Characterization of a Fluorescent Lipid Fraction From Cancer-bearing Animals.* (18308)

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In 1947 Penn and Kaplan(1) confirmed Penn's previous isolation of a "methylcholanthrene-like" substance from cancerous human livers(2). Because of the great interest attached to the isolation of endogenous carcinogens, other authors have extended Penn's investigations. Jones and Jamieson (3) prepared fractions by methods "which parallel quite closely those employed by Penn", but were unable to detect in them the typical ultraviolet spectra of polynuclear aromatic hydrocarbons. They imputed the fluorescence observed by Penn to iso-anhydrovitamin A which they detected in some of their preparations. Tuttle and Jacobs(4) were unable to find the typical ultraviolet absorption of methylcholanthrene in crude fractions supplied by Penn. Since far smaller amounts of material can be detected by fluorescence spectrophotometry than by ultraviolet absorption this work does not preclude the presence of a very small amount of a fluorescent "methylcholanthrene-like" substance in Penn's extracts. The present study was undertaken to investigate further the fluorescent components of liver lipids extracted by Penn's procedure.

Materials and methods. Two human livers were obtained through the kindness of Dr. E. Stern. The tissue was digested, extracted and saponified according to the directions of Penn, except that a second hydrolysis of the unsaponifiable fraction was omitted after it was demonstrated that this procedure did not remove any further material. The crude unsaponifiable fraction was further purified by treatment with phthalic anhydride and Girard's reagent T according to the directions of Dobriner, Lieberman and Rhoads(5). This procedure was shown not to affect the blue fluorescence of the material under investigation, and it offered the advantage of eliminating more contaminating compounds than the digitonin treatment employed by Penn. The small amount of tan oil remaining was further fractionated by chromatography on alumina (Alcoa F-20, washed with 5% acetic acid and reactivated at 240°) and silicic acid (Merck, screened to 140 mesh and activated at 350°) from petroleum ether (30-60°, redistilled from sulfuric acid) solution.

Rabbit tissues. Rabbit livers and blood, from animals implanted with the Brown-Pearce carcinoma, were obtained through the courtesy of Dr. E. M. Jacobsen. Only uninvolved tissue was used. The livers were extracted exactly as was the human material. Rabbit blood was poured into 4 volumes of a 1:3 ether-ethanol mixture. The suspension was filtered and the precipitate re-extracted with fresh ether-ethanol mixture. The solvents were removed on the steam bath and the residue extracted into ether. The residue after evaporation of the ether was treated further like the liver lipids. Unsaponifiable lipids, freed of hydroxylic and ketonic compounds, were chromatographed on alumina from petroleum ether. Blue-fluorescent material was eluted with 3-10% ether in petroleum ether until contamination with brown-fluorescent material became evident. The eluates were then re-chromatographed from petroleum

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1. Penn, H. S., and Kaplan, J., *Nature,* 1947, v160, 18.

2. Penn, H. S., *Nature,* 1942, v149, 193.

3. Jones, R. N., and Jamieson, J. R., *Nature,* 1947, v160, 677.

4. Tuttle, W. P., and Jacobs, T. L., unpublished data.

5. Dobriner, K., Lieberman, S., and Rhoads, C. P., *J. Biol. Chem.,* 1948, v172, 248.

ether on silicic acid and the column washed thoroughly with petroleum ether. Elution with 0.5% ether in petroleum ether brought down the blue-fluorescent zone which could not be further purified by chromatography.

Results. Fluorescent material was isolated from human liver, rabbit livers and blood (both from normal and from cancer-bearing animals) and normal hog blood. In "mixed chromatograms" on alumina and on silicic acid the human liver material formed single sharp zones with hog and rabbit blood fractions. A systematic attempt to compare the properties of the blue-fluorescent lipid fractions with authentic methylcholanthrene was undertaken:

1. When methylcholanthrene was mixed with the fluorescent material from "cancerous" rabbit blood and the mixture chromatographed on silicic acid, the two constituents separated. The methylcholanthrene was more strongly adsorbed on the column and the lipid could be washed out selectively. The eluate showed the fluorescence spectrum of the lipid material only.

2. Since Dimter(6) and Stanger, Steiner and Bolyard(7) had isolated hepene (a highly unsaturated terpene precipitable by the addition of ethereal hydrogen chloride) from human livers, the effect of this reagent on methylcholanthrene and our fluorescent liver fraction was investigated. No precipitate formed in either case. The blue fluorescence of the rabbit lipid was quenched instantaneously and could be completely restored by neutralization of the solution with base. Under the same conditions the fluorescence of authentic methylcholanthrene persisted for about a minute and then was gradually replaced by a canary-yellow fluorescence which surpassed the original blue fluorescence in intensity. Neutralization resulted in a blue fluorescence of much lower intensity than the original sample showed.

3. Jones and May(8) attempted unsuccessfully to concentrate the active fluorescent constituent of human livers by precipitation as the picric acid complex. We, too, failed to obtain any adsorption on the precipitate with our lipid fractions. But authentic methylcholanthrene also could not be concentrated from solutions showing equal fluorescence in which the concentration of the carcinogen was about 10^{-8} M.

