URIDINE 3',5'-MONOPHOSPHATE (CYCLIC UMP)

I. ISOLATION FROM RAT LIVER EXTRACTS

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SUMMARY: A compound isolated from the cold acid soluble fraction and from hot water extracts of rat liver has been identified as 3',5'-cyclic UMP by comparison with the authentic compound with respect to the following characteristics: Rf values in nine solvent systems; electrophoretic mobility in three buffers; UV spectra; nmr spectrum; acid and enzymatic hydrolysis; and effect on L-1210 cell growth in vitro.

INTRODUCTION: We have recently described the isolation of cytidine 3',5'-monophosphate (cyclic CMP) from the cold acid soluble fraction of intact leukemia L-1210 cells and from hot water extracts of disrupted L-1210 cells (1). We have also demonstrated the presence of cyclic CMP in normal rat liver, and have found from 50-200 times more cyclic CMP in regenerating than in normal liver (2). Similarly, we were able to demonstrate (by paper chromatography) the presence of cyclic CMP in 24 hr urine specimens from 5 patients with acute leukemia, but did not detect the cyclic nucleotide in 5 urine samples from normal subjects (3). We have also shown that cyclic CMP can initiate the growth of staged leukemia L-1210 cells in vitro, and that it can overcome the 24 hr lag which ensues when cyclic AMP is added to the cells (4).

During the chromatographic separation of cyclic CMP from the cold acid and the hot water extracts of rat liver, a second UV absorbing material was encountered which we have now identified as 3',5'-cyclic UMP.

EXPERIMENTAL:

Materials: Male Sprague-Dawley rats, approximately 3 months old and weighing 150-200 g, were used for all experiments. Uridine 3',5'-cyclic monophosphoric acid-Na salt and uridine 2',3'-cyclic monophosphoric acid were purchased from the Sigma Chemical Co., St. Louis, Mo. and were purified before use by chromatography in the solvents B and I. 3',5'-cyclic UMP was also prepared by us by deamination of cyclic CMP (5) by reacting it at room temperature with sodium nitrite in a solution of 25% aq. acetic acid, followed by purification through Dowex 50-H^+. Cyclic UMP was obtained as the free acid, and a portion of the compound was converted to the sodium salt by passage through Dowex 50-Na^+. 3'-Ribonucleotide phosphohydrolase (E.C. No. 3.1.3.6), partially purified from rye grass (14 u/mg; 2'-nucleotidase activity 0.5%; 5'-nucleotidase activity < 0.2%), and 5'-ribonucleotide phosphohydrolase (E.C. 3.1.3.5) partially purified from Crotalus adamanteus venom (27.6 u/mg) were purchased from Sigma. Crystalline bovine pancreatic ribonuclease (E.C. 3.1.4.22; 40 u/mg) was purchased from Boehringer Corp., New York. Uracil, UMP, UDP and UTP were purchased from Sigma.

UV Spectrophotometry: The UV spectra were recorded on a Aminco DW-2 spectrophotometer. Before assay, the compounds were dissolved in distilled deionized water at pH 7.0 or pH 11.0, as adjusted with 1N NaOH.

Paper Chromatography: The following chromatographic systems were used for the separation and identification of the extracted material.

(A) Ethanol-1M ammonium acetate (7:3 v/v; pH 6.6); (B) Isobutyric acid-2N ammonium hydroxide (66:34; v/v); (C) n-Butanol-glacial acetic acid-water (50:25:25; v/v); (D) Isoamyl alcohol-5% aq. Na_2HPO_4 (1:1, v/v); (E) Isopropanol-conc. NH_4OH-H_2O) (7:1:2; v/v); (F) Isopropanol-conc. NH_4OH - 0.1 M H_3BO_3 (60:10:30; v/v); (G) Na_2HPO_4 13.8 g into 900 ml H_2O adjust pH to 6.8 with H_3PO_4, add H_2O to 1 l, add 600 g $(NH_4)_2SO_4$ and 20 ml n-propanol; (H) Dissolve compounds in 0.1 ml of 0.1M boric acid, apply to chromatogram, air dry and develop in solvent A. (I) n-Propanol-H_2O (60:40, v/v). All separations were made by ascending chromatography at 20° C for 18 hr on Whatman 3 MM papers.

