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SESSION I

BIOCHEMISTRY AND METABOLISM

(*Chairman:* DR. OSWALD SAVAGE)

EFFECTS OF ALLOPURINOL IN RELATION TO PURINE BIOSYNTHESIS

BY

GEORGE H. HITCHINGS

Tuckahoe, New York, 10707, U.S.A.

It is appropriate to begin a homily with a reading from the scriptures. I should like to take as my text the eighth proposition of Garrod (1863):

> The causes which predispose to gout . . . are either such as to produce an increased formation of uric acid in the system, or which lead to its retention in the blood.

In the century since these words were written their fundamental validity has remained unchallenged. It is still a matter of controversy whether a reduced renal clearance of uric acid is a cause or a consequence of prolonged hyperuricaemia, but the overproduction of purine end-product by a substantial proportion of gouty subjects has been firmly established through the use of isotopically labelled precursors.

Therapy, until recently, consisted of anti-inflammatory agents and uricosuric drugs. The first may be regarded as strictly palliative. Uricosuric drugs successfully reduce serum urate concentrations in a large segment of the gouty population, but leave something to be desired in patients with impaired renal function, urate stones, and nephropathies, and are unable to cope with the large surges of urate which may follow the therapy of certain malignant conditions. There have been a few attempts to deal with urate overproduction at its source, but these were directed toward the suppression of purine biosynthesis at an early step, using azaserine or diazo-oxo-norleucine, and no selectivity in the effects could be achieved (Zuckerman, Drell, and Levin, 1957; Grayzel, Seegmiller, and Love, 1960).

Allopurinol was designed to reduce the formation of uric acid from its immediate precursors, hypoxanthine and xanthine, through inhibition of the enzyme xanthine oxidase, and thus to avoid any deep-seated blockade of purine anabolism. This proposal seems simple and obvious, but it raised a number of important questions. In the first place it was by no means certain that the classical picture of uric acid formation was the true one. Indeed, at the Ciba Conference on Purines in 1956, the possibility that xanthine oxidase is not involved in uric acid formation was seriously debated (Greenberg, Bergel, Lewin, and Buchanan, 1957).

Traditionally, uric acid was viewed as the end-product of the action of xanthine oxidase on hypoxanthine and xanthine, which were formed in turn by the de-amination of the adenine and guanine of the nucleic acids (Fig. 1, overleaf). The discovery by Greenberg and Jaenicke (1957) and Buchanan (1960) that inosinic acid (hypoxanthine ribonucleotide) is the primary and core substance of purine biosynthesis was followed by the elaboration of the biochemical pathways whereby purine interconversions take place at the ribonucleotide level as shown in Fig. 2, overleaf (Buchanan, 1960; Gutman and Yü, 1965). It was thus tempting to postulate that transformations might occur mainly in this way, and xanthine oxidase, in this view, would be involved only secondarily with small quantities of free purines liberated through hydrolytic mechanisms.

Both views seem to be half correct. Recent

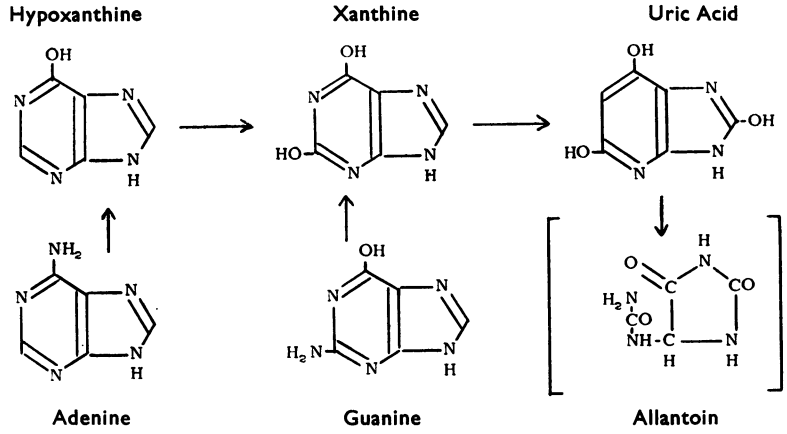

Fig. 1.—Pathways of catabolism of free purines.

