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METABOLISM OF PUROMYCIN AMINONUCLEOSIDE IN THE RAT

FORMATION OF NUCLEOTIDE DERIVATIVES*

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Abstract—6-Dimethylamino-9-(3′-amino-3′-deoxy-β-D-ribofuranosyl) purine, the aminonucleoside of puromycin (PAN), is metabolized by liver slices to the 5′-monophosphate of the nucleoside, 6-methylamino-9-(3′-amino-3′-deoxy-β-D-ribofuranosyl) purine. The product is readily identified in extracts of liver obtained from rats given an intravenous injection of PAN 90 min prior to sacrifice. The metabolite may be responsible for the rapid disappearance of liver glycogen which is known to occur during this time period. Incubation of the aminonucleoside of puromycin with slices of rat kidney cortex suggested that the nucleoside is not metabolized by this tissue.

6-DIMETHYLAMINO-9-(3′-amino-3′-deoxy-β-D-ribofuranosyl) purine, the aminonucleoside of puromycin (PAN), is known to be demethylated by liver microsomal enzymes[1] and the resulting compounds are then rapidly excreted together with other purine catabolic products.[2, 3] PAN or its metabolic products produce several biochemical and physiological effects in rats, such as the rapid depletion and subsequent accumulation of liver glycogen,[4] the alteration of glucose 6-phosphate-dehydrogenase, lactic dehydrogenase and malic dehydrogenase activities[5] and of substrate-induced kidney respiration,[6] and the production of an experimental nephrotic syndrome.[7, 8] Available evidence derived from studies in L-cells[9] and in Ehrlich ascites cells[10] indicates that demethylation of PAN should permit the formation of mono- and polyphosphate derivatives, depending upon whether one or both methyl groups on the 6-amino group of the purine moiety have been removed. These findings are supported by studies with purified liver adenosine kinase and muscle myokinase.[10, 11] Interestingly, polyphosphates of 3′-amino-3′-deoxyadenosine, the completely demethylated derivative of PAN, may act as potent inhibitors of RNA synthesis, presumably by preventing chain elongation.[12] The results obtained with the various test systems suggest that the biochemical and physiological responses are initiated by a metabolite rather than by the parent compound. This paper is concerned with the identification of the phosphorylated products of PAN metabolism in rat liver. Some of these results have been presented elsewhere in a preliminary form.[13]

EXPERIMENTAL

Male Sprague–Dawley rats were used in all experiments. Liver slices, prepared in

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the cold with a Stadie–Riggs microtome, were incubated routinely at 37° in the high K^+ medium of Ashmore *et al.*[14] equilibrated with a mixture of O_2:CO_2 (95:5) before use. Usually, 4–5 g tissue was added to 8·0 ml of ice-cold medium containing PAN (6 μmoles/ml). Incubation was carried out with shaking in 125-ml Warburg vessels gassed with the O_2:CO_2 gas mixture for the first 15 min. After incubation, the slices were rinsed with cold fresh medium, followed by a second rinse in cold physiological saline, then homogenized in about 5 vol. of cold 6% $HClO_4$ followed by centrifugation. The incubation medium and rinses were combined, made 5% with respect to $HClO_4$, and then clarified by centrifugation. The resulting supernatants were neutralized with KOH to remove excess ClO_4^- and stored at −20°.

Analysis of acid-soluble components from medium and tissue was accomplished by a combination of column and paper chromatography. Soluble tissue nucleotides were separated on Dowex-1 and an ammonium formate gradient.[15] Nucleosides were fractionated on a Dowex-50 NH_4^+ (100–200 mesh, ×4) column, 0·9 × 10 cm, using the gradient developed between 500 ml of 0·05 M ammonium formate, pH 3·5, in the mixer flask and 2·0 l. 0·2 M ammonium formate, pH 8·0, in the reservoir. Ultraviolet absorbance at 265 mμ was monitored continuously with a Gilson detector and fractions collected under each peak were combined, concentrated *in vacuo* with a rotary evaporator at 30°, and then lyophylized.

