A Novel Nucleotide Found in Human Erythrocytes, 4-Pyridone-3-carboxamide-1-β-D-ribonucleoside Triphosphate*[S]

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We report the identification of a hitherto unknown nucleotide that is present in micromolar concentrations in the erythrocytes of healthy subjects and accumulates at levels comparable with the ATP concentration in erythrocytes of patients with chronic renal failure. The unknown nucleotide was isolated and identified by liquid chromatography with UV and tandem mass detection, ^{1}H nuclear magnetic resonance and infrared spectroscopy as 4-pyridone-3-carboxamide-1-β-D-ribonucleoside triphosphate (4PYTP), a structure indicating association with metabolism of the oxidized nicotinamide compounds. Subsequently, we demonstrated formation of 4PYTP in intact human erythrocytes during incubation with the chemically synthesized nucleoside precursor 4-pyridone-3-carboxamide-1-β-D-ribonucleoside (4PYR). We noted preferential accumulation of monophosphate of 4PYR (4PYMP) over 4PYTP as well as a decrease in erythrocyte ATP concentration during incubation with 4PYR. Both the 4PYR phosphorylation and ATP depletion were blocked by an inhibitor of adenosine kinase. Plasma concentration of 4PYR was detectable but very low (0.013 ± 0.006 μM) in contrast with the high daily urine excretion of this compound (26.7 ± 18.2 μmol/24 h) in healthy subjects, indicating much greater renal clearance than other nicotinamide metabolites, nucleosides, or creatinine. We also noted a 40-fold increase in 4PYR plasma concentration in patients with chronic renal failure (0.563 ± 0.321 μM). We suggest that 4PYTP formation in the erythrocytes is a hitherto unknown process aimed at sequestering potentially toxic 4PYR in a form that could be safely transported and subsequently released and excreted during passage of erythrocytes through the kidney.

Nucleotides play a vital role in almost every process within living cells, including transfer of genetic information and energy metabolism and regulation. An optimal nucleotide pattern is essential for normal cell function, and disturbances lead to serious clinical consequences covering the full spectrum of human disease (1). Changes in the nucleotide pattern are crucial to various phases of the cell cycle or differentiation, and pharmacological alterations of the nucleotide profile are components of anticancer, anti-inflammatory, immunosuppressive, antiviral, and anti-ischemic therapies (2–4). The erythrocyte plays a central role in nucleotide metabolism related not only to its own requirement but also to the transport of nucleotide precursors and catabolites between their sites of formation, utilization, and excretion (5, 6).

The chemical nature of all physiological nucleotides is believed to be well characterized (2). Therefore, it was very surprising for us to note an unknown nucleoside triphosphate that was present at high levels in the erythrocytes of patients with renal failure but also at low concentrations in the erythrocytes of healthy subjects (7, 8). A correlation between concentrations of this unknown nucleotide in erythrocytes and the plasma concentration of a nicotinamide catabolite N-methyl-2-pyridone-5-carboxamide (Met2PY)[2] suggested that both compounds could be related (7, 8). However, further comparison of chemical properties indicated that this is not the case. The present study was undertaken to fully characterize this novel nucleotide, to search for its possible precursors and to suggest its possible function.

MATERIALS AND METHODS

Collection of Blood from Patients and Control Subject and Preparation of Erythrocyte and Plasma Extracts—To isolate and characterize the novel nucleotide, heparinized blood was obtained with written informed consent and Guy's Hospital Ethical Committee approval from adult patients with severe chronic renal failure either hospitalized or attending renal outpatient clinics. All patients had end-stage renal failure (creatinine 300–700 μmol/liter) and were on either hemodialysis or

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[2] The abbreviations and trivial names used are: Met2PY, N-methyl-2-pyridone-5-carboxamide; Met4PY, N-methyl-4-pyridone-3-carboxamide; 4PY, 4-pyridone-3-carboxamide; 4PYR (PCNR), 4-pyridone-3-carboxamide-1-β-D-ribonucleoside; 4PYTP, 4-pyridone-3-carboxamide-1-β-D-ribonucleoside triphosphate; 2PY, 2-pyridone-5-carboxamide; 4KP, 4-pyridone-2-carboxamide; HPLC, high performance liquid chromatography.

on continuous ambulatory peritoneal dialysis). The kidney function, defined as glomerular filtration rate was <5 ml/min. For comparison, we used blood from adult controls (healthy laboratory staff). Plasma and erythrocyte extracts were prepared using trichloroacetic acid as we have described previously (9). Blood was centrifuged at 4000 × *g* for 5 min immediately after collection. The plasma fraction was collected, and for erythrocyte extracts, the top layer of packed cells comprising platelets and lymphocytes was discarded. The remaining erythrocytes were washed twice with 0.9% w/v saline, and 100 μl of packed washed erythrocytes was added to a 1.5-ml microcentrifuge tube containing 200 μl of 10% trichloroacetic acid and vortexed vigorously. After centrifuging for 2 min at 15,800 × *g*, the supernatant was extracted with water-saturated diethyl ether. Extracts, when not used immediately, were frozen and kept at −20 °C. Urine collection for assessment of daily excretion was performed as we have described previously (10).

