A cell surface-localized glycoprotein that exhibits alkaline phosphatase activity was induced by treatment of mouse L-cell cultures with dibutyryl cyclic AMP. Treatment of cells with 1.5 mM dibutyryl cyclic AMP for a period of 7 days resulted in a ~2000-fold increase in the specific activity of the enzyme. Enzyme induction was dependent upon de novo RNA and protein biosynthesis since this induction was completely suppressed when actinomycin D (0.5 μg/ml) or cycloheximide (5 μg/ml) was administered with dibutyryl cyclic AMP. Further, the overall rates of incorporation of either [3H]glucosamine or [3H]leucine into macro molecules were identical in the presence or absence of dibutyryl cyclic AMP. Alkaline phosphatase was immunotitrated in 0.5% Triton X-100-solubilized cell extracts with antiserum prepared against purified native enzyme and the results indicated that dibutyryl cyclic AMP stimulated the de novo synthesis of the enzyme. Analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis of specifically immunoprecipitated protein from cells incubated with either [35S]methionine or [6-3H]glucosamine demonstrated that dibutyryl cyclic AMP induced a 76,000-dalton glycoprotein that was characterized as alkaline phosphatase by its identity with native alkaline phosphatase that had been labeled with 32P in its active site. Electrophoretic analysis of specifically immunoprecipitated translation products from an in vitro protein-synthesizing system supplemented with L-cell RNA isolated from uninduced and cAMP-induced cells indicated that dibutyryl cyclic AMP induced the production of alkaline phosphatase-specific mRNA. These results suggest that dibutyryl cyclic AMP directly or indirectly influences the regulation of transcription of the alkaline phosphatase gene in L-cells.

The metabolic effector, cyclic adenosine 3'5'-monophosphate, has been implicated as an important hormonal regulatory modulator in a variety of biological systems (2, 3). These studies established that cAMP-mediated processes control many of the differentiated functions of cultured cells. For example, morphological and growth rate changes have been observed in several types of cultured mammalian cell lines after exposure to dibutyryl cyclic AMP or cAMP (4-7). Also, the expression of specific genes may be controlled through cAMP-mediated processes such as the increased rate of de novo synthesis of tyrosine aminotransferase (8-10), phosphoenolpyruvate carboxykinase (11), the α subunit of human chorionic gonadotropin (12), and murine serum albumin (13).

Alkaline phosphatase is induced by cAMP or its derivative, dibutyryl cyclic AMP, in several mammalian tumor cell lines. In a hybrid cell line developed from mouse mammary tumor cells and Chinese hamster cells, alkaline phosphatase was induced by BtZcAMP almost 300-fold (14), while Nose and Katsuta (7) observed that alkaline phosphatase activity can be induced more than 10-fold by BtZcAMP in aneuploid rat liver cells. In both of these studies, the induction processes were repressed by inhibitors of RNA and protein synthesis, suggesting that the induction process involves de novo synthesis of alkaline phosphatase protein from newly synthesized mRNA. However, direct immunoechemical evidence was lacking in each study. In a recent study, BtZcAMP was shown to induce the de novo synthesis of alkaline phosphatase in BeWo choriocarcinoma cells. The induction was analyzed by a radioimmunooassay and an increase in the amount of alkaline phosphatase protein was clearly demonstrated (12). However, direct evidence for the induction of mRNA for alkaline phosphatase was lacking in the study.

In this paper, we present evidence for a BtZcAMP-mediated induction of the de novo synthesis of alkaline phosphatase protein in cultured mouse L-cells. The induction of translatable mRNA for alkaline phosphatase in cells exposed to BtZcAMP accounted for the induction of enzymatic activity. The present results also indicate that the induced alkaline phosphatase is a plasma membrane-associated glycoprotein. BtZcAMP apparently also affects other cellular processes since exposure to this metabolic effector caused profound morphological changes in the cells.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

**Cellular Morphology**—A profound morphological change was observed when confluent mouse L-cell cultures were exposed to 1.5 mM BtZcAMP. Control cells appear rounded for the most part, while cells exposed to BtZcAMP appear to be fibroblastic with extensive cytoplasmic elongations. The morphological change occurred in the first 12 h of exposure; however, the cells returned to their rounded shape within 24 h after removal of BtZcAMP from the medium. The effect

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1 The abbreviations used are: BtZcAMP, cyclic N6,O2-dibutyryladenosine 3'-5'-monophosphoric acid; MEM, minimum essential medium; TBS, Tris-buffered saline composed of 12.1 g of Tris, 90 g of NaCl/liter, and HCl was added until the buffer was pH 7.3; SDS, sodium dodecyl sulfate; Con A, concanavalin A, Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

2 Portions of this paper (including "Experimental Procedures," Figs. 1 to 8, and Tables I and II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 80M508, cite author(s), and include a check or money order for $2.55 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
Induction of Alkaline Phosphatase

Alkaline Phosphatase Induction—The morphological change that occurred in L-cells after exposure to Bt2cAMP suggested that other metabolic processes were altered. After a lag of 5 h, alkaline phosphatase was induced ~2000-fold in confluent L-cell monolayers over a period of 7 days of continuous exposure to 1.5 mM Bt2cAMP (Fig. 1). The induction process was apparently mediated through CAMP since the induction of alkaline phosphatase after 24 h was synergistically increased following the addition of various concentrations of the phosphodiesterase inhibitor, theophylline, to the incubation medium (data not shown).

