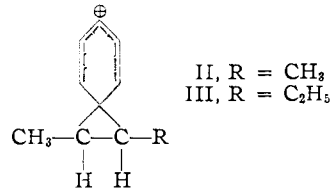
crete intermediate which may be represented by structure I.

I

The σ-value of a substituent can be regarded as a measure of electron availability which it creates due to a combination of inductive and resonance effects.[15] The fact that the curves are concave upward is the result of the ability of electron-releasing groups to increase through resonance the capacity for positive charge. This is especially true for the para substituents, which therefore produce higher rates than do the meta. The discontinuity in the para curve between the halogens and carbethoxy reflects the inability of the latter to stabilize a cation to such an extent that it falls below the meta curve. Now if cleavage of the carbon–boron bond were important in the rate-determining step it would be facilitated by electron withdrawing groups, particularly when in the ortho or para positions. In this event one would expect the point for *p*-carbethoxy to fall above the meta curve.

The fact that one of the reacting species in the rate-determining step is probably a quadricovalent boron derivative does not alter the argument. It would be expected that the electron-releasing groups would decrease the concentration of such an intermediate. In such a case the effect on the brominolysis rate would be the reverse of that actually observed. Thus this factor must be relatively unimportant in determining the relative rates.

Convincing evidence for a discrete intermediate in aromatic electrophilic displacement reactions has been obtained by other investigators. In aromatic nitration tritium is replaced at the same rate as hydrogen.[16] The stereochemistry of the acetolysis of the tosylates of 3-phenyl-2-butanol (II) and 2-phenyl-3-pentanol (III) has been shown by Cram to require the assumption of an intermediate such as II.[17]



II, R = CH_3
III, R = C_2H_5

Figure 3 is a log–log plot of our rate constants *versus* those for aromatic nitration. It is apparent that, whereas the para substituents fall on a straight line, the meta substituents do not.[18] The probable similarity of the transition state complexes in the two reactions might lead one to anticipate a linear relation for both meta and para substituents. However, a number of other factors, such as overall mechanism and solvent, for example, intrude to make prediction difficult. An extension of this work which is in progress may make possible a delineation of the important factors.

(15) Reference 3, p. 196.

(16) L. Melander, *Acta. Chem. Scand.*, **3**, 95 (1949); *Nature*, **163**, 599 (1949); *Arkiv. Kemi*, **2**, 213 (1950).

(17) D. J. Cram, THIS JOURNAL, **71**, 3863, 3875 (1949).

(18) The rate constant for the *m*-chloro substituent in nitration is estimated on the basis of about 0.3% meta nitration of chlorobenzene; A. Hollemann, *Rec. trav. chim.*, **19**, 304 (1900). This has been confirmed by the isotopic dilution method of analysis (private communication from Dr. J. D. Roberts).

DURHAM, NEW HAMPSHIRE

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WASHINGTON]

The Isolation of Dipalmitoleyl-L-α-glycerylphosphorylcholine from Yeast. A New Route to (Dipalmitoyl)-L-α-lecithin[1]

BY DONALD J. HANAHAN AND MICHAEL E. JAYKO[2]

RECEIVED MAY 6, 1952

An individual, completely unsaturated lecithin, dipalmitoleyl-L-α-glycerylphosphorylcholine, has been isolated for the first time from fresh baker's yeast. A reproducible, modified adsorption technique, which uses aluminum oxide as the adsorbent, is described for the isolation of this lecithin. Catalytic hydrogenation of this unsaturated compound gives the corresponding individual, saturated lecithin, (dipalmitoyl)-L-α-lecithin.

In a previous report from this Laboratory,[3] it was shown that the lecithins of egg could be isolated by passage of an ethanol solution of the mixed phospholipides through a column of aluminum oxide. The ethanolic eluates contained only the lecithins, with approximately 25% of their fatty acids being unsaturated. As a supply of a more highly unsaturated lecithin was also desired for enzyme studies, other sources of phospholipides were considered. A very promising source appeared to be yeast (*Saccharomyces cerevisiae*). Salisbury and Anderson[4] reported that the phospholipides obtained from yeast, grown under conditions designed to prevent the production of hydrocarbon impurities, were composed of 4 parts lecithin and 1 part cephalin. Approximately 86% of the fatty acids of these lecithins were unsaturated. In confirmation of these observations, we have found a similar composition of the fatty acids in the mixed phospholipides of commercial baker's yeast.

(1) This work was performed under Contract No. N8-onr-52004 between the University of Washington and the Office of Naval Research, United States Navy Department.

