LOCALIZATION OF RIBONUCLEIC ACID IN THE CYTOPLASM OF LIVER CELLS*

By EUGENE L. OPIE, M.D., and GEORGE I. LAVIN, Ph.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

Plates 7 and 8

(Received for publication, February 21, 1946)

The structure of nucleic acid derived from thymus on the one hand and that derived from yeast on the other were finally established when it was shown by Levene and Jacobs (1) that the former was characterized by a pentose, dextroribose, whereas the other contained (Levene and London, 2) a reduced sugar, desoxyribose. A color reaction described by Feulgen (3) applicable to this sugar has been widely used for the chemical identification of desoxyribonucleic acid and has later been applied (Feulgen and Rossenbeck, 4) with much exactness to the microchemical recognition of this substance in tissues. Numerous observations indicate that the Feulgen reaction is limited to the cell nucleus and our observations are wholly in accord with this conclusion.

In 1904 Kohler (5) described a microscope equipped with a fused quartz lens system. The reason for using such a microscope is twofold. One, since the tissue components such as nucleic acid, nucleoproteins, and proteins have absorption bands in the ultraviolet region of the spectrum, unstained sections can be used. Also the resolution is about twice that obtained with visible light. The technique of ultraviolet microscopy has recently been simplified (Lavin, 6) and it is this technique that has been used to obtain the microphotographs shown in this paper. The microphotographs of unstained sections have an astonishing resemblance to photographs of sections stained with nuclear dyes.

Caspersson (7) described procedures by which it was possible to obtain absorption spectra of nucleic acid from different parts of the cell and as he believes to measure quantitatively with some exactness its distribution. He and his coworkers studying ultraviolet absorption spectra of growing yeast, of root tips of plants, of rapidly dividing cells of *Drosophila* larva (Caspersson and Schultz, 8), and of chick embryos (Caspersson and Thorell, 9) have found maximum absorption of ultraviolet radiation at about 2600 Å whereas the cytoplasm of mature tissues has absorption more closely resembling that of protein.

Ultraviolet Photographs of Tissue Containing Basophile Substance

In a preceding publication (10) changes in the basophile substance of the cytoplasm of liver cells preceding the production of tumors has been discussed. In the present study the attempt has been made to determine by means of ul-

* This study has been conducted with the aid of grants from The Jane Coffin Childs Memorial Fund for Medical Research and The Anna Fuller Fund.

traviolet photographs if the material that is stained by nuclear dyes absorbs ultraviolet radiation of wave length 2537 Å and to learn if it has other characters that serve to identify it.

Disappearance of basophile material (chromatolysis) and its reappearance under the influence of dimethylaminoazobenzene (butter yellow) used to produce tumors of the liver has afforded favorable opportunity for study of the distribution of this substance in cells and its relation to mitochondria. The basophile material occurs in two forms. (1) Minute structures usually elongated and pointed at one end are formed by deeply basophile material that surrounds an unstained oval space of the approximate size of a mitochondrium. These bodies may clump to form coarse particles analogous to the Nissl bodies of cells of the nervous system or may assume a palisade-like arrangement at the margins of liver cell columns. (2) A more diffuse basophile stain of the cytoplasm defines the mitochondria by staining a narrow rim that surrounds an unstained oval space. It is probable that the deeply basophile material of the basophile body represents an excess of that in the mitochondrial rim, for when with advanced chromatolysis caused by butter yellow basophile material of both basophile bodies and mitochondria disappears, mitochondria remain as oval bodies with a rim of material that is now acidophile surrounding a central unstained space. It is possible though not demonstrable that this unstained center consists of the phospholipid known to constitute a considerable part of the substance of mitochondria.

Tissues prepared for ultraviolet photography have been fixed in a mixture of alcohol formalin and acetic acid (see later description) or in formalin alone. They have been embedded in paraffin and, after removal of the paraffin, sections mounted in glycerine between fused quartz slide and cover slip have been photographed unstained.

