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Research Article

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On exposure of cyclic AMP-^{3}H to a crude homogenate of the epithelial cells scraped from the mucosal face of the bladder, the principal metabolite was inosine-^{3}H, whereas 5′-AMP-^{3}H was either absent or present in undetectible amounts. However, when the homogenate included added 5′-AMP (2×10^{-2} mole/liter), substantial quantities of 5′-AMP-^{3}H were recovered.

Metabolism of cyclic AMP-^{3}H by homogenates of the epithelial cell scrapings from the bladder was strongly stimulated by alkalinization over the range in pH of 6-9. Theophylline inhibited metabolism of cyclic AMP only to a limit of 50%, the inhibition being limited to the OH^--stimulated component. These results suggest the possibility that a second pathway for metabolism of cyclic AMP may exist. If such is the case, its relationship, if any, to the ultimate biological effects of cyclic AMP within cells will be studied.

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Metabolism of Adenosine 3',5'-Monophosphate by Epithelial Cells of the Toad Bladder

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A B S T R A C T When whole urinary bladders of the toad were incubated with adenosine 3',5'-monophosphate-³H (cyclic AMP-³H) > 95% of the radioactivity could be extracted from the tissue with trichloroacetic acid (TCA). The TCA-soluble radioactivity was separable by cation-exchange (Dowex-50) chromatography into residual cyclic AMP-³H, 5'-AMP-³H, adenosine diphosphate (ADP)-³H and inosine-³H. Thus, neither substantial tight binding of cyclic AMP to TCA-precipitable cell constituents nor any novel metabolite of cyclic AMP were found.

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Metabolism of cyclic AMP-³H by homogenates of the epithelial cell scrapings from the bladder was strongly stimulated by alkalinization over the range in pH of 6–9. Theophylline inhibited metabolism of cyclic AMP only to a limit of 50%, the inhibition being limited to the OH⁻-stimulated component. These results suggest the possibility that a second pathway for metabolism of cyclic AMP may exist. If such is the case, its relationship, if any, to the ultimate biological effects of cyclic AMP within cells will be studied.

INTRODUCTION

The antidiuretic action of vasopressin is mediated within the target cell by adenosine 3',5'-

monophosphate (cyclic AMP) (1, 2). Vasopressin acts by activation of adenyl cyclase, the enzyme which catalyzes formation of cyclic AMP from adenosine triphosphate (3, 4). The intracellular level of cyclic AMP is also regulated by the enzyme cyclic nucleotide phosphodiesterase, which catalyzes hydrolysis and inactivation of cyclic AMP to adenosine 5'-phosphate (5'-AMP) (5). The latter reaction is inhibited by the methyl xanthines, caffeine, and theophylline.

Although many features of the over-all antidiuretic pathway (6–9) and of the above enzymatic reactions (10) have been defined, there is no knowledge at present of the chemical steps by which cyclic AMP produces alterations in the resistance of the limiting membrane to flow of water along an osmotic gradient. Although Handler and Orloff demonstrated activation by vasopressin and cyclic AMP of phosphorylase in toad bladder and mammalian kidney (11), they later concluded that the physiological significance of phosphorylase activation relative to antidiuresis is unclear (2).

A question which has not been resolved is whether cyclic AMP or a metabolite of cyclic AMP is the final effector of permeability changes in target cells and whether the active nucleotide acts directly at the locus of change in permeability or rather initiates a further set of reactions which in turn bring about the ultimate effects of vasopressin. To gain insight into this final phase in the antidiuretic pathway we studied the chemical fate of cyclic AMP in one target tissue, the urinary bladder of the toad, and also examined the effects of pH and theophylline on metabolism of cyclic AMP. Observations on this tissue would be relevant to the action of cyclic

AMP in many other hormone-sensitive tissues (10, 12–15).

METHODS

For studies with the intact tissue urinary bladders from *Bufo marinus* [1] were mounted on glass tubing as sacs except that the bladders were everted so that the serosal surface formed the inside of the sac. The bags were filled with 3 ml of a standard serosal solution and were placed into a large reservoir which also contained the standard serosal solution of the same composition as previously described (9) except that glucose was omitted from the medium. Th solution used to fill the bags contained cyclic AMP-³H at a concentration of 1.6×10^{-5} mole/liter (1.1–1.4 c/mmole). The external solution was oxygenated by bubbling vigorously with air. After the bags, in groups of four to six, had been exposed for 1 hr to the cyclic AMP-³H, the exposed area of tissue was excised, blotted lightly, and frozen in liquid nitrogen. Pairs of bladders were minced together and homogenized in a glass homogenizer with 4 ml of 5% trichloroacetic acid (TCA). The precipitate was removed by centrifugation and washed twice with small volumes of 5% TCA. The TCA extract and washes were pooled. In studies WB-3 and WB-4 the TCA pools were applied directly to the cation-exchange columns, whereas in all subsequent studies the TCA pool was first extracted three times with an equal volume of diethyl ether. The pH of the unextracted TCA pool was approximately 0.7, while after ether extraction the pH of the pools ranged from 2.1 to 2.2. Nucleic acids were extracted from the TCA precipitates with 2 ml of 10% NaCl in a water bath at 90°C for 60 min. The original 10% NaCl extract and two subsequent washes were pooled and aliquots were taken for radioassay. The residual precipitate was digested with 1 ml of 1.0 N KOH, and an aliquot was taken for assay of radioactivity.