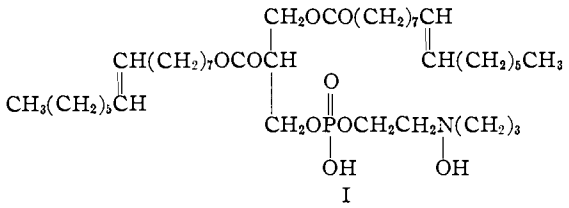
(2) A portion of this paper formed the thesis submitted by Michael E. Jayko to the Graduate School, University of Washington, January, 1952, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry.

(3) D. J. Hanahan, M. B. Turner and M. E. Jayko, *J. Biol. Chem.*, **192**, 623 (1951).

(4) L. F. Salisbury and R. J. Anderson, *ibid.*, **113**, 541 (1936).

On the basis of our previous results on the separation of egg lecithins, the fractionation of a highly unsaturated lecithin from yeast by an adsorption technique appeared possible.

Briefly the following general procedure was adopted for the isolation of yeast lecithin: Fresh commercial baker's yeast was extracted with alcohol–ether at room temperature. The alcohol–ether soluble fraction was removed and concentrated to a small volume at 40° under reduced pressure in an atmosphere of nitrogen. The concentrate was extracted with diethyl ether and the phospholipides isolated by precipitation with acetone. After several precipitations and washings with acetone, the mixed phospholipides were dissolved in 95% ethanol, passed through an aluminum oxide column and the ethanolic eluates were collected. Only one type of lecithin was identified in these ethanolic eluates. This was an optically active, individual lecithin, which was found to be dipalmitoleyl-L-α-glycerylphosphorylcholine (I).



I

Compound I is a pasty, colorless substance, which is quite stable to atmospheric oxidation. Samples of this material have been exposed to air for several days at room temperature without any changes in color or unsaturation. It is easily soluble in diethyl ether, chloroform, ethanol, 90% acetone, difficultly soluble in petroleum ether and forms stable emulsions with water.

Catalytic hydrogenation of compound I at atmospheric pressure gave the corresponding (dipalmitoyl)-L-α-lecithin (II). Its composition, melting point, optical activity and X-ray diffraction pattern established II as identical with synthetic (dipalmitoyl)-L-α-lecithin. The assignment of the alpha configuration to II (and I) was made possible only by comparison with the characteristics of synthetic (dipalmitoyl)-L-α-lecithin, which has been synthesized by Baer and Kates.[5] The present evidence supports the theory of Baer and Kates[6] that only the α-lecithins exist in nature.

Of significance is the fact that the saturated lecithin II is the only compound present in the reaction mixture from the hydrogenation of I. Analysis of this reaction mixture, and of the substance recrystallized from dioxane and diisobutyl ketone, show no differences in composition or optical activity.

Salisbury and Anderson[4] found that the catalytically hydrogenated fatty acids of their yeast lecithin were composed of 63% palmitic acid and 37% stearic acid. However, we find only a single fatty acid associated with the lecithin. These differences may be resolved when it is noted that only 65 to 70% of the choline placed on an aluminum oxide column is found in the eluates. Thus it is conceivable that under the conditions used here, the C_{16}-fatty acid containing lecithin is not absorbed while the C_{18}-fatty acid ones are. However, it is quite possible that differences in the strain of yeast used and the growth medium might account for these variations in results.

It was noted that upon overloading a column, *i.e.*, using amounts of mixed phospholipides greater than 4 g. per 120 g. of aluminum oxide, the isolated lecithin consistently had high P, N and choline values (P, 4.46; N, 2.02; choline, 17.50). This suggested the presence of a contaminant. Upon dialysis of the lipide against distilled water or by decreasing the amount of lipide placed on the column to a value below 4 g. per 120 g. of adsorbent, this contaminant was removed. No evidence as to its structure has been obtained. It is interesting to note that the lecithins isolated by Salisbury and Anderson had similarly high values for these constituents and it is possible that a similar contaminant might have been present in their preparation.

