# Alkaline Phosphatase Content and the Effects of Prednisolone on Mammalian Cells in Culture

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ABSTRACT The alkaline phosphatase content of different tissue culture cell lines has been shown to vary from no detectable activity to high enzyme concentration. Within the epithelial lines studied alkaline phosphatase is either constitutive or inducible. Two epithelial cell strains in which alkaline phosphatase was "absent" could be induced to develop significant amounts of the enzyme when grown in the presence of $\Delta^1$-hydrocortisone. Phosphate did not repress enzyme induction by prednisolone. Under conditions of deadaptation the induced enzyme was diluted by cell multiplication. The mouse fibroblastic L line and several human fibroblastic lines did not contain alkaline phosphatase when grown under the conditions described nor could they be induced to produce the enzyme when cultivated in medium with prednisolone.

$\Delta^1$-Hydrocortisone has other characteristic effects on established mammalian cell cultures which vary among cell lines. Human epithelial lines show reduction in cell multiplication with increase in mitotic index. The cytoplasm is increased and cell volume is nearly doubled. Mouse fibroblasts show a similar reduction in cell multiplication with a decrease in mitotic index. There is no increase in cell cytoplasm. Human fibroblast strains show no inhibition of multiplication or alteration in total cell protein when grown in medium containing prednisolone.

Antisera prepared against "negative" prednisolone-inducible human cell lines and against a positive human line inhibited alkaline phosphatase activity to an equal degree.

## INTRODUCTION

Most human cells capable of continuous cultivation *in vitro* have been found to be similar in their nutritional requirements (1), immunologic reactivity (2), and virus susceptibility (3). The enzyme composition of four established

human cell lines and a primary rabbit kidney cortex tissue culture has been reported to be almost identical (4). Two hypotheses have been advanced to account for this uniformity (5). The first is that similarities between cell lines derived from various species and tissues may be due to the selection of a ubiquitous cell type which is best able to multiply *in vitro*. The second alternative is that the tissue culture environment may either influence different cell types to develop common nutritional and metabolic patterns, or during long term culture select a particular cell type from the initial population. By employing suitable environments, clonal lines with characteristics distinguishing them from the parent cultures may be isolated from human and animal cell cultures (6–10). Cultivation of mammalian cell lines in the presence of compounds having specific biologic actions, such as hormones, drugs, and inducers or repressors of specific protein synthesis might reveal different responses related to the tissue of origin. In particular, hormones are known to have different specific effects depending on the tissue and organ studied (11). Investigations on the action of hydrocortisone and its analogues in animals and in tissue culture have shown inconstant and at times opposite responses (11–16). These paradoxical results may be explained in part by differences between the cell types (epithelial or fibrocyte) and possibly by the characteristics of the tissue from which the line was derived.

Variations in the presence and amounts of certain enzymes and other proteins which are not necessary for multiplication are to be expected in cells growing in culture. Enzymes whose distribution *in vivo* is restricted either to a tissue or to certain stages of cellular differentiation may be of the inducible type. The presence of the enzyme and the factors influencing its formation may differ in cells derived from various tissues. Mammalian cell alkaline phosphatase appears to be a heterogeneous group of enzymes whose physiologic functions and substrates are unknown. Variations in alkaline phosphatase synthesis among embryonic tissues during differentiation (17) and its restricted distribution in adults (18) suggested that this enzyme system might be useful for the study of induction in mammalian cells in culture. Adrenal glucocorticoids are known to induce alkaline phosphatase synthesis during development in amphibian, chicken, and mouse intestine (17, 19). The alkaline phosphatase activity of human leucocytes is markedly increased in patients treated with hydrocortisone (20). On the other hand, renal and liver alkaline phosphatase is apparently not influenced by adrenal glucocorticoids. The phenomenon of induction of alkaline phosphatase in human HeLa cell lines by prednisolone, a hydrocortisone analogue has been reported (21). The present communication amplifies and extends the previous observations.

## MATERIALS AND METHODS

### *Nutrient Media*

Several tissue culture media were used. Medium 199 was employed in most experiments (22). Occasional studies were carried out using either Eagle's basal medium (BME) (23), medium 1066 (24), or Waymouth's medium (25). Eagle's basal medium and medium 199 were purchased from Microbiological Associates, Inc., Bethesda, Maryland. Medium 1066 was supplied by Dr. L. N. Castor, Johnson Foundation, University of Pennsylvania. Waymouth's medium was made in the laboratory of Professor G. Pontecorvo, University of Glasgow, Scotland. Penicillin (50 units per ml) and streptomycin (50 $\mu$g per ml) were added to all media.

### *Sera*

In most experiments 10 per cent human serum which had been previously heated to 56°C for 45 minutes was added to the tissue culture medium. Human serum obtained from healthy donors was purchased from the Philadelphia Serum Exchange. In occasional experiments 10 per cent horse or calf serum previously heated at 56°C for 45 minutes was used in place of human serum. Animal serum was obtained from Cappel Laboratories, West Chester, Pennsylvania. Sera and media were tested for toxicity on renal cell cultures prior to routine use. Within individual experiments the same lot of serum was always used.

### *Hydrocortisone Analogues*

Two hydrocortisone analogues, prednisolone sodium phosphate and prednisolone–21-hemisuccinate, were used. Both these substances had similar effects on cells in culture.

Prednisolone sodium phosphate ($\Delta^1$-hydrocortisone phosphate Merck) was furnished by Dr. A. I. Winegrad (26). The preparation contained 74.4 per cent prednisolone, 6.4 per cent phosphate, and 9.5 per cent sodium. It was readily soluble in water. Prednisolone sodium phosphate was used in a concentration of 0.5 $\mu$g per ml in medium 199 containing 10 per cent human serum. Initially, disodium phenyl phosphate was added in concentration of 0.5 $\mu$g per ml up to 10 $\mu$g per ml to the control medium; experiments indicated that these amounts of organic monophosphate had no demonstrable effects on alkaline phosphatase content of epithelial cells. Therefore, it was omitted from the control medium in the majority of experiments.

Prednisolone–21-hemisuccinate was supplied by Roussel Laboratories Ltd. It is a preparation of the double succinate of prednisolone and sodium produced by adding a solution of sodium bicarbonate to a solution of prednisolone–21-hemisuccinate. A concentration of 0.5 $\mu$g per ml of prednisolone–21-hemisuccinate was used in medium 199 containing 10 per cent human serum. The effects of this preparation on tissue

culture cells were identical with those of prednisolone sodium phosphate which was employed in most experiments.

### *Cell Lines Continuously Cultivated in Vitro*

Table I lists the various cell lines studied, their origin, and the growth medium used in the laboratory from which the cell lines were obtained. Three uncloned HeLa lines, MBA, NIH, and Kline, were furnished by Dr. Lewis Coriell, South Jersey Medical Research Foundation, Camden, New Jersey, who had carried them in culture for 1½ to 3 years prior to our studies.

All cultures were examined at intervals of 9 to 12 weeks for possible contamination by pleuropneumonia-like organisms employing Hayflick's method (43). No evidence of contamination has been detected at any time. Occasional cultures developed gross bacterial contamination and were discarded. Cultures were examined as a routine at monthly intervals for occult bacterial contamination. Beef heart infusion broth containing 5 per cent fresh defibrinated rabbit blood was inoculated with both cells and supernate from each cultured line of cells. After incubation for 48 hours at 37°C a loopful of broth was streaked on the surface of a blood agar plate.

Sixteen months after the initiation of the studies, a slow growing, Gram-negative, bacterial species was isolated from the cell lines. The cells at this time had become granular and were growing poorly. The bacterium was killed *in vitro* by kanamycin in a concentration of 30 μg per ml. Tissue cultures treated with kanamycin for 3 weeks became bacteriologically sterile; however, after several additional passages without added kanamycin over a 5 week period, the bacterium could be isolated again. Prolonged treatment of the cell lines with kanamycin for 4 months has not eradicated the bacterial contaminant.

Most of the experimental data to be reported in the present paper were collected prior to contamination of the cell lines. Repetition of certain experiments indicated that the added kanamycin did not influence the experimental results. Tissue cultures grown in kanamycin (30 μg per ml) have continued to multiply at the same rate as untreated cultures prior to bacterial contamination and no overt evidence of infection has reappeared.

### *Cultural Methods*

1. ESTABLISHED CELL LINES All cell lines were grown in milk dilution bottles employing nutrient media and sera as described above. Except for cell lines designated by a double dagger in Table I, all cultures were adapted to medium 199 with added 10 per cent human serum. The adaptation consisted of gradually reducing the amount of original medium by replacing it with increasing amounts of medium 199 containing 10 per cent human serum. Adaptation from horse serum to human serum was accomplished within two or three passages. In general, cells grown on calf serum required four or five passages over a period of 3 to 4 weeks for adaptation to human

serum. Study of cultures was not initiated until they had become adapted to vigorous multiplication on medium 199 containing human serum. To insure the maximum rate of cell division the cultures were fed every other day by replacing one-half the volume of the supernate with fresh medium. Just prior to the formation of a confluent cell monolayer (3 to 4 day old cultures), the cells were harvested by adding to them a solution of 0.025 per cent trypsin (Nutritional Biochemicals) and 0.02 per cent versene in Puck's saline (44). The yield from one milk dilution bottle, containing between 3 and 8 million cells, was then seeded into three or four milk dilution bottles containing nutrient medium and serum. In the case of media containing human serum, except when noted, at least three passages as described were carried out prior to analysis of cell properties, so that the cells were adapted to rapid growth on glass. Studies using nutrient medium with added animal serum (horse or calf) were carried out only on cell lines that had been continuously grown on the same medium for at least 3 months.

2. PRIMARY CELL CULTURES (TABLE II) Monkey kidney cell cultures were purchased from Microbiological Associates, Bethesda, Maryland, or were supplied through the generosity of Dr. James Prier, Merck Sharpe & Dohme Laboratories, West Point, Pennsylvania. The cultures were grown on medium 199 with added 0.5 per cent lactalbumin hydrolysate and 2 per cent calf serum. As in experiments with established cell lines, the properties of the cells were analyzed when a uniform monolayer was present.

Human kidney cultures were prepared from surgical biopsy specimens. The renal cortex normal in the gross was minced and trypsinized by a modified Youngner technique (45) overnight at 4°C in 0.25 per cent trypsin solution in Hanks' balanced salt solution (46). The cell suspension was centrifuged at 800 RPM for 10 minutes and the supernate was discarded. The sedimented cells were washed twice in Hanks' solution, resuspended in 30 to 40 ml of medium 199 containing 10 per cent human serum, and filtered through gauze. Three or four milk dilution bottles containing 12 ml of nutrient medium were seeded with a cell suspension containing approximately 100,000 to 150,000 cells per ml. When the cells had grown to form a complete monolayer (10 to 14 days), they were trypsinized and used for enzyme determinations. For histochemical staining 100,000 cells were seeded into tubes containing coverslips to which cell attachment occurred. At intervals following inoculation coverslips were removed for staining.

Human pericardium cultures were prepared in 125 mm pyrex tubes by explanting in a chicken plasma clot small pieces of human pericardium obtained surgically from patients with idiopathic pericarditis. Medium 199 containing 10 per cent human serum was used as nutrient. Following initial outgrowth, the cells were trypsinized and seeded into tubes containing coverslips for histochemical study.

Human synovial fluid cultures were obtained in tubes containing a coverslip from Dr. S. Cooperband of the Arthritis Section, Department of Medicine, University of Pennsylvania (47). Cultures were grown on BME with 10 per cent horse serum and were used for enzyme studies when a loose network of cells had developed on the surface of the glass.

