydrolysis and the products were identified by paper chromatography.

chloroform–methyl alcohol–water (100:40:6, v/v) as solvent in a lined chamber; the major improvement over column chromatography was the resolution of A and B, which were eluted together as fraction 1 on silicic acid columns. Thin-layer chromatography was useful, however, as a routine and rapid ancillary technique for monitoring separations on silicic acid columns.

To determine the extent of purification of individual phosphatides obtained by silicic acid chromatography, each of the fractions isolated from samples 92 and 134 (Table IV) was subjected to mild alkaline hydrolysis and the water-soluble products were identified by paper chromatography and in some cases by ion-exchange chromatography. The R_F values of these glycerylphosphoryl products, together with the results obtained with standards and published values (Dawson, 1960; Wheeldon, 1960), are given in Table V. The first fraction was shown to consist of a mixture of phosphatidic acid and an unknown tentatively identified as cardiolipin. While both lipids were present in samples 92 and 134, the proportions varied greatly; cardiolipin was the predominant lipid in 92, but the mixture from 134 consisted almost entirely of phosphatidic acid as shown by ion-exchange chromatography (Fig. 4*A*).

Fraction III (Table IV) was essentially pure phosphatidylethanolamine, but hydrolysates always contained a trace of glycerylphosphorylserine, presumably representing a small quantity of phosphatidylserine in the intact lipid. Mild alkaline hydrolysis also gave an additional product in small yield, corresponding to glycerophosphoric acid on paper chromatograms and ion-exchange columns (Fig. 4*B*); we attribute this to the alternate mechanism for methanolysis of phosphatidylethanolamine and/or phosphatidylserine (Dawson, 1954; Maruo and Benson, 1959).

Fraction IVa was usually observed as a shoulder on the lecithin peak (Fig. 2) and was found to be a mixture of phosphatidylinositol and phosphatidylcholine. Efforts to resolve these components completely by silicic acid were not successful, but analysis of the mixture could be made by ion-exchange chromatography of the mild alkaline hydrolysate.

Fraction IVb consisted mainly of phosphatidylcholine; this component was always contaminated by

TABLE V

PAPER CHROMATOGRAPHIC IDENTIFICATION OF GLYCERYLPHOSPHORYL PRODUCTS FROM MILD ALKALINE HYDROLYSIS OF ADRENAL PHOSPHATIDES

Product	Phenol-H_2O-NH_3			Phenol-H_2O-AcOH-EtOH			MeOH-HCOOH-H_2O		
	Wheeldon (1960)[a]	Silicic Acid Fraction[b]	Dowex Fraction[b]	Dawson (1960)[a]	Silicic Acid Fraction[b]	Dowex Fraction[b]	Dawson (1960)[a]	Silicic Acid Fraction[b]	Dowex Fraction[b]
GPA	0.25 (0.26)	0.25 (I)	—	0.33 (0.38)	0.39 (I)	—	0.74 (0.73)	0.70 (I)	—
polyGPA	0.13	0.10 (I)	0.04–0.21 (VI)	0.28	0.26 (I)	—	0.60	0.62 (I)	—
GPE	0.67	0.64 (III)	0.65 (II)	0.63	0.64 (III)	—	0.47	0.50 (III)	—
GPC	0.90	0.90 (IVa, IVb, VI)	0.90 (I)	0.77	0.82 (IVa, IVb)	—	0.67	0.69 (IVb, VI)	—
GPI	0.09 (0.07)	0.10 (IVa)	0.09 (III)	0.20 (0.23)	0.23–0.28 (IVa)	—	0.45 (0.50)	0.50 (IVa)	—
GP_2I	— (0.03)	—	—	— (0.25)	—	—	— (0.57)	0.58 (IVb)	—
GP_3I	— (0.03)	—	—	— (0.26)	—	—	— (0.60)		
GPS	0.20	0.23 (IVb)	0.25 (IV)	0.30	0.26 (IVb)	—	0.50	0.50 (IVb)	—

[a] Figures in parentheses refer to R_F values obtained in this laboratory with standards. [b] Numbers in parentheses refer to fractions from silicic acid (Table IV) or from anion exchange column (Table VI).