Paper chromatography of the lyophylized eluates was performed on Whatman 3 MM paper in two solvent systems: (1) isopropanol:NH_3:H_2O (7:1:2), and (2) isopropanol:HCl:H_2O (65:16·7:H_2O to 100 ml). Ultraviolet absorbing bands were eluted from the dried paper with 0·05 N HCl for 1–2 hr. The compounds were identified by spectrophotometric comparisons with known compounds.

Acid hydrolysis of isolated compounds was performed at 100° in 1 N HCl for 1 hr except where indicated. 5′-Monophosphate groups were identified enzymatically with 5′-nucleotidase, purified from venom of *Crotalus adamanteus* (Sigma), in 0·1 M Tris buffer, pH 9, at 37° for 2 hr.[16] The components of the reaction mixture were separated by paper chromatography. Substituent 3′-amino groups were determined with picryl sulfonate at 340 mμ.[17] The reaction mixture contained 1·0 ml of 4% $NaHCO_3$, 1·0 ml of neutralized sample and 1·0 ml of 0·1% picryl sulfonic acid (Nutritional Biochemicals Corp.). Incubation was for 2 hr at 37° in the dark. The reaction was terminated by the addition of 1·0 ml of 1 N HCl followed by vigorous shaking to eliminate excess CO_2. Linear results were obtained between 0·03 and 0·8 μmole when PAN was used as the standard. A molecular extinction coefficient of 18,600 was used for the spectrophotometric determination of PAN solutions at 268 mμ and at pH 1.[18]

Liver glycogen phosphorylase assays were performed with crude homogenates at pH 6·1[19] by following the release of inorganic phosphate from glucose 1-phosphate. Phosphate was determined by the method of Fiske and Subbarow.[20]

RESULTS

Studies were undertaken to determine the extent of PAN demethylation, a reaction known to occur in the liver microsome fraction,[1] and the possible conversion of the demethylated product or products to compounds analogous to AMP. For these experiments, acid extracts of liver slices incubated in the presence of PAN were chromatographed on a Dowex-1-formate column (Fig. 1). The elution pattern from the column showed that two prominent ultraviolet-absorbing peaks appeared after

