Biochemical Pharmacology, Vol. 28, pp. 763–768.

0006-2952/79/0315-0763 $02.00/0

INDUCTION OF ALKALINE PHOSPHATASE ACTIVITY IN HELA CELLS

INHIBITION BY XANTHINE DERIVATIVES AND THERMOSTABILITY STUDIES

WALKER WHARTON and BARRY GOZ

Department of Pharmacology, School of Medicine, University of North Carolina, Chapel Hill, NC 27514, U.S.A.

(*Received* 9 *May* 1978; *accepted* 27 *July* 1978)

Abstract—The induction of alkaline phosphate activity in HeLa cells by 5-iodo-2′-deoxyuridine (IUdR) or hydrocortisone was inhibited in a dose-dependent manner by the addition of the xanthine derivatives caffeine, theophylline or 3-isobutyl-1-methylxanthine to the culture medium during the 72 hr of the induction. Pretreatment with theophylline from −24 to 0 hr or treatment from 0 to 24 hr with any of the xanthine derivatives was ineffective in inhibiting alkaline phosphatase induction produced by treatment with IUdR from 0 to 72 hr. The induction of alkaline phosphatase activity produced by treatment with hydrocortisone from 0 to 72 hr was inhibited by pretreatment with theophylline from −24 to 0 hr, although this inhibition was only about 60 per cent as great as that seen with treatment from 0 to 72 hr. As judged by heat inactivation studies, IUdR predominantly increased the heat-labile form of alkaline phosphatase activity, while hydrocortisone predominantly increased the heat-stable form. Regardless of the inducer used, the xanthine derivatives mainly decreased the heat-stable form of alkaline phosphatase activity. Treatment with imidazole over a 72-hr period produced over a 2-fold induction of alkaline phosphatase activity, which could be inhibited completely by concurrent treatment with theophylline.

The addition of 5-iodo-2′-deoxyuridine (IUdR) to the medium of HeLa cells over a 72-hr period results in at least a 3- to 4-fold increase in alkaline phosphatase specific activity [1, 2]. Hydrocortisone will produce a similar effect with the magnitude and time course of the induction similar to that observed with IUdR [3]. Apparently, both of these phenomena are a result of a change in the catalytic efficiency of the enzyme rather than an increase in the total number of enzyme molecules per cell [4, 5], although the alterations in the enzyme responsible for the increase in activity are not known. The action of compounds which inhibit the induction might provide clues as to what processes are involved in the control of alkaline phosphatase activity expression.

Since caffeine has been shown to inhibit the induction of oncogenic viruses in cultured mouse cells by IUdR [6], we decided to investigate whether this compound, along with the related xanthine derivatives theophylline and 3-isobutyl-1-methylxanthine (IBMX), would also inhibit the induction of alkaline phosphatase activity by IUdR or hydrocortisone.

MATERIALS AND METHODS

Materials. Hydrocortisone-21-succinate, 2-amino-2-methyl-1-propanol, *p*-nitrophenylphosphate, caffeine and theophylline were obtained from the Sigma Chemical Co. (St. Louis, MO). 3-Isobutyl-1-methylxanthine was obtained from the Aldrich Chemical Co. (Milwaukee, WI). Imidazole was purchased from Eastman Kodak (Rochester, NY) and 5-iodo-2′-deoxyuridine was obtained from Nutritional Biochemicals (Cleveland, OH). Tissue culture medium and serum were from the Grand Island Biological Co. (Grand Island, NY). All other chemicals were purchased from the Fisher Chemical Co. (Atlanta, GA).

Cell culture. HeLa S3 cells were grown in 25 cm^2 plastic flasks (Corning Glass Works, Corning, NY) in 5 ml of Eagle's minimum essential medium supplemented with 10% fetal calf serum. Cells were detached from monolayers for subculturing by exposure to a minimal volume of 0.02% sodium ethylenediamine tetra-acetate in phosphate buffered saline (PBS) solution (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.6 mM KCl and 1.4 mM K_2HPO_4) and resuspended in prewarmed medium. The cells were seeded at a density of 3 to 4×10^4 cells per cm^2 and were allowed to grow for at least 24 hr prior to the start of an experiment.

Preparation of cell extracts. The cells were harvested by scraping them into 10 ml PBS with a rubber policeman. After centrifugation, the cell pellet was resuspended in 1.0 ml of 50 mM Tris–HCl (pH 8.6) and frozen. Prior to assay, the cells were disrupted with a sonicator (model W-185, Heat Systems-Ultrasonics, Plainview, NY) for two 10-sec bursts.