FIG. 8.—Thin-layer chromatogram of canine adrenal phosphatides on silica gel. Spots *A* and *B*, phosphatidic acid and cardiolipin (order not determined); *C*, phosphatidylethanolamine; *D*, phosphatidylinositol; *E*, phosphatidylcholine (plus a serine-containing phosphatide and inositides); *F*, sphingomyelin; *G*, lysophosphatidylcholine.

phosphatidylserine, or more likely lysophosphatidylserine, and three phosphoinositides (see Fig. 4*C*). We thought perhaps metallic ions such as Ca^{++}, Mg^{++}, or Na^{+} may have been present as salts of some phosphatides and that this factor contributed to tailing and overlapping on the column. Attempts were made to improve the resolution by treatment of the lipids with carboxymethylcellulose prior to silicic acid chromatography, to convert all phosphate to the free acid, but this procedure led to little enhancement of the separations. Nor was a difference observed when mixed phosphatides were washed with dilute acid before chromatography, a technique which was useful in the purification of lysophosphatidylserine (Rathbone *et al.*, 1962).

Fraction V was composed of a mixture of sphingomyelin and phosphatidylcholine, in approximately

TABLE VI
ANALYSES OF CANINE ADRENAL PHOSPHATIDES BY ANION EXCHANGE CHROMATOGRAPHY OF PRODUCTS OF MILD ALKALINE HYDROLYSIS

Fraction	Eluant STB:AF[a] mM	Component	Parent Lipid	Distribution of P[b] (weight %) 92	134
I	5:0	GPC	Phosphatidylcholine and lysophosphatidylcholine	37.0	34.0
II	5:10	GPE	Phosphatidylethanolamine	24.0	28.0
III	5:40	GPI	Phosphatidylinositol	8.7	6.3
IV	5:60	GPS	Phosphatidylserine and lysophosphatidylserine	3.7	3.3
V	5:90	GPA	Phosphatidic acid and other phosphatides	3.2	5.2
VI	5:150	polyGPA	Cardiolipin	5.5	5.6
VII	5:300	GP_2I	Diphosphoinositide	1.1	1.0
VIII	5:300	GP_3I	Triphosphoinositide	3.0	2.6
IX	5:600	Unknown B[c]	—	1.1	1.1

[a] STB is sodium tetraborate and AF is ammonium formate. [b] Total recovery of P averaged 86%. [c] Unknown B is tentatively assumed to be inositol triphosphate.

equal amounts. This was demonstrated by paper chromatography of the mild alkaline hydrolysate, which gave only glycerylphosphorylcholine, and by recovery of alkali-stable, solvent-soluble P after the mild alkaline hydrolysis. Further, thin-layer chromatography of the fraction always gave a spot which corresponded to synthetic sphingomyelin.

Hydrolysis of the final fraction obtained by silicic acid chromatography (fraction VI) produced a single substance which corresponded to glycerylphosphorylcholine, the expected product from lysophosphatidylcholine, on paper chromatograms. This identification must remain tentative, however, because low yields precluded a study of ester-to-phosphorus ratio necessary to establish the fact that it was a lysophosphatide.

Ion-Exchange Chromatography.—The results of ion-exchange chromatography of the products of mild alkaline hydrolysis of total phosphatides are presented in Table VI, and a typical elution pattern is shown in Figure 3. Identifications of some of the fractions were made by actual comparisons with the elution patterns of standards (glycerophosphoric acid and the glycerylphosphoryl esters of inositol, inositol phosphate, and inositol diphosphate) as shown in Figure 5. Some of the fractions, for which standards were not available in this laboratory, were identified on the basis of comparisons with the results reported by Hübscher *et al.* (1960) and on the basis of comparisons of R_P values on paper chromatograms with published values (Dawson, 1960; Wheeldon, 1960) (see Table V). Fractions I, II, III, and IV (Table VI and Fig. 3) were thus shown to be glycerylphosphoryl esters of choline, ethanolamine, inositol, and serine, respectively. Identification of fractions VII and VIII, assumed to be the glycerylphosphoryl esters of inositol phosphate and inositol diphosphate, was based mainly on the results of ion-exchange chromatography; paper chromatography was not entirely satisfactory because of the very low recovery of these products.

