

Patterns of Phosphoribosylpyrophosphate and Ribose-5-Phosphate Concentration and Generation in Fibroblasts from Patients with Gout and Purine Overproduction

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ABSTRACT In the majority of patients with gout and excessive uric acid production, underlying enzyme abnormalities have not been identified. In the present study, measurement of both the rate of generation and concentration of phosphoribosylpyrophosphate (PP-ribose-P) and the concentration of ribose-5-phosphate in cultured cells were undertaken to establish a classification of purine overproducers to direct study of additional enzyme defects. Fibroblasts were cultured from 24 individuals assigned to 4 groups: group 1, 5 normal controls; group 2, 5 patients with gout and normal daily urinary uric acid excretion (gouty controls); group 3, 7 patients with well-defined enzyme abnormalities and excessive urinary uric acid excretion (4 with hypoxanthine-guanine phosphoribosyltransferase deficiency and 3 with excessive PP-ribose-P synthetase activity); and group 4, 7 patients with gout and excessive uric acid excretion but without grossly abnormal activities of the above enzymes in erythrocyte lysates.

In all 14 fibroblast strains from patients showing excessive production of uric acid (groups 3 and 4), rates of purine synthesis *de novo* and PP-ribose-P concentrations exceeded values for cells from control groups. Cells from group 3 patients with hypoxanthine-guanine phosphoribosyltransferase deficiency showed normal PP-ribose-P generation, while those with excessive PP-ri-

bose-P synthetase activity demonstrated increased generation of this regulatory substrate. All strains from group 3 patients had normal ribose-5-phosphate concentrations. Five cell strains from group 4 patients showed one of the two patterns of abnormalities in these measurements seen in strains from group 3 patients: two resembled hypoxanthine-guanine phosphoribosyltransferase-deficient cells, and three resembled cells with excessive PP-ribose-P synthetase activity. Analyses of erythrocyte enzyme preparations from two of these patients in group 4 have led to identification of a kinetic variant of each enzyme as predicted from the foregoing patterns. Two additional group 4 cell lines that showed increased ribose-5-phosphate concentrations in addition to increased PP-ribose-P concentrations and generation were classified in a separate subgroup, since in these individuals excessive purine synthesis appeared to result from increased ribose-5-phosphate concentration, leading to increased availability of PP-ribose-P. No abnormality in either hypoxanthine-guanine phosphoribosyltransferase or PP-ribose-P synthetase has been found in erythrocyte preparations from one patient so classified.

INTRODUCTION

The hyperuricemia of a substantial number of patients with gout results from excessive synthesis of purine nucleotides (1). Although a variety of genetically determined enzyme aberrations that result in purine overproduction have been identified among these individuals (2-5), the underlying metabolic defect or defects in the majority of uric acid overproducers are unknown. De-

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TABLE I
Clinical Characteristics and Laboratory Findings in 24 Skin Biopsy Donors

Group	Patient	Age	Sex	Clinical status	Plasma urate	Erythrocyte lysate enzyme activity					
						Urinary		Hypoxanthine-guanine	Adenine	PP-ribose-P synthetase	Erythrocyte PP-ribose-P
						Uric acid	Creatinine				
					mg/100 ml	mg/24 h		Phosphoribosyltransferase			
								nmol/h/mg protein			nmol/ml cells
Normal*					3.0-7.0	413±78 SD	15-25 mg/kg	81±13 SD	21±4 SD	66±16 SD	2.8±0.5 SD
1	B. A. S.	31	M	Normal	5.3	493	1,418	72	19	49	2.5
	A. F.	34	M	Normal	4.6	437	1,306	88	23	79	2.9
	A. 6.	31	M	Normal	3.8	432	1,108	64	24	70	3.2
	C. J.	29	M	Normal	4.4	511	1,407	77	16	58	3.0
	F. S.	56	M	Normal	5.1	402	1,221	102	22	84	3.6
2	M. S.	39	M	Gout	8.3	475	1,780	69	20	74	2.7
	J. P.	54	M	Gout	8.0	404	1,030	87	19	72	3.3
	A. G.	48	M	Gout	8.8	546	1,660	55	27	53	2.4
	H. A.	63	M	Gout	7.9	448	1,420	77	25	64	2.6
	A. M.	64	M	Gout	8.1	528	1,387	76	21	44	3.0
3	M. P.	13	M	Lesch-Nyhan	8.2	816	380	<0.1	57	53	43.1
	S. M.	15	M	Lesch-Nyhan	9.9	797	490	<0.1	54	—	19.9
	G. B.	44	M	Gout	9.2	870	1,036	0.8	41	71	—
	F. J.	45	M	Gout	12.5†	1,300†	1,130†	1.0†	36	—	—
	T. B.‡	55	M	Gout	10.4	1,405	1,725	76	19	180	5.0
	H. B.‡	45	M	Gout	9.6	1,050	1,550	66	21	184	5.1
	C. B.‡	18	F	Normal	6.2	891	1,636	91	17	187	4.9
4	D. B.	29	M	Gout	11.0	850	1,690	49	36	53	4.4
	S. P.	20	M	Gout	8.1	770	1,360	72	29	69	8.2
	B. P.	34	M	Gout	9.8	1,510	2,200	92	19	83	5.1
	P. B.	39	M	Gout	9.9	878	1,603	81	20	86	3.7
	J. A.	27	M	Gout	8.9	750	2,018	73	23	63	—
	P. G.	43	M	Gout	11.7	1,596	2,469	68	28	64	4.7
	G. A.	27	M	Gout	9.8	670	1,844	88	20	73	3.3

* Data obtained from 28 normal men receiving purine-free diet.

† Data for this patient previously reported (14).

‡ Data for these patients previously reported (11).

iciencies of hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8) activity (3, 4) and excessive activity of phosphoribosylpyrophosphate (PP-ribose-P)¹ synthetase (EC 2.7.6.1) (5, 6), established causes of purine overproduction, appear to be relatively rare (7, 8), and it is estimated that no more than 10% of patients excreting excessive amounts of uric acid in the urine manifest presently identifiable inborn errors of purine metabolism (7).

Our experience in the measurement of the activities of these enzymes of purine metabolism in erythrocyte lysates from nearly 100 individuals with gout and uric acid overproduction has confirmed the rarity of enzyme aberrations detected by standard screening techniques. A different approach to the identification of either additional enzyme abnormalities or subtle variants of pres-

ently known abnormalities has therefore been developed. This approach is based on measurement in cultured fibroblasts of the intracellular concentration and generation of the nucleotide precursor PP-ribose-P and the concentration of ribose-5-phosphate (ribose-5-P). These measurements provide a functional evaluation of the production and (secondarily) utilization of PP-ribose-P and can shed light on the mechanism of purine overproduction in a given individual. The present study indicates their usefulness in classifying patients with uric acid overproduction into subgroups whose specific enzymatic defects may then be corroborated by biochemical and enzymatic techniques.

