tion by endogenous proteases, the inability to fully solubilize the chimeric protein, and the selection of altered plasmids could all be contributing factors to the variability in yield. Although recombinant DNA experiments with chemically synthesized DNA are inherently less hazardous than those with DNA from natural sources, consideration should be given to the possible toxicity of the peptide product. A major factor in the choice of somatostatin was its proven low toxicity (*3*). In addition, the experiment was deliberately designed to have the cells produce not free somatostatin but rather a precursor, which would be expected to be relatively inactive. The cloning and growth of cell cultures were performed in a P-3 containment facility.

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28. The position of an asymmetrically located Hha I site in the 203 base pair Hae III lac control fragment (*9*) allows for the determination of the orientation of the Hae III fragment, now an Eco RI fragment, in these plasmids.
29. This was accomplished by preferential Eco RI endonuclease cleavage at the distal site by partial protection with RNA polymerase of the other Eco RI site localized between the Tc^r and lac promoters, which are only about 40 base pairs apart. After binding RNA polymerase, the DNA (5 μg) was digested with Eco RI (1 unit) in a final volume of 10 μl for 10 minutes at 37°C. The reaction was stopped by heating at 65°C for 10 minutes. The Eco RI cohesive termini were digested with S1 nuclease in a solution of 25 m*M* sodium acetate (p*H* 4.5), 300 m*M* NaCl, and 1 m*M* $ZnCl_2$ at 25°C for 5 minutes. The reaction mixture was stopped by the addition of EDTA (10 m*M*, final) and tris-HCl (p*H* 8) (50 m*M* final). The DNA was extracted with phenol-chloroform, precipitated with ethanol, and resuspended in 100 μl of T4 DNA ligation buffer. The T4 DNA ligase (1 μl) was added and the mixture was incubated at 12°C for 12 hours. The ligated DNA was transformed in *E. coli* strain RR1, and Ap^rTc^r transformants were selected on X-gal–antibiotic medium. Restriction enzyme analysis of DNA screened from ten isolated blue colonies revealed that these clones carried plasmid DNA with one Eco RI site. Seven of these colonies had retained the Eco RI site located between the lac and Tc^r promoters.
30. The alkaline phosphatase treatment effectively prevents self-ligation of the Eco RI–Bam HI treated pBH20 DNA, but circular recombinant plasmids containing somatostatin DNA can still be formed upon ligation. Since *E. coli* RR1 is transformed with very low efficiency by linear plasmid DNA, the majority of the transformants will contain recombinant plasmids (*11*).
31. After 10, 20, and 30 minutes, additional somatostatin DNA (40 ng) was added to the reaction mixture (the gradual addition of somatostatin DNA may favor ligation to the plasmid over self-ligation). Ligation was continued for 1 hour and then the mixture was dialyzed against 10 m*M* tris-HCl (p*H* 7.6).
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33. Supported by contracts from Genentech, Inc. to the City of Hope National Medical Center and the University of California. H.W.B. is an investigator of the Howard Hughes Medical Research Institute. We thank L. Shively, Y. Lu, L. Shih, and L. Directo for their assistance in various aspects of the project, and R. A. Swanson for his assistance and encouragement throughout the design and execution of the project. We also thank D. Gelfand and P. O'Farrell for discussing their unpublished data with us.

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Cytidine 3′,5′-Monophosphate (Cyclic CMP) Formation in Mammalian Tissues

Abstract. *Mammalian tissues possess the capacity to synthesize cytidine 3′,5′-monophosphate (cyclic CMP) via the enzymatic conversion of cytidine 5′-triphosphate to cyclic CMP by cytidylate cyclase. Cyclic CMP formation occurs best in the presence of manganese or iron, at neutral* p*H, at 37°C, in the absence of detergents, and with whole tissue homogenate fractions. Thus, mammalian tissues are capable of synthesizing not only cyclic AMP and cyclic GMP, but also cyclic CMP.*

