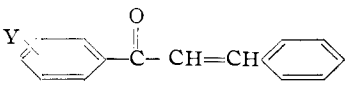
(see Table I). The product of the reaction of acetophenone under these conditions was β-chloro-β-phenylpropiophenone. The condensation of benzaldehyde with *p*-hydroxy-, *p*-methyl-, *p*-amino- and *o*-aminoacetophenones under these conditions did not give the corresponding chalcone.

TABLE I



Group Y	Yield, %	M.p., °C.	Lit. m.p., °C.
p-NO_2	100	146–147	149–150[a]
m-NO_2	100	129–130	131[a]
o-NO_2	98	125–126	128–129[a]
p-$(CH_3)_2N\cdot HCl$	76	165–167	165[b]
p-OH	68	171–174	171–172[c]
p-I	88	112–113	114–115[d]
p-Br	70	102–104	104–105[e]
p-Cl	69	97–98	101[f]
p-F	55	77–79	76–77[f]
m-I	46	83–84	[i]
m-Br	22	92–94	[k]
m-Cl	51	93–94	[l]
m-F	28	62–63	[m]
p-*t*-C_4H_9	54	97–98	98[g]
p-*i*-C_3H_7	68[n]	64–65	65[g]
p-C_2H_5	59[n]	59–61	61[h]
m-CH_3	65[n]	59–60	61[i]

[a] W. Dilthey, L. Neuhaus and W. Schommer, *J. prakt. Chem.*, **123**, 235 (1929). [b] H. Fecht, *Ber.*, **40**, 3902 (1907). [c] J. Simpson and S. Israelstam, *C.A.*, **44**, 5844 (1950). [d] O. Neuhoeffer and D. Roshal, *Ber.*, **86**, 229 (1953). [e] W. Dilthey, *et al.*, *C.A.*, **15**, 1292 (1921). [f] C. Allen, J. Norminton and C. Wilson, *J. Can. Research*, 11, 382 (1934); *C.A.*, **29**, 135 (1934). [g] C. Weygand, L. Mensdorff and F. Strobelt, *Ber.*, **58**, 1832 (1935). [h] J. Michel, *C.A.*, **33**, 7650 (1939). [i] C. Weygand and F. Schacher, *Ber.*, **68**, 232 (1935). [j] *Anal.* Calcd. for $C_{15}H_{11}IO$: C, 53.99; H, 3.32. Found: C, 54.37; H, 3.44. [k] *Anal.* Calcd. for $C_{15}H_{11}BrO$: C, 62.45; H, 3.86. Found: C, 62.05; H, 3.94. [l] *Anal.* Calcd. for $C_{15}H_{11}ClO$: C, 74.23; H, 4.56. Found: C, 73.47; H, 4.91. [m] *Anal.* Calcd. for $C_{15}H_{11}FO$: C, 79.62; H, 4.90. Found: C, 79.29; H, 5.06. [n] These yields were obtained by distillation, at reduced pressure, of the halogen-containing oils which remained after removal of the starting materials.

From the reaction mixtures a non-crystallizable oil was usually isolated in addition to the chalcones. In those cases tested the oils contained halogen which could be removed, with the formation of the chalcone, by distillation under reduced pressure. Thus it is evident that the isolation procedure did not remove completely the hydrogen chloride from the addition compounds of the chalcones. That the hydrogen chloride addition compound was the initial product in every reaction was indicated by the formation of an unstable hydrochloride on treatment of 4′-iodo-chalcone with methanolic hydrogen chloride. 4′-Iodo-chalcone was regenerated by subjecting the hydrochloride to the usual isolation procedure. It is interesting to note that with the exception of the *m*-halogen series those chalcones having the lowest electron density in the vicinity of the carbonyl also formed the least stable hydrogen chloride addition compounds as evidenced by higher yields of chalcones. Thus, a hydrogen chloride-catalyzed condensation for the preparation of chalcones is most successful with acetophenones substituted with electron-withdrawing groups.

