Suppression of Glycine-^{15}N Incorporation into Urinary Uric Acid by Adenine-8-^{13}C in Normal and Gouty Subjects

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ABSTRACT Adenine inhibited the de novo synthesis of purines in both normal and gouty man as shown by inhibition of the incorporation of glycine-^{15}N into urinary uric acid without altering the incorporation of glycine-^{15}N into urinary creatinine. The diminished purine synthesis did not result in a diminution in the 24 hr excretion of uric acid. This observation was explainable in part by the prompt conversion of adenine to uric acid. In addition to this direct conversion, adenine-8-^{13}C provided a slow and prolonged contribution to urinary uric acid.

A feedback inhibition of purine synthesis by nucleotides derived from adenine provides the best interpretation of these results.

INTRODUCTION

A suppression of purine synthesis de novo can be induced by the preferential utilization of exogenously supplied preformed purines in a variety of biological systems. This was observed by Abrams in rabbit bone marrow slices (1), by Gots in bacterial mutants (2), by Nierlich and Magasanik in mouse fibroblasts grown in tissue culture (3), and by Henderson in ascites tumor cells (4). In addition, the administration of the purine precursor 4-amino-5-imidazole carboxamide (AIC) to the human subject has been noted to suppress incorporation of glycine-^{15}N into urinary uric acid (5).

The precise mechanism of the suppression of purine synthesis is not well understood. Caskey, Ashton, and Wyngaarden demonstrated that adenine-containing nucleotides as well as those containing guanine or hypoxanthine exert an inhibitory action on the enzyme responsible for the rate-controlling step of purine synthesis (Fig. 1). This enzyme, phosphoribosylpyrophosphate glutamine amidotransferase, is responsible for the formation of phosphoribosylamine from glutamine and phosphoribosylpyrophosphate (6). The finding that adenine-^{14}C administered in trace quantities was converted to uric acid at a very slow rate (7) suggested the possibility that larger doses of adenine might be used to inhibit, in a physiological manner, the excessive purine synthesis found in some patients with gout without itself being degraded immediately to uric acid.

In the present study we have observed the effect of the administration of adenine on purine synthesis de novo as measured by the incorporation of glycine-^{15}N into urinary uric acid in normal and gouty subjects. Simultaneous determination of the dynamics of the urate pool by means of uric acid-2-^{14}C intravenously administered has permitted the appropriate correction for the extrarenal disposition of uric acid in the various subjects (8). Adenine-8-^{13}C was used in order to

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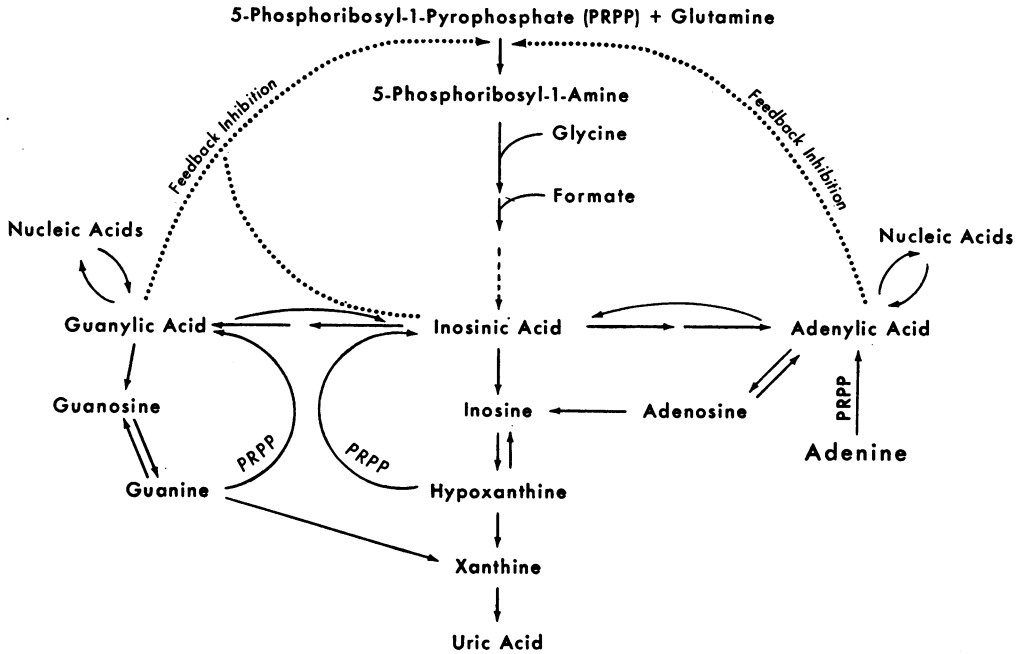


FIGURE 1 Pathway of purine biosynthesis showing feedback inhibition by purine nucleotides.

assess the extent to which exogenous adenine was converted to urinary uric acid.