Fraction VI has not been completely characterized but must result from mild alkaline hydrolysis of polyphosphatidylglycerol (cardiolipin). Hübscher *et al.* (1960) observed a similar peak which was labeled as an unknown. That this substance resulted from the hydrolysis of cardiolipin was concluded on the basis of the following evidence. Partial hydrolysis of the phosphatides eluted from silicic acid by chloroform–methyl alcohol (19:1), fraction I in Figure 2, gave a mixture of water-soluble products, of which one was identical with glycerophosphoric acid while the other gave R_P values identical with those reported for polyglycerophosphate in three solvent systems (Dawson, 1960; Wheeldon, 1960). Chromatography of this mixture on Dowex 1 (formate) gave a peak which corresponded to glycerophosphoric acid and one which corresponded with fraction VI (see Fig. 4*A*). Similar studies of the phosphatidylethanolamine and phosphatidylcholine fractions did not produce any of this component.

Some comparisons may be made between the results obtained by silicic acid chromatography and those of ion-exchange chromatography. The combined yields of phosphatidylcholine, phosphatidylserine, the inositides, and lysophosphatidylcholine, fractions IVa, IVb, and VII (Table IV), from silicic acid should be the same as the yields of P in the partial hydrolysis products from these lipids, fractions I, III, IV, VII and VIII (Table VI), as obtained by ion-exchange chromatography. In both cases the yields were about 50% of total P and thus agreed reasonably well. The yields of glycerophosphoric acid and polyglycerophosphoric acid (fractions V and VI, Table VI) were higher than the yields of phosphatidic acid and cardiolipin (fraction I, Table IV), however, and it was concluded that a portion of the glycerophosphoric acid resulted from partial hydrolysis of other phosphatides, as described by Dawson (1954). The yield of phosphatidylethanolamine from silicic acid differed from the recovery of glycerylphosphorylethanolamine from the ion-exchange column by approximately the same amount as the discrepancy in yields of glycerophosphoric acid and phosphatidic acid, and it may be that most of the latter difference can be accounted for by partial hydrolysis of phosphatidylethanolamine to glycerophosphoric acid.

Fatty acids were liberated from phosphatides as methyl esters by direct methanolysis under previously described conditions for mild alkaline hydrolysis in anhydrous chloroform–methyl alcohol. The results of analyses of these mixtures by gas-liquid chromatography are given in Table VII.

The fatty acids of fraction I, containing cardiolipin and phosphatidic acid in variable proportions, were largely unsaturated and were exceptionally rich in linoleic acid, which accounted for nearly half of the total quantity isolated. It had been demonstrated that fraction I from sample 92 contained mainly cardiolipin, while that from 134 was almost entirely phosphatidic acid. This difference was reflected in the observed fatty acid composition, since it was found that 134 contained nearly three times more arachidonic

acid than 92. The fatty acid pattern found in 574 agreed closely with that of 92, suggesting that this sample, too, was mostly cardiolipin. A typical gas chromatographic record of methyl esters from fraction I is reproduced in Figure 9.

Phosphatidylethanolamine (fraction III) contained approximately equal proportions of saturated and unsaturated fatty acids. Little variation in composition was observed in this mixture, which was composed mainly of stearic, arachidonic, and adrenic acids. The remarkable simplicity of this fraction is readily observed in the chromatographic record (Fig. 10).

Fraction IVa, a mixture of phosphatidylinositol and phosphatidylcholine, contained a mixture of fatty acids in proportions quite unlike those of neighboring fractions (III and IVb), but little comment can be made about the fatty acids of phosphatidylinositol until further purification has been achieved.

Fraction IVb was not pure, but the fatty acids reflected primarily the nature of phosphatidylcholine, since this lipid accounted for about 80% of the total P. Nearly equal proportions of saturated and unsaturated fatty acids were found and palmitic, stearic, oleic, and arachidonic acids were present in substantial concentrations. When this fraction was treated with phospholipase A, fatty acids liberated from the β position (Tattrie, 1959) were almost entirely unsaturated, a finding which agrees with the results obtained routinely in the hydrolysis of lecithin from other sources. A typical pattern of fatty acids of lecithin is shown in Figure 11.

