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AN IMMUNOLOGICAL AND BIOCHEMICAL ANALYSIS OF ALKALINE PHOSPHATASE IN HeLa CELLS EXPOSED TO 5-IODO-2′-DEOXYURIDINE

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Abstract—As ascertained by antibody titration, 5-iodo-2′-deoxyuridine (IUdR) appears not to increase alkaline phosphatase activity by increasing the concentration of the enzyme in HeLa cells but rather more probably by increasing the catalytic activity of the enzyme molecule. However, continued induction of activity ceases when protein synthesis is inhibited. The specific activity attained by the combined use of IUdR and hydrocortisone was equal to or less than the sum of specific activities obtained when either agent was used alone. The moles of phosphate bound per mole of alkaline phosphatase was similar for the enzyme from control cells or cells treated with IUdR or hydrocortisone. Even though each of several compounds tested inhibited alkaline phosphatase activity by 50 per cent at a different concentration, there was no difference between the concentrations needed for each compound to cause 50 per cent inhibition of the enzyme from control or IUdR-treated cells.

The addition of 5-iodo-2′-deoxyuridine (IUdR) to the medium of a HeLa cell culture results in an increased alkaline phosphatase specific activity [1]. Hydrocortisone also increases alkaline phosphatase specific activity in HeLa cells. The increased activity caused by hydrocortisone is due to an increase in the catalytic efficiency of the enzyme rather than an increased number of enzyme molecules [2]. Also, with regard to hydrocortisone induction, Bazzell *et al.* [3] recently reported that the induced form of alkaline phosphatase differs from the control enzyme in having approximately half as many phosphate residues per molecule of enzyme. The authors suggested that the decrease in phosphate residues associated with the enzyme alters its catalytic activity. The following report is a similar investigation of the control and the IUdR-induced forms of alkaline phosphatase. As in the case of hydrocortisone induction, we find that there probably is an altered form of the enzyme rather than more enzyme molecules. However, we were unable to detect a difference in the phosphorylation of alkaline phosphatase from control cells or cells treated with IUdR or hydrocortisone.

MATERIALS AND METHODS

Growth of cells. HeLa S3 cells (obtained from Dr. Paul H. Atkinson of Albert Einstein College of Medicine) were grown either in suspension with Eagle's Minimum Essential Medium modified for suspension culture plus 10% fetal calf serum or in mono-layers with Eagle's Minimum Essential Medium plus 10% fetal calf serum. No antibiotics were used in the media. Routine testing of the cells showed them to be free of mycoplasma contamination. Efforts were made to minimize exposure of cells to light because of the use of IUdR.

Preparation of cell extracts and alkaline phosphatase assay. Appropriate portions of monolayer cells were harvested by scraping into phosphate-buffered saline (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.6 mM KCl and 1.4 mM KH_2PO_4) and centrifugation. Suspension culture cells were simply centrifuged. The pelleted cells were resuspended in phosphate-buffered saline and centrifuged again. After the second centrifugation, the cell pellet was resuspended in 50 mM Tris–HCl (pH 7.8) and frozen until assayed. The thawed cells were disrupted with a sonicator (Heat Systems-Ultrasonics, Inc., Plainview, NY) and assayed for alkaline phosphatase activity and protein content.

Alkaline phosphatase activity was determined by measuring the formation of *p*-nitrophenol from *p*-nitrophenylphosphate. The reaction mixture contained 8 mM *p*-nitrophenylphosphate, 90 mM 2-amino-2-methyl-1-propanol (pH 10.5), 1 mM $MgCl_2$ and cell extract. Incubations were at 37°. *p*-Nitrophenol was measured by absorption at 410 nm. One unit of activity was defined as the formation of 1 nmole *p*-nitrophenol/min/ml.

Purification of alkaline phosphatase. Cells grown in suspension culture were used for enzyme purification. The purification steps were essentially the same as described by Cox *et al.* [2] and Bazzell *et al.* [3]. After appropriate treatment, the cells were harvested by centrifugation and washed by suspension in phosphate-buffered saline and recentrifugation. The cell pellet was resuspended in a solution of ice-cold 50 mM Tris–HCl pH 8.6, at a final cell concentration of 4.0×10^7/ml. A small portion was removed, diluted 1:5 with water and frozen until assay at which time the thawed cells were sonicated and assayed for alkaline phosphatase activity and protein content, as was customary after each step in purification.

