Technique for the Preservation of Tetracycline Fluorescence in Ascites Tumor Cells

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Fluorescence microscopy has a potential application in cellular pharmacology for the study of intra- and intercellular distribution of pharmacological agents. A method is reported for preparing permanent preparations of tetracycline-retaining ascite tumor cells. A combination of glutaraldehyde fixation followed by glycerol dehydration was found satisfactory.

During an investigation concerning tetracycline retention by ascites tumor cells using fluorescence microscopy, it became essential to make permanent preparations of the cells for more detailed observation. It became apparent immediately that application of traditional histologic techniques would not suffice because tetracycline fluorescence is easily quenched or displaced by otherwise suitable fixatives and dehydrating agents. Numerous agents were screened in an effort to develop a satisfactory method for preserving tetracycline fluorescence. Under the conditions reported in this paper, a combination of glutaraldehyde fixation followed by glycerol dehydration proved to be the most satisfactory technique. Fluorescence microscopy has a potential application in cellular pharmacology for the study of intra- and intercellular distribution of pharmacological agents. For this reason, the technique found useful in preserving tetracycline fluorescence is described.

MATERIALS AND METHODS

All microscopic examinations of the cell preparations were made using a Zeiss GFL fluorescence microscope with a mercury light source (HB200). Excitation filters BG-38 and BG-12 were used along with barrier filters 41 and 53. These filters permit the best observation of tetracycline fluorescence. At the same time they eliminate autofluorescence of the ascites cell which may be seen when wider ranges of the U.V. spectrum are employed. Photomicrographs were taken using a Zeiss Ikon camera attachment and 35-mm. Panatomic X film.

The Ehrlich ascites tumor cells are strain ES obtained from Cailleau (1). They are maintained in Swiss mice by weekly intraperitoneal transfer. All cells used in these investigations were removed from the host 6 to 10 days following passage to assure an optimal growth phase.

All chemicals and reagents employed were of C.P. grade or the equivalent. The following substances were selected for evaluation as fixatives: acetone, diethyl ether and alcohol (1:1), formalin, and glutaraldehyde. A wide range of concentrations of each agent was employed to find the most efficient concentration. The following substances were evaluated as dehydrating agents: acetone, diethyl ether, ethanol, and glycerol. Each of these reagents was similarly tested at a variety of concentrations. A variety of exposure times ranging from 2 to 30 min. in the fixatives and dehydrants was employed.

The general procedure was as follows. Several milliliters of ascites cell suspension were withdrawn from the host animal 15 min. following the intraperitoneal injection of 0.1 mg. of tetracycline. This particular dosage and time lapse were selected to assure adequate fluorescence in the tumor cells. The cell suspension was diluted with an equal volume of Hank's balanced saline, then fixed with one of the fixatives. In the case of control preparations, these were examined and photographed immediately following dilution in Hank's saline solution. Ascites cells which were subjected to fixation were exposed for the appropriate time interval, centrifuged lightly, then washed with several changes of Hank's saline solution. The cells were then either examined under the microscope or further treated by exposure to a dehydrating agent through a series of 3 to 5 min. changes in progressively higher concentrations of the dehydrant. At each step samples of the cells were photographed to determine what changes could be observed in the fluorescence pattern in the cells when compared to control specimens.

As this work progressed, it became obvious that fixation in glutaraldehyde dissolved in Sorensen's phosphate buffer was the most satisfactory fixative. The best preservation of fluorescence was obtained when glutaraldehyde fixation was followed by glycerol dehydration. For this reason, the protocol of the glutaraldehyde–glycerol technique is given in more specific detail: fixation is accomplished by diluting the ascites cell suspension with an equal volume of 4% glutaraldehyde (redistilled) in Sorensen's phosphate buffer, pH 7.4, and allowing the cells to remain in the solution for 5 min. Longer fixation seems to produce no deleterious effect, but offers no real advantage. Following fixation and light centrifugation (100 g) the cells are run through a series of 3 min. changes in progressively higher concentrations of glycerol.

RESULTS

As can be seen from the photomicrographs submitted, this technique does not appear to decrease intensity of fluorescence, or displace the tetracycline within the cell. On the other hand, all other com-

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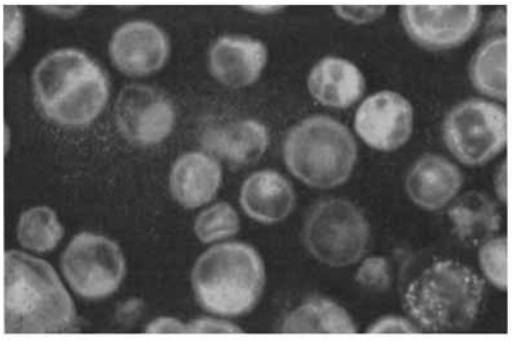

Fig. 1.—Ascites tumor cells freshly removed from the peritoneal cavity of a tetracycline treated mouse. X960.