Alkaline phosphatase assay. The alkaline phosphatase reaction mixture consisted of 90 mM 2-amino-2-methyl-1-propanol (pH 10.5), 1.0 mM $MgCl_2$, 8.0 mM *p*-nitrophenylphosphate and an appropriate dilution of cell extract in a final volume of 2.1 ml. Duplicate tubes were incubated at 37° until the solution turned yellow, at which time the reaction was stopped by the addition of 0.1 ml of 6 N NaOH, and the product of the reaction, *p*-nitrophenol, was measured by absorbance at 410 nm. A unit of alkaline phosphatase activity was defined as the formation of 1 nmole *p*-nitrophenol/ml/min.

Protein content was measured by the method of Lowry *et al.* [7], using bovine serum albumin as the reference standard.

Thermostability studies. Tubes containing cell extract, diluted such that each tube contained approximately 100 μg protein, 94.5 mM 2-amino-2-methyl-1-propanol (pH 10.5) and 1.05 mM $MgCl_2$ in a final volume of 2.0 ml, were heated at 65° in a water bath with gentle agitation. The tubes were removed at the times indicated and cooled rapidly in a salted ice–water slurry. After the samples reached 0–1°, 0.1 ml *p*-nitrophenylphosphate was added such that the final concentration was 8.0 mM and the samples were assayed for alkaline phosphatase activity as described above.

RESULTS

Inhibition of the induction of alkaline phosphatase activity. Figure 1 shows the alkaline phosphatase specific activity measured 72 hr after the addition of either 3 μM IUdR (panel A) or 1.0 μM hydrocortisone (panel B) and various concentrations of caffeine, theophylline or IBMX. All three compounds produced dose-dependent decreases in the inductions produced by either IUdR or hydrocortisone, and at all the dosages tested the order of potency was IBMX >theophylline >caffeine. IMBX produced significant inhibitions at concentrations as low as 0.1 mM, while caffeine and theophylline were not effective until concentrations greater than 0.33 mM. At dosages greater than 0.33 mM, each xanthine derivative appeared to cause a slightly greater inhibition of the IUdR induction than of the hydrocortisone induction.

Alkaline phosphatase in HeLa cells has been referred to as a "self-inducing" enzyme, that is, as the cells grow to confluence, the alkaline phosphatase specific activity increases [8]. When an experiment was begun in which the cells reached confluence at 72 hr, the three xanthine derivatives inhibited this "self-induction" in a dose-dependent manner (for examples see Table 2 and Fig. 5).

Since these xanthine derivatives have been reported to block thymidine transport into cultured cells [9, 10], and since IUdR is an analog of thymidine, it was necessary to examine whether the xanthines were inhibiting the induction of alkaline phosphatase activity by blocking the transport of IUdR into the cells. In these experiments, cultures were treated from 0 to 24 hr with 3 μM IUdR. The ability of IUdR to induce alkaline phosphatase activity in HeLa cells over a 72-hr period has been shown to be the same whether the drug is present from 0 to 24 hr or from 0 to 72 hr [1]. The xanthine derivatives were added to the medium in a final concentration of 1.0 mM either (a) from 0 to 24 hr, when the IUdR was present in the medium, (b) from 24 to 72 hr, after the IUdR had been removed from the medium, or (c) for the entire 72 hr of the experiment. If these compounds were acting only by inhibiting IUdR transport, treatment from 0 to 24 hr, when IUdR was

