Some Effects of Enzymatic Digestions of Fixed Monkey Kidney Tissue Cultures on their Staining Patterns.* (25012)

GEORGE R. DUBES, MARGARET CHAPIN AND ITALO ARCHETTI†
Dept. of Pediatrics and Hixon Memorial Lab., University of Kansas School of Medicine, Kansas City, Kan.

Nearly a half century ago Van Herwerden (1) used a spleen extract containing native ribonuclease to diminish basophilia of sea urchin oöcyte. Many others (see, *e.g.*, 2, 3, 4) have since used ribonuclease and other enzymes to digest cells before staining and microscopic examination, with results sometimes very useful for determining intracellular distribution of cellular materials. The purpose of this paper is to report results obtained by applying such enzymatic digestions to a primate cell grown in tissue culture. Specifically, the effects of digestion of fixed monkey kidney cells with chymotrypsin, ribonuclease, desoxyribonuclease, and some other enzymes on subsequent staining pattern of these tissue culture cells will be given.

Materials and methods. Sources of enzymes and stains. Chymotrypsin (salt free from ethyl alcohol), desoxyribonuclease (1x crystallized), lipase (wheat germ), pancreatin (3x U.S.P. potency), papain, ribonuclease (crystalline), and trypsin (2x crystallized, containing 50% $MgSO_4$) were obtained from Nutritional Biochemicals Corp., Cleveland, O.. Trypsin (1:250) from Difco Laboratories, Detroit, Mich. Pyronin Y and thionin were obtained from National Aniline Division, Allied Chemical and Dye Corp., N. Y., Basic fuchsin from Coleman and Bell Co., Norwood, O. *Purification of pyronin Y and thionin.* For some cultures pyronin Y was used without purification; for other cultures purified preparations were used. Paper chromatography of commercial pyronin Y in solvent composed of 3 volumes *i*-propanol and 1 volume N aqueous formic acid revealed that this stain contained at least 3 colored compounds in addition to pyronin itself, the major rose red constituent at Rf 0.59. Pyronin was partially separated from the other 3 colored compounds (purple one at Rf 0.77, rose at Rf 0.26, and mauve at Rf 0.00) by extracting an aqueous solution of commercial pyronin Y 3 times serially, each time with 0.4 volume *n-amyl* alcohol. These 3 alcoholic extracts were combined and extracted once with 0.11 volume demineralized water. This water extract was shown by paper chromatography to contain the major rose red component largely but not entirely free of the other 3 colored compounds and is referred to herein as purified pyronin. Paper chromatography of commercial thionin in 3 volumes *i*-propanol and 1 volume N aqueous ammonia revealed, in addition to blue spot of thionin itself at Rf 0.40, a less intensely colored mauve spot at Rf 0.69. The blue compound was then separated from the mauve compound by adding 0.05 volume N aqueous ammonia to aqueous solution of the impure material. This ammoniacal solution was then extracted 4 times serially, each time with equal volume of ethyl acetate. Thionin then crystallized upon partial evaporation of final aqueous phase. This crystalline product showed none of the mauve component by paper chromatography and is referred to herein as purified thionin. For some cultures this purified thionin was used whereas for others the impure commercial product was used. *Preparation of fixed monkey kidney tissue cultures*: Kidney cells from rhesus (*Macaca mulatta*) or cynomolgus (*Macaca irus*) monkeys were dispersed with trypsin using the technic of Youngner(5). These cells were grown on cover slip fragments (*ca* 1 cm x 1 cm) placed in 60-mm diameter Petri plates. Growth medium was lactalbumin medium of Melnick(6) and the Petri plates were incubated at 35-37°C in incubator continuously flushed with 3% CO_2 and 97% air. Cover slip fragments with adherent cultures of kidney cells were removed after cells had grown for 3-6 days, at which time usually 30-90% of top surface of fragment was covered with

* Supported by grant from National Fn.

† Present address: Istituto Superiore di Sanità, Roma, Italy.

TABLE I. Effects of Digestion of Fixed Monkey Kidney Cells with Chymotrypsin, Ribonuclease, and Desoxyribonuclease on Their Subsequent Staining with Pyronin, Thionin, and Feulgen Method.