The remaining portion of each sample was mixed with ½ volume of cold *n*-butanol with a Vortex Genie Mixer (Scientific Products, Evanston, IL) for about 3.5 min. The suspension was centrifuged at 15° for 8 min at 2000 rev/min (International Centrifuge model

PRJ, rotor no. 269). The bottom, aqueous layer was carefully removed with a Pasteur pipette and stored in an ice bath. To the remaining frothy layer, a $\frac{1}{2}$ volume of buffer (50 mM Tris–HCl, pH 8.6) was added. The suspension was mixed and centrifuged as before, followed by a second aqueous extraction. The aqueous extracts were combined and dialyzed overnight in the cold against three 2-liter changes of buffer (50 mM Tris–HCl and 50 mM NaCl, pH 7.4). After dialysis, samples were concentrated to a volume of 3–4 ml by blowing air over the dialysis tubing.

Each sample was layered on a 30 × 0.9 cm Sephadex column containing G-200 Sephadex gel (Pharmacia Fine Chemicals, Uppsala, Sweden) previously equilibrated with the eluent buffer of 50 mM Tris–HCl and 50 mM NaCl, pH 7.4. Fractions were collected at room temperature and assayed for alkaline phosphatase activity. The peak fractions were pooled for electrofocusing.

A 110-ml electrofocusing column (LKB, Rockville, MD) was used essentially as described in the LKB instruction manual. Electrofocusing was for 24 hr at 900 V. These conditions were adequate for focusing of proteins, as judged by the sharpness of the alkaline phosphatase band. Fractions were collected, the pH was measured so as to determine the isoelectric point for each preparation (i.e. the pH of the fraction with peak activity) and assayed for alkaline phosphatase activity. Peak activity fractions were pooled and dialyzed extensively against five 2-liter volume of a solution of 10 mM Tris–HCl pH 7.4. After dialysis, samples were concentrated as before to a volume of 5–6 ml for phosphate analysis and/or injection into rabbits for antibody production. As reported by Bazzell *et al.* [3] and other investigators, isolectrofocusing resulted in considerable loss of enzyme activity. Verification of alkaline phosphatase purity was based upon SDS-acrylamide gel analysis performed essentially as described by Laemmli [4]. After isolectrofocusing, the recovered alkaline phosphatase activity corresponded to only a single visible band of protein. No other visible bands of protein except that containing alkaline phosphatase activity were seen.

Phosphate analysis. Bovine pancreatic ribonuclease A and deoxyribonuclease were added at a final concentration of 10 μg/ml each to the purified samples of alkaline phosphatase. The samples were dialyzed for about 16 hr at room temperature against three 1.1 vol. of 10 mM Tris–HCl buffer, pH 7.4. Perchloric acid was added to quadruplicate portions of each preparation at a final concentration of 10 g/100 ml. After incubation for at least 1 hr in ice, the samples were centrifuged for 12 min at 5000 *g* at 4°. The supernatant fluids were carefully removed and the pellets were saved for phosphate [5] and protein [6] assay.

The standards (in 1 to 5-μl volumes) and sample pellets were dried in an oven overnight at 95° with $CaSO_4$. After drying, 0.1 ml of ashing mixture (10 N H_2SO_4 and 4.55 g/100 ml of perchloric acid) was added. The tubes were heated in an oven at 95° for 2 hr and then immediately at 165° for 2 hr. The tubes were allowed to cool to room temperature before adding 1.0 ml of a solution containing ascorbic acid (1.0 g/100 ml), ammonium molybdate (0.225 g/100 ml) and 90 mM sodium acetate. The samples and standards were placed in a water bath at 37° for 2 hr and then their absorption was determined at 820 nm. The calculations for moles of phosphate bound per mole of enzyme assumed a molecular weight of 120,000 as per Bazzell *et al.* [3].

