Hereditary Hemolytic Anemia with Human Erythrocyte Pyrimidine 5′-Nucleotidase Deficiency

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ABSTRACT A severe deficiency of a red cell pyrimidine 5′-nucleotidase was found to be associated with hereditary hemolytic anemia in four members of three kindreds. The syndrome was characterized by marked increases above normal in red cell basophilic stippling, total nucleotides, and GSH and by a fairly severe deficiency of ribosephosphate pyrophosphokinase (EC 2.7.-6.1.). Patient erythrocytes uniquely contained large amounts of pyrimidine 5′-ribonucleotides. In earlier studies, these were erroneously considered to be adenosine phosphates, since all previous investigations of the nucleotides of human red cells and reticulocytes have shown 97% or more to contain adenine. Total nucleotides in patient cells were present in amounts 3–6 times greater than normal, and approximately 80% contained pyrimidine. The ultraviolet spectral curves of deproteinized red cell extracts exhibited a shift in maximum absorbance from the usual 256–257 nm to approximately 266–270 nm, and absorbance at 250, 270, 280, and 290 nm, expressed as a ratio of that at 260 nm, differed greatly from normal. The spectral characteristics of extracts provide the basis of a readily performed screening procedure, which does not require enzyme assay. The nucleotidase activity in deficient red cells assayed less than 14%, and usually less than 10%, of normal and much less in terms of reticulocyte-rich blood, where it was consistently found to be increased. The enzyme has a pH optimum of 7.5–8.0, is inhibited by EDTA, and does not utilize purine 5′-ribonucleotides or β-glycerophosphate as substrates. While comparatively few family members have been available thus far for study, initial data are compatible with an autosomal, recessive mode of transmission of the deficiency. The pyrimidine 5′-ribonucleotides are presumably derived from RNA degradation and, not being diffusible, accumulate when the enzyme catalyzing their dephosphorylation is deficient. It is postulated that the prominent basophilic stippling results from retarded ribosomal RNA degradation secondary to accumulation of degradation products, namely pyrimidine 5′-ribonucleotides. Ribosephosphate pyrophosphokinase deficiency is considered to be an epiphenomenon. The mechanism responsible for increased red cell GSH is unknown.

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INTRODUCTION

In 1972–73, this laboratory reported four subjects with hereditary nonspherocytic hemolytic anemia associated with increased red cell adenine nucleotides GSH, and basophilic stippling, and a fairly severe, though partial, deficiency of ribosephosphate pyrophosphokinase (RPK,[1] 5-phosphoribosyl-1-pyrophosphate synthetase, EC 2.7.-6.1.) (1, 2). In these studies, AMP, ADP, and ATP were measured by a conventional enzymatic procedure (3). The assay had been considered reliable, since normally 97% or more of the nucleotides of both human red cells and reticulocytes are adenosine phosphates (3–11). Nucleotides containing guanosine may range in amount from less than 1 to 3%, and pyrimidine-contain-

[1] *Abbreviations used in this paper:* AK, adenylate kinase; EU, enzyme units; LDH, lactate dehydrogenase; NDPK, nucleoside diphosphate kinase; NMPK, nucleoside monophosphate kinase; PCA, perchloric acid; PEP, P-enolpyruvate; PGK, phosphoglycerate kinase; PK, pyruvate kinase; PRPP, 5-phosphoribosyl-1-pyrophosphate; RBC, erythrocytes; RPK, ribosephosphate pyrophosphokinase; TCA, trichloroacetic acid; TEA, tetraethylammonium.

ing nucleotides are present in negligibly small amounts. In the assay procedures, however, nonadenosine nucleotides would react but relatively slowly. In recent investigations of a new patient with the hemolytic syndrome described above, we became aware of the probable presence of significant concentrations of such "slow-reacting" nucleotides in the deproteinized blood extracts. Reexamination of the recorded assays of the earlier patients revealed identical findings, which were absent in hemolysate extracts of all other hemolytic anemias studied in this laboratory over a period of several years. This report documents subsequent investigations indicating (*a*) the red cells of subjects with this hereditary hemolytic syndrome uniquely contain very large amounts of cytidine and uridine nucleotides, (*b*) the latter are *partially* reflected in the enzymatic assays for nucleotides and were the basis for our initial misinterpretation that adenine nucleotides were increased in patient erythrocytes (1, 2), and (*c*) the genetically determined lesion is a severe deficiency of a pyrimidine-specific 5′-nucleotidase, an enzyme readily demonstrable in normal erythrocytes, that exhibits increased activity in reticulocytes. As will be discussed, RPK deficiency is believed to be an epiphenomenon, secondary to the relatively enormous concentrations of pyrimidine nucleotides in circulating erythrocytes and presumably in marrow nucleated precursor cells.

METHODS

Case material

Patient R. has not been previously reported. This 24-yr-old woman of Jewish ancestry was first seen at the Albert Einstein College of Medicine in 1972. There was no history of neonatal jaundice, but active hemolytic anemia was diagnosed in childhood. She subsequently required cholecystectomy for obstructive jaundice, and later splenectomy. However, chronic hemolysis has continued unabated with marked reticulocytosis (20–45%), and Hb values usually ranged from 8 to 10 g/100 ml of blood. Except for a grade II/VI systolic ejection murmur, a palpable liver edge, and jaundice, physical examination was normal. The Hb was electrophoretically normal, and red cells had normal resistance to osmotic lysis. The serum haptoglobin was reduced in amount. Serum bilirubin was 7.9 mg/100 ml of blood, with 7.0 mg reacting indirectly. At the time of initial study in the Los Angeles laboratory, erythrocyte packed cell volume was 29%, Hb 8.8 g/100 ml of blood, RBC 2.35 million/μl, and reticulocyte count 25%. The leukocyte count was 5,800/μl. The patient's parents are first cousins. Both parents and a sister were clinically well and hematologically normal.

Patients Tr. (1), Vi. T., and Va. T. (2) have been previously reported in detail and were restudied as part of the present investigation.

Materials

All purine and pyrimidine nucleotides, NAD and NADH, P-enolpyruvate (PEP) and the enzymes nucleoside monophosphate kinase (NMPK) and bacterial (*E. coli*) alkaline phosphatase were obtained from Sigma Chemical Co., St. Louis, Mo. Lactate dehydrogenase (LDH), phosphoglycerate kinase (PGK), and 3-phosphoglycerate were purchased from Calbiochem, LaJolla, Calif. Pyruvate kinase (PK), adenylate kinase (myokinase, AK), glyceraldehyde-3-phosphate dehydrogenase, and nucleoside diphosphate kinase (NDPK) were obtained from Boehringer Mannheim Corp., New York. Plasmagel is manufactured by Roger Bellon Laboratories, Neville, France. Each 100 ml contains fluid gelatin 3 g, NaCl 0.7 g, $CaCl_2 \cdot 2H_20$ 0.2 g. 4 mg heparin was added per 100 ml.

Experiments

General. Blood collected in heparin or EDTA was transported to the Los Angeles laboratory in ice. Leukocytes were separated with Plasmagel as the erythrocyte-sedimenting agent. The red cells were washed thrice in saline and adjusted to a concentration of about $3 \times 10^6/\mu$l, the erythrocytes and contaminating white cells enumerated, and the reticulocyte percentage was determined. The suspensions were frozen and thawed three times if hemolysates were required and were deproteinized and neutralized before analysis for content of nucleotides or glycolytic metabolites. GSH was measured as described by Beutler, Duron, and Kelly (12).