For studies of homogenates of the epithelial cell of the bladders the hemibladders were everted, incised, and spread on a flat surface. Epithelial cells were removed in sheets from the mucosal surface of the bladders by lightly scraping with an ordinary glass microscope slide. Epithelial cells from 10 to 12 hemibladders were scraped into the standard serosal solution, pooled, and collected by centrifugation. After centrifugation the pellet of epithelial cells was homogenized using the Polytron [2] device at the number 7 setting for about 1 min. The homogenizing medium contained 96 mmoles of KCl, 2 mmoles of MgCl₂, and 40 mmoles of Tris per liter (titrated to pH 8.5 with HCl). For studies of the identification of metabolites of cyclic AMP-³H by column chromatography, 2-ml aliquots of the epithelial cell homogenates were mixed with 1.5 ml of cyclic AMP-³H (with or without added unlabeled 5′-AMP and inosine) dissolved in water and 0.5 ml of a fourfold concentration of the standard KCl-MgCl₂-Tris homogenizing medium. The final concentration of cyclic AMP in the mixture

was 1.1×10^{-5} mole/liter. When added, the final concentration of 5′-AMP and of inosine was 5×10^{-2} mole/liter for each. The mixture was incubated for 60 min at room temperature and after removal of aliquots for radioactive assay and for direct application to the Sephadex G-10 column,[3] 0.1 volume of 50% TCA was added, and the precipitate was removed by centrifugation. The TCA extract and two subsequent TCA washes of the precipitate were pooled. After extraction of the TCA pool three times with an equal volume of ether the TCA pool was applied to the ion-exchange column. Unless otherwise stated, chromatography was at room temperature.

For studies of the effect of pH and theophylline on metabolism of cyclic AMP-³H by homogenates of the epithelial cells the procedure was the same as in the preceeding paragraph through the step of homogenization of cell scrapings. At this stage we measured into five pairs of small test tubes the following solutions: (a) 0.4 ml of the epithelial cell homogenate, (b) 0.05 ml of 400 MM Tris titrated to pH 6, 6.7, 7.3, 7.9, or 9.0 with HCl, (c) 0.05 ml of cyclic AMP-³H, (d) either 0.1 ml of 50 MM theophylline [4] to the experimental tubes or 0.1 ml of a 1.25 × concentrated solution of the basic KCl-MgCl₂-Tris homogenizing solution. Final concentration of cyclic AMP was 7×10^{-5} mole/liter.

The mixtures were incubated at room temperature for 1 hr after which 0.05 ml of 50% TCA was added. After removal of the precipitate by centrifugation a 10 μl aliquot from each tube was spotted separately on PEI-cellulose [5] thin-layer plates along with a mixture of nonradioactive neucleosides and nucleotides which included adenosine diphosphate (ADP), adenosine 5′-phosphate (5′-AMP), cyclic AMP, adenosine, and inosine.[6] An aliquot of the TCA extract was also taken for assay of radioactivity and after further washing with TCA the precipitate was digested with 1.0 N NaOH. An aliquot was taken from the alkaline digest for assay of radioactivity and for the determination of protein by the Lowry method (16).

The cation-exchange resin used throughout these studies was of the Dowex-50 type (AG-50-X8, 200-400 mesh [7]). The resin was washed in bulk with successive volumes of 1.0 N NaOH, 5.0 N formic acid and water, and then equilibrated with 0.03 M formic acid buffer (made to pH 2.7 with NH₄OH). The size of the columns and the buffer systems used for elution are described in the Result section under individual experimental groups. The anion-exchange resin used throughout was diethylaminoethyl (DEAE)-Sephadex,[8] A-25, coarse. For initial

[3] Pharmacia Fine Chemicals Inc., Piscataway, N. J.

[4] Nutritional Biochemicals Corporation, Cleveland, Ohio.

[5] Polyethyleneimine obtained from Chemirad Corp., East Brunswick, N. J. and cellulose from Macherey, Nagel & Co., Duren, Germany.

[6] Nucleotides and nucleosides from Calbiochem, Los Angeles, Calif. and Schwarz Bio Research, Inc., Orangeburg, N. Y.

[7] Bio-Rad Laboratories, Richmond, Calif.

[1] Tarpon Zoo, Silver Springs, Fla.

[2] Brinkmann Instruments Inc., Westbury, N. Y.

studies the DEAE-Sephadex was prepared by successive washes of 0.5 M NaOH, 0.5 M acetic acid, 0.5 M ammonium acetate, pH 8.9, and 0.01 M ammonium acetate, pH 8.0. Since a simpler method of preliminary washing was found to be equally satisfactory, in later studies the exchanger was prepared by washing with 0.5 M ammonium acetate (pH 8.9) followed by equilibration with the 0.01 M ammonium acetate (pH 8.0) buffer. The size of columns and the buffer systems used for elution are described in the result section under individual experimental groups. The uncharged Sephadex G-10 was prepared simply by exhaustive washing with distilled water until the optical density of the washing water at 260 mμ was negligible.

Cyclic AMP-^{3}H used in these studies was purified before use by passage through a 0.9 × 16 cm Dowex-50 column. This procedure eliminated a small impurity (1–5%) which emerged before cyclic AMP from the column.

Thin-layer chromatography was performed according to the method of Randerath (17) with 0.5% PEI-cellulose plates of 1.0 mm thickness. The plates were developed with a 0.5 M LiCl–5 mM Tris chloride (pH 8.4) solution. Radioactive substances were eluted from the thin-layer plates by scraping the indicated zones from the glass with a razor blade and extracting the fragmented PEI-cellulose in a test tube with 1.0 M ammonium formate (pH 5.5) for several days. The initial ammonium formate extract and two subsequent washings were pooled and aliquots taken for assay of radioactivity. Paper electrophoresis was carried out using Whatman No. 1 paper strips with a voltage source kept constant at 150 v. The buffers used for individual experiments are described in the Result section.

