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FORMATION OF NOVEL NUCLEOSIDES FROM FREE BASE AND SUGAR PHOSPHATE: AQUEOUS REACTION OF 2-AMINOPYRIMIDINE AND RIBOSE-5-PHOSPHATE

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SUMMARY: The facile formation of glycosylamines suggests that a base liberated by depurination might react at the free C_1 position of the sugar phosphate from which it had been hydrolyzed, effectively repurinating the site. Model experiments testing this hypothesis demonstrate that such a reaction does take place. The primary product of a reaction between 2-aminopyrimidine (a model for guanine) and ribose-5-phosphate is characterized by enzymatic and chemical degradation, and UV spectra. It is shown to be a novel nucleoside with the base attached via its exocyclic amino group to the C_1 of the ribose-5-phosphate.

The reaction of ammonia and its derivatives with aldoses is well known (1), extending to amines related to those in DNA (2). The aldoses which undergo the reaction include ribose and 2-deoxyribose (3,4). Such sugars and amines (e.g., adenine, A and guanine, G) are formed as the products of depurination; therefore, they may react to "repurinate" a site in DNA that had just undergone depurination. The result would be formation of a novel nucleotide in the DNA helix, one in which the A or G were bound to the sugar via its exocyclic amino group. This hypothesis has been investigated by studying the reaction of ribose-5-phosphate with 2APy[1] as a highly soluble analogue of G. The results clearly show that they react to produce a novel nucleoside-5'-monophosphate with a structure as predicted.

MATERIALS AND METHODS

Chemicals- Aldrich was the source of 2APy which was then recrystallized from benzene before use. Tris, Tris-Bis-Propane, venom 5'-nucleotidase, and ribose-5-phosphate were obtained from Sigma. Nuclease P1 was obtained from Boehringer-Mannheim. Bacterial alkaline phosphatase (BAP) was obtained from

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[1] 2APY is used for 2-aminopyrimidine.

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Worthington. Other reagents were obtained from J. T. Baker. AG-1-X8 anion exchange resin was obtained from BioRad. Polyethyleneimine (PEI) cellulose sheets (20 x 40) were obtained from Brinkman and used without water washing, being cut to size as needed.

Reaction of Bases and Sugar Phosphate- Ribose-5-phosphate (2.95 g of the Barium salt, 8.1 mmoles) was dissolved in water to a final volume of 5 mls including the addition of 684 µl of concentrated (11.6 M) HCl. 2APy (2.85 g, 30 mmoles) was dissolved in a final volume of 5 mls. Equal volumes of each solution were mixed in a tube which was then sealed with a tight-fitting cap, and incubated at 65° for 60 minutes. The tube was opened, the pH measured at 4.9 by direct immersion of a pH meter probe, the contents poured into ~50 mls of water, the reaction tube well rinsed, and the diluted reaction titrated to pH 8 with ammonium hydroxide (constant mixing). After bringing the volume to 100 mls, the absorbance was read at 303 nM.

The solution was loaded on a column that had been packed with AG-1-X8 anion exchange resin and extensively prewashed with 0.1 M HCl followed by water. The sample was loaded, the column washed with water and then with dilute HCl to effect elution of any bound UV-absorbing nucleotidic compounds. At least 96±5% of the UV absorbing material is unretained by the column and appears in the initial breakthrough. The columns were monitored by means of an LKB UVicord S a type 1 lamp (277 nm).

UV Spectra- UV spectra were obtained using a Gilford Model 2600 spectrophotometer. Scans were taken from 200 to 400 nanometers using, as a blank, column fractions that contained the same concentration of elutant but no UV absorbing material. The pH of the samples and blanks to be scanned were adjusted by adding appropriate volumes of solutions of concentrated HCl, or 1M stocks of K_2HPO_4 or 0.4 M Na_3PO_4, and normalizing the plotting of the UV-absorbance data.

RESULTS

The aminopyrimidine was incubated with ribose-5-phosphate (rather than ribose) to facilitate separation of any covalently attached product from unreacted base. The phosphate moiety also precludes furanose-pyranose interconversion, simplifying product analysis. Figure 1 presents a column chromatograph for a reaction between 2APy and ribose-5-phosphate. After changing from water to 5 mM HCl, a sharp, small peak elutes. This material is as yet uncharacterized. Further elution leads to a major UV absorbing peak, as well as a small third peak (the latter also not yet characterized). A variety of controls show the presence of the principle peak must derive from the co-incubation of 2APy and ribose-5-phosphate, not being an intramolecular reaction of one of the reactants (data not shown).