Ultraviolet photographs of unstained sections of liver resemble those prepared from stained sections with visible light because nuclei, absorbing the ultraviolet, are accurately reproduced. The coarse particles that are stained by basic dyes in the cytoplasm of liver cells adjacent to the central veins are well shown in ultraviolet photographs (Fig. 1). It is noteworthy that the cytoplasm of the cells that contain these clumps is in appropriately stained sections otherwise devoid of basophile material and in the ultraviolet photographs the clumps are surrounded by a relatively clear background of cytoplasm.

The more diffusely stained cytoplasm of cells close to the portal spaces is represented in ultraviolet photographs with high magnification by a fairly uniform dark background closely set with clear oval spaces corresponding to the mitochondria (Fig. 2). It is evident that this picture is not the result of fixation for it is seen in ultraviolet photographs of frozen sections of fresh liver (Fig. 3).

In these photographs the dark background enclosing oval spaces corresponds with the diffuse basophilia of the cytoplasm but may be the result of absorption of ultraviolet radiation not only by nucleic acid but by protein and other

substances as well. However, the extinction coefficient of proteins is only one-sixtieth to one-hundredth that of nucleic acids. It may be assumed that the oval spaces that transmit ultraviolet rays are almost or wholly free from nucleic acid or nucleotides. These spaces presumably correspond with phospholipid of the mitochondria.

In impression preparations made by touching freshly cut liver of rat to the surface of a clean quartz slide liver cells are spread in a thin layer and are in large part partially or completely disintegrated. These preparations offer a favorable opportunity for study of intracellular structures by ultraviolet photography. In them oval bodies with clear central space and opaque rim (Fig. 4) are seen within the intact cytoplasm of a liver cell and within disintegrating cytoplasm. They occur in the surrounding medium as the result of complete destruction of liver cells. Impression preparations stained by the Giemsa method show oval bodies with clear central space and basophile rim. In similar preparations fixed in Regaud's fluid and stained by aniline fuchsin mitochondria are demonstrable in cells and in the surrounding medium, set free by disintegration of most of the cells. They have form and distribution similar to the bodies seen in ultraviolet photographs and in preparations stained by the Giemsa method.

Sections of tissue, Caspersson (7) has pointed out, as the result of protein content or other constituent may absorb so much ultraviolet that definition of nucleic acid of the cells is not obtainable. With the peculiar degenerative change accompanied by chromatolysis evident in the centers of cells (10) the localization of the remaining basophile substance at the periphery of liver cells has been shown in an ultraviolet photograph (Fig. 5). The relative opacity of adjacent liver cells is in contrast with the area in which chromatolysis is advanced.

In a preceding publication (10) the occurrence of conspicuous basophile hyperplasia of newly formed bile ducts in association with the lesion designated cholangiofibrosis has been described. The sharply localized basophilia of these hyperplastic ducts suggests the possibility of its identification in ultraviolet photographs. Comparison of an ultraviolet photograph of this lesion (Fig. 6) with a color photograph of a similar lesion (Fig. 5 of the preceding publication, 10) shows that the basophile material is well defined in the former. Ducts that have not undergone hyperplasia and have not accumulated basophile material are recognizable in the ultraviolet photograph but have absorbed little of the ultraviolet radiation (Fig. 6, *A*). A duct-like structure may be hyperplastic at one part of its circumference when sectioned transversely and here opaque for the ultraviolet whereas at another part it is only obscurely indicated in the photograph (Fig. 6).

The cytoplasm of tumor cells of both hepatomas and of cholangiomas in ultraviolet photographs is fairly uniform. Within a dark background are the light oval spaces that corespond to mitochondria and an ill defined darker rim

may be seen in places immediately about these spaces. Darker particles may give a faintly stippled appearance to the cytoplasm.