Isolation and Sequential Degradation of the Novel Nucleotide—The initial isolation of the novel nucleotide and its quantitative analysis were performed using the anion-exchange HPLC system described in detail previously (9). To obtain the nucleoside, nucleotide fractions collected by anion-exchange HPLC were pooled and concentrated

FIGURE 1. **Identification of 4-pyridone-3-carboxamide-1-β-D-ribonucleoside triphosphate (4PYTP).** *A*, mass analysis of isolated nucleotide showing (from the *top panel*) the full mass spectrum, indicating ion at m/z = 509, MS2 analysis of the 509 ion, and MS3 analysis of the 509→411 transition. *B*, mass and NMR analysis of nucleoside obtained by alkaline phosphate degradation and NMR analysis of the chemically synthesized 4-pyridone-3-carboxamide-1-β-D-ribonucleoside (4PYR) indicating ion at m/z of 270 in full mass spectrum mode and its fragments at m/z = 139 and 122 in MS2 mode. ^{1}H NMR data were similar for isolated nucleoside and chemically synthesized 4PYR: δ_H (D$_2$O) 3.68 (1*H*, dd, *J* 4.0, 12.6 Hz, H-5′a); 3.76 (1*H*, dd, *J* 3.1, 12.6 Hz, H-5′b); 4.10 (1*H*, m, H-4′); 4.14 (1*H*, dd, *J* 3.2, 5.4 Hz, H-3′); 4.20 (1*H*, t, *J* 5.4 Hz, H-2′); 5.52 (1*H*, d, *J* 5.4 Hz, H-1′); 6.58 (1*H*, d, *J* 7.6 Hz, H-5); 7.92 (1*H*, dd, *J* 2.3, 7.6 Hz, H-6); and 8.67 (1*H*, d, *J* 2.3 Hz, H-2). *C*, mass and ^{1}H NMR analysis of base obtained by acid hydrolysis of an unknown nucleotide and ^{1}H NMR analysis of chemically synthesized 4PY, 4KP, and 2PY. Mass analysis of the isolated base indicated ion at m/z = 139 in full mass spectrum and its fragment at m/z = 122 in MS2 mode. ^{1}NMR data for the isolated base and chemically synthesized 4PY were similar: δ_H (D$_2$O) 6.54 (1 H, d, *J* 7.3 Hz, H-5); 7.74 (1 H, dd, *J* 1.2, 7.3 Hz, H-6); and 8.44 (1 H, d, *J* 1.2 Hz, H-2).

by freeze drying. Incubation buffer (0.05 ml) containing 100 mM Tris/HCl, pH 8.0, with 50 mM MgCl$_2$ was then added followed by 2 μl of enzyme solution containing 2 units of alkaline phosphatase. Incubation was carried out for 4 h at 37 °C. The reaction was terminated by the addition of 0.2 ml of 10% trichloroacetic acid, and following centrifugation, the samples were extracted with diethyl ether as described for the preparation of erythrocyte extracts. Post-reaction extracts were purified using the reversed-phase procedure described below, freeze-dried and subjected to chemical analysis by NMR or infrared spectroscopy, or further hydrolyzed to obtain free base. The latter reaction was conducted by reconstitution of HPLC-purified and freeze-dried nucleoside in 60% formic acid and incubation at 140 °C for 1 h (11). The post-reaction mixture was again freeze-dried, reconstituted in water, and purified using the reversed-phase system. This method applied a Hypersil BDS 3-μm column (150 mm/4.6 mm). Buffer A was 5 mmol/liter ammonium formate; the mobile phase B was acetonitrile. A

linear gradient from 100% A to 50% B in 12 min was used for elution, with 2 min of 50% mobile phase B running isocratically and an additional 5 min for re-equilibration with Buffer A before the next injection. The mobile phase was delivered at 1.2 ml/min. Known nucleosides and nucleobases were identified by their characteristic UV light absorption spectra (range, 210–310 nm) and retention time compared with the standards. The peak corresponding to the nucleoside and base peak in the post-reaction mixtures were identified in a chromatogram by its UV spectrum being identical to nucleotide and by molecular weight and fragmentation pattern. The UV spectrum of the nucleoside was identical to that of the nucleotide, but the UV spectrum of the base peak was different.