Paper Electrophoresis: The following buffers were used for the electrophoretic characterization of the extracted material after its purification by paper chromatography:

1) Na_2HPO_4-NaH_2PO_4, 0.02 M, pH 7.8; 2) KH_2PO_4-NaOH, 0.015 M, pH 4.6; 3) Boric acid-sodium borate ($Na_2B_4O_7$. 10 H_2O) (0.62 g: 7.63 g/l, adjust to pH 9.0 with NaOH). Whatman 3 MM papers (15 x 57 cm) were used for all determinations in a water cooled Savant flat plate high voltage electrophoresis unit. A potential of 2500 V was applied for 60 minutes with buffer 1 and 1500 V for 30 minutes with buffers 2 and 3.

Isolation of cyclic UMP from rat liver:

a) Isolation from the acid soluble fraction. The method of Higgins and Anderson (6) was used to remove the median and left lateral lobes from rats anesthesized with nembutal (30 mg/kg). Immediately upon excision, the tissues (250 - 300 g) were cut into small sections and immersed into ice cold 1 N perchloric acid (volume = 3 x tissue weight) contained in a Waring blender. Homogenization was

carried out for 1 min, and the material was kept in the cold acid for an additional 14 min, with occasional stirring. The homogenate was neutralized with 1 N KOH, and the resulting $KClO_4$ together with the tissue debris was removed by centrifugation. The supernatant solution was reduced in vacuo to the smallest possible volume which would allow the solutes to remain in solution, and was applied in narrow bands (0.2 x 40 cm) to 46 x 57 cm Whatman 3 MM papers. After ascending chromatography for 18 hr at 20° in solvent A, a band approximately 5 x 40 cm was cut from each paper at the location of the cyclic UMP marker (the band being removed extending approximately 1.5 cm above and below the marker). The bands were eluted jointly in 2000 ml of distilled deionized water, pH 6.8, and the eluate brought to dryness in vacuo at 30° with a rotary evaporator. The material thus obtained was dissolved in a minimum of water, and was rechromatographed in solvent A, eluted, and rechromatographed twice in solvent B, followed by chromatography in solvent I (2 x). The material was eluted and was used for the various determinations of its identity.

b) Isolation from hot water extracts of disrupted liver cells. To diminish the possibility that the isolated material is an artifact of the acid extraction procedure, rat livers (65-75 g samples) obtained under conditions identical to those described under (a), were frozen at -70° and disrupted by means of a Hughes press. The debris was then extracted for 10-15 min with 1500 ml of boiling water, pH 6.8. The pH did not change during the extraction. The debris was removed by centrifugation, and the supernatant solution was brought to dryness in a rotary evaporator at room temperature. The residue was subjected to repeated chromatography, as described for the acid soluble fraction.

Hydrolytic degradation of the isolated material. An aliquot of the isolated material (7.3 A_{260nm} units, pH 7.0), was heated in 1 ml of 1 N HCl for 2 hr at 100° (5). The solution was cooled and brought to pH 7.0 with 1 N NaOH. To one 0.3 ml aliquot of the neutralized hydrolysate was added 0.1 ml of a solution of 1 mg of 5'-ribonucleotide phosphohydrolase in 1 ml of tris buffer, pH 8.5, and 0.1 ml of 0.1 M $MgCl_2$, and the mixture was incubated at 37° for 1 hr with gentle agitation. To another 0.3 ml aliquot of the neutralized hydrolysate was added 0.1 ml of a solution of tris-buffer, pH 7.5, containing 0.4 mg of 3'-ribonucleotide phosphohydrolase, and the mixture was incubated for 3 hr at 37° with gentle agitation.

Following incubation, the reaction mixtures and the remaining untreated 0.3 ml aliquot were immersed in a boiling water bath for 1 min and their volume was reduced in vacuo to approximately 0.1 ml. The aliquots were applied to a chromatography sheet, which was developed in solvent H, and the dry chromatogram was then photographed under UV light (Fig. 2).

Effect of RNAase on the isolated material. To 3.8 A_{260nm} units, (pH 7.0) of the material isolated from liver, in 0.1 ml of tris buffer, pH 7.0, was added 0.2 ml of a solution of crystalline bovine pancreatic ribonuclease (1 mg/ml of tris buffer, pH 7.0). Incubation with gentle shaking proceeded for 60 min at 37°, after which the reaction vessel was immersed into a boiling water bath for 1 min. The entire mixture was applied to paper, chromatographed in solvent A, and the dry chromatograms were photographed under UV light (Fig. 3). Equivalent aliquots (based on UV absorbance) of authentic 3',5'-cyclic UMP and 2',3'-cyclic UMP were treated identically (7).

