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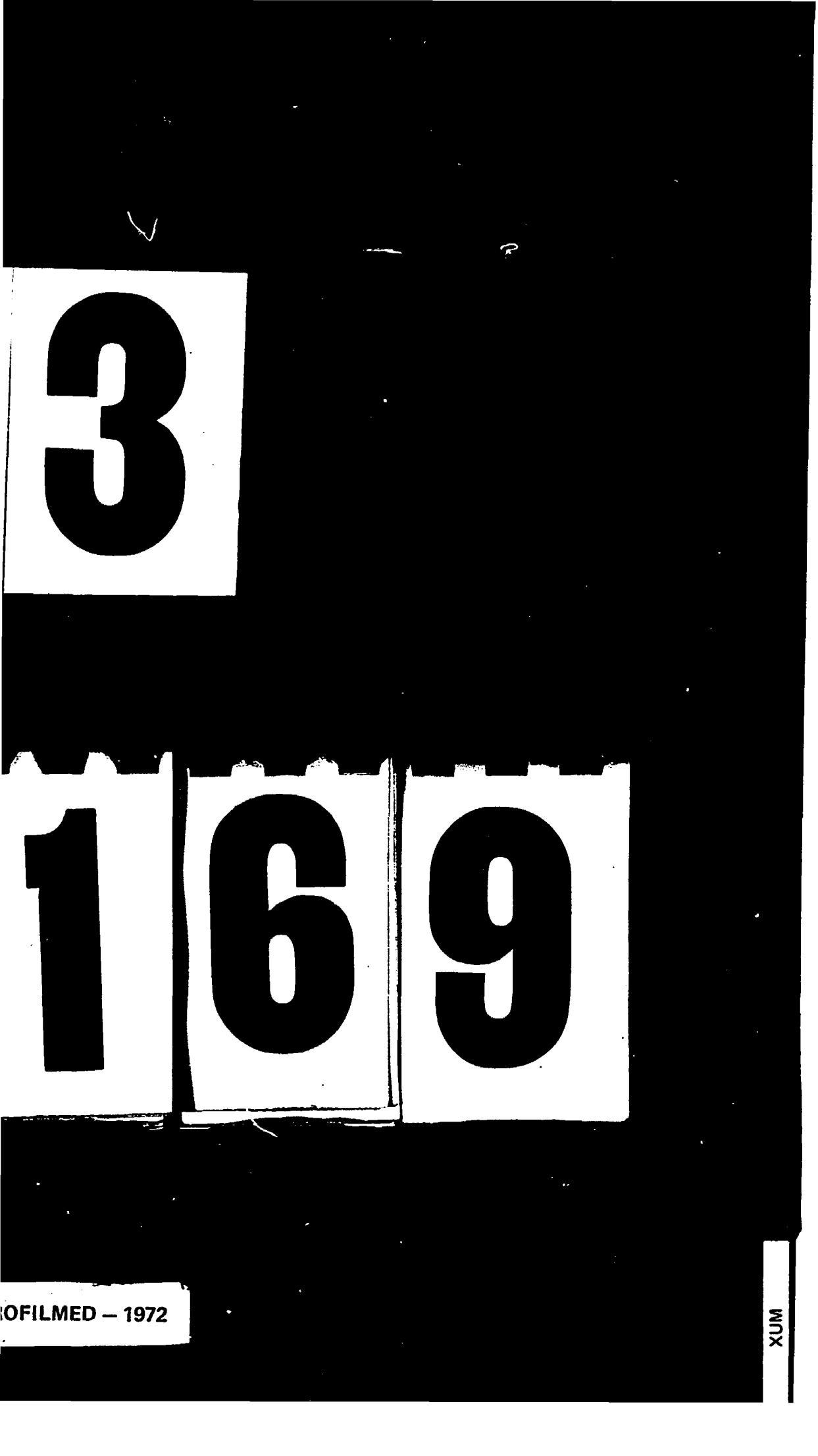

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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

STUDIES ON THE MECHANISM OF INDUCTION OF ALKALINE PHOSPHATASE IN HELA CELLS

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

GARY HOUSTON PRICE

Oklahoma City, Oklahoma

1972

STUDIES ON THE MECHANISM OF INDUCTION OF

ALKALINE PHOSPHATASE IN HELA CELLS

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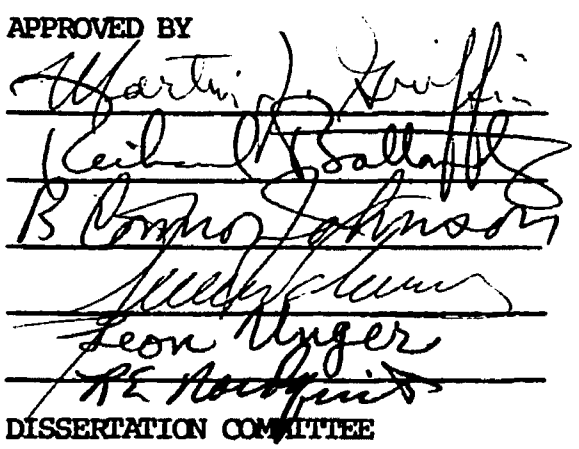

DISSERTATION COMMITTEE

ACKNOWLEDGMENTS

With deep and heartfelt gratitude and appreciation I would like to acknowledge the guidance, encouragement and assistance of all those who have been instrumental in the conduct of this course of study and these investigations.

A special thanks is due Dr. Martin J. Griffin for his patience and understanding as well as for the stimulation provided by many hours of fruitful discussion and for his genuine friendship.

I am also in the debt of Dr. Leon Unger for his wise counsel in selection of an area of research and encouragement when it was most needed, for exchange of ideas and for service on the reading committee. Appreciation is also extended to Dr. Genaro M. A. Palmieri for his interest in the research and stimulating discussion as well as for the kind donation of polypeptide hormones.

To the Department of Biochemistry and Molecular Biology and to the other members of the reading committee, Dr. B. Connor Johnson, Dr. Richard H. Bottomley and Dr. Robert E. Nordquist, I am also indebted.

Special recognition and appreciation goes to Mrs. Kate Sutter for the consistent excellence of glassware preparation and maintenance.

I am also grateful to the United States Army and the Office of The Surgeon General for supporting this work and providing the opportunity to complete my formal training.

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CHAPTER I

INTRODUCTION

Alkaline phosphatases (International Enzyme Classification 3:1:3:1) (1) are enzymes found in a wide variety of tissues and are characterized by the ability to hydrolyze phosphomonoesters in alkaline medium. The designation is somewhat arbitrary, considered within the context of molecular biology, there being no evidence to support localized intracellular alkalinity of an order of the reported pH optima; and, even though no physiological role has been proposed which can be unequivocally demonstrated, alkaline phosphatases have been widely investigated as a result of their usefulness as diagnostic parameters in clinical pathology. Mammalian alkaline phosphatases have been shown to be glycoproteins, containing hexosamines, fucose, mannose, glucose, galactose (2) and sialic acid, which is susceptible to neuraminidase (3), resulting in changes in electrophoretic mobility (4). There is evidence to suggest that there are two identical subunits, and that the active form of the enzyme is a dimer whose molecular weight has been determined to be, by gel filtration, 120,000-130,000, from human placenta or kidney (5); by ultracentrifugation, 116,000, with a monomer of 58,000, from human

placenta (6); by sucrose density centrifugation, 100,000, and by Sephadex G-200 chromatography, 150,000, from HeLa cells (7); and a more highly polymerized form from human placenta of molecular weight 240,000 (8). An alkaline phosphatase of molecular weight 550,000 has been isolated from human chronic granulocytic leukemic leukocytes, another of molecular weight 410,000 from normal human leukocytes and one from leukocytes taken from patients with reactive granulocytosis which has a molecular weight of 470,000 (9). Tightly bound zinc has been shown to be an integral part of the structure of the alkaline phosphatase from swine kidney (10) and its removal by ethylenediaminetetraacetic acid (EDTA) results in loss of activity which can be restored by addition of several combinations of divalent cations, zinc and magnesium, zinc and cobalt, magnesium and cobalt or cobalt alone, to preparations of enzyme from rabbit small intestine (11). Zinc at 4×10^{-4} molar has been shown to strongly inhibit the monoesterase activity of HeLa cell alkaline phosphatase (12) as well as the pyrophosphatase activity which has been found associated with human intestinal alkaline phosphatase (13). Magnesium stimulates the phosphomonoesterase activity of human intestinal alkaline phosphatase (14) but inhibits the pyrophosphatase activity of HeLa cells (7). Higher concentrations of magnesium also inhibit the monoesterase activity of the enzyme from human intestine (15). Magnesium is a more effective activator of rat intestinal alkaline phosphatase than of human (14). Enzyme purified from bovine synovial fluid has no adenosine triphosphatase (ATPase) or pyrophosphatase activity and is activated by millimolar magnesium, and by zinc, strontium and calcium at 10^{-5} molar (16). The phosphomonoesterase activity of urinary alkaline phosphatase is also inhibited by inorganic

phosphate (17). Phosphate has been found to form a covalent ester bond with a hydroxyl group of a serine residue of bovine liver alkaline phosphatase; by radioisotope incorporation studies, this phosphate ester has been shown to be rapidly and reversibly formed at pH 8 and is presumed to be an important factor in the active site (18). The amount of phosphate was calculated to be about one half mole per mole of enzyme. The amino acid composition of human placental alkaline phosphatase includes an abundance of the hydroxyl amino acids, serine and threonine; Ghosh reports 20 moles and threonine and 23 moles of serine residues per 70,000 grams of protein (2).

Several physical and kinetic properties of alkaline phosphatase have been investigated in attempts to distinguish enzyme from different tissues. Alkaline phosphatase in the mouse is localized mainly is the plasma membrane (19), and when cells are lysed the enzyme eventually finds its way into the blood where its stability permits it to remain for some time. A clinician can base a diagnosis of lesion to a particular tissue to a greater extent if he can first detect an increase in total serum alkaline phosphatase and then identify the tissue of origin. One property which has been so employed is the inhibition of some species of alkaline phosphatase by L-phenylalanine. The enzymes from human intestine (14), placenta (20), normal and chronic granulocytic leukemic leukocytes (9), a primary bile duct tumor (21), pleural fluid from pleuritis carcinomatosa (22), a disseminated bronchogenic carcinoma (23), and HeLa cells (24) have been shown to be L-phenylalanine sensitive; preparations from bone (25), liver, kidney, lung or spleen are not (13). The inhibition is an uncompetitive, coupling type which involves a homosteric interaction

with a lysine residue and a high degree of stereospecificity, as D-phenylalanine does not inhibit any of the alkaline phosphatases (14). The mechanism of the inhibition is reported to be the prevention of breakdown of a phosphoryl-enzyme intermediate (26). The other essential aromatic amino acid, L-tryptophan, also inhibits human placental and HeLa alkaline phosphatase, but D-tryptophan does not (25). Cysteine has also been found to inhibit alkaline phosphatase in HeLa cells, probably by forming a chelate with zinc in the active site; this inhibition is reversible by zinc but not by calcium (27). Placental and HeLa alkaline phosphatases are also inhibited by DL-histidine (25). L-leucine inhibits placental enzyme fifty per cent at ten millimolar, and inhibits pleuritis carcinomatosa enzyme (Nagao isoenzyme) sixty per cent at one millimolar (22).

A physical characteristic which may prove of great value in diagnosis of neoplasia is the stability to heating at 56^{O} Centigrade for 30 minutes which has been observed in HeLa (24), placenta (28), Regan isoenzyme (29), Nagao isoenzyme from pleuritis carcinomatosa (22) and primary bile duct tumor (21) alkaline phosphatase. Enzyme from liver, bone (30), Chang liver (12) and leukocytes (9) loses most or all of its activity under similar conditions. Normal human intestinal alkaline phosphatase loses no activity on heating at 56^{O} C for one hour (11).

Electrophoretic mobility on starch gel electrophoresis has also been widely investigated as a means to identify different types of alkaline phosphatase (31), and attempts have been made to definitively correlate mobility with tissue of origin. Treatment with neuraminidase removes terminal sialic acids from alkaline phosphatases from placenta (32), liver (4), kidney (33), HeLa cells (34) and Regan isoenzyme (29) with a

resultant loss in electrophoretic mobility, but the alkaline phosphatase of human intestine is resistant to neuraminidase (3); it may be that the structure of intestinal alkaline phosphatase makes the sialic acid inaccessible to the neuraminidase, as the electrophoretic mobility is altered somewhat on storage at 4^{o} C for four months (35). This is probably due to slow degradation of sialic acid. This study includes a treatment of HeLa 65 alkaline phosphatase with Vibrio cholerae neuraminidase followed by polyacrylamide disc gel electrophoresis, rather than the starch gel electrophoresis used in previous studies. The investigators cited above find no change in enzyme activity after neuraminidase treatment; however, treatment of a preparation from isolated rat liver plasma membranes did result in loss of activity (36).

Several studies have suggested that there are three immunological groups of alkaline phosphatases, the first including bone, liver, spleen and kidney (3), a second which includes placenta, HeLa cells (37), Regan isoenzyme (22) and a primary bile duct tumor (21) and the third, intestine (3).

Optimum pH values have been reported for rat small intestine alkaline phosphatate to be 9.8, using phenylphosphate as substrate, and 8.8, using beta-glycerophosphate as substrate (38); bovine synovial fluid alkaline phosphatase, pH 10.0 at five millimolar para-nitrophenylphosphate and 9.3 at 0.05 millimolar para-nitrophenylphosphate (p-NPP) (16); human placenta, sodium phenylphosphate, pH 10.6 (40); using sodium phenylphosphate as a substrate, chicken liver microsomes, pH 10.0 (39); human kidney, sodium phenylphosphate, pH 10.6 (40); HeLa 71 cells, para-nitrophenylphosphate, pH 10.0 (7); HeLa E cells, sodium phenylphosphate, pH 10.4

(41). Optimum pH is clearly dependent to some extent on the nature and concentration of substrate, the source of enzyme and the buffer system.

Among the substrates used to measure or demonstrate phosphomonoesterase activity are beta-glycerophosphate, para-nitrophenylphosphate, phenyl phosphate, indoxyl phosphate, beta-naphthyl phosphate, phenolphthalein phosphate, phenolphthalein diphosphate and methylumbelliferyl phosphate (42). Cox and Griffin demonstrated the ability of HeLa cell alkaline phosphatase to catalyze the hydrolysis of inorganic pyrophosphate at pH 8.5. Several other alkaline phosphatases from human leukocytes, kidney, intestine, rat bone and calf intestine also display pyrophosphatase activity at pH 8.5, but only HeLa enzyme has more activity as a pyrophosphatase than a monoesterase at that pH. It has been suggested on the basis of these findings that the natural physiological role might be as a pyrophosphatase, and certainly the pH optimum falls within a more plausible physiological region (43). Orthophosphate and pyrophosphate each act as a competitive inhibitor of the other's hydrolysis by human intestinal alkaline phosphatase (15). Identity of the protein in HeLa cells possessing the two activities has been established by immunological assay, concomitant purification, cysteine inhibition and response to hydrocortisone (7). Moss and Eaton (4) have reported that alkaline phosphatases readily hydrolyze adenosine mono-, di- and triphosphates. ATPase activity dependent on calcium has been demonstrated in chick intestinal brush border cells (44); magnesium and potassium-magnesium dependent ATPases have been reported to occur in rat liver plasma membranes (36).

