*STUDIES ON THE MECHANISM OF HORMONE INDUCTION OF ALKALINE PHOSPHATASE IN HUMAN CELL CULTURES, II. RATE OF ENZYME SYNTHESIS AND PROPERTIES OF BASE LEVEL AND INDUCED ENZYMES**

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The alkaline phosphatase activity of certain HeLa cell lines is increased manyfold by growth in medium containing a hormone with adrenal glucocorticoid activity.[1-6] Although the mechanism of this increase in enzyme activity is not known, there are several possibilities such as an increased rate of enzyme synthesis, a decreased rate of enzyme degradation, or a conformational change in the enzyme leading to an increased activity of the individual enzyme molecules. The studies presented in this paper favor the last mechanism.

Materials and Methods.—Cell cultures: The methods used in this laboratory for monolayer and suspension cultures have been described.[2, 6] Two different HeLa cell lines were studied: HeLa S_3, a line with low alkaline phosphatase activity which can be increased from 5- to 20-fold by growth in medium containing prednisolone (Δ^1 hydrocortisone); and HeLa Ch, a cell line constitutive for high levels of alkaline phosphatase which is unaffected by adrenal glucocorticoid hormones.[6]

Media: Cells were grown in monolayer culture using Waymouth's medium containing 10% calf serum.[7] Suspension cultures[8] were grown in Eagle's minimal essential medium (MEM)[9] with 7% calf serum. L-leucine-1-C^{14} was added to the medium at a final concentration of 50 mμc per ml 24 hr after subculturing. Prednisolone was added to induced cultures at the same time as radioactive leucine except when otherwise indicated. The cultures were harvested about 60–70 hr later.

Alkaline phosphatase assay: Alkaline phosphatase activity was measured spectrophotometrically at 410 mμ using as substrate 8 mM p-nitrophenyl phosphate (Sigma 104) in 0.5 *M* 2-amino-2-methyl-1-propanol hydrochloride with 10^{-3} *M* $MgCl_2$ at pH 10.5 by the method of Lowry.[10]

Determination of Michaelis constants: Kinetic studies were carried out using varying concentrations of p-nitrophenyl phosphate (Sigma 104) in 0.5 *M* 2-amino-2-methyl-1-propanol hydrochloride with 10^{-3} *M* $MgCl_2$ at pH 10.5. A Cary recording spectrophotometer was used with a wavelength of 410 mμ at a fixed slit width of 0.053. Full scale deflection of 0.100 OD units was used. The Michaelis constants were determined from the initial velocity of the reactions according to the method of Lineweaver and Burk.[11]

Alkaline phosphatase preparations: Partially purified alkaline phosphatase was prepared by homogenizing about 5×10^7 cells in 6 ml of 0.05 *M* Tris hydrochloride pH 7.4 with 2 ml of n-butyl alcohol.[12] The homogenate was centrifuged at $900 \times g$ for 15 min and the aqueous layer was removed. The frothy n-butanol layer was re-extracted with 0.05 *M* Tris and the aqueous extracts were combined. The recovery of alkaline phosphatase was about 90% when compared to cells lysed in 0.5% sodium deoxycholate.

Purification of alkaline phosphatase by column chromatography: The partially purified enzyme was concentrated by filtration and dialysis using collodion bags with a porosity of less than 5 mμ (Kollodiumhulsen Gottingen). The concentrated enzyme preparation was layered on a 1×15-cm Sephadex G200 column which was developed with 0.05 *M* Tris pH 7.4. Alkaline phosphatase was eluted just after the void volume and came off the column before the second internal volume was collected. The induced enzyme consistently was eluted slightly earlier from Sephadex G200 than the base level alkaline phosphatase (prepared from cells grown in medium without prednisolone) as shown in Figure 1. The peak activity represents a further four- to fivefold purification of the enzyme with a recovery of 65–75%.

Preparation of antibody against HeLa cell alkaline phosphatase: The antigen was alkaline phosphatase prepared from HeLa Ch cells by butanol extraction followed by chromatography on Sephadex G200. The final purification of the antigen varied between 300- and 500-fold. Two white New Zealand rabbits were injected subcutaneously in four sites, interscapularly, and into the lateral thighs with a 1:1 emulsion of enzyme preparation (about 125 μg protein) in complete Freund's adjuvant.[13] They were given two booster injections at 2-week intervals and bled 7 days later. Further booster injections were repeated at 3- to 4-week intervals and bleedings done 7–9 days thereafter. The serum used for these studies was from the fourth postinoculation bleeding. For certain experiments the antiserum was heated to 56°C for 30 min in order to partially inactivate its endogenous alkaline phosphatase activity.