Enzyme assays. All the enzymatic activities of the Embden-Meyerhof pathway, the two-pentose shunt dehydrogenases, glutathione peroxidase, and glyoxalase I and II were assayed in hemolysates as previously described (13–18). The activities of enzymes of the distal pentose shunt, including those of transketolase and transaldolase, were screened in terms of the conversion of ribose-5-phosphate to fructose-6-phosphate. RPK (PRPP synthetase) and adenine phosphoribosyltransferase (EC 2.4.2.7) activities were assayed by methods described elsewhere (19). AK activity was measured essentially as described by Haslam and Mills (20) and ATPase was assayed according to Brewer (21).

NMPK (EC 2.7.4.4.) activity was assayed at 37°C in a system containing the following reagents in micromoles per 3 ml final volume: tetraethyl ammonium (TEA) HCl buffer, pH 7.5, 25, KCl 225, $MgSO_4$ 24, NADH 0.84, PEP 9, ATP 6, UMP 0.9, LDH 18 enzyme units (EU), PK 3 EU, and hemolysate equivalent to approximately 3×10^7 erythrocytes (RBC). Two blanks, one without UMP and one without ATP, were employed. The reaction was initiated by addition of hemolysate and monitored at 340 nm with a Gilford Multi-Sample recorder (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). NMPK catalyzes the reaction ATP + UMP (or CMP) $\rightleftarrows$ ADP + UDP (or CDP). Both CDP and UDP can serve as cofactors in the PK reaction, though UDP is appreciably more active.

NDPK (EC 2.7.4.6.) catalyzes the reaction ATP + CDP (or UDP) $\rightleftarrows$ ADP + CTP (or UTP). The assay screening for NDPK contained the following reagents in micromoles per 2.0 ml final volume: ATP and CDP each 2.5, $MgCl_2$ 50, TEA-HCl buffer, pH 7.5, 200, and hemolysate equivalent to about 6×10^8 RBC. After incubation for 30 min at 37°C, the reaction was terminated by addition of 1 ml of 1.2 N perchloric acid (PCA), and the extracts were neutralized with 5.0 N K_2CO_3, centrifuged, and assayed for ATP. Blanks omitting (*a*) CDP, (*b*) ATP, or (*c*) hemolysate until after PCA addition were included simultaneously. After appropriate subtraction of blank values,

the assay then provided an estimate of the CDP-dependent disappearance of ATP. While effective for screening purposes, the procedure is not quantitative, due to presence of AK in hemolysates.

Pyrimidine 5'-nucleotidase. In the assay procedure finally selected, hemolysates were dialyzed overnight (sometimes with one change) against about 200 vol of saline containing in final concentration 0.01 M Tris-HCl, pH 8.0, and 0.01 M $MgCl_2$. This removed the majority of P_i as well as dialyzable sugar phosphates and nucleotides, permitting low blank values in the final analyses. The procedure was selected only after comparisons between the activities of dialyzed and fresh hemolysates indicated no loss of nucleotidase activity. After dialysis, the Hb content of each sample was measured. The pyrimidine 5'-nucleotidase assay contained the following in micromoles per 1.75 ml final volume: Tris-HCl buffer, pH 8.0, 45, $MgCl_2$ 15, either CMP or UMP 4.0 (final concentration $= 2.3 \times 10^{-3}$ M), and 0.5 ml dialysate. The reaction was terminated after 2 h at 37°C by addition of 1 ml of 20% trichloroacetic acid (TCA). A "time-zero" blank contained TCA added before incubation. A 2-h blank was incubated without nucleotides, which were added after TCA at the end of the incubation period. The deproteinized extracts were filtered, and aliquots employed to measure liberation of P_i from substrate by the method of Fiske and SubbaRow (*22*). Both CMP and UMP were usually employed as substrates, and activity was expressed as micromoles of P_i released per gram Hb per hour. In preliminary studies, the same assay was employed but with fresh, undialyzed hemolysates, and with acetate-barbital, Tris, and glycylglycine buffers at pH's ranging from 5.0 to 9.5. Nucleotidase activity was also determined at pH 5.0–9.5 with AMP, IMP, GMP, XMP, and β-glycerophosphate as substrates. In additional experiments at pH 5.5, α-glycerophosphate, a much better substrate than the β isomer for human erythrocyte acid phosphatase (*23, 24*), was employed.

The enzymatic measurement of nucleotides. Deproteinized extracts of either whole blood or saline suspensions of known numbers of RBC were prepared by adding 2 vol of 0.6 N PCA to 1 vol of blood or cells or, in the case of some suspensions, 1 vol of 1.2 N PCA to 1 vol of cells. The extracts were centrifuged, the supernates were neutralized to about pH 7.5 with 5 N K_2CO_3, and the precipitated perchlorate was removed. In the enzymatic assays of nucleotides (*3*), such extracts normally supply the only source of AMP, ADP, and ATP in the nucleotide-dependent AK, PK, and reverse PGK reactions, respectively. All reactions are readily linked to indicator systems in which the conversion of NADH to NAD is measured as diminished absorbance (A) at 340 nm (*3*). Each assay is complete when the necessary nucleotide is completely utilized. The reactions with adenine nucleotides are very rapid, but to standardize conveniently and assure certain completion of the reaction, we normally record the results in blank and test systems after 5–10 min at 37°C. Since both normal and reticulocyte-rich populations of human erythrocytes contain negligible amounts of other nucleotides (*3–11*), the results are customarily interpreted as reflecting only the concentrations of adenosine phosphates (*3*). Nonetheless, PK reacts, albeit at a slower rate, with UDP, CDP, IDP, XDP, and GDP (*25*). Moreover, the commercially obtained PGK also slowly utilizes UTP and CTP as well as the purine triphosphates GTP, ITP, and XTP. The AK enzyme employed reacts not only with AMP and ADP, but also slowly with CMP and CDP as well, but not with other nucleotides under conditions we have used.

The measurement of CTP and UTP in the presence of ATP. Nucleoside mono- and diphosphates can be largely converted to triphosphates by incubating neutralized extracts with highly active PK, AK, and NMPK. PK converts nucleoside diphosphates to the triphosphates. In the presence of ATP, AK and NMPK convert AMP, CMP, and UMP to diphosphates, which are subsequently further converted to triphosphates by PK. In a typical experiment, 9 EU of PK, 22 EU of AK, and 0.03 EU of NMPK were added to 1.0 ml of neutralized extract containing 2.5 μmol TEA-HCl buffer, pH 7.5, and 3.0 μmol of PEP and incubated 90 min at 37°C. The reaction was terminated with 0.15 ml of 60% TCA, the mixture was centrifuged, and the supernate was extracted five times with ether to remove TCA. In extracts containing low concentrations of adenine nucleotides, the addition to the incubation of ATP at a final concentration of 10^{-4} M facilitates conversion to triphosphates. A blank containing H_2O in place of extract must be incubated simultaneously. With most nucleotides in the triphosphate form, advantage may be taken of the following reactions.

