never failed to develop in an animal exposed to this schedule. Further experiments are in progress to elucidate the conditions necessary and sufficient for this effect.

The methods typically used to produce a sustained diuresis in the intact animal are loading the stomach with water or administering sodium chloride, mannitol, or other diuretic agents. Another method forces intake by making shock avoidance contingent upon licking (*5*). The arrangement described in this report results in voluntary drinking at a sustained high rate in the unrestrained, normal animal, without the various traumatic concomitants of the above methods.

JOHN L. FALK
Department of Nutrition, Harvard University School of Public Health, Boston, Massachusetts

References and Notes

1. This study was supported in part by grants-in-aid from the National Institutes of Arthritis and Metabolic Diseases (grant No. A-2911), the Nutrition Foundation, New York, and the Fund for Research and Teaching, Department of Nutrition, Harvard University School of Public Health.
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10 October 1960

Selective Localization of Tetracycline in Mitochondria of Living Cells

Abstract. The property of tetracycline to induce fluorescence has been used to determine its localization in living cells. It was found that this antibiotic, as well as the related antibiotics oxytetracycline and chlorotetracycline, specifically combines with the mitochondria of living cells, either in tissue culture or in fresh preparations from various organs.

In the search for a fluorophor which would selectively localize and remain localized on mitochondria in living cells, a number of substances were tried with varying results. Benzpyrene and related carcinogens, as used by Graffi (*1*), localized on mitochondria and on fat droplets. Glycerol was employed as a vehicle. Using acridine orange, Wolf (*2*) found that the distribution and size of metachromatic granules in viable cells suggest that these granules may be mitochondria. With this florophor we found a variable fluorescence, which made observations difficult to interpret.

Our attention was drawn to the work of Milch *et al.* (*3*) and Rall *et al.* (*4*), who studied the gross distribution of the tetracycline group of antibotics in normal and neoplastic tissues. The possible use of antibiotics would have the added advantage over use of many fluorescent dyes in that antibiotics would not markedly interfere with the development of living cells, and would simultaneously supply further information regarding the mode of action of tetracyclines.

Milch *et al.* reported that bone fluorescence persisted for at least 10 weeks when a single, small parenteral dose (0.3 mg/kg) of tetracycline was administered to freshly frozen sections of several species of laboratory animals. This induced fluorescence disappeared from all tissues except bone within 6 hours after injection.

In the present study, observations on living cells are reported by means of phase-contrast and fluorescence microscopy.

Monkey kidney tissue-culture cells, grown in medium 199 with 2 percent calf serum and maintained in Eagle's basal medium on submersed coverslips, were exposed to 10 or 20 μg of tetracycline per milliliter of medium. (The amount of tetracycline currently used in the antibiotic mixture added to tissue culture media to suppress bacterial growth is about 10 μg/ml.)

Photomicrographs were made of the same cell, first by phase-contrast (Fig. 1), then by fluorescence microscopy (Fig. 2). The mitochondria showed intense fluorescence. Nuclei, vacuoles, interparticulate cytoplasm, and cell boundaries appeared dark. Some decrease in the intensity of the yellow fluorescence occurred within a few seconds after exposure to ultraviolet radiation. In similar preparations made of the same material, and in the continued presence of the antibiotic, the cells were alive after 4 days and the fluorescence was still localized in or on the mitochondria. At concentrations of 100 μg or more cell damage occurred (*5*). When the tetracycline-treated material was resuspended in tetracycline-free medium, the fluorescence decreased gradually over several hours until the mitochondria were only faintly fluorescent.

No fluorescence was observed in chromosomes in dividing cells which had been previously treated with the antibiotic. Strain L cells showing a centrosome area did not show fluorescence of the centrosome. Similar results were obtained with tissue cultures which were exposed to oxytetracycline or chlorotetracycline (10 to 20 μg/ml of medium).

To test fresh isolates, adult mice were injected intraperitoneally with 1.0 ml of saline containing 2000 μg of tetracycline per milliliter, or approximately 100 μg/g of mouse; after 2 hours the animal was killed, and brain, liver, and spleen fragments were collected in 0.88*M* sucrose. Fresh microscopic preparations of these organs were made. The tetracycline was again localized on the mitochondria. Liver mitochondria