The intracellular localization of alkaline phosphatase has

been reported to be mostly particulate in human liver, e.g., in nuclei, mitochondria and lysosomes, and largely a soluble constituent of intestinal mucosa (45); in the plasma membranes of rat liver (46) and HeLa cells (47).

Gey, Coffman and Kubichek (48) first cultured the human epithelial cell line, HeLa, from tissue taken from a cervical adenocarcinoma. HeLa cells are aneuploid in the original clone and have been subcultured over the years in clones characterized by high or low alkaline phosphatase activity, virus susceptibility, optimum growth in selecting medium containing sera from different animal sources and varying in nutrient content. As long as tissue culture conditions remain constant, virus and mycoplasma infections are avoided, uniform medium employed and no selection by radiation or chemical stress occurs, the clones are reasonably stable over protracted periods of time. The cell lines can be partially characterized by major modal chromosome number and karyotype. The cell line employed in this study is designated HeLa 65, as the majority of the cells from this clone bear 65 chromosomes (49).

One of the chief problems inherent in the study of mammalian genetics at the cellular level is the paucity of marker enzymes which can be followed in culture and used to distinguish somatic chromosome differences. Cells derived from specialized tissue in vivo tend to become generalized or "dedifferentiated" until they develop common metabolic traits, nutritional requirements, morphology and enzyme composition. HeLa cells provide some advantages in that they can be continuously cultured indefinitely with little, if any, change in several readily measurable parameters under relatively well-defined growth conditions.

One of the best studied of these parameters is the total level and specific activity of alkaline phosphatase, which, in cell lines of low alkaline phosphatase activity, can be frequently increased markedly (50) by addition of prednisolone (40), hydrocortisone, dexamethasone or other glucocorticoid analogs (51). This "induction" does not occur by the same mechanism as the classic system of Jacob and Monod (52), as there is not an increase in total alkaline phosphatase protein, as determined by immune precipitation techniques (47), although new protein synthesis is required. Actinomycin D and puromycin block the induction (53). These data imply that either a new protein species of alkaline phosphatase is synthesized by a gene switching mechanism, or that some new factor, which is instrumental in a structural modification of the enzyme as it is newly synthesized, is being made or activated. The primary objective of the present study is to find some molecular distinction between the alkaline phosphatase produced in HeLa 65 in the control state (65C) and that in the hydrocortisone regulated state. Cells are considered to be in the hydrocortisone regulated (65H) state after continuous culture with three micromolar hydrocortisone for six passes. An intermediate "induced" state (65I) in which 65C cells are grown with hydrocortisone for 72 hours is also used to study the transition from 65C to 65H. The increase in alkaline phosphatase activity in HeLa 65 on initial subculture with hydrocortisone is characterized by a twelve hour lag period (54) after which the specific activity rises linearly for about 36 hours. The increase in activity is initiated exclusively in the synthetic phase of the cell cycle. By 72 hours after addition of hydrocortisone, the enzyme level is fully induced and will remain at a constant level as long as

hydrocortisone is maintained in the medium (54). If hydrocortisone is omitted from the medium for several passes, the activity returns to the original control level (40). Addition of retinoic acid to the medium at the time of initial treatment with hydrocortisone blocks the induction of alkaline phosphatase (55) as does D-glucosamine (56). However, glucosamine has been reported to exert a powerful cytotoxic effect on various ascites tumor lines, resulting in decreased viability and transplantability; it also inhibits cell-free ribonucleic and deoxyribonucleic acid synthesis 70 to 85 per cent in 30 minute incubations (57), and de novo transcription and translation has been established as a requisite of the induction. Cysteine added to the growth medium also blocks the hydrocortisone induction (40). In a bacterial system, it was found that growth of Escherichia coli in phosphate deficient medium led to a marked increase in alkaline phosphatase activity (58), but efforts by other investigators (59) and this study to demonstrate the same phenomenon in a mammalian cell culture system failed to show either an increase or decrease in activity by changes in phosphate levels in the growth medium. In broiler chicks, dietary electrolytes have been found to influence the activity of intestinal alkaline phosphatase; high levels of calcium and magnesium repress activity, while phosphate deficiency greatly stimulates it (60). Vitamin D induces alkaline phosphatase as well as calcium dependent ATPase activity in chick intestinal brush border cells (44) and Rasmussen has in fact presented substantial evidence to suggest that both activities are functions of the same protein. HeLa cells grown in suspension culture medium in which calcium concentration is about ten per cent and phosphate concentration ten fold that of monolayer medium have

much lower levels of alkaline phosphatase activity (24). An increase in calcium levels in suspension culture medium leads to a decrease in intracellular sodium and an increase in potassium in HeLa cells (61). Dibutyryl cyclic 3',5'-adenosine monophosphate (DB c-AMP) has been shown to induce alkaline phosphatase activity in a hybrid cell line between mouse and Chinese hamster, but this line does not respond to glucocorticoids (62). Hydrocortisone has also been found to induce tyrosine-alpha-keto-glutarate transaminase in rat hepatoma ascites tumor cells in culture, reaching full level of activity within six hours of addition of hydrocortisone to the medium and requiring rapid de novo protein synthesis, resulting in a net increase in enzyme protein (63). Glucagon, insulin and benzoate also induce tyrosine aminotransferase (64). The induction is blocked by actinomycin D, cycloheximide, puromycin, progesterone and chloramphenicol. The same enzyme is induced five to eight fold in rat hepatoma cells by D-glucosamine, with a concomitant marked reduction in protein and DNA synthesis over a 72 hour period (65). In normal rats, hydrocortisone or tryptophan injected intraperitoneally induces tyrosine aminotransferase in liver homogenates ten fold (66). Adrenocorticotropic hormone (ACTH) injected intramuscularly into patients with chronic myelocytic leukemia, in which leukocyte alkaline phosphatase activity is low, leads to a marked increase in that activity (67). Rat atrial alkaline phosphatase activity is rapidly induced by subcutaneous injection of isoprenaline into the rat (68). The rise in activity can be suppressed by pretreatment of the rats with cycloheximide or actinomycin D. Kaumann (69) has reported potentiation of the effects of isoprenaline and noradrenaline by hydrocortisone in cat heart muscle, and

Clausen and Fomby (70) have demonstrated the stimulation of rat brain synaptic alkaline phosphatase activity by noradrenaline. Progesterone induces rabbit leukocyte alkaline phosphatase (71). A liver enzyme, tryptophan pyrrolase, can be induced in vivo by hydrocortisone or by tryptophan, although there are evidently two separate mechanisms of induction; the inductions are additive, but the hydrocortisone induction is not associated with an increased level of free tryptophan or excretion of tryptophan metabolites by pyridoxine deficient rats. The tryptophan induction does increase the concentration of free tryptophan in liver and increase metabolite excretion. It is notable that the hydrocortisone type induction in rat liver can be stimulated by adrenaline as well (72). Increases in osmolarity by addition of 42 millimolar sodium chloride or 82 millimolar sucrose have been reported to induce an increase in heat stable alkaline phosphatase activity in HeLa 65 (S_3) cells. This induction can be blocked by puromycin (73).

Since HeLa 65 cells show a marked increase in alkaline phosphatase activity after a period of growth in medium containing hydrocortisone, and immunologic techniques have shown that there is no more enzyme protein per cell in 65H than 65C, it has been concluded that the intrinsic specific activity of the enzyme is increased by a chemical modification or configurational change which improves the catalytic efficiency. The amount of zinc in both 65C and 65H alkaline phosphatase has been measured and found to be not significantly different. However, there is apparently a difference in the tightness of the zinc binding to the active site or in the accessibility of the zinc to EDTA, as the initial rates of inactivation of the control enzyme by EDTA are markedly higher

than the hydrocortisone regulated enzyme initial rate of inactivation (47). The electrophoretic mobilities of both types of enzyme are the same both on polyacrylamide disc gel and starch gel electrophoresis. Change in electrophoretic mobility on starch gel after neuraminidase treatment is also the same for both 65C and 65H, indicating the same number of neuraminidase-accessible terminal sialic acid moieties (47). Kinetic studies indicate that both 65C and 65H alkaline phosphatase have the same K_m value (47).

The regulation of enzyme intrinsic specific activity by chemical modification has been reviewed by Holzer and Duntze (74). Glutamine synthetase is converted from the active to the inactive form by cleavage of an adenyl residue; an adenylating enzyme catalyzes the inactivation by re-adenylating the active form. Glycogen phosphorylase and its regulatory enzyme, phosphorylase b kinase, are both activated by phosphorylation of serine residues. Fructose 1,6-diphosphatase and pyruvate dehydrogenase are both inactivated by phosphorylation, and activated by dephosphorylation (74). Radioisotope studies have indicated that labeled adenosine is not incorporated into either 65C or 65H alkaline phosphatase (75).

The first specific objective of this study, then, was to purify both types of alkaline phosphatase to as high a degree of purity as possible and determine if one or both are phosphorylated, and, if so, whether there might be a difference in the degree of phosphorylation. Since D-glucosamine had been implicated in the regulatory process, the amount of hexosamines was determined. The number of free sulfhydryl groups on each variety was measured, since it has been reported that

hydrocortisone increases thiol content in rat liver nuclear proteins (76). Other drugs which have been shown to be related to alkaline phosphatase activity were tested for their ability to induce it in the HeLa system. Other hexosamines and acetylated hexosamines were also tested for their capability to either induce, block the hydrocortisone induction of, or reduce the control level of alkaline phosphatase.

A previous attempt to induce alkaline phosphatase in HeLa cells by addition of theophylline and dibutyryl cyclic 3',5'-AMP failed to demonstrate any change in alkaline phosphatase activity when the drugs were added to the growth medium after 48 hours of growth and the cells harvested four hours after addition of the drugs. In view of the lag period which has been observed in the hydrocortisone induction, it seemed possible that growth of the cells with theophylline and DB c-AMP under the same growth conditions as the hydrocortisone induction might have a different effect. Epinephrine was also used in the earlier study (77) and was tested in this study as well.

CHAPTER II

MATERIALS AND METHODS

Cell Cultures and Medium

Two HeLa cell clones of major modal chromosome numbers 65 and 71, designated HeLa 65 and HeLa 71 (49), were cultured in monolayer in Blake or milk dilution bottles or T-25 flasks (Falcon Plastics). The medium employed was Eagle's Minimum Essential Medium (78) (Auto Pow, Flow Laboratories, Inc., Rockville, Maryland) supplemented with ten per cent (v/v) calf serum (Colorado Serum Company, Denver Colorado), five millimolar glutamine, fifty units per milliliter penicillin, and fifty micrograms per milliliter each of streptomycin and kanamycin. Antibiotics were obtained from Grand Island Biological Company, Grand Island, New York. Prior to inoculation, the sterilized bottles were gassed with a mixture of five per cent carbon dioxide in air to maintain the pH of the medium at about 7.2. The calf serum was inactivated by heating to 56° C for thirty minutes before addition to the medium. To achieve the hydrocortisone regulated (Hcr) state, the cells were subcultured into growth medium containing one microgram per milliliter (three micromolar) hydrocortisone (Sigma Chemical Company, Saint Louis, Missouri) and allowed to grow to confluency. The cells were repeatedly subcultured into medium containing hydrocortisone for at least three weeks (six passages) to ensure the new steady state (Hcr). In those studies involving the induced (65I)

state, the hydrocortisone was added to control cells at the initiation of the experiments which were usually of 72 hours duration. Cell subculture and harvesting techniques have been described (79). Most of the experiments were harvested at a confluency of approximately 12-18 x 10^4 cells per square centimeter, representing an exponentially dividing population of cells in asynchronous growth (80).

Cell Counts

All cell counts were made on a Bright-Line hemocytometer from American Optical Instrument Company, Scientific Instrument Division, Buffalo, New York.

Separation of Glycoproteins

The cell monolayers from Blake bottles were harvested by washing twice with ten milliliter aliquots of saline-Tris (ST) buffer, 0.025 molar Tris hydrochloride in isotonic sodium chloride at pH 7.2. The cells were then scraped into a third ten milliliter aliquot of ST buffer, using a rubber policeman; after combining the cell suspensions, the cells were pelleted by centrifugation at 1000 revolutions per minute for fifteen minutes in an International PR-6 centrifuge equipped with a number 253 head. The cell pellets were resuspended in about one milliliter of ST buffer per ten million cells. The suspension was extracted for three minutes with n-butanol (one milliliter per milliliter of suspension) at maximum speed on a Vortex Genie mixer (Fisher Scientific Company, Pittsburgh, Pennsylvania). The phases were separated by centrifugation at 1700 revolutions per minute for fifteen minutes and the upper, lipid phase was

re-extracted with three volumes of ST buffer. The aqueous phases were combined and dialysed against three changes of four liters of two millimolar Tris, pH 7.5. An ultrafiltration apparatus using a collodion bag (Schleicher and Schuell, Keene, New Hampshire) was employed to reduce the volume until an approximate protein concentration of 20 milligrams per milliliter was reached. Filtration was carried out at a vacuum of fifteen pounds per square inch, using a Millipore pump (Millipore Corporation, Bedford, Massachusetts).

Whole Cell Lysates

Whole cell lysates were prepared by washing the cell monolayer three times with ST buffer, then adding two milliliters of 0.25 per cent sodium deoxycholate to the monolayer to lyse the cells. The flask necks were broken off to facilitate withdrawal, and a Pasteur pipette used to mix and withdraw the lysed monolayer.