(1) $\text{ATP} + \text{3-PGA} \xrightarrow{\text{PGK}} \text{1,3-DPG} + \text{ADP}$ Very rapid

$\text{1,3-DPG} + \text{NADH} \xrightarrow{\text{G-3-PD}} \text{3-PG} + \text{NAD}$

(2) $\text{CTP (or UTP)} + \text{3-PGA} \xrightarrow{\text{PGK}}$

$\text{1,3-DPG} + \text{CDP (or UDP)}$ Slow

$\text{1,3-DPG} + \text{NADH} \xrightarrow{\text{G-3-PD}} \text{3-PG} + \text{NAD}$

(3) $\text{CTP (or UTP)} + \text{ADP} \xrightarrow{\text{NDPK}}$

$\text{ATP} + \text{CDP (or UDP)}$ Relatively rapid

By recording reaction 1 within 2 min of its initiation, ATP conversion to ADP is more than 95% complete. The 2-min reading measures mainly ATP together with a very minimal contribution from CTP and a small contribution from the somewhat more reactive UTP, if pyrimidine nucleotides are present. When PGK, NDPK, ATP, and pyrimidine nucleoside triphosphates are present, ADP formed in reaction 1 is rapidly reconverted to ATP by reaction 3, and ultimately all triphosphates are converted to diphosphates. Diminution in absorbance in an assay system initiated by addition of PGK + NDPK minus the diminution of absorbance in a system initiated with PGK alone and recorded within 2 min of incubation, then, provides a measures of the amounts of CTP and UTP present. While the method readily detects the presence of pyrimidine nucleoside triphosphates, it is not quantitative, due to some contamination of commercially obtained NDPK with AK. It is most nearly so when the ratio of pyrimidine to adenine nucleoside triphosphates is large, as will be shown to be the case in pyrimidine 5'-nucleotidase deficiency, and where CTP is the major pyrimidine-containing nucleotide present. CTP is much less reactive with PGK than is UTP, and its effects are nearly negligible when results are recorded within 2 min of initiating the assay reaction.

The measurement of UDP and CDP in the presence of ADP. When the nucleoside diphosphates in extracts are assayed as cofactors of PK and the reaction allowed to go to completion (about 30 min), the total of ADP, UDP, CDP, and other nucleoside diphosphates is measured. The

assay mixture may then be deproteinized (with PCA), reneutralized, and ATP measured with the PGK assay with results recorded 2 min after initiation of the reaction. If the ATP content of the extract before conversion of diphosphates, the total amount of diphosphates registering in the PK reaction, and the *final* amount of ATP present *after* diphosphate conversion to triphosphate are known, the ratio of ATP-forming and non–ATP-forming nucleoside diphosphates present in the original extract can be computed readily on a nearly quantitative basis.

Extracts prepared for spectral analysis. Either whole blood or washed red cell suspensions having a known Hb content and containing known numbers of erythrocytes were deproteinized with 1 vol of 1.2 N or 2 vol of 0.6 N PCA, neutralized to pH 7.4 with K_2CO_3, and separated from the precipitates. Alternatively, deproteinization was accomplished by adding equal volumes of 10% TCA, centrifuging, removing the TCA with repeated ether extractions, and adjusting the pH to about 7.0 with 5 N K_2CO_3. Whole blood extracts were prepared immediately after withdrawal, and those of cell suspensions in some instances were prepared at the laboratory of origin and in some at the Los Angeles laboratory from specimens shipped in ice. While whole blood extracts and, to a lesser extent, extracts of washed RBC contain some nonnucleotide ultraviolet-absorbable material, this is small and spectral data reflect predominantly the red cell nucleotides. In the case of some extracts, nucleotides were adsorbed on activated charcoal (Norit "A" 10–100 mg/ml in H_2O, American Norit Co., Jacksonville, Fla.), a procedure which does not remove sugar phosphates and is relatively specific for removing nucleotides. 5 ml of extract were treated with 1–5 ml of activated charcoal, centrifuged, and washed thrice with distilled H_2O. After centrifuging, the precipitated charcoal was eluted with 6 ml of a solution consisting of 10.5 ml 95% ethanol, 9.5 ml H_2O, and 1.0 ml concentrated NH_4OH. The ultraviolet absorbance of extracts was measured at pH 2, 7, and 11 at wavelengths from 230 to 310 nm in cuvettes with a 1-cm light path. Extract in amounts from 0.05 to 0.20 ml was added to media of appropriate pH to a final volume of 1 ml. The spectral characteristics of the Norit eluates were essentially identical to those of the original extracts.

Chromatographic and electrophoretic studies on extracts. Some investigations employed native extracts, and others employed extracts in which the nucleotides were enzymatically dephosphorylated to nucleosides. The most successful procedure consisted of incubation of ether-extracted TCA extracts for 2–3 h with nucleotide pyrophosphatase (EC 3.6.1.9.) and up to 90 EU of bacterial alkaline phosphatase (*E. coli*), (EC 3.1.3.1.) at pH 8.5. Nucleoside di- and triphosphates are dephosphorylated by both enzymes, and nucleoside monophosphates are dephosphorylated by the alkaline phosphatase. After incubation, the extracts were again deproteinized with small amounts of 60% TCA, and the latter was removed by six extractions with ether.

The nucleosides were separated by two-dimensional paper chromatography, by the basic technique described previously (26, 27). An amount of extract equivalent to 2.2 *A* U at 260 nm was applied as a small spot on Whatman No. 3 MM filter paper that had been pre-washed by descending chromatography with deionized water for 4 days. For the first separation, a mixture of *tert*-butyl alcohol, methyl-ethyl ketone, water, and ammonium hydroxide (40:30:20:10) was used. The solvent for separation in the second direction consisted of a mixture of isobutyric acid and 1 M ammonium hydroxide (32:19). The finished chromatogram was allowed to stand overnight in a fume hood and subsequently washed twice by chromatography in absolute ethanol in the same direction as that used for the isobutyric solvent to remove excess isobutyrate and some interfering background absorbance. The ultraviolet-absorbing spots were outlined over a shortwave ultraviolet lamp, cut out, and the nucleoside components concentrated to a tip with a 2-day period of elution (28). The tips were subsequently excised and eluted in appropriate amounts of 0.01 N HCl. Spectral curves were obtained with a recording spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif., model DK-2), over a wavelength range of 220–340 nm. The concentration of each nucleoside was calculated with the appropriate molar extinction coefficient at the wavelength of maximum absorbance.

The nucleotides (3.0–3.5 *A* U at 260 nm) were separated on prewashed Whatman No. 3 MM filter paper, by a combination of electrophoresis (Durrum Cell, Durrum Instrument Corp., Palo Alto, Calif., 350 V, 3.5 h; buffer: 7.4 ml acetic acid and 3.68 ml formic acid/liter) and conventional paper chromatography in the same isobutyric-ammonium hydroxide solvent used for nucleoside separation. Excess isobutyrate and some interfering background absorbance were removed by chromatography in absolute ethanol, and nucleotides were subsequently concentrated, eluted, and estimated by the same methodology as described for nucleosides. On some chromatograms, it was recognized from spectral curves that CMP and AMP, and CDP and IMP, were not completely separated, and concentrations of the individual components in these binary mixtures were calculated by two simultaneous equations (29). There was some breakdown of the nucleoside di- and triphosphates to the monophosphate level in the acidic solvent used for electrophoresis, so the overall base composition of nucleotide mixtures was calculated without distinguishing between different levels of phosphorylation.

The chromatographic recoveries were rather low (comparing the total $A_{260\ nm}$ U applied to a given chromatogram with a summation of the $A_{260\ nm}$ U measured in the eluants of the individual spots of that chromatogram), so direct spectrophotometric analyses were also performed. An aliquot of each of the erythrocyte extracts used for chromatographic analyses was also analyzed directly by ultraviolet spectrophotometry. A pair of spectral absorbance curves was obtained for each extract, first in 1 ml of 0.01 N HCl and again after addition of 4 μl of 6 N NaOH to the acidic mixture in the cuvette. For each such pair of curves, the position of absorbance maxima, isosbestic points, ratios of maximal absorbances at acid and alkaline pH's, and the absorbances at 240 nm, 250 nm, 270 nm, 280 nm, and 290 nm, calculated as ratios relative to the absorbance at 260 nm, were tabulated. Each set of curves was also replotted to give an absorbance of 1.0 at 270 nm at pH 2.0. This permitted comparison by direct superimposition of the curves over standard curves similarly prepared from known mixtures of nucleosides, and the best fit between a curve for a given patient and one from a series of artificial mixtures could be determined visually.

