

Regulation of Alkaline Phosphatase in Human Skin Fibroblasts¹ (34596)

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(Introduced by J. L. Irvin)

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Moog (1) has demonstrated that alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) is active in all cells during early stages of embryogenesis. During intermediate stages, the enzyme is undetectable in most tissues, but in later stages it again becomes active in certain characteristic sites (*e.g.*, osteoblasts, proximal kidney tubules, and granulocytes). Fell and Danielli (2) have shown that the activity of alkaline phosphatase in adult skin fibroblasts *in vivo* is normally quite low, but that during early stages of wound healing, skin fibroblasts and associated collagen fibers transiently develop high alkaline phosphatase activity. Similar observations have been made by Bourne (3), in regenerating bone, and by Buck (4), in regenerating tendon.

Although the actual mechanism of activation of alkaline phosphatase is not well understood, it is clear that the activity of the enzyme in certain tissues is affected by adrenal glucocorticoids. Moog (1) has shown that the normal activation of alkaline phosphatase in postembryonic intestinal epithelium is accelerated under conditions of stress or by administration of ACTH or cortisone. It is well known that under conditions of

stress, levels of unconjugated 17-hydroxycorticosteroids increase in human blood plasma (5). Valentine and co-workers (6) have shown that patients under postoperative stress or those treated with ACTH or glucocorticoids exhibit elevated leukocyte alkaline phosphatase activity. Since the activity of alkaline phosphatase in these tissues is apparently increased when glucocorticoid levels are elevated, the question arises as to whether alkaline phosphatase of regenerating connective tissue is similarly controlled. To test whether such a hypothesis is valid requires the demonstration that isolated fibroblasts show increased alkaline phosphatase activity in the presence of physiological levels (10^{-6} M to 10^{-8} M) of glucocorticoid hormones. We have previously reported (7) that addition of prednisolone 21-phosphate (10^{-6} M) to diploid human skin fibroblasts in tissue culture results in significant induction of alkaline phosphatase specific activity. This study was undertaken to define certain characteristics of the induction process with respect to hormonal specificity and to show that the rate of increase in alkaline phosphatase specific activity is dependent upon the concentration of prednisolone phosphate added to the medium.

Materials and Methods. Cells and culture techniques. Human fetal skin fibroblasts were derived from dermal connective tissue of a 4-month-old female (Microbiological Associates, Bethesda, Md.) and a 4-month-old male (Grand Island Biological Co., Grand Island, N.Y.). Human newborn skin fibroblasts were established in our laboratory from a skin biopsy of a 2-week-old newborn male. Human adult skin fibroblasts were established from a skin biopsy of a 35-year-old female (obtained from Hyland Division, Travenol

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Laboratories, Los Angeles, Calif.). Stock maintenance cultures were examined bimonthly for bacterial and fungal contamination with negative results. No pleuropneumonia-like organisms were isolated from cells or media.

Cultures were maintained in cell production roller vessels (Bellco Glass, Inc., Vineland, N. J.) having a growth surface of 840 cm². Cells were dissociated for subculture using 0.25% trypsin in Earle's balanced salt solution. The normal split ratio was 2:1. New cultures were established on Eagle's basal medium with Hanks' balanced salt solution (HBME) for the initial 48 hr. The use of this medium during the first 48 hr helped to promote rapid attachment and growth of cells. Thereafter, Eagle's basal medium with Earle's balanced salt solution (EBME) was employed for maintenance. Both HBME and EBME were supplemented with 9% fetal bovine serum, 2 μ moles per ml L-glutamine, 50 units per ml penicillin, 50 μ g per ml streptomycin and 2.5 μ g per ml amphotericin B. All tissue culture media and supplements were obtained from Microbiological Associates, Bethesda, Md., except amphotericin B which was supplied by E. R. Squibb and Sons, New York, N.Y.

