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RESOLUTION OF THE INTERMEDIATES OF *DE NOVO* PURINE BIOSYNTHESIS BY ION-PAIR REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

ROBERT G. HARDS* and DAVID PATTERSON
Eleanor Roosevelt Institute for Cancer Research, 1899 Gaylord Street, Denver, CO 80206 (U.S.A.)
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SUMMARY

An ion-pair reversed-phase high-performance liquid chromotographic procedure capable of resolving twelve of the fourteen intermediates of *de novo* purine biosynthesis is presented. The method utilizes isocratic elution and detection by ultraviolet light absorption. Separation of all twelve intermediates can be achieved in 90 min.

INTRODUCTION

A simple, single chromatographic separation for the intermediates of *de novo* purine biosynthesis has not been documented until now. While various methodologies have been established to isolate one[1–3] or at best two or three[4–6] of the intermediates no one system was directly applicable to the entire pathway. Resolution of the greatest number of intermediates was accomplished by the methods of Patterson and co-workers[7–10], which were not without certain drawbacks. The methods involved incorporation of radioactive precursors into the intermediates, extraction of the labelled cells, separation of the compounds by TLC*, visualization by autoradiography

* The following abbreviations are used: ADP = adenosine 5′-diphosphate; Ade = adenine; AICAR = 5′-aminoimidazole-4-carboxamide ribonucleotide; AICR = 5′-aminoimidazole-4-carboxylate ribonucleotide; AIR = 5′-aminoimidazole ribonucleotide; AMP = adenosine 5′-monophosphate; AMPS = adenylosuccinic acid; ATP = adenosine 5′-triphosphate; CHO = Chinese hamster ovary; DBAP = dibutyl ammonium phosphate; EDTA = ethylenediaminetetraacetic acid; F12 = Ham's F12 culture media; FAICAR = 5′-formaminoimidazole-4′-carboxamide ribonucleotide; FC = fetal bovine serum; FCM = macromolecular fraction of fetal bovine serum; FGAM = N-formylglycinamidine ribonucleotide; FGAR = N-formylglycinamide ribonucleotide; FH = tetrahydrofolate; GAR = glycinamide ribonucleotide; glu = glutamic acid; $gluNH_2$ = glutamine; GMP = guanosine 5′-monophosphate; HEPES = N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; HPLC = high-performance liquid chromatography; IMP = inosine 5′-monophosphate; NADP = nicotinamide–adenine dinucleotide phosphate, oxidized; NADPH = nicotinamide–adenine dinucleotide phosphate, reduced; PEI = polyethyleneimine; P_i = inorganic phosphate; PRA = phosphoribosylamine; PRPP = 5′-phosphoribosyl-1-pyrophosphate; SAICAR = 5′-aminoimidazole-4-N-succinocarboxamide ribonucleotide; TLC = thin layer chromatography; XMP = xanthosine 5′-monophosphate.

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and finally removal of the compounds from the TLC matrix. The procedures are time consuming, prone to loss of material and require radioactive labelling of the compounds. The method described in this paper utilizes an ion-pair reversed-phase HPLC system employing isocratic elution and detection by ultraviolet light absorption, thereby eliminating the need to remove the compounds of interest from the matrix after detection and the requirement that the intermediates be radioactively tagged.

Because of the large number of compounds that will absorb ultraviolet light in the range used for detection in the system described, "clean" samples are required for analysis. A method for the preparation of such samples is presented.

MATERIALS AND METHODS

Chemicals

The following chemicals were purchased from Sigma: AMP, GMP, XMP, IMP, AMPS and AICAR. The mobile phase components DBAP and acetonitrile were obtained from Aldrich and Burdick & Jackson Labs., respectively. Methanol and ethanol were purchased from Fisher Chemical. All chemicals were of the highest quality available. Chemically synthesized GAR was kindly provided by Dr. S. Benkovic of the Pennsylvania State University.

