top of the resultant pellet was carefully washed with 1.15 percent KCl, and the pellet was suspended in 1.15 percent KCl. Protein concentration was determined by the method of Lowry *et al.* (*10*).

The gas chromatographic analysis is presented in Fig. 1. We observed that the presence of microsomal protein did not significantly influence the quantitative aspects of the extraction of *trans*-dihydrodiol. More than 90 percent of diol was recovered in the presence of 2.5 to 10 mg of microsomal protein over a range of diol concentrations of 20 to 100 μg per 0.4 ml of assay system. Peaks showing low retention times (indicated by arrows) were also present in control extracts obtained from microsomes alone.

The large peak following the C_{32} standard (*B*) was identified by mass spectrometry (*11*) as the trimethylsilyl ether of cholesterol. This compound has the same retention time as the silylated derivative of *cis*-11,12-dihydro-11,12-dihydroxy-3-methylcholanthrene (*cis*-diol). However, under our assay conditions, no *cis*-diol could be detected from the mass spectral fragmentation pattern associated with this peak, indicating that the microsomal reaction is stereospecific.

The major enzyme activity is associated with the microsomal fraction, although the fraction sedimenting at 600 to 20,000*g* had a specific activity which was approximately 30 percent that of the 100,000*g* pellet (4.0 as compared to 11.2 nmole of diol formed per milligram of protein per 10 minutes). A good deal of the former activity may be associated with microsomal contamination. No activity could be found in the 100,000*g* supernatant.

The reaction appeared linear for up to 10 minutes and, consequently, the latter time was chosen as a part of the assay conditions. A study of velocity of the reaction plotted against substrate concentration indicated that 0.44 m*M* oxide (50 μg) was sufficient to saturate the system; a calculated Michaelis constant (K_m) for this substrate was 2.8×10^{-4} *M*. Enzyme activity was also directly proportional to the protein concentration of liver microsomes, up to 10 mg of protein per incubation.

The distribution of activity with respect to rat tissues is indicated in Fig. 2. Liver microsomes had the greatest activity, whereas those from kidney and lung appeared to be 25 and 5 percent as active, respectively. No demonstrable enzyme activity could be detected in microsomes from either spleen or small intestine. Finally, as reported by Oesch *et al.* (*12*) for the hydration of styrene oxide, equimolar quantities of 3,3,3-trichloropropene oxide completely inhibited the formation of the diol from 3-methylcholanthrene oxide (data not shown).

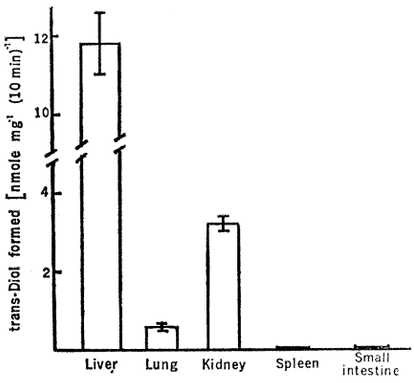

Fig. 2. Epoxide hydrase activity in rat tissues. Microsomes from the tissues of three male rats (approximately 120 g) were prepared as described in the text. Each incubation received 3.0 mg of microsomal protein and the number of nanomoles of *trans*-diol formed were measured. The bars indicate the standard error.

In conclusion, we have demonstrated a relatively simple method for determining epoxide hydrase activity which is based on the conversion of 3-methylcholanthrene-11,12-oxide to the corresponding diol. The gas chromatographic assay is advantageous because (i) it requires only readily available substrates and should be applicable to various arene oxides, and (ii) it is extremely sensitive and can detect less than 0.2 nmole of diol.

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Regulation of Phosphodiesterase Synthesis: Requirement for Cyclic Adenosine Monophosphate–Dependent Protein Kinase

Abstract. *Endogenous cyclic adenosine monophosphate (AMP) and its dibutyryl derivative increase cyclic AMP phosphodiesterase activity in cultured lymphoma cells. This effect is prevented by cycloheximide. A variant population of cells deficient in cyclic AMP–dependent protein kinase contains lower basal phosphodiesterase activity, which cannot be induced by cyclic AMP.*