Anion Exchange Chromatography with Mass Detection—The analytical system used was an LCQ-Advantage or LCQ-Deca XP mass detector linked to a Surveyor chromatography system with an in-line Agilent 1050 or 1100 diode array detector. The
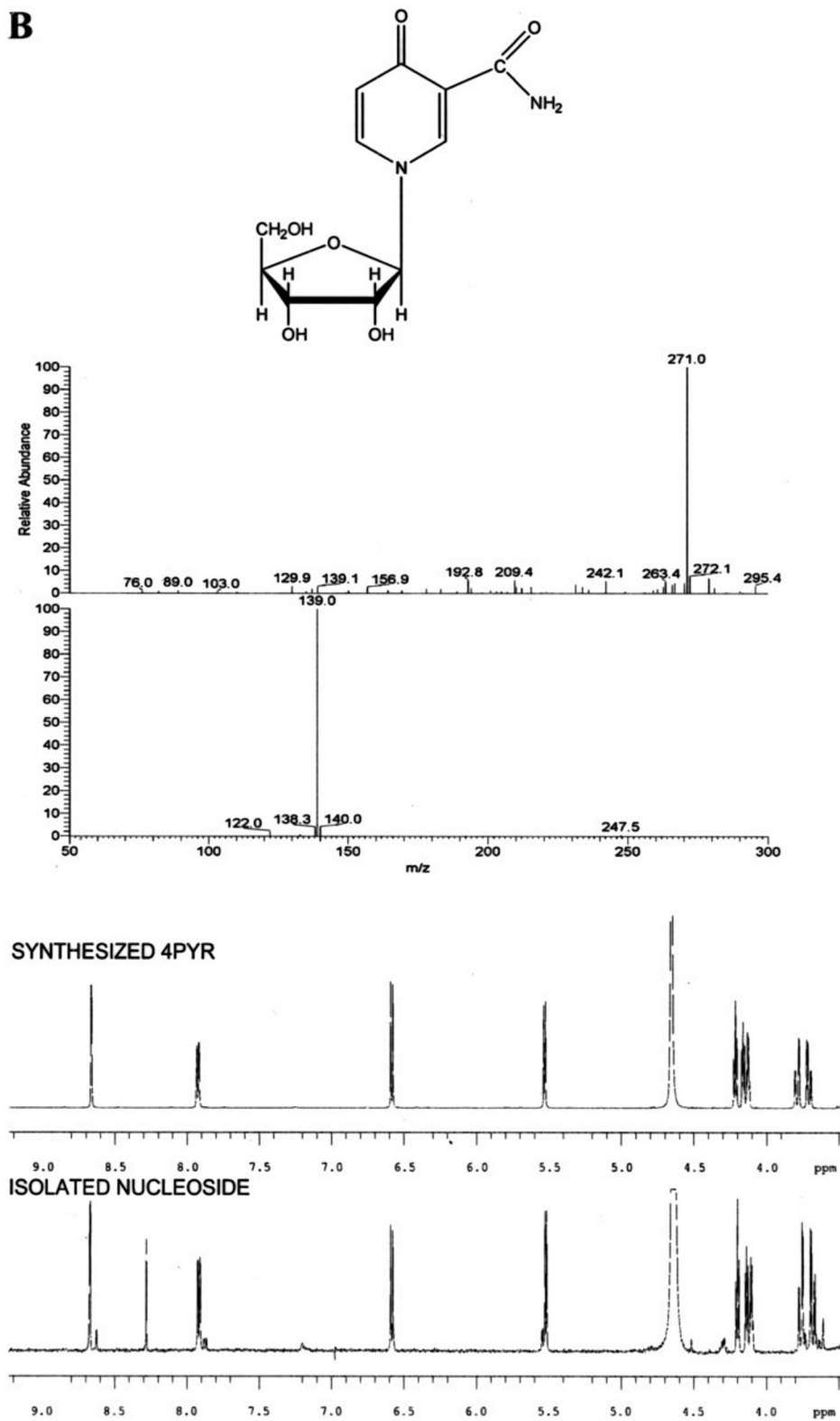
B

SYNTHESIZED 4PYR

ISOLATED NUCLEOSIDE

FIGURE 1—*continued*

profile from 100% buffer A to 100% buffer B in 12 min was used for elution with re-equilibration time of 5 min with 100% buffer A. The unknown nucleotide peak was recognized by its characteristic UV spectrum. The mass detector was operated in negative ion mode. A Thermo-Finnigan Electrospray ion source was used with a 5-kV cone voltage setting and an arbitrary nebulizing gas (nitrogen) flow set at 35%. The heated capillary was maintained at 220 °C. Ion optics parameters were optimized for ATP with standard instrument routines. Helium was used as the collision gas. A collision energy of 28% was used for analysis of the fragmentation pattern.

Reversed-phase Chromatography with Mass Detection—The analytical systems described above incorporating diode array and mass detection were used for characterization and quantitative analysis of the nucleoside and base in extracts and post-reaction mixtures. The reversed-phase method employed a Hypersil BDS 3-μm column, 150/2 mm. Buffers were running at a flow rate of 0.2 ml/min. Buffer A was 5 mmol/liter ammonium formate; the mobile phase B was acetonitrile. A linear gradient from 100% buffer A to 50% B in 12 min was used for elution, followed by 2 min of 50% B and 5 min of re-equilibration at 100% A. Nucleosides and nucleobases were identified by their characteristic UV absorption spectra (range: 210–310 nm) and retention time compared with the standards. The mass detector was operating in the positive ion mode. A Thermo-Finnigan Electrospray ion source was used with a 5-kV cone voltage setting and an arbitrary nebulizing gas (nitrogen) flow set at 35%. The heated capillary was maintained at 250 °C. Ion optics parameters were optimized for adenosine with standard instrument routines. Helium was used as

chromatographic procedure used a chemically stable anion-exchange column (Phenomenex; 3 μm, Luna-NH$_2$, 150/2 mm). Buffer A was 5 mmol/liter ammonium acetate; buffer B was 30 mM N',N',dimethylhexylamine/50 mM ammonium hydroxide delivered at a flow rate of 0.2 ml/min. A convex gradient
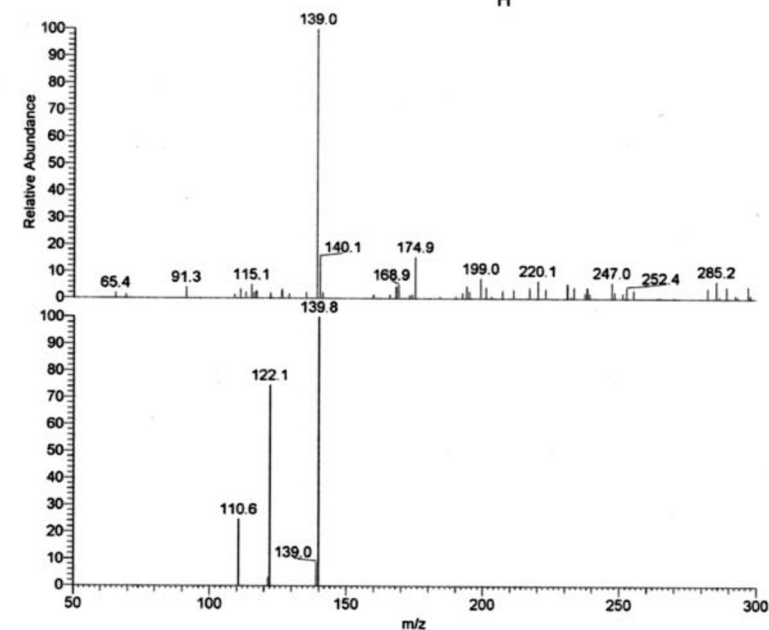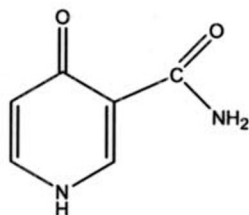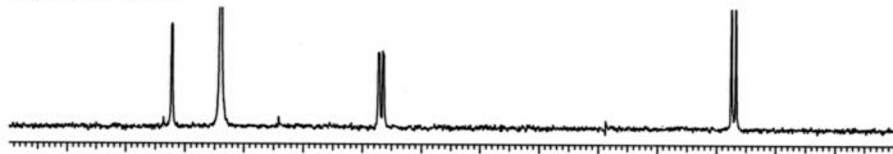
the collision gas. A collision energy of 35% was used to obtain the fragmentation pattern of the nucleoside under investigation.