HPLC separation of purine intermediates

Separation of the intermediates of *de novo* purine biosynthesis was accomplished using a 30 cm × 3.9 mm μBondapak C^{18} reverse phase HPLC column (Waters Assoc.) coupled to a 5 cm × 4.6 mm guard column of Bondapak C_{18}/Corasil (Waters Assoc.). The isocratic mobile phase consisted of the ion-pairing buffer 5 m*M* DBAP, pH 6, plus 1% or 3% acetonitrile. The mobile phase was delivered by a Waters M6000A pump at a flow-rate of 1 ml/min. Eluting compounds were detected by a Model 440 absorbance detector with a 254-nm wavelength filter (Waters Assoc.) connected to a Waters Model 730 DATA module and/or a Model GM770 variable-wavelength detector (Schoeffel) set at 210 mm connected to an Omniscribe recorder (Houston Instruments). In some experiments a Flow One Beta radioactive detector (Radiomatic Instruments) set to measure ^{14}C and connected to a dot matrix printer was used to monitor eluting compounds.

For anion-exchange HPLC a 25 cm × 4.6 mm Partisil-10 SAX column (Whatman, Inc.) was employed, connected to a 5 cm × 4.6 mm guard column packed with pellicular anion exchanger (Whatman). The solvent, 20 m*M* ammonium phosphate, pH 4.2, plus 1% acetonitrile was delivered by a Waters M6000A pump at a flow-rate of 2 ml/min. Detection of the compounds was achieved using the two UV-absorbance systems described above.

Preparation of intermediates

The intermediates of *de novo* purine biosynthesis were obtained from mutant CHO cells defective in one or more steps of the pathway[7,10] using a modification of the procedure described previously[7]. Cells were grown on plastic tissue culture plates (Lux Science Corp.) in F12 (Flow Labs.) plus 8% FC and 0.1 m*M* glycine for 24 to 36 h at 37°C. Cells were harvested prior to confluence by brief trypsinization which was stopped by the addition of 5% FCM. The cells were harvested by centrifugation,

washed three times with F12 minus hypoxanthine and glutamine plus 8% FCM and 20 m*M* HEPES, pH 7.5, and then resuspended in F12 minus hypoxanthine plus 8% FCM, 2 m*M* glutamine, 0.1 m*M* glycine and 20 m*M* HEPES, pH 7.5, at approximately $5 \cdot 10^6$ cells/ml. For the Ade-G cell line which accumulates FGAR and FGAM, 10 μCi/ml of [^{14}C]formate (50 mCi/nmol, New England Nuclear) was added for some experiments. The cells were incubated at 37°C with shaking for 2 h, chilled, washed twice with ice-cold F12 minus hypoxanthine and the intermediates extracted with ice-cold 80% ethanol for 30 min at 4°C with gentle agitation. Cells were pelleted by centrifugation and the supernatant applied to a Sep-Pak silica column (Waters Assoc.). The column was washed with 4 ml of absolute methanol and the compounds of interest eluted with 10 ml of distilled/deionized water. The sample was quickly frozen, lyophilized to dryness, resuspended in 1 ml of 100 m*M* ammonium acetate, pH 8.8, and applied to a 6.5 cm × 0.7 cm column of Affigel 601 (boronate gel, Bio-Rad Labs.). The column was washed with 14 ml of 100 m*M* ammonium acetate, pH 8.8, and the purine intermediates then eluted with 20 m*M* acetic acid (10 ml, of which the first two were discarded). The sample was quickly frozen, lyophilized to dryness and resuspended in a minimum volume of 5 m*M* DBAP, pH 6, plus 1% or 3% acetonitrile. Immediately prior to HPLC analysis the samples were centrifuged at 13 000 *g* for 5 min and the supernatant injected.

Methods of peak identification

Detection of diazotizable aromatic amines and preparation of derivatives of such compounds were carried out using the procedure of Bratton and Marshall[11] as modified by Flaks and Lukens[12,13].