Polyacrylamide Disc Gel Electrophoresis

Separation and purification of alkaline phosphatase from the concentrated butanol extracts was carried out by polyacrylamide disc gel electrophoresis as described by Davis (81) except that the hard gel composition was modified as follows: one part of Solution A (36.3 grams of Tris, 0.23 milliliter of N,N,N',N'-tetramethylethylenediamine, 48 milliliters of one normal hydrochloric acid, water added to total 100 milliliters) and one part of Solution C (28.0 grams of acrylamide, 0.735 grams of methylene-bisacrylamide, water to 100 milliliters) were added to two parts of 0.14 per cent ammonium persulfate. Earlier preparations utilized

a soft sample gel; however, the last two preparations were done by the alternative procedure in which a layer of forty per cent sucrose was overlaid with the hard gel. The spacer gel described by Davis was omitted from these preparations. Electrophoresis was carried out at a constant current of two milliamperes per gel, usually for a period of five hours. Alkaline phosphatase bands were localized by means of a histochemical stain composed of 100 milliliters of a 0.15 molar Tris buffer, pH 10.3, containing one millimolar magnesium chloride and 200 milligrams each of alpha-naphthyl phosphate and Fast Blue BB diazonium dye (Dajac Laboratories, The Borden Company, Philadelphia, Pennsylvania). Staining time averaged about fifteen minutes. In the preparative runs, twelve identical gels were made up, one of which was stained and the electrophoretic mobility of the alkaline phosphatase bands carefully measured. A five millimeter slice corresponding to the enzyme activity was excised from each of the other eleven gels; the discs were then drawn by suction into an empty glass tube of the same size used to form the gels, with solid contact between the discs and with all air bubbles excluded from the tube. The remaining pieces of the gels were histochemically stained to ensure the accuracy of the excision of the alkaline phosphatase band. The gel which had been stained to locate the alkaline phosphatase band was stained again with the Amido-Schwartz stain to visualize the remaining protein bands (82). A four to five inch piece of quarter-inch dialysis tubing was soaked in water and the end stretched over the glass tube containing the excised discs and secured with a rubber band. The tube was filled with 10X glycine-Tris buffer (three grams of Trizma base, 14.4 grams of glycine in one liter of water, final pH 8.3) and tied so as to include about one

milliliter of fluid below the gels. The tube was replaced in the Canalco Model 6 Disc Gel Electrophoresis apparatus in which the anode was connected to the lower reservoir which was filled with 10X glycine-Tris buffer and contained the collecting bag. To compensate for the additional length added to the glass tube by the dialysis tubing, the upper reservoir was elevated by placing four number five rubber stoppers at equidistant points around the circumference, between the upper and lower chambers. Electrodialysis was performed for twenty hours at two milliamperes per gel. The electrodialysates were dialyzed exhaustively against distilled water prior to further analysis.

Alkaline Phosphatase Assay

Alkaline phosphatase activity was measured by incubating up to 0.2 milliliter of sample with 0.4 milliliter of 8 millimolar para-nitrophenyl phosphate (p-NPP, Sigma) in 0.25 molar Tris, pH 10.0, stopping the reaction by addition of 1.0 milliliter of 0.25 normal sodium hydroxide and measuring the absorbance of the liberated para-nitrophenylate ion at 410 nanometers on a Beckman DUR spectrophotometer modified with a Gilford cuvette positioner, power source and optical density converter (83). Since the total assay volume and pH was such that the absorbance at 410 nanometers equals ten times the number of micromoles of para-nitrophenol in solution, the enzyme activity was calculated by the following formula:

$$A_{410} \times 100 \div \text{incubation time in minutes} = \text{nanomoles/minute}$$

One milliunit is defined as one nanomole of para-nitrophenylate ion formed per minute. Aliquots were chosen which would yield an absorbance of 0.050 to 1.0 within ten to sixty minutes of incubation at 37° C.

Protein Assay

Protein was estimated by a modification of the method of Lowry, et al., (84) using fetuin as a standard. Two milliliters of reagent 1 (freshly made prior to assay by combining one milliliter of one per cent copper sulfate with one milliliter of two per cent sodium tartrate and 98 milliliters of two per cent sodium carbonate in 0.1 normal sodium hydroxide) was added to each sample (up to 0.2 milliliter containing up to 100 micrograms), vortex mixed and incubated at 20° C for exactly fifteen minutes. Reagent 2 (Folin-Ciocalteau phenol reagent, Curtin Scientific Company, diluted 1:1 with water), 0.2 milliliter, was added, vortex mixed and incubated for exactly one hour. Absorbance at 690 nanometers was measured and compared with a standard curve ranging from ten to 100 micrograms.

Hexosamine Assay

Hexosamine was estimated by the method of Gatt and Berman (85).

Sulfhydryl Assay

Free sulfhydryl groups were measured by a micro scale modification of the method of Deakin, et al., (86), in which the reaction mixture, in order of addition, consisted of 0.2 milliliter of 0.05 molar phosphate buffer, pH 7.0, 25 microliters of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, Sigma), up to 0.2 milliliter of sample or standard and made up to a final volume of 0.525 milliliter by addition of water. The absorbance of the assay mixture at 412 nanometers was read immediately. Cleland's reagent, dithiothreitol (Pierce Chemical Co., Rockford, Ill.) was used for the standard curve which was linear from one to fifty nanomoles (87).

Neuraminidase Treatment of Alkaline Phosphatase

Aliquots of 150 microliters, each containing approximately 1.5 milligrams of protein, of the concentrated butanol extracts from 65C and 65H were incubated with 25 micrograms of Clostridium perfringens neuraminidase, Type V (Sigma) at 37° C for twenty hours. Duplicate samples were incubated without neuraminidase under identical conditions. After the incubation, aliquots were taken for protein and alkaline phosphatase assay and the remainder was layered in 0.25 milliliter of forty per cent sucrose directly on top of a polyacrylamide gel which had been prepared by the alternative method described above. The sucrose-sample layer was overlaid with a layer of glycine-Tris 10X buffer in forty per cent sucrose (1:1) and electrophoresis carried out at 4° C for five hours in the Canalco Model 6 apparatus. The gels were stained histochemically to localize the alkaline phosphatase bands.

Phosphate Assay

Phosphate assays were carried out in glassware which had been precleaned by boiling thirty minutes in 0.5 normal perchloric acid, followed by three rinses with distilled water, then three rinses of doubly glass distilled water. Samples to be analyzed for inorganic phosphate were exhaustively dialyzed against several changes of doubly distilled water, pyrolyzed at 180° C in seventy per cent perchloric acid until all organic material was completely ashed and the samples dry, then back hydrolyzed in 0.9 milliliter of water and 0.2 milliliter of seventy per cent perchloric acid for one hour at 100° C to hydrolyze any pyrophosphates which might have been formed during the pyrolysis. To the sample was

added 0.4 milliliter of 1.25 per cent ammonium molybdate, with mixing, followed by 0.4 milliliter of five per cent ascorbic acid, with mixing. The reaction mixture was heated at 100° C for seven minutes and the absorbance at 797.5 nanometers measured (88).

Intracellular Phosphate

Replicate cultures of 65C and 65H cells grown to $12 \pm 2 \times 10^4$ cells per square centimeter were washed twice with ST buffer and scraped into five per cent perchloric acid, the protein precipitate pelleted by centrifugation on a Model CL desk top centrifuge (International Equipment Company, Needham Heights, Massachusetts) and the supernatant assayed for inorganic phosphate. A replicate culture of each was trypsinized and the cells counted. The molar concentration of phosphate in each clone was calculated based on an average cell volume of 3.3×10^{-12} liters for 65C and 4.0×10^{-12} liters for 65H (89).

Preparation of Placental Alkaline Phosphatase

A fresh human placenta weighing approximately 1.5 pounds was perfused with ice cold ST buffer to remove as much blood as possible. The spongy tissue was excised and cut into small pieces with scissors. The total tissue volume of 275 milliliters was diluted to 900 milliliters with cold ST and homogenized in a Virtis Model 45 homogenizer for five minutes at about 75 per cent maximum speed. The homogenate was divided into four portions, each of which was extracted with 100 milliliters of n-butanol for five minutes in the Virtis. The extracts were placed into 300 milliliter glass centrifuge jars and centrifuged at 1000 rpm for

fifteen minutes in an International PR-2 centrifuge equipped with a number 533A head. The straw-colored aqueous phase was separated by siphon, using a Pasteur pipet and tygon tubing, from the gelatinous, brownish-red upper phase. The aqueous phase was dialyzed against three changes of 25 millimolar Tris, pH 7.5. It was then centrifuged for thirty minutes at 30,000 x g in a Sorvall RC-2B refrigerated centrifuge and the precipitated material discarded. The supernatant fluid was divided into two portions and lyophilized to dryness and stored at -20° C for further analysis. A portion of the residue weighing 43 milligrams was dissolved in doubly glass distilled water and dialyzed for 4.5 hours against 0.5 per cent LKB Ampholine carrier ampholytes, pH 3-10 (LKB Produkter, AB, Sweden).

Isoelectric Focusing

The isoelectric focusing column used was the LKB type 8102, capacity 440 milliliters, cooled to 4° C by a Neslab Instruments, Inc., (Portsmouth, New Hampshire) cooling system and the power supply was a Vokam Model 2541 from Consolidated Laboratories, Inc., Chicago Heights, Illinois. The column was prepared by the method of Li and Li (90). The gradient was prepared by mixing 100 grams of sucrose (Ultrapure, Schwarz-Mann, Orangeburg, New Jersey) with 150 milliliters of water and four milliliters of LKB 3-10 Ampholine solution, to make up the dense solution. The light solution consisted of the sample solution plus 2 milliliters of Ampholine, and diluted to a final volume of 210 milliliters. The dense solution was placed in the outlet side of the LKB gradient former, with the electric stirrer. After preparation of the column, a constant voltage of 400 volts DC was applied to the column at an initial current ranging

from 10-30 milliamperes, depending on the sample. After a period of 24 to 48 hours, the current dropped to a constant minimum value of 2 to 2.5 milliamperes and the isoelectric focusing was completed. The current was turned off, the center well closed and the contents of the column collected in five milliliter fractions on a Gilson fraction collector. A Corning Model 12 pH meter was used to determine the pH of the fractions. The fractions containing the alkaline phosphatase activity were placed in 5/8" dialysis tubing and dialyzed against 2 millimolar Tris·HCl, pH 7.5, prior to assay for protein and alkaline phosphatase activity.

Hexosamine and Drug Response in Culture

In order to test the response of HeLa alkaline phosphatase total and specific activity and cell growth (as measured by total protein) to a variety of hexosamines and drugs, HeLa cells were inoculated in four milliliter aliquots into T-25 flasks at an inoculum concentration of about one hundred thousand cells per milliliter. The compound to be tested was made up as a sterile stock solution either in water or in complete medium, in those cases where solubility was low and a water solution might introduce dilution effects into the experiment. Aliquots of the stock solutions were added to the growth medium at the time of inoculation, and the cultures were harvested as described above either 24, 48 or 72 hours later. Hexosamines tested were D-glucosamine, D-galactosamine, N-acetylglucosamine, N-acetylgalactosamine and N-acetylmannosamine. D-mannose was also tested. All hexoses used were obtained from Sigma Chemical Company, Saint Louis, Missouri. Inorganic phosphate was Baker Analyzed Reagent. Hydrocortisone, dibutyryl cyclic 3',5'-adenosine monophosphate, theophylline,

imidazole, epinephrine bitartrate, retinoic acid and isoproterenol were also obtained from Sigma. The peptide hormones tested were donated by Dr. G. M. A. Palmieri and were partially purified bovine (TCA extract) parathyroid hormone (Wilson Laboratories, Chicago,Illinois) and synthetic salmon calcitonin (AL-0977, Armour Pharmaceutical Company, Kankakee, Illinois) and all other reagents were of reagent grade.

CHAPTER III

RESULTS

Monosaccharides Added to Growth Medium

Table 1 illustrates the effects of several hexosamines and N-acetylated hexosamines on the specific activity of alkaline phosphatase in HeLa 65 control cultures to which hydrocortisone has been added to the growth medium to a final concentration of three micromolar at the time of inoculation. The value for alkaline phosphatase specific activity in the induced state without hexosamine represents the mean $\pm$ standard error for ten replicate cultures. Five millimolar D-glucosamine almost completely blocks the increase in alkaline phosphatase activity above that observed in control cultures without hydrocortisone. The stereoisomer, D-galactosamine, is about twenty five per cent effective in blocking the induction. N-acetylglucosamine has no effect at three millimolar, but enhances the induction by fifty per cent at five millimolar. N-acetylgalactosamine stimulates the induction by twenty to twenty five per cent at three millimolar and at five millimolar. N-acetylmannosamine was the most effective in enhancing the induction, resulting in about a twenty five per cent increase at two millimolar or greater. The N-acetylated hexosamines did not affect the total amount of protein per culture. Cells grown without hexosamines yielded 0.84 ± 0.02 milligrams of protein per culture (cell count was $3.3 \pm 0.2 \times 10^6$ cells per culture) and cultures grown with five

TABLE 1

EFFECTS OF SEVERAL MONOSACCHARIDES ON
HYDROCORTISONE INDUCTION OF
ALKALINE PHOSPHATASE

Addition	Concentration moles/liter	Alkaline Phosphatase milliunits/mg protein
None	-	23.2 ± 1.6 [a]
D-glucosamine	5×10^{-3}	6.5 ± 0.5
D-galactosamine	5×10^{-3}	17.6 ± 1.1
N-acetylglucosamine	3×10^{-3}	21.4 ± 1.5
"	5×10^{-3}	33.5 ± 1.8
N-acetylgalactosamine	3×10^{-3}	28.7 ± 1.7
"	5×10^{-3}	30.9 ± 1.2
N-acetylmannosamine	2×10^{-3}	32.4 ± 0.9

Hydrocortisone was added to 65C cells at the time of inoculation and the cells were harvested after 72 hours of growth. The data represent the mean of five replicate cultures ± standard error. 65C cells grown without hydrocortisone have alkaline phosphatase specific activity of 4.0 ± 0.3 milliunits per milligram of protein

a- ten replicate cultures

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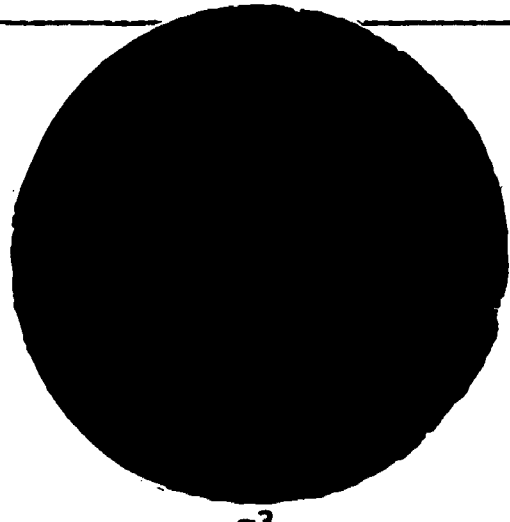

Hydrocortisone was added to 65C cells at the time of inoculation and the cells were harvested after 72 hours of growth. The data represent the mean of five replicate cultures ± standard error. 65C cells grown without hydrocortisone have alkaline phosphatase specific activity of 4.0 ± 0.3 milliunits per milligram of protein

a- ten replicate cultures

millimolar N-acetylglucosamine yielded 0.82 ± 0.02 milligrams of protein per culture. Figure 1 shows the time course of induction of alkaline phosphatase by hydrocortisone and the effect of addition of five millimolar D-glucosamine to the system. The increase in alkaline phosphatase activity above control values is diminished from nearly nine fold without D-glucosamine to only fifty per cent.