Effect of the isolated material on the resumption of leukemia L-1210 cell growth. The techniques used to evaluate the delaying effect which cyclic UMP exerts on the resumption of the growth of leukemia L-1210 cells grown to stationary phase and cooled at 4° for 1 hr, has been described previously (3).

NMR Spectrometry. Approximately 100 μg of the isolated material and of authentic cyclic UMP were dissolved in 0.3 ml aliquots of D_2O and dioxane was added as the internal standard. The spectra were obtained on a Varian Model XL-100 spectrometer operating in the FT mode.

RESULTS: Following extensive chromatographic separation, a UV absorbing material was isolated from the cold acid soluble fraction as well as from boiling water extracts of normal rat liver, which was identified as 3',5'-cyclic UMP on the basis of the following criteria:

1) The material had the same Rf values as authentic cyclic UMP upon paper chromatography in the nine solvent systems listed in the experimental section, and exhibited the same relative mobilities as authentic cyclic UMP upon electrophoresis in the three buffer systems listed in that section.

2) The material has UV spectra (Fig. 1) which are essentially indistinguishable from those of authentic cyclic UMP ($\lambda_{max}^{pH\ 7.0}$ = 261 nm, $\lambda_{max}^{pH\ 11.0}$ = 260 nm).

3) Acid hydrolysis of the isolated material produced two UV absorbing products (Fig. 2) which, in the solvents B, C, F and H had the respective Rf values of 5'-UMP, and 2',(3')-UMP. To confirm the identity of the products, an aliquot of the hydrolysate was treated for 1 hr at 37° with snake venom 5'-nucleotidase. Under these conditions (Fig. 2), the product considered to be 5'-UMP disappeared, and a new UV absorbing material was seen, which, in all four solvents, had Rf values corresponding to uridine. Upon treatment with 3'-nucleotidase, part of the material considered to be 2',(3')-UMP was converted to uridine. The same result was obtained upon hydrolysis of authentic cyclic UMP.

4) Unlike the phosphodiester ring of 2',3'-cyclic UMP, which is opened by

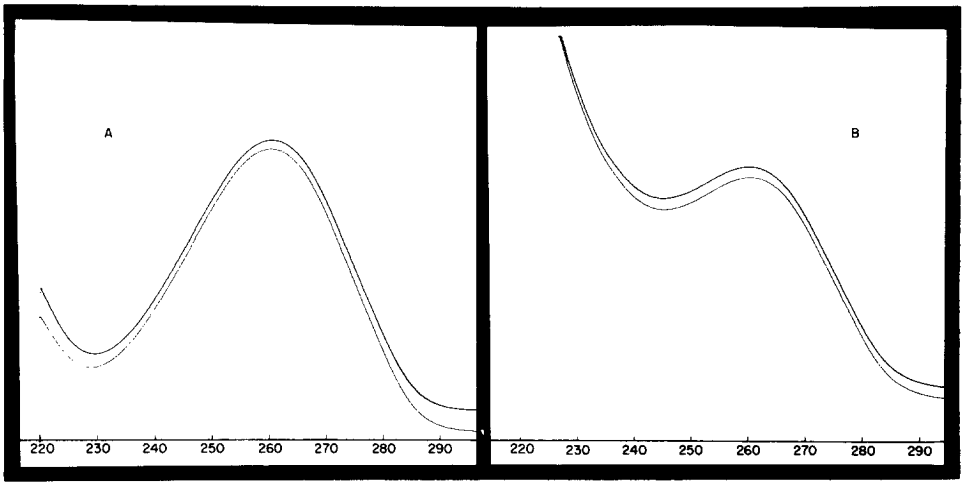

Fig. 1 UV spectra of authentic 3',5'-cyclic UMP at pH 7.0 (A) and pH 11.0 (B) (upper curves), and of the isolated compound at corresponding pH's (lower curves). (The spectra were separated for the sake of clarity, since they overlap closely in the 270-280 nm region.)

treatment with RNAase(7), that of 3',5'-cyclic UMP is not hydrolysed by treatment with this enzyme. Thus, after incubation of 0.5 μmole of 2',3'-cyclic UMP with eight units of RNAase for 60 min at 37°, this nucleotide could no longer be detected upon paper chromatography (Fig. 3). Instead, a new product was encountered which, in the solvents B, F and H had the Rf values of 2', (3')-UMP. Under the same conditions, neither the material isolated from the cells (3.5 A_{260nm} units, pH 7.0) nor authentic 3',5'-cyclic UMP underwent any degradation detectable on the chromatograms (Fig. 3).