The neutralized nucleotidic material of the main peak was bound to a QAE Sephadex column, eluted with triethylammonium bicarbonate, and repeatedly evaporated to concentrate it. The mobility of the desalted compound was then examined by thin layer chromatography. Table I shows the UV-absorbing compound

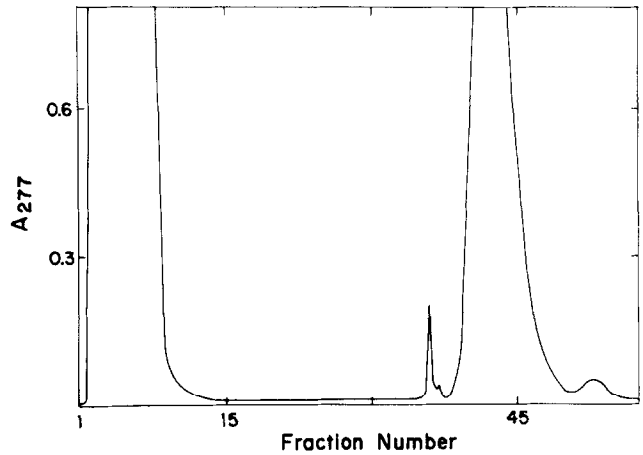

Figure 1 Chromatographic Profile of the Products of the Reaction of Ribose-5-phosphate and 2-aminopyrimidine. The 0.9 x 25 cm anion exchange column was loaded, washed with water and then eluted with 5 mM HCl. Fractions of 23 mls were collected at ten minute intervals. Elution with H_2O began at fraction 6 and elution with 5 mM HCl began at fraction 26. After the main peak eluted, continued elution with 5 mM or 10 mM HCl did not reveal other products.

to have an R_f of 0.57 when the PEI cellulose TLC is eluted with 0.2 M LiCl. Development with water does not move the UV absorbing spot from the origin unless pretreated with BAP (5) or 5'-nucleotidase (6). In contrast, nuclease P1,

TABLE I

Thin Layer Chromatographic Mobility on PEI Cellulose[a]

Sample	Mobilities		
	H_2O	0.20 M LiCl	0.20 M LiCl (boric acid sat.)
Nucleotide	0	0.57	0.41
BAP treated nucleotide	0.60	--	--
rUMP	0	0.44	0.32
P1 treated nucleotide	0	--	--
5'-Nucleotidase treated nucleotide	0.58	--	--
Acid treated nucleotide	0.66	--	--
2-Aminoplyrimidine	0.66	--	--

[a]The BAP treatment was as for Table I. The P1 treatment was 1.0 units of enzyme for 1 hr at 37°C in 20 mM Tris 7.0. Controls (data not shown) demonstrated complete conversion of either 3'dGMP or 3'dAMP to their corresponding nucleosides by this enzyme under these conditions. The 5'-nucleotidase treatment was with 1.0 unit of enzyme for 3 hr. at 37°C in 5 mM $MgCl_2$, 20 mM Tris Bis Propane, pH 9.5. The spontaneous hydrolysis treatment was for 6 days at 37° C, pH 6 (0.02 M NaP_i). The TLC's were for 17 cm on 20 cm PEI cellulose sheets containing fluorescent indicator in the solvent indicated. The 0.20 M LiCl and the 0.20 M LiCl saturated with boric acid were first adjusted to pH 7 with NH_4OH.

an enzyme with potent 2',3'-nucleotidase activity (7), has no such effect. Consistent with this and demonstrating the 2',3' cis hydroxyls, inclusion of boric acid in the 0.2M LiCl retards the mobility (8,9). Taken together, these data show that the compound is a 5'-ribomononucleotide.

Table I also shows that when incubated at pH 6, the nucleotide slowly breaks down to free 2APy. The sugar-base bond is slowly hydrolyzed at acid and alkaline pH as well (data not shown). This not only demonstrates a glycosylic sugar-base bond, but also shows that it is not a typical nucleoside glycosylic bond since it is cleaved at neutral and alkaline pH as well as at acid pH. This behavior is quite characteristic of the glycosylamines of ammonia, primary and secondary amines (10,11,12), but not those of tertiary amines or ring nitrogen attached nucleosides (12). Thus, the structure must be 2APy covalently attached to a ribose-5-phosphate by a bond between the exocyclic amino group of the base and the C_1 of the sugar phosphate as shown in Figure 2.

