Purification and characterization of phthalanilide–lipid complexes from tissues

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ABSTRACT What appears to be a new class of phospholipids has been isolated from dog brain in the form of complexes with a substituted phthalanilide. The complexes were extracted by chloroform–methanol and purified by countercurrent distribution in solvent systems containing water, chloroform, methanol, and Freon 113.

The binding of the phthalanilide congener to lipids has some ionic character. Cations such as H^+ or Ca^{++} displaced the phthalanilide from its lipid complex. The pH for 50% displacement acid was about 3.8 and was independent of the purity of the complex.

Thin-layer chromatography of the lipid yielded four subfractions of lipid, three of which were ninhydrin-positive and all of which yielded a group of unidentified ninhydrin-positive components on hydrolysis. Each lipid subfraction contained nitrogen, phosphorus, fatty acids, and glycerol but in different ratios.

Of the known phospholipids containing nitrogen, none matches the composition and behavior of the lipids isolated as phthalanilide complexes. We have therefore concluded that the phthalanilides bind to a new class of phospholipids characterized by a high content of unidentified ninhydrin-positive components.

KEY WORDS phthalanilide–lipid complexes · dog · brain · new phospholipid class · high nitrogen content · countercurrent distribution · Freon 113 as solvent

THE SUBSTITUTED phthalanilides, designed to be "phosphatide blockers" (1) are chemotherapeutic agents that are highly active against rodent neoplasms (2, 3). Chromatographic isolation procedures (4) were developed to determine the physiological disposition of the phthalanilides in animal tissues (5–7) and leukemia cells (4). However, the analysis of phthalanilides in fatty tissues, e.g. brain, was difficult because the alcoholic extracts contained lipid, which interfered with the chromatography (4). When the lipid extraction procedure of Folch, Lees, and Sloane Stanley (8) was used, the phthalanilides, which stayed in the aqueous phase in the absence of tissues, partitioned to the nonaqueous phase and were associated with lipids in all tissue extracts studied (7).

This paper describes our characterization studies of the lipids in dog brain which complex with the phthalanilides. Preliminary reports have been made by Yesair and coworkers (9–11).

Abbreviations: CCD, countercurrent distribution; NANA, *N*-acetylneuraminic acid; TLC, thin-layer chromatography.

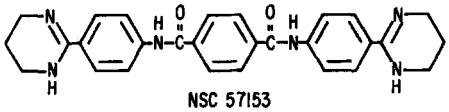
MATERIALS

The substituted phthalanilide congener NSC 57153 (Fig. 1) was synthesized by the Research Institute of Dr. A. Wander S. A., Berne, Switzerland and obtained from the Clinical Branch, Collaborative Research, National Cancer Institute, Public Health Service. Freon 113 (1,1,2-trichloro-1,2,2-trifluoroethane; E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) and all other solvents and reagents were the best grades commercially available. Most of the lipids and all methyl esters of fatty acids were obtained from Applied Science Laboratories Inc., Philadelphia, Pa. Cholesterol, tripalmitin, and taurine-conjugated bile acids were obtained from Mann Research Laboratories, Inc., New York, N.Y. Silica Gel H was obtained from Brinkmann Instruments Inc., Westbury, N.Y., and Celite 545 was obtained from Johns-Manville, Boston, Mass.

METHODS

Estimation of Phthalanilide

The quantity of NSC 57153 was determined by one or more of the following methods. The phthalanilide was



NSC 57153

FIG. 1. Substituted phthalanilide. NSC 57153 is 4′,4″-bis(1,4,5,6-tetrahydro-2-pyrimidinyl)terephthalanilide dihydrochloride.

extracted from tissue with alcohol and levulinic acid (4). It could then be isolated by chromatography and estimated by UV spectrophotometry (4) or be hydrolyzed to yield an aniline derivative which was diazotized and coupled to form a violet azo compound which was estimated by spectrophotometry at 545 mμ (12). In the third method, the drug was extracted from the tissue as hydrophobic drug–lipid complexes by the extraction procedure of Folch et al. (8). After the solvents had been removed, the drug was dissociated from the lipids by the addition of 5 ml of 0.1 N HCl, partitioned to a fresh aqueous phase by adding 5 ml of CH_3OH and 10 ml of $CHCl_3$, and estimated by UV spectrophotometry (3, 6, 7, 9).

Extraction and Analysis of Drug–Lipid Complexes

The drug–lipid complexes were extracted from various tissues and tumor cells, and purified by washing the chloroform–methanol extract with water, extracting the drug–lipid complex from the dried residue into the aqueous phase of a Freon 113–ethanol–water mixture, extracting it back into chloroform, and subjecting it to CCD in Freon 113–methanol–chloroform–water. Details of the procedure for dog brain are as follows.