METHODS

Patients and controls. Fibroblast cultures were derived from upper-arm skin obtained by punch biopsy from the 24 individuals whose clinical findings and chemical values relevant to purine metabolism are recorded in Table I. Plasma and urinary uric acid determinations were made by the enzymatic ultraviolet spectrophotometric method (9), and measurement of urinary creatinine was carried out by

¹ Abbreviations used in this paper: DPBS, Dulbecco's phosphate-buffered saline; FGAR, α -N-formylglycinamide ribotide; KRP, Krebs-Ringer phosphate buffer; PP-ribose-P, 5-phosphoribosyl 1-pyrophosphate; ribose-5-P, ribose-5-phosphate.

the method of Taussky (10). After all medication except colchicine had been withheld for 1 wk, production of uric acid in each patient was assessed by measurement of the daily urinary uric acid excretion during the last 3 days of the week-long administration of a purine-free diet of 2,600 cal containing 1 g protein/kg body weight. Purine overproduction was defined by the urinary excretion of more than 600 mg of uric acid/day (1). Activities of the enzymes hypoxanthine-guanine phosphoribosyltransferase, adenine phosphoribosyltransferase (EC 2.4.2.7), and PP-ribose-P synthetase in erythrocyte lysates were determined radiochemically at saturating concentrations of the respective substrates by previously described methods (4, 11). The labeled substrates used in these enzyme assays were [8-¹⁴C]hypoxanthine (5 mCi/mmol), [8-¹⁴C]adenine 5 mCi/mmol), and [8-¹⁴C]adenine (60 mCi/mmol), respectively (all products of Amersham/Searle Corp., Arlington Heights, Ill.). Erythrocyte PP-ribose-P concentration was measured as previously described (11).

As also shown in Table I, each individual was assigned to one of four groups according to clinical status, daily urinary acid excretion, and the results of the enzyme determinations. Group 1 consisted of five normal male skin biopsy donors (normal controls). Group 2 was composed of five hyperuricemic adult men with episodes of acute monoarticular gout (confirmed by demonstration of monosodium urate crystals in synovial fluid) and normal daily urinary uric acid excretion (gouty controls). Seven patients with excessive urinary uric acid excretion and either marked deficiency of hypoxanthine-guanine phosphoribosyltransferase or excessive PP-ribose-P synthetase activity comprised group 3. Among the hypoxanthine-guanine phosphoribosyltransferase-deficient males, two children had virtually complete absence of enzyme activity and typical features of the Lesch-Nyhan syndrome (3, 12), and two adult patients with gout had less severe deficiencies (4), with up to 3% residual enzyme activity. Two brothers and the daughter of one of these patients had increased PP-ribose-P synthetase activity (6, 11). (Despite absence of clinical symptoms, the 18-yr-old girl with increased PP-ribose-P synthetase activity has previously been shown to excrete excessive amounts of uric acid in the urine [11]). Group 4 was composed of seven male patients with gout and excessive urinary uric acid excretion whose erythrocyte enzyme activities were normal (six patients) or nearly nor-

mal (one patient). In patient D. B., erythrocyte hypoxanthine-guanine phosphoribosyltransferase activity was 60% of the mean for normal individuals, and adenine phosphoribosyltransferase activity was increased approximately two-fold. Although increased adenine phosphoribosyltransferase activity is commonly encountered in erythrocytes from patients with deficiencies of hypoxanthine-guanine phosphoribosyltransferase (13), the deficiency manifested in the latter enzyme activity was milder than has been previously associated with purine overproduction and clinical gout (14), prompting inclusion of this patient in group 4 for further evaluation.

Plan and rationale of study. After classification of the skin biopsy donors, measurements of the rate of purine synthesis *de novo* and of the activities of the previously mentioned enzymes were undertaken in each fibroblast strain to confirm in these strains the results of the urinary uric acid excretion and the erythrocyte lysate enzyme studies, respectively. Determinations of intracellular PP-ribose-P and ribose-5-P concentrations and generation of PP-ribose-P were then carried out in each cell strain to test the prediction that the results of these three measurements would permit classification of individuals with purine overproduction into the four subgroups shown in Table II. The value of the proposed classification in directing further study would then lie in establishing an association of each subgroup with abnormality in a particular enzyme reaction (or group of reactions) as postulated in Table II. Thus, for the fibroblasts of group 3 patients, in whom excessive uric acid production was associated with one or the other of two well-defined enzyme abnormalities, the patterns of proposed subgroups II and III (Table II) were predicted for excessive PP-ribose-P synthetase activity and hypoxanthine-guanine phosphoribosyltransferase deficiency, respectively. Conversely, classification of group 4 patients with uric acid overproduction of unknown etiology was intended to direct intensive enzyme analyses that would confirm or deny the value of the classification procedure in predicting subtle enzyme alterations or new enzyme defects.

Tissue culture methods. Fibroblasts were grown and propagated in monolayer (15) on plastic roller bottles in Eagle's minimal essential medium with 10% fetal calf serum supplemented with 2 mM glutamine, nonessential amino acids, penicillin (50 U/ml), and streptomycin (50 µg/ml). Cells were grown at 37°C under 1 atm of 5% CO₂ in air.

TABLE II
Proposed Classification of Abnormalities Associated with Excessive Purine Production

Subgroup	PP-ribose-P		Ribose-5-phosphate concentration	Example of associated enzyme abnormality
	Concentration	Generation		
I	Increased	Increased	Increased	Glucose-6-phosphatase deficiency*
II	Increased	Increased	Normal or decreased	Increased PP-ribose-P synthetase activity
III	Increased	Normal	Normal	Hypoxanthine-guanine phosphoribosyltransferase deficiency
IV	Normal or decreased	Increased	Normal	Feedback resistant PP-ribose-P amidotransferase*

* Association of enzyme abnormality with pattern of PP-ribose-P and ribose-5-P determinations proposed but not yet demonstrated.

All experiments to be described were carried out with cells grown to confluence. For each cell strain, enzyme determinations and estimations of the rates of purine synthesis were made in duplicate on at least two occasions, and the other biochemical determinations described below were made in triplicate on at least three occasions representing different generations of the cells in culture. In up to 18 generations, the same cell strain showed no major difference in any single study and for this reason, the results of the measurements have been combined for analysis.

Preparation of cell suspensions. The medium was decanted from the bottle, and the cell monolayer was washed four times with 10-ml aliquots of calcium-free Dulbecco's phosphate-buffered saline containing 5.5 mM glucose (DPBS). The cells were then removed from the surface of the roller bottle by incubation for 3 min at 37°C in DPBS containing 0.02% tetrasodium EDTA and 0.05% trypsin. After completion of trypsin treatment, 10 ml of 10% fetal calf serum in DPBS was added, and the cells were transferred to 50-ml plastic centrifuge cups. The roller bottles were washed twice more with the phosphate-buffered saline solution, and the washings were added to the initial washing before centrifugation at 2,000 *g* for 5 min in a Sorvall RC-3 centrifuge (Dupont Instruments, Sorvall Operations, Newtown, Conn.). The supernatant layer was then removed by aspiration, and the cell pellet was washed twice more with DPBS. After completion of the second washing step, the supernatant layer was removed, and the cells resuspended at a density of $5\text{--}10 \times 10^6/\text{ml}$ in Krebs-Ringer phosphate buffer (pH 7.4) with 5.5 mM glucose (KRP). The suspension was incubated at 37°C for 20 min with shaking in a water bath. After this incubation, 100 μl of cell suspension was diluted to 1 ml in KRP containing 0.04% trypan blue, and this suspension was incubated at 37°C for 5–10 min before enumeration of the viable cells on a Spencer Bright-line hemocytometer (American Optical Corp., Scientific Instrument Div., Buffalo, N. Y.). Except for enzyme activities, all values for determinations made in fibroblasts and described in Results are expressed per million viable cells. Suspensions in which less than 90% of cells were viable were discarded before analysis.