A mixing experiment involving extracts from Bt2cAMP-induced cells and control cells indicated that the induction process was not due to the synthesis of an enzyme activator or degradation of an enzyme inhibitor. In data not shown, cellular extracts from cells induced 3 days were mixed in various proportions with control cell extracts (100 μg of total protein) and the resulting specific activity of alkaline phosphatase was additive. Since the control cells exhibited ~500-fold lower specific activity than induced cells, the theoretical additive activity was approximately the same as the activity of the induced cell extract.

The Bt2cAMP-mediated induction of alkaline phosphatase was not due to a general effect of this compound on protein synthesis or protein glycosylation. Bt2cAMP had no effect on either [3H]leucine incorporation or [3H]glucosamine incorporation into 10% trichloroacetic acid-precipitable material. In addition, exposure to Bt2cAMP resulted in no detectable alterations in the rate of [3H]uracil or [3H]thymidine incorporation into macromolecular material, suggesting that the rates of RNA and DNA synthesis were unaffected.

The protein synthesis and RNA synthesis inhibitors, cycloheximide and actinomycin D, respectively, were employed to determine the site of induction of alkaline phosphatase. Exposure to cycloheximide (5 μg/ml) or actinomycin D (0.5 μg/ml) suppressed the induction of alkaline phosphatase when either of the reagents was added to the culture media simultaneously with 1.5 mM Bt2cAMP (Fig. 2), indicating that the induction process is dependent upon new RNA and protein synthesis. During the course of the experiment, the cells remained viable as determined by monitoring the cellular retention of lactate dehydrogenase. When cells were exposed to cycloheximide 20 h after exposure to Bt2cAMP, further induction was immediately suppressed and the level of alkaline phosphatase activity was constant for several days (Fig. 3). This type of effect of cycloheximide has been shown for other induced proteins (33, 34). When cells were exposed to actinomycin D 20 h after exposure to Bt2cAMP (Fig. 3), the induction rate was initially slowed; however, 40 h later, the level of remaining alkaline phosphatase activity was approximately equal to the level of enzyme at 20 h. The continued presence of Bt2cAMP was critical for the maintenance of the induced levels of alkaline phosphatase. Four hours after the Bt2cAMP medium was replaced with control medium, the induced enzyme levels began to decrease and under these conditions, the half-life of alkaline phosphatase was estimated to be ~41 h (Fig. 3).

Partial Purification and Identification of Alkaline Phosphatase—In order to analyze the induction of alkaline phosphatase with immunological methods, the enzyme was partially purified from 10 g of L-cells to a specific activity of 18.1 units/mg by the purification procedure shown in Table I. Alkaline phosphatase was quantitatively solubilized by butanol-1 extraction into an aqueous phase that contained ~13% of the original protein and all of the alkaline phosphatase activity. This preparation was applied to a DEAE-cellulose column and the enzymatic activity was eluted with a linear NaCl gradient of 0 to 0.3 M. Alkaline phosphatase was eluted from the column at ~0.1 M NaCl as determined by conductivity measurements. Subsequently, the pooled activity was applied to a ricin-derivatized Sepharose column and eluted with 2% galactose. The pooled activity from the lectin column was then dialyzed against a buffer containing 10 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 1 mM ZnCl₂, and the preparation was applied to a L-histidylidiazobenzylphosphonic acid-derivatized Sepharose column; an affinity resin for alkaline phosphatase (20). After eluting alkaline phosphatase with a solution containing 10 mM Na₂HPO₄, in 10 mM Tris-HCl, pH 8.0, the pooled fractions were concentrated by pressure dialysis. The final preparation contained ~0.04% of the initial protein.