Six grams of fresh dog brain (Beagle) was homogenized with NSC 57153 (2.4 mg) at 4°C and within 10 min extracted with 90 ml of chloroform–methanol 2:1. The insoluble fraction was removed by filtration and washed with chloroform–methanol 2:1. The final filtrate was diluted to 156 ml with the same solvent mixture. When 45 ml of water, 33 ml of methanol, and 3 ml of chloroform were added to this filtrate, 95–100% of the added drug partitioned to the nonaqueous phase as drug–lipid complexes. Several of these nonaqueous phases were pooled and washed three to six times with the same volume of an equivalent aqueous phase, which removed gangliosides, as judged by TLC. The solvents of the washed nonaqueous phase were removed under vacuum. The residue was then partitioned with 90 ml each of aqueous and nonaqueous phases from the solvent mixture Freon 113–ethanol–water 9.8:5:1. In this step, the drug–lipid complexes dissolved in the aqueous phase; some insoluble material was removed from the interface by filtration. This aqueous phase was washed three to six times with an equal volume of nonaqueous phase, which extracted large amounts of lipids which chromatographed as lecithin, sphingomyelin, phosphatidyl ethanolamine, etc., on TLC. The amount of drug was determined by its UV absorbance after acid displacement from the lipids (3, 6, 7, 9) and the drug was isolated in some cases by chromatography (4).

To make the removal of solvents easier and to eliminate any free drug, we extracted the drug–lipid complexes into chloroform and discarded the aqueous phase, which would contain any free drug (known from earlier work). The solvents were removed under vacuum and purified lipid residues were dissolved in the solvents (specific mixtures of water–methanol–chloroform–Freon 113, 6:25:23 to 43:45 to 25, total volume 99 ml) to be used for CCD. In general, equal volumes of each phase were used. CCD was performed in large separatory funnels or in a 20 tube Craig apparatus manufactured by the E-C Apparatus Corporation (Swarthmore, Pa.).

The individual lipids were isolated by preparative TLC (13). A Desaga apparatus (Brinkmann Instruments Inc., Westbury, N.Y.) was employed with "basic" Silica Gel H and with the developing solvent chloroform–methanol–glacial acetic acid–water 50:25:0.5:3 (14). Phospholipids and their hydrolysis products were detected by methods described by Skidmore and Entenman (15).

The lipids were analyzed chemically as follows. After they had been digested with sulfuric acid, nitrogen was determined as ammonia with ninhydrin by the Moore and Stein procedure (16) and phosphorus was determined by the spectrophotometric method of Chen, Toribara, and Warner (17). *N*-Acetylneuraminic acid was determined by the colorimetric method of Long and Staples (18).

IR spectra of the lipids were determined in KBr discs with the aid of a Perkin-Elmer Model 221 IR Spectrophotometer (Perkin-Elmer Corp., Norwalk, Conn.).

Microdetermination of Glycerol and Fatty Acids

A fraction of a micromole of lipid was hydrolyzed by refluxing with 3 ml of 0.5 N KOH in absolute ethanol for 1 hr. The mixture was cooled, 3 ml of 1 N HCl in ethanol (containing 8% water from concentrated HCl) was added, and the sample was refluxed for another 30 min. The solution was dried under nitrogen. The solids were redissolved in 2 ml of distilled water and the fatty acids were extracted with a total of 2 ml of petroleum ether. After removal of the petroleum ether, the water layer that contained the glycerol was dried again under nitrogen and redissolved in 0.4 ml of distilled water or 1 ml of ethanol.

The extracted glycerol was isolated and identified by TLC and the quantity of glycerol was estimated by two methods, the first being semiquantitative. In this case, thin layers of Brinkmann MN 300 cellulose (Brinkmann

Instruments Inc., Westbury, N.Y.), 300 μ thick, were activated at 115°C for 15 min before use in TLC. The use of two solvent systems made identification more certain. The R_f values for glycerol were 0.66 with isopropyl alcohol–acetic acid–water 40:10:5 and 0.60 with butanol–acetic acid–water 40:1:5. Glycerol was detected as a white spot on a blue background after the plates had been sprayed with freshly prepared 0.028 M $NaIO_4$ in 0.2 M phosphate buffer (pH 7.0), followed by freshly prepared 0.1 M benzidine dihydrochloride in ethanol–acetone 4:1. Glycerol from the sample was compared with five glycerol standards (5, 10, 15, 20, and 25 μg) to yield semiquantitative estimations. Also, recovery experiments which included all the steps (hydrolysis, extraction, and chromatography) were conducted on glycerol and egg phospholipids. Recoveries between 60 and 80% were obtained each time.

The procedure for quantitative glycerol analysis used silica gel plates, 200 μ thick (19). The R_f for glycerol was 0.55 with ethanol–30% ammonia 77:23. After chromatography, the glycerol was extracted from the plates and determined by chromotropic acid assay (20) with good precision and accuracy. For example, we found 0.24 ± 0.035 (SD) μmole of glycerol, in seven determinations for glycerol, in phosphatidyl ethanolamine that contained 0.26 μmole of phosphorus.