Biochemical determinations. The rate of the early steps of purine nucleotide synthesis *de novo* was estimated by measurement of the rate of incorporation of [^{14}C]formate (59 mCi/mmol; Amersham/Searle) into α -N-formylglycinamide ribotide (FGAR) in cells blocked in the further conversion of this compound by the glutamine antagonist azaserine at a concentration of 0.3 mM (16, 17). This method provides a useful estimate of the rate of purine nucleotide synthesis *de novo* (16), and in previous studies in fibroblasts derived from normal individuals and patients with a variety of abnormalities of purine metabolism, results of this method have correlated well with *in vivo* determinations of rates of purine synthesis *de novo* (11, 14, 15). For the present study, the generation and extraction of labeled FGAR was carried out as previously described (15), and a thin layer chromatographic separation of FGAR from other labeled compounds was achieved by the method of Boyle et al. (18).

Fibroblast PP-ribose-P concentration and generation (11) and activities of hypoxanthine-guanine phosphoribosyltransferase (19), adenine phosphoribosyltransferase (20), and PP-ribose-P synthetase (11) were measured by previously described methods. In brief, PP-ribose-P concentration was determined by the conversion of [8- ^{14}C]adenine (60 mCi/mmol; Amersham/Searle) to [^{14}C]AMP in the presence of highly purified adenine phosphoribosyltransferase (21). PP-

ribose-P generation has previously been defined (11) as follows: PP-ribose-P generation = (PP-ribose-P concentration after incubation with adenine + total incorporation of adenine into purine nucleotides and nucleic acids) – PP-ribose-P concentration before addition of adenine.

Incorporation of adenine into purine nucleotides and nucleic acids was measured with [^{14}C]adenine as previously described by Raivio and Seegmiller (22).

Two methods were developed for the measurement of fibroblast ribose-5-P concentrations, and extracts of each fibroblast strain were analyzed for ribose-5-P by each method. Both methods utilized the conversion of ribose-5-P to PP-ribose-P by purified preparations of erythrocyte PP-ribose-P synthetase. In the first assay, PP-ribose-P synthetase, purified 200–1,000-fold (23), converted ribose-5-P in heat-treated fibroblast extracts, prepared as described previously (11), to PP-ribose-P. The PP-ribose-P thus generated was converted in the same reaction mixture to labeled AMP as described above. Ribose-5-P concentration equaled the difference between the total amount of [^{14}C]AMP generated from labeled adenine by fibroblast extracts incubated in the presence of partially purified PP-ribose-P synthetase ("ribose-5-P + PP-ribose-P"), and the amount generated in a parallel incubation mixture in which this enzyme was omitted ("PP-ribose-P only"). The reaction mixture (pH 7.4) contained the following in a volume of 120 μl : Tris-HCl (pH 7.4), 5 μmol ; disodium EDTA, 50 nmol; reduced glutathione, 250 nmol; [^{14}C]adenine, 10 nmol; highly purified adenine phosphoribosyltransferase, 1 μg ; ATP, 106 nmol; magnesium chloride, 750 nmol; sodium phosphate buffer (pH 7.4), 4.2 μmol ; supernate of heat-treated fibroblast extract containing 0.05–1.50 nmol of ribose-5-P and/or PP-ribose-P; and either 0.8 μg of PP-ribose-P synthetase or an equal volume of water.

After incubation for 60 min at 37°C, a portion of the reaction mixture was applied directly to a cellulose thin-layer chromatography sheet (Eastman Kodak Co., Rochester, N. Y.); separation and counting of [^{14}C]AMP was carried out as described previously (24). Agreement between the measurements of PP-ribose-P concentration in the standard assay and in the PP-ribose-P only assay was within 3%, and recoveries of ribose-5-P and PP-ribose-P added to fibroblast extracts as internal controls were over 95%. Standard curves for authentic ribose-5-P and PP-ribose-P were linear to at least 2 nmol in the assay. Fibroblast extract was omitted in the assay blank for the ribose-5-P + PP-ribose-P determination; fibroblast extract and PP-ribose-P synthetase were both omitted in the blank for the PP-ribose-P only assay. Finally, the partially purified preparations of PP-ribose-P synthetase did not catalyze conversion of ribose-5-P in the absence of ATP and did not produce PP-ribose-P from ribose-1-phosphate or from ribulose-5-phosphate.

The alternative method for determination of ribose-5-P concentration was modified from the method of Fox and Kelley (25) and involved measurement of the ribose-5-P-dependent generation of [^{14}C]AMP from [^{14}C]ATP in the presence of an excess of highly purified PP-ribose-P synthetase. More than 1,000-fold purification of the enzyme (23) was found necessary for this assay to avoid ribose-5-P-independent generation of AMP from ATP. The reaction mixture of 60 μl contained the following: [U- ^{14}C]ATP (120 mCi/mmol; Amersham/Searle), 3 nmol; reduced glutathione, 150 nmol; Tris-HCl (pH 7.4), 3 μmol ; disodium EDTA, 25 nmol; magnesium chloride, 300 nmol; sodium phosphate buffer (pH 7.4), 1.8 μmol ; heat-treated fibroblast extract containing 0.05–0.75 nmol of

ribose-5-P and highly purified PP-ribose-P synthetase, 0.8–1.0 μ g. After incubation for 60 min at 37°C, a sample of the incubation mixture was spotted on a cellulose thin-layer chromatography sheet, developed for 3 h in a solvent system consisting of: *n*-butyl alcohol:acetone:glacial acetic acid:5% ammonium hydroxide:water (7:5:3:3:2, vol/vol) (18). The spot corresponding to the AMP marker was cut out and counted in a liquid scintillation counter with Liquifluor-toluene phosphor (Pilot Chemicals, Inc., Watertown, Mass.).

The ATP conversion assay of ribose-5-P was linear to at least 1 nmole of this compound. The agreement between the two assays for ribose-5-P was within 5%. Highly purified preparations of PP-ribose-P synthetase failed to convert ATP to adenylic acid in the presence of sugar phosphates other than ribose-5-P.

Protein determinations were performed by the method of Lowry et al. (26) with bovine serum albumin as standard.