Antibody production and titrations. A volume of 3–5 ml made up of equal volumes of purified enzyme (containing about 3 mg protein) from control cells and Freund's complete adjuvant was injected into a rabbit. Three such injections were made subcutaneously at 2-week intervals, followed within a week by a heart-puncture bleeding. The blood was allowed to incubate for 30 min at room temperature and in the refrigerator for 30 min. Centrifugation was done at 0° for 15 min at 300 *g*. The serum was collected and centrifuged a second time for 5 min, after which it was apportioned into 1.0-ml aliquots and frozen until used.

For antibody titration, 0.2 ml of suitably diluted enzyme samples was incubated at 37° with 0.2 ml of appropriately diluted antiserum for 2 hr, followed by incubation at 4° for 72 hr. The samples were centrifuged at 5000 *g* for 10 min at 4°. The supernatant fluids were collected and assayed for alkaline phosphatase activity. After washing the precipitates twice by centrifugation and resuspension with ice-cold NaCl solution (0.9 g/100 ml), pellets were resuspended in 0.3 ml of 50 mM Tris–HCl buffer, pH 7.4, and assayed for alkaline phosphatase activity. Enzyme sample dilutions were made with water and antiserum dilutions were made with 50 mM Tris–HCl, pH 7.4.

Chemicals. Except as the following specifies, all chemicals were obtained from Fisher Scientific Co., Fair Lawn, NJ or Mallinckrodt Chemicals Works, St. Louis, MO. Sigma Chemical Co., St. Louis, MO, was the source for *p*-nitrophenyl phosphate, 2-amino-2-methyl-1-propanol buffer and hydrocortisone-21-sodium succinate. Beef pancreas deoxyribonuclease was obtained from Miles Laboratories, Inc., Elkhart, IN. Bovine pancreas ribonuclease A was a product of Worthington Biochemical Corp., Freehold, NJ.

RESULTS

Immunological measurement of alkaline phosphatase. Antibody against alkaline phosphatase purified from control cells was used to measure alkaline phosphatase in control and IUdR-treated cells. Of several such experiments which yielded similar results, the experiment depicted in Fig. 1 was chosen for presentation because of the striking 13.7-fold increase in specific activity in the IUdR-treated cells relative to control (usually increments of about 4-fold are found and only occasionally are higher values obtained). Butanol extracts (13.6-fold difference in specific activity, 93 and 95 per cent recovery of total activity compared to sonicated cells for control and IUdR-treated cells, respectively) of these two preparations were incubated at various dilutions with a constant amount of antibody. Figure 1A shows that as increased amounts of protein were added from either control or IUdR-treated cells increased alkaline phosphatase activity was precipitated. At the highest amount of protein used, enzyme activity from both control and treated cells appears to have reached or almost reached a maximum. What is significant to