Nucleoside monophosphates chromatographically isolated from the erythrocyte extracts were subjected to chromatography in an acetone, boric acid, and ammonium hydroxide solvent (32 ml acetone, 18 ml boric acid reagent; the latter consists of 990 ml water, 60 ml ammonium hydroxide, and 16 g boric acid). Standards of 3'- and 5'-ribonucleotides were included on the same chromatogram for direct comparison.

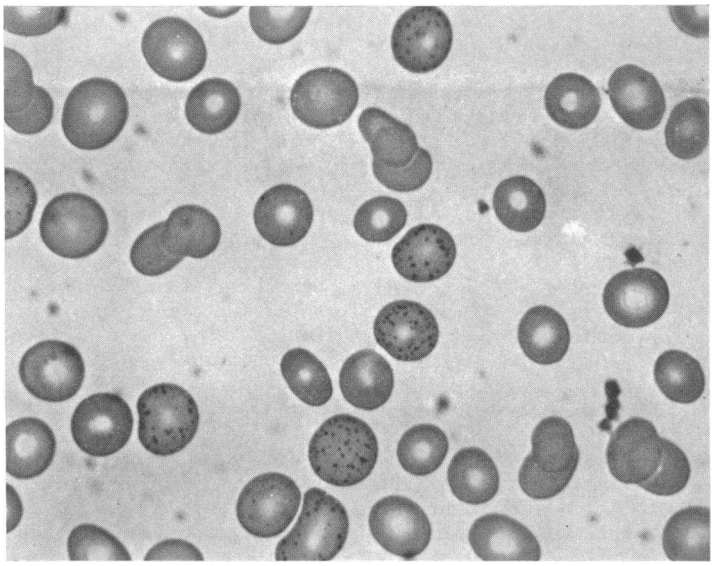

FIGURE 1 Photomicrograph of Wright's-stained blood film (patient Vi. T.) showing prominent basophilic stippling of red cells.

The 5'-ribonucleotides possess a *cis*-glycol group in the ribofuranosyl moiety that can complex with borate, and the chromatographic mobility of the complex is markedly reduced.

RESULTS

General. In patient R., the hematological and biochemical findings coincided with those observed in patients previously reported (1, 2). Hemolysis was marked, and increased basophilic stippling was a very prominent feature of the Wright's-stained blood film (Fig. 1). Acanthocyte-like red cells were common, and some densely staining, spiculated spherocytes were also present. Erythrocyte GSH was greatly increased, and RPK activity was substantially below normal. A comparison of the erythrocyte GSH and RPK activity is presented in Table I for the four subjects in whom erythrocyte pyrimidine 5'-nucleotidase deficiency is documented in this report. In addition, assay for NMPK activity (see Methods) was performed on the cells of all patients and for NDPK on all except those of patient Tr. In every instance, both enzymes were normally active. All patients possess the enzymatic machinery to interconvert pyrimidine mono-, di-, and triphosphates.

TABLE I

GSH and RPK Activity of Red Cells of Patients with Pyrimidine 5'-Nucleotidase Deficiency

Subject	GSH	RPK
	μg/10¹⁰ erythrocytes	
Tr.	1,225	16.5
Vi. T.	1,150	14.2
Va. T.	976	11.3
R.	1,429–1,632	18.0
Normal controls (26)	739±111	30.5±3.8
"High-reticulocyte" controls (10)	715±150	51.6

RPK activity measures PRPP production as previously described (19). "High-reticulocyte" controls all had hemolytic syndromes with 3–15% reticulocytosis. Numbers of subjects in each control group are in parentheses.

Studies with authentic nucleotides. In assays of AMP, ADP, and ATP in hemolysate extracts, the amount of substrate utilized in the AK, PK, and PGK reactions bears a direct molar relationship to amount of nucleotide present. Although all previous investigations of human erythrocytes have indicated that more than 97% of the nucleotides contained adenine (3–11), the lack of absolute specificity of the enzymatic reactions employed permits detection of other purine and pyrimidine nucleotides, should these be present. Indeed, when nucleoside diphosphates were assayed enzymatically in patient red cell extracts, a slowly terminating reaction was observed that suggested the presence of nonadenosine diphosphates. Since review of assays performed over several years failed to disclose similar slow reactions in red cell extracts from any other subjects, it was deemed important to define the nucleotide specificities and reaction kinetics under conditions employed in our laboratory.

As expected, these investigations disclosed the apparent K_m of PK in the nucleoside diphosphate assay to be much higher for UDP and CDP than for ADP.

With attention given to rapid mixing and at a concentration of 2.6×10^{-4} M, the reaction with ADP was complete by the time of initial recording (within 1 min of PK addition). The time for half-completion of the reaction was about 1–2 min with UDP and 5 min with CDP. Full completion of the reaction required approximately 7 min with UDP and 25–30 min with CDP.

In the ATP assay mixture, the apparent K_m's for pyrimidine nucleotides were very high. At concentrations between 10^{-3} and 10^{-4} M, CTP reacted very slowly. UTP, while also slow, was 10 times more reactive. Increasing concentrations of either pyrimidine nucleotide from 1.6×10^{-4} to 10^{-3} M resulted in a sevenfold acceleration of the initial reaction rate. In the assays of ADP and ATP, the concomitant presence of pyrimidine nucleoside di- or triphosphates would clearly result in significant augmentation of values derived from recordings made 5–10 min after initiation of the reaction. Furthermore, their contribution would vary as residual concentrations of nucleotides diminished as the reaction progressed. Fig. 2 depicts reaction curves of ADP, CDP, and UDP in the nucleoside diphosphate assay and also shows the slowly terminating reaction evident with all cell extracts of patient R. Fig. 3 compares ATP, CTP, and UTP in our routine assay of ATP.

The measurement of pyrimidine nucleoside diphosphates in the presence of ADP. It is possible to measure UDP and CDP semiquantitatively in the presence of ADP by utilizing the technique described in detail under Methods. In essence, ADP, CDP, and UDP are measured as a totality and simultaneously converted to their respective triphosphates by the PK of the assay system. Both the amount of ATP present before the assay of the diphosphates and after conversion of the latter to triphosphates can be determined under conditions minimizing contributions of the slower-reacting CTP and UTP. If only adenosine di- and triphosphates were present, the ATP finally assayed should equal the sum of original ATP and that formed from ADP by the PK reaction. As expected, when this procedure was employed with extracts of normal red cells or of cell populations with reticulocytosis up to 30%, we found close agreement between the amount of triphosphates finally present and the sum of initial di- and triphosphates. In contrast, when artificial mixtures of ATP, ADP, CDP, and UDP were similarly assayed, the triphosphates finally measured were appropriately less than the sum of initial ATP plus diphosphates. The difference closely approximated the amounts of CDP and UDP in the artificial mixtures. In the red cell extracts of the patients reported here, non–ATP-forming nucleotides comprised 74% of the nucleoside diphosphates of patient R. and 70% of those of patient Va. T. Similar assays were not performed on the other two subjects.

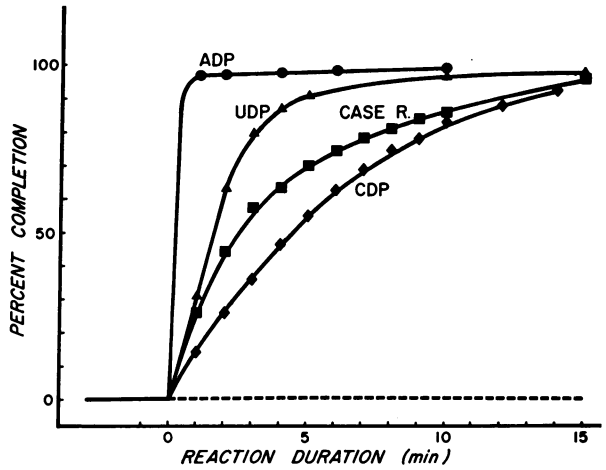

FIGURE 2 Comparison of activity of ADP, UDP, and CDP in the PK assay system. Also shown for comparison is the slow-terminating reaction curve of patient R. (0.1 ml neutralized, deproteinized extract). In experiments with nucleotide standards, final concentrations approximating 10^{-4} M were used.

