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Separation and Quantitation of Purines and Their Anaerobic and Aerobic Degradation Products by High-Pressure Liquid Chromatography[1]

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A high-pressure liquid chromatography method has been developed for the analysis of a number of purines (adenine, 6,8-dihydroxypurine, 2-hydroxypurine, hypoxanthine, purine, uric acid, xanthine) and their anaerobic and aerobic degradation products (4-ureido-5-imidazolecarboxylic acid, 4-amino-5-imidazolecarboxylic acid, 4-aminoimidazole, formiminoglycine, allantoin, ureidoglycolate) using a reversed-phase column. The mobile phases consisted of potassium phosphate buffers in a pH range from 2.7 to 3.7. Rapid separation of the compounds was achieved within 18 min. The decomposition of adenine by cell-free extracts of *Clostridium purinolyticum* was found to proceed via hypoxanthine, xanthine, and the imidazole derivatives mentioned above.

The biological importance of purine compounds is emphasized by their occurrence in nucleic acids, coenzymes, and nucleotides as well as by their participation in biosynthetic reactions. Microorganisms play an essential role in the mineralization of these substances. A number of methods for the analysis of free bases, nucleosides, and nucleotides by high-pressure liquid chromatography (HPLC)[3] has been described in the literature. Isocratic separations on ion-exchange columns (1–3) have proved to be more time consuming than analyses performed by reversed-phase HPLC (4–8). A review of the practical aspects of this technique in biochemical research has recently been published by Brown and Krstulovic (9).

On the other hand separation and detection of metabolites of the aerobic and anaerobic degradation of purines are rather difficult. As products of aerobic decomposition allantoin can be identified by thin-layer chromatography (10) and allantoate and ureidoglycolate have been measured by means of a colorimetric reaction (11). Imidazole derivatives originate from the anaerobic cleavage of the purine ring system and were identified by modifications of the Pauly test and the Bratton–Marshall test (12,13). Formiminoglycine can be determined by a colorimetric assay using ferricyanide–nitroprusside (14). All these procedures require a relatively large amount of sample material and are rather time consuming. This paper describes a rapid and sensitive HPLC analysis for purines and metabolites generated during their degradation using a reversed-phase column and isocratic elution.

MATERIALS AND METHODS

Reagents. Adenine, allantoic acid, formiminoglycine, 2-hydroxypurine, hypoxanthine, purine, ureidoglycolate, and uric acid were purchased from Sigma Chemie GmbH, München, Federal Republic of Germany.

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[3] Abbreviation used: HPLC, high-pressure liquid chromatography.

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6,8-Dihydroxypurine was obtained from Ega Chemie GmbH & Co. KG, Steinheim, Federal Republic of Germany, and allantoin and xanthine were from E. Merck, Darmstadt, Federal Republic of Germany. 4-Nitroimidazole, 4-nitro-5-imidazolecarboxylic acid, and 4-ureido-5-imidazolecarboxylic acid were kindly provided by Dr. H. A. Barker, University of California, Berkeley. 4-Nitroimidazole may also be purchased from BASF Aktiengesellschaft, Ludwigshafen, Federal Republic of Germany. The reduction of the nitroimidazoles to their respective amino compounds was carried out as described by Rabinowitz (15). All other chemicals used were of the highest purity commercially available. Standards were prepared by dissolving substances in glass-distilled water to a concentration of 2 mM just before use and adjusting the pH exactly to a value of 7.5.

Equipment. The HPLC system used consisted of a Dupont solvent-delivery pump, Model 870 (Dupont, Wilmington, Del.), a syringe-loading sample injector, Model 7125 (Rheodyne, Cotati, Calif.) a variable-wavelength ultraviolet detector, Uvicon Model 720 LC (Kontron, Zürich, Switzerland) set at 205 nm, and a printer–plotter, Model C-R1A (Shimadzu, Kyoto, Japan), capable of recording all analyses and determining the peak areas and retention times. Chromatography was performed with a C_{18} reversed-phase column (0.46 cm i.d. × 25 cm, filled with LiChrosorb RP-18, 10-μm particle size, E. Merck) from Kontron. The injected volume was 20 μl for all samples. All operations were carried out at ambient temperature.

