Effects of Some Fixatives on Inclusion Bodies of Infectious Bovine Rhinotracheitis.* (24803)

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Early workers reported that inclusion bodies were not observed in respiratory epithelial cells of cattle infected with infectious bovine rhinotracheitis (IBR) virus(1). Later, other investigators stated that the virus initiated development of intranuclear inclusions both in tissue cultured cells and in necropsy material from infected animals(2). Both laboratories used a hematoxylin and eosin (H & E) stain, but the early group fixed their preparations in neutral buffered formalin while the later workers used Zenker's fluid for cells derived from infected animals, and Bouin's fixative on cells obtained from both sources. Because of conflicting results obtained with different fixatives, it seemed desirable to determine the effects of some commonly used fixing agents and their ingredients on appearance of inclusion bodies induced by IBR virus in infected tissue cultures.

Material and methods. Light microscope examinations were made on primary monolayer tissue cultures of trypsinized bovine kidney epithelium which had previously been exposed to IBR virus (Cooper strain first isolated by TLC from feedlot outbreak of IBR in Colorado in 1956). Cultures were prepared essentially by the method of Youngner(3), and grown in medium consisting of T.C. 199 (Difco) plus 10% IBR antibody free bovine serum (obtained aseptically from newborn calves that had received no colostrum). Two hundred units of penicillin and 200 μg of streptomycin were incorporated into each ml of growth medium. Cells were seeded in one ml amounts (6 x 10^5 cells/ml) into 16 x 125 mm pyrex screw capped test tubes, each tube containing 7 x 22 mm number one coverslip. With no media changes, a satisfactory monolayer usually developed on the coverslip after approximately 6 days' incubation at 37°C. When coverslip cultures were ready for use, the growth medium was poured off, the slips washed with fresh media, and the tubes divided into 2 groups. Into each tube of one group (controls) one ml of new culture medium was placed, while the second group (infected) received $10^{4.5}$ $TCID_{50}$ of IBR virus incorporated into 1 ml of same medium. All cultures were then incubated at 37°C. Our previous work has shown that intranuclear inclusion bodies in infected cells prepared by these methods can best be demonstrated at 20 to 24 hours after inoculation of virus. When infected cultures had been incubated for this period, and examination of a representative infected coverslip under low power revealed typical cytopathological effects, all coverslips were removed from the tubes, washed with warm physiological saline, treated with desired fixatives, and stained with Harris' hematoxylin (pH 2.6) and an acetic acid-ethyl alcohol-eosin^Y solution (pH 4.4).

Results. Initially, a comparative study of some commonly used fixatives was undertaken. Since Bouin's fluid followed with H and E stain was known to demonstrate inclusions in tissue culture, while neutral buffered formalin had not shown them in tissue sections of respiratory epithelium of infected animals, these 2 fixatives were compared. Fixatives also included were absolute ethyl alcohol, absolute methyl alcohol, Carnoy's fluid, and acetone.

Bouin's fixative demonstrated many eosinophilic to amphophilic inclusions of slightly varying density. Many were small, some multiple in individual nuclei, some were surrounded by a distinct halo, and many (in late stages of infection) filled the entire nucleus. Chromatin material and nucleoli were aggregated into clumps and strands, and underwent margination as cellular infection progressed (Fig. 2).

No inclusions were demonstrated with the use of neutral buffered formalin. Cells of the