Experimental studies were carried out on monolayer cultures in disposable 16 \times 150 mm plastic screw cap tubes (Falcon Plastics, Los Angeles, Calif.). In order to minimize effects of selection, cells in roller vessels were trypsinized and pooled in a 500-ml spinner flask (Bellco Glass, Inc.) containing HBME. Aliquots of 2 ml (2 to 4 \times 10⁵ cells) were transferred by means of a repetitive syringe (Manostat Corp., New York, N. Y.) to the plastic tubes. Cultures were well established after incubation for 48 to 72 hr at 37°. HBME was then replaced by EBME (2 ml) with or without hormones. There was no subsequent change of medium.

Disodium prednisolone 21-phosphate of highest purity (Merck, Sharpe and Dohme Research Lab., Rahway, N.J.) was added to EBME in sterile aqueous solution representing 0.1% of the volume of the culture medium. An equivalent amount of sterile water was added to controls. Prednisolone, hydro-

cortisone, cortisone, deoxycorticosterone, estradiol-17 β , testosterone, and progesterone were obtained from Calbiochem., Los Angeles, Calif. and were added to EBME in an amount of ethanol equal to 0.1% of the total volume of medium. Prednisolone and testosterone were USP grade; other free steroids were A-grade and certified chromatographically homogeneous by the suppliers. Ethanol (0.1% of medium volume) was added to cultures serving as controls.

Experimental procedures. Randomly selected experimental and control cultures were harvested at 24- or 48-hr intervals over the course of the induction period (approximately 10–13 days). After medium was drained, the cell surface was washed four times with 2.0 ml of 0.9% saline, and 0.6 ml of 1% sodium deoxycholate (Mann Research Lab., New York, N. Y.) was then added to lyse the cells. The tubes were shaken for 30 min at 4°. Lysates (0.6 ml) were then frozen in the tubes at –20° for subsequent assays.

Alkaline phosphatase was determined by the procedure of Lowry (8). One ml of ice cold solution containing the substrate, 8 mM *p*-nitrophenyl phosphate (Mann Research Lab.) and 2 mM magnesium chloride in 0.5 M 2-amino-2-methyl-1-propanol hydrochloride (buffer pH at 10.6) was added to 0.1 ml of deoxycholate lysate. Incubation was carried out for 30 min at 37° at which time the reaction was stopped by addition of 2.5 ml of 0.25 N sodium hydroxide. Absorbance was measured at 410 m μ and compared to a standard containing 0.1 ml of 1.0 mM *p*-nitrophenol (Eastman Organic Chemicals, Rochester, N. Y.). In the results to be reported, one unit of alkaline phosphatase specific activity is equivalent to 1 nmole of *p*-nitrophenol per min at 37° per mg protein.

Protein was determined by a modification of the procedure of Lowry and co-workers (9) on 0.05 ml of deoxycholate lysate. The sample volume was diluted to 0.6 ml with all reagent volumes doubled. Absorbance was measured at 750 m μ . Samples were compared to standards prepared from twice recrystallized bovine albumin (Nutritional Biochemicals Corp., Cleveland, Ohio).

Results. As shown in Fig. 1 the induction

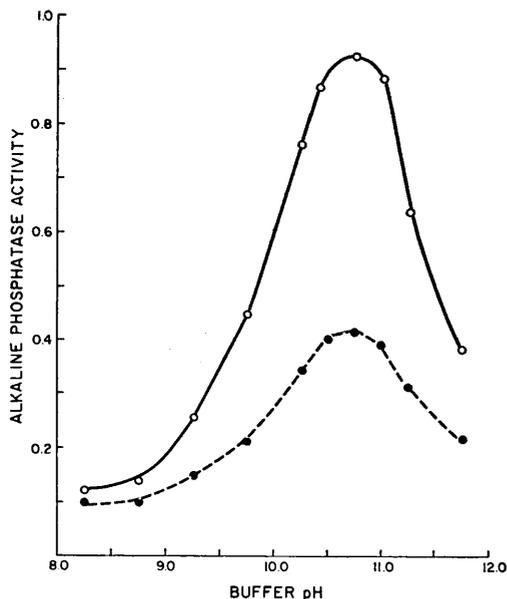


FIG. 1. Alkaline phosphatase pH-activity curves for prednisolone 21-phosphate (10^{-6} M) induced (○—○) and control (○---○) cell lysates. Cultures were harvested 7, 8, and 9 days following addition of prednisolone. Cells were lysed with 1% sodium deoxycholate and pooled. Refer to *Materials and Methods* for lysis and assay procedures. Alkaline phosphatase activity is expressed as micromoles *p*-nitrophenol per ml per 30 min at 37°. Total protein was equivalent in all samples.

of alkaline phosphatase by prednisolone 21-phosphate (10^{-6} M) does not involve a shift in the pH optimum of the enzyme. Therefore, enzyme specific activities in induced and control cultures can be compared directly.