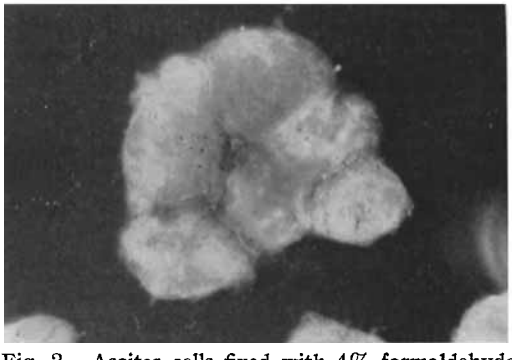

Fig. 2.—Ascites cells fixed with 4% formaldehyde for 2–3 min. X960.

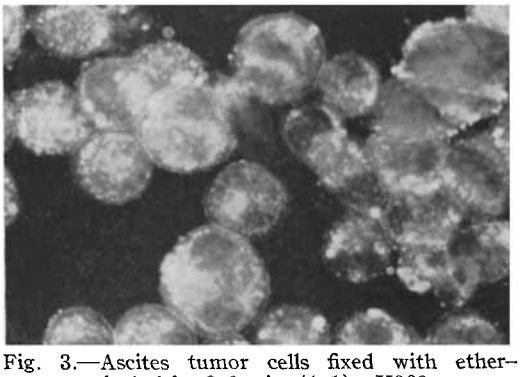

Fig. 3.—Ascites tumor cells fixed with ether–alcohol for 2–3 min. (1:1). X960.

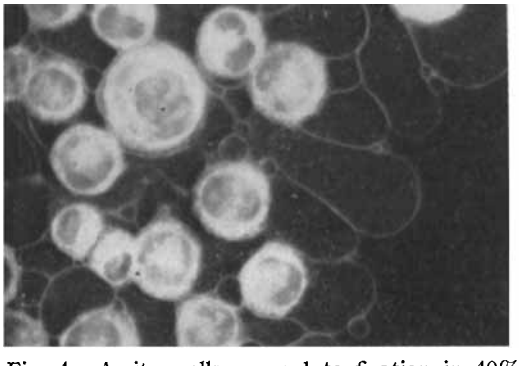

Fig. 4.—Ascites cells exposed to fixation in 40% acetone. X960.

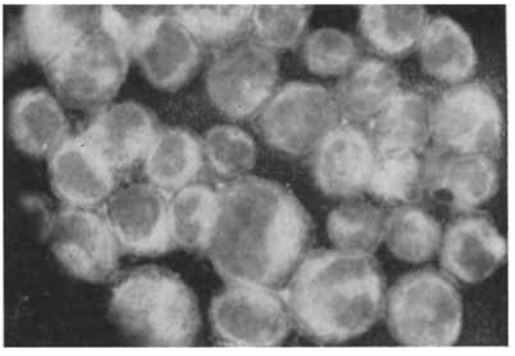

Fig. 5.—Ascites tumor cells fixed in 4% glutaraldehyde. X960.

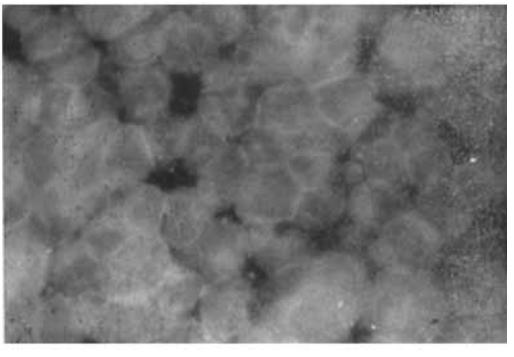

Fig. 6.—Effect of acetone dehydration after glutaraldehyde fixation. X960.

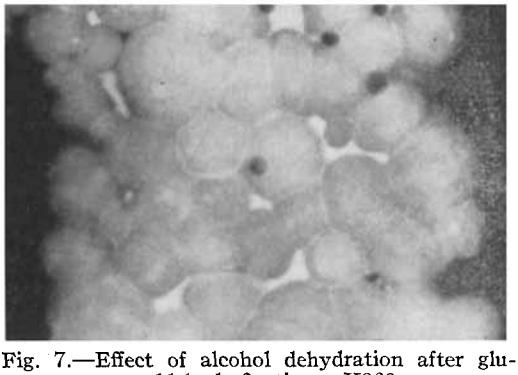

Fig. 7.—Effect of alcohol dehydration after glutaraldehyde fixation. X960.

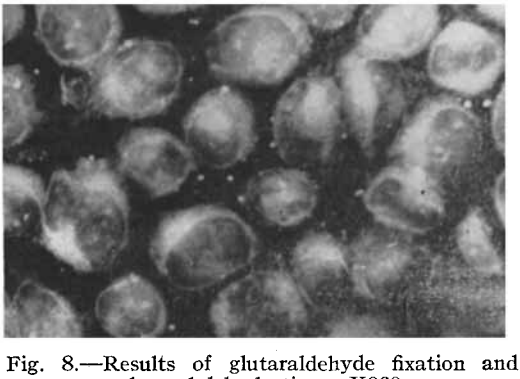

Fig. 8.—Results of glutaraldehyde fixation and glycerol dehydration. X960.

binations of fixatives and dehydrating agents appeared to introduce artifacts, some of these being visible in the photomicrographs.