Immunodiffusion and immunoelectrophoresis: Immunodiffusion was performed in agar as described by Kabat and Mayer.[14]

Immunoelectrophoresis was carried out on 8 × 10-cm glass plates covered with 1% agar in 0.05 *M* barbital buffer pH 8.2 by the method of Grabar and Williams.[15] Immune precipitation was complete after 48 hr at room temperature. For certain studies, the precipitation bands were cut out for enzyme assay and radioactive measurements. The alkaline phosphatase assay was performed on homogenized slices of agar using p-nitrophenyl phosphate as substrate. The reaction was stopped, and the agar dissolved by adding 0.25 *N* NaOH followed by brief heating. Radioactive measurements on the agar strip were carried out by dissolving it in 0.3 ml of hot water and adding a dioxane scintillation solution.[16] A clear solution with negligible quenching was obtained.

Heat stability studies: Column-purified enzyme preparations were diluted in 0.5 *M* Tris at pH 10.2. The protein content of the diluted base level and induced enzymes was about 80 μg per ml. Heat stability of the enzyme preparations was tested by heating to 56°C ± 2° for 30 min. Heat inactivation studies on various alkaline phosphatase preparations also were carried out at 64.5° ± 0.5°C.

Results.—Incorporation of L-leucine-C^{14} into alkaline phosphatase during prednisolone induction: HeLa S_3 suspension cultures were grown with (induced) and without (control) prednisolone in medium containing L-leucine-C^{14}. Figure 1 shows the distribution of protein, alkaline phosphatase activity, and radioactivity obtained upon Sephadex G200 chromatography of the concentrated n-butanol extract of cell lysates. Fractions of 0.5 ml were collected and were numbered starting just after the internal volume was eluted. The two fractions showing the highest alkaline phosphatase activity from each column were pooled, and the enzyme preparation was precipitated with antiserum. Enzyme activity and radioactivity were measured on enzyme preparations from control and induced cultures purified by column chromatography and those purified further by antibody precipitation. The results are shown in experiment I of Table 1. Although the amount of radioactivity incorporated into the protein peaks containing the alkaline phosphatase

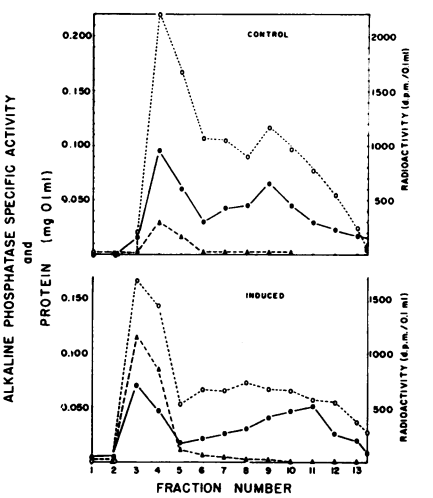

FIG. 1.—Elution from Sephadex G200 of alkaline phosphatase preparations from HeLa S_3 cells grown in medium without prednisolone (control) and cultures grown with prednisolone (induced). △---△ Alkaline phosphatase specific activity μmoles of p-nitrophenol released/15 min incubation at 37°C/0.1 mg of protein; ●—● mg of protein/0.1 ml; O---O radioactivity disintegrations/min above background [av. 55 dpm/0.1 ml]. Counting efficiency 80% for C^{14}.