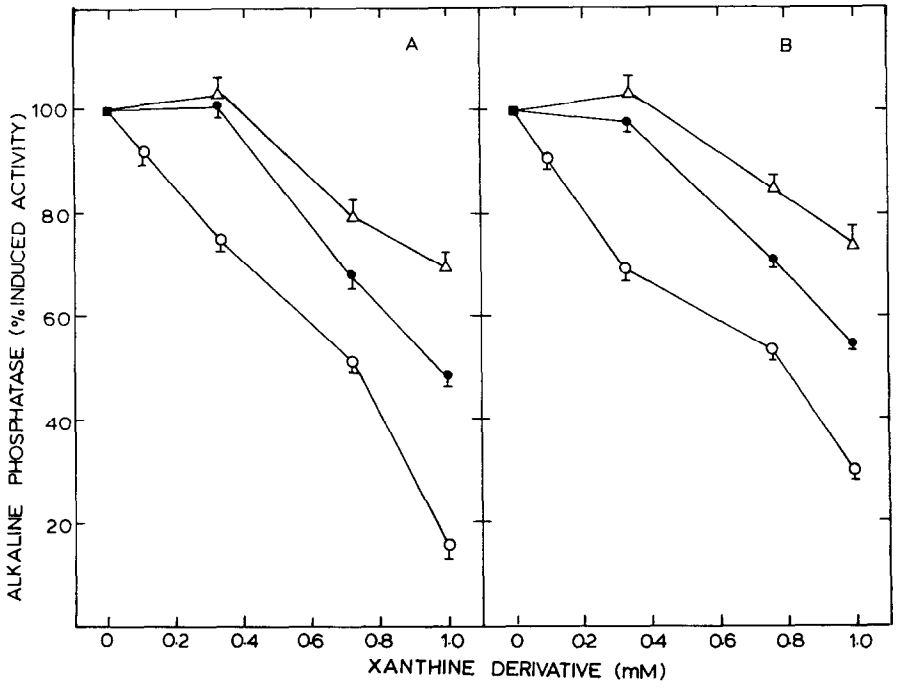

Fig. 1. Inhibition by the xanthine derivatives of the induction of alkaline phosphatase activity by IUdR or hydrocortisone. Inhibition of the induction was produced by treatment with either caffeine (△——△), theophylline (●——●) or IBMX (○——○). Cells were harvested 72 hr after the addition of 3 μM IUdR (panel A) or 1 μM hydrocortisone (panel B) and various concentrations of the xanthine derivatives. In panel A, alkaline phosphatase specific activity for untreated cultures was 12.81 ± 0.87 units/mg of protein and 65.08 ± 1.37 units/mg of protein for cells treated with IUdR. In panel B, untreated cultures had an alkaline phosphatase specific activity of 11.74 ± 0.37 units/mg of protein and 97.7 ± 2.86 units/mg of protein for hydrocortisone treated cells. Each value is the mean ± standard error from triplicate samples.

Table 1. Effectiveness of inhibition by xanthine derivatives of induction of alkaline phosphatase activity relative to the time of addition during induction*

Treatment	Alkaline phosphatase specific activity		
	Theophylline	Caffeine	IBMX
IUdR (0–24) + xanthine (0–24)	124.31 ± 4.10	126.73 ± 3.51	135.14 ± 3.11
IUdR (0–24) + xanthine (24–72)	57.06 ± 2.78	66.35 ± 2.98	25.73 ± 2.01
IUdR (0–24) + xanthine (0–72)	53.04 ± 4.15	68.75 ± 3.66	22.49 ± 1.99

* Cultures were treated from 0 to 24 hr with 3 μM IUdR. In addition, 1.0 mM caffeine, theophylline or IBMX was added for the times indicated. All cultures were harvested and assayed for alkaline phosphatase activity at 72 hr. Cells treated with only control medium had an alkaline phosphatase specific activity of 11.75 ± 1.02 units/mg of protein, while those treated with IUdR alone had a specific activity of 123.11 ± 2.56 units/mg of protein. All values are the mean ± standard error for triplicate samples.

present, should be as effective as treatment for the entire 72 hr, and treatment from 24 to 72 hr should be ineffective. The results of this experiment are shown in Table 1. Treatment with either theophylline or caffeine from 0 to 24 hr caused no inhibition of the induction of alkaline phosphatase activity produced by IUdR, while treatment with IBMX from 0 to 24 hr caused a slight stimulation of the induction. With all three compounds, treatment from 24 to 72 hr was as effective as treatment from 0 to 72 hr. The magnitude of the inhibition and the order of potency of the xanthine derivatives were similar to those seen in Fig. 1. Thus, the inhibition of the induction after treatment with the xanthine derivatives was not due to inhibition of IUdR transport into the cells.