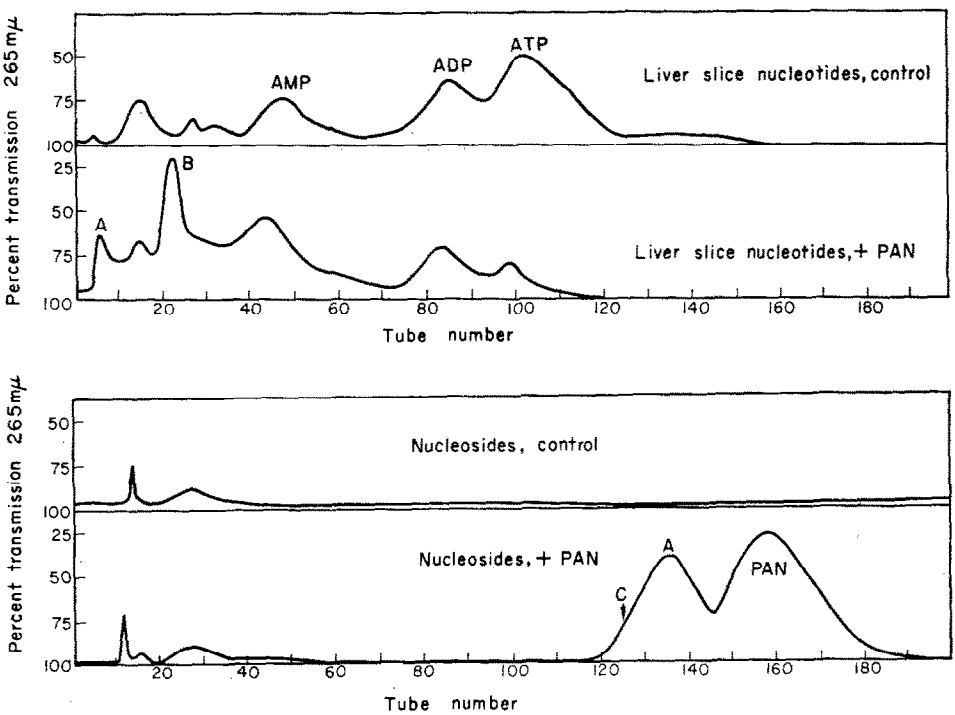

FIG. 1. Column chromatography of nucleotide and nucleoside components after incubation of liver slices in the presence and absence of PAN. Nucleotides: Acid-soluble nucleotides extracted from 4·0 g tissue were fractionated on a column 0·9 × 35 cm containing Dowex-1 resin (200–400 mesh, × 8) in the formate form. Elution was carried out with the ammonium formate gradient described by Hurlbert.[15] Nucleosides: One-half the combined volume of the clarified incubation medium and tissue rinses was adjusted to pH 1·5 with 8 N formic acid and applied to a column of Dowex-50 NH_4^+. Elution was carried out with an ammonium formate gradient as described in the Experimental section.

incubation with PAN. Fractions under each peak were combined and designated A and B for purposes of identification. Similar results were obtained when liver slices were incubated in Krebs–Heinseliet medium[21] containing PAN. It should be noted also (Fig. 1) that the presence of PAN altered the balance of the major nucleotides in liver cells as shown by the increase in the amount of AMP and a decrease in the amount of ATP.

The major component of peak A did not bind strongly to the Dowex-1 column and behaved as a nucleoside on paper chromatography in two solvent systems (Table 1). Absorption maxima were found at 263 and 267 mμ in acidic and basic solution respectively. Acid hydrolysis released a purine base which had chromatographic properties and ultraviolet absorption spectra identical to those of 6-methylaminopurine. Analysis for total phosphorus was negative.

The major component of peak B behaved like a nucleotide on paper chromatography (Table 1). Like A, compound B had a maximum absorption at 263 and 267 mμ in acidic and basic solution, respectively, and released 6-methylaminopurine on acid hydrolysis. The presumptive nucleotide was hydrolyzed by snake venom 5′-nucleotid-

ase to a compound indistinguishable from the monomethyl nucleoside A. In addition, analysis for total phosphate in a sample containing 0·6 μmole of compound B resulted in the formation of 0·61 μmole of inorganic phosphate. This phosphate group was not released by hydrolysis in 1 N HCl at 100° for 20 min. These observations suggest that the compound is a nucleotide containing 6-methylaminopurine with the phosphate group on the 5′-position of the sugar moiety. Polyphosphate derivatives of this compound were not found in the soluble nucleotide fraction derived from tissue slices.

Both A and B failed to give a positive reaction for ribose,[22] but did react positively in the presence of picryl sulfonic acid, indicating the presence of a primary or secondary

TABLE 1. PROPERTIES OF THREE METABOLITES OF PAN OBTAINED BY COLUMN CHROMATOGRAPHY

Fraction	Treatment*	Paper chromatography†		Absorbancy maximum‡	
		R_{f1}	R_{f2}	Acid (mμ)	Base (mμ)
A		0·72	0·29	263	267
	Acid hydrolysis	0·49		267	274
	5′-Nucleotidase	0·72		263	267
B		0·17	0·33	263	267
	Acid hydrolysis	0·49		267	274
	5′-Nucleotidase	0·72	0·29	263	267
C		0·52	0·15	257	259
	Acid hydrolysis	0·51	0·32	262	268

* See text for details.
† R_f values were obtained in two solvent systems: R_{f1}, isopropanol: NH_3:H_2O (7:1:2, v/v), and R_{f2}, isopropanol:HCl:H_2O (65:16·7:18·3, v/v).
‡ Spectrophotometric data were obtained from the sample in 0·1 N HCl or NaOH.

TABLE 2. EQUIVALENCE OF AMINO GROUP AND PURINE CONTENT IN THREE METABOLITES OF PAN

Fraction	Purine (μmoles)	Amino group* (μmoles)
A	1·38	1·43
B	0·62	0·63
C	1·93	1·87
AMP†	1·0	$<$0·01

* Picryl sulfonic acid method; see text for details.
† Other compounds which do not react significantly with picryl sulfonic acid are adenosine, 6-methylaminopurine and 6-dimethylaminopurine.

amine.[17] With PAN as the standard, both A and B formed a chromogen in amounts equivalent to their purine content (Table 2). Adenosine, AMP, 6-methylamino- and 6-dimethylamino-purine do not react significantly with the sulfonate reagent under the conditions employed. In addition, both A and B gave a negative ninhydrin test in 1·5% NaOH either before or after acid hydrolysis, but were positive in the presence of ninhydrin in 3% $NaHCO_3$, a result expected with 3′-amino-3′-deoxyribose.[22]

The analyses are consistent with the conclusion that A is 6-methylamino-9-(3′-

amino-3′-deoxyribofuranosyl) purine (monomethyl-PAN) and that B is the 5′-monophosphate derivative (monomethyl-PAN-5′-P).