Removal of Basophile Substance of Cytoplasm by Ribonuclease

A heat-resistant enzyme obtained by W. Jones (11) from pancreas caused the disappearance of yeast nucleic acid with the appearance of four nucleotides in solution. Dubos and MacLeod (12) found that enzymes prepared from several tissues extracted from pneumococci the substance that reacts with the Gram stain. With similar enzyme acting during 1 hour at 70° Brachet (13) removed the basophile substance from the cytoplasm of cells of the pancreas liver, cardiac glands of the stomach, and central nervous system. It acted, he believed, specifically on ribonucleic acid and left the nucleus unaffected. The enzyme has been used by Gersh and Bodian (14) and by Davidson and Waymouth (15) for the same purpose in a veronal acetate buffer with pH 6.75 for 5 hours at 37°C. Kunitz (16) prepared from the pancreas of beef a crystalline enzyme capable of disintegrating yeast nucleic acid. In a borate buffer it was effective within a range from pH 7.0 to 8.2 with optimum at pH 7.7.

Sections of tissues that have been fixed in a mixture of alcohol, formalin, and acetic acid (90 cc. of 80 per cent alcohol, 5 cc. of formalin, and 5 cc. of glacial acetic acid) and embedded in paraffin have been subjected after removal of paraffin to the action of ribonuclease. We are indebted to Dr. M. Kunitz for the crystalline ribonuclease that has been used in these experiments. It has been dissolved in a buffer in the proportion of 1 mg. in 1 cc. A small quantity of this solution (about 0.3 cc.) has been placed on a slide with narrow strips of glass 1 mm. in thickness cemented to each end. A glass slide with section attached has been inverted and brought into contact with the enzyme solution. During exposure to a temperature of 37° or 56° in a thermostat the preparations have been kept in a closed vessel with moisture to prevent evaporation. After washing with water sections have been stained by the Giemsa method.

It is noteworthy that at a temperature of 37° for 5 hours or 56° for 2 hours certain concentrations of sodium chloride may remove the basophile material from the cytoplasm though they leave the nuclei intact. In molar and half molar solutions of sodium chloride the basophile material has been unaffected but in one-third and in one-fifth molar solutions it has been diminished. In one-sixth molar solution (0.95 per cent) it has been almost completely removed. With one-eighth molar solution basophile material is diminished but after treatment with distilled water it is not less than in untreated control sections.

The buffer solutions that are usually employed with ribonuclease cause some disappearance of basophile material from the cytoplasm. A borate or phosphate buffer with pH 7.7 almost completely removes the basophile material after 2 hours at 56°. Veronal acetate buffers with pH 6.75 and pH 7.66 at 37° for 5 hours have diminished cytoplasmic basophilia but buffers of pH 3.62 and pH 4.66 on the one hand and pH 8.68 on the other have produced no change when comparison is made with untreated control sections. A veronal acetate buffer of pH 6.75 in contact with sections for 2 hours has produced no change.

Crystalline ribonuclease in the proportion of 1 mg. to 1 cc. of veronal acetate buffer with pH 6.75 has removed completely the basophile material of the cytoplasm of liver cells. The diffusely distributed basophile substance that in the normal liver is found in cells about the portal spaces and the basophile bodies that toward the central veins form coarse clumps or have assumed a palisade-like arrangement in double rows are removed completely by ribonuclease. The basophile material that accumulates in the cytoplasm of cells with focal hyperplasia either columnar or tubular, is similarly removed. The cytoplasmic substance that appears in the hyperplastic ducts of the lesion

designated cholangiofibrosis and gives them an intense basophile stain disappears completely when treated with the enzyme. Basophile material in the cells of hepatomas and cholangiomas is removed by the same agent.

When basophile substance has been removed from the periportal areas of normal liver or from foci of basophile hyperplasia, where with the Giemsa method the cytoplasm has a diffuse basophile stain and mitochondria are defined as minute bodies with a basophile rim and unstained central space, the mitochondria now are recognizable as well defined bodies with an acidophile rim (pink with Giemsa stain) surrounding an unstained central space. This relation indicates that the basophile material is present within the substance of the mitochondria perhaps within the differentiated membrane which as Claude (17) suggests on the basis of observations made with the electron microscope may surround these structures.