Fatty acids in the petroleum ether extract were methylated, after evaporation of the solvent, with boron trifluoride–methanol (21). The esters were dissolved in benzene and a portion was injected into a gas chromatograph (F & M, Model 500 with the 609 flame ionization detector, F & M Scientific Corp., Avondale, Pa.). The carrier gas was helium. On one of two 6-ft columns, the stationary phase was 6% SE-30 (silicone gum rubber, General Electric) on Chromosorb W (MicroTek Instruments, Inc., Baton Rouge, La.). The starting temperature was 150°C; after 4 min the temperature was programmed up to 300–325°C at a rate of 7.9° per min. The second column utilized 20% LAC 728 (diethylene glycol succinate polyester) containing 3% H_3PO_4 on Chromosorb W-AW 609 (MicroTek Instruments, Inc., Baton Rouge, La.) at 185°C. Calculations based on peak area measurement showed that recoveries of the methyl ester mixtures K-101, K-102, K-108 and methyl arachidonate (Applied Science Laboratories Inc., State College, Pa.) were 40–50% after hydrolysis, esterification, extraction, and chromatography. Determination of accuracy and precision of each step in this procedure revealed that the low recovery was due mainly to incomplete esterification and was reflected in both the saturated and unsaturated fatty acids, with no apparent selective loss or degradation. Therefore, the incorporation of an internal standard (21:0) to a lipid sample before hydrolysis made quantitative results (90 ± 3% SD) possible.

RESULTS

Purification of Phthalanilide–Lipid Complexes

The extracted phthalanilide–lipid complexes partitioned quantitatively to the nonaqueous phase of a chloroform–methanol–water 2.05:1.63:1 biphasic solvent system. Attempts to purify the complexes by column chromatography with silicic acid or by partition chromatography on Celite 545 (solvent system: water–methanol–chloroform–Freon 113 6:25:23:45) were unsuccessful because the complexes were dissociated by contact with silicic acid and Celite 545. Therefore, new solvent systems containing Freon 113 were used for purification by CCD. Freon 113 had several advantages over ether, hexane, and other common lipid solvents. Freon 113 is nonflammable and its density is greater than water, which permitted the transfer of the organic phase during CCD of the drug–lipid complexes. Because the solubility of phospholipids in Freon 113 is relatively poor compared to their solubility in chloroform or methanol,[1] substitution of Freon 113 for chloroform in the chloroform–methanol–water system displaces the drug–lipid complexes from dog brain to the aqueous phase (Fig. 2). This type of partitioning was also characteristic of phthalanilide–lipid complexes obtained from dog kidney, monkey liver, and P388 leukemia cells after treatment in vivo.

Results of the CCD in biphasic solvents of two NSC 57153–lipid concentrates, which had been isolated from brain and purified, are given in Fig. 3. In addition, a second CCD with a different solvent system was per-

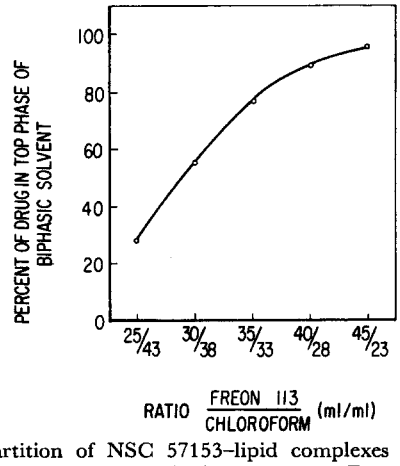


FIG. 2. Partition of NSC 57153–lipid complexes obtained by adding the drug to dog brain homogenate. For these studies, 50 μg of crude drug–lipid complexes was partitioned between 10 ml of each phase of solvent systems containing 68 parts (by volume) of Freon 113 plus chloroform (in the ratios shown), 6 parts of water, and 25 of methanol. Drug in each phase was analyzed by the acid displacement method (3, 6, 7, 9).

[1] Personal experience of one author (D. W. Y.).

formed on complexes obtained from the first CCD. After the second distribution, the TLC pattern of the lipid in all drug-containing tubes was identical. Approximately 95% of the drug was recovered as drug–lipid complexes. The over-all recovery of drug–lipid complexes from dog brain was 80–90% after all isolation steps, including two countercurrent distributions.

Characteristics of the Composite Lipids

Thin-Layer Chromatography. When the method of Skipski, Peterson, Sanders, and Barclay (14) was used, three lipid components were separated from the purified samples (*a* and *b*, Fig. 3). The R_f values of these components were approximately 0.2, 0.5 (subsequently shown to contain two substances), and 0.8. The relative amounts of the components were about 20, 50, and 30%, respectively. Two of the lipid areas (with R_f 0.2 and 0.5) were positive to ninhydrin, but their R_f values did not correspond to those of phosphatidyl ethanolamine (0.7) or phosphatidyl serine (0.3) which were run on the same plate. The two most concentrated lipid areas gave a positive reaction in the molybdic acid test for phosphate.