Adenosine 3′,5′-monophosphate (cyclic AMP) and guanosine 3′,5′-monophosphate (cyclic GMP) are purine cyclic nucleotides that are generally thought to influence or regulate numerous cell functions and biological events. In many instances, however, alterations in cell function cannot be accounted for by corresponding or concomitant alterations in the tissue concentrations of either of the two purine cyclic nucleotides. Therefore, the existence of other endogenous regulatory molecules is constantly being sought. Cytidine 3′,5′-monophosphate (cyclic CMP) was first identified in cells (leukemia L-1210) by Bloch, who demonstrated also that the addition of exogenous cyclic CMP to L-1210 cells in culture abolishes the characteristic temperature-dependent lag phase and stimulates the resumption of growth or proliferation of these leukemic cells (*1, 2*). These experimental findings suggest that cyclic CMP, a pyrimidine cyclic nucleotide, may play a biologic role in the control of proliferation of leukemic cells.

Shortly after the discovery of the natural occurrence of cyclic CMP in certain leukemic cells, an enzyme system capable of forming cyclic CMP from its naturally occurring substrate was found in murine myeloid leukemic tumors and in normal mouse liver and spleen (*3*). Thus, our experimental findings on the capacity of mammalian tissues to synthesize cyclic CMP support those of Bloch on the identification of cyclic CMP in malignant cells.

The properties and biologic importance of cytidylate cyclase in normal and malignant mammalian tissues were recently reported briefly (*4*). At the same time Cailla and Delagge reported on the

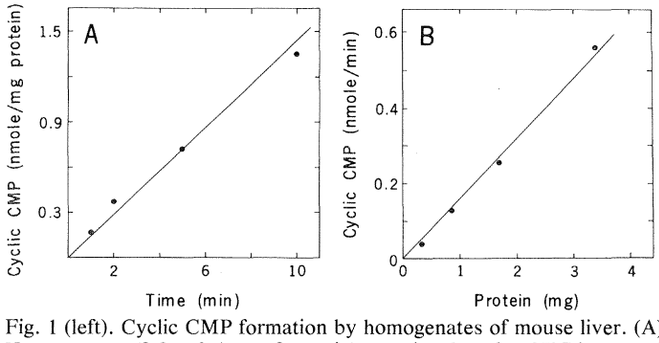

Fig. 1 (left). Cyclic CMP formation by homogenates of mouse liver. (A) Homogenates (2.8 to 3.4 mg of protein) were incubated at 37°C in neutral buffer containing 0.1 m*M* cytidine triphosphate (CTP) and 0.3 m*M* Mn^{2+} in a final volume of 1.0 ml. Incubations were conducted for periods up to 10 minutes. (B) Various amounts of mouse liver homogenate (containing 0.34 to 3.4 mg of protein) were incubated for 10 minutes at 37°C as described above. Data from single representative experiments are shown; three to six additional experiments yielded qualitatively and quantitatively similar data.

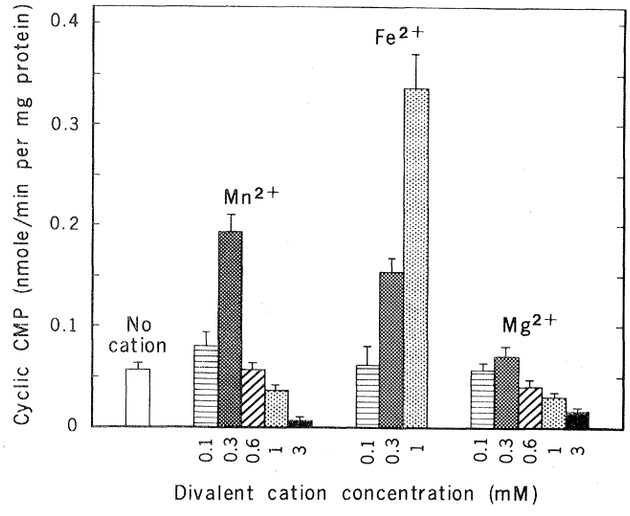

Fig. 2 (right). Effects of divalent cations on cyclic CMP formation by mouse liver homogenates. Homogenates (2.8 to 3.4 mg of protein) were incubated at 37°C for 10 minutes in neutral buffer containing 0.1 m*M* CTP in a final volume of 1.0 ml. The divalent cations tested were Mn^{2+} as $Mn(C_2H_3O_2)_2$, Fe^{2+} as $FeSO_4$, and Mg^{2+} as $Mg(C_2H_3O_2)_2$. Data represent the means ± standard error of the means of 6 to 15 determinations from two to five separate experiments.

development of a sensitive radioimmunoassay which is specific for cyclic CMP (*5*). Further, these investigators provided data confirming the presence of cyclic CMP in leukemic cells and in normal mammalian liver. We now report the first detailed account of the identification of an enzyme system capable of synthesizing cyclic CMP in normal mouse liver.