A search for other possible metabolites of PAN was extended to the medium. Two nucleosides were partially resolved on a Dowex-50 column (Fig. 1) which, after separation by paper chromatography, were identified as unreacted PAN and monomethyl-PAN. The latter is identical to compound A (Table 1) and apparently binds loosely to Dowex-1 resin. During the purification of monomethyl-PAN, a new ultraviolet-absorbing spot appeared on paper chromatograms. Upon isolation, the compound had properties consistent with the nucleoside, 3′-amino-3′-deoxyadenosine (compound C in Tables 1 and 2).

The data in Table 3 summarize the formation of the three derivatives of PAN in rat

TABLE 3. FORMATION OF COMPOUNDS A, B AND C IN LIVER SLICES OVER A PERIOD OF 90 min*

Fraction†	Compound	Time (min) 30	60	90
A	Monomethyl-PAN	2·33	3·68	4·32
B	Monomethyl-PAN-5′-P	0·13	0·21	0·27
C	3′-Amino-3′-deoxyadenosine	0·03	0·16	0·22

* Liver slices (4·5 g) were incubated in 8 ml of medium containing 6 μmoles/ml of PAN. Analytical details as described in the Experimental section, Fig. 1 and Table 1. Values are expressed as μmoles/g wet wt.

† Quantitative spectrophotometric measurements were made in 0·1 N HCl at the wavelength of maximum absorption (Table 1). A molecular extinction coefficient of 16,300 was used for fraction A and B and 15,100 for fraction C.

TABLE 4. EFFECT OF MONOMETHYL-PAN-5′-P* ON LIVER GLYCOGEN PHOSPHORYLASE ACTIVITY

Conditions	Expt. 1 (μmoles P/10 min/mg protein)	Expt. 2
Liver homogenate	2·89	2·26
+ AMP (2 μmoles)	3·38	2·70
+ Monomethyl-PAN-5′-P (1·32 μmoles)	3·40	2·77

* Monomethyl-PAN-5′-P was isolated from combined extracts of liver slices incubated in the presence of PAN. The purified nucleotide was homogeneous after paper chromatography in two solvent systems (see Table 1).

liver slices over a period of 90 min. Results similar to these were also found when rabbit liver slices were incubated in the presence of PAN. Compared to rat liver slices, approximately twice the amount of the monomethyl nucleotide (0·63 μmole) appeared after 90 min of incubation under similar conditions. It was observed in other experiments that PAN was unchanged after incubation with rat kidney slices for 90 min.

One possible metabolic consequence of the formation of the monomethyl-PAN nucleotide in liver may be the rapid disappearance of liver glycogen within the first few hours after an intravenous injection of PAN into rats.[4] The possibility that such a

derivative may be capable of stimulating liver glycogen phosphorylase activity in a manner similar to AMP was therefore investigated (Table 4). It can be seen that the addition of 1·32 μmoles monomethyl-PAN-5′-P to the phosphorylase assay medium increases the phosphorylase activity by an average of 18 per cent. In the control experiment, 2 μmoles AMP produced a similar increase in activity. The physiological significance of these results is strengthened by the observation that monomethyl-PAN-5′-P was identified in acid extracts of liver obtained from rats given an intravenous injection (10 mg PAN/100 g rat) 90 min prior to sacrifice.