Further erythrocyte analyses. Crude and partially (80-fold) purified preparations of erythrocyte hypoxanthine-guanine phosphoribosyltransferase (27) and 80–200-fold purified preparations of erythrocyte PP-ribose-P synthetase (23) from selected group 4 patients were subjected to further analysis. For both enzymes, affinity constants (K_m) for substrates and thermal sensitivities were determined; electrophoretic mobilities of PP-ribose-P synthetase on cellulose acetate gel were also examined. Preparations of comparable degree of purity from normal individuals served as controls for each procedure.

In the kinetic analyses of hypoxanthine-guanine phosphoribosyltransferase, families of double reciprocal plots of initial velocities were generated by varying the concentration of either substrate while holding the other substrate concentration constant. [8-¹⁴C]Hypoxanthine (50 mCi/mmol; Amersham/Searle) and [8-¹⁴C]guanine (6 mCi/mmol) were varied over a concentration range from 2 to 500 μ M, and PP-ribose-P concentration was varied from 10 to 1,000 μ M, with MgCl₂ concentration 5 mM in excess of PP-ribose-P concentration. Kinetic analyses of PP-ribose-P synthetase were performed similarly, as described in detail elsewhere (28), and affinity constants for substrates of both enzymes were determined from least squares analyses of the secondary plots of velocity intercepts versus the reciprocal of the fixed substrate concentrations.

Thermal inactivation of the crude hypoxanthine-guanine phosphoribosyltransferase was studied at 80°C (14) and of partially purified PP-ribose-P synthetase at 55°C (28). Mobility of PP-ribose-P synthetase on cellulose acetate gel was determined by electrophoresis in a buffer system previously described (29). The enzyme was identified by means of an activity stain that depends on the generation of ATP from AMP and PP-ribose-P in a reversal of the usual PP-ribose-P synthetase reaction (30).

RESULTS

Determination of fibroblast enzyme activities confirmed the abnormalities of hypoxanthine-guanine phosphoribosyltransferase and PP-ribose-P synthetase found in erythrocyte preparations from patients in group 3 and the mild deficiency of the former activity in patient D. B. (Table III). Fibroblast adenine phosphoribosyltransferase activities in cell strains from all of these patients were normal, as were all other enzyme activities in cells of patients in groups 1, 2, and 4.

TABLE III
Enzyme Activities in Fibroblast Cell Lines

Group	Cell line	Phosphoribosyltransferase		PP-ribose-P synthetase
		Hypoxanthine-guanine	Adenine	
		nmol/h/mg protein		
1	B. A. S.	132	238	297
	A. F.	133	269	434
	A. 6.	117	219	477
	C. J.	119	244	433
	F. S.	133	199	318
2	M. S.	156	212	299
	J. P.	132	243	267
	A. G.	111	245	418
	H. A.	142	187	456
	A. M.	168	254	431
3	M. P.*	1-4	247	327
	S. M.*	1-5	253	354
	G. B.*	4-7	230	423
	F. J.*	4-6	234	518
	T. B.	148	267	1,495†
	H. B.	152	243	1,383
	C. B.	129	218	1,212
4	D. B.	88	211	362
	S. P.	120	231	304
	B. P.	137	254	412
	P. B.	143	234	419
	J. A.	126	199	286
	P. G.	144	216	489
	G. A.	132	231	391

Fibroblast extracts were prepared as previously described for the respective phosphoribosyltransferase (19, 20) or PP-ribose-P synthetase (11) assays. All assays were carried out at saturating concentrations of substrates, and the final inorganic phosphate concentration in the PP-ribose-P synthetase reaction was 30 mM. Separation of the appropriate labeled nucleotide from the labeled base substrate was, in each case, achieved by cellulose thin layer chromatography (24). Results represent the means of at least two separate determinations for each enzyme in each cell line. Variation between measurements was less than 15% for each enzyme except in the case of hypoxanthine-guanine phosphoribosyltransferase-deficient lines (*), where values for this activity are given in ranges.

† Value previously reported (11).

When compared with strains derived from normal individuals (group 1) or from patients with gout and normal uric acid excretion (group 2), the rates of the early steps of purine synthesis *de novo* were increased in each of the 14 fibroblast strains from patients with excessive urinary uric acid excretion (groups 3 and 4) (Table IV). The magnitude of the increased rate of accumulation of FGAR varied in individual strains from 1.9 to 5.6-fold the rate of the control groups. The rates of purine synthesis *de novo* in cultures from individuals in groups 1 and 2 were indistinguishable.

In intact normal cells, purine synthesis *de novo* is inhibited by incubation with adenine or hypoxanthine (31). The effects on FGAR accumulation of 1 mM and 0.1 mM concentrations of these compounds were studied in the 24 fibroblast strains (Table IV). Purine synthesis *de novo* in cells from group 3 patients with hypoxanthine-guanine phosphoribosyltransferase deficiency was

TABLE IV
*Purine Biosynthesis De Novo in Fibroblast Lines and its Inhibition
by Incubation with Purine Bases*

Group	Cell line	FGAR accumulation <i>cpm/h/10⁶ cells</i>	Inhibition of FGAR accumulation by:			
			Hypoxanthine		Adenine	
			1 mM	0.1 mM	1 mM	0.1 mM
			%	%	%	%
1	B. A. S.	2,200	71	56	90	77
	A. F.	2,640	74	47	92	70
	A. 6.	1,920	69	53	89	76
	C. J.	2,730	66	54	89	75
	F. S.	1,860	70	48	91	79
2	M. S.	2,240	74	56	85	76
	J. P.	2,880	68	62	88	78
	A. G.	1,790	68	54	85	76
	H. A.	2,120	70	49	90	75
	A. M.	1,910	70	46	95	78
3	M. P.	9,280	-18	2	92	76
	S. M.	8,140	-6	-7	90	68
	G. B.	4,950	14	1	87	77
	F. J.	5,320	6	-6	87	73
	T. B.	6,560	29	11	57	25
	H. B.	6,030	32	17	60	23
	C. B.	6,120	24	16	54	18
4	D. B.	6,400	21	10	88	72
	S. P.	5,110	30	9	81	75
	B. P.	8,160	38	15	46	34
	P. B.	6,730	25	12	53	38
	J. A.	5,320	27	16	56	39
	P. G.	11,280	24	14	48	30
	G. A.	4,930	38	29	56	42

Fibroblasts ($1-2 \times 10^6$ viable cells) were incubated in 0.50 ml Krebs-Ringer phosphate buffer, pH 7.4, for 60 min at 37°C with 4 mM glycine, 20 mM glutamine, 5.5 mM glucose, 0.3 mM azaserine, and 1.27 mM [¹⁴C]-formate. In the inhibition studies, hypoxanthine or guanine was added at the final concentrations indicated. All values represent the means of at least two separate experiments for each cell line. In each experiment, analyses were performed in duplicate. Variation within each experiment was less than 10% and between experiments was less than 20%.

resistant to inhibitory effects of incubation with hypoxanthine at these concentrations. In addition, fibroblasts from group 3 patients with increased PP-ribose-P synthetase activity showed relative resistance to inhibition of FGAR accumulation during incubation with either adenine or hypoxanthine. One or the other of these previously described (15, 31) patterns of resistance were seen in the strain from each individual in group 4. Two strains (D. B. and S. P.) showed partial resistance to inhibition by hypoxanthine but not by adenine, a finding also observed in cells with partial deficiency of hypoxanthine-guanine phosphoribosyltransferase activity. The five other strains showed a similar degree of re-

sistance to inhibition by either adenine and hypoxanthine, thus resembling cells with increased PP-ribose-P synthetase activity.