Experimental

Reaction of Benzaldehyde with Substituted Acetophenones in Alcoholic Hydrogen Chloride.—To 30 ml. of anhydrous methanol saturated at 0° with hydrogen chloride was added 4.0 ml. of benzaldehyde and 0.02 ml. of the acetophenone. The reaction mixture in a vented 125-ml. Erlenmeyer flask was allowed to stand at room temperature. The reaction proceeded at different rates with the various acetophenones as evidenced by the time which elapsed after mixing the reagents before precipitation began. For example, *p*-nitroacetophenone started precipitation after 5 min., while *p*-fluoroacetophenone required up to 42 hr. before precipitation began. At the end of 5 days the reactions were stopped by the addition of water, and the mixture was steam distilled to remove any unreacted starting materials. The residue was neutralized with sodium bicarbonate and, if the product crystallized, it was removed by filtration. Otherwise, the residue from the steam distillation was extracted with ether. After drying, the ether solution was distilled, and the residue was crystallized from petroleum ether or ethanol. The products from the reactions of *p*-isopropyl-, *p*-ethyl- and *m*-methylacetophenones failed to crystallize after this treatment and were distilled under reduced pressure and were isolated as the fractions: b.p. 233° at 12 mm., 213° at 4 mm., and 206° at 2 mm., respectively, which crystallized on standing.

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Paper Chromatographic Separation of Phospholipids[1]

BY G. V. MARINETTI AND ELMER STOTZ

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The paper chromatographic separation of phosphatidylcholines and phosphatidylethanolamines has been difficult to achieve. Although Bevan and co-workers[2] have reported the separation of lecithin and cephalin, others[3] have been unable to confirm this work. Recently the chromatographic separation of various phospholipids has been reported.[4–6] Although the isoamyl alcohol–acetone system of Amelung and Bohm[4] can separate fairly simple mixtures of purified lecithin and cephalins, in our hands it was not capable of separating these lipids in a more complex phospholipid mixture extracted from various rat tissues. The reason for this is believed to be the significantly higher R_f values of the naturally occurring cephalins than those of the more saturated purified phosphatidylethanolamines. The paper chromatographic separation of acetal phospholipid from lecithin and cephalin has been difficult to achieve and has not been satisfactorily accomplished to date.

The authors and collaborators[7,8] have found solvent systems capable of separating unmodified phos-

(1) This investigation was supported in part by a research grant H-2063, from the National Heart Institute of the National Institutes of Health, Public Health Service.

(2) T. H. Bevan, G. I. Gregory, T. Malkin and A. G. Poole, *J. Chem. Soc.*, 841 (1951).

(3) C. H. Lea and D. N. Rhodes, *Biochem. J.*, **54**, 467 (1953).

(4) D. Amelung and P. Bohm, *Z. physiol. Chem.*, **298**, 199 (1954).

(5) C. H. Lea and D. N. Rhodes, *Biochem. J.*, **59**, v (1955); C. H. Lea, D. N. Rhodes and R. D. Stoll, *ibid.*, **60**, 353 (1955).

(6) J. W. Dieckert and R. Reiser, *Federation Proc.*, **14**, 202 (1955).

(7) G. Rouser, G. Marinetti and J. F. Berry, *ibid.*, **13**, 286 (1954).

(8) G. Rouser, G. Marinetti, R. F. Witter, J. F. Berry and E. Stotz, submitted for publication.

pholipids on unimpregnated paper. These studies have shown that phospholipids are firmly bound to the filter paper and that the positive charge on the nitrogen atom of these lipids is responsible for a large part of this binding. Therefore it seemed reasonable to assume that the elimination of the positive charge on the nitrogen base of the phospholipids would alter their mobilities and result in better separations.

No simple way is available to remove the positive charge on the quaternary nitrogen atom of phosphatidylcholines without disrupting the entire molecule. However, the positive charge on the nitrogen atom of phospholipids containing a free amino group can be removed easily by acylation under mild conditions. Therefore phosphatidylethanolamine and acetal phospholipid were acylated with either acetic anhydride, benzoyl chloride or 2,4-dinitrofluorobenzene. The N-acyl phospholipid derivatives demonstrated greater mobilities than the corresponding untreated lipids (Table I). Only the R_f values of the N-acetyl derivatives are given since the N-benzoyl and N-dinitrophenyl derivatives possessed high mobilities similar to the N-acetyl compounds. The yellow color of the N-dinitrophenyl compounds permits their detection visibly whereas the absorption of light by the benzene ring should permit the detection and determination of the N-benzoyl and N-dinitrophenyl derivatives in the ultraviolet region. However, the N-acetyl derivatives were better resolved from each other and were easily detected by use of fluorescent dyes.