Adenosine 3′,5′-monophosphate (cyclic AMP) is well established as an intracellular "second messenger" for many hormones, and plays an important role in controlling growth of cells. However, the molecular basis of cyclic nucleotide action is known for only a few of these processes. Protein phosphokinases specifically activated by cyclic AMP control the moment-to-moment activity of several enzyme systems [for example, glycogen synthetase and glycogen phosphorylase (*1*)]. Although similar cyclic AMP–activated protein kinases are present in most mammalian cells (*2*), they have not been directly implicated as mediators of the other effects of cyclic AMP, including induction of the synthesis of specific enzymes (*3*, *4*) and regulation of cell growth and differentiation (*5*). We have reported the selection of a stable population of cultured lymphoma cells deficient in both cyclic AMP–activated protein phosphokinase and its cyclic AMP–binding subunit, and have presented evidence that these cells may prove useful in defining the role of such kinases in mediating the diverse actions of cyclic AMP (*6*). We now present

evidence that cyclic AMP–activated protein kinase may be required for the cyclic nucleotide induction of cyclic AMP phosphodiesterase. The increased activity of cyclic AMP phosphodiesterase, which specifically degrades cyclic AMP, represents a servomechanism by which the nucleotide can regulate its own accumulation within cells.

Cells of the mouse lymphoma line S49.1 TB4 (*6*) die after exposure for 48 hours to N^6-2′-*O*-dibutyryl (DB) cyclic AMP, and are therefore designated DB cyclic AMP–sensitive (S) cells. The DB cyclic AMP–resistant (R) subpopulation contains about 15 percent of the cytoplasmic cyclic AMP–binding activity and nucleotide-dependent histone kinase activity of the S line from which it is derived (*6*). We have found that isoproterenol and prostaglandin E_1 (PGE_1), which stimulate adenylate cyclase activity in both cell types, produce much greater and more prolonged elevation of cyclic AMP content in R than in S cells; these differences were accounted for, at least in part, by a threefold reduction of cyclic AMP phosphodiesterase activity in R, as compared with S, cells (*7*). We therefore imagined that cyclic AMP increases the rate of synthesis of phosphodiesterase in S cells, as has been suggested in other cell lines (*4*), and that in R cells, in which activity of the protein kinase system is diminished, this elevation cannot occur. If this explanation is correct, exogenous DB cyclic AMP and substances that elevate endogenous cyclic AMP content should increase phosphodiesterase activity in S cells, but not in R cells. These predictions are fulfilled (Figs. 1 and 2A).

In S cells, DB cyclic AMP (1.2 m*M*), in the presence of 1.0 m*M* theophylline, causes an increase in cyclic AMP phosphodiesterase activity which reaches a plateau in about 5 hours (Fig. 1). The rise in enzyme activity is completely prevented by cycloheximide at a concentration (0.2 m*M*) that reduces incorporation of radioactive leucine into S cell protein by 76 percent (*8*). Actinomycin D (1.0 μg/ml) also completely blocks the DB cyclic AMP–induced rise in phosphodiesterase (results not shown); however, since actinomycin D also inhibits incorporation of radioactive leucine into S cell protein by 62 percent (*8*), this result does not establish a requirement for RNA synthesis in the phosphodiesterase-inducing effect of DB cyclic AMP. The increase in phosphodiesterase synthesis in S cells is not due to stimulation of general protein synthesis, since the same concentrations of DB cyclic AMP and theophylline (1.2 and 1.0 m*M*, respectively) produced 50 percent inhibition of overall incorporation of [^{3}H]leucine into S cell protein (*8*), as shown previously (*7*).

In the same experiment, DB cyclic AMP and theophylline caused no change in phosphodiesterase activity of R cells (Fig. 1), which remained about 30 percent (per milligram of cell protein) of the value in unstimulated S cells, as previously reported (*7*).

Not only the inhibitor experiments described above, but also other results, suggest that the increase in phosphodiesterase activity in S cells exposed to DB cyclic AMP is caused by an increase in the rate of enzyme synthesis. Under the conditions of the assay (Fig. 1), DB cyclic AMP itself inhibited enzyme activity, as well on a molar basis as did theophylline (50 percent inhibition at 0.2 m*M* concentration of either drug). Cycloheximide (at concentrations up to 6.0 m*M*) did not significantly inhibit phosphodiesterase activity. In addition, the possibility that DB cyclic AMP and theophylline induce synthesis of an endogenous activator of phosphodiesterase (or block synthesis of an inhibitor) was made less likely by the fact that the phosphodiesterase activities of basal and induced S cell preparations were completely additive in mixing experiments (results not shown). Similarly, activities of the enzyme in S and R cell preparations were also completely additive in mixing experiments. Finally, the apparent affinities of basal and induced S cell phosphodiesterase were identical [apparent K_m (Michaelis constant) values of 3 and 12 μ*M* in both preparations, as has been reported for S and R cells (*7*)].