TABLE 1

ALKALINE PHOSPHATASE ACTIVITY AND RADIOACTIVITY OF CHROMATOGRAPHIC FRACTIONS AND ANTIBODY PRECIPITATES OF HELA CELL EXTRACTS

Expt.	Cell line	Fractions assayed	Alkaline Phosphatase[a]		L-Leucine-1-C^{14} Incorporation[b]		Protein[c]		Specific Activity of Enzyme Antibody Precipitate	
			Column fractions	Antibody precipitate	Column fractions	Antibody precipitate	Column fractions	Antibody precipitate	Alkaline phosphatase[d]	Radioactivity[e]
I	HeLa S_3 suspension cultures	#4, #5 (control)	0.020	0.022	1930	480	80	33	0.67	15
		#3, #4 (induced)	0.105	0.087	1510	410	62	23	4.22	18
II	HeLa S_3 monolayer cultures	#4, #5 (control)	0.087	0.060	1090	257	120	18	3.33	14
		#3, #4 (induced)	0.510	0.500	860	260	110	16	31.25	16

[a] μMoles p-nitrophenol liberated per 15 min incubation/1.0 ml enzyme at 37°C.
[b] Disintegrations per min above background (av. 55 dpm) at 80% counting efficiency.
[c] μG protein/1.0 ml.
[d] μMoles p-nitrophenol liberated per 15 min incubation μg protein/0.1 ml suspension.
[e] Dpm/μg protein/0.1 ml suspension.

from control and induced cultures is approximately the same, the enzyme activity is fivefold higher in the protein peak from the induced cultures. Nearly quantitative recovery of alkaline phosphatase activity was found when these chromatographic fractions were precipitated by antiserum (experiment I, Table 1). However, the enzyme-antibody precipitate contained only one fourth of the counts present in the enzyme peaks. This finding indicates that three fourths of the radioactivity associated with the protein peak containing the alkaline phosphatase activity was incorporated into nonalkaline phosphatase protein. The proportion of radioactivity and total protein was the same in enzyme-antibody precipitates from control and induced cultures. This is most clearly seen in a comparison of specific enzyme activity and specific radioactivity of the enzyme antibody precipitates as shown in the right-hand column of Table 1. In another experiment performed with HeLa S_3 cells in suspension culture, not shown in Table 1, the total L-leucine-C^{14} incorporation was lower, but the results were similar in every other respect to those shown in experiment I.

Experiment II, Table 1, was carried out with monolayer cultures of HeLa S_3 cells. Despite a sixfold increase in alkaline phosphatase specific activity in cultures grown in medium with prednisolone, the amount of radioactive leucine-1-C^{14} incorporated into the enzyme-antibody precipitate is the same in control and induced cultures. Moreover, the total protein precipitated and the radioactivity of this antigen antibody precipitate is the same for control and induced cultures.

In another experiment performed on HeLa S_3 cells grown in monolayer culture, 17 mμc per ml of radioactive leucine were added at three time intervals, 17, 31, and 43 hr after prednisolone. In this experiment the amount of radioactivity incorporated into the chromatographic fractions from cells harvested at 69 hr was much less than

when a single dose of 50 mμc per ml of leucine-1-C^{14} was added at the same time as prednisolone. Moreover, when alkaline phosphatase was precipitated by antiserum, smaller but equal amounts of radioactivity were present in the antigen-antibody precipitate from both control and induced cultures.

Specific precipitation of radioactive counts by antiserum: The capacity of undiluted antiserum to precipitate alkaline phosphatase and an estimate of the number of radioactive counts nonspecifically trapped in the antigen-antibody complexes were obtained in the following experiments. Nonradioactive HeLa cell alkaline phosphatase was added as carrier to the radioactive enzyme from control and induced cultures in experiment II and the mixture was reacted with antiserum. Although the resulting antigen-antibody precipitate contained about four times as much protein, the radioactivity of the precipitate was only 10 per cent greater than without added carrier. This suggests that the precipitation of enzyme in all these experiments was carried out with excess antibody and that the radioactive counts in the antigen-antibody precipitate represent specific precipitation of labeled protein rather than a nonspecific trapping of radioactive counts.

Precipitation of base level and induced alkaline phosphatase preparations by increasing concentrations of anti-HeLa alkaline phosphatase antibody: Figure 2 shows the amount of enzyme activity remaining in the supernatant when constant amounts of base level and induced alkaline phosphatase were precipitated by increasing concentrations of antiserum. Despite a four- to fivefold greater enzyme activity in the induced alkaline phosphatase preparations, the induced and base level enzymes were completely precipitated at the same antibody concentration. This evidence suggests that the amounts of enzyme protein in the induced and base level alkaline phosphatase preparations are approximately the same.