METHODS

Adenine-8-^{13}C·HCl·1/2 H_2O, containing 9.89 atom % excess ^{13}C, was synthesized by a modification of the procedure of Clark and Kalckar (9–11). $^{13}CO_2$ was liberated from barium carbonate-^{13}C (71.3 mmole; 55.7 atom % excess ^{13}C, Eastman Kodak, Rochester, N. Y.) by the action of orthophosphoric acid, entrained in carbon dioxide-free nitrogen, and trapped in sodium hydroxide. The solution was cooled to 0°C, titrated to pH 8.5 with sulfuric acid, and the ^{13}C-labeled sodium bicarbonate was reduced to sodium formate by hydrogen (24 hr at 80°C and 3700 psi in the presence of 10 g of pallidized charcoal [1 g of Pd]) (12). Formic acid-^{13}C was isolated by steam distillation, the distillate titrated to pH 8.4 with sodium hydroxide, and finally evaporated to dryness to yield sodium formate-^{13}C.

N(4,6-diamino-5-pyrimidinyl)-formamide-^{13}C was prepared from 4,5,6-triaminopyrimidine (Sigma Chemical Co., St. Louis, Mo.) (11) and converted to adenine-8-^{13}C by heating at 210°C in diethanolamine under nitrogen for 90 min (10). After cooling the reacting mixture on ice we neutralized the diethanolamine by the addition of an equivalent quantity of concentrated hydrochloric acid and isolated adenine as the picrate. Adenine picrate was converted to adenine hydrochloride by treatment with a saturated solution of dry hydrogen chloride in anhydrous acetone. The last traces of picric acid were removed by stirring an aqueous solution of the adenine hydrochloride with Dowex -1×8 (200–400 mesh, Cl^- form). The ion exchange resin was removed by filtration, adenine hydrochloride hemihydrate was crystallized from the filtrate, and recrystallized three times from hot water. The overall yield from barium carbonate was 52%.

The molar absorbency index of the synthesized adenine hydrochloride hemihydrate -8-^{13}C($C_5H_5N_5$·HCl·1/2 H_2O) at pH 2 $\epsilon_{263} = 13{,}345$ and the elemental analysis was as follows:

calculated: C, 33.47; H, 3.90; N, 38.6; Cl, 19.57
found: C, 33.25; H, 3.86; N, 38.9; Cl, 19.41
(Correcting for ^{13}C)

Glycine containing 97.96 atom % excess ^{15}N was synthesized from potassium phthalimide-^{15}N (Iso-met Corporation) (13, 14). Uric acid-2-^{14}C was synthesized as previously described (15), diluted to a specific activity of 12.1 μc/mmole, and converted to the lithium salt by the addition of an equivalent quantity of lithium carbonate. Sterile distilled water and glucose solution were added to give a final concentration of 1 mg of uric acid per ml of 5% glucose (8). This was sterilized by filtration through a Millipore filter, packaged in 10-ml vials, and tested extensively for sterility and pyrogens before use. All vials were stored at -5°C.

All subjects were hospitalized during the entire study at the Clinical Center of the National Institutes of Health. The three gouty subjects selected were all known to have excessive production of uric acid from previous studies of their purine synthesis (8). A diet essentially free of purines, containing 70 g of protein, 350 g of carbohydrate, and 100 g of fat was given for a period of at least 5 days before the administration of isotopically labeled com-

pounds and throughout the period of study. All drugs with the exception of maintenance doses of colchicine were discontinued before starting the equilibration period. A fluid intake of at least 3 liters/day was maintained during the 2-day period immediately after adenine administration in order to minimize the hazard of renal deposition of the 2,8-dioxyadenine formed from adenine by xanthine oxidase action. Such a deposition has been shown to constitute the principal toxic effect of adenine administration in the rat (16). The largest dose of orally administered adenine that could be given to the rat without causing renal deposition of 2,8-dioxyadenine or other toxic manifestations was found to be 25–50 mg/kg body weight. An additional margin of safety in translating these studies to the human subject is the fact that the specific activity of xanthine oxidase in human liver is only one-fiftieth the activity of rat liver (17, 18). Adenine-8-$^{13}C \cdot HCl \cdot 1/2\ H_2O$, 11.25 mg/kg body weight, along with carrier adenine, 3.6 mg/kg body weight, (Sigma Chemical Co.), was dissolved in a minimum volume of water and administered in orange juice with breakfast. Glycine-^{15}N was diluted with carrier glycine (Fisher Scientific Co., Pittsburgh, Pa.) to 60.0 atom % excess in order that data could be directly compared with previous control studies performed on two subjects, G. S. and J. S., (8) and 100 mg/kg body weight was given in breakfast milk immediately after the intravenous administration of 8 mg of lithium urate-2-^{14}C. A period of at least 6 months elapsed betwen control studies with glycine-^{15}N and urate-2-^{14}C and studies with glycine-^{15}N, urate-2-^{14}C, and adenine-^{13}C. Serum urate was determined daily (19). All urine was collected at room temperature with 3 ml of toluene as preservative, analyzed for uric acid (19) and creatinine content (20), and then stored at $-5°$ C until isolation procedures could be carried out. Uric acid was isolated from urine as previously described (21). Creatinine was isolated from urine as the picrate and recrystallized as creatinine zinc chloride (22). The content of ^{13}C and ^{15}N in uric acid and the abundance of ^{15}N in creatinine were determined in a consolidated-Nier mass spectrometer after appropriate digestion of samples (23). Residual ^{15}N found in uric acid during the 2 days before adenine administration was taken as the natural abundance value for the second study. The ^{14}C content of uric acid was determined by plating uric acid on planchets and by counting at 30% efficiency in a shielded end-window gas-flow counter (Nuclear-Chicago Corp., Des Plaines, Ill.) with coincidence circuitry to give a background of less than 2 cpm. Counts of all samples were corrected to a standard thickness represented by 50 mg of uric acid.