5) The identity of the compound isolated was also confirmed by nmr spectrometry. The spectrum of the isolated material was essentially the same as that of authentic cyclic UMP.

6) We have observed (4) that when leukemia L-1210 cells are grown to stationary phase and are then cooled at 4° for 1 hr, growth in the presence of fresh (37°) medium resumes only after a lag of approximately 2 hr. Upon the addition

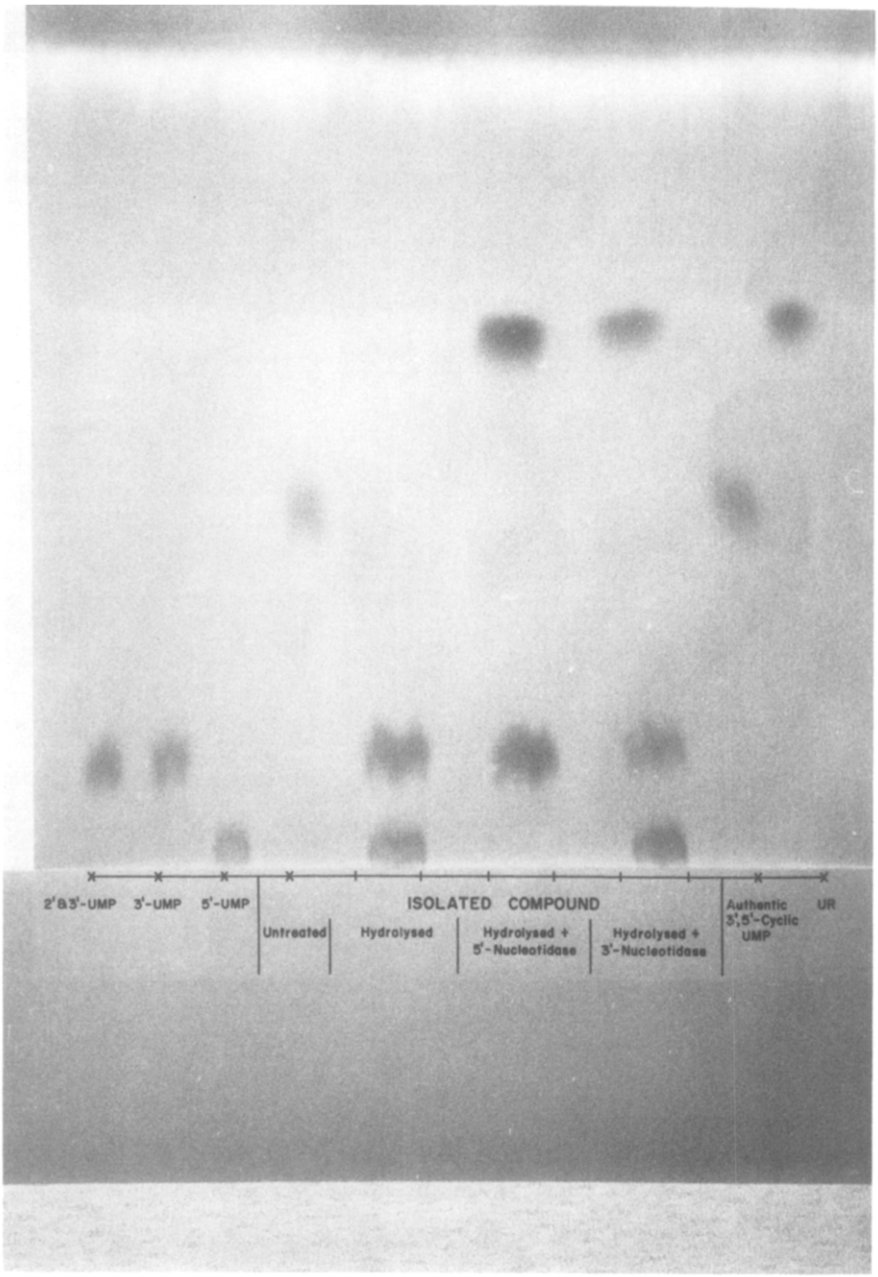

Fig. 2 Paper chromatographic analysis of an acid hydrolysate of the compound isolated from rat liver extracts. 7.3 A_{260nm} units (pH 7.0) of the material were heated in 1 ml of 1 N HCl for 2 hr at 100°. Following neutralization with 1 N NaOH, the hydrolysate was divided into three parts. One was treated with 5'-ribonucleotide phosphohydrolase, another with 3'-ribonucleotide phosphohydrolase and the third served as control. Ascending chromatography of the three reaction mixtures was carried out on Whatman 3 MM paper at 20° for 15 hr in solvent H (see text).