A difference between the inhibitions by theophylline of the inductions produced by IUdR or hydrocortisone was revealed when cells were pretreated with theophylline. Figure 2 shows alkaline phosphatase specific activities measured in cells that were treated either from −24 to 0 hr (pretreatment) or from 0 to 72 hr (continuous treatment) with 1.0 mM theophylline. Alkaline phosphatase activity was induced by treatment with either 3.0 μM IUdR (left panel) or 1.0 μM hydrocortisone (right panel) from 0 to 72 hr. Theophylline inhibited the induction when it was present with either inducer

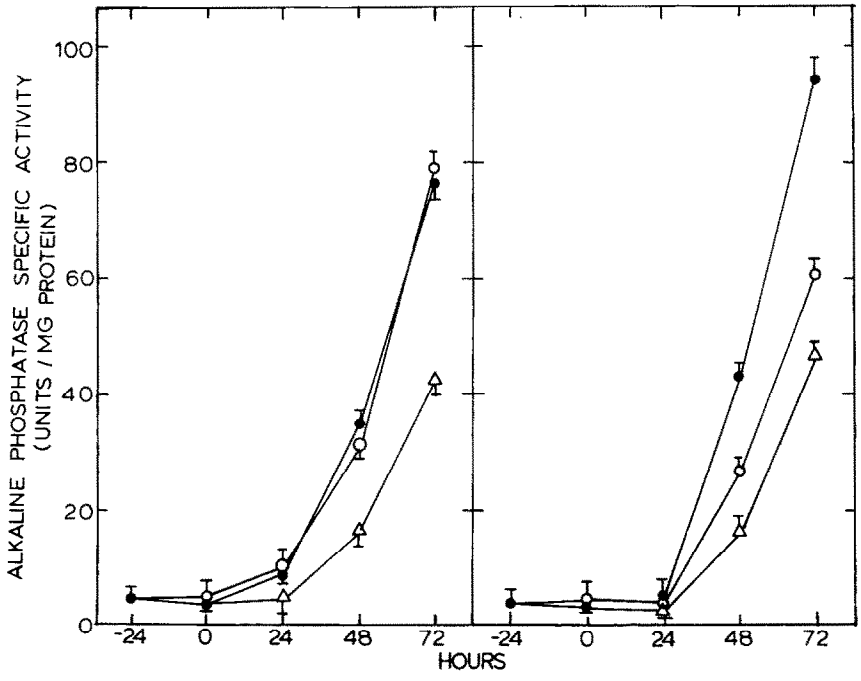

Fig. 2. Effect of pretreatment with theophylline on induction of alkaline phosphatase activity by IUdR or hydrocortisone. Alkaline phosphatase specific activities were induced in cells by treatment from 0 to 72 hr with either 3 μM IUdR (left panel) or 1 μM hydrocortisone (right panel). Cultures were treated either with the inducer alone (●——●), or were also treated either from −24 to 0 hr (○——○) or from 0 to 72 hr (△——△) with 1.0 mM theophylline. Each value is the mean ± standard error of triplicate samples.

from 0 to 72 hr to approximately the same extent as shown in Fig. 1. Although pretreatment for 24 hr with theophylline caused no inhibition in the induction by IUdR, it did produce a significant inhibition of the induction by hydrocortisone. Pretreatment with theophylline, however, was only about 60 per cent as effective an inhibitor of alkaline phosphatase induction by hydrocortisone as theophylline treatment from 0 to 72 hr.

Heat inactivation of alkaline phosphatase activity. HeLa cell alkaline phosphatase is a variant of the placental from of the enzyme [11, 12], and as such is insensitive to heat inactivation at a neutral pH [13]. At an alkaline pH, the enzyme is inactivated in a biphasic manner, with a rapid initial decline in activity followed by a slower steady rate of inactivation [13]. Figure 3 shows the patterns of heat inactivation of alkaline phosphatase activity from both control and IUdR-treated cultures grown with and without 1.0 mM theophylline. Induction with IUdR caused an increase in the proportion of the rapidly inactivated form of the enzyme activity (e.g. that inactivated by 2.5 min) as compared to controls. Addition of theophylline to either control or IUdR-treated cultures further increased the proportion of the more labile form of the activity. A line of best fit by the method of least squares was calculated for each treatment for the more stable form of the enzyme activity (e.g. that remaining after heating for between 2.5 and 15 min). The line was projected back to the ordinate to solve for the percentage of the stable enzyme activity present before heating.

Table 2 shows the original alkaline phosphatase specific activity of both the stable and labile forms of the enzyme calculated as described above. In experiment I (calculations from the data shown in Fig. 3), induction with IUdR caused a 6-fold increase in alkaline phosphatase specific activity, and treatment with 1.0 mM theophylline inhibited both the IUdR induction and the "self-induction" seen in the control cells. Although IUdR increased the specific activity of both the stable and labile forms of the enzyme activity, the labile form was increased over 20-fold, while the stable form was increased only about 4-fold. Treatment with theophylline had effects predominantly on the stable form of the enzyme activity both in control and IUdR-treated cells. In the cells treated with IUdR, the heat-stable form of the alkaline phosphatase enzyme was almost 3-fold lower after treatment with 1.0 mM theophylline. The heat-labile form, however, was only about 25 per cent lower after treatment with the xanthine derivative.