However, in identical studies, with blood from both normal controls and from the mother of patients Vi. T. and Va. T., at least 95% of nucleoside diphosphates was ADP.

The measurement of pyrimidine nucleoside triphosphates in the presence of ATP. As indicated in Methods, it is also possible to convert nucleotides in deproteinized extracts largely to the triphosphate form. The amount of ATP alone and the sum of ATP, CTP, and

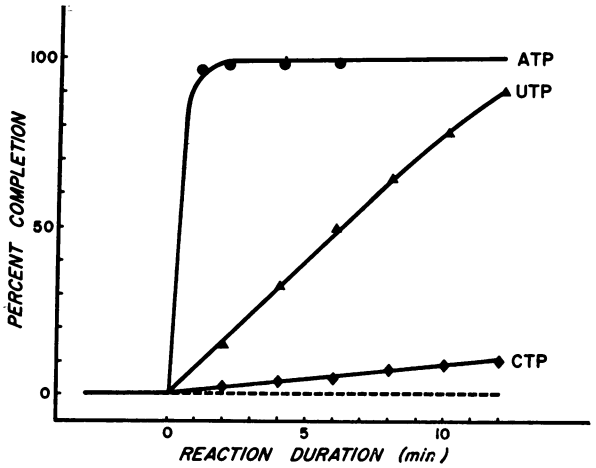

FIGURE 3 Comparison of activity of ATP, UTP, and CTP as nucleotide cofactors of the reverse PGK reaction. The assay mixture contained the following in μmol/1 ml final volume: TEA-HCl buffer, pH 7.4, 80, $MgCl_2$ 3.2, 3-phosphoglycerate 4.8, NADH 0.2, mercaptoethanol 70, ATP, UTP, or CTP 0.3, and glyceraldehyde-3-*P*-dehydrogenase 2.5 EU.

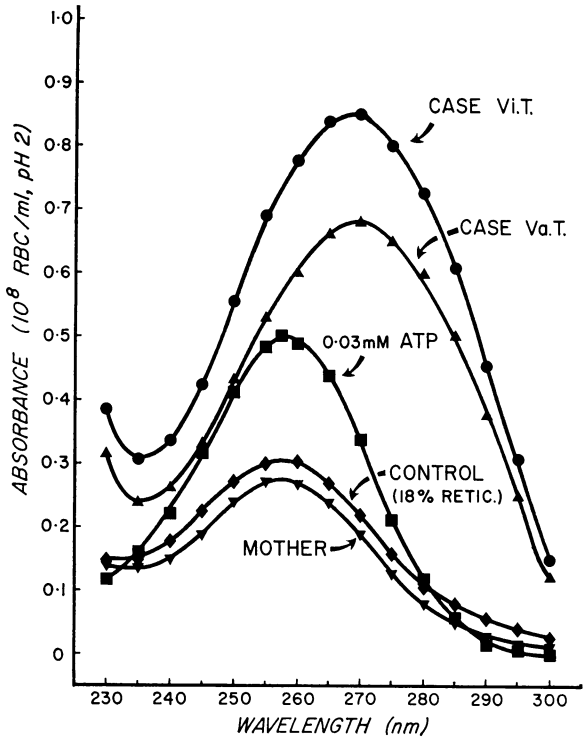

FIGURE 4 A comparison of spectral data on deproteinized red cell extracts of patients Vi. T. and Va. T., their mother, and a representative subject with sickle cell disease and reticulocytosis of 18%. For comparison, spectral data for an aqueous standard solution of ATP are also shown. Absorbances are adjusted to correspond to 10^8 erythrocytes extracted in each instance.

UTP can then be estimated independently, with the difference representing pyrimidine nucleoside triphosphates. In the presence of all three nucleotides, the reaction initiated by PGK alone and recorded after 2 min largely measures the amount of highly reactive ATP. If, in a separate assay, the reaction is initiated by both PGK and NDPK, the ADP formed will be continuously recycled to ATP and back until all pyrimidine triphosphates have been converted to diphosphates. With this procedure, pyrimidine-containing nucleotides were found to comprise approximately 70–80% of the total in patients Vi. T., Tr., and R. In a separate study, about 80% of the nucleoside triphosphates in an unmodified red cell extract of patient Tr. contained pyrimidines. The assay gives only approximate values since a small amount of contaminating AK in commercially obtained NDPK prevents precise measurements. The estimations are most accurate when the ratio of pyrimidine-containing nucleotides to ATP is high, as proved to be the case in subjects reported here. These approximations conformed closely to those obtained by spectral, chromatographic, and electrophoretic analysis.

The assays for total nucleotides also revealed the relatively enormous concentrations of these in patient cells in comparison to those of normal subjects. Since most extracts were prepared from washed red cell suspensions, and since some dephosphorylation of nucleoside di- and triphosphates occurs in extracts with the passage of time, the data do not permit assessment of ratios of mono-, di-, and triphosphates present in cells in vivo. As with adenine nucleotides, only a comparatively small percentage of the pyrimidine ribonucleotides were present in the monophosphate form, however.

Nature of nucleoside phosphates in patient red cells. In separate experiments, authentic adenosine 2′- and 3′-monophosphates were found to be totally unreactive as cofactors for either the AK or NMPK reactions. Cytidine 3′-monophosphate also did not serve as substrate in the NMPK reactions. The monophosphates of extracts of patient erythrocytes were fully active, supporting chromatographic data, to be discussed later, indicating they existed as the 5′-ribonucleotides.

Spectral analyses. Spectral data obtained on deproteinized, red cell extracts of patients and certain members of kindred T. are presented in Table II. Included for comparison are representative data for extracts prepared from a red cell population with marked reticulocytosis. All data are expressed in terms of 10^{10} erythrocytes, and were computed from the red cell num-

TABLE II

Total Nucleotides per 10^{10} RBC in Subjects with Pyrimidine 5′-Nucleotidase Deficiency, Certain Relatives, and a Representative Subject with Reticulocytosis (Based on Spectral Data of Deproteinized Erythrocyte Extracts)

	A U* per 10^{10} RBC	Total nucleotide‡
		μmol/10^{10} RBC
Patient R.	85	8.5
Patient Tr.	88	8.8
Patient Vi. T.	85	8.3
Patient Va. T.	68	6.0
Mother T.	27	1.6
Brother T.	27	1.8
Hb SS disease—patient M.	30	2.0

* At maximum absorbance (255 nm for mother and brother of patients T. and for subject M. with sickle cell disease and 18% reticulocytosis; 270 nm for patients).

‡ Based on average molar absorbancy of 15×10^3 for adenine-containing nucleotides at 255 nm and of 10×10^3 at 270 nm for patients' extracts. The latter value was calculated from molar absorbancy at 270 nm of a nucleotide mixture containing cytidine: uridine: adenosine in ratios of 5:3:2—a close approximation to actual ratios in patients' cell extracts as determined chromotographically.

bers present in the original cell suspensions from which extracts were made. It is immediately apparent that patient erythrocytes possess total nucleotides in amounts 3–6 times those of comparable numbers of normal red cells or of the cells of representative patient M. with sickle cell disease and 18% reticulocytosis.