That intrinsic activators or inhibitors are not involved in the induction of alkaline phosphatase by prednisolone phosphate was demonstrated by an experiment in which equal volumes of lysates with approximately equivalent total protein concentrations were combined from induced and control cultures harvested at the time of maximal induction. The resulting enzyme activity was the expected average value. Also, addition of prednisolone phosphate directly to lysates produced no change in enzyme activity.

Induction of alkaline phosphatase by prednisolone 21-phosphate (10^{-6} M) was observed in the four different human skin fibro-

blast strains studied (two embryonic, one newborn, and one adult). The pattern of induction was consistent in all strains at serial passages ranging from 6 to 40. The only variation in the induction time course was observed in adult skin fibroblasts where induction began about 4 days later than in rapidly growing fetal and newborn strains. The typical time course of alkaline phosphatase induction by prednisolone phosphate in fetal and newborn strains is shown in Fig. 2. Enzyme specific activity in prednisolone treated cultures increases slowly for the first 4 to 5 days and rapidly thereafter. Maximal specific activity in prednisolone-treated cultures is 3 to 8 times that observed in control cultures by the 9th or 10th day.

As shown in Fig. 3, when prednisolone 21-phosphate was added to media in final concentrations of 10^{-8} , 10^{-7} , 10^{-6} , and 10^{-5} M on day 0, the concentration dependence of the rapid phase of induction (4th through 10th day) can be readily observed. Concentrations of 10^{-8} , 10^{-7} , and 10^{-6} M produced increased enzyme specific activity at rates of 5, 10, and 15% per day, respectively. An additional tenfold increase in hormone concentration (10^{-5} M) produced no further increase in rate of induction. At all concentrations of prednisolone phosphate used in these experiments the increase in alkaline phosphatase specific activity was linear with re-

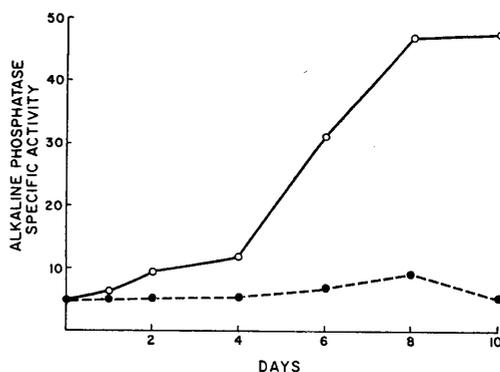


FIG. 2. Time course of alkaline phosphatase induction in human newborn skin fibroblasts following transfer 11. ○—○, Prednisolone 21-phosphate (10^{-6} M) added at zero time; ○---○, Control. Alkaline phosphatase specific activity is expressed as nanomoles *p*-nitrophenol per min at 37° per mg protein.

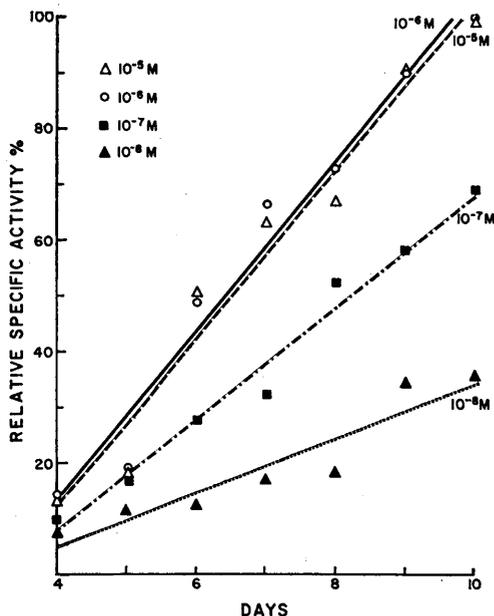


FIG. 3. Rate of induction of alkaline phosphatase specific activity during the rapid phase of increase (days 4-10) in cultures with varying concentrations of prednisolone 21-phosphate. Relative specific activity (%) was calculated by the formula:

$$\frac{SA_t - SA_c}{SA_{t \max} - SA_{c10}} \times 100$$

where, SA_t and SA_c refer to alkaline phosphatase specific activities in induced and control cultures on a given day, and where $SA_{t \max}$ refers to the maximal induced enzyme specific activity observed on the 10th day. SA_{c10} refers to the enzyme specific activity of control cultures on the 10th day.

spect to time.

A number of steroid hormones were compared in order to determine their effect on induction of alkaline phosphatase in human skin fibroblasts. Figure 4 shows that steroids having high adrenal glucocorticoid activity were most effective. The final concentration of all hormones tested was $10^{-6} M$, since it had been determined that this concentration of prednisolone 21-phosphate produced a maximal rate of induction. The relative order (highest to lowest) of effectiveness in induction of alkaline phosphatase by glucocorticoids was prednisolone 21-phosphate, prednisolone (free corticosteroid), hydrocortisone, cortisone, and deoxycorticosterone. Estradiol- 17β , testosterone, and progesterone showed

no significant inductive effect on the specific activity of the enzyme.

Discussion. Bush (10) has reported that the normal level of free hydrocortisone in human plasma is between 10^{-7} and $10^{-8} M$. Gray, Greenaway and Holness (5) report that under conditions of stress, plasma concentrations of nonprotein-bound hydrocortisone frequently reach $10^{-6} M$ (1.1 to $2.2 \times 10^{-6} M$ in nonfatal infection). In our studies prednisolone phosphate at $10^{-6} M$ produced a maximal rate of induction of alkaline phosphatase specific activity in skin fibroblasts. A further 10-fold increase in concentration of prednisolone phosphate (to $10^{-5} M$) produced no greater rate of induction of the enzyme. The specific activity of the enzyme was significantly increased in the presence of prednisolone phosphate at a concentration as low as $10^{-8} M$. By extrapolation, the hormone concentration dependence of the rate of induction of alkaline phosphatase *in vitro*

RELATIVE INDUCTION OF ALKALINE PHOSPHATASE BY EQUI MOLAR CONCENTRATIONS OF DIFFERENT HORMONES

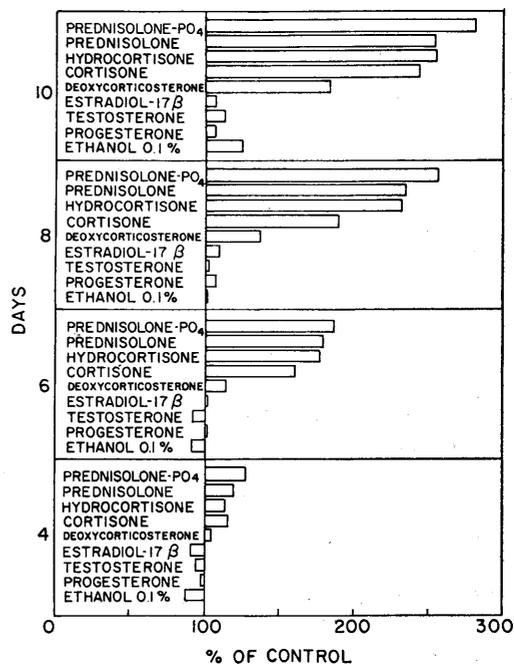


FIG. 4. Effect of $10^{-6} M$ conc of various hormones on the specific activity of alkaline phosphatase as compared to untreated controls. Free steroids were added in absolute ethanol representing 0.1% of the culture medium volume.

indicates that the activity of the enzyme in human skin fibroblasts *in vivo* may be regulated by adrenal glucocorticoids at concentrations known to be present in human plasma.