Enzyme assays of FGAR amidotransferase and AIR synthetase were run as previously described[14]. Briefly [^{14}C]FGAR or [^{14}C]FGAM, isolated using ion-pair reversed-phase HPLC was incubated with CHO-K1 extract in the presence of 50 m*M* HEPES, pH 7, 10 m*M* magnesium chloride, 100 m*M* potassium chloride, 0.1 m*M* EDTA and 2 m*M* ATP in a total volume of 100 ml. For the FGAR amidotransferase assay 5 m*M* glutamine was also present. After incubation for 15 min at 37°C the reactions were stopped by the addition of 100 ml of ice-cold ethanol. Following centrifugation to remove protein, aliquots of the supernatants were spotted on PEI-cellulose TLC plates which were then developed in 0.35 *M* lithium chloride, air dried and placed on X-ray film (Kodak). Radioactive spots were identified by their R_F values.

Ultraviolet and visible absorption spectra of various compounds were obtained using a Beckman DU-50 spectrophotometer equipped with an Epson RX-80 printer.

Identification of the purine intermediate FAICAR involved its conversion to IMP or AICAR. These chemical reactions were accomplished using the procedures of Lukens and Flaks[13].

RESULTS AND DISCUSSION

Purine auxotrophs isolated and characterized by Patterson and co-workers[7–10] were used as the sources for twelve intermediates of *de novo* purine biosynthesis resolved by the ion-pair reversed-phase HPLC system described in this communication (Fig. 1). The retention time for each intermediate and the method(s) used to

confirm its identity are listed in Table I. A description of the cell lines used and the intermediates isolated from each is given below.

Ade-E cells were found to accumulate a compound that eluted at 3.5 min. This compound failed to absorb ultraviolet light above 230 nm and co-eluted with chemically produced GAR when analysed with either ion-par reversed-phase or anion-exchange HPLC. The peak was therefore identified as GAR.

Ade-G cells were found to significantly accumulate two peaks, FGAM and FGAR, which eluted at 4.5 and 10.7 min, respectively (Fig. 2). The peaks were identified by their co-elution with [^{14}C]FGAM and [^{14}C]FGAR that had been prepared and characterized by TLC[14]. In addition, intermediate accumulation with Ade-G cells was carried out in the presence of [^{14}C]formate. The putative FGAM and FGAR were isolated by ion-pair reversed-phase HPLC and subsequently used as