TABLE I

DETECTION OF ALKALINE PHOSPHATASE BY HISTOCHEMICAL STAINS IN ESTABLISHED CELL LINES

| Established cell lines | Tissue of origin | Laboratory from which cultures were obtained | Medium | Serum 10 per cent | Histochemical detection of alkaline phosphatase: α-naphthyl phosphate, Original medium and serum | α-naphthyl phosphate, Adapted to medium 199 and human serum | Gomori, Medium 199 with human serum | Reference |
|---|---|---|---|---|---|---|---|---|
| *Human non-malignant* | | | | | | | | |
| Renal cell | Needle biopsy of a nephrotic kidney | R. Cox | 199 | Human | 2+ to 4+ | 2+ to 4+ | 1+ | |
| Henle intestine | Embryonic jejunum | L. Coriell | 199 | Horse | 4+ | 4+ | 3+ | 27 |
| Chang liver | Fetal liver | Microbiological Associates | 199 | Human | 1+ to 2+ | 1+ to 2+ | 0 | 28 |
| Girardi heart | Adult heart | 1. Microbiological Associates | 199 | Human | 1+ to 2+ | 1+ to 2+ | 0 | 29 |
| | | 2. Merck, Sharpe & Dohme | 199 | Calf | 4+ | * | * | |
| Wish | Amnion | L. Hayflick | BME | Calf | 1+ to 2+ | 1+ to 2+ | 0 | 30 |
| F. L. amnion‡ | Amnion | Merck, Sharpe & Dohme | 199 | Calf | 4+ | * | * | 31 |
| Detroit 98 | Normal bone marrow | Microbiological Associates | 199 | Human | 1+ to 2+ | 1+ to 2+ | 0 | 32 |
| Whil‡ | Fetal lung | L. Hayflick | BME | Calf | 0 | * | 0 | 33 |
| Skin‡ fibroblasts 10 lines | Foreskin of individual donors (10) | G. Pontecorvo | Waymouth | Calf | * | * | * | |
| *Human malignant* | | | | | | | | |
| HeLa MBA§ | Carcinoma of cervix | Microbiological Associates | 199 | Calf | 5+ | 2+ to 4+ | 2+ | 34 |
| HeLa Kline§ | Carcinoma of cervix | Morton Kline | 199 | Horse | 3+ | 2+ | * | 34 |
| HeLa $A_{76}$‡ | Carcinoma of cervix | Merck, Sharpe & Dohme | 199 | Calf | 4+ | * | * | 34 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| HeLa Henle | Carcinoma of cervix | W. Henle | BME | Horse | 0 | 0 | 0 | 34 |
| HeLa NIH§ | Carcinoma of cervix | H. Eagle | 199 | Calf | 0 | 0 | 0 | 34 |
| KB‡ | Carcinoma of mouth | 1. F. Charalampous | BME | Horse | 4+ | 4+ | 3+ | |
| | | 2. Merck, Sharpe & Dohme | 199 | Calf | 4+ | * | * | 35 |
| J111 | Monocytic leukemia | L. Coriell | BME | Calf | 5+ | 5+ | 4+ | 36 |
| H.Ep. No. 2‡ | Epidermoid carcinoma | Merck, Sharpe & Dohme | 199 | Calf | 4+ | * | * | 37 |
| *Mouse non-malignant* | | | | | | | | |
| L cell mouse fibroblast | $C_3H$ adipose tissue | A. Graham | BME | Calf | 0 | 0 | 0 | 38 |
| MCN | Human bone marrow line replaced by L cell | W. Henle | 199 | Horse | 0 | 0 | 0 | 39 |
| *Rabbit non-malignant* | | | | | | | | |
| $MRK_2$‡ | Kidney | Merck, Sharpe & Dohme | 199 | Calf | 4+ | * | * | 40 |
| $MRK_3$‡ | Kidney | Merck, Sharpe & Dohme | 199 | Calf | 4+ | * | * | 40 |
| FRK‡ | Kidney | Merck, Sharpe & Dohme | 199 | Calf | Trace | * | * | 41 |
| *Chicken non-malignant* | | | | | | | | |
| Chicken embryo heart‡ | Embryonic heart | Merck, Sharpe & Dohme | 199 | Calf | 4+ | * | * | 42 |

* Not tested.

‡ Studied only on the medium used in the laboratory from which the cultures were obtained.

§ Obtained from Dr. L. Coriell, South Jersey Research Institute, Camden, New Jersey.

TABLE II

DETECTION OF ALKALINE PHOSPHATASE BY HISTOCHEMICAL METHODS IN PRIMARY CELL CULTURES

| Tissue of origin | Preparation of culture | Laboratory of origin | Medium | Serum | Histochemical detection of alkaline phosphatase | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | α-naphthyl phosphate | | Gomori | |
| | | | | | Original medium and serum | Adapted to medium 199 and human serum | Medium 199 with human serum | Reference |
| Monkey kidney | Trypsinized cortex | Merck, Sharpe & Dohme | 199 | Calf | 0 | 0 | 0 | |
| Human kidney | Trypsinized cortex | R. Cox | 199 | Human | 0 | 0 | 0 | |
| Human pericardium | Explants | R. Cox | 199 | Human | 0 | 0 | * | |
| Joint fluid | Patients with rheumatoid arthritis | S. Cooperband | BME | Horse | 0 | * | * | 38 |

* Not tested.

### *Determination of Cell Numbers*

Trypsinized cells were suspended in an equal volume of nutrient medium with 10 per cent serum. The suspension was vigorously mixed and two separate aliquots were taken for counting employing a Levy hemocytometer. Each aliquot was counted in duplicate and unless counts agreed within 15 per cent, additional aliquots were prepared, counted, and the averages taken. When individual counts varied more than 20 per cent from the mean, the experiment was discarded.

### *Determination of Cell Size*

A trypsinized cell suspension was diluted with an equal volume of medium containing serum and dispensed into a Levy hemocytometer chamber. Cell diameters were measured microscopically using a standardized ocular micrometer.

### *Coverslip Preparations*

Coverslip preparations were made by seeding 50,000 to 100,000 cells into 125 mm pyrex tubes containing a 6 × 30 mm No. 1 Corning coverslip. When confluent cell growth was present, the coverslips were removed. The medium was completely decanted and the monolayer was washed with isotonic saline. Histologic or histochemical stains were then made.

### *Mitotic Indices*

Mitotic indices were determined on cell monolayers grown in tubes containing a coverslip. To insure a high mitotic rate cultures were fed 24 hours before as well as immediately before adding cholchicine to the medium. Incubation with colchicine was for 4 to 6 hours at 37°C. Coverslips were removed from the tubes, dipped in distilled water, and air-dried. The hypotonicity of the distilled water rinse causes the cells to swell and permits good visualization of the mitotic figures. Following fixation in absolute methanol for 10 to 15 minutes, the coverslips were stained by Giemsa's method. The mitotic indices were determined by counting a minimum of 2,000 nuclei per slide.

### *Histochemical Determination of Alkaline Phosphatase*

1. SODIUM α-NAPHTHYL PHOSPHATE METHOD The cell monolayer grown on a coverslip was washed once in isotonic saline, air-dried, and then stored for 1 hour to 10 days at room temperature prior to fixation and staining. Kaplow's modification (49) of the Menten, Junge, and Green method (50) of diazotizing α-naphthyl phosphate was usually employed as alkaline phosphatase stain. The coverslip preparations were fixed in formalin 10 parts and absolute methanol 90 parts at 5°C for 30 seconds. The specimens were then rinsed in water and incubated in substrate at pH 9.75 for exactly 10 minutes and again were rinsed for 10 seconds in running tap

water. Following air-drying they were mounted without counterstain either in gelatin or in 50 per cent polyvinylpyrrolidone.

The specificity of the stain was tested by immersing coverslips in water at 90°C for 2 minutes to destroy the enzyme (51); heated preparations were consistently negative for alkaline phosphatase. Preparations fixed in 80 per cent ethyl alcohol for 24 hours or preserved by immediate freezing and lyophilization showed an intensity and localization of enzyme activity similar to those shown when Kaplow's method was used.

2. CALCIUM PHOSPHATE METHOD In addition to the use of the α-naphthyl phosphate technique, preparations of most cell lines were stained by the Gomori method as modified by Lillie (52). Following overnight fixation in 80 per cent ethyl alcohol the coverslips were incubated with substrate at pH 9.5 for 1 hour at room temperature. No counterstain was used. Following dehydration in acetone and xylol solutions the coverslips were mounted in Canada balsam.

### *Preparation of Mechanically Disrupted Cells*

After the cells had been counted, the suspension was centrifuged at 1,000 RPM for 10 minutes. The supernate was completely decanted and the cells were thrice washed by resuspending in isotonic saline and centrifuging at 1,000 RPM for 10 minutes. The washed cells were suspended in Earle's balanced salt solution (53), pH 7.0 to 7.4, at a concentration of 2 million cells per ml. The cell suspension was transferred to a 10 ml chamber of a Mickle disintegrator together with 2 to 3 gm of size 110 washed glass beads (Minnesota Mining and Manufacturing Company) and 1 drop of octyl alcohol. The cells were disrupted by 90 minutes of agitation at 4°C. Microscopic inspection of the homogenate consistently showed few or no intact cells. The cell homogenate was centrifuged in the cold (5°C) at 1,500 RPM for 30 minutes and the clear middle layer was removed for study. The homogenate was kept refrigerated at 0 to 5°C at all times.

### *Preparation of Sodium Deoxycholate–Lysed Cells*

The cells were washed three times in isotonic saline as described above. They were then suspended at a concentration of 4,000,000 per ml in 1 per cent sodium deoxycholate dissolved in distilled water. The suspension was mechanically shaken for 20 minutes at room temperature and an equal volume of isotonic saline added so that the final suspension was equivalent to 2,000,000 cells per ml. Cell lines which contained large amounts of alkaline phosphatase were diluted to 1 million cells per ml before testing. The deoxycholate lysates were stored at 4°C.

### *Chemical Measurement of Acid and Alkaline Phosphatase*

Alkaline and acid phosphatase activity were determined by the phenolphthalein diphosphate (Sigma Chemical Company) method of Huggins and Talalay (54). For

measurement of alkaline phosphatase pH was adjusted to 9.4, and for acid phosphatase the pH was 4.8 to 5.0. The incubation of substrate with either Mickle homogenate or deoxycholate lysate was at 37°C for 2 hours in order to increase the sensitivity in cases in which low levels of phosphatase were present (54). Colorimetric determinations were made immediately after adding glycine buffer to the reactants, employing a Klett-Summerson photometer with a 540 mμ filter. Duplicate determinations on the same sample usually agreed within 5 per cent and repeated determinations on the sample over several days usually varied less than 10 per cent.

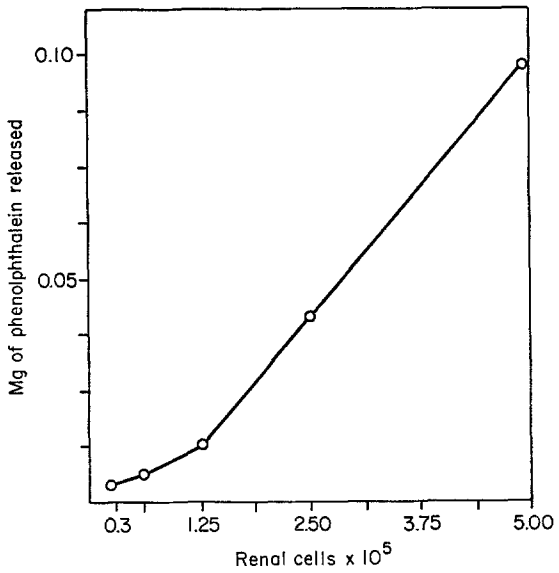


FIGURE 1. Phenolphthalein released by varying concentrations of renal cell alkaline phosphatase at pH 9.75.

All alkaline and acid phosphatase results are expressed as *milligrams of phenolphthalein liberated by 2 million cells during 2 hours of incubation at 37°C.* A value of 0.15 unit indicates that 0.15 mg of phenolphthalein was liberated by 2 million cells during 2 hours' incubation at 37°C. This unit is $\frac{1}{100}$ of a Huggins unit.

The amount of phenolphthalein released in a 2 hour period varied linearly with the concentration of renal cell alkaline phosphatase at concentrations of 0.01 mg phenolphthalein or greater (Fig. 1) but below this amount of phenolphthalein, enzyme activity was not proportional to enzyme concentration.

To control the specificity of the method determinations of alkaline phosphatase were made also by Shinowara's method (55) using 0.5 per cent glycerophosphate as substrate. The inorganic phosphate was measured colorimetrically by Fiske and SubbaRow's (56) procedure. Although the results were similar to those obtained by the phenolphthalein method, Shinowara's method was found to be less sensitive and the results were less reproducible.

### *Protein Determinations*

Protein results are expressed as milligrams of nitrogen per 2 million cells. Protein was measured as total nitrogen by the micro-Kjeldahl technique.

### *Measurement of Deoxyribonucleic Acid and Ribonucleic Acid*

A counted suspension of triply washed tissue culture cells was centrifuged at 1,500 RPM for 20 minutes and the supernatant completely decanted. The cell pellet was washed with ice cold 10 per cent trichloroacetic acid (TCA) and the cell suspension centrifuged at 1,500 RPM in the cold (4°C) for 3 minutes. The TCA was poured off and the precipitate was allowed to drain in the inverted position for several minutes. To obtain reproducible results it was found necessary to carry out the TCA extraction rapidly and at a temperature below 5°C. The pellet may be stored for several days at 4°C without change in the results.