Lysophosphatidylcholine was surprisingly rich in unsaturated fatty acids, a finding which was characteristic of all samples examined. In one case (574) arachidonic acid accounted for 36% of the total fatty acid. These results suggest that lysophosphatidylcholine bears no simple metabolic relationship to adrenal lecithin, particularly in view of marked differences in palmitate levels in the two lipids.

Discussion

Evidence has been presented that canine adrenal gland contains a complex mixture of lipids, of which cholesterol esters and triglycerides are the predominant types. The pool of cholesterol esters stored in this tissue ranged from 85 mg per g of wet tissue to as much as 140 mg. This variation was greater than was expected and may reflect variations in the physiological status of the dog at the time of sacrifice. It has been well documented that stress causes rapid depletion of adrenal cholesterol (Sayers and Sayers, 1948). While free cholesterol was not examined in detail, two samples contained only 6.5 and 8.2% of total cholesterol as free sterol, confirming previous reports (Cook, 1958) that adrenal cholesterol exists almost entirely in the form of esters. The cholesterol esters contained a greater variety of fatty acids than was observed in any other adrenal lipid. The results shown in Table III indicate the major acids, but there were a number of additional components at a trace level, some of which are barely visible in Figure 6. In general the levels of various fatty acids in cholesterol esters were comparable to those reported by Dailey *et al.* (1960), with the exception that we found consistently higher proportions of adrenic acid.

Apparently all of the fatty acids involved as intermediates in the conversion of linoleic to arachidonic acid (Mead and Howton, 1957) are included as components of the cholesterol esters. The presence of significant quantities of adrenic acid, 7,10,13,16-docosatetraenoic acid, suggests that this enzymatic

Table VII
Analyses of Methyl Esters Derived from Individual Adrenal Phosphatides Isolated by Silicic Acid Chromatography

Fraction	I			II		III		IVa			IVb			VI	
Major Component	Phosphatidic Acid and Cardiolipin			Unknown		Phosphatidyl-ethanolamine		Phosphatidylinositol and Phosphatidylcholine			Phosphatidylcholine			Lysophos-phatidylcholine	
Sample	92	134	574	92	134	92	134	574	92	134	574	92	134	574	92	134	574
Fatty Acid																	
14:0	1.5	0.7	1.1	1.2	1.5	—	—	—	1.0	1.5	1.0	1.8	1.2	—	2.5	2.3	—
15:0	3.0	Trace	—	1.0	2.3	—	1.6	—	1.0	2.0	—	—	—	—	1.2	1.0	—
16:0	11.5	6.5	13.8	8.1	9.8	—	7.2	7.4	5.0	5.4	4.7	2.9	45.2	40.8	38.6	18.2	13.5	18.0
16:1	2.0	2.2	1.1	—	1.7	—	2.3	1.5	1.4	—	—	—	—	1.1	0.6	5.6	4.2	—
18:0	4.8	2.1	4.6	21.2	25.2	—	32.5	34.7	33.6	43.0	45.1	45.2	10.5	11.2	13.1	16.7	9.1	13.5
18:1	20.0	20.0	18.3	14.7	13.6	—	8.6	6.8	7.2	6.6	3.6	3.1	16.3	17.5	18.1	24.4	36.5	14.8
(18:2)	45.2	46.0	45.1	19.4	15.4	—	2.9	2.5	2.1	1.4	1.9	1.7	6.0	4.1	5.1	5.6	5.1	7.1
(20:2)	1.4	2.4	2.0	1.8	2.5	—	—	—	0.2	—	—	—	—	1.0	1.4	—	1.1	1.2
20:3	2.3	3.8	3.4	5.0	4.4	—	2.0	2.1	2.3	1.8	1.5	1.5	1.4	2.5	2.0	10.8	5.2	2.3
20:4	4.3	5.2	5.3	15.3	12.3	—	30.8	31.6	35.3	37.6	34.2	35.8	14.3	15.8	17.4	11.5	18.2	35.7
22:4	4.0	11.1	5.3	12.3	11.7	—	13.7	11.8	12.9	2.2	5.5	8.8	4.5	4.8	3.6	3.5	3.8	7.4
Total saturated	21	9	20	32	39	—	40	44	40	50	53	49	57	53	52	39	26	32
Total unsaturated	79	91	80	68	61	—	60	56	60	50	47	51	43	47	48	61	74	68