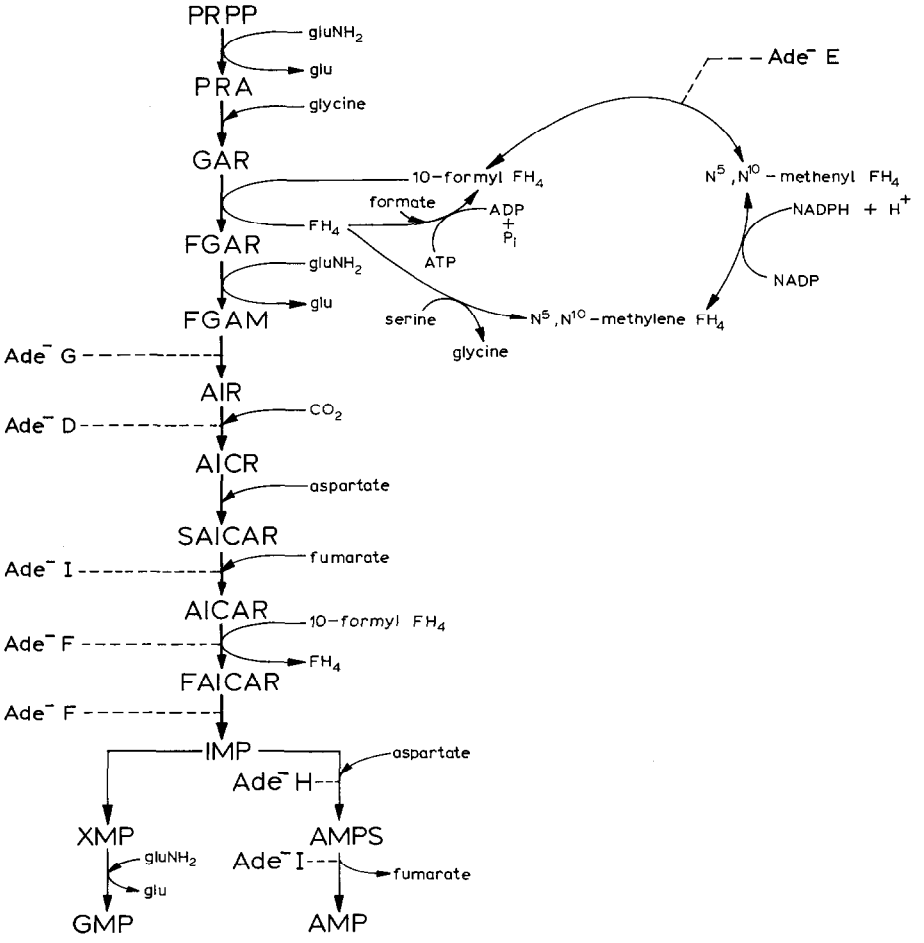

Fig. 1. A simplified representation of purine biosynthesis indicating intermediates and CHO mutant cel lines discussed in text.

TABLE I

RETENTION TIMES OF INTERMEDIATES OF *DE NOVO* PURINE BIOSYNTHESIS USING ION-PAIR REVERSED-PHASE HPLC

Methods of peak identity confirmation: 1 = co-elution with known compound using ion-pair reversed-phase HPLC; 2 = co-elution with known compound using anion-exchange HPLC; 3 = characteristic positive reaction for diazotizable aromatic amine; 4 = comparison of absorption spectrum to known compound; 5 = use as substrate in enzyme assay; 6 = chemical conversion of compound.

Compound	*Retention time (min)*		*Method of peak identity confirmation*
	1% CH_3CN	*3% CH_3CN*	
GAR	3.5		1
FGAM	4.5		1, 5
AIR	5.0	4.5	3
FGAR	10.7		1, 5
AICAR	24.3		1, 2, 3, 4
GMP	31.6	12.0	1, 2, 4
IMP	33.4	13.0	1, 2, 4
SAICAR	42.8	15.9	4, 5
FAICAR	48.7		4, 6
XMP	50.8	18.0	1, 2, 4
AMP	72.1	23.4	1, 2, 4
AMPS		90.6	1, 2, 4

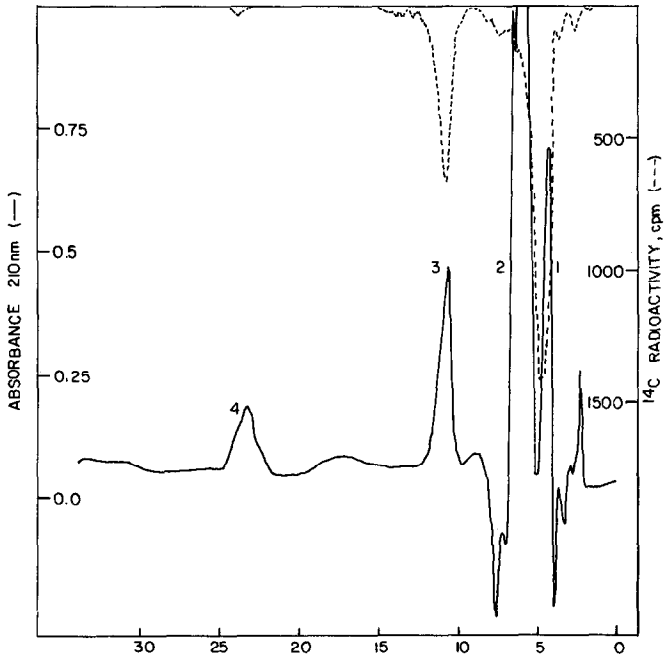

Fig. 2. Simultaneous UV and radioactivity detection of ion-pair reversed-phase HPLC separation of intermediates accumulated by Ade-G cells. Mobile phase: 5 m*M* DBAP, pH 6, plus 1% acetonitrile. Peaks: 1 = FGAM; 2 = acetate; 3 = FGAR; 4 = unknown. Slight offset of radioactive peaks due to time required for flow between detectors.

substrates in AIR synthetase and FGAR amidotransferase assays. The products obtained confirmed the peak assignments.