Specificity of anti-HeLa alkaline phosphatase antibody: An estimate of the specificity of the antiserum was obtained by means of immunodiffusion and immunoelectrophoresis. Immunodiffusion showed a single zone of precipitation with a line of identity between the enzymes prepared from control and induced HeLa S_3 cells and from HeLa Ch cells. Upon immunoelectrophoresis of induced HeLa S_3 alkaline phosphatase preparations, there are three bands of precipitation. The first two cathodal bands contain alkaline phosphatase activity as measured by staining the agar for alkaline phosphatase and by enzyme assay performed on agar strips cut from the electrophorogram. The anodal precipitate has no alkaline phosphatase activity. Chromatographic fractions containing radioactive alkaline phosphatase

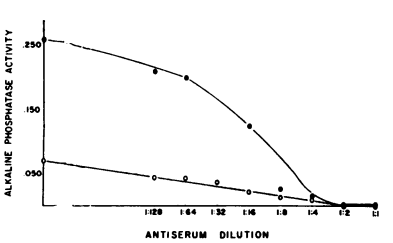

FIG. 2.—Precipitation of alkaline phosphatase preparations derived from induced and control HeLa S_3 cultures by antialkaline phosphatase antibody. The enzymes were prepared by chromatography on Sephadex G200 as described in *Materials and Methods*. Enzyme preparation of 0.1 ml (8.0 μg protein) was mixed with 0.1 ml of diluted antiserum as indicated on the abscissa. The precipitate was removed by centrifuging and the alkaline phosphatase activity remaining in the supernatant was assayed. The alkaline phosphatase activity of the diluted antiserum was subtracted from the total activity to give the remaining induced and control enzyme activity (ordinate). At high antibody concentrations (>1:8) the enzyme activity precipitated was measured in the precipitate. O—O Control (alkaline phosphatase prepared from HeLa S_3 cells grown in medium *without* prednisolone); ●—● induced (alkaline phosphatase prepared from Hela S_3 cells grown in medium *with* prednisolone).

TABLE 2

PHYSICAL AND CHEMICAL CHARACTERISTICS OF ALKALINE PHOSPHATASE FROM HELA CELL CULTURES

Cell line	Heat stability* at 56°C	Inhibition Index† L-phenylalanine	Inhibition Index† L-tryptophane	Michaelis constants (K_m)
HeLa S_3				
Control	98	1.0×10^{-3}	1.0×10^{-3}	4×10^{-4}
Induced	96	1.0×10^{-3}	1.0×10^{-3}	4×10^{-4}
HeLa Ch	73	1.8×10^{-3}	1.6×10^{-3}	

* Per cent of alkaline phosphatase activity remaining after heating to 56°C for 30 min.
† μMoles of inhibitor required to produce a 50% inhibition of alkaline phosphatase activity.

obtained from HeLa S_3 cells in experiment I, Table 1, were diluted with nonradioactive carrier enzyme and analyzed by immunoelectrophoresis. The anodal "contaminant" band had only 12–15 per cent of the total radioactivity, and the remainder of the counts were recovered in the two lower precipitin bands with alkaline phosphatase activity.

Physical properties of alkaline phosphatase from uninduced and induced cell cultures: Alkaline phosphatase isolated from HeLa cell cultures comprises a heterogeneous group of enzymes as shown by electrophoresis.[17] Hormonal induction of alkaline phosphatase in HeLa S_3 cells leads to an increase in the enzymatic activity of all the alkaline phosphatase bands without changing the electrophoretic pattern or rate of migration.[17] Furthermore, both the induced and base level alkaline phosphatases are heat-stable at 56°C, while the consitutive HeLa Ch enzyme loses about 27 per cent of its activity at this temperature as seen in Table 2.[18] We have recently found that the alkaline phosphatase which is induced by prednisolone in cell cultures is inhibited by low concentrations of either L-phenylalanine or L-tryptophane, a trait shared with the hormone-inducible human leukocyte alkaline phosphatase.[17, 19] Table 2 shows the concentration of these amino acids which inhibit the alkaline phosphatase activity by 50 per cent. The induced and base level enzymes have identical inhibition indexes. The Michaelis constants (K_m) were also the same for the base level and induced alkaline phosphatase as seen in Table 2.