Confirmation of the supposed identity of radioactive peaks was obtained by cochromatography of the radioactive unknowns with nonradioactive, pure reference standards. In the case of 5′-AMP and inosine additional support for the identity of the radioactive peaks was obtained by comparing the rate of transformation of both the radioactive peak and pure reference standards (in a mixture) to derivatives using enzymatic transformation or acid hydrolysis. Transformation of 5′-AMP to inosine 5′-phosphate (5′-IMP) was achieved using the highly specific 5′-adenylic acid deaminase prepared from rabbit muscle.[8] Incubation with enzyme was carried out according to the method of Kalckar (18) followed by separation of substrate from product on the cation-exchange column system described above (AG-50-X8). Transformation of the radioactive peak suspected to be inosine along with either pure reference nonradioactive inosine or pure reference nonradioactive hypoxanthine was accomplished by incubating for 1 hr in a water bath at 95–99°C, a 1:1 mixture of the nucleotide solution and concentrated HCl. After cooling and neutralizing the acid with NaOH we chromatographed aliquots on PEI-cellulose plates as described above.

Samples for radioactive assay were pipetted into a 2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene

[8] Sigma Chemical Co., St. Louis, Mo.

(PPO-POPOP) liquid scintillation solution the composition of which has been described (8) and assayed in a liquid scintillation counter.[9] Corrections for quenching were made using internal standards.

RESULTS

Metabolites of cyclic AMP-^{3}H in whole bladders. In order to evaluate the possibility that breakdown of the cyclic AMP-^{3}H might occur during homogenization and extraction of the tissue with TCA during column chromatography, two control studies were performed. In these studies sections of toad bladder frozen in liquid nitrogen, ice cold TCA, and an aliquot of cyclic AMP-^{3}H were added to homogenizing tubes, and the mixture was homogenized at ∿ 0°C. After centrifugation the TCA supernatant was chromatographed on a Dowex-50 column as described under Methods for studies in which the tissues were exposed to cyclic AMP-^{3}H for 1 hr before homogenization. In both control studies greater than 97% of the radioactivity was recovered in a single peak in the position of cyclic AMP. From 1 to 2.5% of the radioactivity emerged early from the column (before cyclic AMP) after a buffer volume approximately equal to the volume of the ion-exchange bed had passed through the column.

The results of four studies on separation of radioactive compounds in the TCA extract of whole bladders which had been exposed for 60 min to cyclic AMP-^{3}H are shown in Table I. Fig. 1 shows the results of study WB-3 in which the tissues contained the largest percentage of radioactivity as metabolites of cyclic AMP. Peak B is the

[9] Packard Instrument Co., Inc., Downers Grove, Ill.

TABLE I

Separation by Column Chromatography on Dowex-50 of Radioactive Metabolites of Cyclic AMP-^{3}H in the Trichloracetic Acid Extract of Whole Toad Bladders

| Study | Radioactive peaks* | | | |
	A	B	C	D
WB-3	27.4	43.9	18.7	10.1
WB-4	6.1	86.0	7.8	Trace
WB-6	Trace	96.0	0	0
WB-8	9.2	71.8	17.7	1.3

Cylic AMP, adenosine 3′,5′-monophosphate.
* Values for each peak are the per cent of total activity recovered in the column effluent.
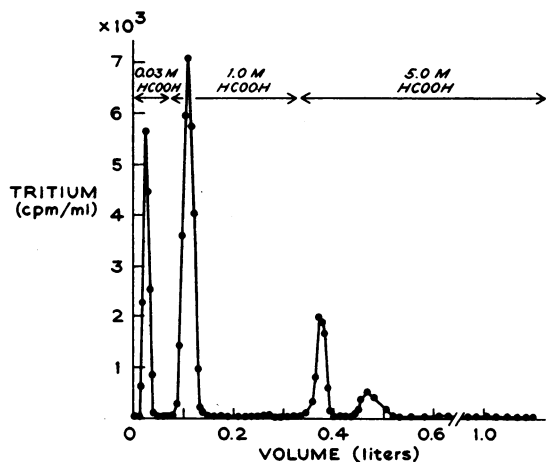
FIGURE 1 Separation of adenosine 3',5'-monophosphate (cyclic AMP)-³H from metabolites in the trichloroacetic acid (TCA) extract of whole toad bladders. Elution of nucleotides from the 0.9 × 30 cm Dowex-50 column was by means of formic acid (HCOOH) solutions. The horizontal axis represents the cumulative volume of buffer used. The peaks of radioactive metabolites in this study (WB-3) and the others listed in Table I are designated A, B, C, and D (from left to right).

position known by previous calibration of columns to be the characteristic elution position for cyclic AMP. Studies on the identity of peaks A, C, and D will be discussed below. In study WB-4 both serosal and mucosal media contained theophylline (2.9 × 10⁻² mole/liter) during the period of exposure to cyclic AMP–³H. Additional studies in the presence of theophylline were not carried out because of the extreme variability in the rate of metabolism found with the intact tissues. Thus, in the three studies without theophylline (WB-3, WB-6, and WB-8) the percentage of radioactivity present as metabolite ranged from as high as 56% to as low as 4%. Further efforts at this stage, therefore, were directed at identifying the nature of peaks A, C, and D.

Of the total radioactivity found in tissues in these studies, 96% was TCA soluble, 3.5% was extracted from the TCA precipitate with hot 10% NaCl, and finally 0.5% was found in the KOH digest after the hot NaCl extraction procedure. Thus, only very small amounts of radioactivity if any were incorporated into nucleic acids and proteins.