Initial experiments indicated that the highest cytidylate cyclase activity is in the whole homogenate fraction of normal mouse liver. Enzyme activity in the homogenate fraction was greater than that in the mitochondrial, microsomal, or supernatant fractions. Liver homogenates were prepared according to standard tissue fractionation techniques (*6*). Earlier studies indicated that, although conversion of cytidine triphosphate (CTP) to cyclic CMP by mouse liver homogenates proceeds in the absence of added divalent cation, the inclusion of either 0.3 m*M* Mn^{2+} or 1 m*M* Fe^{2+} greatly facilitates cyclic CMP formation. Cytidylate cyclase activity is determined by monitoring the formation of cyclic [^{32}P]CMP from [α-^{32}P]CTP (*7, 8*). No detectable degradation of added or generated cyclic CMP is evident after 10 to 20 minutes of incubation at 37°C. Moreover, the inclusion of excess unlabeled authentic cyclic CMP (1 m*M*) does not alter the rate of cyclic CMP formation. Through extensive investigation, cyclic CMP was verified as being the principal product of the enzyme reaction by comparison with authentic cyclic CMP in several systems including column chromatography on Dowex-1-formate, column chromatography on polyethyleneimine cellulose, thin-layer chromatography on polyethyleneimine cellulose, crystallization to constant specific activity, resistance to degradation by added beef heart phosphodiesterase, and specific ultrasensitive radioimmunoassay (*9*).

After identifying the whole homogenate as the most active fraction of mouse liver and conducting pilot experiments to determine the optimal incubation temperature (37°C), *p*H (7.4), and ionic requirements (0.3 m*M* Mn^{2+} or 1 m*M* Fe^{2+}), we made a detailed analysis of the properties of cytidylate cyclase. Cyclic CMP formation drops off sharply at acidic or alkaline *p*H, at 4°C or 45°C, in the presence of 1 percent Triton X-100 or Tween 20 (nonionic detergents), and is negligible when the homogenate is boiled for 5 minutes prior to assay. Cytidylate cyclase activity or cyclic CMP formation, in the presence of 0.3 m*M* Mn^{2+} is linear with time for at least 10 minutes and is dependent on the amount of whole homogenate added (Fig. 1). Qualitatively similar data are obtained when 1 m*M* Fe^{2+} is employed instead of 0.3 m*M* Mn^{2+}.

The ionic requirements of cytidylate cyclase from mouse liver are illustrated in Fig. 2. Although cyclic CMP formation occurs in the absence of added cations, the inclusion of either 0.3 m*M* Mn^{2+} or 1 m*M* Fe^{2+} in the incubation mixture greatly facilitates cyclic CMP formation. The addition of EDTA abolishes formation of cyclic CMP either in the presence or absence of added cations, suggesting that sufficient cation is present in the homogenate to allow the reaction to proceed. Magnesium and calcium (data not illustrated) cannot be used as substitutes at concentrations ranging from 0.1 to 3 m*M*. The finding that 0.3 m*M* Fe^{2+} is nearly as active as 0.3 m*M* Mn^{2+} in stimulating cytidylate cyclase activity is interesting since Fe^{2+} does not stimulate either cyclic AMP or cyclic GMP formation. The reasons for the marked increase and decrease in cyclic CMP formation with increasing concentrations of Fe^{2+} and Mn^{2+}, respectively, are not apparent at this time. In collaboration with Cailla and Delaage (*9*), a specific radioimmunoassay for cyclic CMP was used to verify the formation of cyclic CMP in the presence of 0.3 m*M* Mn^{2+} and a two- to threefold greater formation of cyclic CMP in the presence of 1 m*M* Fe^{2+}.