Mobile phase preparation and column life. Phosphate buffers (final concentration 100 mmol/liter) were prepared from potassium dihydrogen phosphate using only glass-distilled water. The desired pH was adjusted with phosphoric acid (100 mM) or dipotassium hydrogen phosphate (100 mM). Special care was taken to keep the pH of the buffer solutions essentially constant during all experiments. The eluants were filtered under reduced pressure before use and subsequently deaerated by purging with helium. A constant stream of helium was blown through the solvents during the separation. Care was taken to prevent bacterial growth on the column material since a phosphate buffer system was used. The column was stored in methanol when not in use in order to prevent a decrease of column efficiency. During a 4-month period analyses of more than 400 samples were carried out with the same column packing. The operating pressure during the course of this period was found to be increased only slightly (about 10% increase).

Degradation of purines by bacterial cell-free extract. Culture of *Clostridium purinolyticum* (Deutsche Sammlung von Mikroorganismen (DSM) 1384) and preparation of cell-free extracts were performed as described (16). Buffered purine solutions (pH 7.5) were pipetted into plastic reaction vessels, supplemented with EDTA (final concentration 6 mM), and brought to a final volume of 0.9 ml with distilled water. Some experiments were carried out without addition of EDTA. The vessels were closed with serum stoppers (Arthur H. Thomas Co., Philadelphia), gassed with nitrogen for 30 min, and placed into a water bath (37°C). The degradation was started by adding 100 μl of cell-free extract (about 2 mg of protein). Samples (20 μl) were taken at hourly intervals and analyzed by HPLC.

Peak identification and quantitative analysis. Peaks were tentatively identified by comparison of retention times with those of freshly prepared standards. Furthermore, an increase in peak area after addition of a known amount of pure substance to the sample was taken as identification. Finally, the uv absorption spectrum (200–400 nm) was determined of all compounds analyzed and compared to the spectra of the respective pure substances. This operation was performed by means of a scanning supplement