Selected specimens of uric acid were degraded to alloxantin to separate the nitrogen atoms of positions 7 and 9 from those of 1 and 3 (24, 25).

RESULTS

Adenine administration diminished the incorporation of glycine-^{15}N into urinary uric acid in both normal and gouty subjects (Figs. 2 and 3). The maximum enrichment of uric acid with ^{15}N was

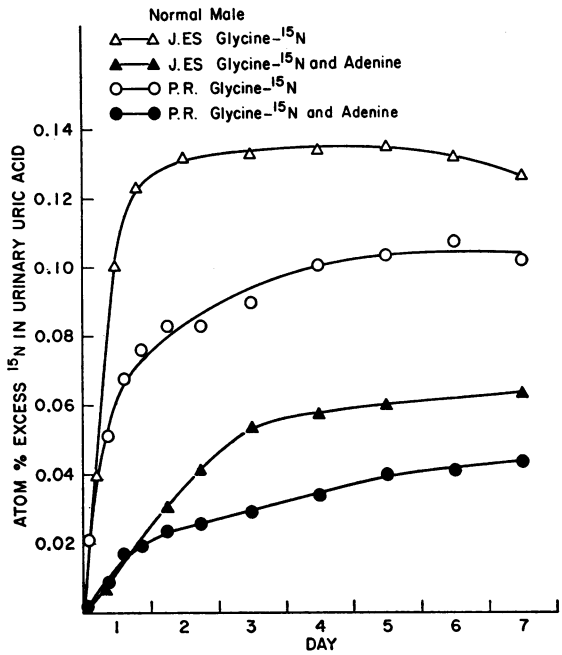


FIGURE 2 Effect of adenine on the incorporation of glycine-^{15}N into urinary uric acid in normal subjects.

reached in control subjects (Fig. 2) by the 3rd–4th day at values of 0.1 and 0.13 atom% excess. Adenine administration resulted in a substantially lower enrichment of ^{15}N in each sample of uric acid. In the control studies of three gouty patients the maximum enrichment was found on the 1st or 2nd day, with maximum values of 0.3–0.8 atom% excess, about three to six times the values found in normal subjects. Adenine administration not only diminished glycine incorporation into urinary uric acid but also changed the time course of ^{15}N enrichment. The early peak of ^{15}N incorporation characteristic of gouty subjects with excessive uric acid synthesis was abolished and the time of appearance of maximum incorporation was delayed. Although adenine administration to gouty patients diminished the atom per cent excess ^{15}N found in uric acid to values approaching those found in the control studies of normal subjects, they nonetheless remained substantially greater than the values found when the same control subjects were given adenine. This effect of adenine was also reflected in the total recovery of ^{15}N in urinary uric acid shown in Table I.

The magnitude of the inhibition of glycine-^{15}N incorporation into urinary uric acid in response

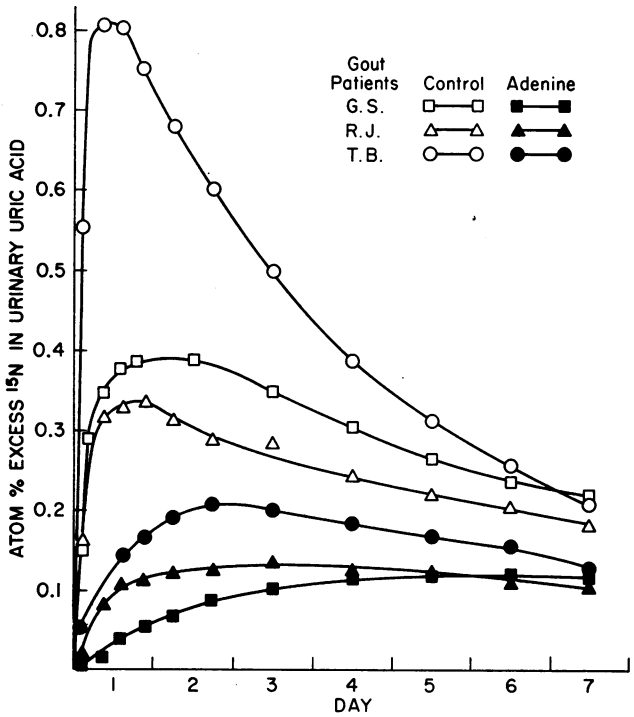


FIGURE 3 Effect of adenine on the incorporation on ^{15}N into urinary uric acid in gouty patients.