The infrared spectra of (dipalmitoleyl)-L-α-lecithin (I), (dipalmitoyl)-L-α-lecithin and palmitoleic acid are shown in Fig. 1. The two lecithins

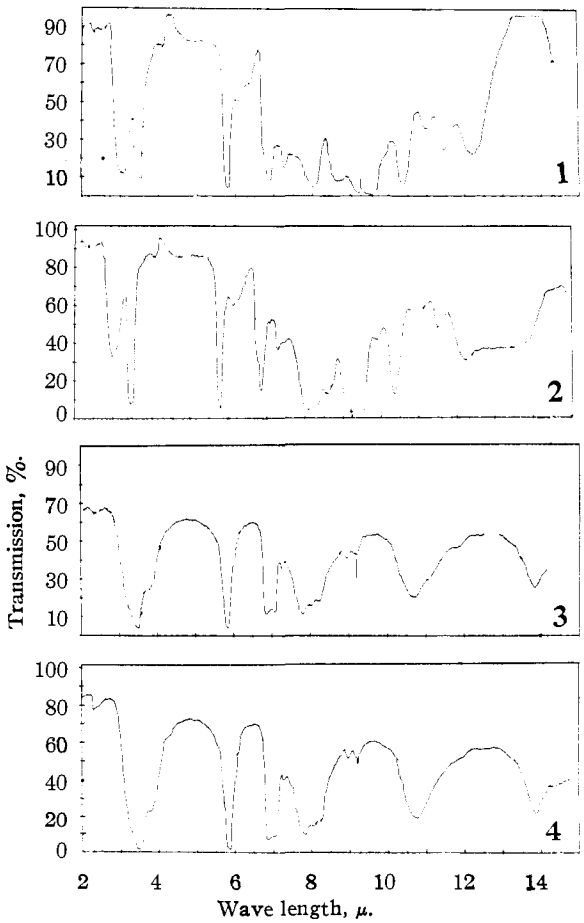


Fig. 1.—Infrared spectra: 1, (dipalmitoleyl)-L-α-lecithin, 8.6% in $CHCl_3$, 0.10-mm. cell; 2, (dipalmitoyl)-L-α-lecithin, 8.6% in $CHCl_3$, 0.10 mm. cell; 3, palmitoleic acid isolated from 1, 0.018-mm. cell; 4, oleic acid obtained from methyl oleate (Hormel), 0.025-mm. cell. Perkin–Elmer recording infrared spectrophotometer, Model 21.

(5) E. Baer and M. Kates, THIS JOURNAL, 72, 942 (1950).
(6) E. Baer and M. Kates, *J. Biol. Chem.*, 185, 615 (1950).

have very similar spectra and show absorption bands characteristic of long chain glycerides.[7] The main difference between these two compounds appeared to be in the presence of an absorption band at 14.7μ in the unsaturated lecithin. Upon hydrogenation of this compound, the band disappeared. It would appear that absorption in this region is due to the presence of the *cis*-acid in the unsaturated lecithin molecule. The spectrum of the fatty acid obtained from I showed absorption bands characteristic of a long chain unsaturated aliphatic acid, *i.e.*, the C–H stretching band at 3.5 μ with a doublet at 6.90 and 7.10 μ, the –COOH band at 7.83 μ, the 10.65 μ band for the –OH of carboxyl group, and the characteristic absorption of a *cis* double bond at 13.87 μ. No absorption band at 10.36 μ for a *trans* component was observed. According to the evidence presented by Shreve, *et al.*,[7] one could predict that oleic and palmitoleic acid should be quite similar in their spectral characteristics. A comparison of the infrared spectra of these two compounds showed a remarkable similarity. Unfortunately no palmitoleic acid from another source was available for comparison.

To date, approximately 400 pounds of baker's yeast have been processed by the procedure described in this communication. Except for difficulties encountered in the initial experiments, all the runs have given the same results and have indicated that this unsaturated lecithin is a naturally occurring component of yeast.

To our knowledge, this is the first report of the isolation of an individual, completely unsaturated lecithin from a natural source. A convenient route to a completely saturated, individual lecithin is presented.

Experimental

Materials and Methods.—Fresh Fleischmann's commercial baker's yeast, without starch additive, was used.[8] In all cases, the yeast was stored at 4° until used and was processed within one day after receipt in the laboratory.

The solvents were chemically pure or reagent grade. Diethyl ether was freshly distilled from sodium. Ethyl alcohol was distilled over potassium hydroxide. Petroleum ether, b.p. 30–60°, was washed with concentrated H_2SO_4 and distilled over solid NaOH.

Aluminum oxide, Merck and Co., Inc., "suitable for chromatographic analysis," was used as the adsorbent in all experiments. Little or no variation was found in the adsorption qualities of aluminum oxide from different lots.