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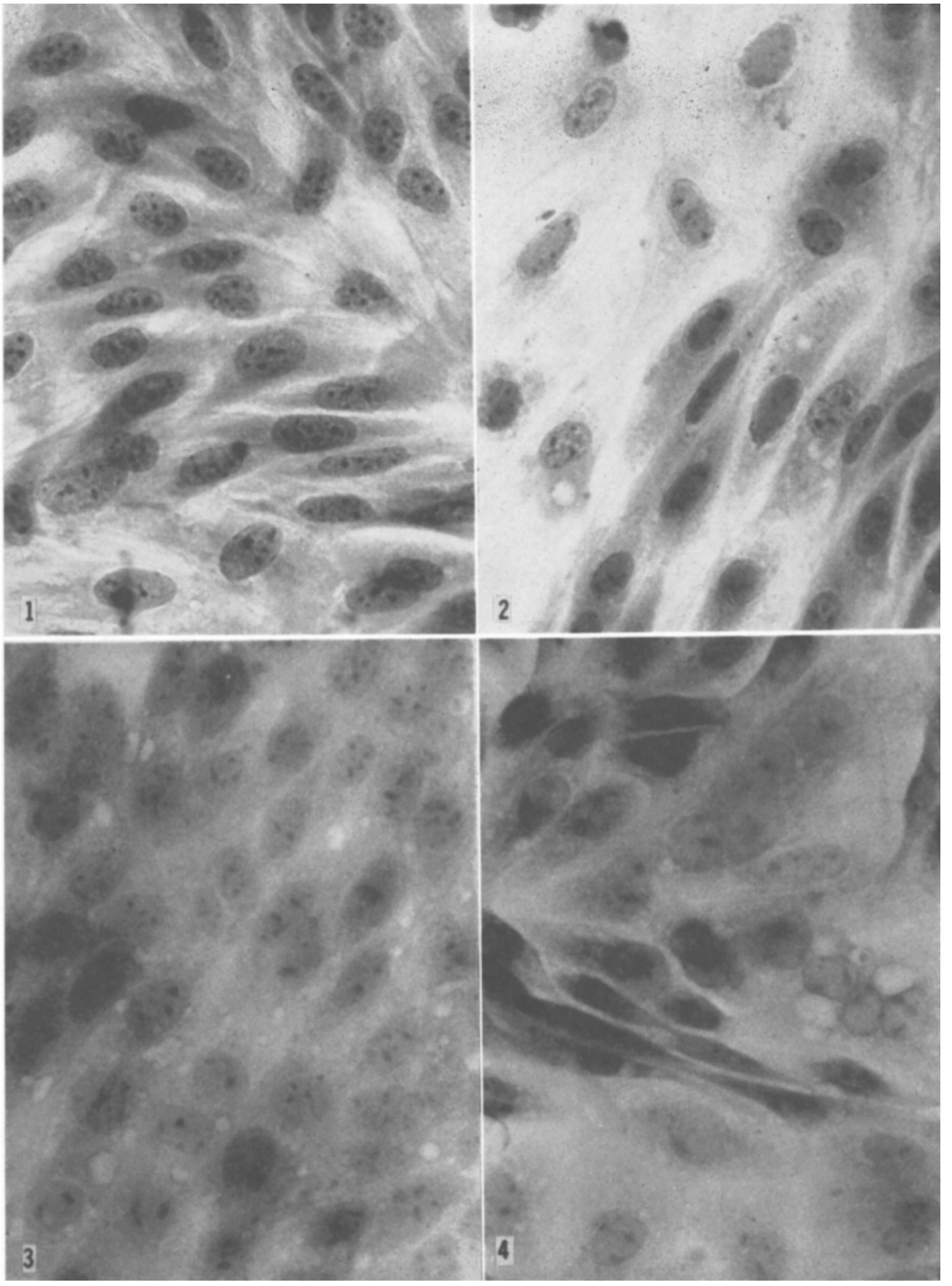

FIG. 1. Uninfected cells fixed with Bouin's fluid.

FIG. 2. Infected cells fixed with Bouin's fluid. Nucleoli and chromatin material are clumped and marginated, and intranuclear inclusion bodies are prominent.

FIG. 3. Uninfected cells fixed with neutral buffered formalin. Cellular and nuclear definition are relatively poor.

FIG. 4. Infected cells fixed with neutral buffered formalin. Nucleoli and chromatin material are clumped and marginated, but no intranuclear inclusion bodies are discernible.

(All fields are of bovine kidney monolayers stained with H and E and photographed at 560 ×.)

TABLE I. Microscopic Observations of IBR Inclusion Bodies in Bovine Kidney Epithelial Tissue Cultures Treated with the Constituents of Bouin's and Zenker's Fixatives and Stained with H & E.

Chemicals used as fixatives	pH	Comparative No. and appearance of inclusions
1. Constituents of Bouin's fluid		
a. Stock Bouin's	1.3	Many eosinophilic to amphophilic well defined inclusions, sometimes multiple in individual nuclei
b. Saturated aqueous picric acid	1.5	None
c. Bouin's with water in place of formalin	1.5	Many inclusions, same appearance as with stock Bouin's fixation
d. Bouin's with sodium acetate in place of acetic acid (same ionic strength)	5.7	Few amphophilic to basophilic, indistinct inclusions
e. Bouin's titrated to pH 11 with 4N sodium hydroxide	11.0	None
f. Bouin's with no acetic acid	1.5	"
g. 5% acetic acid in water	2.2	"
2. Constituents of Zenker's fluid		
a. Stock Zenker's	2.2	Few less inclusions than Bouin's, but similar appearance
b. Stock Helly's fluid	4.5	None
c. Zenker's without acetic acid	3.4	"
d. Zenker's with hydrochloric acid (dilute) in place of acetic acid	2.0	Very few, small inclusions
e. Zenker's with sodium acetate in place of acetic acid (same ionic strength)	5.7	None
3. Formalin plus 5% acetic acid	2.2	Very few, indistinct inclusions

type that had demonstrated inclusions when fixed in Bouin's fluid showed only aggregated and marginated chromatin, and displaced, marginated nucleoli after formalin fixation (Fig. 4). General cellular definition was poor with this fixative, and tinctorial characteristics varied from cell to cell.