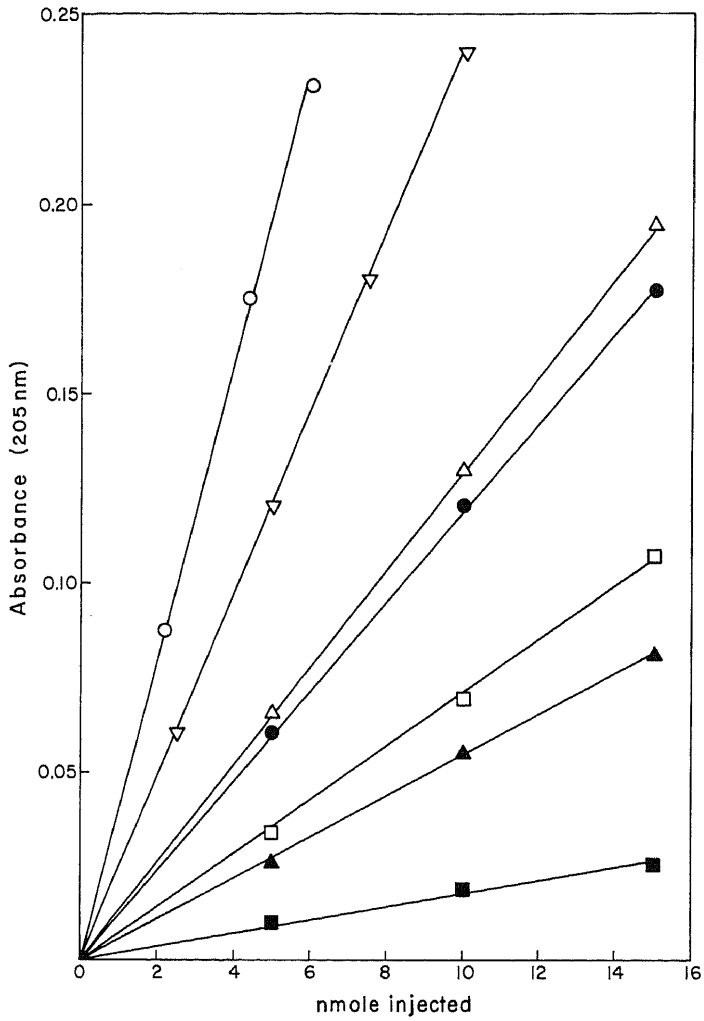

FIG. 1. Calibration curves for the quantitative analysis of several purines and some of their degradation products. The indicated amounts were injected as aqueous solutions in a total volume of 20 μl each. The resulting peak height was monitored at 205 nm. Formiminoglycine (○); 2-hydroxypurine (▽); hypoxanthine (△); xanthine (●); uric acid (□); 4-ureido-5-imidazolecarboxylic acid (▲); 4-aminoimidazole (■). Eluant: potassium phosphate buffer (100 mM, pH 3.7); flow rate: 1.5 ml/min.

of the uv detector. Although retention times and peak areas were routinely determined with a printer–plotter as already described excellent quantitation was also obtained by using maximum peak heights except for 4-amino-5-imidazolecarboxylic acid and purine. Calibration standard curves according to this method were found to be linear. A typical example is given in Fig. 1. All analyses were run at least in duplicate and found to be within 5% of one another. Recoveries were quite similar regardless of whether samples containing cell extract or standard solutions were used. The concentrations of the compounds in unknown samples were calculated from calibration curves determined under the same experimental conditions. Amounts of less than 1 nmol could be

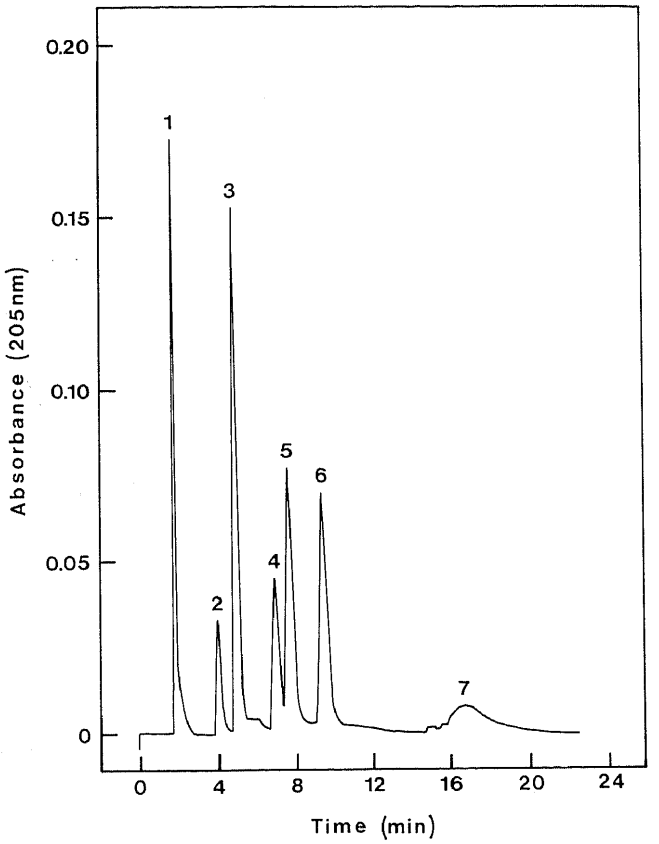

FIG. 2. Separation of purines and 4-ureido-5-imidazolecarboxylic acid in the presence of EDTA: (1) EDTA; (2) 4-ureido-5-imidazolecarboxylic acid; (3) 2-hydroxypurine; (4) uric acid; (5) hypoxanthine; (6) xanthine; (7) purine. Amount injected of each compound was 5.8 nmol. Eluant: potassium phosphate buffer (100 mM, pH 3.7); flow rate: 1.5 ml/min.

determined even in assay samples containing cell-free extract.