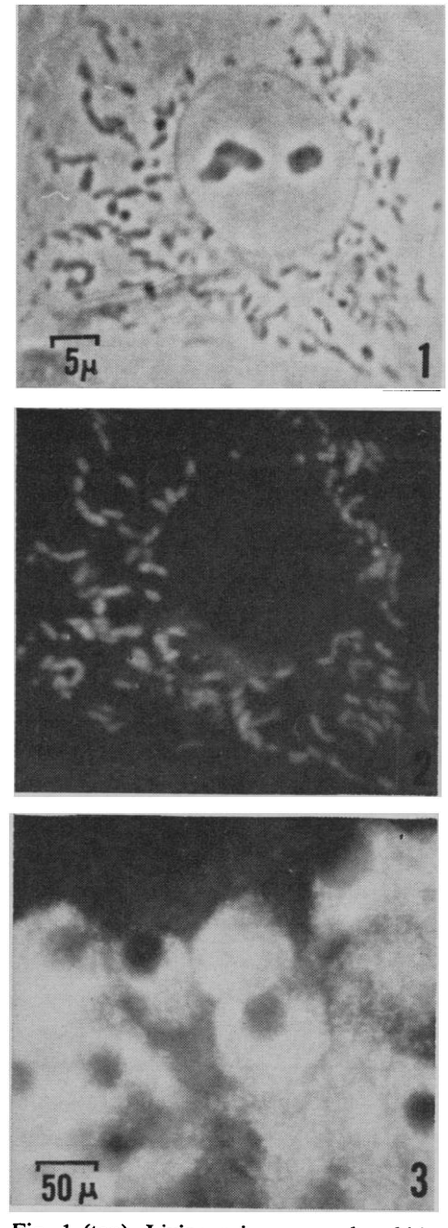

Fig. 1 (top). Living primary monkey kidney cells after a 3-hour exposure to 20 μg of tetracycline per milliliter (Chas. Pfizer). Phase contrast. Fig. 2 (middle). Same field as Fig. 1, showing mitochondrial fluorescence. Zeiss W microscope with mercury HBO-200 light source, used with two BG-12 exciter filters and barrier filter OG-5. Fig. 3 (bottom). Mouse liver tissue fragment mounted in 30 percent sucrose. Nuclei appear black in contrast to strong fluorescence by mitochondria. The animal was injected intraperitoneally with 2000 μg of tetracycline prior to death and observation.

showed strong fluorescence (Fig. 3), whereas the fluorescence of spleen mitochondria was moderate, and that of brain mitochondria weak. When additional tetracycline was added to the brain fragments, the fluorescent staining of the mitochondria was increased.

A similar selective localization was found in subcellular fractions when tetracycline at a final concentration of $5 \times 10^{-5} M$ (or approximately 20 μg/ml of the final solution) was added to a 25 percent mouse brain or liver homogenate, made in 0.25 *M* sucrose. The nuclear, mitochondrial, microsomal, and supernatant fractions were prepared by differential centrifugation. The particulate pellets were resuspended in sucrose with tetracycline, to make the equivalent of a 12.5 percent homogenate. Again, when exposed to ultraviolet radiation, the nuclei showed neither autofluorescence nor the specific fluorescence of tetracycline. This provides a useful way to determine contamination of nuclear preparations with mitochondrial fragments. In the mitochondrial fraction of the brain, the yeastlike "myelin bodies," which always contaminate this fraction, were dark. When crescents occurred in the mitochondria, the crescents showed stronger fluorescence than the remainder of the mitochondrion, which was barely visible. When the mitochondria disintegrated with sodium desoxycholate, the fluorescence became diffuse. Microscopically, the "microsomal" pellet showed a yellow diffuse fluorescence, whereas the fluorescence of the supernatant fluid was very faint, except for contaminating particles. The observation that the "microsomal" fraction showed diffuse fluorescence, whereas the interparticulate cytoplasm of the intact living cell appeared optically empty, seems to support our interpretation, given previously, that the "microsomes" are artifacts (*6*, p. 373) and do not occur as such in living, intact cells. To evaluate further this interpretation, the quantitative aspects of the intensity of fluorescence in various areas of the cell, as compared with that in isolated fractions, are being investigated with varying concentrations of the antibiotics.

An aliquot of the isolated mitochondria, prepared as described, was studied metabolically. (The presence of the usual cofactors and substrates (*7*) did not change the fluorescence of the preparation.) As reported by others (*8*), we also found that, under the conditions of our experiments, oxygen uptake was not changed, but oxidative phosphorylation was decreased (3.35 μmole of magnesium sulfate per milliliter was added to the Warburg vessels), with glucose as substrate for brain mitochondria, and fructose diphosphate for liver mitochondria.

A susceptible microorganism, *Salmonella typhosa* strain Ty 2, growing in nutrient broth, was treated with 50 μg of tetracycline per milliliter. Observations after 10 minutes showed a strong yellow fluorescence in distinct areas within the bacteria. The wall was faintly discernible, and the remainder of the intercellular area was dark. The particulates varied in location, size, and intensity of fluorescence, and appeared similar to those identified as mitochondria with Janus green B by Davis *et al.* (*9*).

Sequential observations of the distribution of the fluorescence in living cells are being made, in order to study the exchange between tetracycline-tagged mitochondria and nuclei during cell division, and changes in mitochondria after virus infection, or after the introduction of deficient media to the cell environment. Also, further information might be obtained about the locus of action of tetracyclines in cases where complications occur resulting from prolonged therapy—for example, liver degeneration (*10*)—or about the primary site of action of these compounds in susceptible microorganisms.

H. G. DU BUY
J. L. SHOWACRE
National Institute of Allergy and Infectious Diseases, Bethesda, Maryland

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28 September 1960