When the basophile material that occurs in coarse clumps in cells in the part of the lobule about the central vein is partially removed by ribonuclease it becomes evident that these clumps are composed of basophile bodies formed by basophile material surrounding clear spaces which have the size and shape of those within mitochondria. The double rows of basophile particles seen in many columns of liver cells are similarly resolved into small bodies of the same character.

Precipitation of Basophile Substance of Cytoplasm by a Lanthanum Salt

Lanthanum salts have been used by Caspersson, Hammarsten, and Hammarsten (18) to precipitate ribonucleic acid and Davidson and Waymouth (19) have used this procedure for the quantitative determination of nucleic acids in tissues. We have undertaken experiments to determine if the basophile substance can be fixed locally by a lanthanum salt so that it cannot be removed by ribonuclease. Paraffin sections after removal of paraffin have been immersed in a fifth molar solution of lanthanum acetate from 2 to 3 hours and after washing with water have been treated with ribonuclease and stained by the Giemsa method as in previous experiments. Basophile substance of basophile bodies, of hyperplastic foci of parenchymatous cells, of hyperplastic ducts of cholangiofibrosis, and of tumor cells retain their basophilia with slight diminution though it disappears from corresponding sections treated with ribonuclease alone.

SUMMARY

The attempt has been made to determine the character of the basophile material that occurs normally in the cytoplasm of liver cells and accumulates in association with the hyperplasia of liver cells and of newly formed bile ducts when the azo dye butter yellow is administered to white rats. This substance in the normal liver cells, in the parenchymatous foci of basophile hyperplasia that are precursors of hepatomas, and in the hyperplastic basophile ducts that precede the cholangiomas produced by butter yellow has the characteristics of

ribonucleic acid. It absorbs ultraviolet radiation of wave length 2537 Å. It does not, like desoxyribonucleic acid, give the Feulgen reaction. It is removed from the cytoplasm by ribonuclease, and precipitation with lanthanum acetate protects it against the enzyme.

BIBLIOGRAPHY

1. Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, 1909, **42,** 1198.
2. Levene, P. A., and London, E. S., *J. Biol. Chem.*, 1929, **81,** 711; 1929, **83,** 793.
3. Feulgen, R., *Z. physiol. Chem.*, 1914, **92,** 154.
4. Feulgen, R., and Rossenbeck, H., *Z. physiol. Chem.*, 1924, **135,** 203.
5. Kohler, A., *Z. wissensch. Mikroskopie*, 1904, **21,** 129, 273.
6. Lavin, G. I., *Rev. Scient. Instr.*, 1943, **14,** 375.
7. Caspersson, T., *Skand. Arch. Physiol.*, 1936, **73,** suppl. No. 8.
8. Caspersson, T., and Schultz, J., *Nature*, 1939, **143,** 602.
9. Caspersson, T., and Thorell, B., *Chromosoma*, 1941, **2,** 132.
10. Opie, E. L., *J. Exp. Med.*, 1946, **84,** 91.
11. Jones, W., *Am. J. Physiol.*, 1920, **52,** 203.
12. Dubos, R. J., and MacLeod, C. M., *J. Exp. Med.*, 1938, **67,** 791.
13. Brachet, J., *Compt. rend. Soc. biol.*, 1940, **133,** 88.
14. Gersh, I., and Bodian, D., in Frontiers in Cytochemistry, (N. L. Hoerr, editor). Biological Symposia, Vol. **10,** Lancaster, The Jaques Cattell Press, 1943, 163.
15. Davidson, J. N., and Waymouth, C., *Proc. Roy. Soc. Edinburgh, Section B*, 1944, **72,** 96.
16. Kunitz, M., *J. Gen. Physiol.*, 1940, **24,** 15.
17. Claude, A., and Fullam, E. F., *J. Exp. Med.*, 1945, **81,** 51.
18. Caspersson, T., Hammarsten, E., and Hammarsten, H., *Tr. Faraday Soc.*, 1935, **31,** 367.
19. Davidson, J. N., and Waymouth, C., *Biochem. J.*, 1944, **38,** 39.