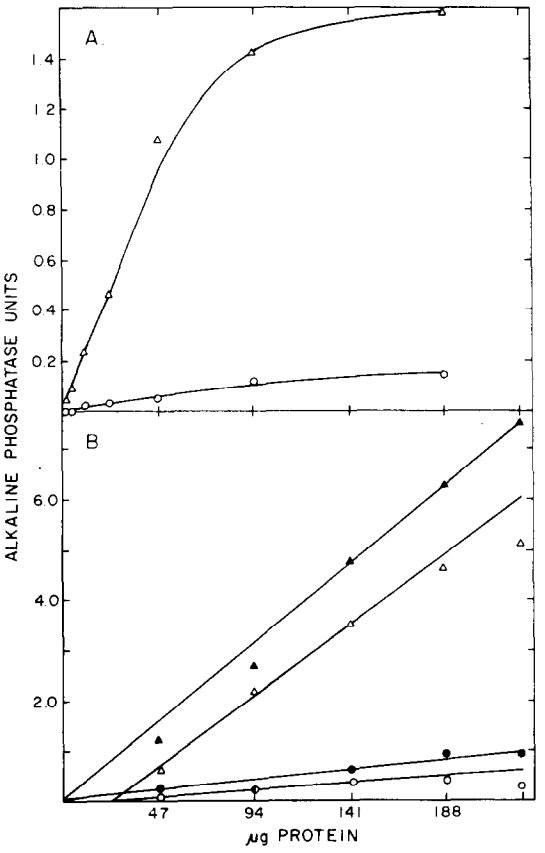

Fig. 1. Antiserum precipitation of alkaline phosphatase from control and IUdR-treated cells. Cells were grown in suspension with or without 3 μM IUdR for 72 hr, as described under Materials and Methods. The respective cultures were harvested and butanol extracts were made of each as described for enzyme purification. The extract from control cells had a specific activity of 4.0 units/mg of protein and that from IUdR-treated cells of 54.3 units/mg of protein. Several dilutions of the extracts from control or IUdR-treated cells were added in 0.2-ml volumes to incubate with 0.2-ml volumes of a 1:4 dilution of antiserum that had been prepared by injecting purified alkaline phosphatase from control cells into rabbits. The highest total amount of enzyme units per incubation with antiserum for control and IUdR extracts was 0.9 and 12.2 units respectively. Incubation conditions are described under Materials and Methods. Precipitated enzyme was separated from soluble enzyme by centrifugation. The alkaline phosphatase activity in the (A) precipitates and (B) supernatant fluids after incubation with antiserum was determined for enzyme from control (○) and IUdR-treated (△) cells. Also shown in (B) is the activity when enzyme from control (●) and IUdR-treated (▲) cells were incubated with 0.2 ml of 50 mM Tris–HCl buffer, pH 7.4, instead of antiserum. For the enzyme incubated with buffer, only the enzyme activity found in the supernatant fluid is depicted, as no activity was precipitated. Also, incubation of either the control or IUdR-treated cell extract with control serum (prepared from a rabbit receiving injections only of the vehicle used to suspend the purified enzyme) resulted in no precipitation of enzyme activity. The abscissa represents the μg of protein of the cell extract used per incubation with the antiserum. The ordinate represents the total units of alkaline phosphatase activity recovered in the (A) precipitate and (B) supernatant fluid. Recovery of added units of activity was modest but comparable for incubations of extracts from either control or IUdR-treated cells, e.g. at 188 μg protein representing 10.2 units of activity for the extract from IUdR-treated cells, 1.6 units were recovered from the precipitate and 4.6 units from the supernatant fluid for a 61 per cent recovery of added activity; recovery from the control at 188 μg protein was 51 per cent.

note is that when equal amounts of cell extract protein from control and treated cells were added, the activity precipitated from the preparation from IUdR-treated cells was 10.6-fold greater than that from control cells. This agrees well with the 13.6-fold difference in specific activities found for these two preparations and indicates that the difference in specific activity was probably due to a more catalytically active molecule rather than a greater number of enzyme molecules. This conclusion is corroborated by the data in Fig. 1B depicting the change in activity found in the supernatant fluid under the same conditions described in Fig. 1A. Despite the 13.6-fold difference in activity, the enzyme activity from both the control and IUdR-treated cells extrapolates back to the same protein concentration on the abscissa.

Interaction of IUdR and hydrocortisone on induction. Cox *et al.* [2] reported that hydrocortisone also induced alkaline phosphatase activity in HeLa cells by increasing the catalytic efficiency of the enzymes. Since both IUdR and hydrocortisone appear to increase alkaline phosphatase specific activity in a similar manner, it would be expected that addition of both agents together should yield an additive or less than additive increment in enzyme specific activity, as compared to each agent used alone. Figure 2 shows that this did happen. At 24 and 48 hr, the specific activity in cells treated with both compounds was about equal to the sum of specific activities in cells in which either compound was used alone. At 72 and 96 hr, the specific activity of alkaline phosphatase from cells treated with both agents was less that the sum of specific activities in cells treated with either agent alone. If these compounds did not cause induction in a similar manner, it might be expected that use of both together would result in a greater than additive increment in specific activity. It should be emphasized that this finding of an additive or less than additive effect of the two agents is certainly not conclusive proof that IUdR and hydrocortisone increase alkaline phosphatase specific activity in a similar manner but is only consistent with this supposition.