Fig. 4 graphically depicts spectral data obtained at pH 2.0 with extracts of cells from patients Vi. T. and Va. T., their mother, and a representative extract prepared from reticulocyte-rich blood, compared with 0.03 mM ATP. Several points deserve emphasis. (*a*) All data pertaining to cell extracts are expressed as absorbance per 10^8 RBC to render them comparable from subject to subject. The markedly increased amounts of total nucleotides present in the cells of Vi. T. and Va. T. are readily appreciated. (*b*) The extract of cells from the patients' mother, who is clinically well and hematologically normal, and that from representative reticulocyte-rich blood have spectral characteristics nearly identical to the ATP reference solution. Similar findings have characterized all of a large number of extracts of normal blood studied, as well as those of more than 35 subjects with marked reticulocytosis of diverse etiologies. In every instance, maximal absorbance was observed between 255 and 260 nm. (*c*) In contrast, the absorption maxima exhibit a bathochromic shift to about 266–270 nm in all four patients. The ratios of absorbance at various wavelengths to that at 260 nm are highly abnormal, and will be discussed in conjunction with data obtained by chromatographic analyses. (*d*) Although not shown, additional spectral curves obtained at neutral and alkaline pH's suggested the presence of large amounts of cytidine nucleotides in the patient extracts. (*e*) Although not shown in Fig. 4, the spectral curves on eluates obtained after nucleotide absorption on activated charcoal were essentially identical to those observed with neutralized PCA or neutralized, ether-extracted TCA extracts.

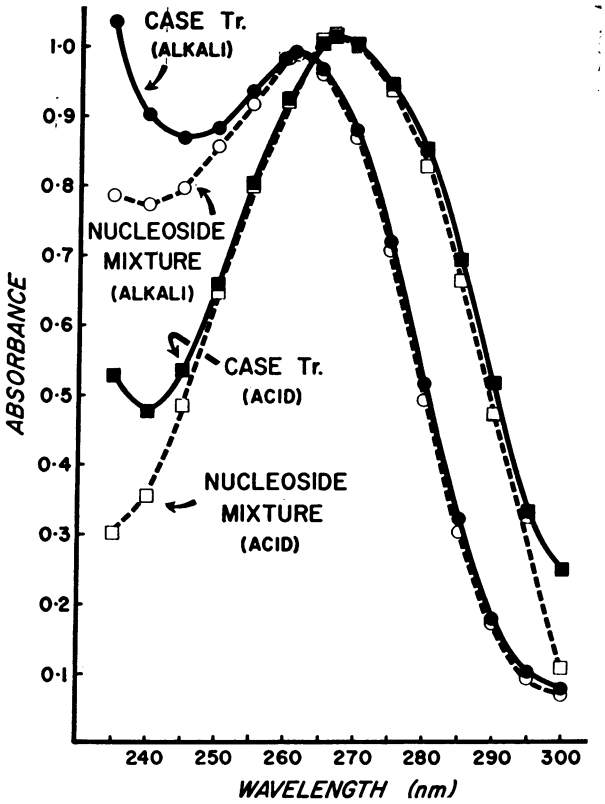

FIGURE 5 UV absorption spectral curves of red cell extract of patient Tr., together with curves for a mixture of pure nucleosides prepared to match exactly the chromatographically determined composition of that extract.

Chromatographic and electrophoretic data. The chromatographic recoveries ranged from 52 to 68%

TABLE III

Chromatographic Analyses (and Spectrophotometric Estimates) of the Purine and Pyrimidine Ribonucleosides or -Nucleotides in Extracts of RBC from Patients with Pyrimidine 5'-Nucleotidase Deficiency*

Patient	No. of chromatograms analyzed	Adenosine	Inosine	Uridine	Cytidine	Total pyrimidines
		%	%	%	%	%
R.	3-nucleosides					
	3-nucleotides	4 (4)	10 (7)	30 (32)	56 (57)	86 (89)
Tr.	2-nucleosides					
	3-nucleotides	13 (13)	4 (4)	32 (32)	51 (51)	83 (83)
Vi. T.	1-nucleotide	10 (10)	12 (10)	31 (31)	47 (49)	78 (80)
Va. T.	1-nucleotide	12 (14)	12 (11)	27 (26)	49 (49)	76 (75)

* Figures in parentheses indicate the composition of the known mixture of nucleosides yielding UV spectral curves most closely approximating those of the original TCA extract from each patient.

TABLE IV

UV-Spectral Data for Nucleotide-Containing Extracts of RBC's in Patients with Pyrimidine 5'-Nucleotidase Deficiency, Certain Relatives, Normal Controls, and Subjects with Reticulocytosis

Subject	*A* at specified wavelengths relative to that at 260 nm (0.01 N HCl)					Wavelength		
	240	250	270	280	290	Max. acid	Max. alkali	Isosbestic*
R. (patient)	0.46	0.71	1.17	1.05	0.66	270	262.5	264.5
Tr. (patient)	0.41	0.69	1.11	0.94	0.58	267.5	262	263
Vi. T. (patient)	0.46	0.74	1.06	0.89	0.53	266.5	261	263.5
Va. T. (patient)	0.44	0.72	1.09	0.92	0.56	266.5	261	265
F. T., mother of Va. T. and Vi. T.	0.63	0.92	0.71	0.32	0.15	255.5	258	
J. T., brother of Vi. T. and Va. T.	0.69	0.95	0.70	0.35	0.20	256	258	
G. N. (normal)	0.67	0.94	0.70	0.33	0.16	255.5	257	281
W. V. (normal)	0.58	0.92	0.68	0.27	0.11	255.5	257	280
S. H. (normal)	0.67	0.94	0.70	0.32	0.15	255.5	257	280
D. M. (retics 32%)	0.65	0.93	0.70	0.33	0.17	256	256.5	278
T. Mc (retics 25%)	0.55	0.87	0.64	0.21	0.07	257.5	257.5	

* Wavelength isosbestic is used here to designate the wavelength at which the two spectral curves obtained in acid and alkali intercept.

for nucleotides and from 66 to 83% for nucleosides. It seemed essential to determine whether the low recoveries may have introduced a significant alteration in the relative proportion of the various bases in the mixtures. This point was checked by working backwards, in effect, and using the results of chromatographic analyses as the basis for preparing known mixtures of pure nucleosides for precise spectrophotometric comparisons with the original erythrocyte extracts, as described under Methods. The results were gratifying, for, in general, mixtures of pure nucleosides made up to match the chromatographically determined composition of a given erythrocyte extract provided spectral absorbance curves that fit those of the original extract quite closely. Such a comparison is illustrated in Fig. 5, where the correspondence is very close except in the lower wavelengths, a region where many types of impurities in biological solutions show strong absorbance. The multipoint comparisons of the spectral curves proved to be quite sensitive, and when the composition of a mixture of pure nucleotides was adjusted to give a best fit to the spectral curves of an extract, the adjusted composition of the prepared mixture closely approximated the chromatographically determined composition of the extract. The results of the chromatographic analyses and of the spectrophotometric checks are summarized in Table III. It is evident that the pyrimidine-containing nucleotides account for 75% or more of the total nucleotides in the patients' red cells. The inosine-containing nucleotides are believed to result from deamination of adenosine-containing nucleotides during the processing of patient cells. Thus the adenosine-plus-inosine–containing nucleotides as determined chromatographically represent the adenine nucleotides originally present. Chromatographic analyses have also been performed with TCA extracts of erythrocytes obtained from F. T. and J. T., the mother and brother, respectively, of patients Vi. T. and Va. T., as well as from three normal subjects and two with pronounced reticulocytosis No pyrimidine-containing nucleotides were observed in those extracts. Spectral data for these seven subjects, together with those for the patients, are given in Table IV.