No photomicrograph is submitted for ascites cells not exposed to tetracycline since at the particular wavelength employed there is no autofluorescence; hence, it is not possible to take a photomicrograph of the cells against the darkfield background. The first five photomicrographs were taken of cells before exposure to dehydrating agents. Figure 1 illustrates the appearance of tetracycline-containing tumor cells freshly removed from the host animal but having been subjected to no treatment other than their removal from the host. They may be considered as control preparations. In such cells the tetracycline seems to be located in the cytoplasmic portion of the cell, with concentration in the perinuclear zone. Figure 2 shows the effect of 4% formalin fixation for 2 min. There is an apparent loss of definition and diffusion of the tetracycline. Higher concentrations of formalin or longer fixation periods further exaggerated these observations. Figure 3 shows the effect of diethyl ether–alcohol fixation for 2 min. The tetracycline complex appears to be precipitated as judged by the altered pattern of fluorescence. The effect of acetone fixation is shown in Fig. 4. There is extensive blebbing with the suggestion that tetracycline has diffused into the karyoplasm. Figure 5 is a photomicrograph of cells fixed with glutaraldehyde. One sees an apparent intensification of the fluorescence. This was a consistent finding in all glutaraldehyde preparations.

The remaining photomicrographs demonstrate the effects of several dehydrating agents. Figure 6 illustrates the result of acetone dehydration following glutaraldehyde fixation. Even when the total dehydrating time is kept to 4 min. there appears to be a reduced fluorescence. The use of ethanol dehydration following glutaraldehyde fixation (Fig. 7) seems to reduce sharpness of detail of the preparation although there is no loss of fluorescence. Areas of bright fluorescence in some of the intercellular spaces suggests a possible leakage of fluorescent substance from the cells. The final photomicrograph (Fig. 8) shows the result of glutaraldehyde fixation followed by glycerol dehydration in accordance with the protocol described in this report. There is no loss of cellular detail, nor any apparent loss or distortion of fluoresence.

REFERENCE

(1) Cailleau, R., and Costa, F., *J. Natl. Cancer Inst.*, **26**, 271(1961).

Hydrolysis of Methylparaben

By N. N. RAVAL and E. L. PARROTT

The hydrolysis of methylparaben in aqueous solution at 70, 80, and 85° in pH range from 6 to 8 has been studied.

SINCE their introduction into the United States in the 1930's, the parabens have been widely used as preservatives in foods, drugs, and cosmetics (1–5). The lack of toxicity and the broad spectrum of preservative activity of methylparaben have been reported (6–8). The correlation of preservative activity with the binding of methylparaben to other substances has led to more effective and intelligent product formulation (9–11).

Methylparaben is stable in air. Aqueous solutions of methylparaben buffered at pH 3 and 6 showed no decomposition when heated for 2 hr. at 100° or for 30 min. at 120° (12). As the removal of the ester portion by hydrolysis yields *p*-hydroxybenzoic acid, which possesses insignificant preservative activity, this investigation was undertaken to study the extent of hydrolysis of methylparaben in a pH range utilized in pharmaceuticals.

EXPERIMENTAL

A 0.1-Gm. quantity of methylparaben[1] was accurately weighed into a 100-ml. volumetric flask and dissolved in a sufficient amount of buffer to bring to a volume of 100 ml. of solution. The solution was filled and sealed into 10-ml. ampuls. The ampuls were placed in appropriate constant-temperature baths, and after thermal equilibrium was attained, an ampul was removed and, by means of iced water, was cooled to 25°. This solution was analyzed for zero-time concentration of methylparaben.

Five milliliters of the solution was withdrawn, adjusted to pH 7, and sufficient distilled water was added to bring the volume to 100 ml. A 10-ml. aliquot was extracted with four 10-ml. portions of anhydrous ether. The ether was evaporated and the residue was dissolved in 100 ml. of distilled water. The absorbance of the solution was measured at 255 mμ using a Beckman DU spectrophotometer. By means of a standard absorption curve, the concentration of methylparaben was determined. Ampuls were removed at definite intervals of time and analyzed by this procedure, which is essentially that of Aalto *et al.* (1).

All chemicals were reagent grade. McIlvaine's buffer was used for pH 6 and 7; Palitzsch's buffer was used for pH 8 and 9 (13). All buffer solutions were adjusted at and the pH determined at the temperature of hydrolysis by means of Beckman Zeromatic pH meter.

RESULTS AND DISCUSSION

The rate of hydrolysis of methylparaben was followed at pH 6, 8, and 9 at 70, 80, and 85°. A plot of the logarithm of concentration against time produced a straight line. The observed velocity constant k can be evaluated by multiplying the slope of the line by 2.303. The half-life or time required

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[1] The methylparaben used was Methyl Parasept, Heyden Newport Chemical Corp.