Analysis of the most highly purified preparation of alkaline phosphatase (Table I) by nondenaturing 5% polyacrylamide gels revealed the presence of two bands by Coomassie blue staining (data not shown). The major band remained at the top of the gel and corresponded with the enzymatic activity when the gel was assayed for alkaline phosphatase activity. Analysis of the same fraction by reducing SDS-polyacrylamide gel electrophoresis revealed two detectable protein bands. In this gel system, the major protein band had a Mr = 76,000 and was identified as alkaline phosphatase by utilizing the [32P]PO₄-active site-labeling procedure of Milstein (26). As shown in Fig. 4 (Lane B), only one protein band (76,000 daltons) was labeled with [32P]phosphate under conditions that trap a phosphorylated form of alkaline phosphatase. The identity of the 76,000-dalton 32P-labeled protein was further confirmed by its co-migration in nonreducing SDS gels with alkaline phosphatase enzymatic activity. Since alkaline phosphatase is active after electrophoresis in nonreducing SDS-polyacrylamide gels (36), the 32P-labeled preparation was subjected to electrophoresis in SDS-polyacrylamide gels under nonreducing conditions with added unlabelled alkaline phosphatase carrier. This gel was analyzed for alkaline phosphatase activity and the segment of the gel that contained the active enzyme was excised. The contents of excised segment were eluted electrophoretically and subsequently subjected to reducing SDS-gel electrophoresis. As shown in Fig. 4 (Lane A), the 76,000-dalton 32P-labeled protein was contained in the portion of the nonreducing SDS gel that was excised on the basis of enzymatic activity. In data not shown, the 32P-labeled protein also bound to a ricin-derivatized Sepharose column and eluted with 2% galactose, indicating that it is a glycoprotein.

Immunoprecipitation of Alkaline Phosphatase—Two lines of evidence indicated that rabbit antibodies were successfully raised against L-cell alkaline phosphatase. Double antibody immunoprecipitates (goat anti-rabbit IgG serum and rabbit serum) were formed with either immune or nonimmune rabbit antialkaline phosphatase serum in the presence of 0.2 unit of partially purified alkaline phosphatase. The immunoprecipitates were incubated with [32P]phosphate, under conditions that radiolabel alkaline phosphatase, and analyzed by reducing SDS-gel electrophoresis. The immunoprecipitate containing antialkaline phosphatase antibodies adsorbed L-cell alkaline phosphatase, while immunoprecipitates that contained nonimmune serum did not precipitate the enzyme.

Alkaline phosphatase activity was also quantitatively immunoprecipitated from Triton X-100-solubilized L-cell extracts and the enzyme remained fully active in the immuno-
precipitate. In data not shown, 0.5% Triton X-100-solubilized L-cell extracts were incubated with rabbit IgG (various combinations of immune and nonimmune) and then adsorbed with goat anti-rabbit IgG antibody-derivatized agarose. With increasing amounts of immune IgG, more alkaline phosphatase was associated with the immunopellet, while the remaining activity was associated with the immunosupernatant. Thus, the antibodies had no effect on the total alkaline phosphatase activity. Since alkaline phosphatase was quantitatively adsorbed to the antibody-derivatized agarose, the immunoprecipitation of the enzymatic activity in the immunopellet represents a direct assay for the amount of alkaline phosphatase in a solubilized L-cell preparation. When goat anti-rabbit IgG serum was substituted for the goat antimonomoglobin-derivatized agarose beads, an equivalent immunoprecipitation was observed.

**Immunotitration of Alkaline Phosphatase from Control and Bt-cAMP-induced Cells**—The immunotitration of alkaline phosphatase from detergent-solubilized preparations of L-cells indicated that the Bt-cAMP-mediated induction of enzymatic activity represented a stimulation in the synthesis of alkaline phosphatase protein. L-cell cultures were exposed to Bt-cAMP for 17, 41, and 89 h and after detergent solubilization of alkaline phosphatase, each sample was immunotitrated as previously described. The immunotitration curves were plotted as a function of alkaline phosphatase activity in the immunoprecipitate versus the amount of immune serum added to the mixture. As shown in Fig. 5, all three immunotitration curves for the solubilized enzyme were superimposable since the percentage of alkaline phosphatase activity immunoprecipitated for a given amount of alkaline phosphatase antibodies was constant for each sample. Thus, in the preparations with an increased level of alkaline phosphatase, an increased amount of antibodies were necessary to immunotitrate a given percentage of activity. These results suggest that the increase in L-cell alkaline phosphatase activity caused by exposure to Bt-cAMP can be totally accounted for by an equivalent increase in the level of immunoprecipitable alkaline phosphatase protein. If the Bt-cAMP-mediated induction of enzymatic activity had been a result of an increase in the catalytic efficiency of alkaline phosphatase, less alkaline phosphatase antibodies would have been utilized in order to quantitatively immunoprecipitate a given percentage of the enzyme activity at the later time points of Bt-cAMP exposure. If this were the case, immunotitration curves for alkaline phosphatase from cells exposed longer periods of time to Bt-cAMP would have been shifted to lower ratios of antibodies/unit of enzymatic activity and the magnitude of the shift would have been proportional to the increase in catalytic efficiency of the enzyme. The results from all immunotitration experiments were consistent with an increase in the amount of immunoprecipitable alkaline phosphatase protein which accounted for the Bt-cAMP-mediated increase in enzymatic activity.