Although prednisolone phosphate was used in experiments to establish the concentration dependence of the induction process, our results indicate that a qualitatively and quantitatively similar induction of alkaline phosphatase was elicited by naturally occurring free corticosteroids, such as hydrocortisone and cortisone. Thompson, Tomkins and Curran (11) have tested hydrocortisone, deoxycorticosterone, estradiol, testosterone, and progesterone for induction of tyrosine transaminase in hepatoma tissue cultures (HTC line). They observed a pattern of hormone specificity very similar to that which we have shown for alkaline phosphatase. Melnykovich (12) has qualitatively defined the hormonal specificity for alkaline phosphatase induction in HeLa epithelioid cell cultures. In his experiments induction occurs only with steroids having hydroxyl groups at both carbon atoms 11 and 21. Berliner and Dougherty (13) have shown that fibroblasts possess an 11 β -hydroxy dehydrogenase enzyme system which carries out the conversion of cortisone to hydrocortisone. It is possible that a similar conversion accounts for the effectiveness of cortisone in inducing alkaline phosphatase in our experiments with human skin fibroblasts. Melnykovich (12) did not observe alkaline phosphatase induction with cortisone and concluded that HeLa cells probably do not convert cortisone to hydrocortisone.

The findings of Griffin and Cox (14) in epithelioid HeLa cell cultures suggest that the induction of alkaline phosphatase by prednisolone may involve a change in the conformational state of the enzyme to enhance its activity. Further studies *in vitro* will be necessary, however, to define the mechanism of the hormonal induction of alkaline phosphatase in human diploid skin fibroblasts.

Summary. The specific activity of alkaline phosphatase in human diploid skin fibroblasts in tissue culture increased following addition

of prednisolone, hydrocortisone, and cortisone. Estradiol-17 β , testosterone, and progesterone had no significant effect on induction of alkaline phosphatase in the same tissue culture system. Using prednisolone-21-phosphate (10^{-5} to 10^{-8} M), we have shown that the phase of rapid increase in enzyme activity is linear with respect to time and that the rate of increase is dependent on concentration of the hormone. Maximal rate of induction occurred at a 10^{-6} M concentration of prednisolone-21-phosphate. Evidence from our *in vitro* system indicates that the activity of alkaline phosphatase in regenerating connective tissue may be effectively regulated by physiological levels of adrenal glucocorticoids present in plasma.

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1. Moog, F., in "Cell, Organism and Milieu" (D. Rudnick, ed.), p. 121. Ronald Press, New York (1959).
2. Fell, H. B., and Danielli, J. F., *Brit. J. Exp. Pathol.* **24**, 196 (1943).
3. Bourne, G. H., *J. Anat.* **82**, 81 (1948).
4. Buck, R. C., *J. Pathol. Bacteriol.* **66**, 1 (1953).
5. Gray, C. H., Greenaway, J. M., and Holness, N. J., in "Hormones in Blood" (C. H. Gray and A. L. Bacharach, eds.), p. 498. Academic Press, London (1961).
6. Valentine, W. N., Follette, J. H., Hardin, E. B., Beck, W. S., and Lawrence, J. S., *J. Lab. Clin. Med.* **44**, 219 (1954).
7. Waters, M. D., and Summer, G. K., *Biochim. Biophys. Acta* **177**, 650 (1969).
8. Lowry, O. H., in "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), p. 371. Academic Press, New York (1957).
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.* **193**, 265 (1951).
10. Bush, I. E., *Brit. Med. Bull.* **18**, 141 (1962).
11. Thompson, E. B., Tomkins, G. M., and Curran, J. F., *Proc. Nat. Acad. Sci. U. S. A.* **56**, 296 (1966).
12. Melnykovich, G., *Biochem. Biophys. Res. Commun.* **8**, 81 (1962).
13. Berliner, D. L., and Dougherty, T. F., *Pharmacol. Rev.* **13**, 329 (1961).
14. Griffin, M. J., and Cox, R. P., *Proc. Nat. Acad. Sci. U. S. A.* **56**, 946 (1966).