		Effect of enzymatic digestion of			
		Formalin-fixed cells			Carnoy's-fixed cells
		On staining with			
Enzyme	Portion of cell	Pyronin*	Thionin*	Feulgen method	
Chymotrypsin	Nucleus	0†	0†	0	Cells removed‡
	Cytoplasm	DD	DD	0	
Ribonuclease	Nucleus	D§	0†	0	0
	Cytoplasm	DDD	DDD	0	0
Desoxyribonuclease	Nucleus	DD‖	DDD‖	DD‖	
	Cytoplasm	0	0	0	Not done

0, no effect; D, slight decrease in intensity of staining; DD, moderate decrease; DDD, large decrease.

* Purified stain in M/10 sodium acetate buffer, pH 4.2.

† Whether or not nucleolar staining was decreased is not known since these results are from cultures treated with hypotonic buffered saline and nucleoli of cultures so treated are usually not readily identifiable.

‡ Chymotrypsin removed Carnoy's-fixed cells from cover slip.

§ This is actually a moderate decrease in nucleolar staining without noticeable decrease in staining of rest of nucleus.

‖ Staining of mitotic chromosomes was similarly diminished.

cells. These freshly harvested cultures were thoroughly washed in phosphate-buffered saline (PBS) (7) at pH 7.2-7.4. Those cultures whose chromosomes were to be examined were then immersed for 10 minutes at 37°C in 6-fold dilution of PBS in demineralized water. Incubation in this very hypotonic solution resulted in marked dispersal of the chromosomes; a similar hypotonic treatment has been used for dispersal by Chu and Giles (8). All cultures were then fixed. Fixation was usually in 4% formaldehyde in 0.075 M sodium phosphate buffer at pH 6.9 for 4-20 hours at room temperature. Formaldehyde was chosen as fixative since Stowell and Zorzoli (9) had shown it satisfactory for studying the action of ribonuclease on fixed rabbit pancreas cells, whereas they found other fixatives quite unsuitable. Carnoy's fixative (60 volumes ethyl alcohol, 30 volumes chloroform, and 10 volumes glacial acetic acid) was used for some cultures which were to be stained by the Feulgen method. Fixation in Carnoy's was for 13-40 hours at room temperature. *Digestion of fixed cells with various enzymes*: Excess formaldehyde or Carnoy's fixative was removed by thoroughly washing the fixed cultures in demineralized water before treating them with enzymes. Digestions with enzymes were at 37°C for one hour unless otherwise stated. When more than one digestion was used, slip cultures were washed with demineralized water between digestions. Control slips were incubated under same conditions in the solvent used for enzyme being studied. Unless otherwise indicated, chymotrypsin was dissolved in PBS, desoxyribonuclease in 0.2 M sodium borate buffer at pH 7.0 with 0.025 M $MgSO_4$, lipase in PBS, pancreatin in PBS, papain in PBS with L-cysteine hydrochloride at 0.020 M, ribonuclease in demineralized water, and trypsin in PBS. Enzymes were usually used at concentration of 0.25%, except for desoxyribonuclease and ribonuclease which were usually used at 0.10%. *Staining.* Slip cultures were washed in demineralized water after digestion. Those to be stained by the Feulgen method were then heated in N HCl at 60°C for 8 minutes, if they had been fixed in Carnoy's, and for 5 minutes if fixed in formaldehyde. Schiff's reagent for the Feulgen method was prepared from basic fuchsin and sodium metabisulfite according to the method of de Tomasi, [see (3)]. Staining with Schiff's reagent was for 30-60 minutes at room temperature. Staining with pyronin (commercial pyronin Y or purified pyronin) or thionin (commercial or purified) was in 0.10 M sodium acetate buffer (pH 4.9) unless otherwise noted, and for 15 minutes (occa-