Identification of acid-soluble metabolites of cyclic AMP–³H in whole bladders. Peaks A from studies WB-3 and WB-8 were lyophilized and fur-

ther chromatographed on DEAE-Sephadex columns. After loading a 1 × 15 cm DEAE-Sephadex column with peak A of WB-3 in 0.01 M acetate (ammonium) buffer, pH 8, the column was developed by serial changes in buffers of increasing acidity and ionic strength. However, the radioactive material emerged (as a single peak) from the column in 5.0 M formic acid only after a combined buffer volume of 1385 ml. Although no cold standards were chromatographed with the radioactive material, the very strong retardation of this metabolite, well beyond the point of emergence of 5'-AMP, strongly suggested that the substance had a high negative charge. We therefore chromatographed peak A from study WB-8 along with nonradioactive ADP on a 0.9 × 40 cm DEAE-Sephadex column. To reduce any possibility of acid breakdown the column was developed using a continuous gradient from 0.10 M ammonium acetate (pH 6) to 1.0 M ammonium acetate (pH 4.4). The radioactivity and the cold ADP (measured by absorption at 260 mμ) emerged as a single symmetrical peak after 1028 ml of buffer had passed through the column.

Identification of *peak B* was also obtained by comparison of the radioactive peaks with nonradioactive reference cyclic AMP on DEAE-Sephadex columns (0.9 × 16 cm). Peaks B from studies WB-3 and WB-4 were chromatographed with cold standards in two separate studies and in both instances the radioactivity and the nonradioactive standards emerged together in the position characteristic for cyclic AMP. Peak B from study WB-3 was also compared to known cyclic AMP by thin-layer chromatography on PEI-cellulose plates. The radioactive material coincided with the pure nonradioactive cyclic AMP, which was clearly separated by the buffers used for development (0.7 M LiCl–10 mM Tris chloride, pH 8.4) from ADP, 5'-AMP, inosine, inosine diphosphate (IDP), and inosine triphosphate (ITP).[10] 5'-IMP in this system has the same mobility as cyclic AMP; however, as 5'-IMP is eluted considerably earlier from Dowex-50 than the radioactive material, this nucleotide was clearly eliminated as a possibility.

The elution position of *peak C* from Dowex-50

[10] Gulyassy, P. F., and M. Neate. Separation of adenosine 3',5'-monophosphate from related nucleotides and nucleosides. Manuscript in preparation.

suggested that this peak was 5'-AMP. Cochromatography of peak C from study WB-4 with reference standard 5'-AMP on Dowex-50 confirmed this possibility as radioactive material, and reference 5'-AMP emerged as a single peak. More definitive support for the tentative identification of peak C as 5'-AMP was obtained in studies illustrated in Fig. 2. In this study peak C from study WB-8 was mixed with a measured amount of pure reference 5'-AMP, and the mixture was incubated for 60 min with the highly specific 5'-adenylic acid deaminase. The substrate (5'-AMP) was separated from the product (5'-IMP) on a Dowex-50 column. Two peaks of both radioactivity and UV absorbing material were recovered in the characteristic positions of 5'-AMP and 5'-IMP. The percentage of the radioactivity which was recovered as metabolite in the 5'-IMP peak was 32.3%; the percentage of the pure reference 5'-AMP converted to 5'-IMP was 36.5%. Because of the high specificity of 5'-adenylic acid deaminase for 5'-AMP and the close correspondence of the fractional conversion of radioactivity and pure reference standard to 5'-IMP, this study provides strong proof of identification of peak C.
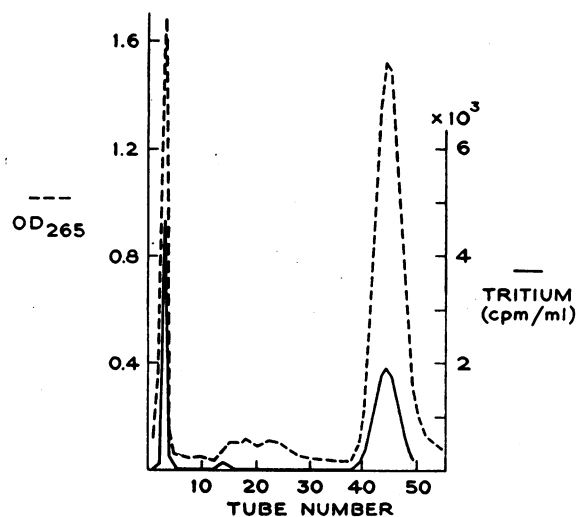
Further chromatography of *peak D* was possible

FIGURE 3 Separation of cyclic AMP-^{3}H from metabolites in the TCA extract of homogenate of epithelial cells of toad bladders. Elution of nucleotides from the 0.9 × 30 cm Dowex-50 column was by means of 0.03 M formate (ammonium) buffer, pH 2.7. The radioactive metabolites in this study correspond (from left to right) in position to peaks A, B, and D in Fig. 1 and Table I.

only in study WB-3 because of the trivial amounts that were obtained in the later studies. When peak D from study WB-3 was chromatographed again on a second Dowex-50 column, it was found to elute after 5'-AMP had emerged from the column but before adenosine emerged. Since the three subsequent studies yielded trace or undetectable amounts of radioactivity in the region of peak D of study WB-3, no further chromatographic analyses were possible. Further information on this peak, however, was obtained in studies described below with homogenates of the epithelial cells of bladders.