RESULTS AND DISCUSSION

The separation of some purines and their first anaerobic degradation product, 4-ureido-5-imidazolecarboxylic acid, using potassium phosphate buffer (pH 3.7) as the eluant can be achieved (Fig. 2). EDTA was included in this experiment because the anaerobic degradation of purines as known from studies with *Clostridium cylindrosporum* proceeds via xanthine cleavage to 4-ureido-5-imidazolecarboxylic acid and may be stopped at this stage by the addition of EDTA (13). Good resolution was obtained for all compounds, but the elution profile for purine showed a flattened peak. 6,8-Dihydroxypurine coeluted with hypoxanthine and adenine proved to have the same retention time as xanthine. In order to separate these purines from the other components various analytical systems were tested. Since retention times of purines are highly affected by changes in pH using reversed-phase systems (17), the pH value was determined at which best resolution of all purine compounds tested was obtained. In the pH range from 2.0 to 2.7 adenine could be separated from

TABLE 1

INFLUENCE OF THE pH VALUE ON THE SEPARATION OF ADENINE FROM OTHER PURINES[a]

pH value	Compound analyzed	Retention time (min)
2.0	Adenine	4.9
	Hypoxanthine	5.6
	Uric acid	6.0
2.3	Adenine	5.1
	Hypoxanthine	5.8
	Uric acid	6.8
2.7	Adenine	5.2
	Hypoxanthine	6.4
	Uric acid	7.0
2.9	Adenine	6.2
	Hypoxanthine	6.7
	6,8-Dihydroxypurine	7.4
3.55	Hypoxanthine	7.2
	6,8-Dihydroxypurine	7.4
	Adenine	8.3
3.7	Hypoxanthine	7.4
	Adenine	9.3
	Xanthine	9.3
4.5	Adenine	12[b]

[a] Eluant: potassium phosphate buffer (100 mM); flow rate: 1.5 ml/min. Amount injected of all compounds: 13.2 nmol.

[b] A precise determination was impossible due to flattening of the peak.

xanthine, hypoxanthine, and 6,8-dihydroxypurine. Adenine was always eluted first. At increasing pH values the peaks of adenine and hypoxanthine started to overlap. Best resolution for these two compounds was achieved at pH 2.7. Retention times for the separation of adenine at various pH values are listed in Table 1. Values higher than 3.0 led to a shift in the retention behavior of hypoxanthine and uric acid. Under these circumstances the latter compound showed shorter retention times than hypoxanthine. These results are in accordance with some data already published; at pH 2.1 hypoxanthine is eluted in advance of uric acid (18) whereas at values of 4.0 and higher the retention behavior of the two substances is reversed (4–8).

6,8-Dihydroxypurine could be well separated from hypoxanthine and xanthine at pH 2.9 (Fig. 3). In the same system also detection of adenine was possible. This compound was eluted just in advance of hypoxanthine with both peaks overlapping to a certain degree. Increasing pH values did not improve the resolution. Starting at the range of 4.3–4.5 the peak representing adenine began to flatten. These data show that no single system could be found which allowed a satisfactory separation of all the purines tested. Nevertheless, a complete analysis was possible in two runs by changing the pH of the eluant. Potassium phosphate buffer was used as the mobile phase, because the resolution of purines in this solvent was found to be better (8) compared to that in ammonium phosphate buffer (4,7). The detection wave-