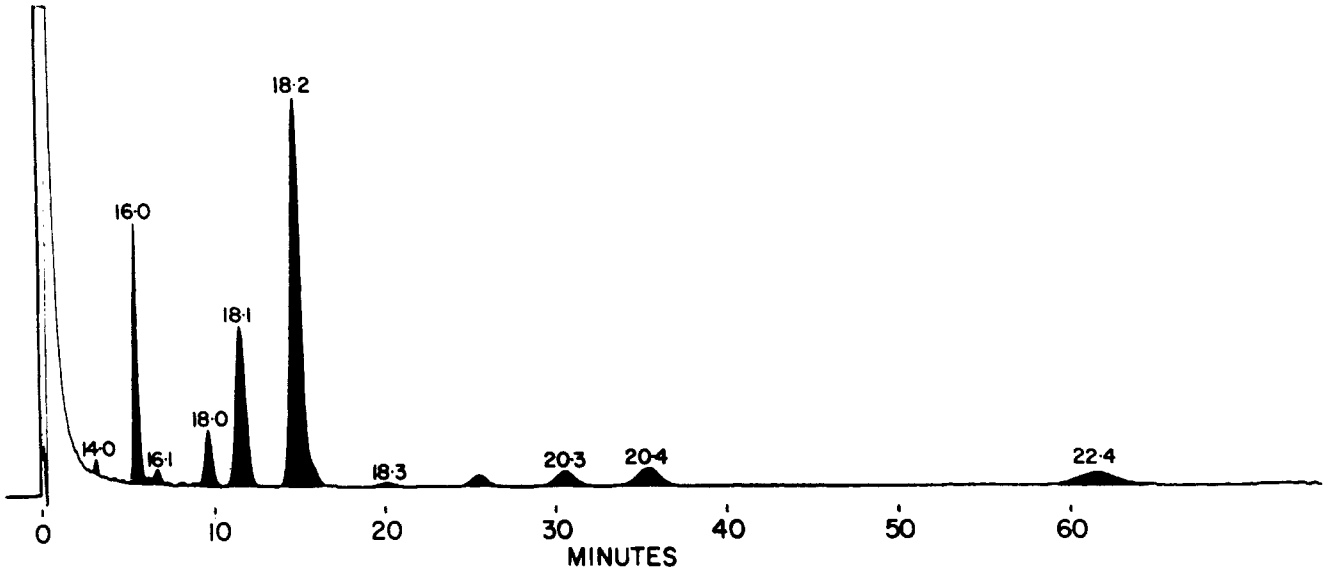


FIG. 9.—Gas-liquid chromatographic analysis of methyl esters of phosphatidic acid and cardiolipin (fraction I, sample 574)