Cyclic CMP formation was studied in several tissues from the mouse and in liver from various species. In the mouse, cyclic CMP formation is highest in liver (0.16 to 0.20 nmole/min per milligram of protein) and lowest in lung (0.08 to 0.09 nmole/min per milligram of protein). The activities of cytidylate cyclase in mouse spleen, kidney, and heart are 0.10 to 0.14, 0.17 to 0.19, and 0.08 to 0.10 nmole/min per milligram of protein, respectively. Cytidylate cyclase activities in liver from rat, guinea pig, and rabbit are 0.17 to 0.19, 0.18 to 0.22, and 0.23 to 0.27 nmole/min per milligram of protein, respectively. The rate of cyclic CMP formation is greater in rapidly proliferating than in slowly proliferating tissues (unpublished result). For example, cyclic CMP formation proceeds two- to threefold more rapidly in regenerating normal rat liver (48 to 72 hours after 80 percent hepatectomy) than in suitable controls. Further, rapidly proliferating murine myeloid leukemic tumors show temporally increasing specific activities of cytidylate cyclase during the first 120 hours

of tumor growth after transplantation to recipient mice (*10*). A kinetic analysis of cytidylate cyclase from these tissues has not yet been done. Preliminary data indicate that the K_m of cytidylate cyclase for CTP, with 0.3 m*M* Mn^{2+} as the cation, is 0.16 m*M* (obtained from a double-reciprocal plot of enzyme reaction velocity as a function of substrate concentration).

The data above represent the first demonstration of cytidylate cyclase activity and the formation of cyclic CMP in biologic tissues. These experimental findings are complementary to those reported by Bloch (*1*) in which cyclic CMP was identified as a natural constituent of malignant murine L-1210 cells in culture. Our findings on cytidylate cyclase activity in normal mammalian liver are supported by the report by Cailla and Delaage of the presence of cyclic CMP in rat liver (*5*). The experimental results reported from these three laboratories suggest that cyclic CMP is associated with stimulation of tissue growth and that this pyrimidine cyclic nucleotide may play a role in the bioregulation of cell proliferation in normal and malignant mammalian tissues.

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6. Livers from BAGG-Swiss male mice (20 g) were homogenized (20 percent, weight to volume) in cold 10 m*M* tris-HCl (*p*H 7.4) containing 10 m*M* NaCl, 10 m*M* KCl, and 0.005 m*M* EDTA (disodium salt) with a Potter-Elvehjem tissue grinder equipped with a Teflon pestle. Homogenates were filtered through Nitex (No. 110) nylon filament bolting cloth (50 μm, pore size) and used 60 minutes after preparation.
7. Cytidylate cyclase assays were conducted in glass tubes at 37°C in a final volume of 1.0 ml. Incubation media consisted of 40 m*M* tris-HCl (*p*H 7.4), 0.1 m*M* CTP, [α-^{32}P]CTP (3×10^5 count/min; 13 to 25 c/mmole; New England Nuclear), 0.3 m*M* $Mn(C_2H_3O_2)_2$, and 0.1 ml of whole homogenate (2.8 to 3.4 mg of protein). Incubations were terminated by the addition of 0.1 ml of cold 60 m*M* EDTA (disodium salt) and cooling samples to 4°C. Cyclic [^{3}H]CMP ($\sim 3 \times 10^4$ count/min; 21 c/mmole: Amersham/Searle) was added to the samples, which were then chromatographed on neutral alumina columns. Alumina column chromatography retains more than 99.99 percent of added CTP and allows for a 60 percent recovery of cyclic [^{3}H]CMP. Chromatography of authentic CTP, CDP (cytidine diphosphate), CMP, and cyclic CMP indicates that only cyclic CMP is recovered in the first 3 ml of eluate after elution with neutral tris buffer. Eluates were added to scintillation fluid, and the radioactivity was measured (*8*). In most instances, neither [α-^{32}P]CTP nor cyclic [^{3}H]CMP required further purification prior to use.
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9. Radioimmunoassays were performed by H. Cailla and M. Delaage from the Centre de Biochimie et Biologie Moleculaire in Marseille, France.
10. Myeloid leukemic tumors, which originated spontaneously in liver and spleen, were obtained as subcutaneous transplants in C57BL mice (Jackson Laboratory), and were transplanted in C57BL/6J or DBA/1J male mice.
11. Supported by a research grant from the Cancer Association of Greater New Orleans.