Figure 2 shows the inhibition of rate of protein synthesis in 65I cultures as a result of addition of five millimolar D-glucosamine. During the 72 hour growth period, the rate of increase in only about half of that without the hexosamine. The diminution of protein increase is markedly less than the inhibition of the increase in alkaline phosphatase activity. Figure 3 illustrates the linear dependence of the inhibition of growth on the hexosamine concentration throughout the range tested. Figure 4 shows that the decrease in alkaline phosphatase activity is also dependent on the concentration of D-glucosamine, and that the level of enzyme can not only be prevented from increasing in the induced system, but can be brought down from the elevated steady state level in the 65H system. Figure 5 shows the response of alkaline phosphatase specific activity to concentration of N-acetylmannosamine in the growth medium.

Electrophoretic Mobility After Neuraminidase Treatment

Figure 6 illustrates the identity of electrophoretic mobility of HeLa 65C and 65H alkaline phosphatases on polyacrylamide disc gel both prior and subsequent to treatment with *Vibrio cholerae* neuraminidase. Neither species of enzyme displayed any change in specific activity (65C, 8.9 milliunits/mg; 65C after NANAase, 11.2; 65H, 35.2; 65H after NANAase,

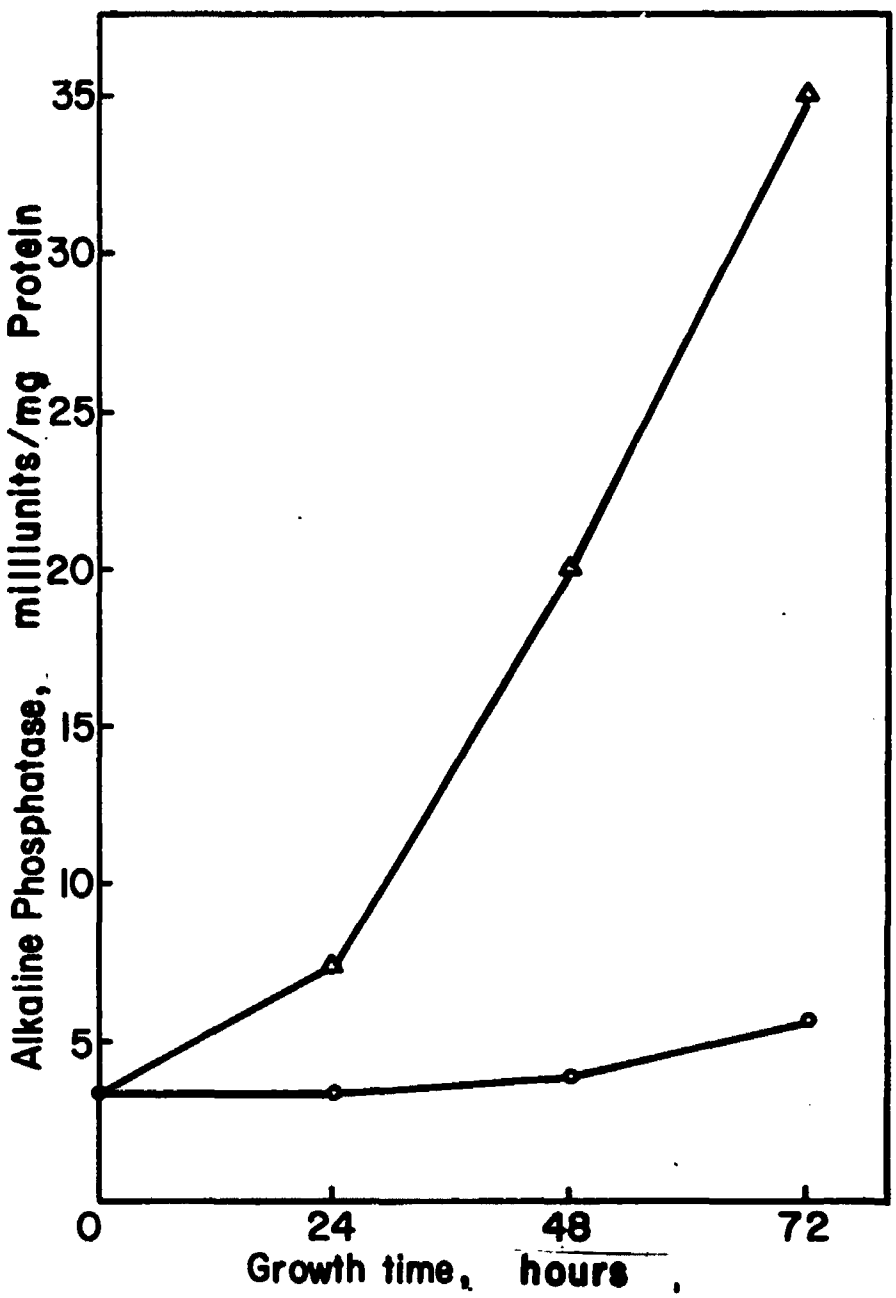

Figure 1. Inhibition by D-glucosamine of hydrocortisone induction in HeLa 65I. Control cells were inoculated with 3 micromolar hydrocortisone (-Δ-) and with five millimolar D-glucosamine plus 3 micromolar hydrocortisone (-o-) and harvested at 24 hour intervals.

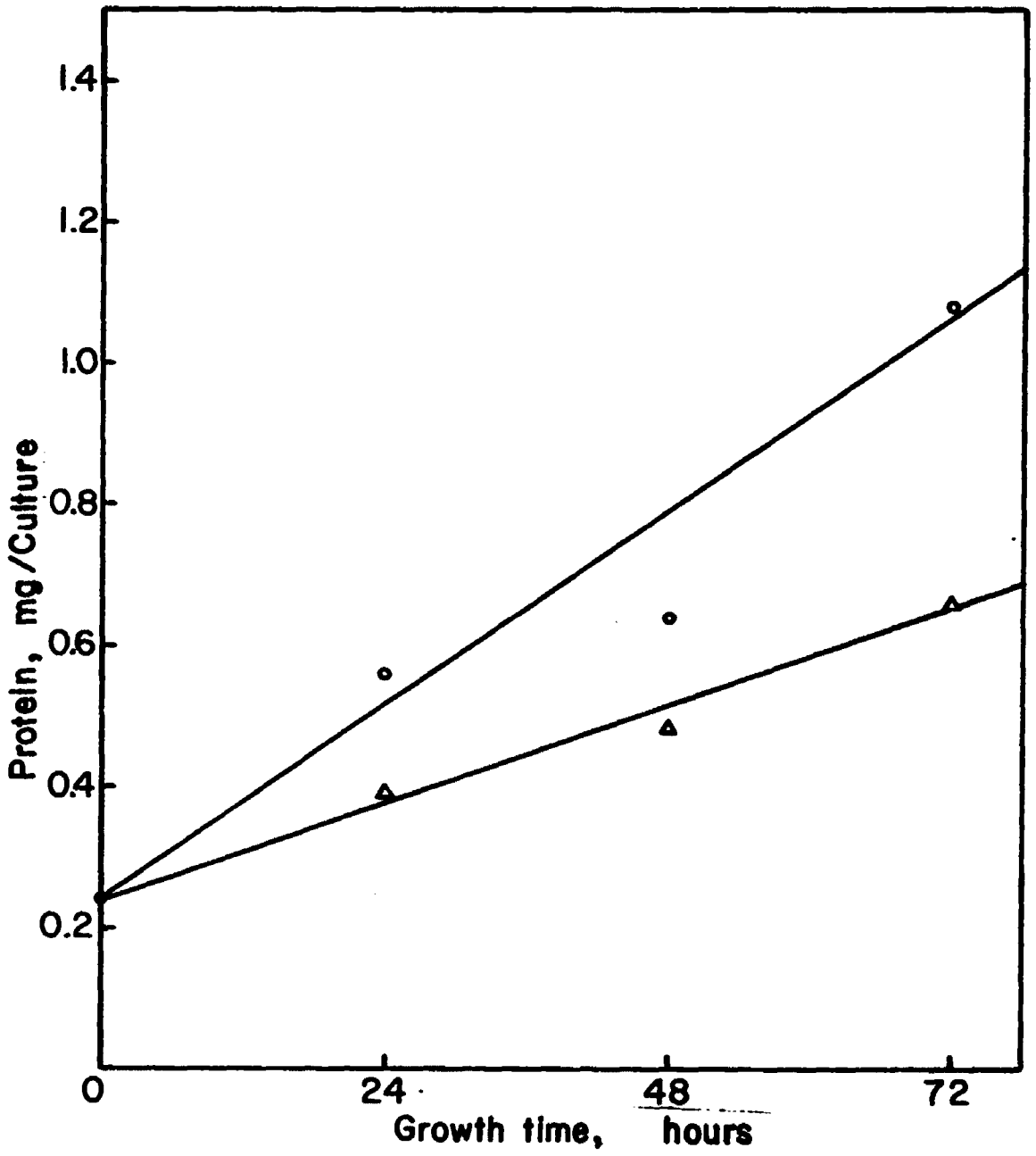

Figure 2. Inhibition of cell growth by D-glucosamine added to 65I cultures. Five millimolar D-glucosamine plus 3 micromolar hydrocortisone (-Δ-) and 3 micromolar hydrocortisone (-o-) were added to 65 control cells at the time of inoculation and cells were harvested after 72 hours.

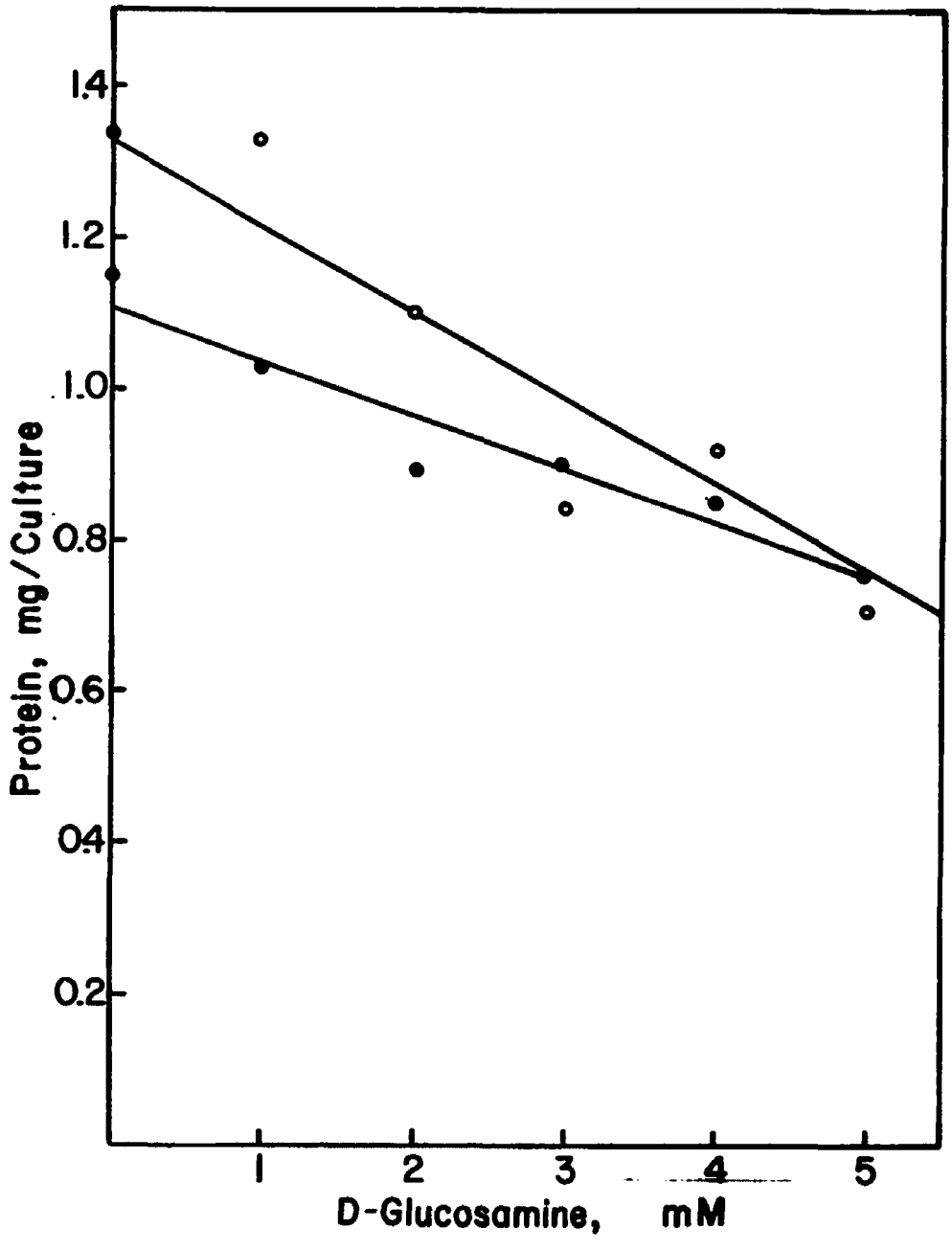

Figure 3. Total cell protein in HeLa 65H and 65I cultures as a function of D-glucosamine concentration. D-glucosamine was added to 65H (-●-) cultures and D-glucosamine plus 3 micromolar hydrocortisone was added to 65C cells (-o-) and cells harvested after 72 hours growth.