In data not shown, the immunotitration curve of alkaline phosphatase solubilized from control cells was essentially identical with the immunotitration curve for alkaline phosphatase solubilized from cells exposed to Bt-cAMP for 72 h. These results further suggest that exposure to Bt-cAMP results in an increased level of alkaline phosphatase protein, as well as suggesting that an inactive form of alkaline phosphatase is not present in control cells. This latter point was further substantiated when a 10-fold excess of protein from detergent-solubilized control cells did not displace the immunotitration curve observed for equivalent extracts of cells exposed to Bt-cAMP for 72 h (data not shown). The lack of competition in the immunotitration analysis from a 10-fold excess of solubilized control protein indicates that the solubilized extract from Bt-cAMP-exposed cells contains a significantly larger amount of alkaline phosphatase protein than an equivalent sample from control cells.

**Electrophoretic Analysis of Immunoadsorbing Material from Cells Exposed to Bt-cAMP**—The results from the immunotitration experiments were confirmed by electrophoretic analysis of alkaline phosphatase-precipitated material from Triton X-100-solubilized cells incubated in the presence or absence of 1.5 mM Bt-cAMP. L-cells were radiolabeled with either [35S]methionine or [3H]glucosamine. As illustrated in Fig. 6, Bt-cAMP induced the synthesis of a 76,000-dalton [35S]methionine-labeled protein that is precipitated by antialkaline phosphatase antibodies. This induced protein was further identified as alkaline phosphatase by its co-migration in reducing SDS-polyacrylamide gels with the 32P-labeled alkaline phosphatase. The induced 35S-labeled protein also bound to L-histidyl-diazobenzylphosphonic acid-derivatized Sepharose affinity resin (data not shown) and was eluted with 10 mM Na2HPO4.

Since alkaline phosphatase is a glycoprotein, specific immunoprecipitated material from [3H]glucosamine-radiolabeled cells also were analyzed by SDS-gel electrophoresis. As shown in Fig. 7, Bt-cAMP induced a 76,000-dalton [3H]glucosamine-labeled glycoprotein that co-migrated with the 32P-labeled alkaline phosphatase. Thus, a 76,000-dalton Bt-cAMP-induced glycoprotein was identified as alkaline phosphatase by the following criteria: 1) immunoadsorption; 2) co-migration with 32P-labeled alkaline phosphatase; 3) adsorption to an alkaline phosphatase affinity resin; and 4) incorporation of [3H]glucosamine. On the basis of these results, we concluded that Bt-cAMP stimulates the de novo synthesis of a glycosylated form of alkaline phosphatase in cultured L-cells.

**Translation of L-Cell RNA**—Total RNA was isolated from cultured L-cell monolayers exposed in the presence or absence of 1.5 mM Bt-cAMP by combining the guanidinium thiocyanate procedure developed by Chirgwin et al. (22) and the CsCl density centrifugation method developed by Glnsin et al. (23). RNA preparations isolated from cells subjected to each type of culture condition were analyzed for their capacity to direct de novo protein synthesis in a cell-free protein-synthesizing system; [35S]methionine was utilized as a tracer for protein synthesis. The production of 10% trichloroacetic acid-precipitable [35S]methionine-containing material in the cell-free protein-synthesizing system was dependent upon the addition of L-cell RNA. Analysis by SDS-polyacrylamide gel electrophoresis of the total mixture of translation products did not reveal major differences between those obtained from Bt-cAMP-treated cells and control cells. However, analysis of the translation products by immunoprecipitation indicated that a marked increase in alkaline phosphatase mRNA occurred in preparations from Bt-cAMP-treated cells (Fig. 8). The immunoprecipitable translation product co-migrated in SDS-polyacrylamide gels with 32P-labeled alkaline phosphatase. These results suggest that Bt-cAMP causes a stimulation in the production of alkaline phosphatase-specific mRNA. Further, the increase in mRNA appears to account for the expression of alkaline phosphatase activity when the L-cells are exposed to Bt-cAMP.

**Intracellular Localization of Alkaline Phosphatase**—In order to determine if the Bt-cAMP-mediated induction of alkaline phosphatase can be utilized as a model system for analyzing the induction of biosynthesis of a plasma membrane-associated glycoprotein, attempts were made to determine the subcellular localization of alkaline phosphatase. The Bt-cAMP-induced alkaline phosphatase is totally membrane-
associated since 98% of the total activity was recovered in the pellet after centrifugation of a cell sonicate at 85,000 × g for 45 min.

Several experiments indicated that ~50% of the alkaline phosphatase in the B2CAMP-induced cells is associated with the plasma membrane. L-cell plasma membranes were isolated by the dextran-polyethylene glycol aqueous two-phase system developed by Brunette and Till (30); K⁺-dependent (Na⁺, K⁺)-ATPase was used as a marker enzyme. As shown in Table II, ATPase activity was enriched ~16-fold in the plasma membrane fraction, while alkaline phosphatase was enriched 8-fold in this fraction. Approximately 50% of the total alkaline phosphatase activity was found in the plasma membrane fraction when the yield was normalized to 100% plasma membrane fraction, while alkaline phosphatase was in Table 11, ATPase activity was enriched ~16-fold in the enzymatic activity was found in the first supernatant fraction (Table II); the alkaline phosphatase activity in this supernatant fraction pelleted when centrifuged at 80,000 × g for 30 min (data not shown).