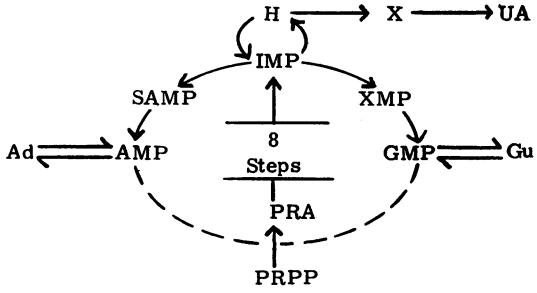

Fig. 2.—Diagrammatic representation of biosynthesis and inter-conversions of purine nucleotides.

H—Hypoxanthine, X—Xanthine, UA—Urate, G—Guanine, A—Adenine, IMP—Inosinate, XMP—Xanthylate, AMP—Adenylate, SAMP—Succinoadenylate, GMP—Guanylate, PRA—Phosphoribosylamine, PRPP—Phosphorylribosylpyrophosphate.

In the centre of the Figure is indicated the sequence of reactions by which the purine ring is completed (in IMP) by stepwise additions of one to three atoms to the first atom of the purine ring—the N atom of PRA. Subsequent conversions of IMP to AMP and GMP proceed *via* intermediates (SAMP, XMP) at the ribonucleotide level.

studies of xanthinuric patients, in whom xanthine oxidase activity seems to be nearly if not entirely absent, show high xanthine to hypoxanthine ratios (Ayvazian, 1964; Engelman, Watts, Klinenberg, Sjoerdsma, and Seegmiller, 1964).[1] These findings suggest that the main pathway to urate is from inosinate to xanthylate, through the action of inosinate dehydrogenase, followed by liberation of xanthine and its oxidation by xanthine oxidase.

Inhibitors of xanthine oxidase are diverse and numerous. During the 1950s, when this approach first came under consideration, Mrs. Lorz in our laboratory tested several hundred purines, pyrimidines, and analogues for their abilities both to serve as substrates for and to inhibit xanthine oxidase (Lorz and Hitchings, 1950, 1956). Among this group there were twenty or thirty strong inhibitors,[2] a few as strong as allopurinol, but the latter had two especially favourable properties. It was not only an inhibitor but also a substrate for the enzyme (Lorz and Hitchings, 1956; Elion, Taylor, and Hitchings, 1964) (Fig. 3), and the product of the reaction was

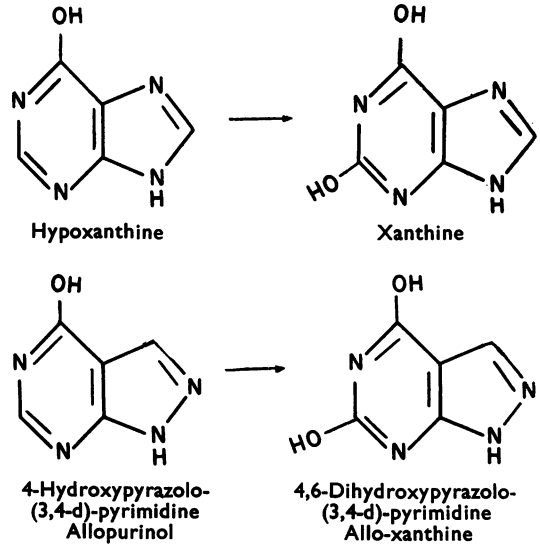

Fig. 3.—Structural formulae of oxypurines and oxypyrazolopyrimidines.

[1] When a xanthinuric patient was treated with high doses of allopurinol, a fall in this ratio occurred (Goldfinger, Klinenberg, and Seegmiller, 1965). This suggests a secondary site of action of allopurinol. This might be competition with hypoxanthine for inosinate pyrophosphorylase, displacement of hypoxanthine from inosine under the influence of inosine phosphorylase (transferase), or possibly at the nucleotide level an inhibition of inosinate dehydrogenase.