The intracellular concentrations of PP-ribose-P were higher in all strains from uric acid overproducers (groups 3 and 4) than in fibroblast strains from normal individuals or individuals with gout and normal uric acid production (Fig. 1). The uniformly increased PP-ribose-P concentration in fibroblasts from patients in group 4 contrasts with previous reports in which erythrocyte PP-ribose-P concentrations were increased in only a small proportion of individuals with uric acid overproduction of unknown etiology (11, 32). Erythro-

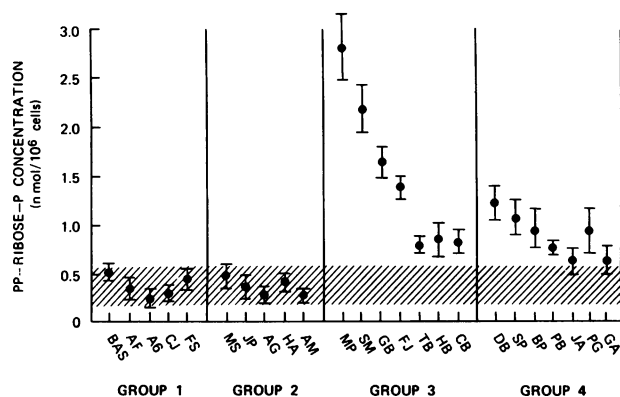


FIGURE 1 PP-ribose-P concentrations in cultured fibroblasts. Solid circle indicates mean and brackets ± 1 SD from mean for each cell strain. PP-ribose-P concentrations were determined in triplicate on at least three different occasions for each strain. Shaded bar represents ± 2 SD from mean determined for strains from normal (group 1) patients.

cyte PP-ribose-P concentrations were measured in the majority of the fibroblast donors in this study and are shown in Table I. In each of the five patients in group 3 in whom this determination was made, erythrocyte PP-ribose-P concentration was more than 2 SD greater than the mean for a large group of normal individuals. Erythrocyte PP-ribose-P concentrations in four of the six patients from group 4 in whom this determination was made were also increased. Despite the high degree of correlation between erythrocyte and fibroblast PP-ribose-P concentrations ($r = 0.92$; $P < 0.01$ by t test) for the entire group studied, however, dissociation between erythrocyte and fibroblast PP-ribose-P concentrations was observed in the remaining two patients in group 4 in whom increased fibroblast concentrations of PP-ribose-P were accompanied by normal erythrocyte concentrations.

Generation of PP-ribose-P by intact fibroblasts is shown in Fig. 2. Compared with PP-ribose-P generation in cells from individuals in the control groups, group 3 patients with excessive PP-ribose-P synthetase activity generated this compound at nearly twice the rate. Generation of PP-ribose-P in cells deficient in hypoxanthine-guanine phosphoribosyltransferase activity, however, was comparable to that of the cells from the control groups. In strains from patients in group 4, PP-ribose-P generation was normal in two (D. B. and S. P.) and above normal in the other five. PP-ribose-P generation among these latter cell strains exceeded that of cells with excessive PP-ribose-P synthetase activity in two cases (B. P. and P. G.) and was comparable or slightly less in the remaining three.

The ribose-5-P concentrations in fibroblasts from individuals in groups 1, 2, and 3 were comparable, and the

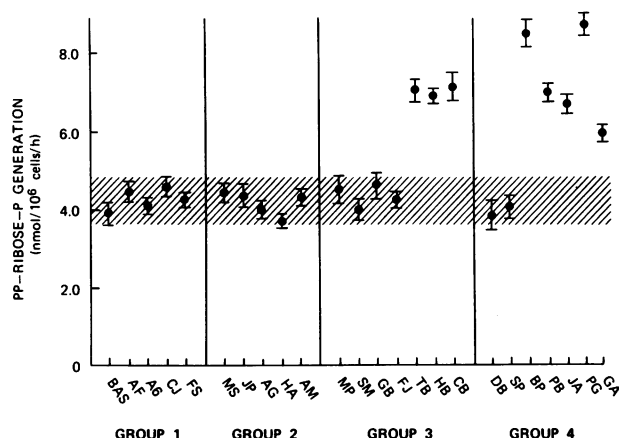


FIGURE 2 Generation of PP-ribose-P in cultured fibroblasts. Symbols are those described for Fig. 1. Determinations were made in triplicate on at least three different occasions for each strain.

range of variation (Fig. 3) was narrow. Fibroblasts from three patients in group 4, however, showed abnormalities in ribose-5-P concentrations: ribose-5-P concentration was more than 2 SD below the mean for groups 1 and 2 in one strain (B. P.) in which increased PP-ribose-P concentration and generation were found; in two other strains (P. G. and G. A.), also with increased PP-ribose-P concentration and generation, the ribose-5-P concentrations were more than 2 SD greater than the mean for the control groups. Concentrations of ribose-5-P were normal in the four additional group 4 strains, two of which showed normal and two of which showed increased PP-ribose-P generation.

Three of the four predicted patterns of variation from the normal values of fibroblast PP-ribose-P concentra-

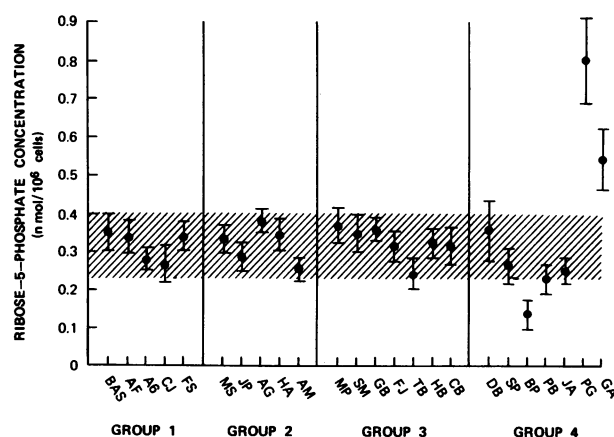


FIGURE 3 Ribose-5-P concentrations in cultured fibroblasts. Symbols are those described for Fig. 1. Determinations were made in triplicate on at least three occasions for each strain.