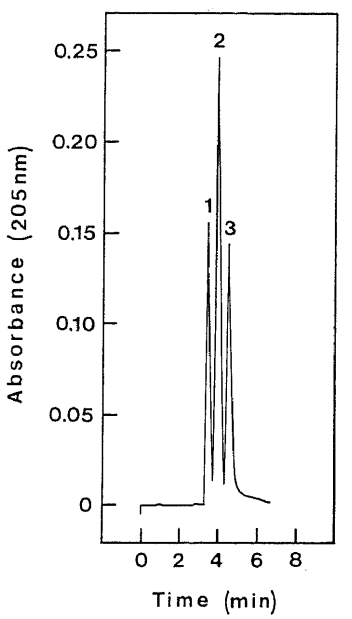

FIG. 3. Separation of hypoxanthine, 6,8-dihydroxypurine, and xanthine: (1) hypoxanthine; (2) 6,8-dihydroxypurine; (3) xanthine. Amount injected of each compound was 13.4 nmol. Eluant: potassium phosphate buffer (100 mM, pH 2.9); flow rate: 3 ml/min.

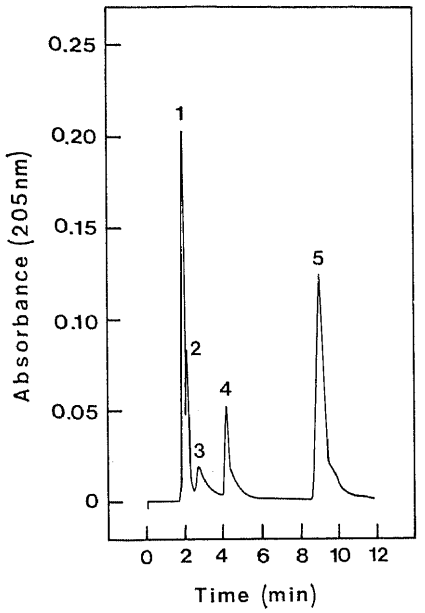

FIG. 4. Separation of intermediates of anaerobic xanthine degradation: (1) formiminoglycine (5.8 nmol); (2) 4-amino-5-imidazolecarboxylic acid (14.2 nmol); (3) 4-aminoimidazole (14.2 nmol); (4) 4-ureido-5-imidazolecarboxylic acid (11.4 nmol); (5) xanthine (11.4 nmol). Eluant: potassium phosphate buffer (100 mM, pH 3.55); flow rate: 1.5 ml/min.

length of 205 nm proved to be optimal, since the uv absorption of hypoxanthine and adenine at 250 nm was much lower. At 230 and 280 nm almost no absorption could be observed. No further improvement in resolution was achieved by using a system consisting of two C_{18} columns in series as described earlier (19).

The products of anaerobic purine degradation (15) could be well separated from each other using potassium phosphate buffer (pH range 3.5–3.7) as the eluant (Fig. 4). Only the peaks of formiminoglycine and 4-amino-5-imidazolecarboxylic acid overlapped to some degree. 4-Imidazolone was not detectable due to its rapid hydrolysis. Even freshly synthesized samples proved to be unstable under the chromatographic conditions used. 4-Amino-5-imidazolecarboxylic acid easily decarboxylated to yield 4-aminoimidazole and was, therefore, prepared fresh daily from 4-nitro-5-imidazolecarboxylic acid (see Materials and Methods). The analysis also could be achieved in the same run in which the purines were analyzed (Fig. 5). Only adenine and 6,8-dihydroxypurine had to be determined by means of a different elution system as described above. Even the end products of anaerobic purine degradation, such as acetate and formate, can be determined by a C_{18} reversed-phase HPLC method (20).

The degradation of purines by aerobic organisms starts from uric acid by a pathway yielding allantoin, allantoic acid, ureidoglycolate, and finally urea and glyoxylate (21). Urea can be determined by an enzymatic procedure using urease, and glyoxylate by colorimetric tests (11) whereas the other intermediates first have to be selectively hydrolyzed to glyoxylate (11). Figure 6 shows the analysis of a mixture of uric acid, allantoin, and ureidoglyoxylate. All compounds are satisfactorily separated. Due to its low absorbance at 205 nm allantoic acid could not be detected.