[2] Feigelson and others (1957b) investigated the 4-amino- and 4-amino-6-hydroxypyrazolo (3,4-d) pyrimidines (Falco and Hitchings, 1956; Robins, 1956), but not allopurinol or allo-xanthine as has been erroneously stated (McCollister and others, 1964). The amino-hydroxy derivative has activity comparable to that of allo-xanthine both *in vitro* and *in vivo* (Elion and others, 1966b).

also an inhibitor (Elion and others, 1964). In addition, neither allopurinol nor its oxidation product, allo-xanthine, seemed to be involved in purine anabolic pathways, since neither inhibited the growth of *Lactobacillus casei* (Hitchings, Elion, Falco, Russell, Sherwood, and VanderWerff, 1950) or other bacteria, and neither exhibited any effect on the growth of transplantable tumours.[3] In a final evaluation, it may be that the unusual pharmacokinetic properties of allo-xanthine will have been found to have contributed rather significantly to the success of allopurinol (Elion, Taylor, and Hitchings, 1966b).

The foreseeable consequences of inhibiting xanthine oxidase *in vivo* included:

(1) The accumulation of precursors;

(2) Interference with other than the target functions of xanthine oxidase;

(3) Possible failure, through the unleashing of enzyme control mechanisms that might regenerate new enzyme as fast as it was inactivated.

In view of its wide distribution (De Renzo, 1956), xanthine oxidase should probably be regarded as a constitutive enzyme; however, adaptive changes in its activity have been reported. Thus, its level is raised during liver regeneration (Feigelson, Feigelson, and Gross, 1957a), folate (Keith, Broach, Warren, Day, and Totter, 1948) and vitamin E deficiencies (Dinning, 1953), and appears to be dependent on dietary protein and minerals (Westerfeld and Richert, 1951). Of more direct moment were reports that xanthine oxidase is, at least in part, inducible by the administration of xanthine (Stirpe and Della Corte, 1965; Feigelson, Feigelson, and Wood, 1954; Dietrich, 1954). It seemed possible, therefore, that inhibition of the enzyme would be followed by raised levels of xanthine which in turn would induce more enzyme, and one would thus follow a helical course culminating in frustration. So far it has not been possible, because of technical difficulties, to follow enzyme activity levels serially during therapy with allopurinol. But it is apparent from both animal and clinical data that no important changes in enzymic activities occur. The data of Table I are illustrative. They show not only that the extent of inhibition of purine catabolism is closely dose-dependent, but also that the degree of inhibition is quickly established and remains relatively constant over a period of several months at least. Similarly, the drug appears to produce consistent effects in patients over protracted periods (Yü and Gutman, 1964; Rundles, Metz, and Silberman, 1966b; Klinenberg, Goldfinger, and Seegmiller, 1965).

TABLE I

EFFECTS OF ALLOPURINOL ON PURINE METABOLISM IN THE DOG*

Dose (mg./kg.)	Time (wks)	Urinary Excretion (mg./day)	
		Allantoin	Oxypurines
0	—	383±45	*c.* 2
30	2	217±56	51±5
	4	233±58	57±10
	6	244±47	79±11
	17	246±29	74±10
90	1	106±13	98±17
	3	111±25	86±8
	5	84±16	120±4
	7	106±31	119±5
	17	123±25	95±9

*Dogs received the drug daily in two divided doses. Five animals on each dose.

Many functions have been attributed to xanthine oxidase (De Renzo, 1956). Not only a wide variety of purines, pyrimidines, pteridines, and other heterocycles, but also aldehydes may be substrates. A release of ferritin iron from liver to plasma *via* the reduction of ferric ferritin by reduced xanthine oxidase has been postulated (Mazur, Green, Saha, and Carleton, 1958). This topic will be discussed elsewhere in this symposium; but it might be worthwhile to mention at this point that there were no significant haemosiderin deposits in the livers of animals on long-term toxicity trials, nor any accumulation of iron in the livers of rats receiving allopurinol over a period of 38 weeks (Bushby and Udall, 1966). Patients receiving the drug have exhibited normal haemodynamics, and no evidence of disturbance of iron metabolism. It is, therefore, difficult to evaluate the report that rats receiving allopurinol and added iron accumulated liver iron (Powell and Emmerson, 1956).