Absolute ethyl alcohol, absolute methyl alcohol, and acetone yielded very few inclusions, but these were lighty stained and indistinct. Cells fixed with Carnoy's fluid showed inclusions, but they were inferior in number and distinctness to those treated with Bouin's fixative. The contrast between cellular basophilic and eosinophilic elements was more pronounced after fixation with Carnoy's fluid, but there was extreme shrinkage of many cells and nuclei.

Zenker's, an acid fixative (pH 2.2) with mordanting agents (bichloride of mercury and potassium dichromate) was then compared to Bouin's which is similar in nature (pH 1.3 and mordanting agent picric acid). Infected cultures treated by each of these solutions were almost identical in appearance. Cells treated with Bouin's, however, demonstrated a few more inclusions.

The constituents of Zenker's and Bouin's fluids were employed separately in an attempt to evaluate the effects of each. Formalin combined with acid, and formalin with mordanting agents of Zenker's and Bouin's was also studied. The chemicals used, and the comparative results observed appear in Table I.

Discussion and conclusions. Microscopic observations of tissue cultured cells infected with IBR virus, fixed by various reagents, and stained with H and E led to the following conclusions: 1. Bouin's and Zenker's fluids were superior to either formalin, ethyl alcohol, methyl alcohol, Carnoy's fluid or acetone in demonstrating IBR inclusion bodies. Bouin's, however, was slightly superior to Zenker's in number of inclusions demonstrated. 2. Formalin, in combinations tested, is of no value. Neutral formalin, acid formalin, Helly's fixative and picric acid with formalin did not show inclusions. Effects of constituents of Bouin's fluid without formalin were identical

to those of stock Bouin's. 3. Highly acidic solutions of mordanting agents in water without acetate ions do not demonstrate inclusions. Use of picric acid and water alone, and bichloride of mercury and potassium dichromate plus HCl and water demonstrated the need for acetate ions. 4. Limited experimentation with pH variations indicated that mordants with acetate ions at pH values somewhat higher than stock fixatives, are probably ineffective. Comparatively poor results obtained with Bouin's fluid at pH 5.7, and negative results with Bouin's at pH 11 and Zenker's fixative at pH 5.7, support this statement. However, Bouin's fluid at pH 11 had a high ionic strength, and was warmed to maintain solubility. 5. Acetate ions in aqueous or formalin solution at low pH without mordants is of little value. This conclusion is indicated by inability of acetic acid in water, and acetic acid in formalin to demonstrate many inclusions. 6. Aqueous solutions of mordants without acetate ions do not demonstrate inclusions. Saturated aqueous picric acid alone, and bichloride of mercury plus potassium dichromate in aqueous solution failed to reveal any inclusions. Therefore it appears that a highly acidic fixative with acetate ions and a mordanting agent is necessary for best demonstration of IBR inclusion bodies in tissue cultured cells.

Any explanation of the phenomena noted must necessarily await a more complete investigation of physical and chemical variables of the fixatives (effects of various fixation times, ionic strengths, and other substituted ions in particular), and a determination of the chemical nature of inclusion bodies. Coupled with conclusions of previous workers(1,2) this study indicates a similarity, both *in vitro* and *in vivo*, of the effects of fixatives on IBR inclusion bodies.

Summary. Primary bovine kidney epithelial cells grown in tissue culture monolayers were infected with infectious bovine rhinotracheitis virus, treated with various common fixatives, and stained with hematoxylin and eosin. Of fixatives used, Bouin's and Zenker's gave the best demonstration of characteristic intranuclear inclusion bodies. Further work with components of these 2 fluids indicated that for best demonstration of inclusions, a highly acidic fixative containing acetate ions and a mordanting agent is needed.

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Elevation of Plasma Fibrinogen Following Lymphosarcoma Tissue Injections in Rats. (24804)

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Bacterial diseases, virus diseases, trauma and cancer generally produce elevation of plasma fibrinogen level. The basic cause of the phenomenon has not been extensively investigated but most observers appear to agree with Foster and Whipple(1) that dead tissue or nonspecific (aseptic) inflammation is required to produce the elevation(2). The present paper is concerned with our observation that subcutaneous injection of cancer tissue homogenates or suspensions will cause elevation of plasma fibrinogen, whereas injection of similar preparations of a number of other tissues will not do so.

Methods. Young adult Carworth-Wistar female rats from Carworth Farms were used, and maintained on Purina dog chow and water *ad lib.* in individual cages. The Murphy-Sturm lymphosarcoma was used. It was carried by subcutaneous transplantation in the