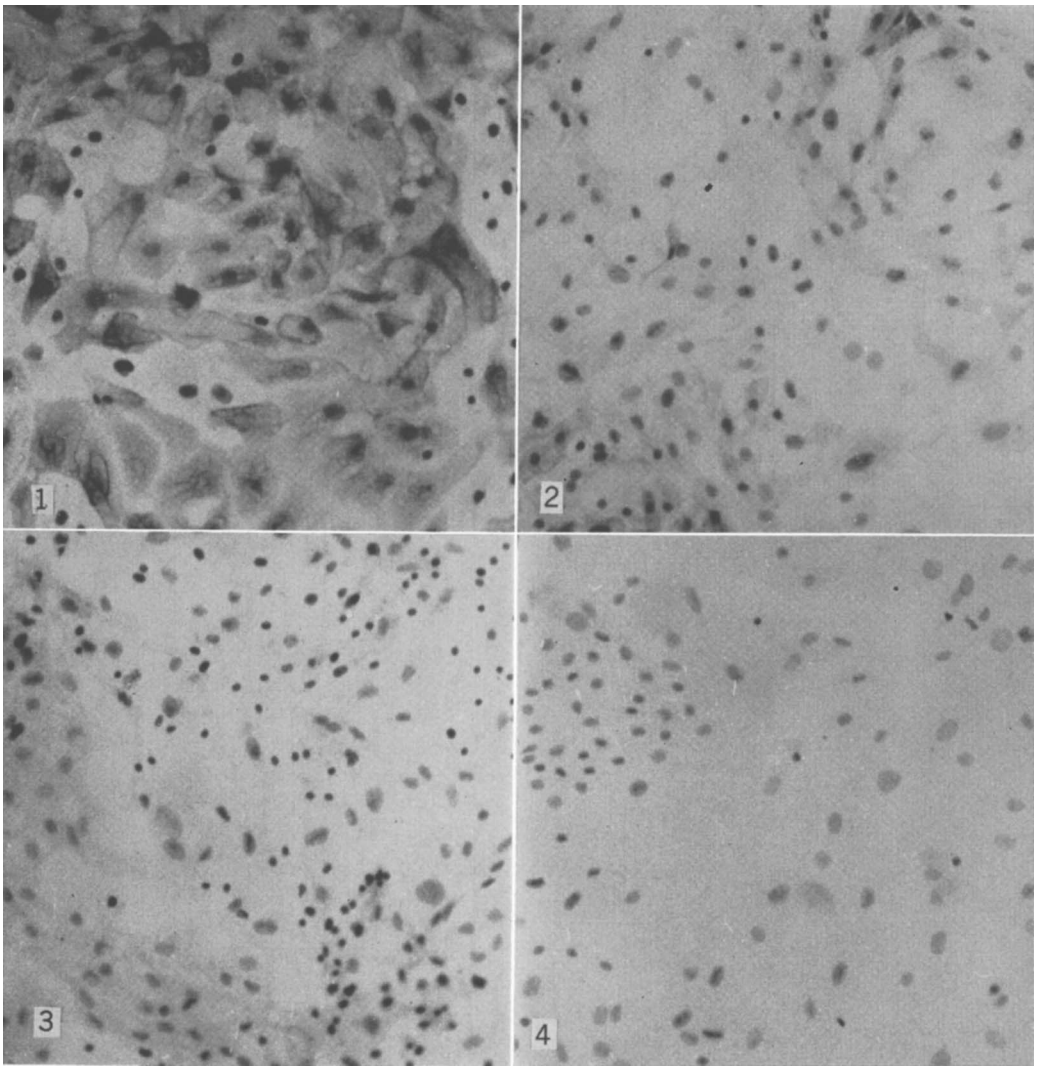

PLATE 1. Reduction in cytoplasmic pyroninophilia as result of digestion with chymotrypsin, ribonuclease, or both. All 4 monkey kidney tissue cultures had been treated with hypotonic saline and then fixed in formalin.

FIG. 1. Control culture, incubated in PBS without $CaCl_2$ and $MgCl_2$.
FIG. 2. Incubated in chymotrypsin dissolved in PBS without $CaCl_2$ and $MgCl_2$.
FIG. 3. Incubated in ribonuclease in same solvent.
FIG. 4. Incubated in a mixture of these 2 enzymes in the same solvent.

All 4 were then stained for 25 min. at room temperature in 0.06% purified pyronin in M/10 sodium acetate buffer, pH 4.2. Magnified about 180 diameters.

sionally 25 minutes) at room temperature. Pyronin Y was usually used at 0.5% but in some experiments at concentration as low as 0.06%; thionin was used at 0.1%. After staining, cultures were washed with demineralized water; they were then dehydrated, cleared, and mounted.