In experiment II, the induction of alkaline phosphatase activity by IUdR was inhibited with 1.0 mM caffeine. The pattern of changes seen in the different form of the enzyme was similar to that seen in experiment I with treatment with theophylline.

In experiment III, in contrast to the case with IUdR, the induction of alkaline phosphatase specific activity with hydrocortisone predominantly increased the heat-stable form of the enzyme more than the heat-labile form, as had been reported earlier [14]. There was almost a 10-fold increase in the heat-stable form of the enzyme activity after treatment with hydrocortisone,

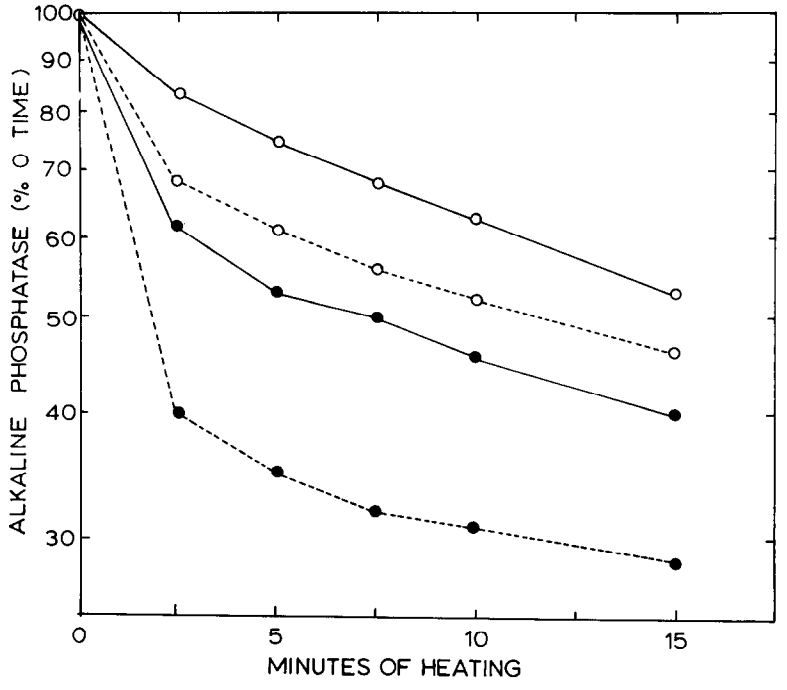

Fig. 3. Heat inactivation of alkaline phosphatase activity. Cultures were harvested 72 hr after the addition of either control medium (open symbols) or 3 μM IUdR (closed symbols). One mM theophylline was also added to either control or IUdR-treated cells (dashed lines). Alkaline phosphatase activity was inactivated by heating at 65° at pH 10.5 as described under Materials and Methods. Each point is the mean of nine samples.

Table 2. Heat-stable and heat-labile alkaline phosphatase activity after treatment of cells with compounds that promote or inhibit induction of enzyme activity*

Treatment	Alkaline phosphatase specific activity		
	Total	Heat-stable	Heat-labile
Experiment I			
Control	20.65	18.62	2.03
IUdR	120.92	77.38	43.59
Theophylline	11.75	8.74	3.01
IUdR + theophylline	59.05	27.51	31.54
Experiment II			
Control	15.86	12.14	3.72
IUdR	86.73	56.20	30.53
Caffeine	9.18	6.07	3.11
IUdR + caffeine	49.18	20.44	28.74
Experiment III			
Control	17.95	12.86	5.09
Hydrocortisone	140.39	119.33	21.06
Theophylline	12.88	8.05	4.83
Hydrocortisone + theophylline	69.31	50.68	18.63

* Cultures were harvested 72 hr after the addition of the drugs indicated and the alkaline phosphatase activity was inactivated by heating samples to 65° at pH 10.5. The amounts of the heat-labile and heat-stable activity were calculated as described under Results. All values are the mean of nine samples.

while the heat-labile form increased only 4-fold. Treatment with theophylline caused reductions only in the heat-stable form of the enzyme, similar to the effects seen in cells induced with IUdR.