EXPLANATION OF PLATES

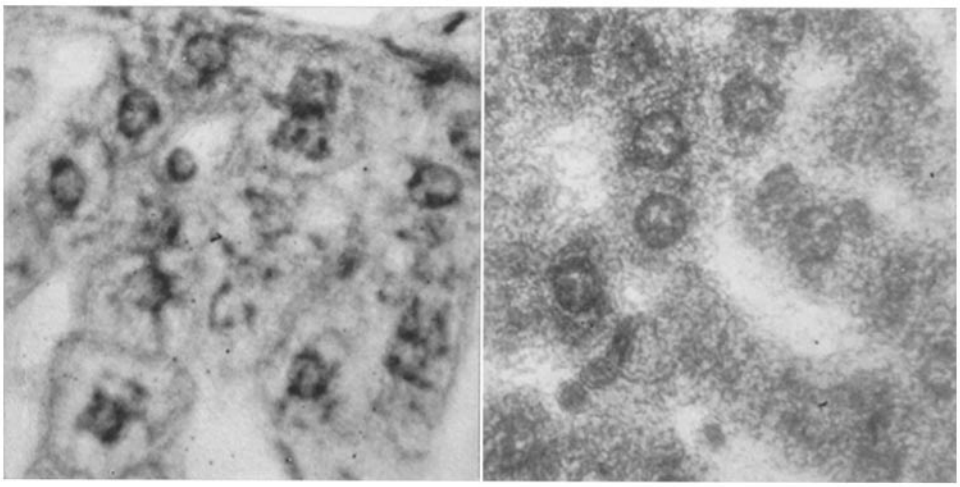
PLATE 7

FIG. 1. Ultraviolet photograph of basophile material in liver cells near central vein. ×750.

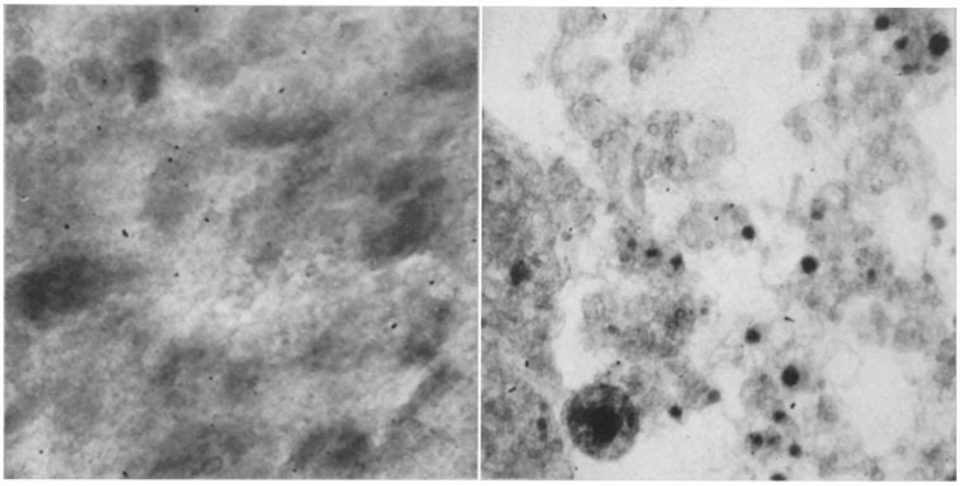
FIG. 2. Ultraviolet photograph showing basophile ground substance of cytoplasm of liver cells near a portal space in a regenerating liver following partial extirpation. The clear oval spaces are occupied by mitochondria surrounded by a rim of material that absorbs ultraviolet rays. ×750.

FIG. 3. Ultraviolet photograph of a section of frozen fresh liver showing ground substance absorbing ultraviolet rays. The clearer oval spaces are at the site of mitochondria. ×750.

FIG. 4. Ultraviolet photograph of impression preparation of liver, showing bodies with clear central space and surrounding dark rim. These are seen in the disintegrating cytoplasm about the isolated nucleus of a liver cell. ×750.