The cell precipitate was suspended in 5 per cent perchlorate at a concentration of 1 million cells per ml and extracted by stirring at 70°C for 15 minutes in a water bath. The extract was then rapidly cooled. Following centrifugation at 1,500 RPM for 10 minutes the supernatant was decanted.

Burton's modification of the Dische diphenylamine method for measuring deoxyribonucleic acid (DNA) was used (57). One ml of diphenylamine acetaldehyde reagent was added to 0.5 ml of the 5 per cent perchlorate cell extract. After mixing the reactants were incubated overnight at room temperature. Optical density was measured in a Beckman DU spectrophotometer at 600 mμ and compared to a standard of highly polymerized calf thymus DNA (Nutritional Biochemicals Corporation, Cleveland, Ohio). Over the range of amounts of DNA employed the optical density was proportional to the concentration. All determinations were carried out in duplicate.

Flacks' modification (58) of Mejbaum's orcinol method (59) for determining ribonucleic acid was used. Six-tenths of an ml of the 5 per cent perchlorate cell extract was diluted with 0.4 ml of distilled water to give a final perchlorate concentration of 3 per cent. One ml of 0.1 per cent ferric chloride in concentrated hydrochloric acid and 0.1 ml of 10 per cent orcinol in 95 per cent ethyl alcohol were added to the 3 per cent perchlorate cell extract. The contents were mixed and the vessel was placed in a boiling water bath for exactly 30 minutes. The reactants were rapidly cooled in an ice bath and the optical density was determined at 670 mμ against a reagent blank. All determinations were carried out in duplicate.

### *Viruses*

Attenuated variants of poliovirus type 1, WCh (60), type 3, WFx (60), and type 2 MEF (61), were obtained from Mr. T. W. Norton of the Wistar Institute as supernates from infected monkey kidney cell cultures. Viruses were stored in sealed ampoules in a dry ice chest at −70°C.

### *Cytopathogenicity of Polioviruses for HeLa Cells*

Washed monolayers of cells grown in tubes on medium 199 and 10 per cent human serum were infected with 0.2 ml of tenfold dilutions of the virus suspensions. Following incubation at 37°C for 1 hour, 2 ml of medium 199 with 5 per cent horse serum was added and the tubes were again incubated at 37°C. The monolayers were examined microscopically daily and the degree of cytolytic change was recorded. The highest dilution of virus which caused complete cellular destruction in 7 days was taken as an indication of the susceptibility of a cell line to the cytolytic effects of the virus.

### *Production of Anti-HeLa Cell and Anti-Renal Cell Rabbit Serum*

HeLa cells of the Henle strain and human renal cells were grown for several passages on glass surfaces in medium 199 containing 10 per cent rabbit serum. The cells were harvested by trypsinization and the suspension was washed three times in Earle's balanced salt solution. The washed cells were resuspended in Earle's solution and mixed with an equal volume of Freund's incomplete adjuvant (arlacel 15 per cent and bayol F 85 per cent) (62). Four rabbits weighing between 2 and 3 kg were injected subcutaneously, two for each cell line, with from 10 to 40 million cells at monthly intervals for 10 months. A total of 280 million cells was given to each of the four rabbits.

The rabbits were bled prior to the initial injection of cells and at 10 to 21 day intervals after each injection. Blood was allowed to clot at 37°C, and was then refrigerated overnight. The serum was separated and stored at 4°C without preservative. All sera were heated to 56°C for 30 minutes before using them in the tests to be described below.

### *Titration of Anti-HeLa Cell Serum for Hemagglutinating Antibodies*

Hemagglutination titers were determined by a modification of Kabat's method (63). Human red blood cells from defibrinated blood were stored in Alsever's solution. Sheep red blood cells were obtained from the Veterinary School, University of Pennsylvania, and stored in Alsever's solution. Mouse red blood cells were separated from defibrinated blood which had been obtained by cardiac puncture. They were used immediately after blood was drawn and were not stored. Before use in hemagglutination tests red cells of all three species were thrice washed in isotonic saline.

Dilutions of serum in isotonic saline solution in a volume of 0.3 ml were pipetted into agglutination tubes measuring 8 × 50 mm. An equal volume of a 2 per cent red cell suspension was added. The tubes were shaken to disperse the red cells and were then incubated in a water bath at 37°C for 1 hour. Agglutination readings were made immediately after removing the tubes from the water bath by observing the pattern of red cells on the bottom of the tube as well as following dispersion of red cells by manually flicking the tube. Both methods gave almost identical endpoints.

The reciprocal of the highest final dilution causing macroscopic agglutination is given as the titer of the serum.

### *Titration of Anti-HeLa Cell Serum for Agglutinins for Tissue Culture Cells*

The technique employed was essentially the same as that used in the titration of hemagglutinating antibodies, with the substitution of tissue culture cell suspensions for erythrocytes. A trypinized cell suspension was prepared in isotonic saline. Following three saline washings, 0.15 ml of the cell suspension and 0.15 ml of serum dilutions were mixed. The tubes were incubated in a water bath at 37°C for 1 hour and readings were then made by obsering the cell pattern on the bottom of the tubes and the pattern on dispersion of the cells upon agitation.

### *Inhibition of Alkaline Phosphatase Activity by Antiserum Prepared against Tissue Culture Cells*

A cell preparation containing 4 to 6 million cells per ml was suspended in 0.25 M sucrose and homogenized in a Mickle disintegrator at 4°C for 90 minutes. The homogenate was centrifuged at 1,500 RPM at 4°C for 60 minutes and the supernate decanted. The supernate was centrifuged in a Spinco preparative ultracentrifuge at 40,000 RPM (average force 105,400 × *G*) for 60 minutes. The microsome-free supernate was stored at 4°C.

Anti-HeLa and anti-human renal cell sera were those described above. They had similar hemagglutinating titers for human type O erythrocytes. The sera were heated to 65°C for 1 hour to inactivate their alkaline phosphatase before adding them to the microsome-free supernate.

Inhibition of alkaline phosphatase by anti-HeLa and anti-renal antibodies was measured by incubating 0.2 ml of microsome-free supernatant from homogenized renal or HeLa MBA cells with 0.2 ml of rabbit antiserum at 37°C for 1 hour. Phenolphthalein diphosphate was then added and the residual alkaline phosphatase was measured by the methods described above.

Control tubes contained normal rabbit serum in place of the antisera.

## EXPERIMENTAL

### *Histochemical Determinations of Alkaline Phosphatase in Various Cell Lines Continuously Grown in Vitro and of Cells in Primary Tissue Culture*

Tables I and II present the results of experiments carried out over a period of 18 months using two alkaline phosphatase stains. Alkaline phosphatase content of different cell lines cultivated under identical conditions varied from no detectable enzymatic activity to marked activity. The $\alpha$-naphthyl phosphate method was found consistently to be more sensitive than Gomori's method. As shown in Tables I and II, ten of the twenty-five cell lines studied

had a high level of cytoplasmic alkaline phosphatase. These established cell lines were derived from human beings, rabbits, mice, and chickens and were grown on various media and sera as indicated in Table I.

The cells were in the logarithmic phase of growth when harvested for histochemical tests. In general, they had had at least three passages on medium 199 with 10 per cent human serum following adaptation to this medium. Except for the established human renal cell line, experiments in which calf or horse serum was used in the growth medium were carried out before the cells had been adapted to medium 199 and added human serum. A few lines were grown and studied only on the medium and animal serum used in the laboratory from which the cultures were obtained. The presence or absence of alkaline phosphatase could not be ascribed to the medium or serum employed in growing the cells.

Five of the cell lines appeared to have low or intermediate alkaline phosphatase content.

Five established laboratory lines and all five lines of primary cultures (Tables I and II) were consistently negative for alkaline phosphatase by histochemical techniques.

It is of particular interest that among the five strains of HeLa cells studied, three produced alkaline phosphatase which was readily detectable by the $\alpha$-naphthyl phosphate method, whereas two lines of HeLa, Henle and NIH, were negative.

### *Histologic Localization of Alkaline Phosphatase in Human Cells in Culture*

Alkaline phosphatase–positive human cell lines studied by the $\alpha$-naphthyl phosphate method showed diffuse cytoplasmic staining with a moderate number of deeply staining granules near the cell membrane and in the perinuclear area. The diffuse staining may result from alkaline phosphatase present on microsomes. Alkaline phosphatase–positive material was present at the outer cell margin and could be removed by brief treatment with 0.025 per cent trypsin solution in buffered saline. Its relationship to the alkaline phosphatase–positive granules in the cytoplasm is unknown since diffusion artifacts were not evaluated.

The nuclei showed no alkaline phosphatase–positive material when stained by the $\alpha$-naphthyl phosphate technique. Round cells stained more deeply than those of epithelial form; it is presumed that many of them were premitotic. Dividing cells were also intensely stained. The chromosomes could be seen to have large amounts of alkaline phosphatase but their morphology was not well visualized because of the intensity of staining. During anaphase the equatorial plane was alkaline phosphatase–negative; however, the spindle

fibers could be seen as alkaline phosphatase–positive threads traversing the equatorial plane.

Gomori's calcium phosphate technique shows a similar cytoplasmic localization of alkaline phosphatase, but the intensity of the staining was less than that observed with the $\alpha$-naphthyl phosphate method. The cell membrane was heavily stained. The nuclear membrane was positive in many of the cells and displayed a beaded appearance. The nucleolus and nucleolar membrane were also positive. The chromosomes in dividing cells were well visualized by their intense alkaline phosphatase staining. Individual chromosomes presented a beaded appearance.

*Chemical Determination of Phosphatase Activity of Mechanically Disrupted Cells*

Table III presents the results of chemically determined alkaline phosphatase on mechanically disrupted cells. There is excellent correlation between the results of the chemically determined alkaline phosphatase activity and the degree of histochemical staining.

Cell lines capable of continuous cultivation *in vitro* may be divided into two groups on the basis of their alkaline phosphatase activity. Lines having constitutive alkaline phosphatase include the human renal, Henle human intestine, Chang human liver, D98 human bone marrow, Wish human amnion, Girardi human heart, two rabbit kidney lines ($MRK_1$ and $MRK_2$), chick embryo heart, J111 human monocytic leukemia, KB human epidermoid carcinoma, and three of the five HeLa lines. These lines are termed "positive."

Cells containing little or no alkaline phosphatase are the L cell mouse fibroblast, MCN (presumably also a mouse fibroblast), Whil, a diploid human fibroblast from fetal lung, several diploid human skin fibroblast lines (Glasgow), and two of the five HeLa lines, HeLa Henle and HeLa NIH. Although small concentrations of alkaline phosphatase are probably present, for convenience in the following sections these lines are designated as negative.

All four primary cell cultures investigated showed little or no alkaline phosphatase activity. Among the positive cell lines, D98, Wish, and Girardi heart have an intermediate concentration of enzyme determined by both histochemical and chemical methods.

Variations in alkaline phosphatase in different experiments with the same cell line appear to be a reflection both of environmental factors and the stage of cell growth. All determinations were made on logarithmically growing cultures; however, the marked adaptability of alkaline phosphatase to environmental factors, as described later in this paper, probably accounts for the range of alkaline phosphatase activity within the same positive cell line.

The relative ineffectiveness of mechanical disruption as compared to deoxycholate lysis in the release of alkaline phosphatase activity, as shown in Table IV, may also explain in part some of the variability of the results.