to adenine varied greatly among the various subjects. In the normal subjects the total recovery of ^{15}N in urinary uric acid was reduced to 26 and 52% of control values, respectively, while in the gout patients it was reduced to 31–55% of control values. The 7 day total recovery in the urine of the intravenously administered uric acid-2-^{14}C was used to correct for the extrarenal disposition of uric acid. The urate pool size and turnover rate (Table II) were not altered significantly by the administration of adenine. The close agreement of the per cent of urate turnover excreted each day (Table II) and the per cent of urate-2-^{14}C recovered in the urine (Table I) provides independent confirmation of the validity of these determinations.

TABLE I

Effect of Adenine on the Recovery over a 7 Day Period of ^{14}C, ^{13}C, and ^{15}N in Urinary Uric Acid of Normal and Gouty Subjects

Subject	Administered uric acid-2-^{14}C U		Administered adenine-8-^{13}C		Administered glycine-^{15}N				
			Uncorrected H	Corrected (H/U) × 100	Uncorrected G		Corrected (G/U) × 100		% of Control (Ad/C) × 100
	C	Ad			C	Ad	C	Ad	
	%		%	%	%		%		
Control									
P.R.	73	80	14.5	18.1	0.099	0.048	0.116	0.060	52
J.S.	65	75	14.0	18.7	0.16	0.044	0.25	0.059	24
Gout									
G.S.	48	38	12.3	32.4	0.40	0.098	0.83	0.26	31
T.B.	78	81	32.0	39.5	1.09	0.46	1.40	0.57	41
R.J.	67	70	24.4	34.9	0.69	0.40	1.03	0.57	55

Lithium urate-2-^{14}C was administered intravenously and glycine-^{15}N orally with and without administration of adenine-8-^{13}C. C, control study; Ad, study with adenine administration; U, recovery of uric acid-2-^{14}C; H, recovery of ^{13}C from adenine-8-^{13}C; G, recovery of ^{15}N from glycine-^{15}N.

TABLE II

Effect of Adenine Administration on the Urate Pool Size and Turnover Rate of Normal and Gouty Subjects

Subject	Serum urate		Urate pool size, *A*		Turnover rate, *K*		Turnover, *KA*		Excretion uric acid, *E*		Turnover Excreted *E/KA* × 100	
	C	Ad	C	Ad	C	Ad	C	Ad	C	Ad	C	Ad
	mg/100 ml		*mg*		*pools/day*		*mg/day*		*mg/day*		%	
Control												
P.R.	4.9	6.3	1088	1670	0.53	0.47	580	786	435	597	75	76
J.S.	4.2	4.8	1008	1230	0.67	0.53	729	650	536	480	74	74
Gout												
G.S.	11.9	11.5	2660	2560	0.49	0.41	1274	1050	529	444	41	42
T.B.	10.3	10.8	2700	3020	0.57	0.60	1540	1820	1242	1453	81	80
R.J.	10.8	10.5	2050	2520	0.80	0.77	1645	1950	1159	1295	70	66

C, control; Ad, study with adenine administration.

Glycine initially contributes primarily to the 7 nitrogen position of the purine ring and only secondarily to the remaining nitrogens through a labeling of the body ammonia and glutamine pools. Suppression of purine synthesis during the period of maximal labeling of the body's glycine pool should, therefore, suppress principally the primary contribution of glycine to position 7 of uric acid. The secondary contribution to the remaining 1, 3, and 9 positions from the glutamine and ammonia pools would be expected to be diminished to a lesser extent since the glutamine and ammonia pools show a more prolonged pattern of labeling with ^{15}N (26, 27). Degradation of uric acid allowed specific determination of ^{15}N enrichment at the 1, 3 and, by difference, at the 7, 9 positions. Adenine administration diminished the specific contribution of ^{15}N to position 7 and 9 to a greater extent than the contribution to positions 1 and 3 of the purine ring in three of four patients Table III). The incorporation of glycine-^{15}N into urinary creatinine was not affected significantly by adenine administration (Table IV).

The suppression of glycine-^{15}N incorporation into urinary uric acid was not reflected in a net decrease in the serum urate concentration or in the 24 hr excretion of uric acid (Fig. 4). In fact, urinary uric acid excretion was substantially greater during the day of adenine administration than during the control study in four of the five subjects.