Physical Analyses. The UV spectrum of the composite lipid in hexane or ethanol had a shoulder at approximately 275 mμ, which suggests a conjugated system. The molar extinction coefficient was approximately 1000 at 275 mμ; the molar concentration of lipid was estimated (see next paragraph) from the number of moles of NSC 57153 which were associated with the lipid before dissociation of the complex. The IR spectra (Fig. 4) were consistent with the interpretation that the lipids contained ester groups (1740 cm^{-1}), primary amino nitrogen atoms (1530 cm^{-1}) (22), many carbon–hydrogen linkages ($—CH_2—$, 2930 cm^{-1}; $—CH_3$, 2960 cm^{-1}), and some unsaturation (—CH + CH—, 3030 cm^{-1}). The absorption at 1660 and 3450 cm^{-1} in the top spectrum (Fig. 4) was probably due to water which was present in this preparation. However, the presence of a weak absorption at 3450 cm^{-1} in the bottom spectrum suggests the presence of hydroxyl and (or) NH groups. Such a low absorption indicates that the percentage of OH or NH groups is very low.

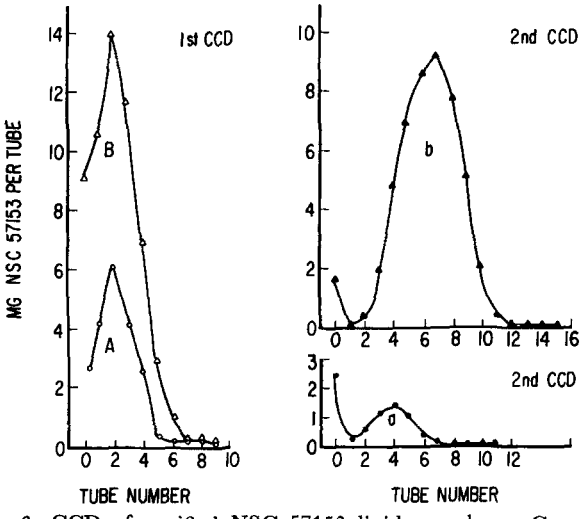


FIG. 3. CCD of purified NSC 57153–lipid complexes. Conventional numbering system was employed; the total number of transfers equaled the number of the last tube. The nonaqueous (lower) phases were transferred. The drug content of each tube was the total quantity of drug in the aqueous and nonaqueous phases. Solvent system: water–methanol–Freon 113–chloroform; for samples *A*, *B*, and *a*, 6:25:30:38; for *b*, 6:25:25:43.

1st CCD: Sample *A* (○—○), 18 mg of complex, 180 ml of each phase; sample *B* (△—△), 58 mg of complex, 250 ml of each phase. 2nd CCD: Sample *a* (●—●), 7 mg of complex from tubes 1, 3, and 4 of sample *A;* 40 ml of aqueous and 80 ml of nonaqueous phase. Sample *b* (▲—▲), 49 mg of complex from tubes 1–5 of sample *B;* 75 ml of each phase. Approximately 95% of the drug in tube 0 of samples *a* and *b* was found in the aqueous phase and was considered to be in the free state.

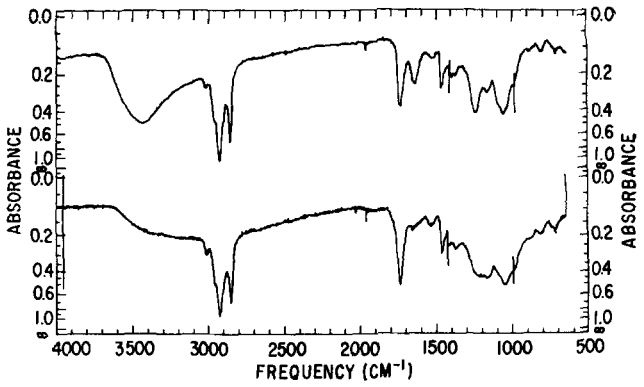


FIG. 4. IR spectra of lipids extracted from drug–lipid complexes. The spectra were of lipid obtained from two different drug–lipid complexes of comparable purity (bottom spectrum: sample *a*, Fig. 3). A similar spectrum was found for sample *b*, Fig. 3.

Chemical Analysis. The composition of the lipids that complex with NSC 57153 is summarized in Table 1. For every mole of drug, there were approximately 4 moles of lipid nitrogen and 1 mole each of phosphorus, glycerol, and fatty acid. The fatty acid pattern showed a predominance of C_{18} fatty acids (55% stearic, 30% oleic), some palmitic acid (10%), and small quantities (5%) of other unidentified fatty acids.

After base and acid hydrolysis of samples *a* and *b* (Fig. 3) followed by extraction with petroleum ether, three ninhydrin-positive materials and one periodate-positive material were found in the aqueous phase. The R_f of the periodate-positive material corresponded to that of glycerol.