Three alkaline phosphatase–containing cell lines (Henle human intestine, human renal cell, and HeLa MBA) and six negative cell lines (HeLa Henle,

TABLE III

ALKALINE PHOSPHATASE ACTIVITY IN HOMOGENATES OF MECHANICALLY DISRUPTED CELLS

| Cell line | Medium | Serum | Mean alkaline Phosphatase/$2.10^6$ cells | Range alkaline Phosphatase/$2.10^6$ cells |
|---|---|---|---|---|
| *Human non-malignant* | | | | |
| Renal cell | 199 | Human | 0.10 | 0.15–0.06 |
| Henle intestine | 199 | Human | 0.18 | 0.52–0.06 |
| Chang liver | 199 | Human | 0.04 | 0.04 |
| Girardi heart | 199 | Human | 0.02 | |
| Wish amnion | 199 | Human | 0.02 | |
| Bone marrow D98 | 199 | Human | 0.05 | 0.07–0.03 |
| *Human malignant* | | | | |
| HeLa MBA | 199 | Human | 0.18 | 0.31–0.08 |
| HeLa MBA | 199 | Calf | 0.61 | 0.66–0.56 |
| HeLa Kline | 199 | Human | 0.02 | |
| HeLa $A_{75}$ | 199 | Calf | 0.16 | |
| HeLa Henle | 199 | Human | <0.01 | |
| HeLa NIH | 199 | Human | <0.01 | |
| KB | BME | Horse | 0.14 | |
| J111 | 199 | Human | 0.38 | 0.40–0.36 |
| *Mouse cell lines* | | | | |
| L Cell | 199 | Human | <0.01 | |
| MCN | 199 | Human | <0.01 | |
| *Rabbit cell lines* | | | | |
| $MRK_2$ | 199 | Calf | 0.255 | |
| $MRK_3$ | 199 | Calf | 0.270 | |
| *Chicken cell* | | | | |
| Embryo heart | 199 | Calf | 0.68 | |
| *Primary culture* | | | | |
| Monkey kidney | 199 | Calf | <0.01 | |

HeLa NIH, L cell, MCN, Whil, and several strains of human skin fibroblasts) were selected for further study.

### *Phosphatase Activity of Cells Lysed by Sodium Deoxycholate*

Deoxycholate lysis of tissue culture cells was found to disrupt them more effectively and render their contents soluble as compared to mechanical homogenization. Table IV compares deoxycholate lysis to mechanical

disruption on replicate samples of pooled renal cells. In this experiment a 12-fold increase of alkaline phosphatase, a doubling of acid phosphatase, and a threefold increase in soluble protein resulted from deoxycholate lysis. Other experiments showed as much as an 18- to 30-fold increase in alkaline phosphataes upon deoxycholate lysis. The increase in protein ranged from three- to fourfold.

Table V summarizes the results of a series of experiments on the three positive cell lines, Henle human intestine, human renal cell, and HeLa MBA, and on the six negative lines, HeLa Henle, HeLa NIH, L cell, MCN, human fibroblast strain Whil, and several human skin fibroblast strains. In general, the levels of alkaline phosphatase of the positive cell lines vary considerably and tend to be higher in older cultures. Acid phosphatase and total protein concentrations remained relatively constant.

TABLE IV

COMPARISON OF PHOSPHATASE ACTIVITY OF MECHANICALLY DISRUPTED CELLS AND CELLS LYSED BY DEOXYCHOLATE

| Renal cells | Alkaline phosphatase* | Acid phosphatase* | Protein nitrogen |
|---|---|---|---|
| | *units* | *units* | *mg* |
| $3.10^6$ cells mechanically disrupted | 0.33 | 0.11 | 0.07 |
| $3.10^6$ deoxycholate lysis | 4.2 | 0.23 | 0.21 |

* Milligrams of phenolphthalein released by $2.10^6$ cells in 2 hours at 37°C.

Cell lines that were negative for alkaline phosphatase by histochemical techniques as well as following mechanical disruption remained negative upon deoxycholate lysis or appeared to have enzyme activity so small in amount that its detection bordered on the limit of sensitivity of the method. When the number of cells per milliliter that were exposed to deoxycholate lysis was increased threefold (from 2,000,000 to 6,000,000), the amount of protein nitrogen and acid phosphatase increased proportionately; however, the level of alkaline phosphatase remained below the sensitivity of the method.

The acid phosphatase content in all cell lines tended to be relatively constant between 0.04 and 0.10 unit with the possible exception of the Henle HeLa line which appears to have a little more activity (0.08 to 0.14 unit).

### *Effect of Magnesium Ion on Tissue Culture Cell Alkaline Phosphatase*

The addition of magnesium sulfate in a final concentration of 0.03 M to the reactants (cell lysate and phenolphthalein phosphate) doubled the alkaline phosphatase activity of the positive cell lines. However, the negative cell

lines were unaffected except for an occasional Henle HeLa culture which showed activity that was at the lower limit of sensitivity (0.01 unit). In-

TABLE V
SUMMARY OF EXPERIMENTS USING DEOXYCHOLATE LYSIS OF ESTABLISHED CELL LINES

| Cell line | Time incubation | No. of experiments | Mean alkaline phosphatase | Range of alkaline phosphatase | Mean acid phosphatase | Range of acid phosphatase | Mean protein nitrogen | Range of protein nitrogen |
|---|---|---|---|---|---|---|---|---|
| | *hrs.* | | *units* | *units* | *units* | *units* | *mg.* | *mg.* |
| Henle intestine | Total | 14 | 0.52 | 1.0–0.23 | 0.07 | 0.10–0.04 | 0.12 | 0.14–0.08 |
| | 24 | 3 | 0.32 | 0.33–0.30 | 0.07 | 0.08–0.06 | 0.12 | 0.18–0.08 |
| | 90–120 | 4 | 0.60 | 0.80–0.36 | 0.08 | 0.10–0.08 | 0.12 | 0.14–0.11 |
| | 140–170 | 7 | 0.70 | 1.0–0.28 | 0.06 | 0.08–0.04 | 0.13 | 0.14–0.08 |
| Renal | Total | 15 | 0.70 | 2.5–0.13 | 0.06 | 0.09–0.04 | 0.10 | 0.12–0.05 |
| | 24 | 3 | 0.30 | 0.75–0.13 | 0.05 | 0.08–0.04 | 0.11 | 0.13–0.09 |
| | 40–50 | 3 | 0.40 | 0.62–0.16 | 0.07 | 0.08–0.06 | 0.11 | 0.11–0.10 |
| | 70–100 | 9 | 1.00 | 2.5–0.3 | 0.06 | 0.09–0.04 | 0.09 | 0.12–0.06 |
| HeLa MBA | Total | 10 | 0.32 | 0.77–0.14 | 0.04 | 0.04–0.02 | 0.10 | 0.14–0.08 |
| | 24 | 1 | 0.19 | 0.19 | 0.04 | 0.04 | 0.11 | 0.11 |
| | 70–100 | 5 | 0.36 | 0.50–0.14 | 0.04 | 0.04–0.03 | 0.10 | 0.12–0.08 |
| | 120–145 | 4 | 0.43 | 0.77–0.24 | 0.03 | 0.04–0.02 | 0.09 | 0.10–0.08 |
| HeLa Henle | Total | 8 | <0.01 | | 0.12 | 0.14–0.08 | 0.08 | 0.10–0.07 |
| | 40–50 | 1 | <0.01 | | 0.12 | 0.12 | 0.07 | 0.07 |
| | 70–100 | 5 | <0.01 | | 0.12 | 0.14–0.10 | 0.09 | 0.10–0.09 |
| | 120–216 | 2 | <0.01 | | 0.09 | 0.10–0.08 | 0.10 | 0.10 |
| HeLa NIH | Total | 6 | <0.01 | | 0.07 | 0.10–0.04 | 0.10 | 0.12–0.09 |
| | 24 | 1 | <0.01 | | 0.04 | | 0.09 | |
| | 70–100 | 3 | <0.01 | | 0.08 | 0.10–0.04 | 0.11 | 0.12–0.09 |
| | 120–150 | 2 | <0.01 | | 0.08 | 0.08–0.07 | 0.10 | 0.10–0.09 |
| Whil | 60–160 | 4 | <0.01 | | 0.10 | 0.11–0.09 | 0.12 | 0.14–0.10 |
| Human skin fibroblast | 120–160 | 14 | <0.01 | | 0.12 | 0.14–0.11 | Not done | Not done |
| L cell | 90–160 | 8 | <0.01 | | 0.07 | 0.10–0.03 | 0.10 | 0.14–0.08 |
| MCN | 115 | 1 | <0.01 | | 0.07 | | 0.12 | 0.10 |

creasing the ionic strength of the reactants with sodium chloride did not enhance enzyme activity significantly. In none of the determinations described in this report was $Mg^{++}$ added as a routine.

It has been suggested (64) that liver alkaline phosphatase is composed of $Mg^{++}$-sensitive and $Mg^{++}$-insensitive enzymes. Rat hepatomas have been characterized as having increased $Mg^{++}$-insensitive alkaline phosphatase as

compared to normal rat liver. If $Mg^{++}$-sensitive and insensitive alkaline phosphatases exist in established cell lines, it would appear from the results obtained that the cells studied possess nearly equal quantities of these enzymes since addition of $Mg^{++}$ caused an approximate doubling of activity.

### *Absence of Alkaline Phosphatase Inhibitor in Negative Cell Lines*

Deoxycholate lysed negative cell lines, HeLa Henle, HeLa NIH, and L cells, were mixed with lysates of positive HeLa MBA cells and the alkaline phosphatase was measured. No inhibition was demonstrated.

### *Effects of Heat and Storage on the Alkaline and Acid Phosphatases of Tissue Culture Cells*

Alkaline phosphatase activity of HeLa MBA, Henle intestine, and the renal cell was stable when deoxycholate lysates in 1 per cent veronal buffer at pH 7.4 were heated at 47°C for 45 minutes. There was no appreciable loss of activity when lysates of the cells were stored at 4°C for as long as 60 days.

Acid phosphatase was found to be more labile; incubation at 37°C for 1 hour resulted in the loss of 95 per cent of the enzyme activity. There was no significant reduction of acid phosphatase when incubation was carried out at 25°C for 1 hour. Activity was unchanged following storage at 4°C for 60 days.

### *pH Optimum of Tissue Culture Cell Phosphatases*

Following heat inactivation of acid phosphatase the human renal cell alkaline phosphatase was determined over a pH range from 6.0 to 11.0. Maximum activity occurred at about pH 9.4. At pH 6.0 and at pH 11.0 activity was markedly decreased.

Acid phosphatase activity of renal cells was maximal at pH 5.0. At pH 4.0 and 6.1 approximately 50 per cent of the maximum enzyme activity was present. Between pH 6.5 and 7.6 there was precipitation of buffer salts so that pH could not be controlled adequately. At pH 7.6 alkaline phosphatase dominated and obscured acid phosphatase activity.

Alkaline phosphatase activity of cell lysates is strongly inhibited by inorganic phosphate ions. Inhibition is nearly complete in the presence of 0.1 M phosphate. Acid phosphatase is less affected by inorganic phosphate.

## FACTORS INFLUENCING ALKALINE PHOSPHATASE CONTENT OF CELLS

Alkaline phosphatase stains on positive cell lines indicated a difference in the intensity of staining from one cell to another in the same culture. Some cells contained large amounts of enzyme and stained darkly; others showed only a faint tinge of color indicating small quantities of enzyme. When the renal cell line was cloned, it was found that in a colony derived from a single cell there can be wide variation in the intensity of alkaline phosphatase staining from one cell to another. The most intensely stained cells were round and the spindle-shaped cells had variable but reduced intensity of staining. The round cells were in mitosis and the darker staining may reflect a thicker optical section. However, the variation in alkaline phosphatase within the spindle-shaped cells of a clone suggests that other factors are also important in determining enzyme content. In certain protozoans no alkaline phosphatase is found in starved animals (65). Immediately after taking food phosphatase appears in the vicinity of food vacuoles. Very shortly afterwards phosphatase appears in nuclei and throughout the cytoplasm. Perhaps similar processes are responsible for the variation in alkaline phosphatase among cells of a clone.

### *Effect of Various Media on Alkaline Phosphatase Activity of Cell Cultures Grown Continuously in Vitro*

Cell lines were examined for alkaline phosphatase following propagation on the medium and serum customarily used in the laboratory from which they were obtained. Histochemical studies on the cells grown on their original medium and serum showed complete correlation with the alkaline phosphatase content of the cells when adapted to medium 199 and 10 per cent human serum (Table I). Henle HeLa, HeLa NIH, MCN, and L cell were alkaline phosphatase–negative on both the original medium and animal serum as well as following their adaptation to medium 199 and 10 per cent human serum. The other cell lines including HeLa MBA, HeLa K, Henle intestine, J111, and KB were positive for alkaline phosphatase on their original medium and following adaptation to medium 199 and human serum.

### *Effect of Culture Media on Alkaline Phosphatase Content*

Phosphatase activity was unchanged in supernatant medium obtained from confluent cultures of alkaline phosphatase–positive and negative cell lines.

The intrinsic phosphatase activity of serum did not influence either alkaline or acid phosphatase concentrations of cells grown in it.