As might be expected from the re-

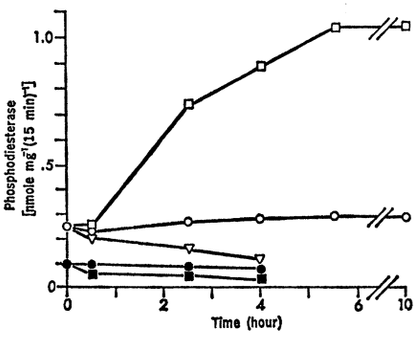

Fig. 1. Cyclic AMP phosphodiesterase in S (open symbols) and R (closed symbols); lymphoma cells at various times after exposure to no drug (circles); DB cyclic AMP (1.2 m*M*) and theophylline (1.0 m*M*) (squares); or DB cyclic AMP and theophylline plus cycloheximide (0.2 m*M*) (triangles). Both S and R cells were propagated as described (*6*). Drugs were added to suspensions of cells in the logarithmic phase of growth (0.5×10^6 to 1.0×10^6 cell/ml) in Dulbecco's minimal essential medium with heat-inactivated horse serum (10 percent) (*6*). At various times 4-ml portions of cell suspension were removed from the incubation flasks, cooled in ice, and centrifuged at 2000*g* for 5 minutes. The supernatant was removed, and the sediment was resuspended in ice-cold 0.25*M* sucrose and subjected to sonication (*7*). Phosphodiesterase activity of the sonicated crude cells was assayed as described (*12*) by conversion of [^{3}H]cyclic AMP (New England Nuclear) to labeled 5′-AMP, which in turn is subsequently converted to [^{3}H]adenosine by *Crotalus atrox* phosphatase. The labeled adenosine is then separated from other nucleotides by ion-exchange chromatography. In these experiments each reaction mixture contained 40 m*M* tris-HCl, *p*H 7.5, 2.0 m*M* $MgCl_2$, and 1.0 μ*M* [^{3}H]cyclic AMP (substrate), plus appropriate amounts of enzyme, in a final volume of 0.15 ml. Under these conditions the rate of conversion of cyclic AMP to 5′-AMP was linear with increasing time (0 to 60 minutes) and increasing amounts of enzyme protein (0 to 50 μg per tube). Each value represents the mean of two determinations, differing by not more than 8 percent. Protein was determined by the method of Lowry *et al.* (*13*).

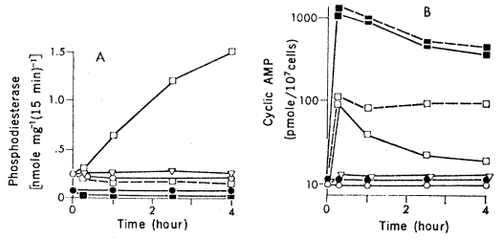

Fig. 2. Phosphodiesterase activity (A) and cyclic AMP content (B) of S and R cells (open and closed symbols, respectively) exposed to the following: no drug (circles); isoproterenol, 1.0 μ*M* (squares with solid line); isoproterenol, 1.0 μ*M*, plus propranolol, 2.0 μ*M* (triangles); isoproterenol, 1.0 μ*M*, plus cycloheximide, 0.2 m*M* (squares with broken line). Incubation media and the assay of phosphodiesterase are described in the legend to Fig. 1. For measurement of cyclic AMP, 1×10^6 to 2×10^6 cells were centrifuged at 2000*g* and the supernatant was discarded. The cell pellet was resuspended in 0.6 ml of ice-cold 5 percent trichloroacetic acid (TCA) and subjected to two cycles of freezing and thawing; the TCA was then extracted, and the cyclic AMP was determined by the competitive protein-binding method of Gilman (*9*). Each cyclic AMP value is the mean of duplicate determinations, differing by not more than 10 percent.

sults with added DB cyclic AMP, isoproterenol—a potent stimulator of endogenous production of cyclic AMP in both S and R cells (*7*)—caused an increase in S cell phosphodiesterase activity (sixfold at 4 hours), but no change in R cells (Fig. 2A). The increased phosphodiesterase activity in S cells was preceded by an initial prompt rise in cyclic AMP content (Fig. 2B). Both the rise in cyclic AMP and the later increase in phosphodiesterase were prevented by propranolol, a β-adrenergic antagonist (Fig. 2). Cycloheximide (0.2 m*M*) also completely prevented the isoproterenol-induced increase in S cell phosphodiesterase, suggesting that endogenously generated cyclic AMP, like exogenous DB cyclic AMP, acts by inducing increased synthesis of the enzyme (Fig. 2A).