No further studies were conducted with whole toad bladders for two reasons. First, the percentage of metabolism as shown in Table I is extremely variable and unpredictable; second, the results of radioautographic studies [11] became available at this time showing that a large fraction of the tissue radioactivity was present outside of the epithelial cells in the large quantity of connective tissue, muscle, and blood vessels present in the intact bladder. The radioautographic studies also showed that the substantial fraction of tissue radioactivity, present in the whole bladder after incubation with cyclic AMP-^{3}H for 60 min, which could not be eluted from the bladder with subsequent prolonged saline washing (9), was almost entirely localized in muscle bundles. Since this large quantity of radioactivity outside the epithelial cells was not related to the physiological effect of interest to us, i.e., changes in rate of movement

FIGURE 2 Chromatography (0.9 × 30 cm Dowex-50 column) of a mixture of peak C from study WB-8 (tritiated metabolite) and 10 μmoles of nonradioactive reference adenosine 5'-phosphate (5'-AMP) after incubation with adenylic acid deaminase. The peak at tubes 2–4 is inosine 5'-phosphate (5'-IMP) and the peak at tubes 39–51 is 5'-AMP. The column was eluted with 0.03 M formate (ammonium) buffer, pH 2.7.
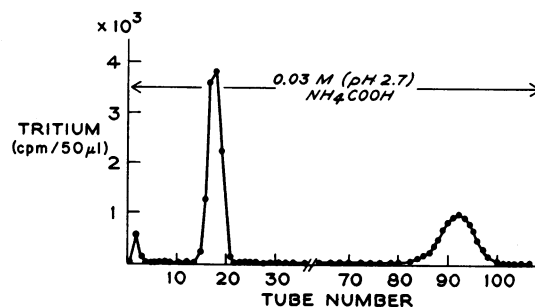
[11] Bogoroch, R. Unpublished observations.

of water across the bladder wall, we turned to studies of the epithelial cells per se.

Metabolism of cyclic AMP-³H by homogenates of epithelial cells. Since the primary goal of the present studies was to obtain information concerning the biochemical events which relate to regulation of permeability of the epithelial cells to water, in the next series we studied the metabolism of cyclic AMP-³H by homogenates of epithelial cells scraped from the mucosal surface of bladders. As outlined under Methods the epithelial cells were homogenized, and cyclic AMP-³H was incubated with the crude homogenate of the epithelial cells of the bladder. As shown in Fig. 3 the pattern of radioactivity in the TCA extract of the homogenate of the epithelial cells showed a strikingly different pattern from that noted with the whole bladder. In this study (and in an additional similar study) one minor and two major radioactive peaks were seen, which corresponded to the positions designated A, B, and D, respectively in the studies on the metabolism of cyclic AMP by the whole toad bladder. The amount of radioactivity in *peak A* was not sufficient for further studies to be conducted; however, it should be noted that the total quantity was no more than that encountered in the two control studies mentioned in the opening paragraph under Results.

The identity of *peak B* from study HEC-2 was tested in several ways. This peak eluted simultaneously with pure reference cyclic AMP from a second Dowex-50 column. An aliquot of the cyclic AMP-³H: epithelial cell homogenate mixture was taken after the 60 min incubation (before addition of TCA) and centrifuged at 4°C for 1 hr at 105,-000 g. The supernatant was applied to a 0.9 × 36 cm column of Sephadex G-10 and eluted with water at 4°C. Only a trivial amount of radioactivity appeared in the void volume of the column, followed by two distinct, large radioactive peaks (Fig. 4). The first of the major radioactive peaks at 1.6 × the void volume was rechromatographed on a Dowex-50 column and coincided with reference cyclic AMP. Chromatography of the TCA extract of the homogenate on PEI-cellulose likewise showed that one of the two major radioactive peaks coincided with the position of cyclic AMP (Fig. 5).

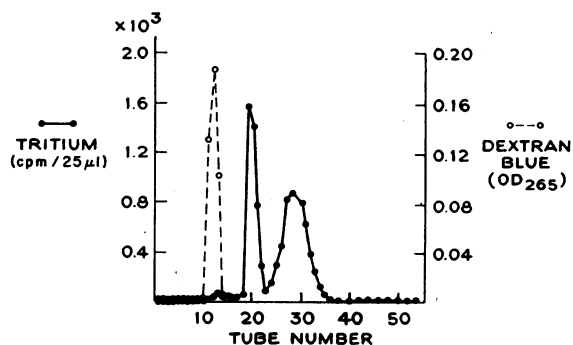Further identification of *peak D*, the second major peak from study HEC-2, was obtained by sev-

FIGURE 4 Passage of 105,000 g supernatant from cyclic AMP-³H: epithelial cell homogenate incubation mixture (study HEC-2) through an 0.9 × 36 cm column of Sephadex G-10 at 4°C. The column was developed with water, and 1.0 ml fractions of elute were collected. The void volume of the column had been determined previously with Dextran blue (OD₂₆₅--O--). The first radioactive peak is cyclic AMP-³H and the second a ³H metabolite.