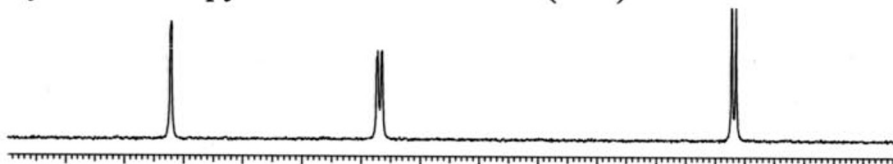*NMR and Infrared Spectroscopy*—A Varian Unity plus 500 MHz NMR spectrometer was used for nucleoside and base
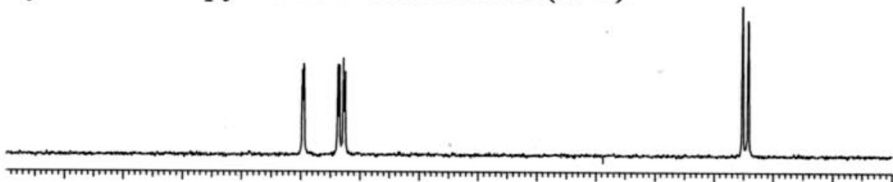
Isolated base

Synthesized 4-pyridone-3-carboxamide (4PY)

Synthesized 2-pyridone-5-carboxamide (2PY)
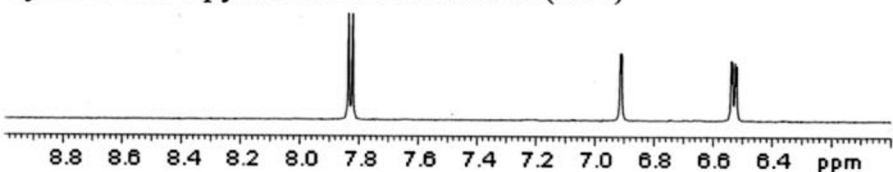
Synthesized 4-pyridone-2-carboxamide (4KP)

FIGURE 1—*continued*

analysis with ^{1}H NMR in D_2O as the solvent at room (22 °C) temperature and with chemical shifts assigned according to the residual signal of water assuming its position at 4.64 parts/million. The purified nucleoside was also analyzed by infrared spectroscopy. Full details are provided in the supplemental material.

Chemical Synthesis of Pyridone Derivatives—Chemical synthesis of pyridone derivatives was performed as previously described. 4-Pyridone-3-carboxamide (12) was prepared from commercially available 4-chloropyridine-3-carboxylic acid and ribosylated under Vorbrüggen's protocol (13). In the case of 4-pyridone-2-carboxamide (14–16), 2-picolinic acid was used as a starting material. Details of the chemical synthesis procedures, methods for structural characterizations, and the spectral properties of the intermediates and final products are provided in the supplemental material.

Incubation of Healthy Human Erythrocytes with 4PY and 4PYR—Incubation of healthy human erythrocytes with 4PY and 4PYR was performed as we have described previously (9, 17). Erythrocytes of healthy humans were used for this experiment and were obtained and washed as described above. The erythrocytes were suspended in Hepes-buffered Krebs medium at 20% hematocrit. 4PY or 4PYR was added at 30–1000 μM concentration as indicated in the legend to Fig. 2. Incubation was carried out for 3, 6, or 12 h at 37 °C. Incubation was terminated by the addition of trichloroacetic acid, and extraction and analysis was completed as described under "Materials and Methods."

Reagents—Alkaline phosphatase was obtained from Amersham Biosciences. Chemicals for the synthesis were obtained from Lancaster and Aldrich. All commercially available nucleotides, nucleosides, and bases were obtained from Sigma. Chromatographic columns were obtained from Phenomenex. HPLC grade solvents and buffer salts were obtained from VWR.

TABLE 1

Concentration of 4-pyridone-3-carboxamide-1-β-D-ribonucleoside triphosphate (4PYTP) and other nucleotides in the erythrocyte extracts of healthy subjects and patients with end-stage chronic renal failure

Values are expressed in μmol/liter packed erythrocytes and represent mean ($\pm$S.D.). *, $p < 0.05$; **, $p < 0.001$ *versus* control.

|	4PYTP	ATP	GTP	NAD
	μmol/liter erythrocytes			
Healthy subjects ($n = 8$)	13.0 ± 4.7 (range: 6–18)	1132 ± 182	34.4 ± 8.1	57.5 ± 9.9
Patients with chronic renal failure ($n = 10$)	162 ± 189* (range: 45–670)	1855 ± 429**	114 ± 33**	69.4 ± 15.7

Statistical Analysis—Data are presented as mean ± S.D. Student's t test or one-way analysis of variance followed by the Dunett test was used to compare two or more groups, respectively. $p < 0.05$ was considered a significant difference.