Ion-pair reversed-phase HPLC analysis of Ade-D cell extract revealed only a single significant peak which eluted at 5.0 min. The compound did not absorb UV light above 230 nm and when tested for the presence of a diazotizable aromatic amine produced an orange coloured compound (absorption maximum of 500 nm). This is a definitive test for the compound AIR.

Extract from Ade-F cells showed a large peak eluting at 24.3 min as well as a less pronounced peak eluting at 48.7 min. The first peak exhibited a UV absorbance spectrum identical to that of commercially produced AICAR, co-eluted with commercially produced AICAR when run on either ion-pair reversed-phase or anion-exchange HPLC and gave a positive reaction when tested for diazotizable aromatic amines with an absorption maximum of 540 nm. The compound eluting at 24.3 min was thus identified as AICAR. The second compound, retention time 48.7 min, had an absorbance maximum at 264 nm. When treated with 0.1 *M* sodium hydroxide for 20 min at 38°C, this compound was converted to another that eluted at 34.0 min and co-eluted with IMP. Treatment in 0.2 *M* sulfuric acid for 3 min at 100°C converted the 48.7-min peak to a compound that co-eluted with AICAR at 25.0 min. These results confirm the identity of the peak eluting at 48.7 min as FAICAR.

In addition to the compounds discussed above the purine auxotrophs grown in hypoxanthine deficient medium were found to accumulate low levels of AMP, IMP, GMP and XMP. These minor peaks were identified by comparison to commercially available compounds with respect to UV absorbance spectra and co-elution on ion-pair reversed-phase and anion-exchange HPLC.

The final purine auxotroph studied was Ade-I and resolution of the accumulated compounds required a slight modification of the chromatographic protocol. Due to the affinity exhibited by one of the accumulated products for the column matrix it was necessary to increase the acetonitrile concentration to 3%. Three major peaks at 4.5, 15.9 and 90.6 min were observed (Fig. 3). The earliest eluting peak failed to exhibit an absorption spectrum above 230 nm and when tested for diazotizable aromatic amines gave a positive result with a maximum absorbance of 500 nm. Thus the peak at 4.5 min would seem to be AIR. The compound eluting at 15.9 min showed an absorption maximum at 267 nm and gave a positive reaction for diazotizable aromatic amines only when tested at 4°C in the presence of 10 *M* sulfuric acid. These results confirm the peak's identity as SAICAR. When run on the ion-pair reversed-phase column with a mobile phase of 1% acetonitrile SAICAR was found to have a retention time of 42.8 min. The compound eluting at 90.6 min was identified as AMPS by comparison to the commercially prepared compound. Not only were the absorption spectra identical but the biologically and commercially prepared compounds co-eluted when run on either an ion-pair reversed-phase or anion-exchange column. In addition to these major accumulations minor peaks corresponding to IMP, GMP, XMP and AMP were also observed and identified as discussed previously.