In view of the fact that the mobilities of lecithin and sphingomyelin were not affected by the acylation treatment, a complete separation of phosphatidylcholines from the amino-containing phospholipids was achieved. Furthermore, with the N-acetyl derivatives a complete separation of cephalin from acetal phospholipid was accomplished. Although the separation of these latter compounds was possible with unmodified lipids using a purified acetal phospholipid, there is evidence that acetal phospholipid purified by alkaline treatment is structurally different from the native acetal phospholipid.[9] Native acetal phospholipid occurring in rat tissues possessed a greater mobility than the altered purified acetal phospholipid and indeed moved with the cephalin fraction. However, it was separated from cephalin by the acylation technique.

Although the separation of lecithin and sphingomyelin from cephalin can be accomplished without acylation as evidenced by their R_f values in Table I, these separations are possible only when very small amounts (10–20 μg.) of lipids are used. When larger amounts of untreated phospholipids are used the various lipid spots become elongated and fuse together. The use of very small amounts of phospholipids makes their identification very difficult by ordinary chemical methods and does not easily permit their isolation for further study. Furthermore, it makes the study of the specific activity of P^{32}-labeled phospholipids difficult since the analysis of P in such small amounts of lipids is subject to considerable error. However, all of these objections are overcome by the acylation technique because complete separation of phosphatidylcholines from phosphatidylethanolamines is achieved even when much larger amounts (300–400 μg.) of material are used.

(9) G. Schmidt, B. Ottenstein and M. J. Bessman, *Federation Proc.*, **12**, 265 (1953); E. Klenk and M. Debuch, *Z. physiol. Chem.*, **296**, 179 (1954).

TABLE I

R_f VALUES[a] OF THE PHOSPHOLIPIDS

Phospholipid	Solvent systems[b] A	B	C
L-α-Distearoylcephalin[d]	0.32	0.12[c]	0.15[c]
N-Acetyl-L-α-distearoylcephalin	.78	.83	.85
Acetal phospholipid[d]	.00	.00	.00
N-Acetyl-acetal phospholipid	.90	.93	.90
L-α-Distearoyllecithin[d]	.45	.34	.40
Sphingomyelin[d]	.49	.33	.40

[a] The R_f, values varied slightly from one run to another. [b] The composition of the solvents is given in the Experimental section. [c] Exhibited some streaking forward. [d] The R_f values of these non-acylated phospholipids have been taken from a more extensive table which will be published elsewhere.[8]

The application of the acylation technique to the study of lipid mixtures extracted from animal tissues will be reported in a forthcoming paper.

Experimental

Acylation of the Phospholipids.—Synthetic L-α-distearoylcephalin[10] (I) and purified acetal phospholipid[11] (II) were acetylated as follows: 3 mg. of each lipid was treated separately with 0.8 ml. of redistilled pyridine and 0.2 ml. of acetic anhydride and heated at 60° for 0.5 hour in a water-bath. The solutions were evaporated to dryness under nitrogen at 40° and the residues dissolved in isoamyl alcohol–benzene 1:1 to give a concentration of 50 μg. of each lipid per 20 μl. In addition the following mixtures were acetylated in the same manner: I + II; I + L-α-distearoyllecithin[10] (III); I + sphingomyelin[12] (IV); II + III; II + IV; I + II + III; and I + II + IV. Under these conditions the N-acetyl derivatives of only I and II were obtained. These latter phospholipids gave a positive ninhydrin test before acylation but a negative ninhydrin test after acylation, and there resulted a marked increase in their R_f values (Table I). The R_f values of III and IV were not affected by the acylation treatment. It was further found that synthetic samples of L-α-dimyristoylcephalin[10] and L-α-dipalmitoyllecithin[10] behaved in a similar manner to I and III, respectively.