Induction of alkaline phosphatase activity by imidazole. Figure 4 shows alkaline phosphatase specific activities in cultures treated from 0 to 72 hr with various concentrations of imidazole, a compound which has been shown to stimulate cyclic nucleotide phosphodiesterase [15]. Addition of imidazole in a final concentration of between 0.05 and 2.5 mM caused a dose-dependent increase in alkaline phosphatase specific activity. At the peak of the dose–response curve (2.5 mM), the alkaline phosphatase specific activity was approximately 225 per cent of control values.

Figure 5 shows the inhibition of the induction of alkaline phosphatase activity by imidazole when the cultures were also treated with theophylline. Imidazole alone produced approximately a 3-fold induction in activity at 72 hr. Both the induction by imidazole and

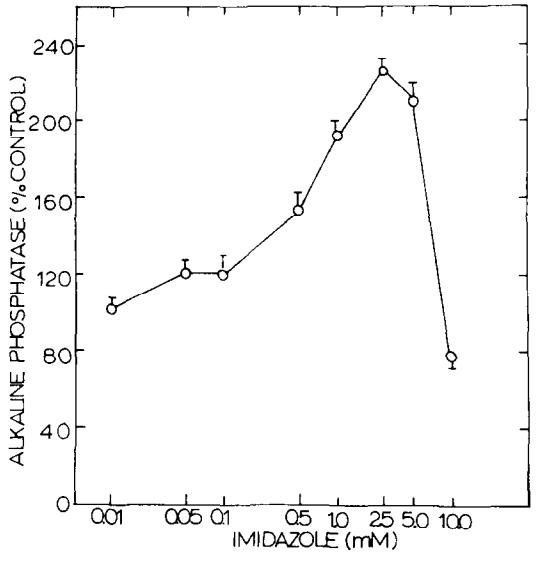

Fig. 4. Induction of alkaline phosphatase activity by imidazole. Alkaline phosphatase activity was measured in cells 72 hr after addition of the indicated concentration of imidazole to the culture medium. Control samples had an alkaline phosphatase specific activity of 13.11 ± 1.04 units/mg of protein. Each point is the mean ± standard error of six samples.

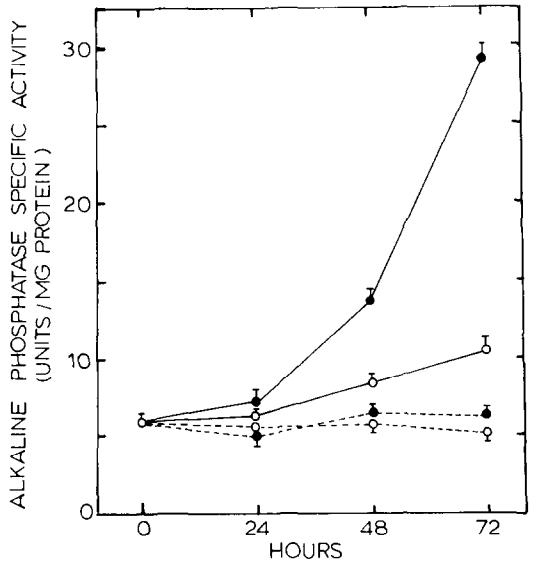

Fig. 5. Inhibition by theophylline of the induction of alkaline phosphatase activity by imidazole. Alkaline phosphatase specific activity was measured at various times after the addition of either control medium (open symbols) or medium containing 2.5 mM imidazole (closed symbols). One mM theophylline was added to either control or imidazole-treated cultures (dashed lines). Each point is the mean of triplicate samples.

the "self-induction" seen in the control cells were inhibited completely by the addition of theophylline.

DISCUSSION

In these experiments we have shown that the three xanthine derivatives, caffeine, theophylline and IBMX, inhibited the induction of alkaline phosphatase activity produced by either IUdR or hydrocortisone. These compounds produced their effects in a dose-dependent manner and at concentrations that had little or no effect on cellular growth rates. The xanthines seemed to specifically alter the expression of the heat-stable component of the enzyme regardless of the agent that produced the induction. These effects are not due to alterations in the amount of total protein per cell, since when enzyme activity is expressed as units/10^6 cells, the results are identical. The forms of alkaline phosphatase with differing heat stabilities which were differentially affected by treatment with either the inducers or the inhibitors cannot be separated by polyacrylamide electrophoresis or isoelectric focusing (unpublished data), and the molecular basis of these differences remains unknown.