Proteolytic susceptibility of alkaline phosphatase in intact cells also suggested that at least 50% of the B2CAMP-induced alkaline phosphatase activity is located in the plasma membranes. L-cells were used for 24 h and digested with either chymotrypsin or papain. In data not shown, ~50% of the total alkaline phosphatase activity was solubilized by chymotrypsin prior to general cellular disruption as determined by the leakage of lactate dehydrogenase into the soluble fraction. Chymotrypsin did not inactivate alkaline phosphatase during the time course of the experiment. Papain also solubilized 50% of the alkaline phosphatase in 4 h with no effect on lactate dehydrogenase leakage (data not shown); however, alkaline phosphatase was inactivated by 30% during the course of the experiment. The rate of the papain-mediated inactivation of alkaline phosphatase was the same for a soluble preparation of alkaline phosphatase, as well as for alkaline phosphatase associated with the plasma membranes on intact cells.

**Lectin Affinity of Alkaline Phosphatase—Solubilized alkaline phosphatase from B2CAMP-induced L-cells bound to a variety of lectin affinity resins. The partially purified preparation of alkaline phosphatase solubilized by chymotrypsin** prior to general cellular disruption as determined by the leakage of lactate dehydrogenase into the soluble fraction. Chymotrypsin did not inactivate alkaline phosphatase during the time course of the experiment. Papain also solubilized 50% of the alkaline phosphatase in 4 h with no effect on lactate dehydrogenase leakage (data not shown); however, alkaline phosphatase was inactivated by 30% during the course of the experiment. The rate of the papain-mediated inactivation of alkaline phosphatase was the same for a soluble preparation of alkaline phosphatase, as well as for alkaline phosphatase associated with the plasma membranes on intact cells.

**Lectin Affinity of Alkaline Phosphatase—Solubilized** alkaline phosphatase from B2CAMP-induced L-cells bound to a variety of lectin affinity resins. The partially purified preparation of alkaline phosphatase solubilized by chymotrypsin prior to general cellular disruption as determined by the leakage of lactate dehydrogenase into the soluble fraction. Chymotrypsin did not inactivate alkaline phosphatase during the time course of the experiment. Papain also solubilized 50% of the alkaline phosphatase in 4 h with no effect on lactate dehydrogenase leakage (data not shown); however, alkaline phosphatase was inactivated by 30% during the course of the experiment. The rate of the papain-mediated inactivation of alkaline phosphatase was the same for a soluble preparation of alkaline phosphatase, as well as for alkaline phosphatase associated with the plasma membranes on intact cells. The large induction, ~2000-fold after 7 days, results, in part, from the extremely low levels of alkaline phosphatase observed in the control cells. In the maximally induced state, the specific activity of alkaline phosphatase is 0.15 unit/mg of cellular protein. This amount of alkaline phosphatase is approximately equal to the specific activity found in B2CAMP-induced choriocarcinoma cells at maximal stimulation (40).

The induced L-cell alkaline phosphatase was identified as a plasma membrane-associated 76,000-dalton glycoprotein based on the following criteria. Analysis by gel electrophoretic techniques of antialkaline phosphatase immunoadsorbing material from detergent-solubilized cells indicated that a 76,000-dalton [³⁵S]methionine-labeled or [³H]glucosamine-labeled protein is induced by exposure to B2CAMP. The induced protein bound to L-histidinylbenzoylphosphonic acid derivatized Sepharose affinity resin is a specific for alkaline phosphatase (20) and is eluted from the resin by inorganic phosphate. Further, a single protein in a partially pure preparation of alkaline phosphatase (specific activity ~ 18.1 units/mg) is labeled with [³²P]phosphate under conditions that have been shown to trap a phosphorylated form of alkaline phosphatase (26). The [³²P]-labeled protein co-migrated in reducing SDS-polyacrylamide gels with the B2CAMP-induced 76,000-dalton protein described above. The induced alkaline phosphatase was identified as a glycoprotein since the detergent-
Induction of Alkaline Phosphatase

solubilized or butanol-1-solubilized enzyme bound to various lectin affinity columns that are specific for carbohydrates. This result was confirmed by an electrophoretic characterization of [3H]galactosamine-labeled immunosorbent-protein from [3H]galactosamine-radio-labeled L-cells. In addition, the 32P-labeled alkaline phosphatase bound to ricin-derivatized Sepharose and was eluted with 2% galactose. It was concluded that the induced alkaline phosphatase is associated with the plasma membrane since it cofractionated with the L-cell plasma membrane marker, K⁺-dependent (Na⁺,K⁺)-ATPase.

These results indicate that Bt2cAMP induces an increase in ribonucleic acid-binding efficiencies of L-cell alkaline phosphatase and the solubilized Bt2cAMP-induced enzyme and the solubilized control enzyme bind to a variety of lectin affinity columns that are specific for carbohydrates.