TABLE V
Characteristics of Erythrocyte Hypoxanthine-Guanine Phosphoribosyltransferase and PP-Ribose-P Synthetase Activities from Selected Uric Acid Overproducers

Patient	Sub-group	Hypoxanthine-guanine phosphoribosyltransferase				PP-ribose-P synthetase			
		K_m^*			Residual activity after 8 min at 80°C†	K_m^*		Residual activity after 14 min at 55°C*	Relative electrophoretic mobility‡*
		Hypoxanthine	Guanine	PP-ribose-P		ATP	Ribose-5-P		
		μM	μM	μM	%	μM	μM	%	R_f
Normal	—	3-9	14-26	12-37	23-52	20-38	100-120	12-21	0.18-0.20
P. G.	1	5	19	24	30	29	116	19	0.19
B. P.	2	8	18	35	42	36	26	20	0.08
D. B.	3	34	79	28	16	23	111	21	0.19

* Procedure carried out on partially purified preparations of the appropriate enzymes (23, 27).

† Procedure carried out on dialyzed erythrocyte lysates. For thermal inactivation studies, the single points indicated here are representative of the response to heating, carried out long enough in each case to inactivate 95% of activity.

‡ Cellulose acetate gel electrophoresis (29).

tion and generation and ribose-5-P concentration (Table II) were seen in cells from the uric acid overproducers of groups 3 and 4. Two of these categories were typified by the cells with well-known enzyme abnormalities included in group 3: deficiency of hypoxanthine-guanine phosphoribosyltransferase activity and increased PP-ribose-P synthetase activity. In the former, PP-ribose-P concentration was increased while generation of this compound and ribose-5-P concentration were normal (subgroup III); in the latter, PP-ribose-P generation as well as concentration was increased and ribose-5-P concentration was normal (subgroup II). Five of the seven strains derived from group 4 patients showed one of these two patterns, and on this basis were classified as follows: D. B. and S. P. in subgroup III, and B. P., P. B., and J. A. in subgroup II. The two additional strains from group 4 patients, however, showed increased concentrations of ribose-5-P as well as increased PP-ribose-P concentrations and generations, and these individuals (P. G. and G. A.) were classified in subgroup I.

The value of this classification of uric acid overproducers in directing further analysis of the basic defects in individual patients was assessed by more detailed study of hypoxanthine-guanine phosphoribosyltransferase and PP-ribose-P synthetase in erythrocytes from three of the seven patients (D. B., B. P., and P. G.), one each from the first three subgroups shown in Table II. These studies (Table V) established abnormal kinetic characteristics for hypoxanthine-guanine phosphoribosyltransferase and PP-ribose-P synthetase in the cells of patients D. B. and B. P., respectively. The abnormal thermal sensitivity and the fourfold diminished affinity of the hypoxanthine-guanine phosphoribosyltransferase of patient D. B. for the substrates hypoxanthine and guanine, and the electrophoretic alteration and the increased affinity for the substrate ribose-5-P of the PP-ribose-P

synthetase of patient B. P. indicate abnormalities in enzyme structure that explain the biochemical abnormalities and increased rates of purine synthesis *de novo* in the fibroblasts of these patients. No abnormality in either of these enzymes was detected in erythrocytes from patient P. G.

DISCUSSION

The identification of a sensitive method for directing study of the enzymatic bases of uric acid overproduction in individuals without gross enzyme aberrations was the aim of this study. The measurements employed were chosen after consideration of contemporary hypotheses concerning regulation of the rate of human purine synthesis *de novo*. The most widely discussed hypothesis regards the antagonistic effects of PP-ribose-P and purine ribonucleotides on the quaternary structure and thus the activity of the enzyme PP-ribose-P amidotransferase (EC 2.4.2.14) as the molecular basis of this regulation (33). Although no evidence of diminished purine ribonucleotide concentrations as a cause of purine overproduction in a disease state in man has yet been presented and although a recent study (34) has questioned the significance in vivo of purine nucleotide feedback inhibition of PP-ribose-P amidotransferase activity, a stimulatory effect of PP-ribose-P on purine synthesis *de novo* has been demonstrated in extensive pharmacological (18, 35-41), clinical (5, 6, 14, 15, 24, 42-45), and kinetic studies (24, 33, 39, 46). For this reason, determination of fibroblast PP-ribose-P concentration is a critical measurement for a study of the biochemical correlates of purine overproduction.

Concentrations of PP-ribose-P are increased in both hypoxanthine-guanine phosphoribosyltransferase deficiency (15, 24, 45) and excessive PP-ribose-P synthetase activity (5, 6), but the mechanisms for the increased PP-ribose-P concentration appear to differ in the two

cases. In hypoxanthine-guanine phosphoribosyltransferase deficiency, PP-ribose-P production is normal (11, 31) so that total utilization of this compound must be diminished; in contrast, in excessive PP-ribose-P synthetase activity, the rate of PP-ribose-P production is increased (5, 6). Thus, by measurement of PP-ribose-P generation, excessive purine nucleotide synthesis *de novo* due to excessive PP-ribose-P synthesis should be distinguishable from that due to PP-ribose-P accumulation resulting from substantially diminished utilization of PP-ribose-P in other pathways of nucleotide synthesis in which PP-ribose-P serves as a substrate. Finally, since the concentration of ribose-5-P is potentially a determinant of the rate of the PP-ribose-P synthetase reaction in vivo (43), the possibility that excessive concentrations of this substrate can result in increased concentration and generation of PP-ribose-P provides the rationale for the measurement of the intracellular ribose-5-P concentration.

Several observations relevant to understanding regulatory defects among patients with uric acid overproduction have emerged from analysis of the patterns of deviation from normal for the three determinations in fibroblasts from these patients. The increased PP-ribose-P concentration in all 14 strains showing excessive rates of purine syntheses *de novo* is evidence for a mechanism of overproduction common to most if not all overproducers. Erythrocyte PP-ribose-P concentrations in two of six patients from group 4 were normal, despite uniformly increased fibroblast PP-ribose-P concentrations. The basis of this discrepancy is unknown, but this observation may explain the difference between the high frequency with which increased PP-ribose-P concentration was found in fibroblasts from group 4 individuals and the low frequency of this finding in erythrocytes from previously studied patients with uric acid overproduction of unknown cause (11, 32). The normal erythrocyte PP-ribose-P concentrations of some patients with partial deficiencies of hypoxanthine-guanine phosphoribosyltransferase activity (45, 47), in whom excessive PP-ribose-P concentration would be expected to underlie the observed uric acid overproduction and gout are a further suggestion of the limitations of PP-ribose-P measurements in erythrocytes.

The finding of increased PP-ribose-P concentration in all group 4 patients suggests that in none of these individuals is uric acid overproduction likely to be underlain by increased activity of PP-ribose-P amidotransferase, an enzyme not presently measurable in fibroblast extracts. Although such abnormality has been postulated in two patients as a result of mutations to states of relative insensitivity to purine nucleotide feedback inhibition (31), both of these individuals (patients T. B. and B. P.) have proved to have structurally altered PP-ribose-P

synthetase and increased PP-ribose-P concentrations in erythrocytes and fibroblasts. If such mutations in PP-ribose-P amidotransferase do occur in association with purine overproduction, PP-ribose-P concentrations are likely to be normal (or diminished) and the pattern to be expected from the measurements described here is likely to be that of subgroup IV in Table II.