RESULTS AND DISCUSSION

Identification of the Unknown Nucleotide—Fig. 1 presents the results of the key identification steps that revealed the structure of the novel nucleotide and allowed its identification as 4-pyridone-3-carboxamide-1-β-D-ribonucleoside triphosphate (4PYTP). Liquid chromatography/mass spectrometry analysis of the chromatographic peak corresponding to the novel nucleotide revealed a negative ion at $m/z = 509$ corresponding to a molecular weight of 510. Similar analysis of ATP or GTP showed ions $m/z = 506$ and 522, respectively, as expected. The fragmentation pattern obtained in MS2 and MS3 modes suggested that it is the base that is unique, as only fragments larger than ribosetriphosphate were different in m/z ratio to fragments generated in MS2 and MS3 mode from ATP (not shown). This molecular weight was consistent with the suggestion that an oxidized metabolite of nicotinamide is the base constituent of the novel nucleotide, assuming that ribose is the sugar.

To obtain the nucleoside constituent for analysis, we treated the HPLC effluent fractions containing the novel nucleotide pooled from several runs with alkaline phosphatase, an enzyme that nonspecifically releases nucleosides from nucleotides. The UV spectrum of the nucleoside was identical to the parent nucleotide. Liquid chromatography/mass spectrometry analysis of the nucleoside peak revealed a positive ion at $m/z = 271$ corresponding to a molecular weight of 270, and analysis of its fragments in MS2 mode revealed ions at $m/z = 139$ and $m/z = 122$ that corresponded to sequential neutral loss of ribose and an amino group. Analysis with infrared spectroscopy and ^{1}H NMR revealed further details of the structure. Our chemically synthesized 4-pyridone-3-carboxamide-1-β-D-ribonucleoside (4PYR) was identical with the isolated biological compound with regard to chromatographic retention time, UV spectrum, mass, ^{1}H NMR, and infrared spectra.

Liquid chromatography/mass spectrometry analysis of the base peak obtained by acid hydrolysis of the nucleoside revealed a positive ion at $m/z = 139$ corresponding to a molecular weight of 138. MS2 mode analysis revealed an ion at $m/z = 122$ corresponding to neutral loss of an amino group. However, we had an insufficient amount of isolated material to perform heteronuclear two-dimensional NMR analysis. To confirm its identity, we therefore synthesized several of the most likely isomers suggested by ^{1}H NMR analysis, including 4-pyridone-3-carboxamide (4PY), 4-pyr-
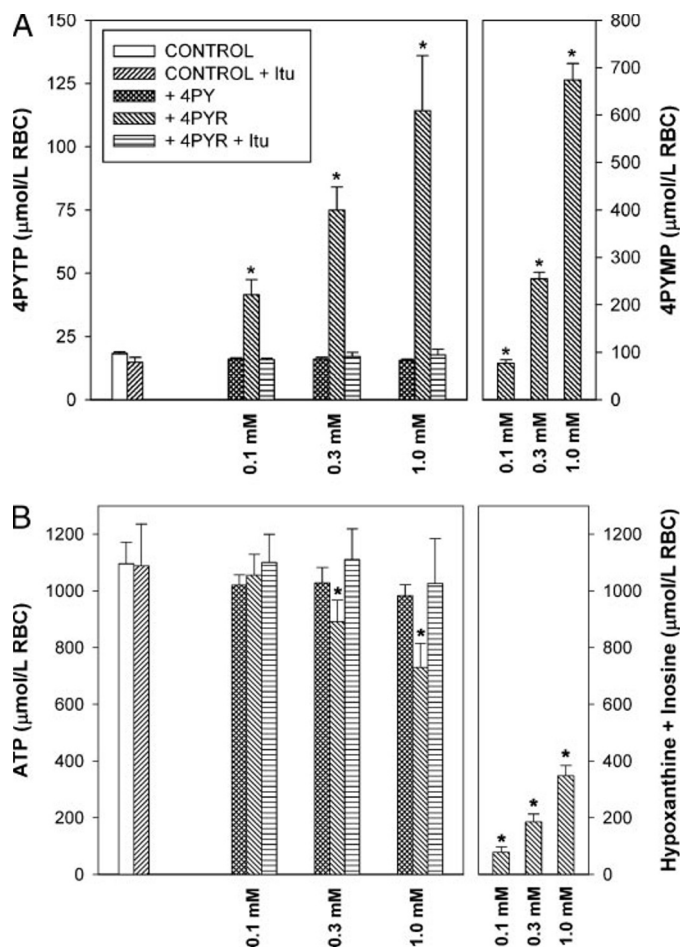
FIGURE 2. **Concentrations of 4PYTP and 4PYMP (*A*) or ATP and hypoxanthine + inosine (*B*) in human erythrocyte suspension.** Concentrations were incubated at 37 °C in Hepes-buffered Krebs medium at 20% hematocrit under control conditions and with 4PY or 4PYR at 0, 0.1, 0.3, and 1 mM concentration as indicated on the *x*-axis for 6 h in the presence of 5 μM 5′iodotubercidin (*Itu*) where indicated. 4PYMP was below 1 μM, and hypoxanthine + inosine was below 15 μM in control, control + Itu, 4PY, and 4PYR + Itu incubations. Values are expressed in μmol/liter packed erythrocytes and represent mean ± S.D., $n = 6$. *, $p < 0.05$ *versus* control.

idone-2-carboxamide (4KP), and 2-pyridone-5-carboxamide (2PY). These chemically synthesized compounds were then compared with the base obtained from the novel nucleotide with regard to UV spectrum, chromatographic retention time, and NMR spectrum. This indicated that synthesized 4PY is identical to the biologically isolated material.