These compounds have been shown to inhibit cyclic nucleotide phosphodiesterase from adipose tissue in the same order of potency that they were found here to inhibit the induction of alkaline phosphatase activity [16]. The most obvious implication of these data is that cyclic nucleotides were involved in the induction or its inhibition. The observation that the phosphodiesterase stimulator imidazole induced alkaline phosphatase activity is also consistent with the hypothesis that alterations in intracellular cyclic nucleotide concentrations are involved in the expression of the enzyme. Since the degree of phosphorylation of alkaline phosphatase has been shown to change when monolayer cultures were treated with hydrocortisone [5], the role of cyclic nucleotides becomes of even more interest. On the other hand, we were unable to find a difference in phosphorylation between control enzyme or enzyme induced by either IUdR or hydrocortisone in cells grown in suspension culture [4].

The xanthine derivatives used in these experiments have been reported to cause only short-lived changes in cellular cyclic AMP concentrations in HeLa cells [10, 17, 18], although a detailed time course of cellular cyclic AMP changes for the extended periods of treatment reported here has not been described in the literature.

Even if these compounds do not cause long-term increases in total cellular cyclic nucleotide concentrations, the inhibition of the induction of alkaline phosphatase activity still might be at least partially mediated by cyclic nucleotides. There are biological processes which have been shown recently to be mediated by cyclic nucleotides, but where the size of the pool of nucleotide responsible for these changes was not reflected by the size of the total intracellular pool. Testosterone production stimulated in Leydig cells after treatment with hCG does not correlate well with cellular cyclic AMP concentration [19], although the bound pool of the nucleotide does reflect the production of the steroid [20].

Further studies measuring changes in cellular cyclic nucleotide concentrations after treatment with the xanthine derivatives and investigating the role that cyclic nucleotides might play in mediating the expression of alkaline phosphatase activity are now underway.

Acknowledgements—This investigation was supported by Grant CA16460, awarded by the National Cancer Institute, DHEW.

REFERENCES

1. B. Goz, *Cancer Res.* **34**, 2393 (1974).
2. B. Goz and K. P. Walker, *Cancer Res.* **36**, 4480 (1976).
3. R. P. Cox and C. M. McLeod, *Nature, Lond.* **190**, 85 (1961).
4. B. Goz and K. P. Walker, *Biochem. Pharmac.* **27**, 431 (1978).
5. R. P. Cox, N. A. Nelson, S. H. Tu and M. J. Griffin, *J. Molec. Biol.* **58**, 197 (1971).
6. H. Yoshikura, *Nature, Lond.* **252**, 71 (1974).
7. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
8. H. Nitowsky and F. Herz, *Biochem. biophys. Res. Commun.* **11**, 261 (1961).
9. J. R. Sheppard and P. G. W. Plagmann, *J. cell. Physiol.* **85**, 163 (1975).
10. C. E. Zeilig, R. A. Johnson, E. W. Sutherland and D. L. Friedman, *J. Cell Biol.* **71**, 515 (1976).
11. N. A. Elson and R. P. Cox, *Biochem. Genet.* **3**, 549 (1969).
12. B. Beckman, L. Beckman and E. Lundgren, *Hum. Hered.* **20**, 1 (1970).
13. F. Herz and H. M. Nitowsky, *Archs Biochem. Biophys.* **96**, 506 (1962).
14. G. Melnykovych and C. K. Sylber, *Proc. Soc. exp. Biol. Med.* **121**, 165 (1966).
15. R. W. Butcher and E. W. Sutherland, *J. biol. Chem.* **237**, 1244 (1962).
16. J. A. Beavo, N. L. Rogers, O. B. Crawford, J. H. Hardmann, E. W. Sutherland and E. V. Newman, *Molec. Pharmac.* **6**, 597 (1970).
17. J. B. Kurz and D. L. Freidman, *J. Cyclic Nucleotide Res.* **2**, 405 (1976).
18. H. Hilz, E. Kaukel, U. Weigers and U. Fuhrmann, *Biochem. biophys. Res. Commun.* **64**, 519 (1975).
19. W. R. Moyle and J. Ramachandran, *Endocrinology* **93**, 127 (1973).
20. M. Dufan, T. Tsuruhara, K. Horner, E. Podesta and K. Catt, *Clin. Res.* **25**, 462 (1977).