Results. Effects of chymotrypsin, ribonuclease, and desoxyribonuclease: The effects of treating fixed monkey kidney tissue cultures with chymotrypsin, ribonuclease, or desoxyribonuclease on some subsequent staining properties of cells are summarized in Table I. After treatment with chymotrypsin or ribonuclease, cytoplasm did not stain usual violet hue with thionin: the small amount of cyto-

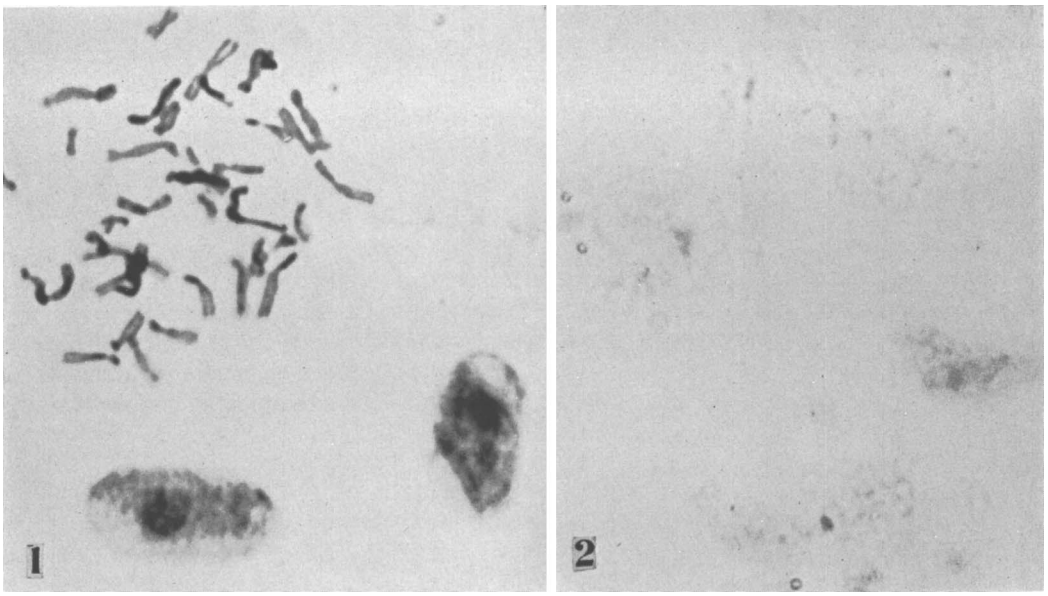

PLATE 2. Reduction in nuclear thioninophilia as a result of digestion with desoxyribonuclease. Both monkey kidney tissue cultures treated with hypotonic saline, fixed in formalin, then digested with mixture of chymotrypsin and ribonuclease in PBS without $CaCl_2$ and $MgCl_2$ to decrease interfering cytoplasmic thioninophilia.

FIG. 1. Control culture whose second incubation was in M/5 sodium borate buffer, pH 7.0, with 0.025 M $MgSO_4$.

FIG. 2. Culture had its second incubation in desoxyribonuclease dissolved in above borate buffer with $MgSO_4$.

Both were then stained 15 min. at room temperature in 0.1% unpurified thionin in M/10 sodium acetate buffer, pH 3.9. The rather vague mitotic chromosomes of Fig. 2 are clearer than those in most mitotic cells which had been treated similarly. Magnified about 2300 diameters.

plasmic staining of enzyme-treated cells was irregularly distributed and blue. When cells were treated with both chymotrypsin and ribonuclease, either simultaneously or serially, subsequent cytoplasmic staining with pyronin or thionin was decreased more than when cells had been treated only with ribonuclease; the action of this pair of enzymes reduced cytoplasmic staining with pyronin or thionin almost to zero. The effects of each enzyme alone and of the pair of enzymes on subsequent staining with pyronin are illustrated by Plate 1. The effect of desoxyribonuclease in reducing subsequent nuclear staining with thionin is shown in Plate 2.

Ribonuclease was effective when dissolved and used in demineralized water but was much less effective in either borate buffer containing $MgSO_4$ or in PBS; in PBS from which $CaCl_2$ and $MgCl_2$ had been omitted, however, ribonuclease was as effective as in demineralized water. Treatment for 15 minutes or 1 hour at 37°C with 0.10% ribonuclease in demineralized water reduced cytoplasmic pyroninophilia by large amount; reduction in cytoplasmic pyroninophilia after treatment under same conditions with 0.010% ribonuclease was slightly less.