The analytical systems described above were used to determine the pathway of adenine degradation by *C. purinolyticum*, recently described as a new species of obligate purine-fermenting bacteria (16). Cell-free extracts were allowed to act upon purine solutions for several hours (see Materials and Methods). In the presence of EDTA adenine was converted to hypoxanthine and xanthine, and hypoxanthine was transformed only into xanthine, whereas xanthine remained unchanged (Table 2). This indicated that xanthine is the intermediate which undergoes ring cleavage in *C. purinolyticum* as is the case in *C. acidiurici* and *C. cylindrosporum* (21). Ring cleavage does not seem to start directly from uric acid, because no products such as allantoin and ureidoglycolate could be detected after prolonged incubation. Since oxygen is only one possible electron acceptor in the urate oxidase re-

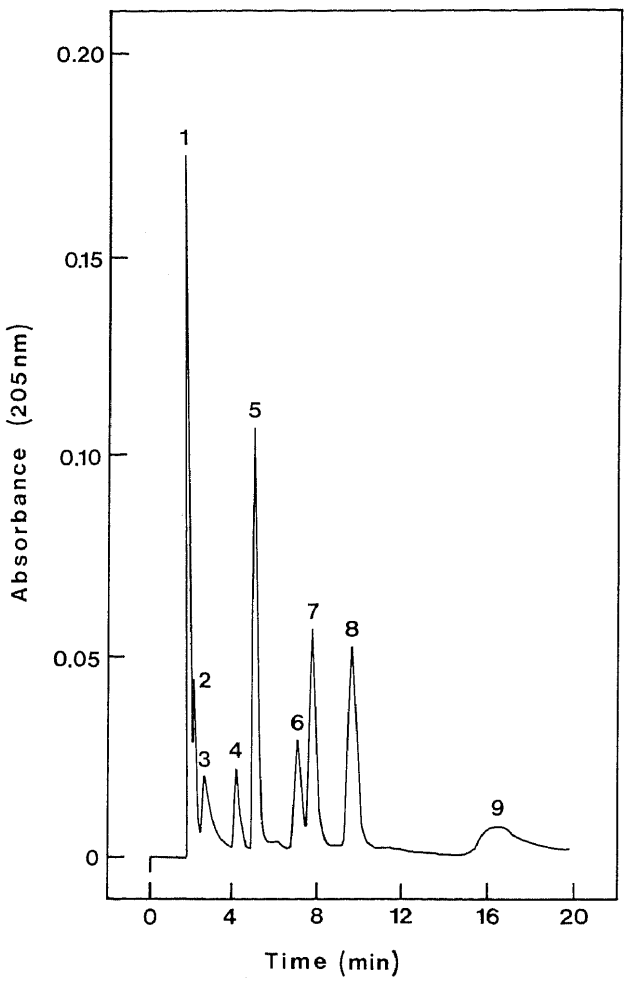

FIG. 5. Separation of purines and their anaerobic degradation products: (1) formiminoglycine (4.4 nmol); (2) 4-amino-5-imidazolecarboxylic acid (10 nmol); (3) 4-aminoimidazole (11.2 nmol); (4) 4-ureido-5-imidazolecarboxylic acid (4.4 nmol); (5) 2-hydroxypurine (4.4 nmol); (6) uric acid (4.4 nmol); (7) hypoxanthine (4.4 nmol); (8) xanthine (4.4 nmol); (9) purine (4.4 nmol). Eluant: potassium phosphate buffer (100 mM, pH 3.7); flow rate: 1.5 ml/min.

