ISOLATION OF ADENOSINE 3',5'-MONOPHOSPHATE AND GUANOSINE 3',5'-MONOPHOSPHATE FROM RAT URINE

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The carbon-nitrogen skeleta and the phosphate moieties of most nucleotides of mammalian bodies are excreted by the kidney as separate units, the non-phosphate portion usually appearing in degraded form. One exception is urinary excretion by man of an intact cyclic nucleotide, adenosine 3',5'-monophosphate (3',5'-AMP) (Butcher and Sutherland, 1962).

Earlier evidence had suggested presence of unidentified organic phosphate compounds in normal urine at a level approximating 1% of the total phosphate (Rae, 1937).

The existence of only a certain type of intact nucleotide in urine represents a selective biological behavior that may prove of interest in relation to rather general biochemical and biophysical characteristics of a system. This communication describes the finding of two cyclic nucleotides in the urine of Fisher rats.

Materials. The following chromatographic solvents were used.

A. Isobutyric acid: 0.5 M NH_4OH (5:3)
B. t-Amyl alcohol: H_2O: 89% formic acid (6:3:1, upper phase)
C. n-Butanol: acetic acid: H_2O (4:1:5, upper phase)
D. Isopropanol: conc. NH_4OH: 0.1 M H_3BO_3 (7:1:2)
E. Saturated ammonium sulfate: water: isopropanol (70:19:2)

Washed Whatman 3 MM paper sheets of 46 x 57 cm were employed. The procedures used for desalting solutions of nucleotides (Norite adsorption, elution with ethanol: conc. NH_4OH: H_2O of 40:2:58) and detection of u.v.-absorbing and radioactive deposits have been reported (Tsuboi and Price, 1959). Synthetic

3',5'-AMP was obtained from Schwarz Laboratories, Mount Vernon, N.Y. A cyclic nucleotide phosphodiesterase that hydrolyses nucleoside 3',5'-monophosphates to nucleoside 5'-phosphates (Drummond and Perrott-Yee, 1961) was kindly donated by Dr. G.I. Drummond, as were synthetic cyclic 3',5'-monophosphates of guanosine, cytidine and uridine (Smith, et al., 1960).

Isolation. In small scale experiments, each of four healthy rats was given an intraperitoneal injection of approximately 0.4 mc phosphate-P^{32} to label nucleotides of rat body origin. The normal pellet diet was replaced by a 10% dextrose solution. Urine was collected for 24 hours in the frozen state. After thawing, the urine was adjusted to pH 4, ether extracted (ether rejected), the pH lowered to 2, and the nucleotides adsorbed on Norite. The eluate was evaporated to dryness in vacuo and the residue dissolved in 2 ml of the eluting solution. 0.2 or 0.4 ml was applied to paper as a 1.5 cm diameter deposit, and the chromatogram developed first dimension in solvent A (ascending flow for 1 day) and second dimension in solvent B (descending flow for 3 days). In the resulting chromatograms, only two localized regions characterized by both P^{32}-activity and absorption of light of 254 mμ could be detected. When aliquots of the Norite eluate were cochromatographed with approximately 100 μg of synthetic cyclic nucleotides, it was found that the labeled compound with highest mobility in solvent A moved with 3',5'-AMP; the compound with lower mobility moved with cyclic guanosine 3',5'-monophosphate (3',5'-GMP). Mobilities of the two compounds in several solvents are presented in part A of Table I.

In a larger scale experiment 20 rats were used, and the Norite eluate chromatographed as applied bands of 75 cm total length. Reference compounds and radioautography aided location of the bands of 3',5'-AMP and 3',5'-GMP after developments in solvents A and B. Final purification was achieved by rechromatography of eluates of the bands in 2 dimensions with the same solvents.

Results. Inspection of Table I shows that parallel studies of the isolated nucleotides and their synthetic counterparts revealed no significant differences between them in chemical, physical or enzymologic properties.