Chymotrypsin was ineffective when dissolved and used in demineralized water but was effective in either PBS or in PBS without $CaCl_2$ and $MgCl_2$. Treatment for 1 hour at 37°C with 0.25% chymotrypsin in PBS gave a large decrease in cytoplasmic pyroninophilia; similar treatments with somewhat lower concentrations gave smaller less striking decreases.

Effects of treatment with trypsin and other treatments. Difco 1:250 trypsin had an effect similar to that of chymotrypsin; however, crystalline trypsin usually was ineffective though sometimes it gave a slight decrease in

cytoplasmic pyroninophilia. In limited tests ethylene diamine tetraacetic acid, lipase, 4N HCl, pancreatin, and papain would not substitute for chymotrypsin in a decrease in cytoplasmic pyroninophilia.

Staining. Fixed cells which had been digested with ribonuclease and chymotrypsin were stained with thionin (unpurified) in M/10 sodium acetate buffer at several pH's 3.8-5.7. Within this range amount of staining was greater, the higher the pH; but the contrast between nuclear and cytoplasmic staining did not vary noticeably with pH. Similarly treated cells were stained with pyronin (unpurified) in M/10 sodium acetate buffer at pH 3.9 and 4.9. Amount of staining, especially of cytoplasm, was greater at higher pH; the contrast between nuclear and cytoplasmic staining was somewhat higher at lower pH.

Effects of chymotrypsin, ribonuclease, and desoxyribonuclease in reducing pyroninophilia were much more strikingly shown when purified pyronin was used than when unpurified pyronin was used. Even after digestion with both chymotrypsin and ribonuclease, the cytoplasm stained considerably with unpurified pyronin but much less with purified stain. The effects of these 3 enzymes in reducing thioninophilia were shown about equally clearly with unpurified thionin as with purified thionin, the hues obtained, however, were bluer with purified stain.

Chromosomes of rhesus and cynomolgus monkeys. Many mitotic cells in cultures which had undergone treatment with hypotonic PBS and had been digested with ribonuclease and chymotrypsin had their chromosomes sufficiently well spread and well stained for counting (Fig. 1 of Plate 2). Counts of some of these were made: the value of 42 chromosomes/cell was found for each species. This count for the rhesus monkey is the same as that reported by Darlington and Haque (10).

Discussion. The mechanism by which chymotrypsin decreases cytoplasmic pyroninophilia and thioninophilia of formalin-fixed monkey kidney cells is not clear. That chymotrypsin preparations were effective in this regard because of contamination with ribonuclease is not indicated since chymotrypsin was more effective in PBS than in demineralized water whereas ribonuclease was more effective in demineralized water than in PBS. It seems reasonable to assume that chymotrypsin was effective because of its proteolytic activity; but whether this proteolysis was effective (a) through hydrolyzing pyroninophilic thioninophilic protein(s), (b) through removing ribonucleic acid from cells, or (c) in some other way cannot be said. Similarity of action of chymotrypsin to that of ribonuclease in removing evenly distributed cytoplasmic violet-staining thioninophilic material but not the irregularly distributed cytoplasmic blue-staining thioninophilic material suggests that mechanism (b) above may obtain. Since crystalline trypsin was ineffective or nearly so, the chymotrypsin-like activity of Difco 1:250 trypsin may have been due to contaminating chymotrypsin.

The effect of ribonuclease in decreasing cytoplasmic pyroninophilia and thioninophilia is very probably due to its hydrolysis of cytoplasmic ribonucleic acid. Brachet(11) used this enzyme as early as 1940 for cytochemical detection of ribonucleic acid.