Renal cells grown on Eagle's basal medium (BME) and human serum from a single person showed a marked reduction histochemically in alkaline phosphatase content when compared to renal cells grown on medium 199 and the same human serum. Table VI presents the alkaline phosphatase activities of renal cells grown on various media as compared to replicate cultures grown on medium 199 and the same human serum. Renal cells propagated in BME showed a slightly increased generation time as compared to the cells grown on medium 199 and a moderate reduction in alkaline

TABLE VI

EFFECT OF VARIOUS MEDIA ON RENAL CELL PHOSPHATASE ACTIVITY*

| Media | No. of cell generations | Generation time | Alkaline phosphatase‡ | Acid phosphatase‡ | Protein‡ |
|---|---|---|---|---|---|
| | | *hrs.* | | | |
| 199 | 500+ | 28 | 100 | 100 | 100 |
| BME | 2 | 32 | 40 | 95 | 100 |
| BME | 5 | 30 | 35 | 98 | |
| BME | 35 | 37 | 25 | 100 | |
| BME, enriched | 3 | 36 | 30 | 100 | |
| BME, with added nucleosides | 2 | 30 | 40 | 100 | 100 |
| BME, with added nucleosides | 5 | 36 | 50 | 100 | |
| 1066 | 2 | 50 | 55 | 110 | 110 |
| 1066 | 5 | 48 | 45 | 100 | |

* Expressed as per cent of activity of replicate culture grown on medium 199 with 10 per cent human serum.

‡ Deoxycholate lysate.

phosphatase. Acid phosphatase and cell nitrogen content were unchanged. The reduced alkaline phosphatase activity of renal cells grown on BME persisted for thirty-five generations over a 2 month interval. Doubling the concentration of amino acids and vitamins in BME (BME, enriched) did not increase the alkaline phosphatase content of renal cells grown in this medium. Addition of nucleosides to BME likewise failed to enhance enzyme activity.

An unexpected finding was that renal cells grown in medium 1066 and human serum showed a prolonged generation time, the cells appeared granular, and the alkaline phosphatase content was reduced as compared to replicate cultures grown in medium 199 and the same lot of human serum.

HeLa MBA when grown on BME and 10 per cent human serum showed

reduced alkaline phosphatase activity compared to replicate cultures grown in medium 199 and the same lot of human serum. Table VII presents the enzyme activity and protein content of HeLa MBA grown on various media. The results are similar to those described for the human renal cell. Alkaline phosphatase of HeLa MBA grown in BME is approximately one-third that of replicate cultures grown in medium 199. The acid phosphatase content and protein of HeLa MBA cells grown in BME or medium 1066 were not significantly altered.

Henle intestine cells grown in BME also showed reduction in alkaline phosphatase activity to approximately one-third the amount produced by replicate cultures propagated in medium 199.

TABLE VII

EFFECTS OF VARIOUS MEDIA ON HELA MBA PHOSPHATASE ACTIVITY*

| Media | No. of cell generations | Generation time | Alkaline phosphatase‡ | Acid phosphatase‡ | Protein‡ |
|---|---|---|---|---|---|
| | | *hrs.* | | | |
| 199 | 200 | 28 | 100 | 100 | 100 |
| BME | 2 | 30 | 40 | 85 | 110 |
| | 5 | — | 30 | 90 | 100 |
| | 15 | 32 | 40 | 90 | 105 |
| 1066 | 8 | 30 | 100 | 95 | 100 |

* Deoxycholate lysate.

‡ Expressed as per cent of activity of replicate culture grown on medium 199 with 10 per cent human serum.

### *Influence of Inorganic Phosphate on the Formation of Phosphatases in Epithelial Cell Lines*

HeLa MBA, a cell line containing constitutive alkaline and acid phosphatase, when grown in a phosphate-deficient medium containing 0.05 mM phosphate, showed no increase in phosphatase activity. The addition of excess concentrations of phosphate (5 mM) to the culture medium did not suppress formation of either acid or alkaline phosphatase.

HeLa NIH, a cell line in which alkaline phosphatase is prednisolone-inducible, when cultivated in phosphate-deficient medium (0.05 mM phosphate), did not develop alkaline phosphatase activity. Acid phosphatase activity of HeLa NIH was unaffected by variation in phosphate concentration.

### *Effects of Cell Injury on Alkaline Phosphatase Content of Renal Cells*

To determine the effect of adverse environmental factors on the alkaline phosphatase activity of our established renal cell line, cultures were incubated at room temperature for 10 to 14 days on medium 199 containing human serum. In most experiments moderate degeneration of the cell monolayer was observed. The medium was then renewed and cultures were reincubated at 37°C. Several days following reincubation, the cells became spindle-shaped and mitoses were seen. The cells were harvested when the monolayer had become confluent. One-half of the cells was seeded into a milk dilution bottle for further passage; the other half was inoculated into tubes containing coverslips for alkaline phosphatase staining.

Renal cells in the spindle phase of growth were uniformly alkaline phosphatase–negative. During the third or fourth generation (second or third passage) the cells resumed their usual epithelioid appearance. Alkaline phosphatase staining of these cultures showed only traces of activity. Six to eight generations (six passages) appear to have been required before there was a return of normal enzyme activity. The cells multiplied rapidly after the third or fourth passage; however, the return of normal alkaline phosphatase activity lags by several generations. As a rule, normal levels are regained abruptly between the sixth and eighth passages.

In other experiments in which cell injury was less severe, as judged by persistence of an intact cell monolayer, the epithelial appearance of the cells was regained during the first passage (one or two generations) and enzyme levels returned to normal within three or four passages (four or five cell generations).

## EFFECTS OF PREDNISOLONE ON GROWTH AND ALKALINE PHOSPHATASE CONTENT OF MAMMALIAN CELL LINES IN CULTURE

### *Effect of Prednisolone on Cell Multiplication*

Multiplication of epithelial cell lines and L cells was greatly reduced in tissue cultures incubated in medium containing 0.5 μg prednisolone per ml. The fourth column in Table VIII shows the per cent reduction in cell numbers of cultures grown in medium with prednisolone for various time intervals, as compared to replicate cultures grown in the same medium without prednisolone. The decreased multiplication of prednisolone-treated cultures became apparent after 24 hours. Following 40 or more hours of incubation with prednisolone, inhibition of multiplication appears to be maximal. Cultures maintained on prednisolone for as long as 60 days continued to exhibit a multiplication rate approximately one-half that of untreated cultures.

TABLE VIII

EFFECT OF PREDNISOLONE ON PHOSPHATASE ACTIVITY OF CELLS IN CULTURE*

| Cell line | Time of incubation | No. of experiments | Change in multiplication of prednisolone-treated cultures | Alkaline phosphatase/2.10⁶ cells | | Changes in alkaline phosphatase | | Acid phosphatase/2.10⁶ cells | | Change in acid phosphatase | | Protein nitrogen/2.10⁶ cells | | Change in cell nitrogen per cell |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Cultures grown in absence of prednisolone | Cultures grown in prednisolone | Based on cell count | Based on cell nitrogen | Cultures grown in absence of prednisolone | Cultures grown in prednisolone | Based on cell count | Based on cell nitrogen | Cultures grown in absence of prednisolone | Cultures grown in prednisolone | |
| | *hrs.* | | *per cent* | | | *per cent* | *per cent* | | | *per cent* | *per cent* | | | *per cent* |
| Henle intestine (human epithelial) | 24 | 3 | −10 | 0.30 | 0.37 | +20 | 0 | 0.07 | 0.08 | +10 | −10 | 0.130 | 0.160 | +20 |
| | 40–100 | 4 | −50 | 0.35 | 0.90 | +250 | +80 | 0.08 | 0.13 | +60 | +10 | 0.130 | 0.185 | +40 |
| | 100–240 | 9 | −55 | 0.60 | 1.40 | +140 | +45 | 0.07 | 0.11 | +55 | − 5 | 0.110 | 0.180 | +60 |
| Renal cell (human epithelial) | 24 | 2 | −20 | 0.12 | 0.20 | +60 | +25 | 0.04 | 0.06 | +50 | +10 | 0.100 | 0.130 | +30 |
| | 40–100 | 8 | −40 | 0.92 | 1.10 | +20 | −15 | 0.07 | 0.08 | +15 | −20 | 0.110 | 0.150 | +35 |
| | 100–120 | 3 | −55 | 0.17 | 0.24 | +40 | −15 | 0.04 | 0.07 | +70 | + 5 | 0.090 | 0.150 | +65 |
| HeLa MBA (human epithelial) | 24 | 2 | −20 | 0.13 | 0.55 | +400 | +250 | 0.04 | 0.05 | +25 | +15 | 0.110 | 0.120 | +10 |
| | 40–100 | 4 | −40 | 0.34 | 1.12 | +320 | +260 | 0.04 | 0.06 | +50 | +20 | 0.107 | 0.133 | +25 |
| | 100–150 | 3 | −40 | 0.44 | 1.60 | +350 | +280 | 0.03 | 0.04 | +30 | 0 | 0.095 | 0.125 | +30 |
| HeLa Henle (human epithelial) | 40–100 | 10 | −45 | <0.01 | 0.08 | | | 0.12 | 0.25 | +110 | +45 | 0.098 | 0.140 | +45 |
| | 120 | 1 | −50 | <0.01 | 0.10 | | | 0.09 | 0.21 | +110 | +50 | 0.094 | 0.150 | +60 |
| HeLa NIH (human epithelial) | 24 | 1 | −25 | <0.01 | 0.01 | | | 0.05 | 0.07 | +40 | +10 | 0.085 | 0.108 | +25 |
| | 40–100 | 4 | −40 | <0.01 | 0.05 | | | 0.08 | 0.175 | +210 | +40 | 0.110 | 0.175 | +60 |
| | 100–150 | 2 | −30 | <0.01 | 0.06 | | | 0.07 | 0.12 | +70 | +30 | 0.100 | 0.130 | +30 |
| L cell (mouse fibrocyte) | 40–100 | 4 | −40 | <0.01 | <0.01 | 0 | 0 | 0.08 | 0.09 | +10 | 0 | 0.115 | 0.120 | +5 |
| | 100–150 | 4 | −60 | <0.01 | <0.01 | 0 | 0 | 0.08 | 0.09 | +10 | 0 | 0.088 | 0.094 | +5 |
| MCN (mouse fibrocyte) | 144 | 1 | −40 | <0.01 | <0.01 | 0 | 0 | 0.10 | 0.10 | 0 | 0 | 0.120 | 0.110 | −10 |
| Whil (human lung fibroblast) | 65–100 | 2 | +10 | <0.01 | <0.01 | 0 | 0 | 0.11 | 0.11 | 0 | 0 | 0.129 | 0.129 | 0 |
| | 100–160 | 3 | 0 | <0.01 | <0.01 | 0 | 0 | 0.15 | 0.16 | 0 | 0 | 0.120 | 0.120 | 0 |
| Human skin fibroblast | 120–180 | 7 | 0 | <0.01 | <0.01 | 0 | — | 0.08 | 0.08 | 0 | — | Not done | Not done | — |

* Deoxycholate lysis.

Multiplication of a human lung fibroblast strain Whil and several human skin fibroblast lines was not reduced by cultivation in medium containing either 0.5 μg per ml or 50.0 μg per ml prednisolone.

There was no evidence that an increased rate of cell degeneration occurs in prednisolone-treated cultures. Examination of supernatant medium from the cultures did not show an increase of detached cells as compared to the controls. Except for an increase in cell size in human "epithelial" tissue cultures, as described below, the cell monolayer appears normal.

*Mitotic Indices of Prednisolone-Treated Cultures*

The mitotic index of prednisolone-treated human epithelial cells was increased on an average of 2 to 3 per cent over replicate cultures grown in medium without prednisolone. Change in the mitotic index of prednisolone-treated mouse-derived fibrocytes strains L and MCN was not consistent but in general there appeared to be a slight decrease which was of the order of 0.5 per cent. Although prednisolone caused an increased mitotic index in the epithelial lines and possibly a slight decrease in the mitotic index of the mouse-derived fibrocytic lines, nonetheless it induced decreased multiplication of similar magnitude in both of these cell types (Table VIII). The Whil strain of human fibroblasts showed no reduction in cell multiplication and no change in mitotic indices when grown in medium with added 0.5 μg per ml or 50.0 μg per ml prednisolone. It seems apparent, therefore, that the effects of prednisolone upon cell multiplication are not paralleled by changes in mitotic index.