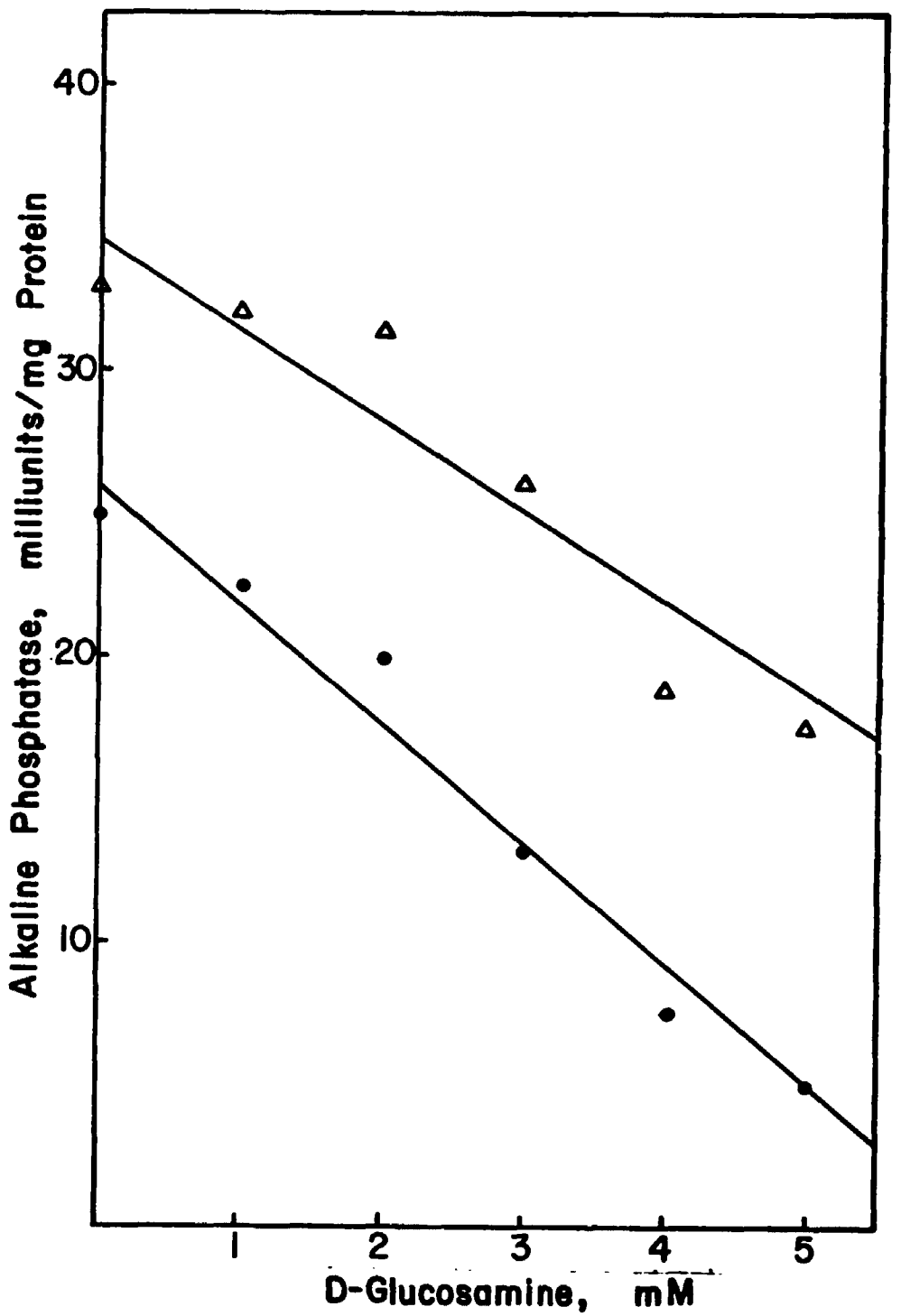

Figure 4. Specific activity of alkaline phosphatase in 65H (-Δ-) and 65I (-●-) cultures as a function of D-glucosamine concentration. Cells were harvested after 72 hours growth.

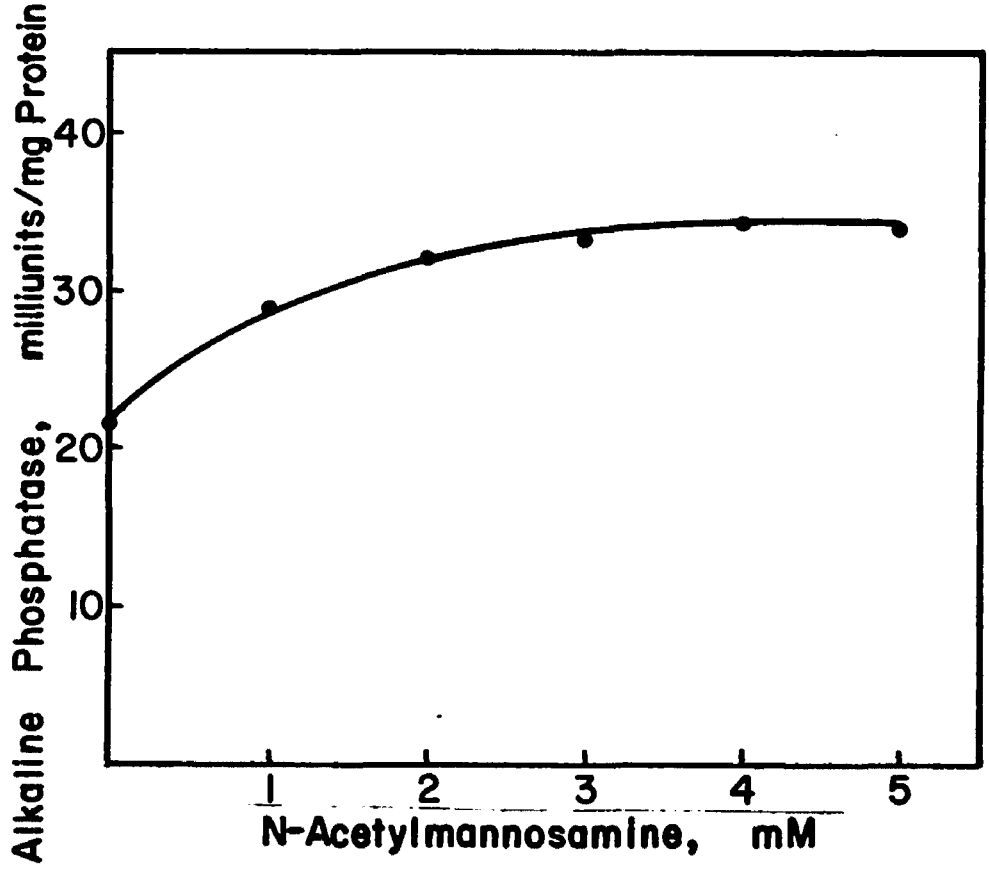

Figure 5. Alkaline phosphatase activity in 65I cultures as a function of N-acetylmannosamine concentration. Growth time was 72 hours.

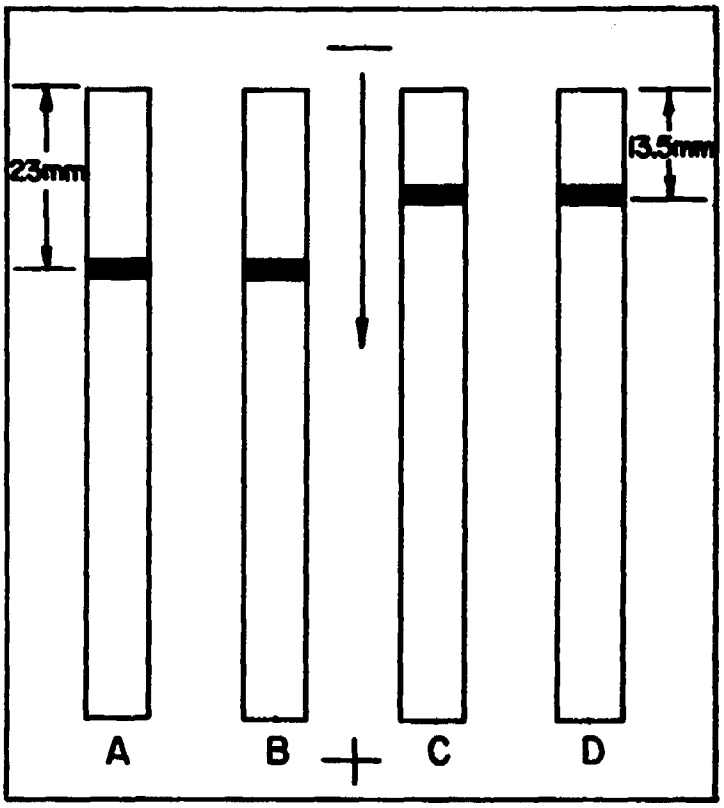

Figure 6. Effect of treatment with neuraminidase on disc gel electrophoretic mobility of HeLa 65C and 65H alkaline phosphatases. A = 65C, B = 65H, C = 65C + NANAase, D = 65H + NANAase. Arrow denotes direction of migration. Cathode at top of gel.

35.7) after cleavage of terminal sialic acid residues, and the electrophoretic mobility of both 65C and 65H alkaline phosphatase is reduced forty per cent after neuraminidase treatment.

Purification of Alkaline Phosphatase from HeLa 65C and 65H

Alkaline phosphatases from HeLa 65C and 65H as well as from human placenta were purified from butanol extracts by isoelectric focusing as described in Materials and Methods. Table 2 shows the recoveries of activity and protein and the extent of purification of the HeLa enzymes. The purifications achieved in these systems does not compare well to published data from other, more elaborate methods. However, analysis by disc gel electrophoresis of the isolated enzyme which was contained in one to three fractions (of 100 collected) yielded a single band of enzyme activity and a single band of protein as visualized by the Amido-Schwartz stain. Preparations of alkaline phosphatase from disc gel electrophoresis are low in specific activity as well, and recoveries of active protein are in the range of a tenth of one per cent. However, polyacrylamide gels containing separated proteins from butanol extracts and stained by Amido-Schwartz show no protein bands closer than three millimeters to the alkaline phosphatase bands, and the histochemically stained gel remnants from which the alkaline phosphatase bands had been excised showed no remaining activity. This evidence suggests that the excision of the bands is a precise and reproducible procedure which ensures the homogeneity of the alkaline phosphatase preparation by this method. For both methods of purification, the losses in protein and activity are similar for both 65C and 65H enzymes at each stage of purification.

TABLE 2

PURIFICATION OF HELA 65C AND 65H
ALKALINE PHOSPHATASE

Purification Step	Protein mg	%recovery	Activity mUnits	%recovery	Sp Act mUnits/mg	Purification Fold
Deoxycholate lysate						
65C	120		580		4.8	
65H	110		2430		17.3	
n-Butanol extract						
65C	106	88	560	97	5.3	1.1
65H	80	73	2200	91	58.5	3.4
Isoelectric focusing						
65C[a]	1.3	1.1	29	5	21.5	4.4
65H[a]	1.4	1.3	236	10	165.8	9.5
Gel electrophoresis[b]						
65C	0.115	0.1	3.4	0.6	29.5	6.1
65H	0.143	0.13	4.8	0.2	33.6	1.9

a- peak fraction only
b- following n-butanol extraction, as an alternative to isoelectric focusing

Isoelectric Points of Alkaline Phosphatases

The isoelectric points of the alkaline phosphatases of various HeLa clones and human placenta are shown in Table 3. HeLa 65C displayed three distinguishable enzyme peaks, two small peaks at pH 4.0 and 4.6 and a major peak at pH 5.2. Only the isoenzyme at pH 4.0 was observed in HeLa 65H. In the HeLa clone containing constitutive amounts of alkaline phosphatase, HeLa 71, both the control and hydrocortisone regulated enzymes had the same isoelectric point, focusing at pH 4.4. Human placental alkaline phosphatase was observed to have its isoelectric point at pH 4.7.

Amount of Hexosamines on Alkaline Phosphatases

Alkaline phosphatase from HeLa 65C and 65H was purified by disc gel electrophoresis as described in Materials and Methods. Total hexosamine in HeLa 65C and 65H was assayed and determined to be eight nanomoles of hexosamine per 115 micrograms (one nanomole) of enzyme protein. HeLa 71C and 71H alkaline phosphatase purified by isoelectric focusing yielded four and eight nanomoles of hexosamine per 115 micrograms of protein, respectively.

Free Sulfhydryls on Alkaline Phosphatase

Alkaline phosphatase purified by isoelectric focusing was assayed for free sulfhydryl groups as described in Materials and Methods and thiol content was determined to be four nanomoles per 115 micrograms of protein in both 65C and 65H.

TABLE 3

ISOELECTRIC POINTS OF SEVERAL
ALKALINE PHOSPHATASES

Type	Isoelectric point(s)
HeLa 65C[a]	4.0 4.6 5.2[b]
HeLa 65H	4.0
HeLa 71C	4.4
HeLa 71H	4.4
Placenta	4.7

a- three distinct isoenzyme peaks observed
b- major peak, analyzed for phosphate (Table 4)

Tightly Bound Phosphate on Alkaline Phosphatase

Alkaline phosphatase from 65C and 65H cells was purified by disc gel electrophoresis (five experiments) or by isoelectric focusing (one experiment). After exhaustive dialysis against doubly glass distilled water, the samples of alkaline phosphatase were assayed for protein and phosphate as described in Materials and Methods. Table 4 presents the number of nanomoles of phosphate per 115 micrograms of protein. The enzyme from 65C cells is seen to contain almost four times as much tightly bound phosphate as that from 65H. This phosphate was not removed by disc gel electrophoresis at pH 8.3, exhaustive dialysis at pH 7.5 or by isoelectric focusing at pH 4.0-5.2. A single experiment in which HeLa 71C and 71H alkaline phosphatases were purified by isoelectric focusing indicated that those enzymes contained or were associated with 110 and 11 nanomoles, respectively, of phosphate per 115 micrograms of protein. An earlier disc gel purification of 71C enzyme yielded 120 nanomoles of phosphate per 115 micrograms of protein. In the case of 65C and 65H, the single value derived from the isoelectric focusing experiment is in good agreement with the five from disc gel electrophoresis.

Effect of Phosphate in Growth Medium

The finding that there was more phosphate in 65C alkaline phosphatase than 65H led to the speculation that perhaps an increase in inorganic phosphate in the growth medium could exert some influence either on the control level of alkaline phosphatase activity or inhibit the induction in our cell system. Growth of 65C and 65I cultures with added phosphate ranging from one to ten millimolar showed no increase or decrease in cells

TABLE 4

AMOUNT OF TIGHTLY BOUND PHOSPHATE ON
ALKALINE PHOSPHATASE PURIFIED
FROM HELA 65C AND 65H

Experiment	Method of Purification	Nanomoles P_i / 115 micrograms protein	
		65C	65H
1	Disc gel	22.7	5.0
2	"	20.9	5.4
3	"	25.4	5.2
4	"	22.8	8.1
5	"	26.5	8.1
6	Isoelectric focusing	23.9	7.4
	Mean $\pm$ standard error	23.7 ± 0.8	6.5 ± 0.6

($3.3 \pm 0.6 \times 10^6$) per culture, protein (0.85 ± 0.02 milligrams) per culture or in alkaline phosphatase specific activity (65C, 4.8 ± 0.2 milliunits/mg protein; 65I, 24.6 ± 0.3 milliunits/mg protein). The free intracellular phosphate concentration was determined to be 6.5 millimolar in HeLa 65C and 7.0 millimolar in HeLa 65H.