Inhibition of protein synthesis and induction. Cycloheximide was added to control and IUdR-treated cells 24 hr after addition of the analog (Fig. 3). Addition of this inhibitor of protein synthesis caused what appears to be an immediate cessation of enzyme induction. There are two obvious interpretations of this effect. First, IUdR may increase alkaline phosphatase activity by inducing the synthesis of a modifier protein and thus inhibition of further synthesis of modifier protein prevents further increases in enzyme activity. Second, modification of alkaline phosphatase to increase activity may only be made on newly synthesized enzyme and thus inhibiting protein synthesis prevents further supply of newly synthesized alkaline phosphatase for modification. The second alternative has also been advanced by Bazzell *et al.* [3] in explanation of the finding that hydrocortisone induc-

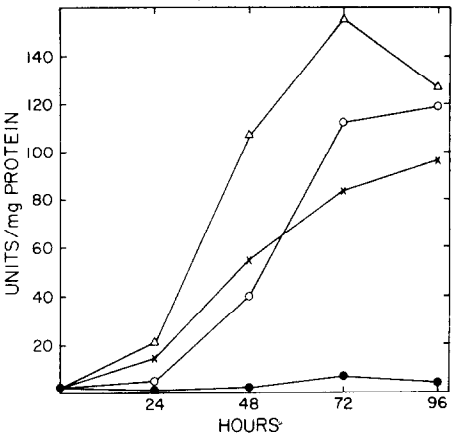

Fig. 2. Induction of alkaline phosphatase activity by IUdR and hydrocortisone alone and in combination. At time zero, 3 μM IUdR (○) and 1 μM hydrocortisone (×) or both 3 μM IUdR and 1 μM hydrocrotisone (△) were added to monolayer cultures. One set of cultures received no drug (●). Cultures were harvested at the indicated times for assay of alkaline phosphatase activity and protein concentration. The abscissa is the time in hr that the cells were treated and the ordinate the alkaline phosphatase specific activity.

tion is also blocked by inhibiting protein synthesis. It is also possible that both explanations are correct, i.e. a modifier protein is made that can alter only newly synthesized alkaline phosphatase.

Alkaline phosphatase associated phosphate. In light of the report by Griffin *et al.* [7] that alkaline phosphatase from hydrocortisone-treated cells had less bound phosphate than the enzyme from control cells, we examined the phosphate content of alkaline phosphatase from IUdR-treated and control cells. The enzyme was purified from suspension cultures by the procedures detailed under Materials and Methods. Cells were treated with 3 μM IUdR for 24 hr at which time

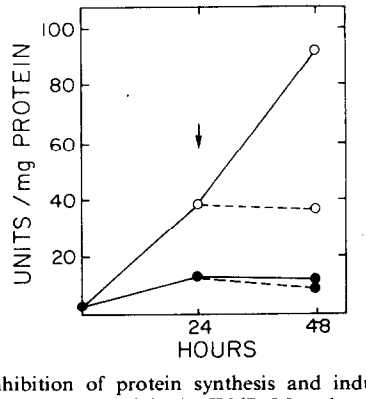

Fig. 3. Inhibition of protein synthesis and induction of alkaline phosphatase activity by IUdR. Monolayer cultures were incubated with (○) or without (●) 3 μM IUdR. At 24 hr, as indicated by the arrow, cycloheximide (30 μg/ml, final concentration) was added to a set of control and IUdR-treated cultures (------). One set of control and IUdR-treated cultures received no further additions (——). At 48 hr, the remaining cultures were harvested and all the samples were assayed for alkaline phosphatase activity and protein concentration. The abscissa and ordinate are as described in Fig. 2.

an equal volume of drug-free medium was added. At 48 hr, the culture volume was again doubled by addition of another equal volume of drug-free medium. Changes in the medium volume for control cells were made in a like manner. Cells were harvested for purification and analysis at 72 hr. A similar procedure was followed for cultures treated with 1 μM hydrocortisone with the exception that the fresh medium added at 24 and 48 hr contained 1 μM hydrocortisone.