action, such a degradation might occur even under anaerobic conditions. In *C. purinolyticum* the ring cleavage reaction by xanthine amidohydrolase seemed to be inhibited by EDTA, whereas in *C. cylindrosporum* the subsequent reaction (catalyzed by 4-ureido-5-imidazolecarboxylate amidohydrolase) is prevented by EDTA (13). The small amount of xanthine transformed in the presence of EDTA might be due to an insufficient concentration of this compound. In the absence of sequestering agents xanthine was rapidly degraded via intermediates known for the anaerobic purine decomposition by *C. cylindrosporum* (15). The data for the recovery of the imidazole derivatives (Table 2) clearly show that all xanthine is fermented via these compounds. Thus, the following scheme summarizes the pathway of adenine degradation to formiminoglycine by *C. purinol-*

TABLE 2

DEGRADATION OF PURINES BY CELL-FREE EXTRACTS OF *C. purinolyticum*[a]

Experiment	Substrate	Concentration (mM)	Addition	Incubation time (h)	Detected compound	Concentration (mM)	Recovery (%)
1	Adenine	4	EDTA (6 mM)	16	Adenine	1.37	
					Hypoxanthine	0.19	77
					Xanthine	1.54	
2	Hypoxanthine	2	EDTA (6 mM)	6	Hypoxanthine	0.52	56
					Xanthine	0.61	
3	Xanthine	2	EDTA (6 mM)	4	Xanthine	1.68	84
4	Xanthine	2	None	0.004	4-Ureido-5-imidazole-carboxylic acid	0.47	
					4-Amino-5-imidazole-carboxylic acid	0.30	
					4-Aminoimidazole	0.89	102
					Formiminoglycine	0.38	

[a] All analyses were performed with potassium phosphate buffer (100 mM) at flow rates of 1.5–3 ml/min. The pH values for the determinations were as follows: 2.7 (Expt 1), 3.7 (Expt 2), 3.7 (Expt 3), and 3.55 (Expt 4).

yticum as determined by the present method:

adenine
↓
hypoxanthine
↓
xanthine
↓
4-ureido-5-imidazolecarboxylic acid
↓
4-amino-5-imidazolecarboxylic acid
↓
4-aminoimidazole
↓
formiminoglycine

Although the decomposition of xanthine proceeded via the same intermediates as in *C. cylindrosporum* there were remarkable differences in purine interconversions. Hypoxanthine could not be oxidized directly to xanthine by *C. cylindrosporum*, but had to be transformed first into 6,8-dihydroxypurine and then into uric acid before being converted into xanthine (22). This might be due to a more pronounced substrate specificity of the xanthine dehydrogenase of *C. cylindrosporum*, which is the enzyme catalyzing redox reactions at the purine ring system. The results obtained for *C. purinolyticum* show clearly that a direct oxida-

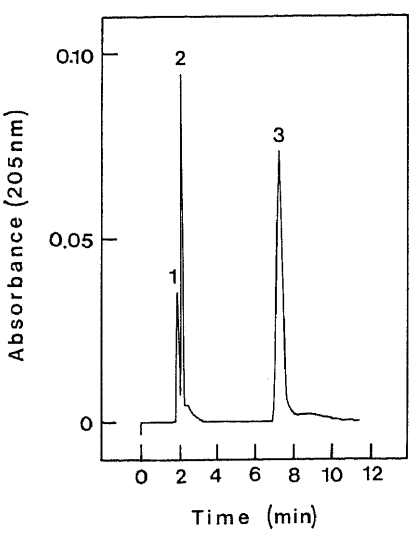

FIG. 6. Separation of uric acid and its aerobic degradation products: (1) ureidoglycolate (20 nmol); (2) allantoin (10 nmol); (3) uric acid (10 nmol); Eluant: potassium phosphate buffer (100 mM, pH 3.1); flow rate: 1.5 ml/min.

tion of hypoxanthine takes place in this organism. This corresponds to the fact that the xanthine dehydrogenase is very active with hypoxanthine as the substrate (16).

The method presented in this paper allows a rapid, sensitive, and reliable separation and detection of a variety of purines and their biological degradation products. It offers, therefore, certain advantages compared to the techniques known so far. Furthermore, it may be used to investigate in detail the enzymes responsible for the purine decomposition.

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