eral means. First, as shown in Fig. 4, this material was a smaller molecule than cyclic AMP as it emerged later from Sephadex G-10. Chromatography of the TCA extract of study HEC-2 on PEI-cellulose as shown on Fig. 5 indicated a high mobility in this system; radioactivity traveled just behind the front at the leading edge of a large spot of unknown UV absorbing material extracted
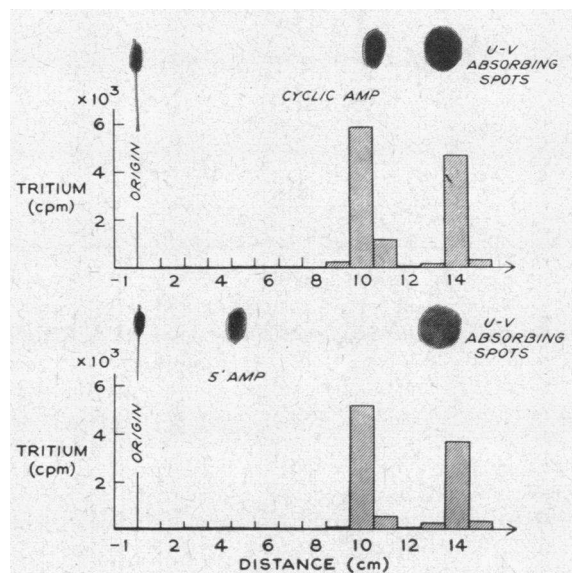

FIGURE 5 Thin-layer chromatography of TCA extract of cyclic AMP-³H: epithelial cell homogenate incubation mixture (study HEC-2) on PEI-cellulose.⁵ Nonradioactive reference cyclic AMP was added to the TCA extract for one separation and 5'-AMP for the other.

from the tissue. The second radioactive peak emerging from the Sephadex G-10 column (see Fig. 4), which corresponded to peak D in the TCA extract, was then lyophilized and further studied by paper electrophoresis. At both pH 3 (0.05 M sodium formate buffer) and pH 7.4 (0.05 M sodium phosphate buffer) the electrophoretic mobility of the radioactive material was trivial, the only movement being due to buffer drift. On electrophoresis at either pH 3 or pH 7.4 the radioactive material remained inseparable from radioactive inosine, which is one potential derivative of cyclic AMP which we could anticipate from the results shown in Figs. 3–5. The second peak from the Sephadex G-10 column (Fig. 4)
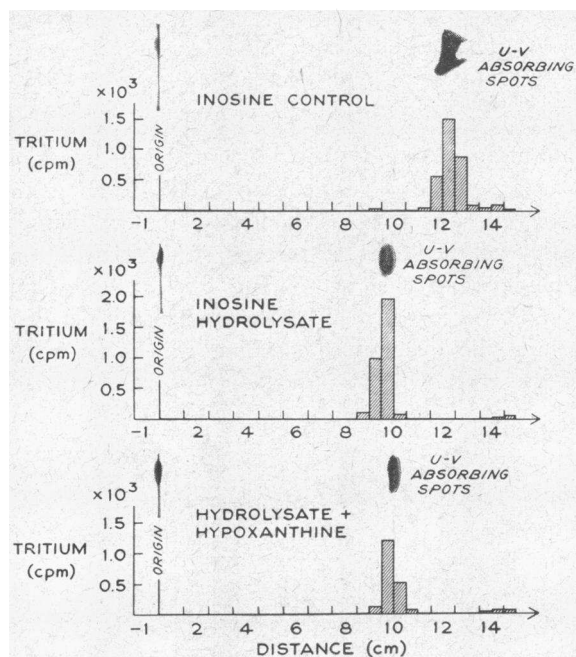

FIGURE 6 Thin-layer chromatography on PEI-cellulose of an ³H metabolite (and its product after acid hydrolysis) which had been separated from cyclic AMP-³H on Sephadex G-10 (Fig. 4). In the control study (upper graph) a portion of the second radioactive peak (³H metabolite) obtained from the Sephadex G-10 column (Fig. 4) was mixed with nonradioactive reference inosine and chromatographed. In the middle graph is shown study in which a mixture of nonradioactive reference inosine, and a portion of the second radioactive peak from the Sephadex G-10 column was hydrolyzed for 60 min with hot HCl, neutralized, and then chromatographed. In the lower graph is shown the study in which a portion of the second radioactive peak from the Sephadex G-10 column was hydrolyzed for 60 min with hot HCl, neutralized, and, after addition of nonradioactive reference hypoxanthine, chromatographed.

was also compared with pure reference inosine by means of thin-layer chromatography on the PEI-cellulose plates, and reference standard and radioactivity proved to have identical mobilities in this system. The final study in an attempt to prove the identity of the major metabolite in study HEC-2 to be inosine is shown in Fig. 6. Hydrolysis of the radioactive metabolite and of pure reference inosine was complete with hot hydrochloric acid, and both the radioactivity and the pure reference standard were quantitatively converted to material that chromatographed in the position of pure reference hypoxanthine.

Failure to detect 5'-AMP-³H in these studies could have been the result of a low concentration of endogenous 5'-AMP in the incubation medium and rapid transformation of 5'-AMP-³H by one or more enzymatic reactions. We, therefore, performed two additional studies (HEC-14 and HEC-15) by the same protocol as for study HEC-2 (see above section) except that added 5'-AMP and inosine (both at 2 × 10⁻² mole/liter) were present throughout the incubation. The distribution of total radioactivity in these two studies were as follows: cyclic AMP-61.7%, 51.5%; inosine-11.0%, 11.7%; 5'-AMP-21.9%, 29.8%, and an unidentified, strongly retarded substance-5.5%, 7.0%.

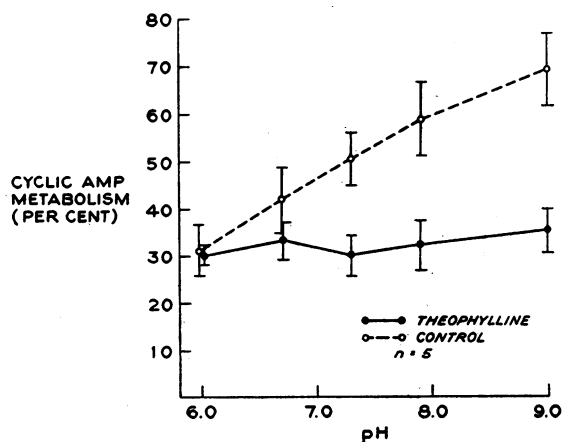*Effects of pH and theophylline on metabolism of cyclic AMP.* In order to study the pH de-

FIGURE 7 The per cent of cyclic AMP metabolized in 60 min by a homogenate of the epithelial cells of toad bladders. Five pairs of studies were performed at each pH value. In the experimental pair of each group theophylline (10 mmoles/liter) was present during incubation. The length of the bars represent ±1 SEM.