Although the value for enzyme associated phosphate of 7.1 moles phosphate/mole of enzyme from control cells is in reasonably good agreement with those found by Bazzell *et al.* [3], the 8.0 moles phosphate/mole of enzyme from IUdR-treated cells is not less than that found for enzyme from control cells (Table 1). Analysis of enzyme from hydrocortisone-treated cells failed to reveal less phosphate associated with enzyme (9.5 moles phosphate/mole of enzyme) (Table 1). Thus, we are unable to confirm this finding of Griffin *et al.* [7] and Bazzell *et al.* [3]. Also, we find no difference between the isoelectric points of alkaline phosphatase from control, IUdR- or hydrocortisone-treated cells. All had isoelectric points at pH 4.4, as reported by Bazzell *et al.* [3] for enzyme from control cells.

Inhibition studies. In an attempt to discern a difference between control and induced enzyme, several known inhibitors of alkaline phosphatase were tested. The results are given in Table 2. For any compound tried, inhibition of both enzymes by 50 per cent was at approximately the same concentration. There appears to be a difference between control and induced enzyme for the 50 per cent inhibitory concentration of phenylalanine but the range of values or variability was great enough so that statistical analysis indicated no significant difference.

DISCUSSION

We have shown by immunological methods (Fig. 1) that the increase in alkaline phosphatase activity induced in HeLa cells by IUdR is more probably a result of the formation of more catalytically active enzyme molecules than a result of a higher concentration of enzyme molecules. Consistent with these results are data that show that the enzyme inductive effects of IUdR and hydrocortisone used together are additive or less than additive compared to the sum of the induction by either agent alone (Fig. 2). Cox *et al.* [2] have previously demonstrated that corticosteroid induction of alkaline phosphatase activity in HeLa cells is also a result of increased catalytic efficiency rather than increased synthesis or concentration of alkaline phosphatase molecules. Thus, two compounds each increasing the catalytic activity of alkaline phosphatase might be expected to give additive or less than additive induction. This is in contrast to a case where one agent increases the concentration of enzyme molecules and thus might be expected to give a greater than additive effect when used in combination with an agent that increases the catalytic activity of the enzyme.

Our previous data [8] failed to show a correlation between the amount of incorporation of IUdR and

Table 1. Alkaline phosphatase associated phosphate*

Preparation	Moles phosphate/mole alkaline phosphatase†	Alkaline phosphatase specific activity‡	Fold stimulation; Treated/control
(1) C	6.5	3.7	
I	7.0	11.7	3.2
(2) C	7.0	1.6	
I	ND	3.6	2.3
(3) C	6.7	1.0	
I	6.7	13.1	13.1
(4) C	ND	2.9	
I	9.7	13.0	4.5
H	8.4	23.7	8.2
(5) C	7.8	2.2	
I	8.0	9.5	4.3
H	9.2	19.6	8.9
(6) C	8.6	4.6	
I	10.6	20.6	4.5
H	16.2	81.8	17.8
(7) C	7.2	1.7	
I	8.4	4.4	2.6
H	8.4	15.4	9.1
(8) C	5.7	7.6	
I	5.5	32.1	4.2
H	5.3	112.8	14.8

* Suspension cultures were grown and treated as described in the text. Purification procedures and phosphate analysis are described under Materials and Methods. Key: (C) control; IUdR-treated; (H) hydrocortisone-treated; and (ND) not done.

† The statistics for moles phosphate/mole of alkaline phosphatase are expressed as the mean ± S. E. for preparations 1 through 8: C = 7.1 ± 0.2; I = 8.0 ± 0.6; and H = 9.5 ± 1.8.

‡ All specific activities (units/mg of protein) were determined on sonicated cells with the exception of preparation 6 in which the butanol extracts were used for specific activity determination.

the extent of induction of alkaline phosphatase activity, although we did provide evidence that some sort of IUdR incorporation into DNA was correlated with or required for induction. The lack of correlation between the extent of IUdR incorporation into DNA and induction may at least in part be accounted for by the finding that an altered enzyme is found. There may be a better correlation between IUdR incorporation and the change in whatever cellular entity is responsible for bringing about the altered alkaline phosphatase molecule.