A possible explanation for the transient increase

TABLE III

Distribution of ^{15}N in Positions 1, 3 as Compared to 7, 9 of Uric Acid

Subject	Atom per cent excess ^{15}N						Ratio ^{15}N position 7, 9/ position 1, 3	
	Total		Position 1, 3		Position 7, 9			
	C	Ad	C	Ad	C	Ad	C	Ad
Control								
P.R.	0.0837	0.0355	0.0284	0.0169	0.1390	0.0541	4.89	3.20
J.S.	0.117	0.0325	0.0370	0.0177	0.197	0.0473	5.30	2.67
Gout								
G.S.	—	0.0873	—	0.0585	—	0.1161	—	1.98
T.B.	0.6008	0.2070	0.3651	0.1440	0.8365	0.2700	2.29	1.88
R.J.	0.2899	0.1354	0.1535	0.0626	0.4263	0.2082	2.78	3.33

C, control study; Ad, study with adenine. Atom per cent excess ^{15}N in position 1, 3 was determined in alloxantin derived from uric acid, while that in position 7, 9 was calculated by difference.

TABLE IV

Effect of Adenine Administration on the Recovery of Glycine-^{15}N in Urinary Creatinine

Subject	Per cent ^{15}N recovered in urinary creatinine over a 7 day period	
	Control	Adenine
Control		
P.R.	0.27	0.28
J.S.	—	0.24
Gout		
G.S.	—	0.22
T.B.	0.30	0.31
R.J.	0.27	0.23

in urinary uric acid excretion was found in the prompt conversion of adenine-8-^{13}C to urinary uric acid shown in Fig. 5. The magnitude of the ^{13}C enrichment of urinary uric acid was similar for both normal and gouty subjects. As a result, the total recovery of ^{13}C in urinary uric acid was largely determined by the daily uric acid production (Table I). The atom per cent excess ^{13}C in urinary uric acid reached a maximum on the 1st or 2nd day in each patient and the subsequent decline in isotope abundance between the 3rd and 7th day followed first-order kinetics, as shown by the linear relationship when the values for isotope abundance were plotted on a logarithmic scale with time (Fig. 6). Comparison of the decline in isotope abundance for both ^{13}C and ^{14}C determined on the same samples of uric acid shows that the ^{13}C values declined much more slowly than did the ^{14}C values in each patient. The numerical values calculated for the first-order rate constant for decline in ^{13}C abundance is shown in Fig. 6. This constant was the same in the normal subjects and in two of the three gouty patients. However, one of the gout patients, T. B., showed a value almost twice that found in control subjects, which indicated a more rapid turnover of the pool labeled by adenine.

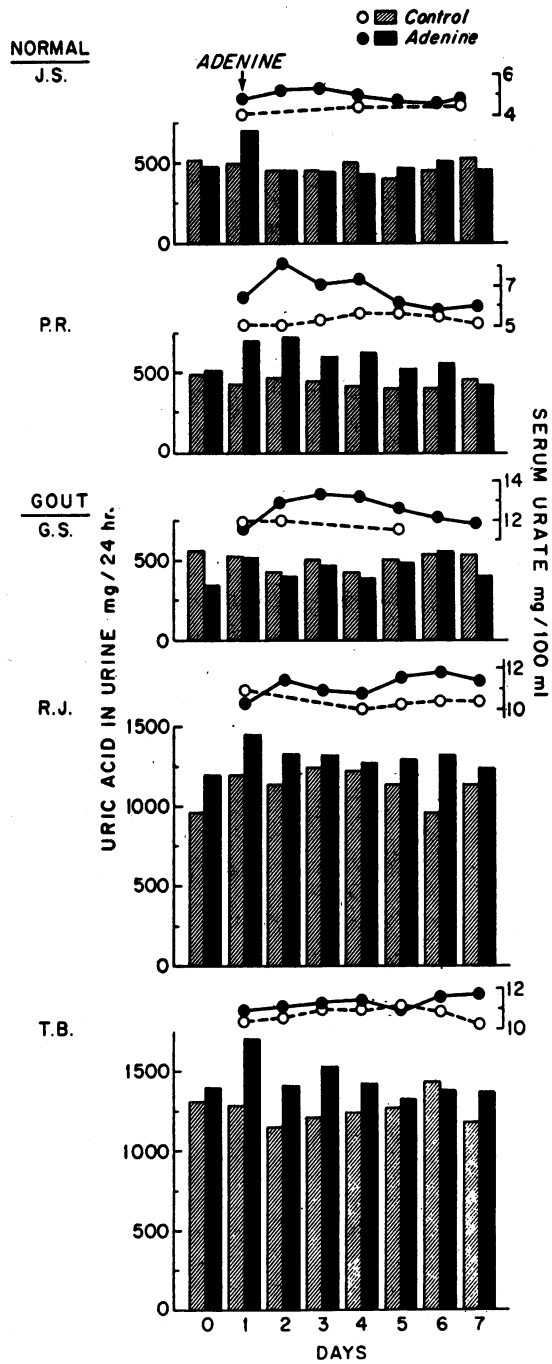


FIGURE 4 Serum urate concentration and urinary uric acid excretion during study periods with and without adenine administration.