*Effect of Prednisolone on Cell Size*

Tissue cultures derived originally from human tissue, including Henle intestine, renal cell, and three HeLa lines, when grown in medium with added 0.5 μg prednisolone per ml, exhibit marked heterogeneity in cell size with an increase in average cell diameter. The average cell volume is nearly double that of untreated cultures. The cell population in prednisolone-propagated cultures varies in size from giant cells up to 40 mμ in diameter to cells of normal size of 16 to 18 mμ in diameter. Table IX presents a representative experiment showing the approximate percentage of cells of different diameters in prednisolone-treated as compared to untreated replicate cultures. The increased average cell size and the heterogeneity in size of the cell population in the presence of prednisolone are apparent. Cultures of human epithelial cells grown in the presence of 0.5 μg per ml of prednisolone for as long as 60 days continued to show an increase in average cell size.

The effect on cell size in human tissue cultures is reversible. Prednisolone-

treated cultures returned to normal size following 65 hours of incubation in medium without the steroid.

The L cell (mouse fibroblast) and certain cell lines which have probably been "contaminated" and replaced by the L cell (human bone marrow strain MCN, Wistar C3H mouse lung, and Wistar C3H mouse kidney) do not show significant increase in average cell size when grown in medium containing 0.5 μg of prednisolone although there may be a few more giant cells than in the control cultures. These mouse-derived cell lines are as much inhibited in their multiplication as are human cell lines but, in contrast, do not have increased cytoplasmic growth.

In the case of Whil, a human lung fibroblast, it was difficult to determine whether prednisolone caused a change in cell size because the untreated

TABLE IX

VARIATION OF CELL SIZE IN HUMAN RENAL CELL CULTURES GROWN IN COMPLETE MEDIUM WITH ADDED 0.5 μg PREDNISOLONE PER ML

| Cell diameter | Control cultures Per cent of cells | Prednisolone-treated cultures Per cent of cells |
|---|---|---|
| μ | | |
| 15 | 25 | |
| 18 | 60 | 15 |
| 21 | 10 | 20 |
| 24 | 4 | 40 |
| 27 | 4 | 15 |
| 30 | | 5 |
| >35 | 2 | 5 |

cell population is heterogeneous and many of the cells are ameboid. Inspection of trypsinized cell suspensions of the Whil line did not indicate significant change in cell size. The same was true for human skin fibroblasts.

### *Effects of Prednisolone on Cell Protein, Ribonucleic Acid (RNA), and Deoxyribonucleic Acid (DNA)*

Table VIII (right-hand columns) shows the change in total nitrogen of cells grown in medium with added prednisolone. Within 24 hours protein in human epithelial cell cultures is increased on the average by 10 to 30 per cent. In general, cell protein nitrogen appears to increase slightly with continued incubation in prednisolone medium.

The mouse L cell and strain MCN on prednisolone-containing medium showed no increase of protein as compared to untreated cultures. The human lung fibroblast line, Whil, likewise showed no change in cell nitrogen.

Human epithelial cell lines propagated on medium with prednisolone showed an average of 30 to 40 per cent increase of ribonucleic acid (Table X). The increased content of RNA roughly paralleled the increased cell protein. The mouse L cell showed no significant alteration in RNA content.

The deoxyribonucleic acid (DNA) concentration in prednisolone-treated human epithelial cultures varied only slightly from that in untreated cultures. The total number of experiments is small but the results suggest an increase of 10 to 15 per cent in DNA content. These findings suggest that the increased cell size of epithelial cultures is mainly due to an increase in cytoplasm with only moderate change in DNA content.

TABLE X

EFFECT OF PREDNISOLONE ON THE NUCLEIC ACID CONTENT OF CELLS IN CULTURE

| Cell line | No. of experiments | Change in RNA of cultures grown in medium with prednisolone | Change in DNA of cultures grown in medium with prednisolone |
|---|---|---|---|
| | | *per cent* | *per cent* |
| Renal cell | 4 | +40 | +16 |
| HeLa MBA | 3 | +42 | +7 |
| HeLa NIH | 2 | +35 | +12 |
| L cell | 3 | +8 | +4 |

*Effects of Prednisolone on the Phosphatase Content of Tissue Cultures*

Alkaline phosphatase activity varies widely in cell lines derived from different animal species and tissues and also among lines of the same cell (HeLa) propagated in different laboratories. Summarized in Table VIII are changes in alkaline and acid phosphatase of cell lines grown in the presence of 0.5 $\mu$g of prednisolone per ml. Human epithelial cell lines showed a consistent increase of alkaline phosphatase activity, except for occasional experiments with the renal cell in which alkaline phosphatase per cell was unchanged. When the content of alkaline phosphatase of human epithelial cell lines grown on prednisolone and those grown on medium without the added steroid was expressed on the basis of total cell nitrogen, there was a significant increase in enzyme in all epithelial lines except the renal cell. This difference in responsiveness of the renal cell to prednisolone may indicate a vestige of enzymatic differentiation since the alkaline phosphatase of embryonic kidney does not increase with adrenal glucocorticoids whereas in embryonic intestine the enzyme increases markedly (17).

The elevation in alkaline phosphatase activity of the prednisolone-inducible cell lines, HeLa strains NIH and Henle, when grown in medium with prednisolone indicates a potential which is not expressed when the cells

are cultivated in media lacking the hormone. The absence of enzyme activity from the L cell lines and from the human fibroblast strains is not affected by the growth of the cultures in medium with prednisolone.

The variability in alkaline phosphatase content of positive cell lines and the influence of various environmental factors result in variations of enzyme content and in the percentage change in enzyme activity from one experiment to another. It should be emphasized, however, that within each experiment all factors were controlled in so far as possible: the cultures of each cell line were replicate, the medium and serum were of the same batch, and

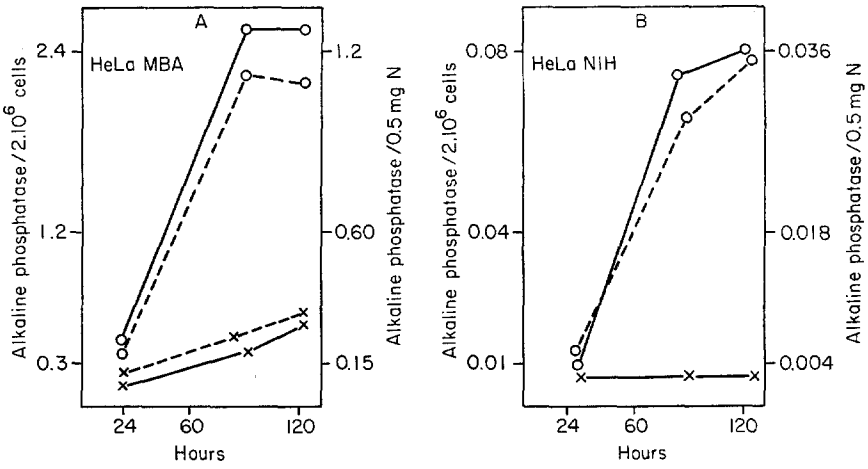


FIGURE 2. Alkaline phosphatase activity of cultures grown with and without prednisolone 0.5 μg/ml: (A) HeLa MBA an example of a line with constitutive activity rising about eight-fold with prednisolone induction. (B) HeLa NIH an example of a strain without detectable constitutive alkaline phosphatase which develops activity by prednisolone induction. ×, without prednisolone, ○, with prednisolone. Solid line, alkaline phosphatase activity per 2 million cells. Broken line, alkaline phosphatase activity per 0.5 mg N.

the cultures were treated in a similar way at all times, except for the addition of prednisolone.

Alkaline phosphatase activity of mammalian cells is independent of phosphate ion concentration in the culture medium. Reduction of phosphate to 0.05 mM does not enhance the alkaline phosphatase content in cell lines that contain the enzyme nor does it induce alkaline phosphatase in the prednisolone-inducible negative HeLa cells. Increase of phosphate ion concentration of the medium to 5.0 mM does not repress prednisolone induction of alkaline phosphatase nor does it alter the enzyme activity of cells with "constitutive" alkaline phosphatase.

Figs. 2 A and 2 B depict the influence of prednisolone on alkaline phos-

phatase of human epithelial cells grown for various time intervals. Fig. 2 A presents a representative experiment with HeLa MBA, alkaline phosphatase positive cell line, and shows the increase in alkaline phosphatase based on the amount per 2 million cells as well as on the basis of total nitrogen in the prednisolone-treated cultures. The increase in alkaline phosphatase of the HeLa MBA line grown in the presence of prednisolone is sixfold. Replicate cultures grown in the same medium without prednisolone show a modest increase in alkaline phosphatase content as the cell monolayer becomes confluent.

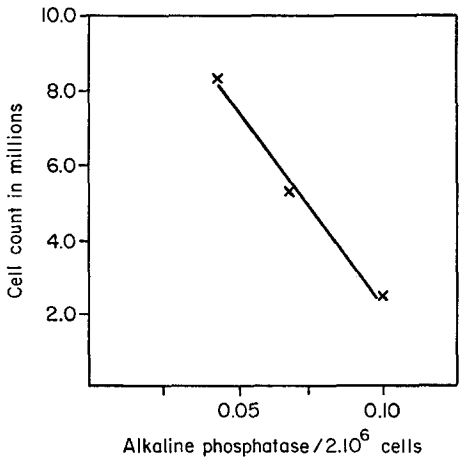


FIGURE 3. HeLa Henle under conditions of deadaptation. Prednisolone induced-alkaline phosphatase is diluted by cell multiplication when the hormone inducer is removed by washing.

Fig. 2 B represents the effects of prednisolone on alkaline phosphatase levels in a cell line, HeLa NIH, which in the absence of added prednisolone is negative. The increase in alkaline phosphatase activity of cultures grown in medium with added 0.5 $\mu$g of prednisolone is unequivocal. Increasing the concentration of prednisolone added to the culture medium from 0.5 to 50.0 $\mu$g per ml did not further enhance the alkaline phosphatase activity of the "inducible" epithelial cell lines.

Under conditions of deadaptation alkaline phosphatase appears to be diluted out by cell multiplication. Fig. 3 shows an experiment with HeLa Henle, a line which is negative in the absence of added prednisolone. Replicate cultures were grown in the presence of prednisolone for 96 hours. The medium was decanted and the cell monolayer was thoroughly washed with a buffer solution. Medium without prednisolone was then added and a culture was harvested at once and at 24 hour intervals until the cell monolayer became

confluent. As shown in Fig. 3 enzyme activity decreases approximately in proportion to the increase in cell numbers for at least three cell generations.

Acid phosphatase in the alkaline phosphatase positive cell lines increased in proportion to the increase of cell protein. In the alkaline phosphatase negative and in prednisolone-inducible cell lines the acid phosphatase content may be increased slightly when expressed on the basis of a unit of nitrogen.

### *Effect of Prednisolone on Acid Production*

Detailed metabolic studies on human tissue cultures grown in medium with added steroid were not carried out. However, acid production of prednisolone-treated epithelial and L cell cultures appeared to be decreased. These cells grown in medium with prednisolone did not become acid as rapidly as control cultures. Human fibroblasts which were grown in medium with 0.5 $\mu$g per ml prednisolone appeared to produce the same amount of acid as control cultures.

### *Delay in the Cytolytic Effects of Type 1 Poliovirus Infection on Cells Incubated in Medium Containing Prednisolone*

Tubes of HeLa Henle cultures grown in medium 199 with 10 per cent human serum were washed and infected with serial tenfold dilutions of type 1, attenuated poliovirus, strain WCh. One-half the tubes were then reincubated in medium 199 with 5 per cent horse serum and the remainder in medium 199 with 5 per cent horse serum and added prednisolone 0.5 $\mu$g per ml. The cytolytic effects of poliovirus infection were recorded daily. In general the prednisolone-treated cultures showed a 24 to 36 hour lag in the development of cytological evidence of infection. Complete cell destruction occurred about 24 hours later in the prednisolone-treated cultures when compared to replicate untreated cultures. The highest dilution of stock virus producing complete cell destruction was the same in prednisolone-treated cultures as in the untreated cultures.

## STUDIES ON HELA SUBLINES

Studies of five HeLa lines, each obtained from a different laboratory, showed that three of them (HeLa MBA, HeLa Kline, and HeLa $A_{75}$) contained large amounts of alkaline phosphatase. Two other HeLa lines (HeLa Henle and HeLa NIH) showed no demonstrable alkaline phosphatase activity unless grown in the presence of prednisolone. In order to study further the differences between these five lines, characters other than phosphatase con-

tent were investigated. These include histologic appearance, susceptibility of HeLa lines to poliovirus infection, chromosomal numbers and morphology, and immunologic relation between the HeLa lines.