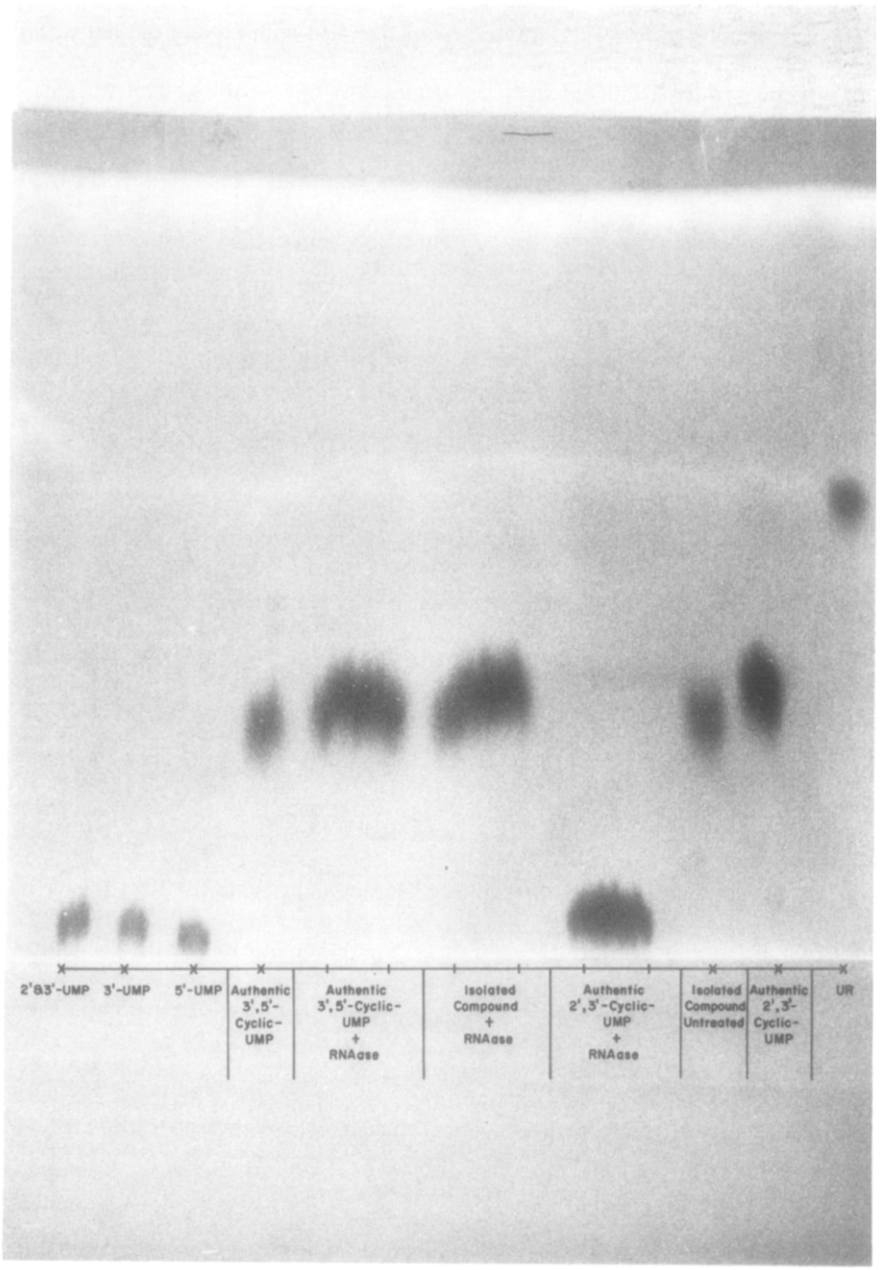

Fig. 3 Lack of susceptibility of authentic 3',5'-cyclic UMP and of the isolated material to cleavage of the phosphodiester bond by RNAase. In contrast 2',3'-cyclic UMP is acted upon by the enzyme.

of 1×10^{-5} M authentic cyclic UMP, the lag is extended by approximately 1.5 hr. The same prolongation of the lag period was produced by the equivalent concentration of isolated cyclic UMP. This additional delay is not effected by uracil, uridine, 2',3'-cyclic UMP, 5'-UMP, 5'-UDP, or 5'-UTP.