DISCUSSION

Incorporation of glycine-^{15}N into uric acid could be diminished by several different mechanisms: (*a*) Alteration in the dynamics of the glycine pool. Such an alteration in the glycine pool should be reflected in a change in the incorporation of glycine into other glycine metabolites such as creatinine. The fact that adenine administration did not alter the incorporation of glycine-^{15}N into

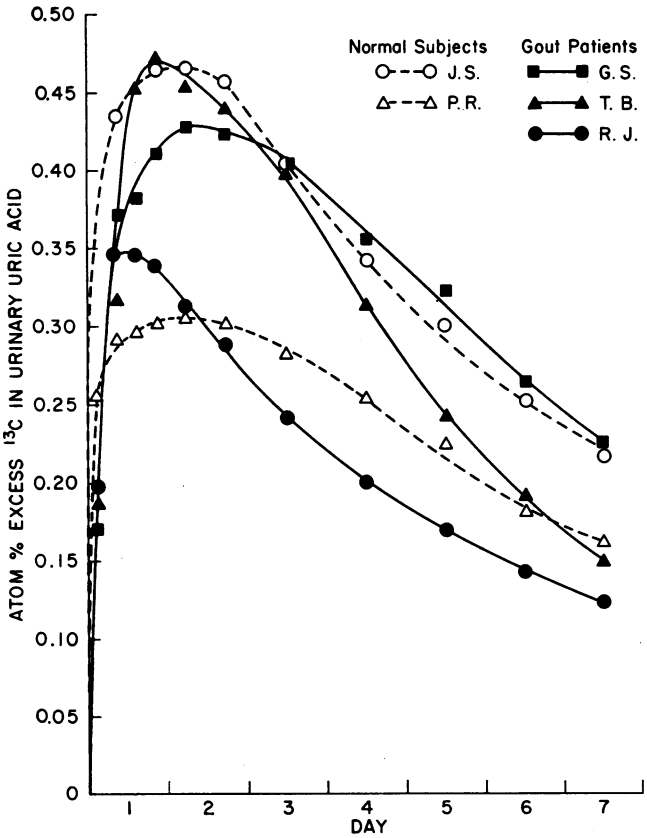


FIGURE 5 The incorporation of adenine-^{13}C into urinary uric acid in normal subjects and gouty patients. The enrichment of urinary uric acid with ^{13}C is shown for the 7 days after its administration.

urinary creatinine provides evidence against this mechanism.

(*b*) A simple dilution of isotopically labeled uric acid precursors by the added adenine. Such a dilution should not alter the relative contribution of the ^{15}N to the 1 and 3 positions as compared to the 7 and 9 positions. The ^{15}N enrichment at the 7,9 positions was decreased to a greater extent than at the 1,3 positions in three of the four subjects. Furthermore adenine administration altered the time course as well as the magnitude of ^{15}N incorporation into urinary uric acid.

(*c*) Depletion of intracellular 5-phosphoribosyl-1-pyrophosphate (PRPP) by adenine administration. Recent studies have shown that administration of much larger quantities of orotic acid, another substrate known to utilize PRPP in its metabolism, did not alter the incorporation of ^{15}N glycine into uric acid.[1]

(*d*) Inhibition of purine synthesis de novo. Although repression of enzymes of purine synthesis has been shown in bacterial systems, such a mechanism for control of purine synthesis in mammalian cells has not yet been demonstrated. Evidence for the existence of a feedback control mechanism has been presented (4–6) and activation of such a mechanism offers the most likely explanation for our results.

The suppression of glycine-^{15}N incorporation which resulted from adenine administration was comparable to that produced by administration of 4-amino-5-imidazole carboxamide (AIC) (5). The early peak of ^{15}N incorporation which characterizes gout patients who have excessive uric acid production was abolished by both substances.[2] The response of the gout patients to adenine and to AIC shows that the excessive purine synthesis found in these patients is capable of being modulated. The derangements leading to the excessive purine synthesis in these patients must, therefore,

[1] Kelley, W. N., F. M. Rosenbloom, and J. E. Seegmiller. Effect of orotic acid on incorporation of glycine N^{15} into urinary uric acid. In preparation.

[2] Seegmiller, J. E., and L. Laster. Effect of 4-amino-5-imidazole carboxamide on the incorporation of glycine N^{15} into urinary uric acid in gout. In preparation.