Growth with Dibutyryl Cyclic 3',5'-AMP

Since other enzymes whose active and inactive forms contain different amounts of phosphate are regulated through the action of cyclic AMP, it seemed very relevant to attempt to induce or repress alkaline phosphatase in our system by cyclic AMP or its dibutyryl derivative, DB c-AMP, and to test the effects of other drugs known to be involved in cyclic AMP-mediated systems. Cultures of 65C cells were prepared to which DB c-AMP (ranging from 0.4 to 1.6 millimolar in the growth medium) was added, and a similar set of cultures to which 3 micromolar hydrocortisone was added in addition to the DB c-AMP. Figure 7 illustrates the increase in alkaline phosphatase activity which can be produced by DB c-AMP and the additive increase in activity due to the combination of DB c-AMP and hydrocortisone. There was a marked inhibition of cell growth (total protein) at one millimolar DB c-AMP (Table 5). This may account for the increased lag time noted in the induction by DB c-AMP (Figure 8) compared with that observed in the hydrocortisone induction.

Theophylline, a methylated xanthine derivative, usually enhances the effect of cyclic AMP by inhibiting the phosphodiesterase which hydrolyzes it. Hoping to demonstrate such a synergistic effect, we added theophylline at 0.1, 0.5 and 1.0 millimolar to the growth medium of cultures

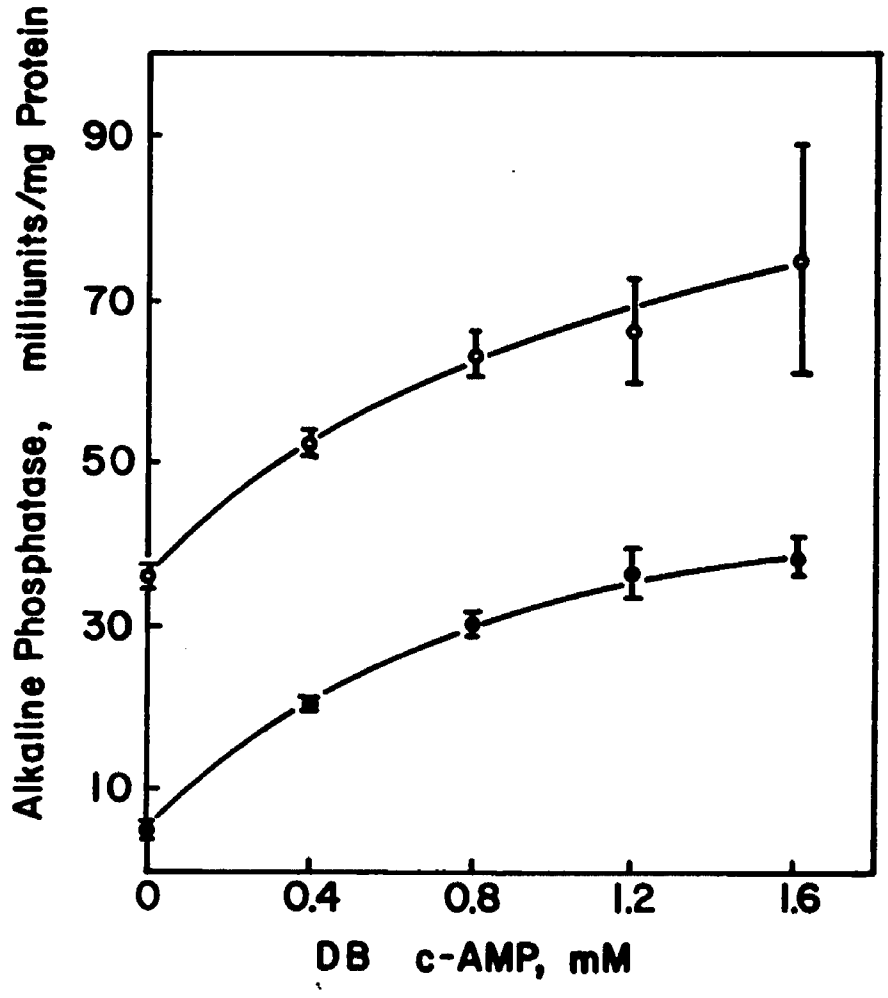

Figure 7. Effect of DB c-AMP (-●-) and 3 micromolar hydrocortisone plus DB c-AMP (-o-) on alkaline phosphatase activity. Growth time was 72 hours. Vertical bars represent standard error.

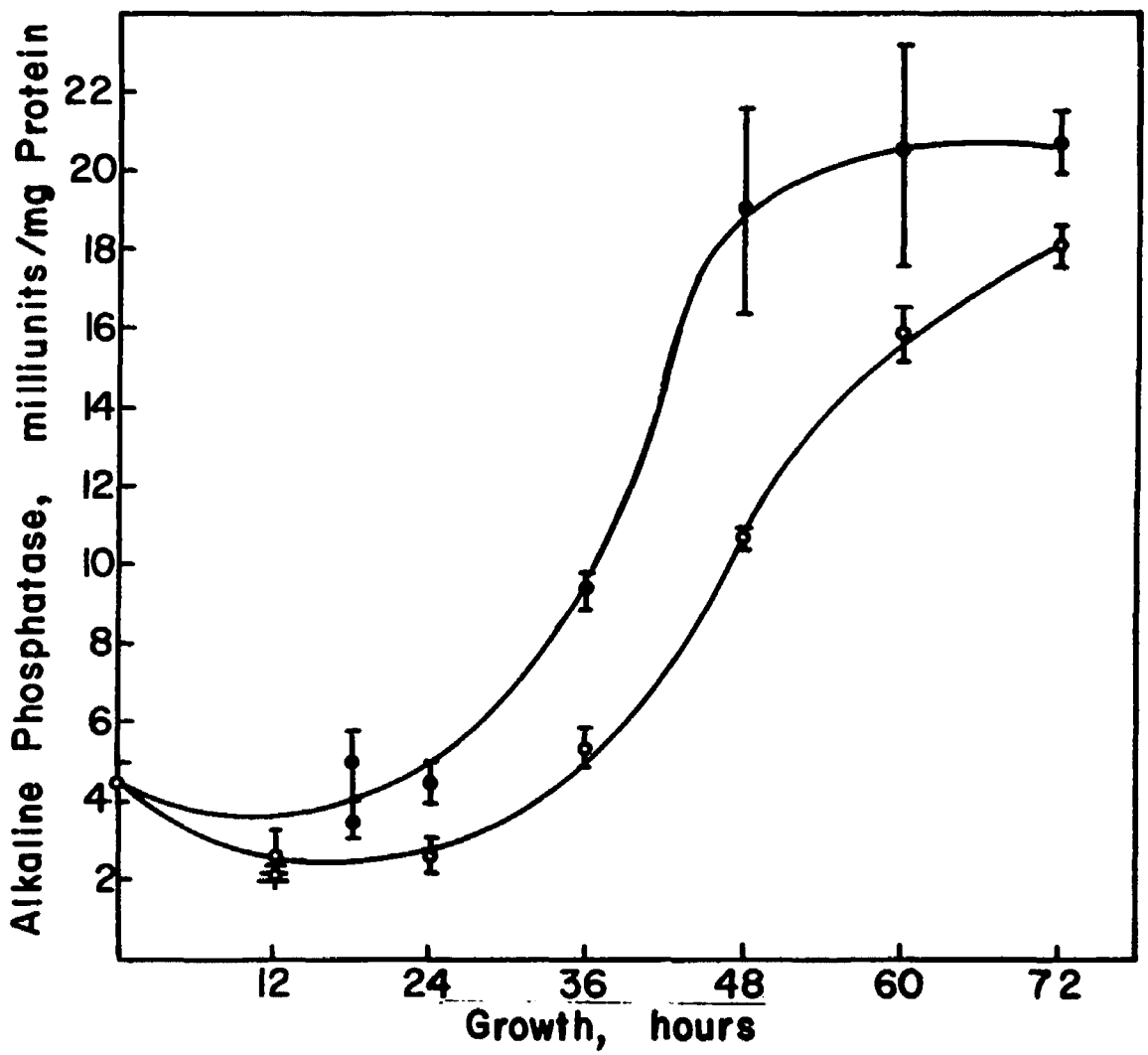

Figure 8. Time course of induction of alkaline phosphatase by hydrocortisone (-●-) and by DB c-AMP. Cells were grown with 3 micromolar hydrocortisone or with one millimolar DB c-AMP (-o-).

of 65C and 65I cells. Figure 9 shows that, rather than enhancing the induction, theophylline markedly represses alkaline phosphatase activity. It seemed reasonable that either alkaline phosphatase might be inhibited by theophylline, and, if so, that sufficient amounts of theophylline might remain in the cell lysates to account for the reduced activity; or, that theophylline might influence a physiologic or metabolic process which determines or modulates alkaline phosphatase activity. To test the first possibility, a series of alkaline phosphatase assays were performed with added theophylline ranging from 0.2 to 1.0 millimolar in the reaction mixture. Figure 10 shows that theophylline at 0.6 millimolar inhibits the phosphomonoesterase activity about fifty per cent. Serial dilution experiments on lysates of cultures grown with hydrocortisone, with and without theophylline at 1.0 millimolar, show that a fifteen fold dilution of theophylline free lysate gives an increase in relative specific activity of thirteen per cent, and similar treatment of a lysate from a culture grown with theophylline yields a relative specific activity increase of twenty eight per cent. These data might seem to suggest that residual theophylline in the lysates was responsible for the reduced activity. However, when the total cell volume of the monolayer is compared with the final diluted volume in the assay, it becomes clear that the cell would have to concentrate theophylline about 670 fold in order to yield a final theophylline concentration in the assay mixture of one millimolar, or enough to produce a seventy per inhibition on activity. If theophylline bound irreversibly to alkaline phosphatase, or irreversibly inactivated it, the possibility would remain that the lysis and subsequent dilution into the assay would not reduce the theophylline concentration below the effective

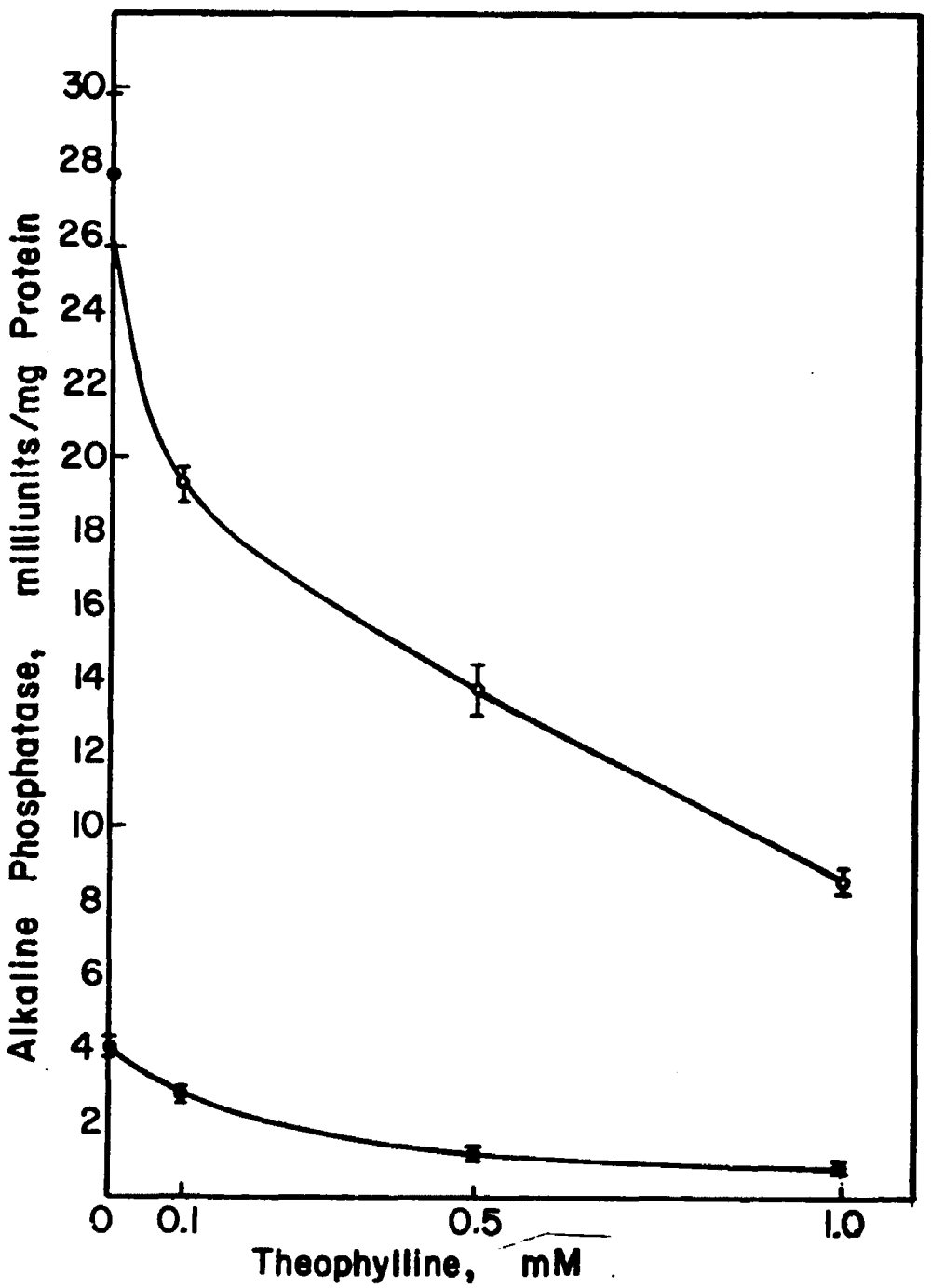

Figure 9. Effect of theophylline on alkaline phosphatase specific activity in cultures of 65C (-●-) and 65I (-o-). Growth time was 72 hours.