Despite these similarities between the base level and induced alkaline phosphatase preparations, the two enzymes differ with respect to certain other properties. For instance, when heat stability is studied at 64.5°C rather than 56°C, the induced enzyme was found to be considerably more labile than the base level enzyme. As seen in Figure 3, the base level activity is reduced by only 50 per cent while the induced alkaline phosphatase loses nearly 95 per cent of its enzymatic activity and the amount of alkaline phosphatase activity remaining approaches the activity of the base level enzyme. Figure 4 shows that at any given time the percentage of enzyme molecules inactivated at 64.5°C is significantly higher for the induced enzyme.

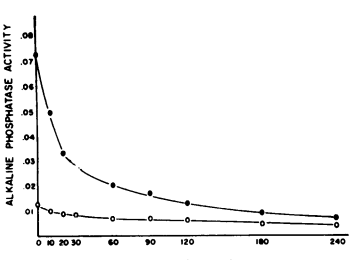

FIG. 3.—Heat inactivation of alkaline phosphatase preparations from control and induced cultures at 64 ± 0.5°C. Enzyme preparations were Sephadex G200 column fractions with the peak alkaline phosphatase activity. The protein content of both base level (control) and induced alkaline phosphatase was 80 μg/ml. O—O Control (base level) alkaline phosphatase prepared from cells grown in meduim *without* prednisolone; ●—● induced enzyme (alkaline phosphatase prepared from cells grown in medium *with* prednisolone).

A further difference between the base level and induced enzyme preparations is seen in Figure 1. The induced alkaline phosphatase preparation consistently elutes slightly earlier from Sephadex G200 than the base level enzyme, suggesting that the induced enzyme has a greater molecular size. Table 3 shows a third difference between the induced and base level alkaline phosphatases. The initial velocity (V_0) of the induced alkaline phosphatase is four to five times that of the base level enzyme at all the substrate concentrations tested.

TABLE 3

INITIAL VELOCITY OF INDUCED AND BASE LEVEL ALKALINE PHOSPHATASE AT VARIOUS SUBSTRATE CONCENTRATIONS

Substrate concentration	Enzyme preparations V_0 base level*	V_0 induced*
4×10^{-3}	0.946	4.36
1×10^{-3}	0.694	3.30
7×10^{-4}	0.538	2.83
1×10^{-4}	0.204	1.02
2×10^{-5}	0.052	0.245

Initial velocity was read from a continuous spectrophotometer recording carried out in a Cary model 15 spectrophotometer.

* Mμmoles of p-nitrophenol liberated/min/μg of protein at 25°C.

Discussion.—The findings presented in this paper suggest that neither an increased *de novo* synthesis of alkaline phosphatase nor a hormone-mediated decrease in the catabolism of the enzyme is responsible for the induction of increased alkaline phosphatase activity. Both these processes would produce an increase in total enzyme protein in induced cultures. However, the radioactive leucine incorporated into HeLa S_3 alkaline phosphatase and the total amount of protein precipitated by antibody were the same in cells whether prednisolone was present or not. Furthermore, the same concentration of antibody completely precipitated both the base level and the induced alkaline phosphatase. It would therefore appear that despite a fivefold increase in enzyme activity in induced cultures, the concentration of alkaline phosphatase is the same in induced and uninduced HeLa S_3 cultures. These conclusions are supported by the following evidence on the specificity of the antibody for HeLa cell alkaline phosphatase:

(1) The radioactive counts in the antigen-antibody precipitate represent a specific precipitation of radioactive protein by antibody rather than nonspecific trapping of radioactive counts since increasing the amount of precipitate fourfold by adding unlabeled alkaline phosphatase as carrier increased the radioactivity of the precipitate by only 10 per cent.

(2) Immunodiffusion in agar shows a single band of precipitate with lines of identity formed between base level and induced alkaline phosphatase from HeLa S_3 cultures.

(3) Immunoelectrophoresis of alkaline phosphatase prepared from HeLa S_3 cultures grown in the presence of radioactive leucine shows two bands with alkaline phosphatase activity containing 80 per cent of the radioactivity and a third band without alkaline phosphatase activity containing less than 20 per cent of the radioactive counts. Each of these bands has a single line of precipitation, indicating that they are each predominantly a single species of protein.[14] The above evidence makes it unlikely that radioactive-contaminating protein in the antigen-antibody precipitate can explain the results.