TABLE II

Concentration of Protein in Studies on the Effect of pH and Theophylline on Metabolism of
Cyclic AMP by Homogenates of Epithelial Cells of the Urinary Bladder of the Toad

pH	6.0	6.3	6.7	7.3	9.0
Control	0.92 ± 0.11*	0.87 ± 0.11	0.88 ± 0.12	0.90 ± 0.15	0.95 ± 0.12
Theophylline	0.87 ± 0.12	0.93 ± 0.10	0.89 ± 0.11	0.92 ± 0.13	0.94 ± 0.12

Cyclic AMP, adenosine 3',5'-monophosphate.
* Values are mean ± standard error of the mean in milligrams per milliliter for five pairs of studies at each pH.

pendence and the effect of theophylline on metabolism of cyclic AMP by the homogenates of epithelial cells of the toad bladder we took advantage of the speed and simplicity of the method of thin-layer chromatography on PEI-cellulose. Control studies with pure reference standards showed that it was possible with this method to separate cyclic AMP cleanly from all the metabolites of cyclic AMP, namely inosine, 5'-AMP, and ADP, which we had detected by the more complex column chromatography on Dowex-50. When cyclic AMP-^{3}H (mixed with nonradioactive cyclic AMP) in 5% TCA was chromatographed in three studies on PEI-cellulose $\geq 98\%$ of the added radioactivity was recovered on elution of the cyclic AMP spot. In each of five paired studies (Fig. 7) the homogenate pool of the epithelial cells was aliquoted into five control tubes and five experimental tubes containing theophylline at a concentration of 10 mmoles/liter. The pH of pairs varied from 6 to 9.0. After incubation for 60 min $< 1\%$ of the added radioactivity came down with the precipitate after addition of TCA. As shown
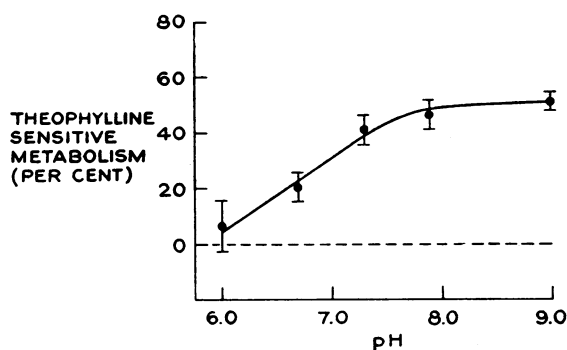

FIGURE 8 The per cent of total metabolism of cyclic AMP by a homogenate of epithelial cells of toad bladders which is inhibited by theophylline as a function of pH. The original data from which these values were derived are illustrated in Fig. 7. The length of the bars represents ±1 SEM.

in Fig. 7 there was marked stimulation of metabolism of cyclic AMP with alkalinization from pH 6 to 9 in the control homogenates, resembling the response of purified cyclic nucleotide phosphodiesterase extracted from other tissues (5, 19).

Theophylline had no effect at pH 6, a borderline effect at pH 6.7, but inhibited metabolism of cyclic AMP progressively up to pH 9. It is noteworthy that despite the presence of a concentration of theophylline (10 mmoles/liter) which inhibits by better than 95% the action of purified phosphodiesterase, there was a residual rate of metabolism of cyclic AMP in the presence of theophylline at all pH values tested, which amounted to 30–35% of the added cyclic AMP. The concentration of protein in each sample pair was reasonably similar in experimental and control groups (see Table II). The percentage of metabolism which was sensitive to the inhibitor theophylline was nevertheless expressed referable to the protein concentration in each individual experimental tube. As shown in Fig. 8, there was a clear linear increase in fractional inhibition of cyclic AMP metabolism by theophylline from pH 6 to pH 7.3, but a saturation effect was reached at pH 7.9 with no further increment above the maximum value of approximately 50% at pH 9.

DISCUSSION

The only chemical transformation of cyclic AMP known to occur in vivo is its hydrolysis to 5'-AMP, a reaction which is catalyzed by a specific cyclic nucleotide phosphodiesterase. Since the possibility of transformation of cyclic AMP to metabolites other than 5'-AMP and the possibility of binding of cyclic AMP to cell membranes has not been studied we searched for such reactions in the present experiments. When the intact urinary bladder of the toad was exposed for 1 hr to cyclic AMP-^{3}H, 97% of the radioactivity in the

whole tissue could be extracted with TCA solution. The small amount of radioactivity which was found in the TCA precipitate did not differ in quantity from that recovered in control experiments. The radioactivity in the TCA extract could be separated by chromatography on the cation-exchange resin Dowex-50 into cyclic AMP and three metabolites. Two of the three metabolites, which proved to be 5'-AMP and ADP on further analysis, are the primary and secondary metabolites resulting from the action of phosphodiesterase and adenylate kinase. The third metabolite present in the intact bladder could not be further identified nor could the effects of theophylline on metabolism because of the extremely variable rate of metabolism of cyclic AMP by the intact tissue. Similar extreme variations have also been noted in the effects of cyclic AMP on sodium and water transport across the toad bladder (1, 7).