This result was confirmed by an electrophoretic characterization of the alkaline phosphatase from control and Bt2cAMP-exposed cells and identical. Second, the enzyme from either source may be titrated with antisera in a similar manner, suggesting that the enzymes have most of their antigenic recognition sites in common. Third, both the solubilized Bt2cAMP-induced enzyme and the solubilized control enzyme bind to a variety of lectin affinity columns which strongly suggest that the enzyme from either source is a glycoprotein. Fourth, the rates of heat inactivation at 56°C and the pH optima for the enzymes are similar, and finally, the L-histidinodiazobenzyl phosphonic acid-binding efficiencies are similar for each preparation of alkaline phosphatase.

These results indicate that Bt2cAMP induces an increase in the rate of synthesis of a pre-existing alkaline phosphatase rather than the de novo synthesis of a unique form of the enzyme.

Currently available evidence suggests that the synthesis of membrane glycoproteins is initiated in the rough endoplasmic reticulum by translation of mRNA on membrane-bound ribosomes (41, 42). While in the rough endoplasmic reticulum, the processing of the polypeptide backbone and the attachment and processing of core oligosaccharide side chains occur in cotranslational or post-translational events (43-48). Presumably at this stage, the nascent glycoprotein is incorporated into the membrane structure. Subsequently, the peripheral sugars are added to the oligosaccharide side chain in the Golgi elements and transported to the plasma membrane (49). It appears that alkaline phosphatase is synthesized by this general mechanism in mouse L-cells.

We are continuing our studies on the molecular and cellular events involved in the biosynthesis of alkaline phosphatase in an effort to further elucidate the regulation of the biosynthesis and mobilization of membrane glycoproteins. In a companion paper, we describe a role for glycosylation in the Bt2cAMP-mediated expression of alkaline phosphatase.

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REFERENCES
Induction of Alkaline Phosphatase

The Cyclic AMP-mediated Induction of Alkaline Phosphatase in Mouse Cells by Gary L. Fruton and Edward C. Nash

EXPERIMENTAL PROCEDURES

Materials.

The mouse L cell culture was a generous gift from Dr. Dave (Department of Pharmacology, University of California, San Francisco). The cell culture and cell extracts were purchased from Grand Island Biological Company (Grand Island, NY). The cell culture plates were transferred to Falcon (Becton, Dickinson). 

Dibucityl AMP (5',5'-AMP), p-nitrophenyl phosphate, cytochrome c, 125I, and protein A-derivatized Sepharose were obtained from Sigma Chemical Co. (St. Louis, MO). (3,5-Double) triglycine sulfate (3,5-TGS) was obtained from Research Products International Corp. (Elk Grove Village, IL). Con A-derivatized Agarose, Tulmon-derivatized Agarose and wheat germ agglutinin-derivatized Agarose were purchased from Affi-Gel (Affi-Gel). While purified ricin was prepared by the method of Mitchell, Ed et al. [10].

Preparation of Cell Extracts.

Cells were harvested by removing the medium from the culture flasks, washing the cells twice with TBS (0.01 M NaCl, 0.005 M KCl, pH 7.3), and disrupting the cells by a rubber policeman. The cells were suspended in TBS containing 0.3 g/ml of freeze and stored at 4°C for 2 min. The cell pellets were resuspended in a small volume of TBS (100 to 150 ml) and washed by resuspending at 4°C for 30 seconds, and all of this preprovision was utilized for the cell extracts as indicated.

Assay of Alkaline Phosphatase.

Alkaline phosphatase activity was monitored by the release of p-nitrophenol from p-nitrophenyl phosphate. The assay system contained 0.5 M 3-triiodothyronine, 0.05 M 3,5,3'5'Triiodothyronine, 0.04 M potassium phosphate, 0.1 M of the above enzyme preparation, and 0.04 ml of wheat germ agglutinin (1 mg/ml) as a receptor. The incubation mixture was harvested by centrifugation at 0°C and 0.5 ml of the supernatant was collected by centrifugation at 0°C for 2 min. After the mixture was incubated at 37°C for 24 hours (maintaining the linearity of the assay with time), 0.5 ml of 0.5 M NaCl was added to the supernatant of p-nitrophenol. The absorbance was measured spectrophotometrically at 405 nm. Enzyme activity is defined as that amount that releases the formation of one mole of p-nitrophenol in one minute. Alkaline phosphatase was assayed in nonradioactive, SDS-polyacrylamide gels (absorbed) immediately after electrophoresis by the method of the subalgal with the alkaline phosphatase assay mixture containing p-nitrophenyl phosphate.

Electrophoresis.

Polyacrylamide gels were cast in a 5.2 x 0.5 x 0.25-in. slab gel apparatus as described by O'Farrell [16] and Sammons [17]. The gelling gels contained a linear gradient of polyacrylamide from 6% to 12% and of glycerol from 0% to 10%. Electrophoresis was performed at 25 W per gel for 6 to 8 hours. Gels were stained, dried and subjected to autoradiography as previously described [18]. Non-reducing SDS-polyacrylamide gels were cast as described above, except that the sample buffer was free of sulfhydryl reducing agents.