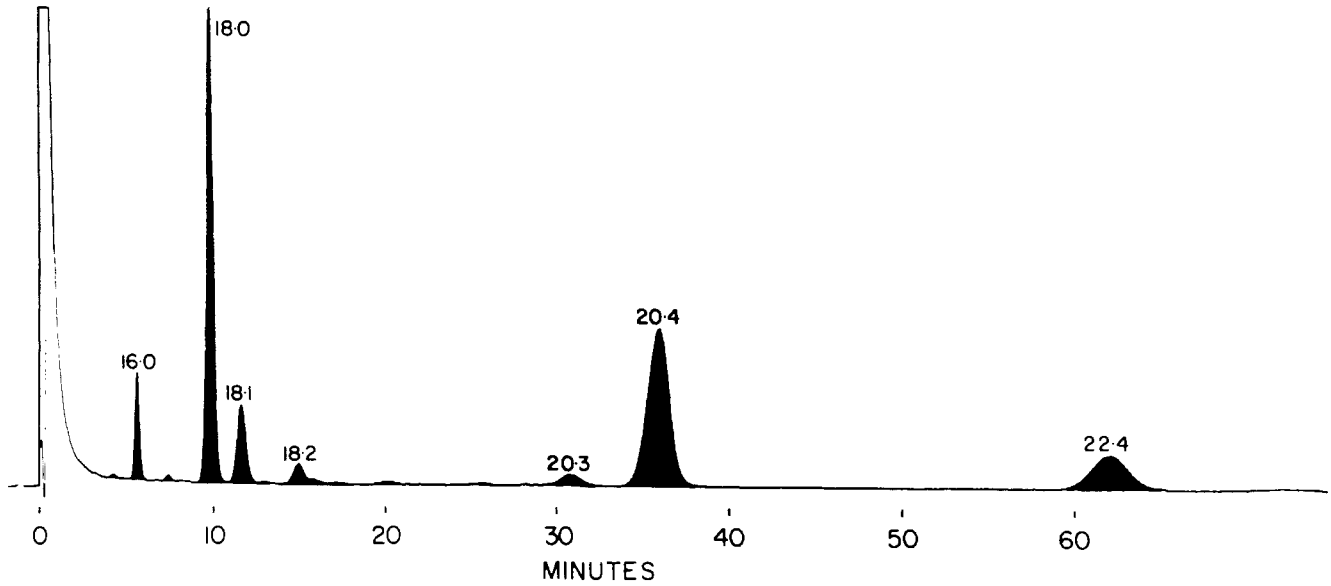


FIG. 10.—Gas-liquid chromatographic analysis of methyl esters from phosphatidylethanolamine (fraction III, sample 574)

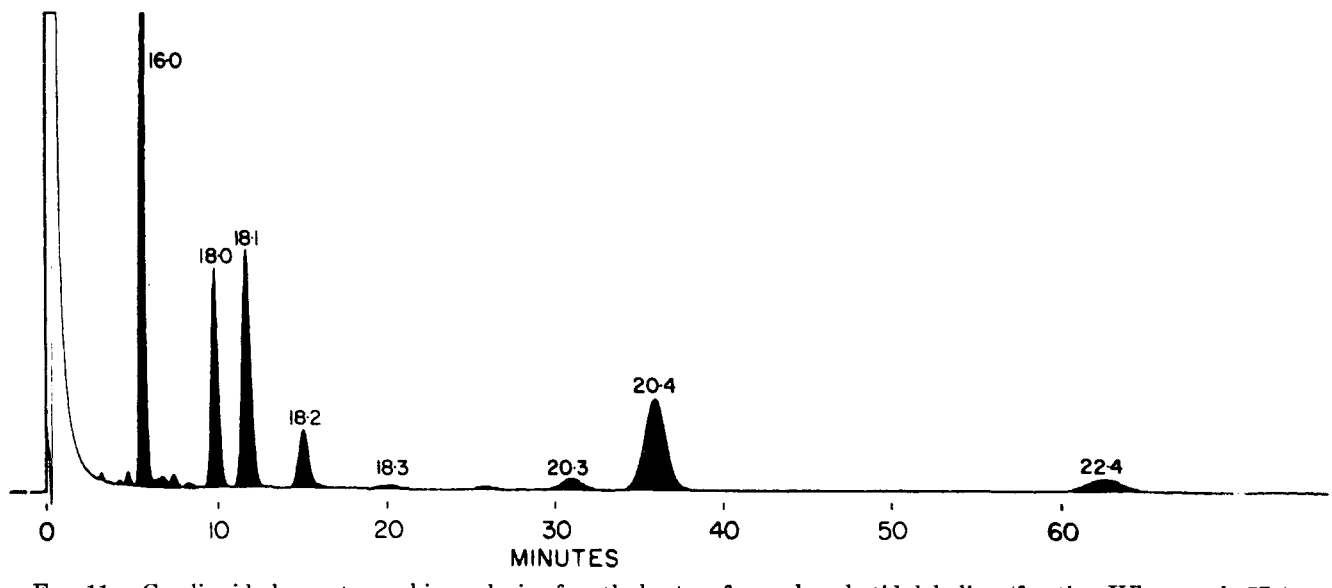


FIG. 11.—Gas-liquid chromatographic analysis of methyl esters from phosphatidylcholine (fraction IVb, sample 574).