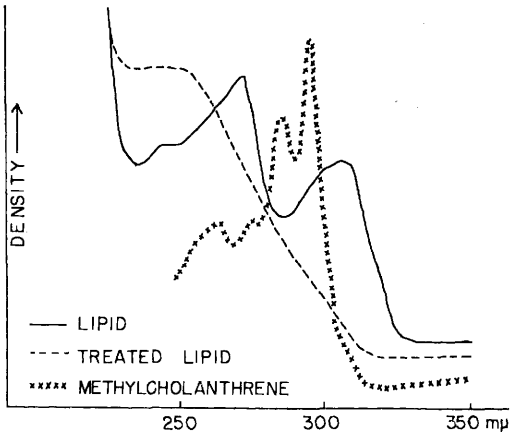

FIG. 1. Ultraviolet Spectra.

All spectra were determined on a Beckman DU spectrophotometer. The ordinate represents optical density plotted in arbitrary units so as to obtain comparable absorptive intensity for the three substances represented. Lipid refers to the unsaponifiable material which was eluted from a silicic acid column as a single sharp zone. Treated Lipid refers to the same material after treatment with potassium permanganate in acetone solution. Methylcholanthrene was obtained from the Eastman Kodak Company. It was purified by chromatography on silicic acid and was then treated with permanganate in acetone under the same conditions as described for the lipid.

4. The ultraviolet absorption spectrum of the fluorescent lipid was determined. Pooled samples from human and rabbit sources gave rise to peaks at 272 mμ and 306 mμ. (Fig. 1.) The curve did not resemble the spectrum of methylcholanthrene at all. Treatment of the lipid fraction with potassium permanganate in acetone did not affect the blue fluorescence of the lipid, but it completely eliminated the absorption peaks in the ultraviolet spectrum. Similar treatment of a sample of authentic methylcholanthrene left the absorption of this hydrocarbon completely unchanged.

5. Since Jones and Jamieson(3) had sug-

6. Dimter, A., *Z. physiol. Chem.*, 1941, v271, 293.

7. Stanger, D. W., Steiner, P. E., and Bolyard, M. N., *J. Am. Chem. Soc.*, 1944, v66, 1621.

8. Jones, R. N., and May, C. D., *Cancer Res.*, 1944, v4, 304.

gested that the banded fluorescence of Penn's liver extracts might arise from a carotenoid compound, a sample of pure dehydro-vitamin A, obtained through the kindness of Dr. E. Schantz of Distillation Products, Inc., was investigated. It differed from the lipid material and from methylcholanthrene both in showing only a weak yellow fluorescence and in its great instability toward atmospheric oxygen, toward which the tissue extracts and methylcholanthrene were entirely stable.

6. Penn first investigated the fluorescence of lipid fractions from cancerous and normal humans. He found marked banding in the spectra of the "cancerous" lipids and only diffuse fluorescence in normal lipids. Visually the two types could not be differentiated. Krichesky and Jacobsen(9) observed similar banding in the fluorescent spectra of unsaponifiable fractions of rabbit blood. In the present study only general blue fluorescence could be obtained from human liver and hog blood lipids. One of the four pools of blood from Brown-Pearce carriers also showed no banding. The other three pools of "cancerous" blood and the one sample of normal rabbit blood investigated gave rise to three distinct bands to the long wave length side of the 365 mμ mercury line. The intensity of the fluorescence from normal rabbit blood seemed to be somewhat lower than that from the blood of Brown-Pearce carriers. Methylcholanthrene gave bands which were very similar in general appearance to those obtained from the banded lipids, but the bands observed with the lipid fractions were shifted 34 mμ toward the ultraviolet.

Discussion. The lipid fractions here investigated obviously are still mixtures of several chemical compounds. Penn and Kaplan reported that most of their material did not absorb hydrogen on catalytic microhydrogenation, and thus could not give rise to the fluorescence observed. Penn(10) found high vacuum fractionation useful for purification of the lipid fraction, but it did not remove the saturated companion substances.

The inhomogeneity of the fractions makes it difficult to evaluate the significance of our findings. However, the separation of added methylcholanthrene from rabbit blood lipid on a silicic acid column and the marked differences in behavior toward ethereal hydrogen chloride preclude identity of the substances. The total absence of the typical methylcholanthrene ultraviolet spectrum in the most highly purified sample of lipid sets an upper limit to the concentration of the methylcholanthrene which could conceivably be present. The banded fluorescent spectrum of the lipid fractions differs significantly from that of authentic methylcholanthrene. The shift of 34 mμ precludes identity of the two substances. Finally it is interesting to note that the compound giving rise to the banding in the fluorescent spectrum is not invariably a constituent of the tissues of cancer-bearing animals. It also occurs in analogous fractions from normal animals. In this respect it resembles the equally sporadic carcinogenic factor isolated from human livers by Steiner (11).

Conclusion. While the blue-fluorescing material first isolated by Penn from human livers has not been identified, several differences between it and methylcholanthrene have been demonstrated. The material occurs in the tissues of both normal and cancer-bearing animals, and its relationship to carcinogenesis is doubtful.

9. Krichesky, B., and Jacobsen, E. M., unpublished data.

10. Penn, H. S., private communication.

11. Steiner, P. E., *Cancer Res.*, 1943, v3, 385.

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