 Incorporation of [3H]-Phosphosphate into Microsome.

Microsomal pellets were prepared by centrifugation and homogenization of 60 mg of liver tissue. To a final volume of 1 ml of 0.25 M sucrose were added 2 mg of mitochon-dial membranes and 2 mg of mitochondrial membranes. The membranes were incubated at 37°C for 20 min. The reaction was stopped by the addition of 5 M HCl. The membrane pellets were washed three times at 4°C and dissolved in 1 M NaOH. The released [3H]-phosphates were then measured by the method of Jackson and colleagues [19].

Transfection of L Cell Total RNA.

RNA from L cells grown in the presence and absence of 1.5 mM Br-EGTA was isolated by chloroform-phenol extraction method developed by Chirgwin, et al. [20] and the 32P labeling method developed by Stahl, et al. [21]. Briefly, cells harvested from 1% of cell monolayers were isolated in 9 ml of 5 M guanosine trisodium-phosphate/50 mM NaCl/0.3% sodium citrate/0.5 mM lithium chloride, pH 7, and immediately concentrated by centrifugation. The supernatant was removed and the cell pellets was resuspended in 3 ml of 0.5 M sodium chloride, pH 7, and dialyzed against 3 changes of 0.1 M Tris buffer, pH 7.5, and then dialyzed against 3 changes of 0.1 M Tris buffer, pH 7.5. The extracted RNA was then eluted with 8 M urea and 0.1 M sodium acetate buffer (17). The immuno-precipitated protein was analyzed by SDS polyacrylamide gel electrophoresis.

Antibodies.

L-5-Methyladenosine-3',5'-monophosphoryl acid-derivatized Serum was synthesized by the method of Land et al. [22]. Alkaline phosphatase was applied to the column in the presence of 10 ml of 3% TCA, pH 0.1, 10 ml of 3% Tris, pH 7.4, and eluted with a solution containing 10 ml of 3% TCA, pH 0.1, 10 ml of 0.5 M Tris, pH 7.4, and 10 ml of 0.5 M acetic acid, pH 3.0. Alkaline phosphatase was then applied to the same column and eluted with a solution containing 10 ml of 3% TCA, pH 0.1, 10 ml of 0.5 M Tris, pH 7.4, and 10 ml of 0.5 M acetic acid, pH 3.0. Alkaline phosphatase was then applied to the same column and eluted with a solution containing 10 ml of 3% TCA, pH 0.1, 10 ml of 0.5 M Tris, pH 7.4, and 10 ml of 0.5 M acetic acid, pH 3.0. Alkaline phosphatase was then applied to the same column and eluted with a solution containing 10 ml of 3% TCA, pH 0.1, 10 ml of 0.5 M Tris, pH 7.4, and 10 ml of 0.5 M acetic acid, pH 3.0. Alkaline phosphatase was then applied to the same column and eluted with a solution containing 10 ml of 3% TCA, pH 0.1, 10 ml of 0.5 M Tris, pH 7.4, and 10 ml of 0.5 M acetic acid, pH 3.0. Alkaline phosphatase was then applied to the same column and eluted with a solution containing 10 ml of 3% TCA, pH 0.1, 10 ml of 0.5 M Tris, pH 7.4, and 10 ml of 0.5 M acetic acid, pH 3.0.
with 1% sodium tetrade in 0.1 M sodium cacodylate, pH 7.2 for 1 hr and washed 10 times in the same buffer. The fixed cells were vacuum coated with carbon and gold palladium and examined in a Cambridge S-4 Scanning Electron Microscope at 45° tilt.

Pulverized 5-7% film was used for the micrographs.

**Assay for χ-dependent Na⁺ ATPase.** The assay mixture contained 20 μl of sonicated cell extracts, 0.1 M Tris-HCl, pH 7.5, 2 μM ouabain, 60 μM NaCl, 5 μM KCl, 0.1 M ethyleneamine-tetraacetate and 4.6 μl [32P]ATP (360 cpm.μl) in a total volume of 100 μl. The reaction was allowed to proceed at room temperature and terminated after 30 or 60 minutes by incubation at 100°C for several minutes. A 10-μl portion of the reaction mixture was spotted on P81-colla-

lous strips and chromatographed in 1 M formic acid for 1 hr. The chromatograms were stained with ninhydrin and scanned in a Cambridge 5-4 Sterorcan Electron Hlcmrcope at 45° tilt.

**Stability of alkaline phosphatase activity after interruption of Bt2cAMP-mediated induction.** Cell cultures were exposed to 1.5 mM Bt2cAMP (A) and after 22 hours the culture media were mixed with media containing the following: no additions (A); 5 μg/ml cyclhexa-

mine (B); or 0.5 μg/ml aminoglycoside D (O). At the indicated intervals cells were harvested and analyzed for alkaline phosphatase as described in the text.