Preparation of Mixed Phospholipides.—Ten pounds of fresh yeast was crumbled into 8 liters of 95% ethyl alcohol and allowed to stand at room temperature for 24 hours with occasional stirring.[9] Sufficient peroxide-free diethyl ether was added to make a 3:1 alcohol–ether mixture. After an additional 24 hours at room temperature, the mixture was filtered, the filtrate set aside and the residue resuspended in 6 liters of 3:1 alcohol–ether mixture for an additional 24 hours. The mixture was filtered and the residue discarded. The filtrates were combined and concentrated to a small volume at 45° under reduced pressure in an atmosphere of nitrogen. The concentrate was extracted with two volumes of diethyl ether. Five volumes of acetone were added to the ether soluble fraction. The precipitated phospholipides were recovered by centrifugation and washed successively with 6 charges of fresh acetone. The precipitate was dissolved in a minimum amount of ether and the acetone precipitations and washings described above were repeated. Finally the phospholipides were dissolved in a sufficient amount of 95% ethyl alcohol to make a 1.5% solution. At this step in the procedure, a considerable amount of alcohol insoluble material was obtained; upon standing or by warming at 45°, this insoluble fraction may slowly dissolve. The average yield of alcohol soluble mixed phospholipides from ten pounds of wet yeast was 7 to 8 g. *Anal.* Found: P, 4.18; N (Kjeldahl), 2.80; choline,[10] 11.4; N/P molar ratio, 1.48; choline/P molar ratio, 0.70; iodine number,[11] 59.0.

One gram of the mixed phospholipides was emulsified in 50 ml. of 5 *N* HCl and refluxed for 6 hours at 95–100°.[12] The hydrolyzate was cooled immediately under nitrogen in an ice-bath and the fatty acids were isolated by extraction with petroleum ether. The extract was washed 3 times with one-half volume of water and dried over anhydrous sodium sulfate. Found: 650 mg. (65%); iodine number, 85.0; neut. equiv., 256.0; unsaturated fatty acids,[13] 91.0% of total; iodine number, 93.0; neut. equiv., 254.0; saturated fatty acids,[13] 9.0% of total; iodine number, 0; neut. equiv., 259.0.

Dipalmitoleylglyceryl-L-α-phosphorylcholine. (1) **Preparation of Adsorption Column.**—An all glass adsorption column, which measured 12 mm. in diameter and 700 mm. in length, was used. It was equipped with a 200-ml. reservoir at the top and fitted with a No-Lub stopcock (Scientific Glass Apparatus Co.) at the base outlet. One hundred and twenty grams of aluminum oxide, suspended in 95% ethanol, was poured into the column, which contained a mat of cotton under a mat of glass wool at the base outlet. The outlet was opened and, while the solvent was allowed to run through, the column was tapped gently from time to time to promote uniform packing.

(2) **"Chromatographic" Procedure.**—Three grams of yeast-mixed phospholipides in 200 ml. of 95% ethanol was poured onto a column of 120 g. of aluminum oxide. A constant head of alcohol was then maintained on the column and the eluates were collected. The first 300 ml. of effluent contained no phosphorus compounds; the 400 through 600 ml. eluates contained 95% of the lipide removed from the column.[14] These fractions were combined and concentrated to dryness at 45° under reduced pressure in a nitrogen atmosphere. The residue was dissolved in diethyl ether and precipitated with 5 volumes of acetone. The precipitate was dissolved in ether, precipitated once more with 5 volumes of acetone, and finally dissolved in diethyl ether; yield 1.4 g. (65% recovery, based on choline).

Anal. Calcd. for $C_{40}H_{78}O_9NP$ (748): P, 4.13; N, 1.87; choline, 16.2; iodine number, 67.8; N/P, 1.00; choline/P, 1.00. Found: P, 4.15; N, 1.86 (Kjeldahl); choline,[9] 16.0; iodine number,[10] 67.2; N/P, 0.99; choline/P, 0.99; $[\alpha]^{25}D$ +6.62°; $[M]_D$ +49.5°; unsaturated fatty acids, 100% (of total); iodine number, 99.5.

(3) **Hydrolysis Product.**—A solution of 1.000 g. of dipalmitoleyl-lecithin in 40 ml. of ethanol and 40 ml. of 2 *N* aqueous potassium hydroxide was refluxed for 6 hours at 95–100°. The solution was concentrated to approximately 40 ml. in a stream of nitrogen and acidified with 10 ml. of 10 *N* sulfuric acid. The fatty acid was extracted in the same manner as described for the mixed phospholipides.