Since IUdR incorporation into DNA correlates with or is required for the increased alkaline phosphatase activity [8], it seems reasonable to assume that another protein(s) is involved in the alteration of alkaline phosphatase. What that protein is can only be speculated upon at present. Bazzell *et al.* [3] have proposed two modes by which alkaline phosphatase may be altered that relate to their finding that corticosteroid-induced enzyme has about half the phosphate residues per molecule of enzyme as control. The idea that altered enzyme activity is related to a change in enzyme associated phosphate moieties was attractive, particularly since one mechanism that Bazzell *et al.* [3] postulated involved corticosteroid inhibition of protein kinase. It was easy to envision how IUdR induction could occur as a result of a reduction in protein kinase synthesis, thus affording the same end result as from hydrocortisone induction, a less phosphorylated alkaline phosphatase. Unfortunately, we have as yet been unable to find any decrease in protein kinase activity in HeLa cells treated with

Table 2. Inhibition of alkaline phosphatase from control and IUdR-treated cells*

Inhibitor	Concn (mM) for 50 per cent inhibition	
	Control	IUdR-treated
l-Phenylalanine	2.1 ± 0.3	1.2 ± 0.3
l-Cysteine	0.15	0.13
l-Tryptophan	1.2	1.0
l-Histidine	14.6	13.1
p-Arsanilic acid	24.3	25.2

* Inhibition measurements were performed with two sets of butanol extracts from control and IUdR-treated cells. The specific activities (units/mg of protein) for extracts from control cells were 11.3 and 15.4 and the respective specific activities from extracts of IUdR (3 μM)-treated cells were 54.4 and 126.1. The values given are the average of two experiments with the exception of phenylalanine for which five determinations were done and a S. E. was calculated.

IUdR (W. Wharton and B. Goz, unpublished observations). More importantly, as reported here (Table 1), we are unable to find any difference in alkaline phosphatase associated phosphate in enzyme purified from control, IUdR- or hydrocortisone-treated cells. On the basis of our findings, we are presently forced to exclude the possibility that the extent of enzyme phosphorylation is the cause of enzyme induction. It should be noted that our finding of 7.1 moles phosphate/mole of control enzyme is in reasonably good agreement with the figure of 6.5 moles phosphate/mole of control enzyme reported by Bazzell *et al.* [3] and also not greatly different from the earlier figure of 12 moles phosphate/mole of enzyme reported by Griffin *et al.* [7]. Perhaps the difference in our results on enzyme associated phosphate from that reported by Bazzell *et al.* [3] is due to the fact that our cells were grown in suspension while theirs were grown as monolayers. This is certainly possible since we have noted, as have Griffin and Cox [9] before us, that the specific activity of alkaline phosphatase from control cells grown in suspension culture is generally substantially lower than that of comparable cells from monolayer cultures. In addition, the high inorganic phosphate concentration is suspension medium may also influence the properties of the enzyme. Also, they were using the cloned subline, HeLa 65. However, since in both their and our reports there was enzyme induction, we would conclude that the difference in phosphorylation of the enzyme found by Bazzell *et al.* [3] either may not be related to the increase in enzyme activity or there may be more than one mechanism for increasing alkaline phosphatase activity. It is interesting that there is a relatively high number of phosphate residues associated with this enzyme, as compared to the much studied *Escherichia coli* alkaline phosphatase. In this regard, Gable and Thomas [10] have presented evidence for the association of polyphosphate with chicken intestinal alkaline phosphatase. Before leaving the issue of enzyme phosphorylation, two other facts should be mentioned, although we cannot say how and whether they may be important in this instance. First, placental alkaline phosphatase which is believed to be identical or almost identical to a form of HeLa alkaline phosphatase, has recently been reported to have phosphoprotein phosphatase activity [11]. Second, relative to the possibility of polyphosphate associate with the enzyme, HeLa alkaline phosphatase apparently also can hydrolyze pyrophosphate [12].

Schlessinger *et al.* [13] have reported the interesting finding that *E. coli* alkaline phosphatase isozymes differ only by a single amino terminal arginine moiety and that one form is convertible to the other by protease action. Perhaps a similar simple change is true for the HeLa enzyme. Such a post transcriptional change could be brought about by IUdR by reduced synthesis of an enzyme responsible for adding amino acids (e.g. arginyl-tRNA protein transferase that is found in HeLa cells as well as other eukaryotic cells [14]) or a protease that removes amino acids.

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