Establishment of characteristic patterns of PP-ribose-P concentration and generation and ribose-5-P concentration in fibroblasts from individuals with gross enzyme aberrations (group 3) provides a framework for the identification in other patients of subtle forms of abnormalities in these enzymes. The analyses required to confirm the significance of the seemingly very mild hypoxanthine-guanine phosphoribosyltransferase deficiency in D. B. and to identify the abnormalities in substrate-binding affinities in his enzyme and in the PP-ribose-P synthetase of patient B. P. were directed by the results of the fibroblast studies shown here. Final assessment of the reliability of PP-ribose-P and ribose-5-P measurements in predicting enzyme aberrations with the degree of accuracy implied by these first two analyses will await detailed studies in the remaining patients.

The identification of increased ribose-5-P concentrations as well as increased PP-ribose-P concentrations and generation in two strains derived from group 4 patients suggests that, in certain patients, excessive ribose-5-P production may underlie purine overproduction. This finding supports several previous studies implying such a relationship (2, 39, 43, 44) but adds to these direct measurement of ribose-5-P concentration. Purine overproduction due to increased availability of ribose-5-P leading to increased PP-ribose-P generation is a mechanism proposed to explain the increased rate of purine synthesis *de novo* in patients with glucose-6-phosphatase deficiency (type 1, glycogen storage disease) (2) and the accelerated PP-ribose-P production in erythrocytes from a high proportion of gout patients in whom normal activities of PP-ribose-P synthetase were measured (43, 44). Although the intracellular inorganic phosphate concentration may exert an important and perhaps primary control over the activity of PP-ribose-P synthetase in vivo (48), the increase in the rate of purine synthesis *de novo* during incubation of intact fibroblasts with the electron donor methylene blue, which stimulates the pentose phosphate pathway (49) and increases intracellular ribose-5-P² and PP-ribose-P (39) concentrations, provides evidence for a significant role of ribose-5-P in determining the rate of PP-ribose-P production. The nature of the abnormalities leading to increased ribose-5-P concentrations in the cells of patients P. G. and G. A. will require careful analysis of

² Becker, M. A. In preparation.

enzyme activities and intermediate concentrations in the pathways of ribose-5-P production.

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REFERENCES

- Seegmiller, J. E., A. I. Grayzel, L. Laster, and L. Liddle. 1961. Uric acid production in gout. *J. Clin. Invest.* **40**: 1304-1314.
- Alepa, F. P., R. R. Howell, J. R. Klinenberg, and J. E. Seegmiller. 1967. Relationships between glycogen storage disease and tophaceous gout. *Am. J. Med.* **42**: 58-66.
- Seegmiller, J. E., F. M. Rosenbloom, and W. N. Kelley. 1967. Enzyme defect associated with a sex-linked human neurological disorder and excessive purine synthesis. *Science (Wash. D. C.)* **155**: 1682-1684.
- Kelley, W. N., F. M. Rosenbloom, J. F. Henderson, and J. E. Seegmiller. 1967. A specific enzyme defect in gout associated with overproduction of uric acid. *Proc. Natl. Acad. Sci. U. S. A.* **57**: 1735-1739.
- Sperling, O., P. Boer, S. Persky-Brosh, E. Kanarek, and A. de Vries. 1972. Altered kinetic property of erythrocyte phosphoribosylpyrophosphate synthetase in excessive purine production. *Rev. Eur. Etud. Clin. Biol.* **17**: 703-706.
- Becker, M. A., L. J. Meyer, A. W. Wood, and J. E. Seegmiller. 1973. Purine overproduction in man associated with increased phosphoribosylpyrophosphate synthetase activity. *Science (Wash. D. C.)* **179**: 1123-1126.
- Becker, M. A., and J. E. Seegmiller. 1974. Genetic aspects of gout. *Annu. Rev. Med.* **25**: 15-28.
- Yü, T.-F., M. E. Balis, T. A. Krenitsky, J. Dancis, D. N. Silvers, G. B. Elion, and A. B. Gutman. 1972. Rarity of X-linked partial hypoxanthine-guanine phosphoribosyltransferase deficiency in a large gouty population. *Ann. Intern. Med.* **76**: 255-264.
- Liddle, L., J. E. Seegmiller, and L. Laster. 1959. The enzymatic spectrophotometric method for determination of uric acid. *J. Lab. Clin. Med.* **54**: 903-913.
- Taussky, H. H. 1954. A microcolorimetric determination of creatinine in urine by the Jaffe reaction. *J. Biol. Chem.* **208**: 853-861.
- Becker, M. A., L. J. Meyer, and J. E. Seegmiller. 1973. Gout with purine overproduction due to increased phosphoribosylpyrophosphate synthetase activity. *Am. J. Med.* **55**: 232-242.
- Lesch, M., and W. L. Nyhan. 1964. A familial disorder of uric acid metabolism and central nervous system function. *Am. J. Med.* **36**: 561-570.
- Rubin, C. S., M. E. Balis, S. Piomelli, P. H. Berman, and J. Dancis. 1969. Elevated AMP pyrophosphorylase activity in congenital IMP pyrophosphorylase deficiency (Lesch-Nyhan disease). *J. Lab. Clin. Med.* **74**: 732-741.
- Kelley, W. N., M. L. Greene, F. M. Rosenbloom, J. F. Henderson, and J. E. Seegmiller. 1969. Hypoxanthine-guanine phosphoribosyltransferase deficiency in gout. *Ann. Intern. Med.* **70**: 155-206.
- Rosenbloom, F. M., J. F. Henderson, I. C. Caldwell, W. N. Kelley, and J. E. Seegmiller. 1968. Biochemical bases of accelerated purine biosyntheses *de novo* in human fibroblasts lacking hypoxanthine-guanine phosphoribosyltransferase. *J. Biol. Chem.* **243**: 1166-1173.
- Henderson, J. F. 1962. Feedback inhibition of purine biosynthesis in ascites tumor cells. *J. Biol. Chem.* **237**: 2631-2635.
- Levenberg, B. E., and J. M. Buchanan. 1956. Formylglycinamide ribotide and 5-aminoimidazole ribotide—intermediates in the biosynthesis of inosinic acid *de novo*. *J. Am. Chem. Soc.* **78**: 504-505.
- Boyle, J. A., K. O. Raivio, M. A. Becker, and J. E. Seegmiller. 1972. Effects of nicotinic acid on human fibroblast purine synthesis. *Biochim. Biophys. Acta.* **269**: 179-183.
- Kelley, W. N., and J. C. Meade. 1971. Studies on hypoxanthine-guanine phosphoribosyltransferase in fibroblasts from patients with the Lesch-Nyhan syndrome. Evidence for genetic heterogeneity. *J. Biol. Chem.* **246**: 2953-2958.
- Kelley, W. N. 1971. Studies on the adenine phosphoribosyltransferase enzyme in human fibroblasts lacking hypoxanthine-guanine phosphoribosyltransferase. *J. Lab. Clin. Med.* **77**: 33-38.
- Thomas, C. B., W. J. Arnold, and W. N. Kelley. 1973. Human adenine phosphoribosyltransferase. Purification, subunit structure and substrate specificity. *J. Biol. Chem.* **248**: 2529-2535.
- Raivio, K. O., and J. E. Seegmiller. 1973. Adenine, hypoxanthine and guanine metabolism in fibroblasts from normal individuals and from patients with hypoxanthine phosphoribosyltransferase deficiency. *Biochim. Biophys. Acta.* **299**: 273-282.
- Fox, I. H., and W. N. Kelley. 1971. Human phosphoribosylpyrophosphate synthetase. Distribution, purification, and properties. *J. Biol. Chem.* **246**: 5739-5748.
- Wood, A. W., M. A. Becker, and J. E. Seegmiller. 1973. Purine nucleotide synthesis in lymphoblasts cultured from normal subjects and a patient with the Lesch-Nyhan syndrome. *Biochem. Genetics.* **9**: 261-274.
- Fox, I. H., and W. N. Kelley. 1972. Human phosphoribosylpyrophosphate synthetase. Kinetic mechanism and end product inhibition. *J. Biol. Chem.* **247**: 2126-2131.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193**: 265-275.
- Arnold, W. J., and W. N. Kelley. 1971. Human hypoxanthine-guanine phosphoribosyltransferase. Purification and subunit structure. *J. Biol. Chem.* **246**: 7398-7404.
- Becker, M. A., P. J. Kostel, and L. J. Meyer. 1975. Human phosphoribosylpyrophosphate synthetase. Comparison of purified normal and mutant enzymes. *J. Biol. Chem.* **250**: 6822-6830.
- Becker, M. A., P. J. Kostel, L. J. Meyer, and J. E. Seegmiller. 1973. Human phosphoribosylpyrophosphate synthetase: increased enzyme specific activity in a family with gout and excessive purine synthesis. *Proc. Natl. Acad. Sci. U. S. A.* **70**: 2749-2752.
- Johnson, M. G., S. Rosenzweig, R. L. Switzer, M. A. Becker, and J. E. Seegmiller. 1974. Evaluation of the role of 5-phosphoribosyl- α -1-pyrophosphate synthetase