The chief hazard appeared to be the accumulation of xanthine. Xanthine (in contrast to hypoxanthine, allopurinol, and allo-xanthine) is only a little more soluble than uric acid (Krakoff and Meyer, 1965; Klinenberg and others, 1965). Furthermore, the clearance of oxypurines at normal plasma levels appeared to be only slightly higher than that of uric acid (Gjørup and Poulsen, 1955). The possibility therefore existed that oxypurines, more particularly xanthine, might accumulate at levels approaching the saturation levels with respect to solubility. Indeed, in small animals, the limiting factor with

[3] Unpublished data from Wellcome Research Laboratories and CCNSC, and Cancer Chemotherapy Screening Data in *Cancer Res.* (1958), **18**, 102; and (1963), **23**, 1112.

respect to toxicity of allopurinol was crystallization of xanthine in the renal tubules. Xanthine precipitation has not occurred in subjects treated with allopurinol. Serum oxypurines are elevated only slightly during therapy since the renal clearances of these are much higher than previously supposed.[4] Urinary purines are divided among three principal forms: uric acid, hypoxanthine, and xanthine; each of these exerts its own or a slightly raised solubility in the presence of the others. The major difference, however, between man and the lower species is found in the vastly greater purine metabolism of the latter in terms of either body weight or water flux. This is illustrated by the data of Table II. The total purine end-product in a 10-kg. dog is essentially equal to that of a 70-kg. man, and is excreted in approximately one-sixth the volume of urine. Moreover, the bulk of the oxypurine excreted is xanthine which, with even a 50 per cent. inhibition of purine catabolism, becomes a major excretory product and its concentration exceeds the solubility limit. The difference is even more striking in the rat and still more in the mouse. Superficially, therefore, allopurinol is a substance that exhibits a progressively greater toxicity the smaller the species in which it is tested. It is apparent that deletion of xanthine oxidase would be a lethal mutation in any of these lower species; indeed the deletion of uricase in the hominidae required many compensatory adjustments in renal physiology. One might speculate that this deletion occurred in a species with a relatively low purine metabolism and high water flux and that the deletion of uricase as well as that of xanthine oxidase could not be tolerated in species with high purine metabolism.

TABLE II

PURINE METABOLISM IN RELATION TO WATER FLUX IN VARIOUS SPECIES
URINARY PURINE END-PRODUCT

Species	Weight (kg.)	End-Product (mg.)	Urine Volume (ml.)	Xanthine* (mg./ml.)
Man	70	420	1500	0·10
Dog	10	400	250	0·56
Rat	0·1	24	5	1·68

*50 per cent. inhibition; xanthine = 70 per cent. of decrease (35 per cent of total).

One cannot feel completely sanguine about the urinary xanthine levels in man. In the example given in Table II, the xanthine concentration is perhaps 70 per cent. of the saturation level, and higher levels could presumably occur. It is reassuring that, in xanthinuric subjects, xanthine stone formation occurs only at levels considerably higher (Seegmiller, 1965) than those reached even by vigorous therapy with allopurinol (Rundles, Elion, and Hitchings, 1966a) and that xanthine deposition in soft tissues does not occur even in xanthinuria. Nevertheless, one should regard xanthine renal stone formation as a possibility and every effort should be made to maintain a high water flux in patients receiving the drug. (Total urinary volume is more important than *p*H in maintaining xanthine in solution, since its solubility begins to rise appreciably only as the *p*H approaches the pKa value of the substance, 7·7).

The possible effect of elevations of systemic xanthine levels on tumour growth also had to be considered. Bergel, Bray, Haddow, and Lewin (1957) had found tissue of the Bittner mammary tumour to have a lower xanthine oxidase content than control mammary tissue. Similarly, Wheeler, Alexander, and Morris (1964) found an inverse correlation between xanthine oxidase activity and rate of growth of a series of hepatomata. A possible interpretation was that such tumours utilize purine end-products for growth and that the levels of these would be regulated by the xanthine oxidase content of the tissue. This view received some support from the findings of Bergel and others (1957) and Haddow, deLamirande, Bergel, Bray, and Gilbert (1958) that the growth of some mouse tumours could be inhibited by the administration of large quantities of the enzyme to host animals, and the demonstration that this did indeed raise the xanthine oxidase activity of the host liver (Haddow and others, 1958). In contradistinction to the above, Feigelson, Davidson, and Robins (1957b) found a direct correlation between xanthine oxidase inhibition and tumour inhibition in a series of pyrazolopyrimidines.