The ultraviolet spectra of the nucleotide at alkaline, neutral and acid pHs are presented in Figure 3. They are similar to those of 2APy and 2-methylaminopyrimidine with a protonation occurring in the pH 2-4 range (data not shown) as for 2APy (13) and for 2-methylaminopyrimidine (14). The ribosyl moiety is expected to have a spectral effect similar to that of a methyl group (15). Since the spectra and pK_a for N_1-methyl-2-aminopyrimidine are radically different (16), this also excludes attachment of the ribose to a ring nitrogen.

DISCUSSION

In this laboratory, initial investigation of the "repurination" reaction focused on ribose-5-phosphate and 2APy as a highly soluble analogue of guanine.

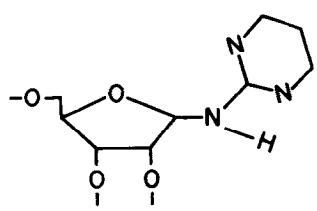

Figure 2 The Proposed Structure for the Isolated Nucleotide. Non-amino protons are omitted for clarity. The α and β anomers are not distinguished as this point has not been investigated. No attempt has been made to represent the bond distances and angles to scale.

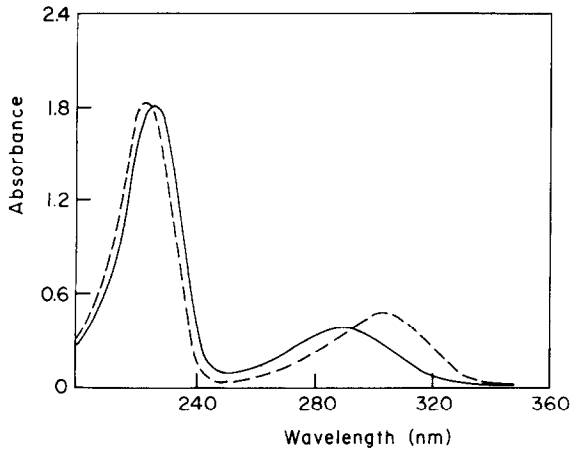

Figure 3 Ultraviolet Scans of 2-aminopyrimidine neo ribonucleoside-5'-monophosphate. The scans were as outlined in Materials and Methods. The pH 1 spectrum (broken line) was taken in 0.12 M HCl, while the pH 6 and pH 12 spectra (indistinguishable and given by the continuous line) were taken in 20 mM pH 6 KPO_4 and 20 mM pH 12 $NaPO_4$, respectively. The maxima are 224 and 302 at pH1, 228 and 291 at pH 6 and pH 12. The minima are 251 at pH 1 and 252 at pH 6 and pH 12.

The data obtained clearly show that 2APy and sugar phosphate participate in a bi-molecular reaction to yield a novel nucleoside-5-monophosphate. The data from the UV spectra, chromatographic behavior, and enzymatic analysis show this "neo" nucleotide to involve attachment of the base to the C_1 of the sugar phosphate through the exocyclic amino group of 2-aminopyrimidine.

It is presumed that reaction occurs directly between the C_1 and the amino group of the base moiety (10). Initial reaction at a ring nitrogen followed by rearrangement is a formal alternative possibility, although this is unlikely since the reactions are carried out in conditions under which 2-amino-1-methyl-pyrimidine is stable. In addition, while the reaction is titrated to pH 8 after dilution, alkali-induced rearrangement of the 1-methyl- to the 2-methylamino derivative (16) would be extremely slow at this pH (17).

The biological significance of these findings remains to be established. Adenine and guanine are relatively insoluble at neutral pH and physiological temperature, but a similar reaction can be demonstrated for them at lower pH (manuscripts in preparation). In addition, the high concentration of base and sugar-phosphate used in the experiments herein reported may seem problematic (the equilibrium constant for the reaction is only $\sim 10^{-2} M^{-1}$). Until the depurinated base diffused from the helix however, the effective concentration of

reactants would be high. A free purine (especially G) would have strong interactions with the bases of adjacent intact residues in the DNA helix and such forces would substantially lengthen the half-time for diffusion of the free base from the AP site, making quite probable the type of glycosylation reaction reported.

There are $\sim 10^4$ depurinations per day per haploid human genome (18). If a few percent give rise to the repurination reaction, the level of formation of these neo nucleotides would be substantial. This would necessitate a repair system whether the lesion could template DNA synthesis or were a block to DNA synthesis (unless the products happened to template DNA synthesis just like their parent nucleotides).

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