- in congenital hyperuricemia and gout: a simple isotopic assay and an activity stain for the enzyme. *Biochem. Med.* 10: 266-275.
31. Henderson, J. F., F. M. Rosenbloom, W. N. Kelley, and J. E. Seegmiller. 1968. Variations in purine metabolism of cultured skin fibroblasts from patients with gout. *J. Clin. Invest.* 47: 1511-1516.
 32. Fox, I. H., and W. N. Kelley. 1971. Phosphoribosylpyrophosphate in man: biochemical and clinical significance. *Ann. Intern. Med.* 74: 424-433.
 33. Holmes, E. W., J. B. Wyngaarden, and W. N. Kelley. 1973. Human glutamine-phosphoribosylpyrophosphate amidotransferase. Two molecular forms interconvertible by purine ribonucleotides and phosphoribosylpyrophosphate. *J. Biol. Chem.* 248: 6035-6040.
 34. Bagnara, A. S., A. A. Letter, and J. F. Henderson. 1974. Multiple mechanisms of regulation of purine biosynthesis de novo in intact tumor cells. *Biochim. Biophys. Acta.* 374: 259-270.
 35. Seegmiller, J. E., J. R. Klinenberg, J. Miller, and R. W. E. Watts. 1968. Suppression of glycine-¹⁵N incorporation into urinary uric acid by adenine-8-¹⁴C in normal and gouty subjects. *J. Clin. Invest.* 47: 1193-1203.
 36. Schulman, J. D., M. L. Greene, W. Y. Fujimoto, and J. E. Seegmiller. 1971. Adenine therapy for Lesch-Nyhan syndrome. *Pediatr. Res.* 5: 77-82.
 37. Kelley, W. N., M. L. Greene, I. H. Fox, F. M. Rosenbloom, R. I. Levy, and J. E. Seegmiller. 1970. Effects of orotic acid on purine and lipoprotein metabolism in man. *Metab. (Clin. Exp.)*. 19: 1025-1035.
 38. Kelley, W. N., and J. B. Wyngaarden. 1970. Effects of allopurinol and oxipurinol on purine synthesis in cultured human cells. *J. Clin. Invest.* 49: 602-609.
 39. Kelley, W. N., I. H. Fox, and J. B. Wyngaarden. 1970. Essential role of phosphoribosylpyrophosphate (PRPP) in regulation of purine biosynthesis in cultured human fibroblasts. *Clin. Res.* 18: 457. (Abstr.)
 40. Fox, I. H., J. B. Wyngaarden, and W. N. Kelley. 1970. Depletion of erythrocyte phosphoribosylpyrophosphate in man. A newly observed effect of allopurinol. *N. Engl. J. Med.* 283: 1177-1182.
 41. Kelley, W. N., I. H. Fox, and J. B. Wyngaarden. 1970. Regulation of purine biosynthesis in cultured human cells. I. Effects of orotic acid. *Biochim. Biophys. Acta.* 215: 512-516.
 42. Jones, O. W., Jr., D. M. Ashton, and J. B. Wyngaarden. 1962. Accelerated turnover of phosphoribosylpyrophosphate, a purine nucleotide precursor, in certain gouty subjects. *J. Clin. Invest.* 41: 1805-1815.
 43. Hershko, A., C. Hershko, and J. Mager. 1968. Increased formation of 5-phosphoribosyl-1-pyrophosphate in red blood cells of some gouty patients. *Isr. J. Med. Sci.* 4: 939-944.
 44. Sperling, O., R. Ophir, and A. de Vries. 1971. Purine base incorporation into erythrocyte nucleotides and erythrocyte phosphoribosyltransferase activity in primary gout. *Rev. Eur. Etud. Clin. Biol.* 16: 147-151.
 45. Greene, M. L., and J. E. Seegmiller. 1969. Elevated erythrocyte phosphoribosylpyrophosphate in X-linked uric aciduria: importance of PRPP concentration in the regulation of human purine biosynthesis. *J. Clin. Invest.* 48: 32a. (Abstr.)
 46. Holmes, E. W., J. A. McDonald, J. M. McCord, J. B. Wyngaarden, and W. N. Kelley. 1973. Human glutamine phosphoribosylpyrophosphate amidotransferase. Kinetic and regulatory properties. *J. Biol. Chem.* 248: 144-150.
 47. Sperling, O., G. Eilam, S. Persky-Brosh, and A. de Vries. 1972. Simpler method for the determination of 5-phosphoribosyl-1-pyrophosphate in red blood cells. *J. Lab. Clin. Med.* 79: 1021-1026.
 48. Hershko, A., A. Razin, and J. Mager. 1969. Regulation of the synthesis of 5-phosphoribosyl-1-pyrophosphate in intact red blood cells and in cell-free preparations. *Biochim. Biophys. Acta.* 184: 64-76.
 49. Brin, M., and R. H. Yonemoto. 1958. Stimulation of the glucose oxidative pathway in human erythrocytes by methylene blue. *J. Biol. Chem.* 230: 307-317.