Isolation and Characterization of Individual Lipids

Preparative TLC (13) of large quantities of the lipid from sample *a* (Fig. 3) revealed that the lipid fraction with an R_f of 0.5 contained two components. UV light was used to locate the lipids. The faster-moving subfraction charred more with H_2SO_4, but gave less color with

TABLE 1 LIPID COMPOSITION OF NSC 57153–LIPID COMPLEX* FORMED BY DOG BRAIN IN VITRO

Analysis of Lipid		μmoles NSC 57153 Displaced from Lipid	Ratio of Lipid Constituent to Drug Displaced
Assay	μmoles Found		
Nitrogen	2.6	0.67	3.9
	2.8	0.67	4.2
Phosphorus	0.75	0.77	0.97
	0.75	0.77	0.97
Glycerol†	1–1.5	1.4	0.7–1
Fatty Acids			
16:0	0.2	1.4	0.1
18:0	0.9	1.4	0.6
18:1	0.5	1.4	0.3
Others	<0.1	1.4	<0.1
Total lipid	1.5–1.7	1.4	1–1.1

* Sample *a*, Fig. 3.

† Glycerol from the hydrolyzed sample was compared with five glycerol standards which were chromatographed on the same plate. The quantity of glycerol being estimated was bracketed between two glycerol standards and was corrected for the recovery of glycerol which was run simultaneously through the same procedures.

ninhydrin than the slower-moving one. Lipid on the plate was subdivided as follows: fraction 1, R_f 0–0.3; fraction 2, R_f 0.3–0.5; fraction 3, R_f 0.5–0.7; and fraction 4, R_f 0.7–0.9. The cross-contamination by adjacent fractions was estimated to be less than 25%.

The results of analyses of these isolated lipid fractions are summarized in Table 2. Each fraction was rich in nitrogen.

The fatty acid pattern for each of the four lipid fractions was similar to that of the composite lipid preparation, but there were several noteworthy exceptions. High molecular weight fatty acids were found in fraction 4. Two of the three were identified as 24:0 and 26:0. Polyunsaturated fatty acids were found in fractions 1, 2, and 3. Linolenic acid (18:3) was present in fractions 1, 2, and 3; arachidonic acid (20:4) was found in fraction 3.

TLC of the hydrolysis products of the individual lipids showed that glycerol and ninhydrin-positive compounds were also present in each lipid fraction. When an acidic solvent system was used (Table 3), three ninhydrin-positive compounds were found in fractions 1 and 4; only two of the three compounds were seen in fractions 2 and 3. With a basic solvent system, only two ninhydrin-positive areas were observed in each isolated lipid fraction.

Ionic Displacement of Drug from Lipid

The binding of the substituted phthalanilide to lipids involves ionic forces. Hydrogen or calcium ions were equivalent in effecting displacement of drug from a crude drug–lipid complex. But as can be seen in Table 4, much higher concentrations of other cations were required to displace equivalent amounts of drug. All of these cations at higher concentrations quantitatively displaced the drug from crude complexes.

Fifty per cent of the drug was displaced from both the crude and a purified drug–lipid extract with hydrochloric acid at pH 3.5–3.8 (Table 4 and Fig. 5). Thus, extensive purification could be accomplished without changing the apparent "pK" of the complex[2] significantly (pH 3.5–3.8). During the progress of purification, equimolar quantities of hydrogen or calcium ions displaced the same quantity of drug. It was found that the quantity of calcium ions required to displace 50% of the drug became constant for complexes which were highly purified but were not of equivalent purity (Fig. 6). Only 22 moles of calcium ions were required to displace 1 mole of drug from these purified drug–lipid preparations (1.1 μmoles displaced 0.05 μmole, Fig. 6) as opposed to approximately 180 moles of calcium required for the crude preparations (Table 4 and Fig. 6).

Complex Formation with Known Lipids

Since cations displaced the drug from the lipid and the drug is cationic at neutral pH, it is likely that the lipid contains anionic sites, e.g., phosphate, carboxyl, or sulfate. As can be seen in Table 5, three of the four phos-

TABLE 2 ANALYSIS OF LIPID SUBFRACTIONS OF DRUG–LIPID COMPLEX

Fraction*	R_f*	Amount of Lipid Constituent Per Sample			
		Phosphorus	Nitrogen	Glycerol†	Fatty Acid
		μmoles			
1	0–0.3	1.0	3.9	0.8	0.5
2	0.3–0.5	1.9	8.1	0.9	1.3
3	0.5–0.7	1.0	7.7	2.3	1.3
4	0.7–0.9	1.5	ca. 13	3.9	3.0
Sum‡		5.4	ca. 32	7.9	6.1
Sum (above) normalized to glycerol equals 1.0		0.7	ca. 4	1.0	0.8
Actual analysis of total lipid from Table 3		1.0	4.1	0.7–1	1–1.1

Approximately 6 μmoles of drug was displaced from the complex (sample *a*, Fig. 3).

* On preparative TLC. The cross-contamination by adjacent fractions was estimated to be <25%.