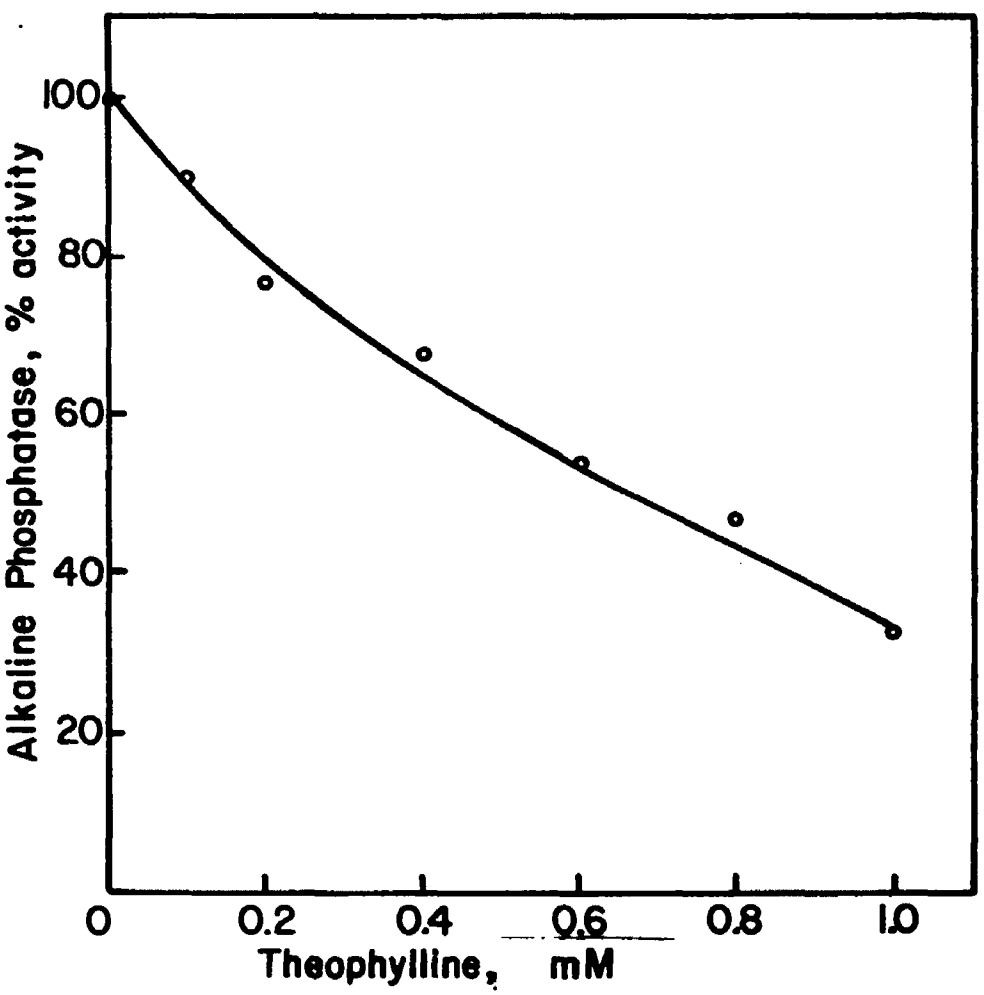

Figure 10. Inhibition of alkaline phosphatase activity by addition of theophylline to the assay.

concentration of one millimolar. In order to determine whether the inhibition was reversible or irreversible, samples of alkaline phosphatase were made up with and without one millimolar theophylline and dialyzed overnight against doubly glass distilled water and assayed for alkaline phosphatase activity. The sample which had never been exposed to theophylline had the same activity (28.0 $\pm$ 0.3 milliunits/mg) as the one from which the theophylline had been dialyzed. From these data it is concluded that the reduced activity is not due to inhibition by residual theophylline in the cell lysates.

Effects of Several Cyclic AMP-Related Drugs

Table 5 illustrates the effects of several drugs which have been shown to affect either the hydrocortisone induction of alkaline phosphatase or the activity of adenyl cyclase, the enzyme which catalyzes the production of 3',5'-cyclic AMP. Retinoic acid, a known inhibitor or alkaline phosphatase induction by hydrocortisone (55), was tested again for that ability as well as its effect on the DB c-AMP induction. It is clear that retinoic acid, which inhibits the hydrocortisone induction by fifty per cent at ten micromolar, has little or no effect on the DB c-AMP induction. Imidazole, which has been shown to stimulate phosphodiesterase (91), thereby reducing the intracellular level of cyclic AMP, lowered the hydrocortisone mediated induction by twenty five per cent. Theophylline reduced the hydrocortisone induction fifty per cent at 500 micromolar and the DB c-AMP mediated induction 100 per cent at this concentration. When used alone, theophylline at 500 micromolar reduced 65C alkaline phosphatase activity seventy per cent. Epinephrine bitartrate, a known stimulator of adenyl

TABLE 5

EFFECTS OF VARIOUS DRUGS ON ALKALINE PHOSPHATASE ACTIVITY AND ON CELL GROWTH

Additions	Final Concentration micromolar	Alkaline Phosphatase munits/mg	Cell Growth % of control
None	--	4.0 ± 0.3	100
DB c-AMP	1000	26.0 ± 1.4	48
Hydrocortisone	3	28.0 ± 1.9	74
DB c-AMP + Hydrocortisone	1000 3	63.3 ± 2.5	82
Retinoic acid + Hydrocortisone	10 3	12.4 ± 0.9	86
Retinoic acid + DB c-AMP	10 1000	24.1 ± 2.2	42
Imidazole + Hydrocortisone	10 3	21.7 ± 0.1	100
Imidazole + Hydrocortisone	1000 3	21.0 ± 0.4	110
Theophylline	500	1.2 ± 0.1	100
Theophylline + Hydrocortisone	500 3	13.7 ± 0.8	105
Theophylline + DB-cAMP	500 1000	3.3 ± 0.3	16
Epinephrine bitartrate	320	19.0 ± 2.0	30

Drugs were added to the growth medium at the time of inoculation and cells were harvested after 72 hours growth at 37° C. Cell growth was estimated from the ratio of cell protein in an experimental culture to cell protein in control cultures using an approximation of similar amounts of protein per cell.

cyclase (92), resulted in a fourfold increase in enzyme activity despite inhibiting cell growth. The effects of the various drugs on cell growth are also shown in Table 5. Hydrocortisone alone, at a concentration of three micromolar, led to a twenty five per cent decrease in total cell protein per culture and DB c-AMP decreased protein by fifty per cent at one millimolar. The two drugs in combination were slightly less inhibitory toward cell growth or total protein than hydrocortisone alone. Theophylline, either alone or in combination with three micromolar hydrocortisone, had no effect on cell protein at 500 micromolar; but in combination with DB c-AMP at one millimolar, cell growth was completely blocked.

Effects of Polypeptide Hormones

Two polypeptide hormones, parathyroid hormone and calcitonin, whose functions in calcium metabolism have been recently reviewed (93), were added to the growth medium of cultures of HeLa 65C (Figure 11). Synthetic salmon calcitonin at fifty milliunits per milliliter induced about a three fold increase in alkaline phosphatase activity per milligram of total cell protein and bovine parathyroid hormone at fifty micrograms per milliliter depressed the alkaline phosphatase activity by about forty per cent. Neither hormone affected total protein per culture.

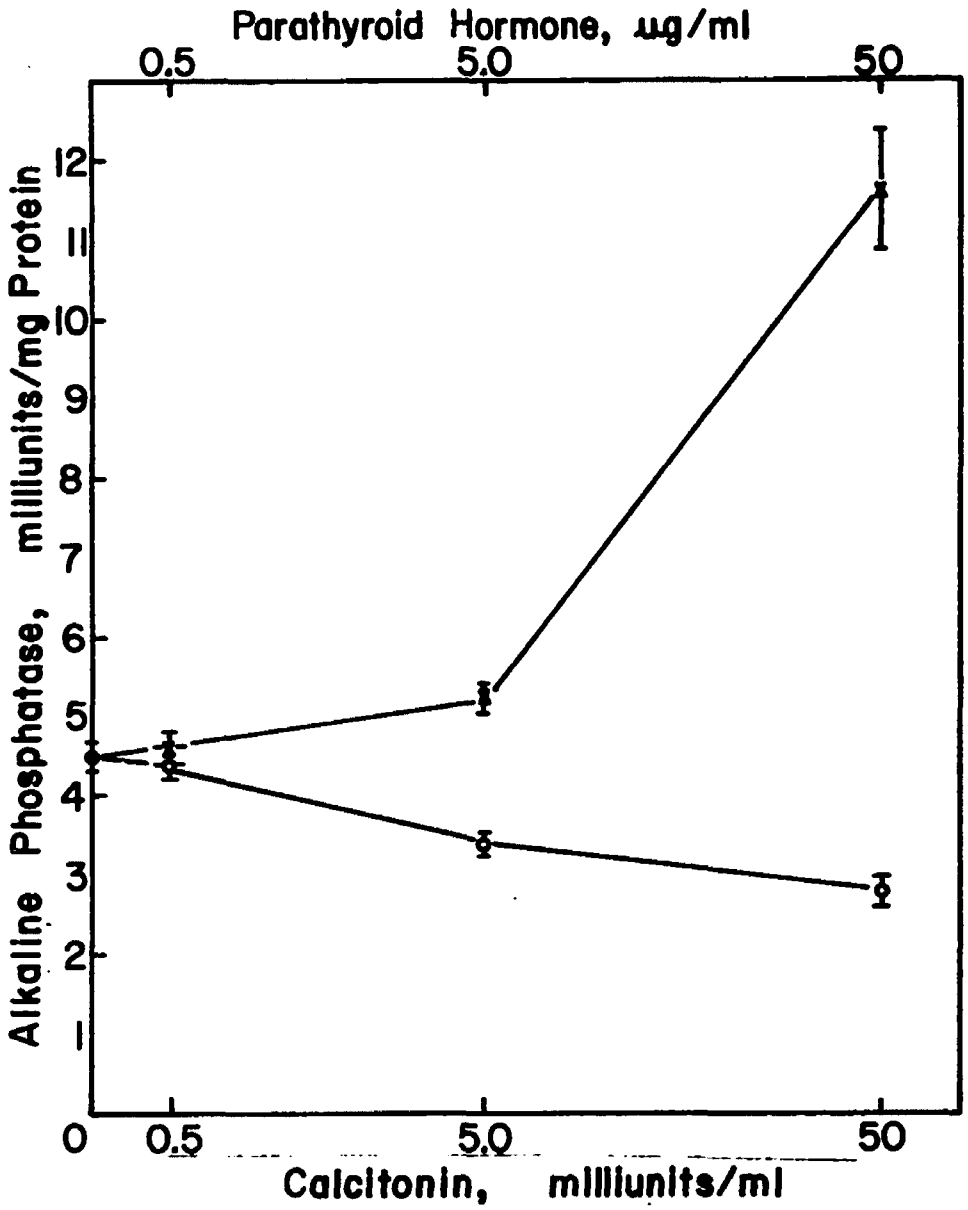

Figure 11. Effects of calcitonin (-x-) and parathyroid hormone (-o-) on alkaline phosphatase specific activity in 65C cells in culture. Growth time was 72 hours.

CHAPTER IV

DISCUSSION

Alkaline phosphatase has been shown to increase four to ten fold in HeLa 65 cultures when cultivated with three micromolar hydrocortisone in the medium (50). The induction occurs by an increase in specific activity of the enzyme rather than by an increase in the amount of enzyme concentration in the cell (47). Melnykovych and Costlow (56) showed that the induction is blocked by D-glucosamine and stated that twenty millimolar glucosamine is growth inhibitory to various cell cultures. We found in our system that the inhibitory effects of glucosamine on total cell protein are seen when the glucosamine concentration in the medium is as low as one millimolar and that the effect in linear up to five millimolar, which was the upper limit of our test conditions. Bosmann has studied the effects of D-glucosamine and D-galactosamine on RNA and protein synthesis in mouse leukemic L5178Y cells and has reported that D-glucosamine at 100 micromoles per milliliter inhibits RNA, DNA, protein and glycoprotein synthesis 95 per cent (94). Our study shows a nearly quantitative inhibition of alkaline phosphatase induction by a much lower concentration; in the mouse cell culture system, D-galactosamine at 100 millimolar inhibits the synthetic processes by 23 per cent and in the HeLa system we have shown that at five millimolar, the inhibition of alkaline phosphatase induction

is about twenty five per cent. The relative effectiveness of the two stereoisomers, then, is about the same in both systems, and that ratio may be accounted for by the interconversion of the two hexosamines by an isomerase. Bosmann also tested D-mannose and found that it inhibited the syntheses by forty five per cent, but in the HeLa system, we found that the ten fold induction of alkaline phosphatase activity by hydrocortisone was unaffected by five millimolar mannose. In our system, the N-acetyl hexosamines enhanced the alkaline phosphatase activity, whereas in the Bosmann study N-acetylglucosamine inhibited synthesis ten per cent, N-acetylmannosamine thirteen per cent, and N-acetylgalactosamine had no effect. The concentration of N-acetylated hexosamines used in Bosmann's study was 100 millimolar, which was several times higher than the highest used in our study, and the differences in effect are probably best accounted for by postulating that our growth medium was somewhat deficient in the hexosamines and the levels we added were sufficient to make up the deficiency, but below the level necessary for toxicity. Bosmann also found that the inhibitions were reversible by addition of uridine nucleotides (uracil and uridine were ineffective) to the growth medium. This suggests that the mechanism of inhibition involves depletion of these nucleotides by formation of the uridyl monosaccharides. Since it has been established that new messenger and protein synthesis is required for the hydrocortisone induction, it seems most likely that the blocking of translation or transcription is the mechanism of inhibition of alkaline phosphatase hydrocortisone induction by D-glucosamine. It would be interesting to add uridine nucleotides to cultures of 65I cells treated with five millimolar glucosamine and learn whether the induction then proceeds.

The observation that neuraminidase reduced the disc gel electrophoretic mobility of 65C and 65H alkaline phosphatase to exactly the same degree is consistent with earlier results using starch gel electrophoresis (49) as is our observation that neuraminidase treatment results in no change in activity. Probably the sialic acid residues are far enough removed from the active site that they do not affect the substrate affinity nor do they appreciably affect the conformation of the enzyme which is believed to underlie the difference in control and induced activity (24).

The accuracy of the data presented concerning the amount of phosphate bound to alkaline phosphatase is dependent upon the degree of purity of the enzyme. The judgment of purity of the enzyme is based primarily on the fact that there were no proteins, as visualized by the Amido-Schwartz stain, other than alkaline phosphatase, within three millimeters on either side of the center of the alkaline phosphatase band. The histochemical staining of the gel remnants would have detected an error in cutting the gels; that is, if the alkaline phosphatase band had a somewhat different mobility on one gel, or if the five millimeter excision had been displaced by three millimeters or more, the alkaline phosphatase band or a portion of it would have remained in a remnant and the purity of the preparation compromised. This was not the case; the gel remnants, in every case, showed no histochemical staining. Another possible source of error could have been the phosphate assay itself; however, after the precleaning procedure was initiated, the occasional false highs due to a bit of detergent residue in the tube were eliminated, and consistent, reproducible standard curves ranging from two to one hundred nanomoles of phosphate were produced routinely. It should be pointed out that the

nature of the mode of attachment of the phosphate measured in this study is quite different from the highly labile, readily exchangeable phosphate described by Engstrom (95) which was removed from the enzyme at pH 8. The total amount of phosphate was calculated to be only 0.5 moles of phosphate per mole of enzyme. The phosphate determined in this study stayed on the enzyme through disc gel electrophoresis at pH 8.3, and there was a substantially greater amount present.