The base that we identified as a constituent of the novel nucleotide is biologically present only in the methylated form as 1-methyl-4-pyridone-3-carboxamide (Met4PY) (18–20). The nucleoside 4-pyridone-3-carboxamide-1-β-D-ribonucleoside (4PYR) has long been identified in human urine and plasma

with the most commonly used name ribosylpyridin-4-one-3-carboxamide (PCNR) (21–23). Furthermore, the two anomers α-1 and β-1 of 4-pyridone-3-carboxamide ribonucleoside have been described (24), but the UV and NMR spectra of α anomer were different from the nucleoside we isolated and synthesized. In addition, isomers such as 2-pyridone-5-carboxamide-1-β-D-ribonucleoside were also described (23). Changes in plasma 4PYR concentration or urine excretion received special attention in the context of cancer treatment (23) and were found to predict early death in patients with active AIDS (22). Increase in plasma 4PYR could reflect cell damage, but on the other hand, it may cause elevation of 4PYTP in cells of the immune system and contributes to the loss of immune function. Interestingly, nicotinamide is known to delay disease progression in AIDS (25). Because high dose nicotinamide therapy emerges as an effective treatment in a variety of other pathological conditions such as diabetes, brain ischemia, or renal failure (26–29), it is important to take into account the effect of this treatment on

cellular 4PYTP concentration, as this may be related to therapeutic or toxic effects.

Formation of 4PYTP in the Erythrocytes and Its Effect on ATP Concentration—Identification of the structure and spectral properties allowed accurate quantitative analysis of the concentration of 4PYTP in erythrocytes in different clinical and experimental conditions. Concentrations of the 4PYTP, ATP, and related metabolites in erythrocyte extracts of healthy subjects and patients with chronic renal failure are presented in Table 1. These data indicate that the healthy human erythrocyte concentration of 4PYTP is significant, corresponding to ~2% of the ATP level. Massive accumulation of 4PYTP was observed in patients with renal failure, up to 10% of ATP on average or up to 30% in advanced chronic renal failure, which is consistent with our previous suggestions (7, 8). Fig. 2 presents the results of the incubation of healthy human erythrocytes with 4PY or with 4PYR. Incubation with 4PYR resulted in progressive accumulation of 4PYTP, whereas no change in 4PYTP was observed during incubation with 4PY or in controls (Fig. 2A). Formation of 4PYTP from 4PYR in the erythrocytes was dependent on adenosine kinase activity as it was inhibited by its specific inhibitor 5′-iodotubercidin (Fig. 2A).

These results together with data from our earlier study (7, 8) provide evidence for the pathway by which 4PYTP is formed in the erythrocytes. Because specific inhibitor totally blocked this process, nucleoside phosphorylation mediated by adenosine kinase is the most likely step leading to incorporation of 4PYR into the nucleotide pool. The source of 4PYR *in vivo* is uncertain, but as mentioned above, this compound has been identified in human plasma and urine previously, and our data shown in Table 2 confirm its presence in plasma in nanomolar concentrations in healthy subjects and demonstrate massive accumu-

TABLE 2
Plasma concentration and daily excretion of
***N*-methyl-4-pyridone-3-carboxamide (Met4PY) and**
4-pyridone-3-carboxamide-1-D-D-ribonucleoside (4PYR) in healthy subjects and patients with end-stage chronic renal failure
Values represent mean ($\pm$S.D.). *, $p < 0.02$; **, $p < 0.001$ *versus* healthy subjects.

	Plasma concentration	
	Met4PY	4PYR
	$\mu mol/liter$	
Healthy subjects ($n = 9$)	0.76 ± 0.35	0.013 ± 0.006
Patients with chronic renal failure ($n = 11$)	3.55 ± 2.29**	0.574 ± 0.622*
	Daily excretion	
	$\mu mol/24\ h$	
Healthy subjects ($n = 9$)	28.9 ± 22.0	26.7 ± 18.2