1 2



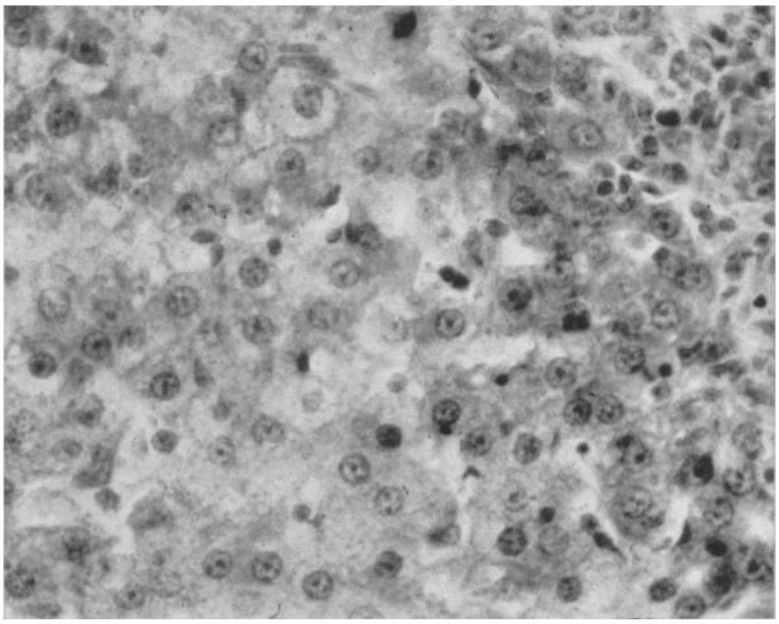
3 4

(Opie and Lavin: Ribonucleic acid in cytoplasm of liver cells)

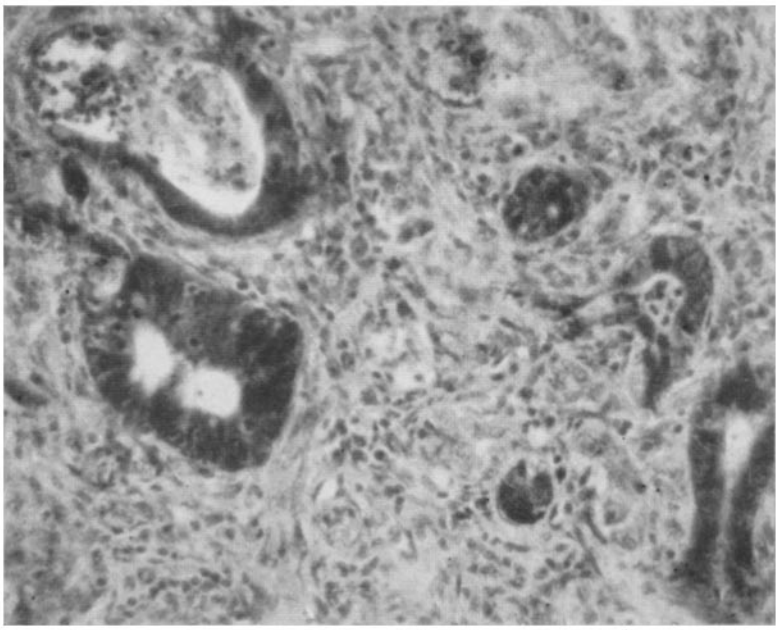
PLATE 8

FIG. 5. Ultraviolet photograph of the liver of a rat that has received butter yellow during 8 months. Chromatolysis with disappearance of basophile material in the central part of cells has occurred. Liver cells in contact with a portal space absorb ultraviolet rays more conspicuously than elsewhere. ×450.

FIG. 6. Ultraviolet photograph of cholangiofibrosis with hyperplasia of newly formed bile ducts. Hyperplastic ducts absorb ultraviolet rays whereas ducts that have not undergone hyperplasia are less sharply defined (*A*). Compare ultraviolet with color photograph of a similar lesion (Fig. 5 of the preceding publication, 10). Hyperplastic ducts with intense basophilia are opaque to ultraviolet rays. ×300.



5



6

A A

(Opie and Lavin: Ribonucleic acid in cytoplasm of liver cells)