DISCUSSION

Experiments with liver slices have shown that PAN is demethylated and phosphorylated to the 5′-nucleotide of 6-methylamino-9-(3′-amino-3′-deoxyribofuranosyl) purine. A second demethylation of PAN also occurs to form the nucleoside, 3′-amino-3′-deoxyadenosine. The demethylated nucleosides appear readily permeable to liver cells, since they are found predominantly in the incubation medium, and thus may be excreted in the urine. Additional degradation products of PAN found in the urine were identified as allantoin, uric acid, xanthin and several hydroxyaminopurine derivatives.[3, 23] The fate of the amino sugar moiety is not known.

The work of Lindberg *et al.*[11] showed that a partially purified preparation of adenosine kinase from rabbit liver was capable of phosphorylating compounds closely resembling PAN, such as N^6-methyladenosine, ($K_m = 1{\cdot}6 \times 10^{-6}$ M), 3-deoxyadenosine ($K_m = 4{\cdot}7 \times 10^{-4}$ M), N^6-methyl-3′-deoxyadenosine ($K_m = 5{\cdot}4 \times 10^{-4}$ M) and 3′-amino-3′-deoxyadenosine ($K_m = 6{\cdot}1 \times 10^{-4}$ M). PAN was found to be neither a substrate nor an inhibitor of the enzyme. These results suggest that the monomethyl nucleoside of PAN formed by liver cells may be a substrate for adenosine kinase, as suggested also by results obtained with Ehrlich ascites cells.[10] A sample of the monomethyl-PAN isolated from rat liver slices and tested in the adenosine kinase system by Dr. B. Lindberg was found to be phosphorylated by the enzyme ($K_m = 1{\cdot}2 \times 10^{-4}$ M). The results obtained with the adenosine kinase preparation suggest that triphosphate derivatives could be formed with the participation of myokinase. However, di- and triphosphate derivatives were not found in liver extracts. On the other hand, 3′-amino-3′-deoxyadenosine is readily phosphorylated to the di- and triphosphate in the presence of adenosine kinase and muscle myokinase.[11] These phosphorylated compounds have been isolated from intact mammalian cells.[12] The evidence suggests that the formation of polyphosphate derivatives of these compounds is a function of substituent groups on the 6-amino position of the purine and that the formation of 3′-amino-3′-deoxyadenosine by the demethylation of PAN should result in the synthesis of the polyphosphate derivatives in liver. This aspect is under investigation at the present time.

The accumulation of the monomethyl nucleotide derived from PAN may either compete with or be synergistic to naturally occurring adenosine nucleotides. For example, glycogen phosphorylase activity is stimulated to a level comparable to that observed in the presence of AMP. This observation may explain the known rapid decrease *in vivo* of liver glycogen levels after PAN administration[4] at a time when the nucleotide is known to be present in large amounts.

In addition, the decrease observed in the level of ATP may reflect utilization in the formation of monomethyl-PAN-5′-P as catalyzed by adenosine kinase, or the mono-

methyl-PAN-5′-P may act as a competitive inhibitor of oxidative phosphorylation, or both. PAN itself does not appear to affect cytochrome c oxidase activity,[24] respiratory control[25] in kidney mitochondria, and the respiration of kidney slices.[26]

PAN is known to be a nephrotoxic compound responsible for the onset of a nephrotic syndrome specifically in rats, monkeys and humans[2, 27, 28] and the monodemethylated nucleoside has the same toxicity in rats.[2] It has been suggested that the differential toxicity of PAN may be related to different rates of demethylation, although qualitatively similar urinary metabolites are found in the rat, mouse and guinea pig.[23] However, PAN and the demethylated nucleosides are excreted rapidly in the urine,[23] while proteinuria associated with the onset of the nephrotic syndrome occurs after a latent period of about 5 days.[6] Since the present experiments have shown that PAN is unchanged by kidney cells, it is suggested that demethylated PAN from liver is made available to the kidney where the compound is either excreted or retained. At least a portion of the demethylated nucleoside may be retained as nucleotides. For example, the synthesis of the 5′-nucleotide of monomethyl-PAN from the nucleoside precursor is known to occur in kidney slices.[29] This nucleotide may be an active metabolite of PAN and its formation in sensitive animals may lead to the production of a nephrotic syndrome-like disease. This hypothesis is supported by the fact that 6-methylaminoadenosine successfully antagonizes the action of PAN in rats[30] and that various purine analogs must be converted to the nucleotide in order to be biologically active.[12, 31, 32]

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