Previous studies using inhibitors of protein synthesis have shown that *de novo* synthesis of alkaline phosphatase is required for prednisolone induction in HeLa S_3 cells.[6] Furthermore, after adding this hormone to cultures of HeLa S_3 cells, 15–24 hr elapsed before the alkaline phosphatase activity increased.[2, 5, 6] Moreover,

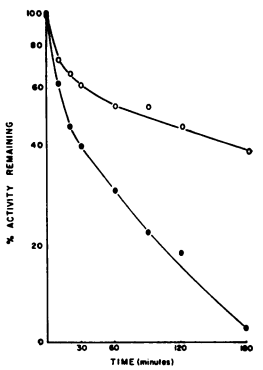

FIG. 4.—Alkaline phosphatase activity remaining after heating to 64 ± 0.5°C. See legend Fig. 3.

adding prednisolone to cell lysates or homogenates does not change their alkaline phosphatase activity.[2, 3] These data suggest that protein synthesis is required for the induction of alkaline phosphatase. An activation of apoenzyme or a simple configurational change in preformed alkaline phosphatase would not be consistent with the findings presented above.

The rate of synthesis of the alkaline phosphatase protein appears to be the same in cells grown in the presence or absence of prednisolone. However, under conditions of induction an altered enzyme with higher specific activity is formed. The alkaline phosphatase prepared from cells grown in medium with prednisolone also differs from the base level enzyme with regard to heat inactivation at 64.5°C and speed of elution from Sephadex G200 columns. The induced and base level enzymes, however, are similar in their electrophoretic mobility both on starch gel and polyacrylamide disk gel,[17] the degree of inhibition produced by low concentrations of L-phenylalanine and L-tryptophane,[17] and their Michaelis constants.

Enzyme induction occurs either by increasing the number of enzyme molecules or by increasing the catalytic efficiency of the enzyme. It is well known that an increased rate of enzyme synthesis or a decreased rate of enzyme degradation can induce an increase in enzyme concentration. For example, certain substances can stabilize pre-existing enzyme protein and delay its degradation. Such a mechanism has been reported for substrate induction of tryptophane pyrrolase by tryptophane in rat liver.[20] A second mechanism for enzyme induction is an increased rate of enzyme synthesis. Hydrocortisone induction of tryptophane pyrrolase, glutamic alanine transaminase, and tyrosine transaminase in rat liver involves an increase in the rate of synthesis of these enzymes.[20–23] Both of these mechanisms lead to an increase in the amount of enzyme protein. Our findings that the amount of enzyme and the rate of synthesis both appear to be the same in control and prednisolone-induced HeLa cells seem to exclude these two mechanisms. A third mode of induction is an increase in the activity of individual enzyme molecules. Hormones have been shown to alter the configuration of previously synthesized enzyme molecules and to change the substrate specificity of these enzymes *in vitro*.[24, 25] This does not seem to be the case with prednisolone induction of alkaline phosphatase in HeLa cells since 15–20 hr of growth in medium containing the steroid are required before enzyme activity increases and enzyme synthesis is necessary for induction.[6] Our findings suggest that prednisolone mediates a configurational change in the enzyme during its synthesis. This altered configuration or assembly of the enzyme which occurs during synthesis leads to an increase in the number of catalytic sites or a lowering of the energy level of the enzyme-substrate transition state. These changes in enzyme structure are accompanied by differences in certain physical properties of induced enzyme when compared to base level alkaline phosphatase.

Cline and Bock have recently proposed that configurational changes which occur in protein during synthesis may alter the function of these proteins or their rate of synthesis.[26] The interactions of specific substances such as hormones or metabolites

with polypeptide chains during their synthesis may affect the configuration or assembly of the protein and thereby influence gene expression. Our findings on prednisolone induction of alkaline phosphatase provide evidence for a translational control of enzyme induction in HeLa cell cultures. Alternatively, prednisolone might induce the synthesis of another substance which interacts with alkaline phosphatase to enhance its enzyme activity.

Summary—The induction of alkaline phosphatase by prednisolone in HeLa cell cultures appears to occur at the level of protein synthesis (translation) as a result of a steroid-induced change in the conformational state of the enzyme during its synthesis.

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