We have suggested (*7*) that the decreased accumulation of cyclic AMP produced by isoproterenol in S cells, as compared with the R population, is due to the greater capacity of S cells to degrade cyclic AMP via the phosphodiesterase. In our experiment, the time course of cyclic AMP accumulation after exposure to isoproterenol (Fig. 2B) suggests, in addition, that the induction of phosphodiesterase is in part responsible for a fall in S cell cyclic AMP, which followed an early peak. Thus, at 2.5 hours, when phosphodiesterase activity had increased more than fourfold, the cyclic AMP content had decreased to near baseline. Cycloheximide, which blocked the increase in phosphodiesterase, also prevented the fall in cyclic AMP in S cells, so that even at 4 hours, cyclic AMP was still elevated.

In contrast, cyclic AMP in R cells exposed to isoproterenol (Fig. 2B) rose to a much higher initial level, and fell more slowly. The subsequent fall of R cell cyclic AMP was not affected by cycloheximide, presumably because induction of phosphodiesterase synthesis did not occur and therefore could not be inhibited.

In summary, our experiments suggest that the protein kinase system, including its cyclic AMP–binding regulatory subunit, is indeed involved in cyclic nucleotide–mediated enzyme induction in lymphoma cells. If the only role of the regulatory subunit is to control protein kinase activity, then it appears unlikely that cyclic AMP promotes enzyme induction in mammalian cells in a way comparable to that in bacteria. In bacteria, a cyclic AMP–binding protein regulates gene transcription, apparently without the involvement of a protein kinase (*9*).

In mammalian cells, indirect experiments in other systems suggest that cyclic AMP promotes enzyme induction primarily by stimulating specific posttranscriptional events (*3*). What these events are, however, is unknown. Although the present experiments do not define the locus of regulation, they show that protein phosphorylation may be involved. Cyclic nucleotide stimulation of the phosphorylation of both nuclear histones (*10*) and certain ribosomal proteins (*11*) suggests that either of these structures might be involved. More work is certainly necessary to answer this question.

Finally, induction of phosphodiesterase by endogenously generated cyclic AMP provides a mechanism by which a cell could modulate its response to continued or repeated hormonal stimulation of adenyl cyclase. Although similar increases in phosphodiesterase activity after hormonal stimulation have been observed in other cultured cells (*4*), we do not know whether this is a general phenomenon. Possibly, induction of phosphodiesterase synthesis could serve as an effective protective mechanism for cells which, like those studied here, are killed by cyclic AMP.

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Zinc: A Trace Element Essential in Vitamin A Metabolism

Abstract. *Zinc is necessary to maintain normal concentrations of vitamin A in plasma. By using animals deficient in both zinc and vitamin A, it was demonstrated that zinc is necessary for normal mobilization of vitamin A from the liver. These results suggest that cases of depressed vitamin A in plasma, unresponsive to vitamin A therapy, may respond to zinc supplementation.*

Forty years ago it was reported that certain cirrhotic patients with impaired dark adaptation did not improve after vitamin A therapy, which suggests that another factor may be involved in vitamin A metabolism (*1*). It is probable that those patients had insufficient metabolizable zinc, since patients suffering from cirrhosis have been shown to have depressed concentrations of zinc in plasma (*2*). Zinc-deficient swine have been reported to have low concentrations of vitamin A in serum, and treatment with massive oral doses of vitamin A was of no apparent benefit (*3*). More recently, zinc supplementation was necessary for maximum efficacy of vitamin A therapy to lambs deficient in both zinc and vitamin A (*4*). We have also noted, after studying 61 Baltimore, Maryland, children, 5 years of age, that plasma zinc and vitamin A were positively correlated ($P < .05$). The results of the above investigations suggest an interrelationship between vitamin A and zinc.

In animal studies designed to elucidate such a possible interrelationship, two groups of 15 conventional weanling rats each were employed. One