The effect of desoxyribonuclease in decreasing nuclear pyroninophilia and thioninophilia is very probably due to hydrolysis of desoxyribonucleic acid. The results obtained on similar cells using Feulgen stain for desoxyribonucleic acid (Table I) are not in disagreement with this interpretation. Others have obtained similar results. Catcheside and Holmes(12) reported abolition of basophilia in bean-root chromosomes with either a noncrystalline desoxyribonuclease preparation from spleen (only if used after digestion of bean cells with ribonuclease, however) or with a noncrystalline desoxyribonuclease preparation from pancreas (with or without preceding digestion with ribonuclease). Brachet and Shaver(13) found that noncrystalline desoxyribonuclease prepared from beef pancreas would abolish basophilia in chromosomes if Mg^{++} were present but not in the absence of Mg^{++}; no ribonuclease was needed. More recently, crystalline preparations of desoxyribonuclease have been found very effec-

tive in decreasing nuclear basophilia [see, *e.g.*, (4)].

The finding that unpurified pyronin revealed the actions of enzymes much less clearly than purified pyronin suggests that at least one of the contaminating reddish pigments stains cellular materials different from those stained by pyronin itself. On the other hand, the mauve contaminant in unpurified thionin appears to stain the same cellular materials as does the bluer thionin since the only noticeable effect of its removal was a shift in hues of stained cellular materials toward the blue.

Use of digestions with these and other enzymes may prove useful in cytochemical studies of virus-infected cells in tissue culture.

Summary. A large decrease in cytoplasmic pyroninophilia and thioninophilia resulted from digestion of formalin-fixed monkey kidney tissue culture with either chymotrypsin or ribonuclease. This pair of enzymes decreased cytoplasmic pyroninophilia and thioninophilia more than either enzyme alone. Digestion of similar cultures with desoxyribonuclease gave a large decrease in nuclear pyroninophilia and thioninophilia. The effects of these 3 enzymes on pyroninophilia of these cells were more clearly shown when a purified pyronin was used.

The authors are indebted to Dr. Herbert A. Wenner for criticism of the manuscript, to Vernon Scholes for skillful assistance in photomicrography, and to Otis Tolbert for the paper chromatography.

1. Van Herwerden, M. A., *Arch. Zellforsch.*, 1913, v10, 431.

2. Brachet, J., *Biochemical Cytology,* Academic Press, N. Y., 1957, pp. 13-18.

3. Pearse, A. G. E., *Histochemistry Theoretical and Applied,* Little Brown and Co., Boston, Mass., 1953, pp314-340.

4. Kurnick, N. B., *Internat. Rev. Cytol.*, 1955, v4, 221.

5. Youngner, J. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 202.

6. Melnick, J. L., *Ann. N. Y. Acad. Sci.*, 1955, v61, 754.

7. Dulbecco, R., Vogt, M., *J. Exp. Med.*, 1954, v99, 167.

8. Chu, E. H. Y., Giles, N. H., *J. Nat. Cancer Inst.*, 1958, v20, 383.

9. Stowell, R. E., Zorzoli, A., *Stain Tech.*, 1947, v22, 51.

10. Darlington, C. D., Haque, A., *Nature,* 1955, v175, 32.

11. Brachet, J., *Comptes rend. séances l'Acad. des Sci.,* 1940, v133, 88.

12. Catcheside, D. G., Holmes, B., *Symp. Soc. Exp. Biol.*, 1947, v1, 225.

13. Brachet, J., Shaver, J. R., *Stain Tech.*, 1948, v23, 177.

Received May 4, 1959. P.S.E.B.M., 1959, v101.

Long-Term Culture of Tissue Cells in Magnet Stirred Fluid Suspensions. (25013)

ROSLYN WALLACE, ARIS AND HERALD R. COX

Viral and Rickettsial Research Section, Lederle Labs., Amer. Cyanamid Co., Pearl River, N. Y.

One of the most important contributions to development of tissue culture technics has been the observation that tumor cells can multiply as freely suspended cell populations in fluid media(1,2). This discovery served to dispel the concept that tissue cells require anchorage to a solid substrate for multiplication, and methods were subsequently developed by Earle and co-workers(3) for first practical cultivation of animal cells on a large scale. Using 1-liter boiling flasks on Brunswick rotary shakers, these investigators successfully propagated massive fluid-suspensions of a number of cell lines and studied growth characteristics and metabolism of these free cell populations(4,5,6). There have been numerous subsequent reports on cultivation of animal cells in suspension(7-13), and other systems have been used. Preliminary experiments* indicated that tissue cells may be

* P. A. Eichorn and A. Der Aris (unpublished data), Amer. Cyanamid Co., Pearl River, N. Y.