Pyrimidine 5'-nucleotidase activity. Pyrimidine nucleoside monophosphates from erythrocyte extracts were isolated from chromatograms and rechromatographed in an acetone-ammonium hydroxide-boric acid solvent, with adjacent standards of 3'- and 5'-ribonucleotides included on the same chromatogram. The nucleotides from the red cells and the 5'-ribonucleotides exhibited markedly reduced chromatographic mobility due to borate complexing. This provided additional evidence that the pyrimidine nucleotides in patient erythrocytes existed as 5'-ribonucleotides. In further investigations, a pyrimidine-specific 5'-nucleotidase was shown to be present in normal erythrocytes, and in higher concentration in reticulocytes, but was markedly deficient in patient red cells. This enzyme had the following characteristics: (*a*) Activity was uniformly greater (usually about 20–30%) with UMP than with CMP. (*b*) While the pyrimidine ribonucleoside 5'-monophosphates were actively dephosphorylated, AMP, IMP, GMP, and XMP, as well as the 2'- and 3'-ribonucleotide derivatives of adenine, did not serve as substrates under the assay conditions. (*c*) The pH range of activity was broad, the optimum being at pH 7.5–8.0. At pH 5–6, 75–80% of maximal activity was still present. Above pH 8.5, activity rapidly

diminished, but about 30% of the maximum activity was retained at pH 9.5. (*d*) Hemolysates had virtually no activity when the nonspecific phosphatase substrate β-glycerophosphate was employed. Slight activity was observed at pH 5.0 with both β-glycerophosphate and IMP as substrates, but activity with the latter was never greater than with β-glycerophosphate. At the pH optimum, 7.5–8.0, of the pyrimidine 5′-nucleotidase, no discernible dephosphorylation of either purine mononucleotides or β-glycerophosphate occurred. (*e*) When α-glycerophosphate, a preferred substrate for human erythrocyte acid phosphatase (23, 24) was employed, patients' hemolysates exhibited entirely normal activity at pH 5.5. The latter observation provided further evidence that the 5′-nucleotidase, deficient in patient cells, was distinct from red cell acid phosphatase. (*f*) The activity with UMP or CMP as substrate was nearly linear in incubations at 37° C up to 4 h, with only modest diminution during the final hour of incubation. (*g*) Activity varied linearly over a fivefold range of hemolysate concentration during a 60-min incubation. (*h*) The enzyme could be inactivated by EDTA, but omission of Mg^{++} in the assay mixture had little if any influence on activity. (*i*) The nucleotidase activity was stable during dialysis for 17–18 h (at 4° C) with conditions described under Methods.

Patient hemolysates were repeatedly assayed in each instance. Representative data are shown in Table V. The patient hemolysates possessed 14% or less of the normal nucleotidase activity, and much less than this when appropriately compared to the much greater activity of reticulocyte-rich bloods. Actually, in the cases of patients R. and Tr., who were initially studied with undialyzed hemolysates, no nucleotidase activity could be demonstrated. Undialyzed hemolysates possess endogenous phosphorylated compounds, such as ATP, that apparently can undergo sufficient dephosphorylation during incubations to produce high blank values, which often mask the very minimal nucleotidase activity. Nucleotidase activity was readily measurable, however, in undialyzed hemolysates prepared from normal blood. When patient hemolysates were dialyzed almost free of P_i, nucleotides, and other organic phosphates, the presence of minimal nucleotidase activity could then be demonstrated. No nucleotidase activity could be detected at any point over a 5-h incubation when the undialyzed hemolysate of patient Tr. was assayed. This was true despite the fact that no additional endogenous dephosphorylation occurred in blanks after the 2nd h. In contrast, normal control hemolysate released P_i from substrate UMP progressively with time, with a 50% yield of P_i observed at the end of 5 h. In additional experiments, 1:1 mixtures of patient and control hemolysates possessed nucleotidase activity corresponding to approximately one-half that of the control hemolysate alone.

TABLE V

*Pyrimidine 5′-Nucleotidase Activity of Erythrocytes: Comparison of Cells of Patients, Certain Relatives, Normal Controls, and Subjects with Reticulocytosis**

Subject	P_i from substrate		% of normal mean	
	UMP	CMP	UMP	CMP
	μmol/gHb/h			
Patient R.	0.78	0.78	11	14
Patient Tr.	0.44	—	6	—
Patient Vi. T.	0.20	0.20	3	4
Patient Va. T.	0.60	0.31	8	5
Mother of R.	4.7	3.8	64	67
Sister of R.	5.8	4.2	79	74
Mother of Vi. T. and Va. T.	5.6 3.9	3.8 3.3	77 53	67 58
Brother of Vi. T. and Va. T.	7.4	5.5	100	96
Normal controls (Mean)	7.1	5.6		
Range UMP (19) 5.0–8.5				
Range CMP (16) 4.3–6.8				
Controls with reticulocytosis				
Range UMP (7) 8.8–17.9				
Range CMP (7) 7.0–12.7				

* All data obtained with dialyzed hemolysates. Undialyzed hemolystates of patients R. and Tr. were repeatedly assayed and no activity was discernible (see text). Number of subjects in control groups are given in parentheses. Patients had reticulocytosis ranging from 9 to 25%.

Although the number of family members available for study is small, the data in Table V strongly suggest that the heterozygous state is detectable by nucleotidase assay. Thus, the obligate heterozygote (if the disorder is autosomally transmitted) mother of patient R. exhibits about two-thirds of the normal mean nucleotidase activity with both UMP and CMP, and this level is below the normal range. The mother of Vi. T. and Va. T. was studied twice. Activity with UMP as substrate ranged from 53–77% that of the normal mean; with CMP, these values were 58 and 67%, respectively. All values but one were lower than the normal range. A sister of patient R. has nucleotidase activity well below the normal mean but with UMP as substrate within the normal range. The cells of a brother of patient Vi. T. and Va. T. exhibit normal activity in all respects.

DISCUSSION

The very large concentrations of pyrimidine nucleotides (initially misinterpreted as representing adenine nucleotides) present in the erythrocytes of patients with the heritable hemolytic syndrome reported here are unique and thus far have not been observed in any other disease state. These observations provide the basis for a simple screening procedure easily accessible to most laboratories. For screening purposes, the grossly aberrant spectral data obtained with deproteinized extracts of red cells at wavelentghs of 240–300 nm suffice.

To our knowledge a pyrimidine-specific 5′-nucleotidase with characteristics described here has not been previously demonstrated in human erythrocytes, reticulocytes, or other tissues. The nucleotidase-deficient subjects discussed in this report had no discernible clinical manifestations involving tissues other than the erythrocyte, but there has not been opportunity thus far to make suitable comparative studies. The failure to observe other manifestations of deficiency except a hemolytic syndrome could be due to isozymes active in other tissues but not present in the erythrocytes. Alternatively, other body cells may be endowed with an array of nucleotidases and/or phosphatases, at least some of which may include among their specificities the capacity to dephosphorylate pyrimidine nucleoside monophosphates, among other substrates. In any event, the maturing reticulocyte must possess mechanisms for disposing of ribosomes and RNA, and accumulation of pyrimidine-containing nucleotides is not characteristic of reticulocytosis *per se*. The remarkable accumulation of pyrimidine nucleotides in these nucleotidase-deficient subjects gives some insight into normal mechanisms of RNA disposal. The findings suggest that (*a*) erythrocyte ribosomal RNA is normally degraded intracellularly, presumably by ribonucleases, (*b*) the chief end products are 5′ rather than 2′ or 3′-ribonucleotides, and (*c*) the nondiffusible pyrimidine nucleotides are normally dephosphorylated by a specific 5′-nucleotidase, inactive with purine nucleotide substrates or β-glycerophosphate. The resulting nonphosphorylated nucleosides presumably can be eliminated by simple diffusion, or possibly by further degradation to diffusible pyrimidine bases and ribose.