† Glycerol from the hydrolyzed lipid sample was estimated by the chromotropic acid procedure after chromatographic separation of the glycerol (see Methods).

‡ Each subfraction was about 25% of the total lipid and we have assumed that each fraction contributed equally to the sum.

[2] "pK" is the pH required for 50% displacement of drug into the aqueous phase of a chloroform–methanol–water, 10:5:5, system.

TABLE 3 TLC OF WATER-SOLUBLE, NINHYDRIN-POSITIVE HYDROLYSIS PRODUCTS* OF LIPID SUBFRACTIONS†

Fraction	R_f, Color‡ (Concentration‡)		
	In isopropanol–acetic acid–H_2O 40:10:5		
1	0.1, purple (+)	0.4, pink (+)	0.8, blue-purple (++)
2	0.1, purple (++)	0.4, pink (+)	
3		0.4, yellow (+++)	0.8, blue-purple (+)
4	0.1, purple (+)	0.4, pink (+)	0.8, blue-purple (++)
	In ethanol–concd NH_4OH 77:23		
1	0.1, yellow (+)	0.4, purple (++)	
2	0.1, yellow (+)	0.4, purple (++)	
3	0.1 trailing to 0.4, yellow (+++)	0.5 purple (+)	
4	0.1, yellow (+)	0.4, purple (++)	

* After base and acid hydrolysis of the fractionated lipids and extraction by petroleum ether, these aqueous solutions were dried under nitrogen, extracted with water, diluted to yield comparable concentrations, and applied to glass plates (20 × 20 cm) coated with cellulose MN 300. The plate had been previously activated for 1 hr at 115 °C.

† See Table 2.

‡ The chromatograms were dried in air, the plates were sprayed with ninhydrin, and color was developed under an IR lamp. The relative concentrations are on an arbitrary scale of + to +++.

TABLE 4 RELATIVE EFFECTIVENESS OF CATIONS TO DISPLACE 50% OF NSC 57153 FROM A CRUDE DRUG–LIPID COMPLEX

Displacing Agent	Moles of Cation Required Per Mole of Drug Displaced
Proton (HCl)	180 (pH 3.5)
Calcium ($CaCl_2$)	180
Magnesium ($MgCl_2$)	890
Potassium (KCl)	28,000
Sodium (NaCl)	34,000

To 0.1 μmole of drug as lipid complex was added 5 ml of aqueous solution which contained displacing agent, 5 ml of methanol, and 10 ml of chloroform. Both phases were analyzed for drug and for the amount of cation required to displace 50% of the drug. The pH of the aqueous phases was 6–7 except in the case of acid displacement.

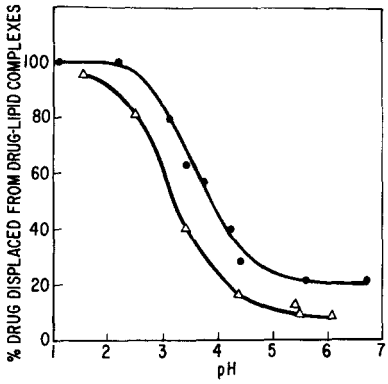


FIG. 5. Relationship between pH and the quantity of NSC 57153 displaced from partially purified drug–lipid complexes from brain (●—●) and from drug–sulfatide complex (△—△). Acid (5 ml) was added to 0.1 μmole of drug as lipid complex and mixed. After the addition of 5 ml of CH_3OH and 10 ml of $CHCl_3$, the displaced drug was measured in the aqueous phase and complexed drug was determined in the nonaqueous phase.

pholipids studied did not complex with the drug. NSC 57153 complexed only with phosphatidyl serine. The complex had a mole ratio of 1:2–3 and a "pK" of 5.5–6.0. It could be dissociated by the addition of phosphatidyl choline or by sodium ions at concentrations similar to the concentration of protons required for displacement.

Both sulfatides and taurine conjugates of bile acids complexed with the drug (Table 5). The mole ratio of the drug–sulfatide complex was approximately 1:2. The "pK" of the NSC 57153–sulfatide complex was approximately 3.2 (Fig. 5) and 0.1 N HCl displaced 95% of the drug, which is consistent with the acidity of the sulfatide lipids (23). Other nonlipid sulfate and sulfonic compounds can complex with the phthalanilide (24–26). Cerebrosides and several other common lipids did not

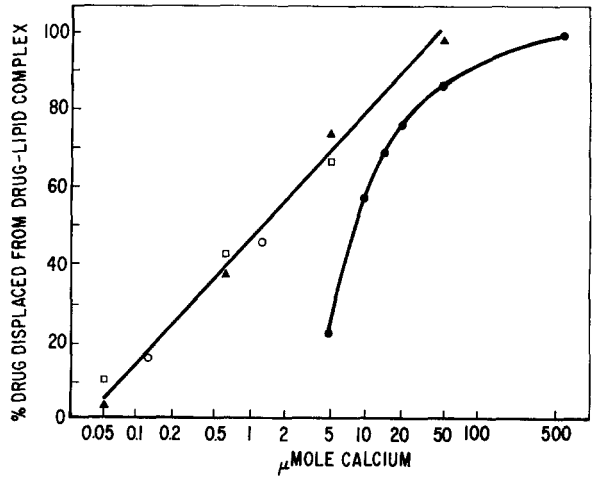


FIG. 6. Relationship of calcium-ion displacement to the purity of the NSC 57153–lipid complexes from brain. One-tenth μmole of drug as lipid complex was used in an experimental design similar to that described in Fig. 5, but at pH 7.0.