In previous studies we noted that, after preloading intact bladders for 60 min with cyclic AMP-^{3}H, 40% of tissue radioactivity could not be eluted despite prolonged washing of the tissue in saline (9). The present studies indicate that the trapped radioactive metabolite in these previous studies did not represent incorporation of some metabolite of cyclic AMP into nucleic acids or tight binding of cyclic AMP to cell membranes. Since 5'-AMP and ADP, which were shown in the present studies to be the principal metabolites of cyclic AMP in the intact bladder, are polyvalent anions at the pH of intracellular fluids, it is most likely that these nucleotides accounted for the trapped metabolite in the previous studies. Preliminary radioautographic studies, however, indicated that the trapped radioactivity in the intact tissue was present in muscle bundles and blood vessels [11] rather than in the cells of interest, i.e., the epithelial cells lining the mucosal surface of the bladders. We, therefore, turned to studies with the epithelial cell layers scraped from the mucosal surface of the bladder. Whether not only studies of degradation reactions but other types of experiments (e.g. effects of hormones on metabolism or activities of enzymes) may also be misleading in the intact tissue requires further study.

Using homogenates of the epithelial cells we were able to establish that the third metabolite found in the intact tissue was inosine. Thus, the only metabolites recovered and identified to date (ADP, 5'-AMP, and inosine) are known normal constituents of most nucleated cells and are present at much higher concentrations than endogenous cyclic AMP. It seems very unlikely that any of these metabolites relate directly to the physiological action of cyclic AMP.

The conversion of cyclic AMP to inosine involves at least three separate chemical reactions, i.e., hydrolysis of two phosphate ester bonds and deamination of the purine ring. Transformation of cyclic AMP to inosine could proceed by several pathways. The most likely sequence based on current information would be initial hydrolysis of the 3'-phosphate bond by cyclic nucleotide phosphodiesterase followed by removal of the phosphate and deamination. Since we used a crude homogenate of the epithelial cells it is highly likely that various phosphatases and either adenylic acid deaminase or adenosine deaminase were present in substantial quantities in the incubation mixture. When the homogenate of epithelial cells was used without added 5'-AMP, this nucleotide was not detected as a metabolite. However, later studies on metabolism of cyclic AMP-^{3}H in the presence of 2×10^{-2} M added 5'-AMP yielded substantial quantities of 5'-AMP-^{3}H. These results suggest that failure in the early studies to find the product of the phosphodiesterase reaction, 5'-AMP, was due to the combined effects of low concentrations of endogenous 5'-AMP and extremely rapid metabolism of 5'-AMP-^{3}H.

We explored in detail the effects of both pH and theophylline on the metabolism of cyclic AMP by the crude homogenate of the epithelial cells of the toad bladder. The control studies shown in Fig. 7 demonstrated stimulation of metabolism of cyclic AMP by alkalinization over the range in pH 6–9.0, a pattern which resembles closely that found for partially purified phosphodiesterase preparations from several tissues. These findings provide support for our previous speculation that inhibition of the effect of cyclic AMP on water flow across the toad bladder by alkalinization from pH 7 to 8.4 was due to stimulation of phosphodiesterase (9).

Previous studies with the methyl xanthines, theophylline, and caffeine by other investigators have been conducted at a single pH value. We,

therefore, explored the effects of theophylline over a wide range of pH. It is of considerable interest that theophylline had no inhibitory effect on the metabolism of cyclic AMP at pH 6, and that inhibition by theophylline above pH 6 up to pH 9 was limited to the hydroxyl-stimulated component of metabolism. The theophylline-sensitive portion of metabolism (Fig. 8) exhibited a saturation pattern reaching a maximum of only 50% of total metabolism. In contrast to the present studies with *crude* homogenates, Butcher and Sutherland found that at the same concentration of theophylline used in the present studies (10 mmoles/liter) the partially *purified* phosphodiesterase from beef heart was 95% inhibited (5). In studies on a *crude* preparation from rat brain Cheung likewise found only 50% inhibition by caffeine (20 mmoles/liter) of metabolism of cyclic AMP (20).

These data on the effect of theophylline raise the possibility that in addition to the phosphodiesterase reaction an additional pathway for metabolism of cyclic AMP may exist in tissues. One possibility is that cyclic AMP may interact initially with some cell membrane component via a phosphate bond with formation of an adenosine-phosphate-membrane complex. If the next step were the hydrolysis of the phosphate ester bond between adenosine and phosphate the products would be free adenosine and phosphate labeling of the membrane. Deamination of adenosine then would yield the other major metabolite found in these studies with homogenates, i.e., inosine. The failure to find significant quantities of radioactivity bound to membranes or soluble protein does not negate such an alternative mechanism, since such a membrane-nucleotide complex might be extremely labile. As an alternative to tracing the fate of the phosphorus in cyclic AMP (^{32}P-labeled cyclic AMP is not available at present) a detailed study of the time course of change of specific activities of metabolites of cyclic AMP-^{3}H (in the presence of appropriate additions of "cold" nucleosides and nucleotides) would provide an indirect test of this postulate.

The nature of the metabolite formed at pH 9 in the presence of theophylline and the cellular components in the homogenate which lead to its formation are interesting questions which remain to be studied. Should the theophylline-resistent component of metabolism prove to be mediated by an enzyme other than cyclic nucleotide phosphodiesterase, it would be of great importance to locate such activity within subcellular components and to attempt to define its relationship to the biological action of cyclic AMP in this and the many other tissues in which it is now clearly known to be the mediator of the action of biologically important hormones.

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