The same amounts of the above lipids and lipid mixtures were also acylated by either dissolving in a solution of 0.8 ml. of chloroform and 0.2 ml. of benzoyl chloride and letting stand at room temperature for two hours or by dissolving in 1 ml. of 0.1 *M* 2,4-dinitrofluorobenzene[13] in 0.1 *M* ammoniacal methanol and letting stand overnight at room temperature. The solutions were evaporated to dryness under nitrogen and the residues dissolved in isoamyl alcohol–benzene 1:1 to give a concentration of 50 μg. of each phospholipid per 20 μl. It was noted that when either I or III was allowed to stand in the benzoyl chloride–chloroform solution for several days or longer at room temperature, the R_f values of these lipids increased and were similar to that of phosphatidic acid (R_f 0.95). These products do not correspond to N-acyl derivatives. This effect may be similar to the diazomethanolysis of phospholipids reported by Baer and Maurukas.[14]

Chromatography of the Phospholipids.—Chromatograms were run in all glass cylinders, 6″ i.d. × 18″, using the as-

(10) Donated by Dr. E. Baer, The University of Toronto, Toronto, Canada.

(11) Donated by Dr. S. J. Thannhauser, Pratt Diagnostic Institute, and Tufts Medical School, Boston, Mass.

(12) Prepared as described by G. Marinetti and E. Stotz, THIS JOURNAL, **76**, 1347 (1954).

(13) The acylation of phospholipids with this reagent has been reported by G. L. Ellman and H. K. Mitchell, *ibid.*, **76**, 4028 (1954), and by F. D. Collins and L. W. Wheeldon, *Nature*, **175**, 772 (1955).

(14) E. Baer and J. Maurukas, *J. Biol. Chem.*, **212**, 39 (1955).

cending technique. The phospholipids were applied to the paper (5″ × 16″) at a concentration of 50 μg. each per 20 μl. of isoamyl alcohol–benzene 1:1. Since mixtures were run the total amount of lipid applied to the paper varied from 50 to 150 μg. Good separations were also obtained using 300–400 μg. of total lipids.

Whatman No. 1 filter paper was used which had previously been washed successively with 1 *N* acetic acid, water and methanol. The papers were first dried in air and then in an oven at 100° for 15 minutes. The solvent systems used were as follows: solvent A, lutidine–methanol 3:2; solvent B, methanol–lutidine–acetic acid 4:16:1; solvent C, 2-octanol–lutidine–acetic acid 90:5:5.

In the chloroform–lutidine–acetic acid system reported previously,[7] which gave useful separations of unmodified phospholipids, the N-acetyl derivatives of I and II moved with an R_f value of 0.93 and were not separated from each other but were completely resolved from phosphatidylcholines. On the other hand, solvent systems A, B and C were able to resolve the N-acetyl derivatives of I and II.

The solvent mixtures were prepared on a volume to volume basis. The lutidine used was obtained from the Eastman Kodak Co. (T4908), and was a mixture of lutidines. Pure 2-octanol, absolute methanol and J. T. Baker analyzed glacial acetic acid were used. In preparing solvents B and C the acetic acid and lutidine were first mixed, cooled and then the third component added. The chambers were lined internally with filter paper and equilibrated with 150 ml. of the developing solvent for 8 hours before use. All chromatograms were run at about 27°. The solvent front was allowed to run about 14–15″.

Detection of the Phospholipids on Chromatograms.—The chromatograms were air-dried, washed with distilled water for 10 minutes, dried and then immersed in an aqueous solution of 0.001% Rhodamine B or G (National Aniline Division, Allied Chemical and Dye Corp.) for 10 minutes. The excess dye was washed out with distilled water and the papers air-dried. The lipid spots were observed under ultraviolet light as fluorescent areas. In addition, the phosphatidylcholines can be detected by the method of Levine and Chargaff[15] and the lipids containing a free amino group by spraying with a 0.3% solution of ninhydrin in acetone–lutidine 95:5. The blue color resulting from the latter reaction was allowed to develop at room temperature in the dark.

(15) C. Levine and E. Chargaff, *J. Biol. Chem.*, **192**, 465 (1951).