(a) **Fatty Acid.**—Yield calcd. 680 mg.; found, 670 mg. (98.7% recovery). Calcd. for palmitoleic acid: iodine number, 99.8; neutral equivalent, 254.4; saponification equivalent, 254.4. Found: iodine number, 99.5; neut. equiv., 255.0; sapn. equiv., 253.2. *p*-Bromophenacyl ester, 39.0–39.5° (acc. to Baudart,[15] 39.5–40.0°); dihydroxypalmitic acid, 86.0–86.5° (reported value,[15] 86.0–

(7) O. D. Shreve, M. R. Heether, H. B. Knight and D. Swern, *Anal. Chem.*, **22**, 1498 (1950).

(8) This yeast was generously supplied by Standard Brands, Inc., Sumner, Washington.

(9) Extraction carried out at 55 to 60° was found to be quite damaging to the phospholipides.

(10) D. Glick, *J. Biol. Chem.*, **156**, 643 (1944).

(11) M. Yasuda, *ibid.*, **94**, 401 (1931–1932).

(12) Hydrolysis of the crude mixed phospholipides proceeded more smoothly in an acid medium, while alkaline hydrolysis worked best with the more purified fractions.

(13) The separation of the fatty acids into the saturated and unsaturated components was accomplished by the method of E. Twitchell, *J. Ind. Eng. Chem.*, **13**, 806 (1921), as modified by J. Folch, *J. Biol. Chem.*, **174**, 439 (1948).

(14) Analysis of every 25 ml. of the eluates showed all to contain the same compounds with no peak being evident.

(15) P. Baudart, *Bull. mat. grasses inst. colonial Marseille*, **29**, 75 (1945).

86.5°); $n^{25}D$ 1.4535. Two hundred milligrams of palmitoleic acid in 95% ethanol was hydrogenated at atmospheric pressure in the presence of 10 mg. of platinum oxide (Adams catalyst). In 15 minutes the adsorption of hydrogen was complete and 17.0 ml. of hydrogen (corrected for catalyst) had been consumed (calculated for reduction of a single double bond fatty acid, 17.5 ml. hydrogen). The catalyst was removed by centrifugation and washed once with warm 95% ethanol. The alcohol soluble fractions were combined and concentrated to dryness *in vacuo* at 45°. The saturated acid was recrystallized once from warm 60% ethanol and once from 10% aqueous acetone; m.p. 60–61°; no depression on admixture with pure palmitic acid; neutral equivalent: calcd., 256.4, found, 255.8.

(b) **Glycerophosphate.**—The water-soluble fraction and the washings from the fatty acid isolation were combined and the glycerophosphate was isolated by the method of Folch.[16] Barium glycerophosphate: calcd., 411.7 mg., found, 345.0 mg.; 84% recovery. *Anal.* Calcd. for $C_3H_7O_6PBa$ (307.3): C, 11.73; P, 10.1; glycerol, 29.3. Found: C, 11.6; P, 10.1; glycerol,[17] 28.0. Over 99% of the water-soluble phosphorus assayed as glycerophosphate by the peroxidative method.[18]

(Dipalmitoyl)-L-α-lecithin.—One gram of dipalmitoleylglycerylphosphorylcholine in 95% ethanol was hydrogenated in the presence of 100 mg. of platinum oxide (Adams catalyst). In 20 minutes the uptake of hydrogen was complete and 58.5 ml. of hydrogen (cor. for catalyst) had been consumed (calculated for the lecithin, 59.6 ml. of hydrogen). The catalyst was removed by centrifugation and washed once with warm ethanol. The filtrates were combined and concentrated to dryness at 40° under reduced pressure in a nitrogen atmosphere. The residue was dissolved in a minimum amount of warm dioxane (60°) and upon slow cooling to room temperature, crystallization occurred. This substance was recrystallized twice more from warm dioxane, and once from diisobutyl ketone. The dipalmitoyllecithin was dried for two days at room temperature over P_2O_5 and paraffin shavings, yield 850 mg. (85% of theory).

This compound formed transparent droplets at 89–92° and upon further heating (25°/min. to 210°, then at 10°/min.), formed a meniscus at 234.8–235.0°; $[\alpha]^{25}D$ +6.62 in chloroform–methanol (1:1), *c*; $[M]_D$ +49.8; reported by Baer and Kates[5] as $[\alpha]^{23}D$ +6.6 in $CHCl_3$–methanol (1:1).