TABLE I

Properties of the Isolated and Known Cyclic Nucleotides

		3',5'-AMP		3',5'-GMP	
		Isolated	Known	Isolated	Known
A.	Mobilities, $R_{phos.}$[1]				
	Solvent A	1.9 (c)[2]	1.9	0.99 (c)	0.99
	Solvent B	0.44 (c)	0.44	0.39 (c)	0.39
	Solvent C	0.71 (c)	0.71	0.28	0.28
	Solvent E	0.11 (c)	0.11	0.19 (c)	0.19
	Electrophoresis[3]	0.46	0.46	0.51	0.51
B.	Fluorescence[4]	-	-	+ (Blue)	+ (Blue)
C.	Spectra pH 1				
	λ_{max}, mμ	258	258	258	258
	E_{250}/E_{260}	0.83	0.83	0.87	0.87
	E_{280}/E_{260}	0.25	0.24	0.71	0.68
	Spectra pH 7				
	λ_{max}, mμ	---	---	254	254
	E_{250}/E_{260}	---	---	1.07	1.08
	E_{280}/E_{260}	---	---	0.70	0.69
	Spectra pH 13				
	λ_{max}, mμ	261	261	262	262
	E_{250}/E_{260}	0.74	0.76	0.89	0.84
	E_{280}/E_{260}	0.22	0.21	0.67	0.68
D.	$Ba(OH)_2$ Hydrolysis Products	5'-AMP 3'-AMP	5'-AMP 3'-AMP	--- ---	--- ---
E.	Brain Phosphodiesterase Hydrolysis Products	5'-AMP adenosine phosphate 5'-IMP inosine	5'-AMP adenosine phosphate 5'-IMP inosine	5'-GMP guanosine phosphate --- ---	5'-GMP guanosine phosphate --- ---

1 Chromatographic mobility relative to that of orthophosphate as 1.00.

2 Indicates that mobility was observed by cochromatography of radioactive urinary nucleotide with a large excess of the corresponding known nucleotide.

3 Paper electrophoresis done with water-cooled apparatus using phosphate buffer of pH 7.1 and ionic strength 0.05. Mobilities in this row only are relative to the corresponding nucleoside 5'-monophosphate.

4 Both samples of 3',5'-GMP fluoresce to light of 254 mμ (and not to light of 365 mμ) when in the strongly acid solvent B. In weaker acids (e.g., solvent A of pH 3.6) or alkali no visible fluorescence is observed.

It may be seen that electrophoretic mobility (A) of a cyclic nucleotide at pH 7 was near half that of the corresponding nucleoside 5'-monophosphate, connoting existence of one negative charge per molecule. Deposits of 3',5'-

GMP in a solvent of pH 2 exhibit the bright bluish fluorescence (B) characteristic of other guanosinium derivatives (Smith and Dunn, 1959). The isolated nucleotides have ultraviolet spectral properties (C) conforming to the designated structures, and the isolated 3',5'-AMP treated with 0.4 N $Ba(OH)_2$ at 100° yielded the expected products (D) (Lipkin, et al., 1959); chromatography in solvents D and E revealed no 2'-AMP.

Degradation of 3',5'-nucleotides with cyclic nucleotide phosphodiesterase theoretically yields only the corresponding 5'-nucleotide (Drummond and Perrott-Yee, 1961; Butcher and Sutherland, 1962). The expected 5'-nucleotide was the main product (E) in all of our experiments, but minor yields of other identified compounds revealed that the particular enzyme preparation employed contained traces of a 5'-nucleotide phosphomonoesterase and adenylate deaminase.

The daily excretion of 3',5'-AMP in urine was determined by the reverse isotope dilution method, and found to be 18 μg per 100 g rat body weight. The value for 3',5'-GMP was estimated at 13 μg/100 g based on an assumption that the isolation procedure extracts the two cyclic nucleotides from the urine with equal efficiency. Possibility that excretion rates may be affected by the 24 hour glucose-water diet has not been examined to date.

Discussion. This study appears to provide the first evidence that guanosine 3',5'-monophosphate exists in biological systems. The indications of prior and present results are that the 3',5'-AMP and 3',5'-GMP are synthesized inside body cells, traverse at least some cell walls, are fairly stable in the blood plasma, and penetrate the glomerular membrane into the urine. The single negative charge of these cyclic nucleotides at physiological pH apparently contributes to their permeability property. In support of this hypothesis, it is notable that the only other identified urinary organophosphate is a one-charge bis-phosphodiester formed consequent to administration of β-naphthylamine (Troll, et al., 1959). Also, singly-charged nucleoside 5'-sulfates are per-

meable and excreted in urine (Arnold and Price, 1963), in contrast to their doubly-charged nucleoside 5'-phosphate counterparts.

Recent evidence indicates that 3',5'-AMP is an essential intermediate in hormonal regulation of function of some cells (Rall and Sutherland, 1961). It is possible that future investigations will reveal regulatory roles for 3',5'-GMP.

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Erratum

Biochem. Biophys. Res. Commun. 10, 333 (1963), in the communication "Increase in Liver Acetyl / Coenzyme A during Ketosis" by O. Wieland and L. Weiss:

Page 337, Table II, Footnote b, "100 mg of dexamethasone . . . " should read:

"100 μg of dexamethasone . . . "