● Crude drug–lipid complexes; O sample *A* (Fig. 3); □ sample *a* (Fig. 3); ▲ drug–lipid complex of comparable purity to O (both contained a small quantity of extraneous lipids).

TABLE 5 ABILITY OF PURIFIED LIPIDS TO FORM COMPLEXES WITH NSC 57153

	Maximum Moles of Lipid Added Per Mole of NSC 57153	Complex Formed*
Phospholipids		
Phosphatidyl serine	11	Yes
Phosphatidyl choline	13	No
Phosphatidyl ethanolamine	ca. 19	No
Sphingomyelin	ca. 13	No
Other Lipids		
Cerebroside	ca. 20	No
Sulfatide	ca. 5	Yes
Taurine–bile acid conjugates	5	Yes†
Palmitic acid	4.5	No
Tripalmitin	10	No
Cholesterol	10	No

* Each lipid in 1 ml of $CHCl_3$–CH_3OH 2:1 was added to 0.28 μmole of NSC 57153 in 0.15 ml of water and the solvents were removed in vacuo. The residue was dissolved in 33 ml of $CHCl_3$–CH_3OH 2:1 and this solution was made biphasic by the addition of 13.5 ml of H_2O, 8.5 ml of CH_3OH, and 1.5 ml of $CHCl_3$. Both phases were analyzed for drug by UV spectrophotometry. Drug was considered to form a complex with the lipid if the drug partitioned to the nonaqueous phase. Free drug partitioned to the aqueous phase.

† This complex was insoluble in water and could be recrystallized from alcohol–water mixtures.

complex with NSC 57153 (Table 5); nor did the lipids in hen's egg yolk (27), or in mammalian blood.

Gangliosides contain neuraminic acid which has a carboxyl group with "pK" 2.5 (28). Although the difference between this "pK" and the "pK" of 3.8 for the dissociation of drug–lipid complexes makes it difficult to consider that a lipid that contains neuraminic acid binds NSC 57153, the possibility was tested by analyzing the contents of all tubes from a CCD of a crude NSC 57153–lipid complex from brain for *N*-acetylneuraminic acid. The data in Table 6 indicate a lack of correspondence between the distribution of *N*-acetylneuraminic acid and drug. The lack of correspondence was not due to the effect that extraneous lipids have on the partitioning of crude drug–lipid complexes, because the CCD of highly purified drug–lipid preparations (samples *A* and *B*, Fig. 3) were also significantly different from the distribution of lipids containing *N*-acetylneuraminic acid (Table 6).

TABLE 6 DISTRIBUTION OF *N*-ACETYLNEURAMINIC ACID RELATIVE TO THAT OF PHTHALANILIDE

Tube number	0	1	2	3	4	5	6	7
μmole NANA	0.59	0.29	0.17	0.10	0.08	0.04	0.03	0.03
μmole NSC 57153	0.04	0.08	0.32	0.72	0.98	0.60	0.24	0.04
Drug/NANA ratio	0.08	0.25	2	7	12	15	8	1

Each tube was charged with 10 ml of nonaqueous and aqueous phase of the solvent system water–methanol–Freon 113–chloroform 6:25:30:38. A lipid–NSC 57153 concentrate (2.2 mg of NSC 57153) from brain was used. NANA and NSC 57153 were determined in the aqueous phases of the CCD.

The difference in the chromatographic pattern of the ninhydrin-positive compounds in acidic and basic solvents (Table 3) suggests that the compounds have ionizable groups and that they may be amino acids. However, amino acids such as serine or threonine were eliminated because the lipid-nitrogen compounds were not oxidized by periodate. The presence of other amino acids as tetrapeptides (to account for the high nitrogen content relative to phosphorus, glycerol, and fatty acids) seemed unlikely because the tissue lipids did not have sufficient amide absorption at 1650 cm^{-1} (Fig. 4). In the synthetic mixtures, one amide bond (sphingomyelin) in the presence of three ester bonds (synthetic phosphatidyl serine) was easily detected by IR spectroscopy in KBr discs. Nevertheless, polybasic amino acids could still be present, because amino acids can be esterified to the glycerol of lipids (29, 30). Arginine that had been subjected to the same base and acid hydrolysis used for the lipids gave R_f = 0.1 in the acidic solvent (see Table 5) and R_f = 0.7 in the basic solvent (see Table 5).