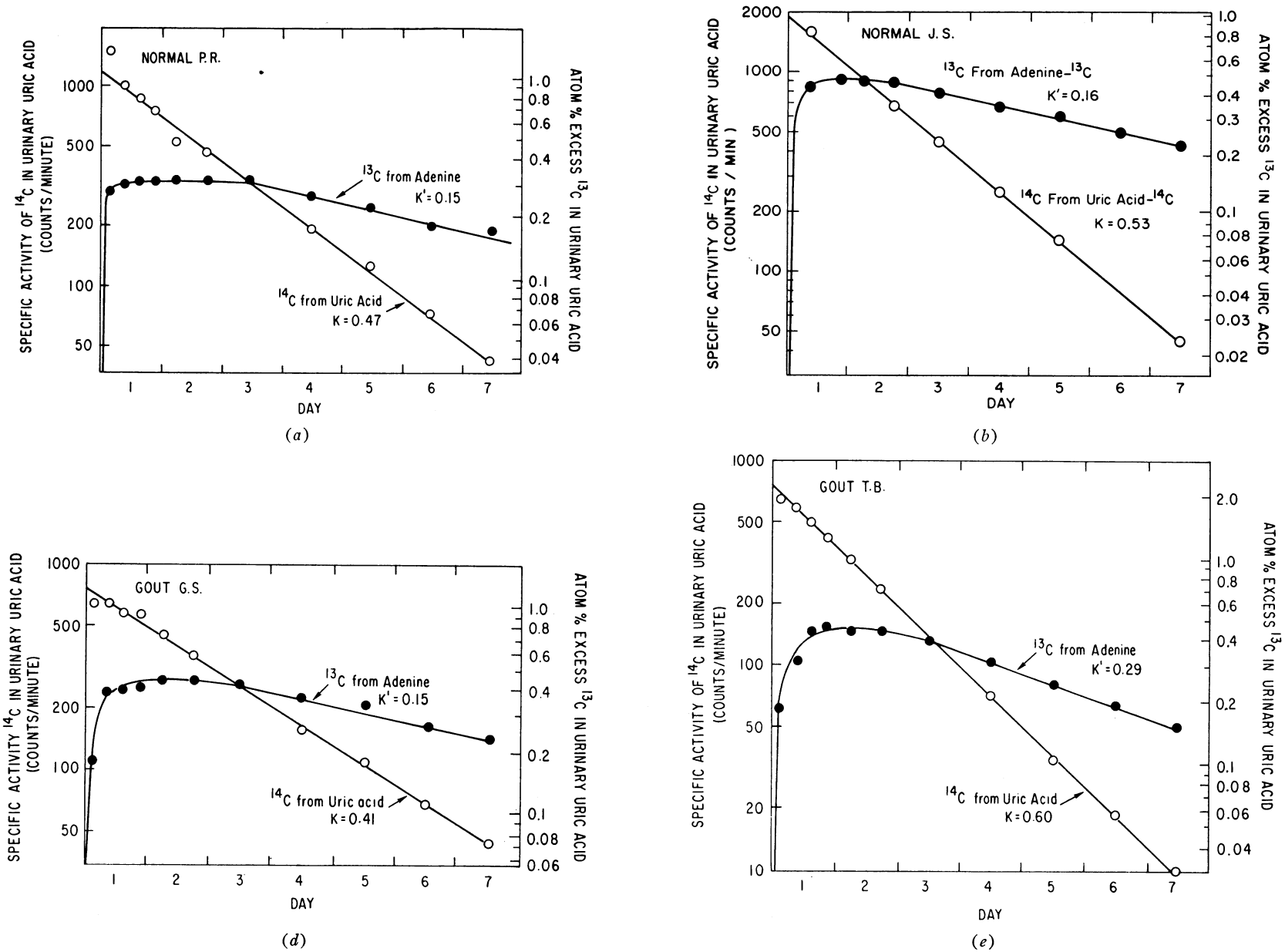
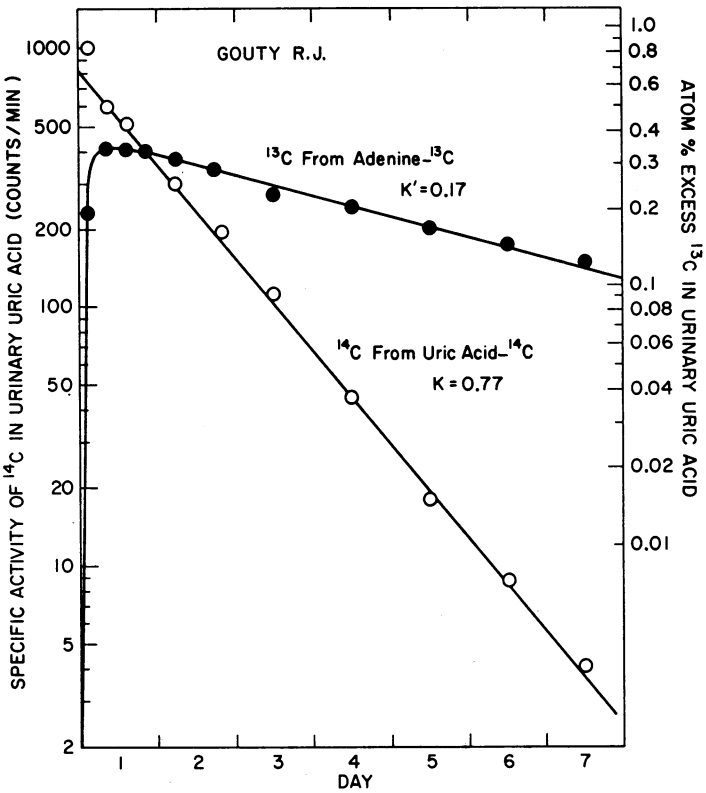