The amount of cyclic UMP isolated from the acid soluble and the hot water extract of normal rat liver ranged from approximately 3-6 nmole/g (wet weight).

DISCUSSION: Whereas the identity of the isolated material as 3',5'-cyclic UMP appears firmly established by these studies, the question arises whether this compound is an artifact of the extraction procedure. The fact that the compound was isolated both from cold acid as well as from hot water extracts diminishes the possibility that the cyclic nucleotide was formed by non-enzymatic cyclization. We have also detected the presence of cyclic UMP in the urine of leukemic patients (3), the isolation of the compound from the urine involving only paper chromatographic separation. Further, we have varied the sequence in which we employ the solvents for chromatography, using for instance, solvent I rather than solvent A for initial separation, without any apparent effect on the amount of cyclic UMP obtained. We have observed, however, that subsequent chromatography in solvent B is required before UV spectra exactly superimposable with those of authentic cyclic UMP are obtained, and before the Rf values in some of the solvents systems used for chromatographic identification of the material coincide accurately with the marker. Solvent B separates cyclic CMP from cyclic UMP and from two other components, one faster and one slower than the cyclic nucleotides, suggesting that the two cyclic nucleotides are bound to other cell components which may interfere with their ready detection by direct methods such as immunoassay (8).

The possibility that cyclic UMP is derived from cyclic CMP during the isolation procedure is diminished by the fact that addition of 1 mg of cyclic CMP to cold 1 N perchloric acid, followed by the chromatographic work-up outlined above, did not furnish any detectable cyclic UMP. Nevertheless, ultimate proof that the cyclic nucleotide is a bona fide cell constituent will have to await the identification of enzymes which catalyse the formation of cyclic UMP from appropriate precursors.

The fact that cyclic UMP like cyclic AMP (9) and cyclic GMP (10) delays the onset of the growth of staged L-1210 cells (4), whereas cyclic CMP initiates their proliferation, could imply that cyclic UMP, like cyclic AMP and cyclic GMP (11-14), participates in the regulation of the cell cycle.

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REFERENCES

1) Bloch, A. (1974) Biochem. Biophys. Res. Comm. 58:642-659.
2) Bloch, A., Hromchak, R., and Leonard, R.J. in preparation.
3) Bloch, A., Hromchak, R., and Henderson, E.S. (1975) Proc. Am. Assoc. Cancer Res., in press.
4) Bloch, A., Dutschman, G., and Maue, R. (1974) Biochem. Biophys. Res. Comm. 59:955-959.
5) Smith, M., Drummond, G.I., and Khorana, H.G. (1961) J. Am. Chem. Soc. 83 :698-706.
6) Higgins, G.M., and Anderson, R.M. (1931) Arch. Path. 12 : 186-202.
7) Brown, D.M., Dekker, C.H., and Todd, H.R. (1952) J. Chem. Soc. 2715-2721.
8) Steiner, A.L., Wehman, R.E., Parker, C.W., and Kipnis, D.M. (1972), In: Advances Cyclic Nucleotide Research, 2 : 21-80.
9. Sutherland, E.W. and Rall, T.W. (1957) J. Am. Chem. Soc. 79 :3608.
10. Ashman, D.F., Lipton, R., Melicow, M.M., and Price, T.D. (1963) Biochem. Biophys. Res. Comm. 11 :330-334.
11. Otten, J., Johnson, G.S., and Pastan, I. (1972) J. Biol. Chem. 247:7082-7087.
12. Johnson, G.S., and Pastan, I. (1972) J. Natl. Cancer Inst. 48 :1377-1383.
13. Millis, A.J.T., Forrest, G., and Pious, D.A. (1972) Biochem. Biophys. Res. Comm. 49 :1645-1649.
14. Goldberg, N.D., O'Dea, R.F., and Haddox, M.K. (1973) In: Advances Cyclic Nucleotide Research, 3 : 155-223.