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24-Hydroxycholane-3,7,12-trione

BY ROBERT H. MAZUR AND EDWARD A. BROWN

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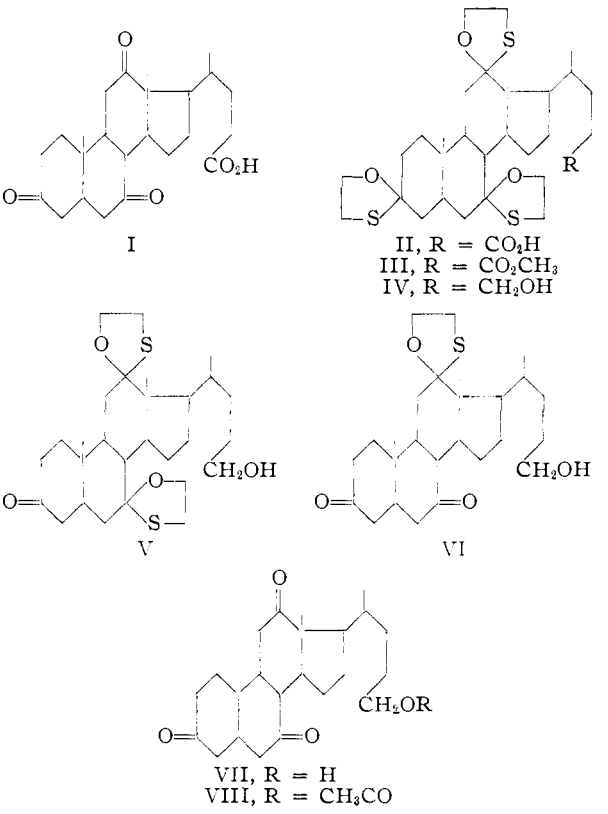
In view of the physiological activity of dehydrocholic acid (I)[1] it seemed desirable to prepare 24-hydroxycholane-3,7,12-trione (VII) for pharmacological evaluation. A suitable synthesis was found by protection of the three keto groups of dehydrocholic acid, reduction of the carboxylic acid (in the form of its ester) and regeneration of the keto groups. The commoner reagents for blocking ketones are ethylene glycol[2] and ethanedithiol.[3] In the present case, ethylene glycol failed even on long refluxing to form a triethylene ketal. Although ethanedithiol reacted readily, the trimercaptol was attacked by lithium aluminum hydride to yield unidentifiable sulfur-containing products. The use of β-mercaptoethanol[4,5] proved successful and gave the desired dehydrocholic acid trihemithioethylene ketal II. The latter was



esterified with ethereal diazomethane and the resulting ester III reduced with lithium aluminum hydride to the alcohol IV. It was found possible to hydrolyze IV selectively so that the mono-, di- and triketo alcohols were prepared. Structures were assigned on the basis of the known relative reactivities of the keto groups of dehydrocholic acid.[6] The 3-ketone V was obtained by acid-catalyzed methanolysis while hydrolysis with sulfuric acid in aqueous dioxane gave a separable mixture of the 3,7-diketone VI and 24-hydroxycholane-3,7,12-trione (VII). A more convenient procedure was to hydrolyze IV with concentrated hydrochloric acid in acetic acid with simultaneous acetylation to yield VIII. The latter could readily be saponified to VII. Compounds II–VIII had the expected infrared spectra (taken at 0.5% concentration in a potassium bromide disc).

Experimental[7]

Dehydrocholic Acid Trihemithioethylene Ketal (II).—A mixture of 82 g. of dehydrocholic acid, 88 g. of β-mercaptoethanol and 1 g. of *p*-toluenesulfonic acid monohydrate in 1.8 liters of toluene was heated under reflux (continuous water separator) for 18 hours. The toluene was distilled and the residue dissolved in 900 ml. of 90% aqueous methanol containing 80 g. of potassium hydroxide. The solution was heated under reflux for two hours, poured into an excess of cold, dilute hydrochloric acid and the product taken up in benzene. The benzene was distilled to a small volume and the residue diluted with one liter of

(1) V. A. Drill, "Pharmacology in Medicine," McGraw-Hill Book Co., Inc., New York, N. Y., 1954, sec. 43, p. 6.

(2) M. B. Fernholz, U. S. Patent 2,378,918; *C. A.*, **39**, 5051 (1945).

(3) H. Hauptmann, THIS JOURNAL, **69**, 562 (1947).

(4) J. Romo, G. Rosenkranz and C. Djerassi, *ibid.*, **73**, 4961 (1951).

(5) C. Djerassi and M. Gorman, *ibid.*, **75**, 3704 (1953).

(6) L. F. Fieser and M. Fieser, "Natural Products Related to Phenanthrene," 3rd Ed., Reinhold Publ. Corp., New York, N. Y., 1949, p. 125.

(7) We are indebted to Robert T. Dillon and his associates for analyses and determinations of physical properties.