The isoelectric focusing procedure does not treat the alkaline phosphatase as harshly as the disc gel electrophoresis, based on the highest specific activities resulting from the two procedures. However, we were concerned less with high specific activity of the final product than with protein homogeneity, and enzyme preparations purified from placenta, HeLa 65C and 65H by isoelectric focusing each yielded a single protein band on disc gel electrophoresis.

The activation of an enzyme by removal of phosphate or inactivation by phosphorylation is illustrated by at least three published examples, fructose 1.6-diphosphatase, pyruvate dehydrogenase and phosphorylase a phosphatase. Mendicino (96) reported that cyclic AMP increased the rate of inactivation of purified fructose 1,6-diphosphatase by a crude prepation from porcine kidney, ATP and magnesium. Torres and Chelala (97) found that phosphorylase a phosphatase partially purified from avian skeletal muscle was inactivated at a greater rate when cyclic AMP was added. Reactivation of inactivated pyruvate dehydrogenase from pig heart muscle is stimulated by incubating the polyenzyme complex with cyclic AMP (74). These findings suggest that cyclic AMP might play a key role in the regulation of alkaline phosphatase activity. Cox (77) had

attempted to induce an increase in enzyme activity by addition of dibutyryl cyclic AMP to the growth medium after 48 hours of growth and harvested the cells four hours after addition of the drug. Apparently that length of incubation with the drug was insufficient to permit the protein synthesis required for the induction (53) and is considerably less than the lag period previously described (54). Our effort was directed toward duplication of the conditions under which induction by hydrocortisone occurs, that is, growth with the drug for 72 hours. The inductive effects of dibutyryl c-AMP and hydrocortisone were additive and the time courses similar, suggesting that the two mechanisms are the same or closely related. Adenyl cyclase in leukocytes from asthmatic and nonasthmatic subjects has been shown to be stimulated by hydrocortisone and isoprenaline (98). It would be expected, if the mechanism of alkaline phosphatase induction is primarily through stimulation of adenyl cyclase, resulting in increased intracellular c-AMP, that the lag period would be reduced if the inducing drug were DB c-AMP rather than hydrocortisone. However, cyclic AMP has been reported to inhibit protein synthesis in rat muscle fibers (99) and this study indicates that the same is true for HeLa cells. Bär and Hechter (100) propose that there is a single adenyl cyclase in fat cell ghost membranes and that its stimulation by different effectors such as glucagon, secretin, adrenocorticotropin, thyroid-stimulating and luteinizing hormones, epinephrine, and other agents is mediated by specific proteinaceous receptors at the outer surface of the plasma membrane. This might explain why Koyama (62) was able to induce alkaline phosphatase with dibutyryl c-AMP but not with glucocorticoids; it may be that the adenyl cyclase of the mouse-hamster hybrid cell, lacking the receptor

protein specific for hydrocortisone, was unresponsive to it. The fact that retinoic acid blocks the induction of alkaline phosphatase by hydrocortisone but not by DB c-AMP adds some weight to the postulate that the action of cyclic AMP follows that of hydrocortisone; possibly retinoic acid alters the protein receptor in such a way that hydrocortisone does not bind to it, or that retinoic acid inhibits adenyl cyclase directly. The possibility that hydrocortisone acts by inhibiting phosphodiesterase rather than stimulating adenyl cyclase was tested by Logsdon, et al. (98), who found that at concentrations of 10^{-6} molar or less, hydrocortisone had no detectable effect; however, at 10^{-4} to 10^{-2} molar, there was significant inhibition. Another piece of evidence which supports cyclic AMP mediation of hydrocortisone induction is the moderate inhibition of that induction by imidazole. The inhibition of induction is the same in magnitude whether ten micromolar or one millimolar imidazole is used in the medium. Probably the lower concentration is sufficient to saturate the phosphodiesterase in the cells. Experiments which must be done in the future to further elucidate the mechanism of induction are to measure a) effect of hydrocortisone on HeLa adenyl cyclase, b) intracellular level of c-AMP in 65C, 65H and DB c-AMP induced cells and c) the amount of tightly bound phosphate on DB c-AMP induced alkaline phosphatase.

The requirement for new RNA and protein synthesis raises several questions about the sequence of events or rate limiting steps in the induction process. Cox (77) suggested that the lag phase is due to the accumulation of an intermediate, possibly messenger RNA, during the synthetic phase of the cell cycle, since cells grown with cycloheximide, a protein synthesis blocking agent, will start immediately to increase in

alkaline phosphatase activity as soon as the cycloheximide is removed. This suggests that the alkaline phosphatase which is of higher specific activity is only the newly synthesized material, which must be structurally modified (dephosphorylated) during or shortly after synthesis, and that once the enzyme has been incorporated into the plasma membrane, it is not altered in activity. It is also possible that the modifying enzyme or enzymes may be a structural component of the smooth endoplasmic reticulum, and that the newly synthesized alkaline phosphatase only has one period of exposure to it or them. The question is whether cyclic AMP induces the synthesis of a phosphatase which cleaves phosphate from phosphoserine residues on the enzyme, represses the synthesis of a kinase which would otherwise phosphorylate the alkaline phosphatase, or by some gene switching mechanism leads to the synthesis of a more active phosphatase or less active kinase. Another possible mechanism would be the simple allosteric activation or inhibition of an already extant phosphatase or kinase, assuming that the requirement for new protein synthesis applied only to alkaline phosphatase itself, and not to some modifier protein. The evidence presented by Griffin and Ber (54) suggests that the alkaline phosphatase induction is initiated only in the synthetic phase of the cell cycle, but it does not necessarily follow that the requirement for new messenger implies a requirement for a different messenger. The simplest mechanism of alkaline phosphatase induction by hydrocortisone may be as follows: hydrocortisone binds to a protein receptor associated with adenyl cyclase in such a way that the cyclase is stimulated and the intracellular level of cyclic AMP is increased. Cyclic AMP may inhibit the phosphorylation of serine residues on alkaline phosphatase

which is being synthesized in the smooth endoplasmic reticulum, or stimulate the removal of phosphate from phosphoserines on the enzyme. Theophylline may stimulate the phosphorylation of the enzyme, but it is more reasonable to postulate that it might inhibit the dephosphorylation, since the functions of theophylline thus far reported have been inhibitory. The alkaline phosphatase conformation resulting from the smaller number of phosphates on the enzyme leads to an enhancement of its specific activity. If theophylline did inhibit the dephosphorylation, it would make the regulatory process more elegant if alkaline phosphatase were the protein phosphatase which by extrinsic or intrinsic autocatalysis promoted its own dephosphorylation. The only firm evidence to support this possibility is the fact that theophylline inhibits the phosphomonoesterase activity of alkaline phosphatase very efficiently. If that were the case, it should be possible, under the proper conditions, to stimulate an autocatalytic dephosphorylation in vitro, unless there were some aspect of conformation which required that the modification take place during or immediately after synthesis.

The regulation of alkaline phosphatase activity may be quite a different mechanism in HeLa 71, although this study does not include sufficient statistical evidence to do much more than indicate or suggest a different mode of regulation. The observation which is consistent with the HeLa 65 data is the lowering of the amount of tightly bound phosphate in the Hcr state; however, the activity of the 71H enzyme is somewhat lower than that of 71C, and the number of hexosamines per molecule of the 71C enzyme is half that of 71H. It may be that the control level of enzyme activity is so high that a mechanism which tends to increase the

activity triggers a separate process involving modification of the polysaccharide side chain, lowering the specific activity. It should be remembered that the control level of alkaline phosphatase activity in HeLa 71 is two orders of magnitude higher than that of 65H.

The role of alkaline phosphatase in clinical diagnosis has been recently reviewed (42,101) and falls outside the scope of this study. A variety of physiological roles have been proposed for alkaline phosphatase such as the transfer of phosphates from a phosphoester to an alcohol (102) hydrolysis of pyrophosphate (43), function as a 5'-nucleotidase (39) and various other functions involving conservation or regulation of nucleic acids, sugars and other small molecules from which phosphate can be removed under assay conditions. An early major hypothesis concerning alkaline phosphatase function in vivo was proposed by Robison (103) who postulated that phosphatase in bone was responsible for the liberation of free phosphate from phosphate esters and that the local increase in free inorganic phosphate concentration led to the deposition of calcium phosphate salts. This theory involved the belief that biological calcification occurred through the precipitation of solutes from supersaturated solutions.

It may very well be that the primary mode of action in vivo is to regulate one of the divalent cations whose function and concentration have been linked to those of phosphate. The most obvious of these is calcium, since so much of the body's mineral weight consists of calcium phosphate. It might be best to look at some of the demonstrated interrelationships between calcium and some of the other factors which influence or or related to alkaline phosphatase. MacManus and his coworkers have

found (104) that the epinephrine stimulated rat thymocyte proliferation mediated by cyclic AMP is enhanced by calcium, through stimulation of adenyl cyclase by calcium. Chi and Francis (105) demonstrated that large increases in calcium but not sodium outflow could be stimulated in a cellular slime mold by addition of 0.1 millimolar cyclic AMP. In frog sartorius muscle, Borys and Karler (106) have shown that addition of ten millimolar caffeine, an inhibitor of phosphodiesterase, stimulates an increase in mitochondrial calcium content and a decrease in microsomal calcium. The intracellular distribution of calcium is critical in regulating a number of enzymic processes as well as influencing muscle contraction and synaptic transmission; Rasmussen (107) has thoroughly reviewed a number of interactions between calcium and cyclic AMP in a wide variety of tissues and organisms. He has also found that in rachitic chicks, a significant increase in both intestinal calcium absorption and intestinal alkaline phosphatase activity occurred following a single physiological dose of vitamin D_3 (44). He also found an increase in calcium activated ATPase activity and presented evidence to suggest that the alkaline phosphatase activity was another function of the same protein as the calcium ATPase activity, both activities being inhibited by L-phenylalanine but not D-phenylalanine.

It would be of no particular benefit to the cell to cleave ATP, which the cell generates at considerable expense in internal metabolic machinery and external energy input, unless a concomitant event occurred against an energy gradient and that event was beneficial to the cell. The cytoplasmic concentration of calcium in mammalian cells is in the range of 10^{-5} to 10^{-8} molar, and plasma concentration is about 10^{-3} molar

(44) and this differential must surely be maintained at considerable expense of energy, whose source is probably ATP. It seems very reasonable that an enzyme whose ability to hydrolyze a high energy compound while associated with calcium, and localized at the outer boundary of the cell, may possess that activity for the purpose of pumping calcium out of the cell against the concentration gradient. It is possible to postulate a feedback system of regulation of the activity of the enzyme based on the following evidence: influx of calcium into the cell is associated with an increase in calcium-ATPase activity and alkaline phosphatase activity (44); calcium stimulates adenyl cyclase activity (104) which increases the level of cyclic AMP, increasing the activity of alkaline phosphatase (calcium-ATPase?). This scheme raises the question of whether hydrocortisone will induce the activity of the calcium-ATPase, and whether identity of the two activities can be demonstrated in a highly purified enzyme preparation and whether alkaline phosphatase activity can be induced by addition of calcium to cells grown in calcium deficient medium. There is some evidence to support the latter, as HeLa cells grown in spinner medium with 0.4 millimolar calcium have only about ten per cent as much alkaline phosphatase activity (24) as do cells grown in monolayer medium which contains 2.7 millimolar calcium (78). MacManus (104) showed a sharp plateau in the effect of calcium on cyclic AMP, with the break at 1.2 millimolar.

Our final experiment implicates an interrelationship between alkaline phosphatase activity and calcium regulation by showing that alkaline phosphatase is induced three fold by calcitonin at fifty milliunits per milliliter in the growth medium. Calcitonin is produced by the

parafollicular cells clustered immediately outside the cells that border the thyroid follicle and causes hypocalcemia by inhibiting bone resorption by osteoclasts (93). Bird, et al. (108), observed marked hypocalcemia and hypophosphatemia in rats injected with imidazole, while theophylline induced hypophosphatemia without significantly affecting plasma calcium. In rats which had been pretreated for four days with cortisone, the imidazole induced hypocalcemia was reduced, while theophylline exerted a marked hypocalcemic effect. These findings are consistent with our model, in that hypocalcemia, which is analogous to a reduced pumping of calcium out of the cell into the medium (plasma) is induced by the same compound, imidazole, which reduces the inducibility of alkaline phosphatase in HeLa cells. In both systems, the effects of imidazole and the glucocorticoid are antagonistic.

The results of this study seem to implicate a key role of alkaline phosphatase in the regulation of calcium transport.

CHAPTER V

SUMMARY

Alkaline phosphatase activity in HeLa cells, an aneuploid line of epithelial origin derived from an adenocarcinoma, is inducible by the addition of hydrocortisone to the growth medium. The induction begins after an eighteen hour lag period, increases linearly for about thirty six hours, and reaches a plateau or new steady state level about six fold higher than the control level. The elevated alkaline phosphatase activity remains constant as long as three micromolar hydrocortisone is maintained in the growth medium. The increase in activity is due to an increase in the intrinsic specific activity rather than to an increase in the amount of enzyme present in the cell. The induction can be blocked by puromycin, actinomycin D or by D-glucosamine, which has been shown to inhibit synthesis of protein, glycoprotein and nucleic acids.

The more active form of the enzyme, from cells grown with hydrocortisone, is found to have four times less tightly bound phosphate than the enzyme from control cells. Dibutyryl adenosine cyclic 3',5'-monophosphate also induces a six fold increase in cellular alkaline phosphatase activity when added to the medium at one millimolar, and this induction is additive to that caused by three micromolar hydrocortisone. Imidazole partially blocks the induction by hydrocortisone, and theophylline lowers the control level of alkaline phosphatase activity in addition to markedly

reducing the amount of induction by hydrocortisone. The combination of dibutyryl cyclic AMP and theophylline exhibits a powerful cytotoxic effect. Theophylline alone permits normal cell propagation, while dibutyryl cyclic AMP alone blocks cell growth about fifty per cent. Retinoic acid blocks the induction by hydrocortisone but not that stimulated by dibutyryl cyclic AMP. Theophylline inhibits the phosphomonoesterase activity of alkaline phosphatase when added to the assay.

Two polypeptide hormones which are important factors in calcium regulation also show some effect on cellular alkaline phosphatase activity. Parathyroid hormone slightly reduces the control level of alkaline phosphatase activity, while calcitonin induces a marked increase in activity. Neither hormone affects cell growth. Evidence is presented to suggest that alkaline phosphatase activity is regulated by phosphorylation and dephosphorylation, that the regulation is mediated by cyclic AMP, and that the *in vivo* function of the enzyme may be to regulate the level of calcium in the cytoplasm.

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