FIGURE 6 Comparison of the ^{13}C and ^{14}C content in urinary uric acid during the 7 day period after the intravenous administration of lithium urate-2-^{14}C and oral administration of adenine-8-^{13}C. Isotope content is plotted on a logarithmic scale. ^{14}C counts in uric acid were all corrected to a standard planchet thickness of 50 mg.



(*c*)

FIGURE 6 Continued.

constitute a difference in quantitative aspects of the regulatory mechanism rather than a qualitative loss of the ability to respond to these agents.

Support for this view has come from studies in which excessive purine synthesis has been associated with a complete absence of the enzyme hypoxanthine phosphoribosyltransferase (PRTase) (E. C. 2.4.2.8) in children with a familial neurological disease (28) and with a partial deficiency of the same enzyme in certain patients with gout who produce excessive quantities of uric acid (29). Patient R. J. had such a partial deficiency. The activity of the enzyme PRTase in his erythrocytes was only 1% of normal values (29). This enzyme deficit interferes with the reutilization of hypoxanthine and guanine for nucleotide formation (Fig. 1) and may be correlated with the earlier appearance of maximal ^{13}C labeling of urinary uric acid in this patient and an earlier decline in isotope abundance than was observed in the other patients (Fig. 5). The relative contribution of ^{15}N from the glutamine pool was significantly greater in this patient and in the other gout patients, than in normal subjects, as shown by the lower ratio of isotope abundance in positions 7 and 9 of uric acid as compared to 1 and 3 during control studies and after adenine administration (27). This type of ^{15}N distribution has been taken as indirect evidence of a postulated deficiency of glutaminase (30). However, no defect in glutaminase has yet been found (31). The presence of this lower ratio, in a patient who has a deficiency of PRTase, suggests that the abnormal distribution of ^{15}N is related to the presence of excessive uric acid production in some less specific manner that is not immediately apparent.

Despite the decrease in glycine-^{15}N incorporation into urinary uric acid the total amount of uric acid appearing in the 24 hr urine was not decreased by adenine administration in the present study. On the contrary, a transient increase in urinary uric acid above values found in control subjects was observed during the 2 days after adenine administration in four of the five subjects

studied, and this was greater than could be accounted for by the fraction of administered adenine which appeared in urinary uric acid. Possible explanations of this observation include a mild uricosuric effect induced by adenine or its metabolites, or an enhanced degradation of tissue nucleotides induced by adenine. An increase in urinary uric acid was also found during the 2 days after AIC administration and was attributed to a direct conversion of AIC to uric acid (5).

The rapid conversion of adenine-8-^{13}C to uric acid–^{13}C found in this study is different from the slow conversion of adenine-^{14}C to urinary uric acid previously reported (7). This discrepancy is undoubtedly related to the fact that a trace dose of adenine-8-^{14}C was used in the earlier study whereas a loading dose of adenine-8-^{13}C was used in the current study. Evidently under the conditions we used, a substantial portion of the adenine was degraded by a fairly direct pathway to uric acid to account for the prompt appearance of ^{13}C in urinary uric acid as well as the increased uric acid excreted on the day adenine was administered. As shown in Fig. 1, there is no adenine deaminase to catalyze the direct deamination of free adenine to hypoxanthine in mammalian tissue. The degradation to uric acid must, therefore, have occurred after conversion of the adenine to its nucleotide by adenine phosphoribosyltransferase (A-PRTase) or to its nucleoside by purine nucleoside phosphorylase. Although patient R. J. has only 1% of normal PRTase activity, he has a very active A-PRTase and so would be expected to incorporate adenine into nucleotides very effectively.

In addition, the incorporation of a portion of adenine-8-^{13}C into tissue nucleic acids and other tissue nucleotides is a possible explanation for the continued slow contribution of ^{13}C to urinary uric acid. A similar slow contribution of ^{13}C to urinary uric acid was observed when AIC-^{13}C was administered (5). The decline in abundance of ^{13}C in urinary uric acid after adenine administration followed an exponential decline described by the formula $dI/dt = K'I$. Where I is isotope abundance, K' is a constant, and t is time. All subjects showed K' values in the same range except T. B., who showed a value almost twice that of either control subject or the other gout patients. This discordance in K' values as well as the fact that G. S. and T. B. each had a PRTase activity in the normal range provides evidence of metabolic heterogeneity among even those gout patients who have an excessive production of uric acid.

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