lation of 4PYR in patients with chronic renal failure. However, the exact source and pathway leading to the formation of 4PYR in plasma is less certain. We suggested earlier that the activity of aldehyde oxidase is essential for generation of the 4PYR, as no 4PYTP accumulation was observed in an erythrocyte extract of a patient with molybdenum cofactor deficiency who developed chronic renal failure (7, 8). Because aldehyde oxidase is not present in the erythrocytes (liver is the major site of its expression in humans (30)), formation of erythrocyte 4PYTP may depend on the provision of 4PYR into the circulation by the other organs, such as the liver. Most cells express a full spectrum of enzymes for upstream and downstream metabolism of nicotinamide compounds (31), and the exact nicotinamide-containing substrate that is oxidized remains to be identified. This question has been discussed broadly in our follow-up
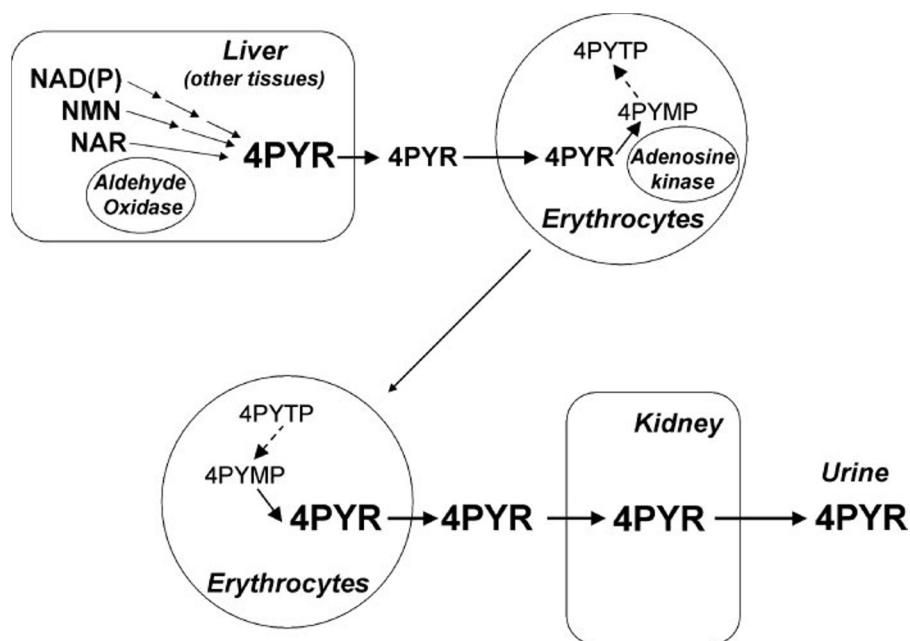
FIGURE 3. **Hypothetical pathway leading to formation of erythrocyte 4PYTP and urine excretion of 4PYR in humans.** 4PYR is produced in liver and other tissues possibly involving aldehyde oxidase from NAD, NADP, or metabolites such as NMN, or nicotinamide riboside (*NAR*). 4PYR is then released, enters the erythrocytes and is phosphorylated via adenosine kinase to 4PYMP and, subsequently, to 4PYTP. This process could then be reversed during passage of the erythrocytes through the kidney, resulting in increased local concentration of 4PYR and its enhanced urine excretion.

study in children with chronic renal failure[3] and considered by other authors (32).

Significance of 4PYTP and Its Precursors—Our discovery of 4PYTP in human cells has several potential implications. The presence of this compound in the erythrocytes of healthy subjects at significant concentrations indicates that this is part of a normal physiological mechanism. 4PYTP may be necessary for specific metabolic processes. However, our data indicating that 4PYR phosphorylation into the monophosphate (4PYMP) occurs much faster than its subsequent metabolism into 4PYTP (Fig. 2) seems to contradict this possibility. This rapid nucleoside phosphorylation and slow further metabolism within the erythrocytes suggests that this process is designed to remove 4PYR from the circulation and not to make 4PYTP. 4PYR could be toxic for nucleated cells by interference with ATP metabolism, by disruption of RNA or DNA synthesis, either directly or after phosphorylation, or through the potential for making an oxidized NAD by analogy with tyrazofurin and benzamide riboside. One evidence for potential toxicity is shown in our Fig. 2*B*, demonstrating decrease in erythrocyte ATP concentration during incubation with 4PYR. Although this occurred at relatively high concentrations, it may occur *in vivo* during prolonged exposures at lower levels or in specific cell types. Trapping of 4PYR in phosphorylated form within erythrocytes could prevent this potentially deleterious process.

Our results demonstrated massive accumulation of 4PYR in the plasma of patients with chronic renal failure (Table 2). This could be a major factor that contributes to the accumulation of 4PYTP in the erythrocytes in patients with chronic renal failure. However, we made several additional important observations. The relative increase in plasma 4PYR in subjects with chronic renal failure by far exceeded that of Met4PY (Table 2) or any other known metabolite, including creatinine. We noted a >50-fold increase in plasma concentration of 4PYR comparing our group of patients with advanced renal failure to the controls, whereas increase in Met4PY was <10-fold, similar to the relative increase in plasma creatinine in these patients (data not shown). Another important observation was a disproportionately high excretion of 4PYR in urine, compared with its plasma concentration in healthy subjects. Our measurements of both the plasma concentration of 4PYR and its urinary excretion are close to earlier estimates of these values (21–24). Although plasma levels and urine excretion of 4PYR in our study were not performed in the same subjects to allow exact renal clearance calculations, even estimates indicate that this value is almost one order of magnitude greater than in the case of other nicotinamide metabolites or nucleosides. Renal clearance was found to be close to creatinine clearance in this study for Met4PY (data from Table 2), in our previous report for Met2PY (10), and in studies of other authors for pseudouridine (33). Such a massive accumulation of 4PYTP in the erythrocytes and 4PYR in the plasma of patients with chronic renal failure to levels that by far exceed what could be expected from reduced renal filtration suggests that there is an additional mechanism that enhances 4PYR excretion in healthy people. We could

speculate that, during passage of erythrocytes through the kidney, there is a reverse process in which 4PYTP is broken down to 4PYMP and further to 4PYR. High local concentration of 4PYR would ensure its effective excretion (Fig. 3). Such a mechanism could explain the disproportionately high excretion of 4PYR in urine in contrast to its extremely low plasma concentration in healthy adults. We could not exclude an active kidney excretion mechanism for 4PYR, but such a process has never been described for any nucleoside. An important practical aspect of such significant changes in plasma 4PYR in renal failure is that this measurement could become very sensitive and an early marker of renal dysfunction. Although this hypothesis still needs further experimental evidence, we propose that formation of 4PYTP in human erythrocytes is an element of a novel excretion pathway for oxidized nicotinamide metabolites.