With substrate concentrations and assay conditions used in these studies, there has been no evidence of significant dephosphorylation of AMP. This does not preclude dephosphorylations under other circumstances, but obviously the preservation of energetically important adenine nucleotides is important to the relatively metabolically impoverished red cell. The mechanism of disposal of guanosine nucleotides, presumably also degradation products of RNA, remains unexplained, since GMP is not a substrate for the deficient nucleotidase and also does not accumulate more than minimally in patient or normal cells (3–11). Red cells possess the enzymatic machinery to deaminate AMP to IMP. The latter, once formed, cannot be reconverted in human erythrocytes to AMP (30). Since inosine and xanthosine slowly accumulate in stored blood (5), dephosphorylation of IMP must occur. It is known, of course, that red cells contain phosphatases that might nonspecifically utilize nucleoside monophosphates. Of these, an acid phosphatase most active at pH 5.7 is best known (23, 24). In our experience, alkaline phosphatase activity in normal human erythrocytes is minimal, if present at all. At the substrate concentrations used for assay of the specific pyrimidine nucleotidase, virtually no nonspecific phosphatase activity was demonstrable within a pH range of 5–9 with β-glycerophosphate, a substrate widely employed for nonspecific phosphatases. However, at pH 5.5, α-glycerophosphate, which is much preferred to the β-isomer as substrate by erythrocyte acid phosphatase, was dephosphorylated actively. Red cells deficient in pyrimidine 5′-nucleotidase possessed an active acid phosphatase with α-glycerophosphate but not with nucleotides as substrate. In any event, in the near absence of the pyrimidine-specific 5′-nucleotidase, the elimination of phosphorylated pyrimidine nucleosides from the cell most proceed exceedingly slowly. At this point it is unclear whether the nucleotidase deficiency results from lack of synthesis of the enzyme protein or from the synthesis of a catalytically inefficient or highly unstable mutant protein. In the latter case, the traces of activity detectable could be confined to a small subpopulation of young reticulocytes with somewhat older red cells being essentially devoid of activity.

The prominent basophilic stippling seen on the Wright's-stained blood films of all subjects with the syndrome prompts the speculation that it could be a reflection of retarded degradation of ribosomal RNA, secondary to feedback inhibition resulting from the high concentrations of pyrimidine nucleotides. Such stippling has been shown by studies employing electron microscopy to be the result of aggregations of undegraded or partially degraded ribosomes (31).

The partial deficiency of RPK (PRPP synthetase) observed in these nucleotidase-deficient subjects is believed to be an epiphenomenon. This was discussed as a likely possibility in an earlier report (2), since a logical relationship of RPK deficiency to the observed metabolic phenomena and hemolytic syndrome was not apparent and since all efforts to detect a carrier state proved fruitless. It is well established that both purine and pyrimidine nucleotides inhibit the synthesis of PRPP mediated by the enzyme (32, 33). However, in our measurement of RPK activity, the concentration of endogenous nucleotides in the assay mixture is much too low to be demonstrably inhibitory. Further, the results of experiments in which cytidine and uridine nucleotides were preincubated with normal hemolysates did not support the hypothesis that depressed RPK activity resulted from direct inhibition of the reaction by pyrimidine nucleotides. An attractive but speculative alternative is that high concentrations of pyrimidine nucleotides within nucleated precursor cells would actually inhibit the synthesis of RPK. The mature erythrocyte would thus be

endowed with a less than normal initial complement of the enzyme protein. Inhibition of enzyme synthesis of this type is well established in microorganisms (34, 35) but thus far has not been unequivocally demonstrated in man. It is also possible that RPK is normally stabilized by its substrate ATP and that the bizarre nucleotide pattern present in patient erythrocytes is less effective in terms of enzyme stabilization.

The data for each of the four subjects with this hemolytic syndrome indicate that cytidine nucleotides are in pronounced excess of those with uridine as the pyrimidine nucleoside component. If both are derived from erythrocyte RNA as suspected, their ratios do not necessarily reflect those in the RNA itself. UTP is a precursor of CTP in a glutamine-dependent amination reaction mediated by a CTP synthetase. At this time, the possible occurrence of CTP synthetase in human erythrocytes has not been adequately investigated. If present, cytidine nucleotides might be derived directly from RNA degradation or from amination of UTP. Both patient and normal human red cells possess the NMPK and NDPK necessary for interconversion of pyrimidine nucleoside mono-, di-, and triphosphates.

The genetics of the described hereditary hemolytic syndrome cannot be regarded as established with certainty. As pointed out in Results, initial data strongly suggest that it is transmitted as an autosomal recessive disorder, homozygotes having severe nucleotidase deficiency and impaired ability to rid themselves of intraerythrocytic pyrimidine nucleotides. Unfortunately, the number of family members available for study to date has been small. In kindred R. the parents of the proband are first cousins. In kindred T. there is no known consanguinity. However, both sets of grandparents of the probands emigrated from Norway to the United States and settled in neighboring communities. Both had the same surname. Although all five known subjects with the disorder are women, it is improbable that the syndrome is X-chromosome-linked and lethal in men. Such a mode of transmission would not be favored by consanguinity, and there is no known evidence favoring the concept. It appears more probable that detection of the syndrome initially in women has occurred by chance in the small number of subjects yet identified. Additional family studies are required, however.

The high GSH content of patient erythrocytes also remains unexplained. The two synthetic enzyme reactions resulting in GSH formation are ATP-dependent and inhibited by ADP. The possible effects of altered ATP/ADP ratios in patient cells on the net reactions of synthesis and degradation is totally unknown, as indeed is the influence, if any, of high concentrations of pyrimidine nucleoside di- and triphosphates.

The mechanism by which a hemolytic syndrome is produced in subjects with nucleotidase deficiency and the consequential large accumulations of intracellular pyrimidine nucleotides must indeed be complex and at the moment can only be treated speculatively. However, there are several obvious possible mechanisms of interference with metabolically important reactions. First of all, the presence of uridine and cytidine di- and triphosphates introduces competitive cofactors capable of occupying binding sites of enzymes such as hexokinase, pyruvate kinase, and phosphoglycerate kinase, where ADP and ATP are preferred and much more efficient. Secondly, ATP itself interacts with the pyrimidine nucleotides via the nucleoside monophosphate and diphosphate kinases present in human erythrocytes. In the normal erythrocyte such reactions, which are in effect competitive with other ATP-dependent activities, do not exist for want of appropriate pyrimidine substrates. The effects of such competitive reactions on ATP/ADP ratios and their net influence on ATP- or ADP-dependent reactions cannot be currently assessed. Finally, the possibility of deleterious feedback inhibitions, conceivably mediated by cytidine and uridine nucleotides, has been virtually unexplored. Nor can it be stated whether the partially deficient PRPP synthesis has significant adverse effects. The presence of this unusual experiment of nature, however, provides new opportunities to explore in the future additional nuances of red cell metabolism.

ADDENDUM

Since the preparation of this manuscript, we have had the opportunity to study two additional subjects with hemolytic anemia, nucleotidase deficiency, and large accumulations of pyrimidine nucleotides in the red cell. We are indebted to Dr. J. M. Bennett of the University of Rochester School of Medicine for assistance in restudying previously reported patient B. (2). We also are indebted to Dr. G. Richard Lee of the Department of Medicine of the University of Utah School of Medicine for referring the new patient L., who has proved to have the identical syndrome. With the cooperation of referring physicians, it has now been possible to study two children of patient B. and three of patient L. All have nucleotidase activity values compatible with heterozygosity for the disorder, strongly supporting an autosomal mode of transmission.

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