*Morphologic Appearance of HeLa Sublines*

All five of the HeLa lines are epithelial in character, and when stained by Giemsa's method have very similar appearing nuclei and prominent nucleoli. Cells of the HeLa Henle line flatten and spread more when growing on glass surfaces than the four other HeLa sublines. A confluent monolayer of HeLa Henle contains between 3 and 5 million cells per milk dilution bottle, whereas the other HeLa lines yield 5 to 8 million cells. The cell volumes of the five

TABLE XI

SUSCEPTIBILITY OF HeLa CELL LINES TO CYTOLYTIC EFFECTS OF POLIOVIRUSES

| HeLa line | Highest dilution of poliovirus producing complete cellular destruction in 7 days | | |
|---|---|---|---|
| | Type 1 WCh | Type 2 $MEF_1$ | Type 3 WFx |
| HeLa Henle | $10^{-6}$ | $10^{-7}$ | $10^{-7}$ |
| HeLa NIH | $10^{-3}$ | $10^{-4}$ | $10^{-3}$ |
| HeLa MBA | Not tested | Not tested | $10^{-7}$ |
| HeLa K | Not tested | Not tested | $10^{-7}$ |
| $A_{75}$ | Not tested | Not tested | $10^{-7}$ |

HeLa lines were nearly the same when measured in suspension. On trypsinization of the HeLa Henle line the cells form clumps which are difficult to disperse, while trypsinization of the other lines results in suspensions consisting mainly of single cells with occasional small clumps.

*Poliovirus Susceptibility of HeLa Cell Lines*

All five HeLa cell lines were found to be susceptible to poliovirus. However, HeLa NIH shows marked resistance to the cytolytic effects of all three types of poliovirus. In Table XI are shown the highest dilutions of poliovirus which caused complete cellular destruction of the HeLa monolayers within 7 days. Virus titrations on supernates from these cultures were not performed. HeLa NIH when infected with high dilutions of poliovirus ($10^{-6}$ to $10^{-7}$), developed either incomplete cellular destruction or showed no evident cytopathic changes, whereas the same amounts of virus caused complete destruction of the cells of the other sublines. It was necessary to increase the inoculum to a dilution of $10^{-3}$ or $10^{-4}$ in order to produce comparable cytolytic effects

on the NIH subline. This subline, therefore, may be said to have relative resistance to the cytolytic effects of the polioviruses.

A preexisting latent virus infection of HeLa NIH could not be demonstrated by inoculating susceptible HeLa lines with cell extracts or medium derived from confluent HeLa NIH cultures. It seems reasonable to ascribe the decreased poliovirus susceptibility of HeLa NIH to an intrinsic resistance of these cultures rather than interference by a latent virus, although the latter possibility is not ruled out by our tests.

### *Chromosome Studies on HeLa Cell Lines*[1]

Preliminary examination of the chromosomes from cells of the HeLa lines NIH, MBA, and Henle revealed no departure from expectations for material

TABLE XII

CAPACITY OF RABBIT ANTISERUM TO HELA CELLS TO AGGLUTINATE SUSPENSIONS OF VARIOUS TISSUE CULTURE CELLS

| Cell line | Reciprocal of titer of preimmunization serum | Reciprocal of titer of HeLa antiserum |
|---|---|---|
| HeLa Henle | 16 | 1024 |
| HeLa NIH | 32 | 2048 |
| HeLa MBA | 16 | 512 |
| HeLa Kline | <8 | 64 |
| Renal cell | 16 | 512 |
| Henle intestine | 16 | 256 |
| L cell | 8 | 8 |
| MCN cell | 8 | 16 |

of human origin. All three cell lines were essentially similar. Chromosome numbers were in the mid-seventies. Chromosomes were predominantly metacentric. The acrocentrics present were identifiable as Nos. 13, 14, 15, 21, and 22 of the human karyotype nomenclature.

### *Immunologic Relations between HeLa Lines*

Anti-HeLa serum produced in rabbits following injection of the Henle subline was tested for hemagglutination against O, A, and B erythrocytes, sheep erythrocytes, and mouse erythrocytes. For human red cells, the reciprocals of the titers ranged from 256 to 2048, and were approximately the same for

[1] Chromosome studies were carried out by Dr. Paul Moorhead, Wistar Institute, Philadelphia.

A, B, and O cells. Hemagglutinating titers against sheep and mouse red blood cells were less than 16 in both pre- and postimmunization bleedings.

Table XII presents the agglutinating titers of the anti-Henle HeLa cell serum for eight tissue culture cell lines. All cell lines of human origin reacted strongly with the antiserum but not with serum from the normal bleeding. The two mouse lines, L and MCN, showed essentially no titer difference when tested against the pre- and postimmunization sera.

It is not known whether the differences in the titers of the serum against the various human cell lines are of immunological significance or whether they are technical in origin. It is possible that absorption studies would reveal the basis for the observed differences; however, these were not carried out.

*Inhibition of Alkaline Phosphatase from Cultured Cells by Antiserum Prepared against Two Different Cell Lines*

Antiserum of similar hemagglutinating titer for human O erythrocytes was prepared in rabbits by immunization with the human renal cell line (alkaline phosphatase–positive) and the HeLa Henle line (alkaline phosphatase–negative unless grown in presence of prednisolone). Prednisolone was not added to the cultures used for immunization. Rabbit antisera prepared against MCN, a poliovirus-resistant fibrocyte probably of mouse origin, and H.Ep. No. 1, a poliovirus-sensitive epithelial cell line derived from a human epidermoid carcinoma, were generously supplied by Dr. V. Defendi of the Wistar Institute. These antisera had high cytotoxic antibody titers against the homologous cell line but did not cross-react (66). Rabbit antiserum prepared against mouse red blood cells was furnished by Dr. J. Palm of the Wistar Institute. In addition, a rabbit antiserum prepared by immunization with whole human serum was supplied by Dr. Neva Abelson, Hospital of the University of Pennsylvania. This antiserum precipitated all human serum fractions and in addition had hemagglutinating titer of 32 against human O erythrocytes.

Table XIII presents the alkaline phosphatase–inhibiting capacity and the hemagglutinating titer for human O erythrocytes of the various antisera. The anti-renal cell and anti-HeLa Henle cell antisera inhibited the human renal cell and HeLa MBA cell alkaline phosphatase activity to about the same degree. It is worth noting that although the HeLa Henle line is alkaline phosphatase–negative by our tests, antiserum prepared against it inhibits alkaline phosphatase as strongly as antisera prepared against the strongly positive renal line.

The specificity of the enzyme inhibition is indicated by the failure of anti-MCN serum to inhibit the enzyme activity of cells of human origin. The

absence of inhibition from the H.Ep. No. 1 cell antiserum suggests that the cell line used in immunizing either lacked alkaline phosphatase or that the methods used in preparing the antisera inhibited the antigenicity of the enzyme. Unfortunately, the H.Ep. No. 1 line was no longer available, so that its phosphatase content could not be determined.

TABLE XIII

INHIBITION OF ALKALINE PHOSPHATASE ACTIVITY BY ANTISERUM PREPARED AGAINST RENAL CELL (POSITIVE LINE) AND HELA HENLE (NEGATIVE LINE)

| Antiserum | Microsome-free supernatant | Alkaline phosphatase | Inhibition of enzyme activity | Hemagglutinating titer for human O RBC |
|---|---|---|---|---|
| | | | *per cent* | |
| 701 Preimmunization | Renal | 0.30 | | 4 |
| | HeLa MBA | 0.07 | | |
| 701 Anti-renal | Renal | 0.20 | −30 | 256 |
| | HeLa MBA | 0.03 | −45 | |
| 704 Preimmunization | Renal | 0.30 | | <4 |
| | HeLa MBA | 0.06 | | |
| 704 Anti-renal | Renal | 0.12 | −60 | 256 |
| | HeLa MBA | 0.03 | −50 | |
| 702 Preimmunization | Renal | 0.33 | | 4 |
| | HeLa MBA | 0.085 | | |
| 702 Anti-HeLa Henle | Renal | 0.15 | −55 | 256 |
| | HeLa MBA | 0.013 | −85 | |
| 703 Preimmunization | Renal | 0.33 | | <4 |
| | HeLa MBA | 0.075 | | |
| 703 Anti-HeLa Henle | Renal | 0.14 | −60 | 32 |
| | HeLa MBA | 0.015 | −80 | |
| Anti-human serum | Renal | 0.31 | −8 | 16 |
| | HeLa MBA | 0.06 | −20 | |
| Anti-H.Ep. No. 1 | Renal | 0.30 | −10 | 512 |
| | HeLa MBA | 0.08 | +5 | |
| Anti-mouse RBC | Renal | 0.31 | −8 | 4 |
| | HeLa MBA | 0.08 | +5 | |
| Anti-MCN (L cell) | Renal | 0.31 | −8 | 4 |

## DISCUSSION

Our results indicate that marked differences in alkaline phosphatase content occur among established tissue culture cell lines derived from different tissues, and among HeLa lines obtained from different laboratories. Contrary to previous reports, established cell lines are not enzymatically identical but may be separated into alkaline phosphatase positive and negative groups.

Alkaline phosphatase concentrations within a positive cell line may vary several fold from one experiment to another. The amount found is partially

dependent on the individual human serum used, on the length of incubation, on whether the cell monolayer is confluent or not, and upon other factors. The fluctuations in alkaline phosphatase content of positive cell lines are small, however, when compared to the differences between positive and negative cell lines.

Differences in alkaline phosphatase content between positive and negative cell lines were found to be independent of the growth medium or the rate of cell multiplication. For these studies all cell lines were cultivated under uniform conditions on the same medium and human serum. When adapted to medium 199 and human serum all grew vigorously. Each cell line was also investigated while growing in the medium and serum customarily employed by the laboratory from which the cultures were obtained. The alkaline phosphatase content of the cells on their original medium and serum correlated with the enzyme activity following adaptation to medium 199 and human serum. In certain cell lines the alkaline phosphatase concentration appeared slightly decreased following propagation on human serum and medium 199; however, all positive lines remained positive. The alkaline phosphatase negative cell lines were negative in their original medium as well as following adaptation to our medium. Chemical and histochemical determinations of alkaline phosphatase corresponded closely within each cell line.

Although the presence or absence of alkaline phosphatase was not dependent on the type of medium or serum in which the cultures were grown, the amount of enzyme activity was markedly influenced by the culture medium. A comparison of alkaline phosphatase positive cultures grown in Eagle's basal medium containing human serum with replicate cultures grown in medium 199 and the same human serum showed that the latter had two to three times more alkaline phosphatase activity than cultures grown in Eagle's basal medium. Neither increasing the concentration of amino acids and vitamins, nor the addition of nucleosides to Eagle's basal medium resulted in renal cell alkaline phosphatase activity equivalent to that of replicate cultures grown in medium 199. The generation times of cultures grown in the two media were nearly the same.

Lines of HeLa cells maintained in different laboratories for several years have as great a variation in their alkaline phosphatase content as do cells derived from different tissues. Three of these lines HeLa MBA, Kline, and NIH were supplied by Dr. Lewis Coriell who had cultivated them for over 1 year in his laboratory. Unknown selecting factors during cell cultivation would appear to be responsible for the alkaline phosphatase variations among the different HeLa lines. The marked differences of alkaline phosphatase content in HeLa lines from various laboratories emphasize that cell lines presumed to be the same from different laboratories may have pronounced

differences in enzymatic composition and in other characters. Therefore, caution should be exercised in applying the findings of experiments performed in one laboratory to those with the same cell line in another laboratory.

The acid phosphatase levels of tissue culture cells are relatively constant. It has been suggested (17) that acid phosphatase is a constitutive enzyme and alkaline phosphatase is adaptive. Our experimental results with established cell lines in culture tend to confirm this idea since acid phosphatase concentration varies only slightly between different cell lines, while alkaline phosphatase displays marked differences and, moreover, its concentration in cells is very susceptible to various environmental influences.

Alkaline phosphatase is not excreted into the culture medium but appears to be closely associated with cell particles. On mechanically disrupting the cells, only a small proportion of the alkaline phosphatase is extracted. Deoxycholate lysis of the cell renders soluble a much larger fraction of the enzyme activity. On the other hand the acid phosphatase is effectively liberated by mechanical disruption of the cells. The alkaline and acid phosphatase activity of tissue culture cells is not dependent on the intrinsic phosphatase activity of the serum in which they are grown.