DISCUSSION

The substituted phthalanilides designed to be "phosphatide blockers" (1) had been shown previously to complex with tissue lipids (3, 7, 9, 10, 31, 32). Booth, Boyland, and Gellhorn (32) showed that most of the phthalanilides that complexed with liver lipids was associated with the phospholipid fraction.

During the purification of the drug–lipid complex, the pH for 50% displacement of drug remained relatively constant. The "pK" as thus defined[2] was 3.5 in crude extracts and 3.8 in highly purified drug–lipid preparations. Throughout the purification of the drug–lipid preparation, protons and calcium ions were equivalent on a molar basis in their ability to displace drug. Furthermore, the same lipid pattern after TLC was obtained for several lipid preparations from brain and the distribution of the lipids after CCD in different solvent systems corresponded to the distribution of drug. These data indicate that one type of lipid was involved, and are consistent with the similarity in chemical composition of the four lipid subfractions to that of the total lipid.

Several different types of known lipids were evaluated experimentally for complex formation. Of these only sulfatide, taurine-conjugated bile acids, and phosphatidyl serine complexed with 4′,4″-bis(1,4,5,6-tetrahydro-2-pyrimidinyl)terephthalanilide dihydrochloride (NSC 57153). These three compounds and other candidate lipids have been eliminated for the reasons summarized in Table 7. The elimination of peptides (Table 7) does

TABLE 7 ELIMINATION OF KNOWN LIPIDS AS POSSIBLE CONSTITUENTS OF THE COMPLEX

Tissue Lipid Characteristic	Lipid Eliminated
Cations displaced the drug	Neutral lipids
Phosphorus was found in all four lipid subfractions	Lipids not containing phosphate, including sulfatides, cerebrosides, gangliosides, and bile salts
Nitrogen was found in all four lipid subfractions	Lipids not containing nitrogen, e.g., cardiolipin
Only one periodate-positive compound, glycerol, was found in the four lipid subfractions	Lipids containing other periodate-positive compounds, e.g., inositol, serine, ethanolamine, sphingosine, and sugars
The "pK" for 50% displacement of drug[2] was 3.8 for the purified composite lipids	Phosphatidyl serine complexes ("pK" 5.5–6.0) and sulfatide complexes ("pK" 3.2)
R_f values of the three ninhydrin-positive lipids were 0.5 (two lipids) and 0.2	Phosphatidyl serine (R_f 0.3) and phosphatidyl ethanolamine (R_f 0.7)
Amide absorption area (1650 cm^{-1}) in IR spectrum was negligible	Lipids containing peptides and sphingolipids
Lipid was hydrolyzed by relatively mild conditions	Sphingosine-containing lipids (33), e.g., sphingomyelin, sulfatides, gangliosides

not necessarily eliminate amino acids, since they can be esterified to glycerol in the lipids (29, 30). However, if amino acids were the unique binding force, we should expect to find a considerable quantity of drug complexed with protein. This does not occur to any great extent between similar phthalanilides and bovine serum albumin (12) or proteins of human plasma (34).

The phosphorus, glycerol, and fatty acid contents of the composite lipid indicate that the lipid contains a lysophosphatidic acid nucleus. The analyses of the four lipid subfractions indicate that some may contain a cardiolipin-like backbone. It is reasonable to speculate that these individual lipids were parts of a more complex lipid and were produced by the acid displacement of drug from the complex lipid. This suggestion may explain the loss of "solubilizing activity" of liver phospholipids after silicic acid chromatography (32). Silicic acid chromatography did displace NSC 57153 from lipid complexes and is reported to disrupt complex lipids that are labile to acid (35–38).

The characteristic features of the four lipid subfractions are the nitrogen compounds which become visible under UV light and are positive to ninhydrin, and the variation in the mole ratios of glycerol, phosphate, fatty acid, and nitrogen. Similar ninhydrin-positive compounds have been observed in lipids that were isolated as NSC 60339[3]–lipid complexes from P388 lymphocytic leukemia cells (11).

Equimolar concentrations of hydrogen or calcium ions displaced the same amount of drug, whereas identical concentrations of other cations (Mg^{++}, K^{+}, Na^{+}) displaced much less drug. The reasons for these differences are unknown. From these results and the "pK" data, we conclude that ionic forces contribute to the formation and stability of the drug–lipid complexes. High concentrations of sulfatide and phosphatidyl serine are found in brain tissues (39, 40). Although complexes with these lipids and NSC 57153 were formed in vitro (Table 5), none (Table 7) was formed in the presence of brain tissue. This suggests that other forces are involved in the formation and stability of the phthalanilide–phospholipid complexes in the extracts of brain and extracts of P388 lymphocytic leukemia cells (11), and we believe that the moieties of high nitrogen content probably contribute to the binding of the phthalanilide derivative to a new class of phospholipids. Further work is underway to establish this and to identify the nitrogenous compounds.

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[3] NSC 60339 is 2-chloro-4′,4″-di(2-imidazolin-2-yl)terephthalanilide.

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