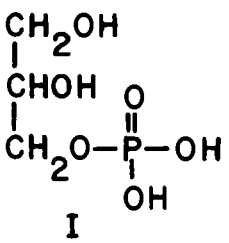
process proceeds one step further in adrenal gland by the addition of a C_2 unit to the carboxyl-terminal end of arachidonic acid.

Triglycerides, like cholesterol esters, represented a major proportion of total lipid. Individual variation in yields was observed but may be of little significance because extraglandular fat might not have been removed entirely in some cases. In contrast to the cholesterol esters this fraction contained almost none of the C_{20}- and C_{22}-polyenoic acids which accounted for 30 to 40% of the fatty acids esterified with cholesterol.

The phosphatides from adrenal tissue presented a more difficult problem of isolation and characterization than did the neutral lipids. Following more or less standard methods of silicic acid chromatography, it was not possible to obtain complete resolution of the mixture. It was evident from the earliest studies made that phosphatidylethanolamine and phosphatidylcholine were the major constituents, with much lower levels of what appeared to be phosphatidic acid, sphingomyelin, and lysophosphatidylcholine. These conclusions were later supported by the results of thin-layer chromatography on silica gel. Examination of the products of mild alkaline hydrolysis by ion-exchange chromatography confirmed the identifications made by silicic acid chromatography and provided evidence for several additional components. Identifications were made, and confirmed by paper chromatography, of the deacylated products of a serine-containing phosphatide and phosphatidylinositol. Two additional components were identified tentatively as the deacylated products of diphosphinositide and triphosphoinostide.

Some doubt remains concerning the nature of the serine-containing phosphatide eluted with phosphatidylcholine. Rathbone (1962) and others (Marinetti *et al.*, 1958; Rouser *et al.*, 1961) have shown that salts of phosphatidylserine are adsorbed somewhat more strongly to silicic acid than the zwitterionic form of the lipid and are eluted with phosphatidylcholine rather than with phosphatidylethanolamine, as is the case generally. That this explanation could account for our results seemed doubtful, since prior chromatography of the phosphatides on carboxymethylcellulose failed to alter the pattern of elution of the serine-containing phosphatide. We are inclined to believe that the fractions contained lysophosphatidylserine, which one would expect to be eluted with phosphatidylcholine, according to recent studies (Rathbone *et al.*, 1962).

Cardiolipin has been identified tentatively as a constituent of the first fraction from silicic acid columns. This component presented an interesting problem. In general, the products of mild alkaline hydrolysis were eluted from Dowex 1 (formate) in order of decreasing pK_a (or pI in the case of zwitterionic products), as one would expect. Since the polyglycerophosphoric acid (indicated as unknown A in Figures 3 and 4*A*) was eluted from the column after glycerophosphoric acid (I) it may be concluded that this substance is more strongly acidic than I. If adrenal cardiolipin were to be represented by the molecular structure suggested by Macfarlane and Gray (1957), hydrolysis would give structure II, which contains two acidic hydrogen atoms. On the other hand, hydrolysis of the original structure proposed by Pangborn (1947) would give III, which contains three acidic hydrogen atoms. Since the polyglycerophosphoric acid was more acidic than glycerophosphoric acid (two acidic hydrogen atoms) we believe that Pangborn's structure is more accurate in the case of adrenal cardiolipin.