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Hunger in Humans Induced by 2-Deoxy-D-Glucose: Glucoprivic Control of Taste Preference and Food Intake

Abstract. *Intracellular glucopenia induced by 2-deoxy-D-glucose (2DG) administration in man produces increased hunger ratings and magnitude estimates of pleasantness for sucrose solutions. Augmented food intake substantiates these changes in affective behavior and relieves experimentally induced hunger. Intracellular glucopenia activates counterregulatory mechanisms to raise plasma glucose concentrations. Inducing hunger experimentally with 2DG provides a useful method for studying appetitive behavior in humans. The neurohumoral control of pituitary hormone release and other hypothalamic functions may be examined after 2DG infusion.*

Hypoglycemia (*1*) or intracellular glucopenia (*2*) activates feeding behavior in most mammals. Previous studies have not described the hormonal, metabolic, and behavioral correlates of feeding during experimental hunger. In humans, manipulations of internal states by food loads (*3, 4*) or insulin administration (*5*) and by changes in body weight (*4, 6*) all modify affective behavior directed toward the sweet taste of sugar. In addition, insulin-induced hypoglycemia increases feelings of hunger in humans (*7, 8*).

Administration of 2-deoxy-D-glucose (2DG) competitively inhibits intracellular glucose utilization by inhibiting glucose transport (*9*) and glucosephosphate isomerase and hexokinase activity (*10*). The use of this glucose analog in humans has been limited to investigations of its antitumor effects in cancer patients (*11*) and to the study of metabolic and hormonal responses to glucopenia in several clinical models (*12*). In animal species, 2DG administration usually but not always increases food intake (*13*). Whether this is the case for humans is unknown. Since 2DG infusions increase plasma glucose but not insulin and glucagon concentrations in humans (*12*), the effects of intracellular glucopenia on hunger may be studied independently of these hormonal changes which may, by themselves, affect hunger and satiety (*14*). The use of 2DG facilitates a multidimensional evaluation of appetitive behavior in humans since 2DG induces metabolic, hormonal, and behavioral changes of longer duration than those produced by insulin (*15*).

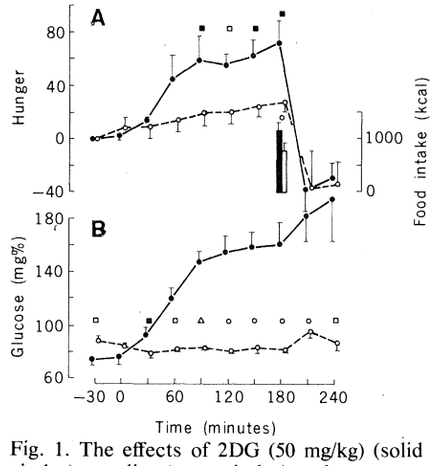

Fig. 1. The effects of 2DG (50 mg/kg) (solid circles) or saline (open circles) on hunger ratings from a visual analog scale (A) and on blood glucose concentrations (B). Bars (A) indicate food intake 180 minutes after 2DG infusion (solid bar) or normal saline infusion (open bar). Means and standard errors of the mean are plotted for a group of five males serving as their own controls. Levels of significance by paired *t*-tests are indicated as follows: $P < .05$, ■; $P < .01$, □; $P < .002$, ○; and $P < .001$, △.

Five healthy, college-age male volunteers of normal weight gave informed consent to receive 2DG (Grand Island Biological) or a sham intravenous infusion of normal saline. The subjects did not know the order of the infusions. They knew that they might experience such side effects as changes in mood, vigor, hunger, thirst, body temperature, and sweating; they did not know the direction of these changes. The subjects were asked to eat additional carbohydrates in the form of bread, potatoes, pasta, and other starches for 3 days before testing to ensure adequate glycogen stores and uniformity of metabolic status the day of infusion. No food, stimulants, or medications were permitted after 8 p.m. the evening before the infusions,