Anal.[19] Calcd. for $C_{40}H_{82}O_9NP$ (752): C, 63.9; H, 11.0; N, 1.86; P, 4.12. Found: C, 63.7; H, 11.06; N, 1.89 (Dumas), 1.82 (Kjeldahl); P, 4.15.

Five hundred milligrams of this saturated lecithin was hydrolyzed and the fatty acid isolated in a manner similar to that described for the unsaturated lecithin; yield of palmitic acid, calcd., 340 mg.; found, 330 mg. (97% of theory). The analytical composition, melting point and neutral equivalent were in excellent agreement with pure palmitic acid and with the palmitic acid isolated by hydrogenation of palmitoleic acid.

An X-ray diffraction powder pattern of the hydrogenated lecithin was made and compared with that reported for synthetic (dipalmitoyl)-L-α-lecithin.[20] The spacings of the yeast lecithin were in excellent agreement with the synthetic compound. No extraneous lines were observed.

(16) J. Folch, *J. Biol. Chem.*, **174**, 439 (1948).
(17) G. Blix, *Mikrochim. Acta*, **1**, 75 (1937).
(18) C. F. Burmaster, *J. Biol. Chem.*, **164**, 233 (1946).
(19) Microanalyses were performed by Elek Micro Analytical Laboratories, 4763 West Adams Boulevard, Los Angeles, California.
(20) We are indebted to Dr. L. H. Jensen for the X-ray analysis.

SEATTLE, WASHINGTON

[CONTRIBUTION FROM THE MCARDLE MEMORIAL LABORATORY, UNIVERSITY OF WISCONSIN, THE DIVISION OF PURE CHEMISTRY, NATIONAL RESEARCH COUNCIL OF CANADA, AND THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF ALBERTA[1]]

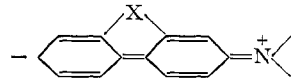
Ultraviolet Spectra and Carcinogenic Activities of Some Fluorene and Biphenyl Derivatives

BY REUBEN B. SANDIN, RUSSELL MELBY, ALLAN S. HAY, R. NORMAN JONES, ELIZABETH C. MILLER AND JAMES A. MILLER

RECEIVED JUNE 2, 1952

The ultraviolet absorption spectra of the following compounds have been determined, *viz.*, 4,4′-dinitrobiphenyl, 2,7-dinitrofluorene, 4-amino-4′-nitrobiphenyl, 2-amino-7-nitrofluorene, 4-acetylaminobiphenyl, 2-acetylaminofluorene and 2- and 2′-methyl-4-acetylaminobiphenyl. Each fluorene compound absorbs at longer wave length than its biphenyl analog without the methylene bridge, whereas the introduction of a methyl group into biphenyl has the opposite effect. The carcinogenic activities of the last four compounds have been determined and these activities have been correlated with the corresponding absorption spectra.

The interesting work of Haddow, Harris, Kon and Roe[2] on the growth-inhibitory and carcinogenic properties of 4-aminostilbene and derivatives has indicated that one of the features necessary for activity is an unbroken conjugation of the amino group with both nuclei, enabling the compound to assume a resonating quinonoid structure. Evidence, which was mainly obtained from ultraviolet spectroscopy, suggested that there was a close parallel between lack of biological activity and steric interference with the planar arrangement of the molecule. Recently Miller, Miller, Sandin and Brown[3] have considered it possible that the greater carcinogenic activity of derivatives of fluorene and its oxygen and sulfur analogs *versus* biphenyl derivatives is associated with the $-CH_2-$, $-S-$ and $-O-$ groups which help maintain a coplanar arrangement of the benzene nuclei. The molecule would then be represented to a greater extent by the resonating structures such as



X = CH_2, O, S

In this paper the absorption spectra of several pairs of biphenyl and fluorene derivatives have been compared in order to determine the effect of the methylene and methyl groups. These compounds include 4,4′-dinitrobiphenyl, 2,7-dinitrofluorene, 4-amino-4′-nitrobiphenyl, 2-amino-7-nitrofluorene, 2-acetylaminofluorene, 4-acetylaminobiphenyl and 2- and 2′-methyl-4-acetyl-

(1) Published as Contribution No. 2820 from the Laboratories of the National Research Council of Canada.
(2) A. Haddow, R. J. C. Harris, G. A. R. Kon and E. M. F. Roe, *Trans. Roy. Soc. (London)*, **A241**, 147 (1948).
(3) E. C. Miller, J. A. Miller, R. B. Sandin and R. K. Brown, *Cancer Research*, **9**, 504 (1949).