REFERENCES

1. Simmonds, H. A., Duley, J. A., Fairbanks, L. D., and McBride, M. B. (1997) *J. Inherit. Metab. Dis.* **20,** 214–226
2. Smolenski, R. T., and Yacoub, M. H. (1996) in *Purines and Myocardial Protection* (Abd-Elfattah, A. S. A., and Wechsler, A. S., eds) pp. 56–80, Kluwer Academic Publishers, Norwell, MA
3. Fairbanks, L. D., Bofill, M., Ruckemann, K., and Simmonds, H. A. (1995) *J. Biol. Chem.* **270,** 29682–29689
4. Ruckemann, K., Fairbanks, L. D., Carrey, E. A., Hawrylowicz, C. M., Richards, D. F., Kirschbaum, B., and Simmonds, H. A. (1998) *J. Biol. Chem.* **273,** 21682–21691
5. Berman, P. A., Black, D. A., Human, L., and Harley, E. H. (1988) *J. Clin. Investig.* **82,** 980–986
6. Berman, P. A., and Human, L. (1990) *J. Biol. Chem.* **265,** 6562–6568
7. Carrey, E. A., Smolenski, R. T., Edbury, S. M., Laurence, A., Marinaki, A. M., Duley, J. A., Zhu, L. M., Goldsmith, D. J., and Simmonds, H. A. (2004) *Nucleosides Nucleotides Nucleic Acids* **23,** 1135–1139
8. Carrey, E. A., Smolenski, R. T., Edbury, S. M., Laurence, A., Marinaki, A. M., Duley, J. A., Zhu, L., Goldsmith, D. J., and Simmonds, H. A. (2003) *Clin. Chim. Acta* **335,** 117–129
9. Simmonds, H. A., Duley, J. A., and Davies, P. M. (1991) in *Techniques in Diagnostic Human Biochemical Genetics: A Laboratory Manual* (Hommes, F., ed) pp. 397–424, Wiley-Liss, New York
10. Slominska, E. M., Rutkowski, P., Smolenski, R. T., Szutowicz, A., Rutkowski, B., and Swierczynski, J. (2004) *Mol. Cell. Biochem.* **267,** 25–30
11. Jenner, A., England, T. G., Aruoma, O. I., and Halliwell, B. (1998) *Biochem. J.* **331,** 365–369
12. Bernofsky, C. (1979) *Anal. Biochem.* **96,** 189–200
13. Vorbruggen, H., Krolikiewicz, K., and Bennua, B. (1981) *Chem. Ber.* **114,** 1234–1255
14. Meigh, J.-P., Alvarez, M., and Joule, J. A. (2001) *J. Chem. Soc. Perkin. Transl.* 2012–2021
15. Meyer, H., and Graf, R. (1928) *Chem. Ber.* **61,** 2202–2215
16. Talik, T., and Plazek, E. (1961) *Rocz. Chem.* **35,** 463–473
17. Marlewski, M., Smolenski, R. T., Szolkiewicz, M., Aleksandrowicz, Z., Rutkowski, B., and Swierczynski, J. (2000) *Nephron* **86,** 281–286
18. Abelson, D., and Boyle, A. (1963) *Nature* **197,** 460–462
19. Abelson, D., Boyle, A., and Seligson, H. (1963) *J. Biol. Chem.* **238,** 717–718
20. Chang, M. L., and Johnson, B. C. (1959) *J. Biol. Chem.* **234,** 1817–1821
21. Dutta, S. P., Crain, P. F., McCloskey, J. A., and Chheda, G. B. (1979) *Life Sci.* **24,** 1381–1388
22. Intrieri, M., Calcagno, G., Oriani, G., Pane, F., Zarrilli, F., Cataldo, P. T., Foggia, M., Piazza, M., Salvatore, F., and Sacchetti, L. (1996) *J. Infect. Dis.* **174,** 199–203
23. Schram, K. H. (1998) *Mass Spectrom. Rev.* **17,** 131–251
24. Chheda, G. B., Patrzyc, H. B., Tworek, H. A., and Dutta, S. P. (1995) *Nucleosides Nucleotides* **14,** 1519–1537
25. Murray, M. F. (2003) *Clin. Infect. Dis.* **36,** 453–460

---

[3] Synesiou, E., Fairbanks, L. D., Simmonds, H. A., Slominska, E. M., Smolenski, R. T., and Carrey, E. A., submitted for publication.

26. Gale, E. A., Bingley, P. J., Emmett, C. L., and Collier, T. (2004) *Lancet* **363,** 925–931

27. Knip, M., Douek, I. F., Moore, W. P., Gillmor, H. A., McLean, A. E., Bingley, P. J., and Gale, E. A. (2000) *Diabetologia* **43,** 1337–1345

28. Maiese, K., and Chong, Z. Z. (2003) *Trends Pharmacol. Sci.* **24,** 228–232

29. Takahashi, Y., Tanaka, A., Nakamura, T., Fukuwatari, T., Shibata, K., Shimada, N., Ebihara, I., and Koide, H. (2004) *Kidney Int.* **65,** 1099–1104

30. Moriwaki, Y., Yamamoto, T., and Higashino, K. (1997) *Histol. Histopathol.* **12,** 513–524

31. Bieganowski, P., and Brenner, C. (2004) *Cell* **117,** 495–502

32. Mrochek, J. E., Jolley, R. L., Young, D. S., and Turner, W. J. (1976) *Clin. Chem.* **22,** 1821–1827

33. Motyl, T., Traczyk, Z., Ciesluk, S., Daniewska-Michalska, D., Kukulska, W., Kaluzny, Z., Podgurniak, M., Orzechowski, A., and Debski, B. (1993) *Eur. J. Clin. Chem. Clin. Biochem.* **31,** 765–771