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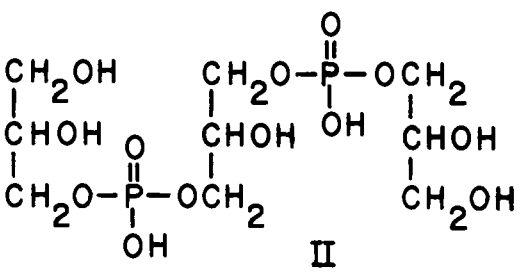

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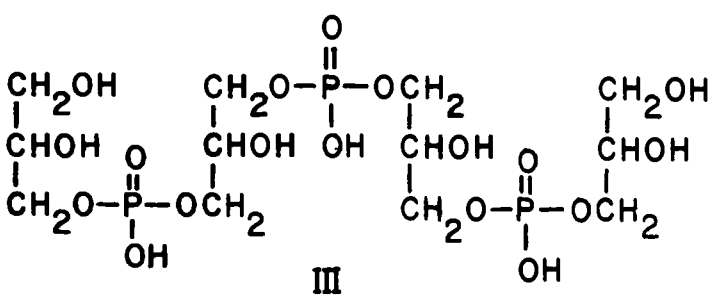
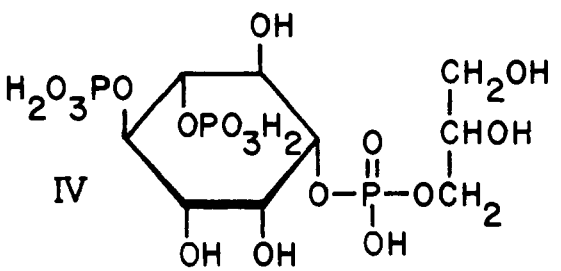


III



IV

By similar reasoning some conclusions may be drawn regarding the structure of fraction IX (Table VI), indicated as unknown B in Figure 3, which was the most highly acidic fraction obtained. Since it was eluted after glycerylphosphorylinositol diphosphate[5] (IV), which has five acidic hydrogen atoms, this substance must have at least five and probably more acidic groups. We can think of only two possibilities to account for this requirement, glycerylphosphorylinositol triphosphate and inositol triphosphate. Of these the latter is more likely, since Dawson and Dittmer (1960) have reported that a low yield of inositol triphosphate results from mild alkaline hydrolysis of triphosphoinositide, and since a tetraphosphoinositide has not heretofore been reported in mammalian tissues.

[5] Based on the structure of triphosphoinositide, from beef brain, determined by C. E. Ballou and colleagues.

Adrenal lipids are relatively rich in total inositides, presumably of the general types described as constituents of brain lipids (Brockerhoff and Ballou, 1961). Molar ratios of inositol to total P, calculated from yields of inositol-containing products of mild alkaline hydrolysis, ranged from 0.076 to 0.102 in this study, as compared with published values from 0.020 to 0.086 for a variety of mammalian tissues (Taylor and McKibben, 1953). The identifications assigned in this study are tentative, however, and full structural characterization of the adrenal inositides must await their further purification.

Arachidonic acid and adrenic acid were distributed throughout the various phosphatides. Phosphatidylethanolamine contained the highest concentrations of these polyenoic acids, which together represented nearly 50% of the total fatty acid. To our knowledge high levels of adrenic acid in cholesterol esters and phosphatidylethanolamine have not previously been reported with lipids isolated from any other mammalian tissue.

An interesting specificity for the type of saturated fatty acid was observed with phosphatidylethanolamine and phosphatidylcholine, which contained stearic and palmitic acids, respectively, as the major saturated fatty acid. In general the characteristic patterns of fatty acids found in triglyceride, cardiolipin plus phosphatidic acid, phosphatidylethanolamine, and phosphatidylcholine, were entirely different. It should be emphasized that neither Kennedy's (1957) scheme for the biosynthesis of triglycerides and phosphatides, involving phosphatidic acid and diglyceride as common intermediates, nor the mechanism for converting phosphatidylethanolamine to lecithin by successive enzymatic methylations (Bremer and Greenberg, 1961; Gibson *et al.*, 1961) can account for specificities of lipids for particular groups of fatty acids, as observed with the canine adrenal lipids as well as previously reported studies with other tissues. Whether these differences may be attributed to special pools of fatty acids in the cell available for the synthesis of individual classes of lipids or, alternatively, whether they may be due to partial metabolic turnover of the fatty acids after synthesis of the phosphatides cannot be answered at the present time. This question remains as one of the perplexing and unexplored problems in lipid metabolism.

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