The occurrence of cytoplasmic alkaline phosphatase in a variety of tissue culture cell lines derived from different tissues is somewhat surprising since the enzyme has limited distribution in adult organs (18, 67). No direct correlation is evident between cell lines containing high levels of alkaline phosphatase and the enzymatic activity of the original tissue. The degree of differentiation has been shown to be related to the alkaline phosphatase content of developing embryos (17, 68). In early stages of chicken and mouse embryogenesis all cells are alkaline phosphatase–positive. Enzyme content decreases in most tissues to undetectable levels during the intermediate stages of development. As the final events in differentiation occur alkaline phosphatase rises to high levels in those organs which characteristically contain the enzyme in postembryonic life. Substrate and hormone "inducers" of alkaline phosphatase in embryos can be effective only after certain critical stages in differentiation have occurred (17). Further evidence for the importance of "cell modulation" in determining alkaline phosphatase content of cells is provided by studies on wound healing and on tumors. During the early stages of wound healing cells and collagen fibers are transiently alkaline phosphatase–positive (69). Tumors derived from certain tissues which do not contain cytoplasmic alkaline phosphatase may have high levels of this enzyme (70, 71).

Histologic localization of alkaline phosphatase in a HeLa cell culture has been reported previously by Gropp (72) and our results are in agreement. In neither study, however, were diffusion artifacts evaluated. Therefore, the significance of alkaline phosphatase–positive cytoplasmic granules and

alkaline phosphatase–positive material on the cell surface cannot be assessed.

Recently, variations in alkaline phosphatase activity between several epithelial cell lines and among clones derived from certain cell strains have been briefly reported (73, 74). Our results agree with these findings.

Cell lines with very low levels of alkaline phosphatase, or those in which the enzyme cannot be demonstrated grow as vigorously as positive cells. Therefore, it would appear that cytoplasmic alkaline phosphatase is not directly concerned with cell multiplication; moreover, the presence of high concentrations of alkaline phosphatase in certain epithelial lines does not appear to interfere with the vital metabolic processes which are dependent on a variety of phosphate esters.

Alkaline phosphatase synthesis in *E. coli* is subject to regulation by a specific repressor, orthophosphate, and alkaline phosphatase is not formed until the inorganic phosphate concentration is limiting (75). On the other hand, the alkaline phosphatase of epithelial cells in tissue culture does not appear to be influenced by inorganic phosphate concentration either in cells in which alkaline phosphatase is usually present or in lines that are prednisolone-inducible. It is not surprising that mammalian cell alkaline phosphatase is independent of inorganic phosphate concentration. Phosphate is a normal environmental constituent of mammalian cells and alkaline phosphatase regulation might be expected to require mechanisms other than limiting inorganic phosphate.

Further evidence that alkaline phosphatase is an "adaptive" enzyme in tissue culture cells is provided by experiments in which cell lines were held at room temperature. Following "injury" by incubation at room temperature for several days renal cells displayed signs of degeneration, and alkaline phosphatase content was greatly decreased. Upon reincubation at 37°C, multiplication rate and cell morphology returned to normal prior to the restoration of normal alkaline phosphatase activity. Similar results have been reported (76) in rats with sublethal uranium poisoning. Recently regenerated renal proximal tubule cells have been found histochemically to be alkaline phosphatase–negative.

Prednisolone in concentration of 0.5 μg per ml has pronounced activity on certain cell lines in tissue cultures while certain other cell lines are not affected. In the past, conflicting results have been recorded following treatment with hydrocortisone and its analogues (12–16, 77, 78). In general, inhibition of multiplication (13–16), depressed migration of cells away from explants (14, 15), and enhanced aerobic glycolysis (77) have been reported with concentrations of cortisone ranging from 500 to 10 μg per ml (15, 16). In one study prednisolone (meticortelone), 12.5 μg per ml, inhibited L cell multiplication, but 5 μg per ml was ineffective (76). The effectiveness of prednisolone phosphate and prednisolone-21-hemisuccinate *in vitro* in

doses as low as 0.5 μg per ml may be attributable to their water solubility. This concentration of prednisolone is equivalent to 2.0 to 2.5 μg per ml of cortisone.

In previous reports on the action of adrenal steroids upon cultured cells, the results observed *in vitro* have occurred in the presence of high concentrations of hormone (15, 16, 77, 78). The significance of these results as related to those observed in intact animals has been questioned because of the very high concentrations of hormone used (77). In the present experiments the results reported have been obtained with concentrations of steroid easily achieved therapeutically.

The tissue culture lines employed in these studies, which were derived from different species and tissues, did not respond to prednisolone in a uniform manner. Human epithelial cell lines exhibit reduced multiplication with increased cytoplasmic growth and an increase in mitotic index. Fibrocytic mouse cell lines (strains L and MCN) showed a similar reduction in multiplication when grown in medium containing prednisolone but there was no alteration in average cell size. The mitotic index of "fibrocyte" mouse cells may be slightly decreased. Human fibroblast strains show no reduction in multiplication, no increase in cytoplasmic growth, nor alteration in alkaline phosphatase when grown in medium containing either 0.5 μg per ml or 50.0 μg per ml prednisolone.

In a recent review, Bass refers briefly to unpublished observations on a cortisone-treated HeLa cell line (78). The cells became larger in size and showed an increase of protein and RNA and a slight increase of DNA. The mitotic index in the cortisone-treated HeLa line was slightly increased with a disproportionate number of dividing cells in metaphase. Our observations confirm those of Bass and extend them to other epithelial human cell lines. However, our findings in mouse fibroblasts (strains L and MCN) and in human fibroblasts provide evidence that the changes are not the same in all established cell lines. Conflicting results of previous reports may be explained in part by the different action of adrenal corticoids on different tissues and cells (12–16) as well as by the nature of the hormone used, especially its solubility and possibly its rate of inactivation.

Certain of the observations made with cultured mammalian cells correlate with findings obtained *in vivo*. The reduced multiplication of prednisolone-treated L cells resembles the decreased cell growth reported in ear epithelium of rats and mice (79, 80) treated with cortisone. Increased cytoplasm of established human epithelial tissue cultures grown in medium with prednisolone is similar to the enlargement of liver cells described in cortisone-treated rats (81) and rabbits (82). Following partial hepatectomy in rats, the observation that restoration of liver weight was not affected by cortisone but that cell multiplication was significantly reduced may also be associated

with increased cell size (81). Increased total nitrogen content has been reported in intestinal cells of chick embryos injected with cortisone (17). That adrenal steroids may have a different effect on various tissues has also been reported in man and in experimental animals. The myeloid cells of the bone marrow proliferate with cortisone administration (83, 84), whereas reticulum cells, fibrocytes, and certain malignant cells undergo atrophy and degeneration (85, 86).

Our data on the effects of prednisolone on mitosis of human epithelial cell lines are not sufficiently clear-cut to permit firm conclusions to be drawn. Cells grown in medium containing prednisolone for over 60 days continued to multiply at rates approximately one-half those of control cultures and showed an increase in mitotic index. Multinuclear cells appeared to be present in prednisolone-treated cultures in slightly increased numbers but mitotic figures did not show an increase in ploidy when compared to those of the controls. The reversibility of the prednisolone effect within several days after its withdrawal and similarity in generation time of cultures treated with prednisolone for short or long intervals, suggest that prednisolone does not act in a manner similar to colchicine.

Different human epithelial cell lines, when analyzed for differences in alkaline and acid phosphatases and for changes in enzyme activity induced by growing in medium with added prednisolone, behave in dissimilar but characteristic manner. Cell lines containing high concentrations of alkaline phosphatase generally show a definite increase in enzyme content per cell. However, the human renal cell which is alkaline phosphatase–positive, does not show increased enzyme content when activity is expressed on the basis of cell nitrogen. On the other hand, the positive intestinal epithelial line and HeLa MBA line show elevation in alkaline phosphatase activity, based on cell nitrogen, when they are grown in medium containing prednisolone. It is possible that the differences in alkaline phosphatase activity of human cell lines treated with prednisolone may be related to the observation that high levels of alkaline phosphatase develop in the intestines of embryos injected with adrenal corticoids whereas alkaline phosphatase activity of embryonic liver or kidney tissue does not increase upon cortisone treatment (17). In this respect, it is of interest that mature rats subjected to total adrenalectomy show a decrease in intestinal and kidney alkaline phosphatase (87). *In vivo* human leucocytes exhibit increased alkaline phosphatase activity when patients are treated with adrenocorticotrophic or adrenal cortical hormones (20).

The variable response of alkaline phosphatase in different tissues *in vivo* is paralleled by our experience with established tissue culture lines. This may imply that differentiation to a variable degree persists in cells grown continuously *in vitro* for several years. Such findings may encourage further

study of the adaptations that occur when cells become "altered" and capable of continuous growth *in vitro*.

The demonstration of prednisolone induction of alkaline phosphatase in two negative HeLa lines, strains NIH and Henle, is of considerable interest. When HeLa NIH and Henle are grown in medium without prednisolone their alkaline phosphatase activity is below the sensitivity of the methods used for its measurement even though lysates containing 4 to 6 million cells per ml were employed in the tests. However, when these HeLa strains are grown in medium containing 0.5 $\mu$g per ml prednisolone significant concentrations of alkaline phosphatase are demonstrable. The mechanism responsible for prednisolone induction of alkaline phosphatase in HeLa NIH and Henle is not known. However, unlike bacterial alkaline phosphatase (75) the induction of the mammalian cell enzyme appears to be independent of phosphate repression. Prednisolone-induced alkaline phosphatase in the absence of the hormone inducer is diluted out by cell multiplication. The disappearance of alkaline phosphatase during deadaptation is similar to that described for induced bacterial enzymes (88) and for induced HeLa cell glutamyl transferase (89).

Most human skin fibroblast strains do not form alkaline phosphatase when grown as described in the present paper even though prednisolone is added to the culture medium. Most of these strains, however, can be induced to synthesize substantial amounts of alkaline phosphatase when grown in the presence of phenyl phosphate, a putative substrate of the enzyme (90). In tissue culture the factors regulating induction of alkaline phosphatase synthesis differ between the human skin fibroblasts and the human epithelial cell cultures. Most of the former are induced by substrate but not by prednisolone, while most of the human epithelial cell cultures are induced by the hormone but not by substrate. The mouse fibroblast (L cell) is not induced to form alkaline phosphatase by either prednisolone or phenyl phosphate.

The reduced acid production of epithelial cell and L cell tissue cultures treated with prednisolone may reflect enhanced aerobic glycolysis with decreased lactic acid production as has been previously reported (77). Several human fibroblast strains showed no significant diminution of acid production when grown in medium with prednisolone.

The delay in the appearance of cytolysis by poliovirus in cultures of HeLa Henle cells maintained in prednisolone-containing medium may be similar to the inhibition of replication of influenza virus and prolongation of survival time described in cortisone-injected chicken embryos 10 to 11 days old (91).

The data on the HeLa lines indicate that the two prednisolone-inducible lines, HeLa Henle and NIH, can be separated from one another. HeLa

NIH can be distinguished with confidence from HeLa Henle because of its increased resistance to the cytopathic effects of all three types of poliomyelitis virus. In addition, the tendency of HeLa Henle cultures to grow in whorls and to be more spindle-shaped than HeLa NIH provides a morphologic difference between these negative cell lines. The chromosomal numbers and morphology are very similar in the three HeLa lines studied. The hypotetraploid number, the metacentric character of most of the chromosomes, and the morphology of the acrocentric chromosomes suggest their human origin.

The reason for low or undetectable amounts of alkaline phosphatase in the negative cell lines is not understood. No inhibitor of alkaline phosphatase in these cell lines could be detected in mixtures of cell lysates possessing alkaline phosphatase activity and cell lysates from the three negative cell lines. HeLa Henle and HeLa NIH cells when grown in the presence of prednisolone acquire the capacity to synthesize alkaline phosphatase. Whether prednisolone acts on alkaline phosphatase repressors or modifies a nonfunctioning enzyme so as to produce small quantities of active enzyme is not clear from our investigations. Evidence for the presence of alkaline phosphatase or an immunologically similar protein in HeLa Henle cells is furnished by experiments on inhibition of soluble alkaline phosphatase by antiserum prepared against the negative HeLa Henle line. The inhibition of enzyme activity equaled that observed with antiserum prepared against the markedly positive renal line, which suggests that a protein immunologically similar to alkaline phosphatase is present in the negative cell line.

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