As expected analyses of extracts from a non-mutant CHO cell line did not indicate accumulation of any intermediates of *de novo* purine biosynthesis. The largest peak observed eluted at approximately 6 min with both 1% and 3% acetonitrile and was identified as acetate. Minor peaks were detected at later elution times

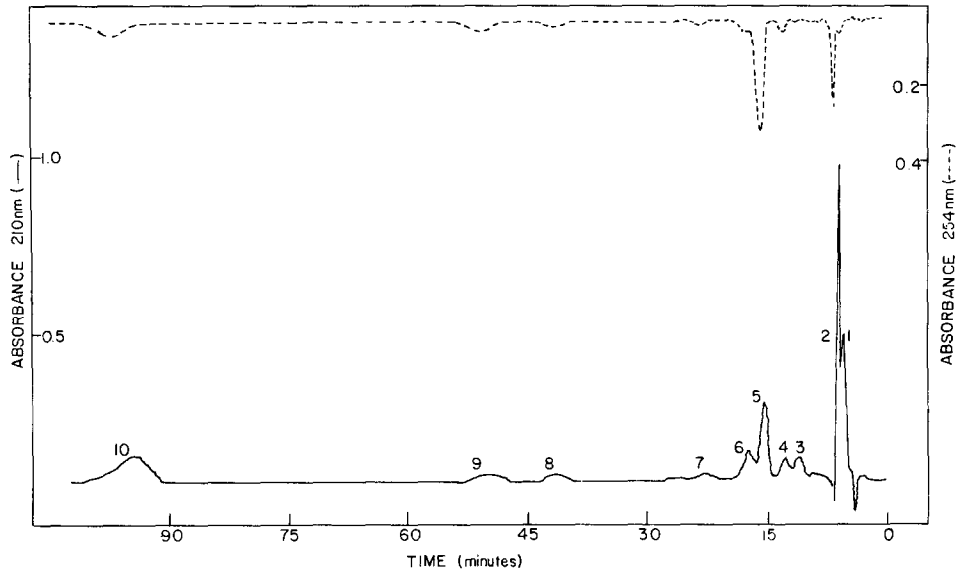

Fig. 3. Ion-pair reversed-phase HPLC separation of intermediates accumulated by Ade-I with UV detection at 210 and 254 nm. Mobile phase: 5 m*M* DBAP, pH 6, plus 3% acetonitrile. Peaks: 1 = AIR; 2 = acetate; 3 = IMP; 4 = GMP; 5 = SAICAR; 6 = XMP; 7 = AMP; 8, 9 = unknown; 10 = AMPS. Slight offset of peaks detected at 254 nm due to time required for flow between the two detectors.

and were shown to correspond to AMP, IMP, GMP and XMP. These compounds which are also observed in analyses of the mutant cell lines grown in hypoxanthine deficient media, are likely components of the normal equilibrium between the cells' nucleoside mono-, di- and triphosphate pools. Levels of the minor peaks were approximately equal in mutant and non-mutant lines.

Two intermediates of the *de novo* purine biosynthetic pathway were not resolved using the system described. The first, PRA, is known to be very unstable[15,16] with a half life of 33 to 35 s at 37°C and pH 7.5. Such a high degree of instability makes it impossible to biologically prepare this compound and isolate it. Failure to resolve the second compound, AICR, is also probably due to a stability problem. Mutants of *Salmonella typhimurium* likely defective in the conversion of AICR to SAICAR do not accumulate AICR but its decarboxylation product AIR[17]. An identical situation seems to have occurred with the Ade-I auxotroph which accumulates SAICAR and AIR, the two intermediates that bracket AICR. It is most probable that the AIR present in Ade-I extract is due to breakdown of AICR. Attempts to minimize this breakdown during the described procedure were to no avail.

The single methodology for the separation of the intermediates of *de novo* purine biosynthesis presented here will be valuable in the study of the pathway be it in regards to regulation by intermediate metabolites or end products[5,18], identification of defective enzyme activities in clinical[2,19–23] or laboratory[1,7,9,24] situations or determining the sites of action for potential therapeutic agents[7,19,25,26]. The methodology described can also be used, in conjunction with available mutants that accumulate the intermediates, as a preparative tool to yield biologically active substrates for

the enzymes of the pathway. While the system described does not require radioactive tagging for detection, if labelled intermediates are required it is a simple matter to include labelled precursors during the 2-h incubation period during which accumulation occurs. The labelled intermediates generated may still be detected using ultraviolet light absorption or by radioactivity if so desired.

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