**Induction of Alkaline Phosphatase**

**Figure 1.** The time dependent effect of Bt2cAMP on the expression of alkaline phosphatase. Confluent mouse L cell cultures in 75 cm² flasks were incubated in the presence (B) or absence (O) of 1.5 mM Bt2cAMP. At the indicated intervals, cells were harvested and analyzed for alkaline phosphatase as described in the text.

**Figure 2.** The effect of cyclhexamine and aminoglycoside D on the Bt2cAMP-mediated induction of alkaline phosphatase. L cell cultures were simultaneously exposed to 1.5 mM Bt2cAMP and either 5 μg/ml of cyclhexamine (O) or 0.5 μg/ml of aminoglycoside D (O). At the indicated intervals, cells were harvested and analyzed for alkaline phosphatase and for the retention of lactate dehydrogenase as described in the text.

**Figure 3.** Stability of alkaline phosphatase activity after interruption of Bt2cAMP-mediated induction. L cell cultures were exposed to 1.5 mM Bt2cAMP (A) and after 22 hours the culture media were mixed with media containing the following: no additions (A); 5 μg/ml cyclhexa-

mine (B); or 0.5 μg/ml aminoglycoside D (O). At the indicated intervals cells were harvested and analyzed for alkaline phosphatase as described in the text.

**Figure 4.** Autoradiographic analysis of the [32P]phosphate-labeled alkaline phosphatase. A partially pure preparation of alkaline phosphatase was labeled in vitro with [32P]phosphate and analyzed by SDS polyacrylamide gel electrophoresis (lane O). This same preparation, with added unlabeled enzyme was subjected to non-reducing SDS-gel electrophoresis. Alkaline phosphatase was assayed and the portion of the gel which corresponded to the enzymatic activity was cut out. The contents of this slice were eluted electrophoretically and analyzed by reducing SDS-gel electrophoresis (lane A). The gels were stained, dried, and exposed to film as described in the text. The molecular weight standards are r-galactosidase (120,000 daltons), phosphorylase A (94,000 daltons), bovine serum albumin (66,000 daltons), ovalbumin (43,000 daltons), and 4.46 kD immunoglobulin light chain (25,000 daltons).

**Figure 5.** Immunonitration of alkaline phosphatase from extracts of L cells exposed to Bt2cAMP for various periods. L cell cultures were exposed to 1.5 μM Bt2cAMP for 17 hours, 41 hours and 89 hours and, after harvesting the cells, alkaline phosphatase was solubilized in 0.5% triton X-100. The immunonitration of the enzyme was monitored by assaying double antibody immunoprecipitate material for enzymatic activity. The per cent alkaline phosphatase activity in the immuno-

precipitate was plotted as a function of the volume (μl) immune serum per unit of alkaline phosphatase added to the original incubation mixture.
Induction of Alkaline Phosphatase

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Figure 6. Immunoadsorption of alkaline phosphatase from extracts of cells incubated in the presence or absence of Bt2cAMP. L Cell cultures were incubated in the presence or absence of 1.5 mM Bt2cAMP in medium containing 100 uCi [32P]orthophosphate. Harvested cells were solubilized with 0.5% Triton X-100, treated with 25 ug of either immune IgG or non-immune IgG, and the immunoprecipitated material isolated and analyzed by gel electrophoresis as described in the text. The autoradiograms were scanned with a densitometer at a wave length of 540 nm. Panel A, 32P-labeled alkaline phosphatase; Panel B, Bt2cAMP treated cells immunoprecipitated with immune IgG; Panel C, Bt2cAMP treated cells immunoprecipitated with non-immune IgG; Panel D, control cells immunoprecipitated with immune IgG; and Panel E, control cells immunoprecipitated with non-immune IgG. The molecular weight standards are 8-galactosidase (120,000 daltons), phosphorylase A (94,000 daltons), and bovine serum albumin (68,000 daltons).

Table 1

<table>
<thead>
<tr>
<th>Purification of Alkaline Phosphatase from L Cells Exposed to Bt2cAMP</th>
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<tbody>
<tr>
<td>Pretreatment</td>
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<tr>
<td>Crude Solvent</td>
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<td>KCl Phase, Butanol Extraction</td>
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<td>DEAE-Cellulose Chromatography</td>
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<td>Bio-Gel-Agarose Chromatography</td>
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<td>L-Histidylalaninephosphonic Acid-Agarose Chromatography</td>
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Table 2

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<tr>
<th>Cell Fractionation of Bt2cAMP Treated L Cells: Specific Activities and Recovery of Na+, K+-ATPase and Alkaline Phosphatase</th>
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<tbody>
<tr>
<td>Alkaline Phosphatase</td>
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<td>L-Cell Homogenate</td>
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<td>Interface (Plasma Membranes)</td>
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<td>Pellet (Nucleolus, Cell Fragments, Internal Membranes)</td>
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<td>Supernatant Fraction from Two-Phase System</td>
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1 No detectable activity.