In fact, allopurinol had been found not to influence the growth rates of a substantial spectrum of transplantable rodent tumours[3] (Skipper, Robins, Thomson, Cheng, Brockman, and Schabel, 1957). A more definitive experiment has been carried out by Alexander, Wheeler, Hill, and Morris (1966). These authors selected a slowly-growing rat hepatoma which has a high xanthine oxidase activity, and compared the rates of tumour growth in controls, and in animals treated chronically with sufficient allopurinol to inhibit purine catabolism in the tumour. The rates of growth of the tumours were identical in both groups. Perhaps it is pertinent to point out that oxypurine levels remain low even with a substantial inhibition of xanthine oxidase activity.

[4] The low renal clearances for oxypurines reported by Gjørup and Poulsen (1955) reflect the low levels of these substances that occur naturally, and possibly the difficulties inherent in the determination of the quantities of the bases present in serum, and the rapidity with which their amounts are augmented in drawn blood through catabolism of the adenine nucleotides of the erythrocytes (Petersen, Jørni, and Jørgensen, 1965).

The primary route of metabolic inactivation of 6-mercaptopurine (6-MP) and related substances was found to be *via* oxidation of the purine ring in the 2 and 8 positions (Elion, Bieber, and Hitchings, 1954; Hamilton and Elion, 1954; Elion, Mueller, and Hitchings, 1959), catalysed by xanthine oxidase. Metabolic studies with 6-MP and other purines provided an opportunity to test the possibility of inhibiting xanthine oxidase *in vivo* (Elion, Callahan, Nathan, Bieber, Rundles, and Hitchings, 1963a). In the mouse, the conversion of 6-MP to thiouric acid (TU) was inhibited by allopurinol in a dose-dependent fashion, and this was accompanied by increased tumour-inhibitory activity. Moreover, allopurinol-treated animals excreted large amounts of oxypurines, principally xanthine. The effect on 6-MP metabolism, therefore, was clearly metabolic in origin rather than a result of its renal clearance (Elion, Callahan, Rundles, and Hitchings, 1963b). Similarly, the administration of allopurinol was followed by significant increases in the excretion of oxypurines in the rat and dog. This encouraged trials in man. These could be conveniently carried out in patients with chronic granulocytic leukaemia where the effects of allopurinol on the metabolism of 6-MP could be compared with the effects on endogenous metabolism, and the various parameters related to xanthine oxidase inhibition could be explored with a low degree of patient risk (Hitchings, 1963; Rundles, Wyngaarden, Hitchings, Elion, and Silberman, 1963). The effectiveness of allopurinol in inhibiting the formation of TU from 6-MP was confirmed and concomitant measurements of serum and urinary urates also confirmed effects on endogenous purine metabolism.[5] Furthermore, rapid resolution of raised leucocyte counts was not accompanied by raised serum or urinary uric acid values (Rundles and others, 1963). Dose-response curves were explored and it was possible to observe patients carefully for the appearance of side-effects. The results were wholly reassuring and led rapidly to more extensive explorations of allopurinol therapy for hyperuricaemias (Krakoff, 1965; Yü and Gutman, 1964; Rundles and others, 1966b).

A dose-response curve carried out on one patient is shown in Fig. 4. As the allopurinol dosage was increased, the urinary uric acid fell progressively from an initial value of nearly 600 mg. to an average value of 265 mg. On a log-dose scale, a straight line drawn through the points could be extrapolated to a value of nil uric acid at a dose of about 5 g. allopurinol per day. It is significant that the slope of the line representing oxypurine increase is lower than that for uric acid decrease. At the dosage level of 800 mg./day, the oxypurines excreted amounted to 170 mg. The total purine end-product, therefore, was 435 mg. compared with an initial value of about 600 mg. The lowering of total purine excretion as a result of therapy has been a matter on which there has been some disagreement, since it was found by Yü and Gutman (1964) and Rundles and others (1966b) but not by Klinenberg and others (1965) or Vogler, Bain, Huguley, Palmer, and Lowrey (1966). In general, as suggested by Yü and Gutman (1964), a lowering of total purine excretion was found more frequently among patients who were classified as "hyper-excretors" than among those classed as "normo-excretors".

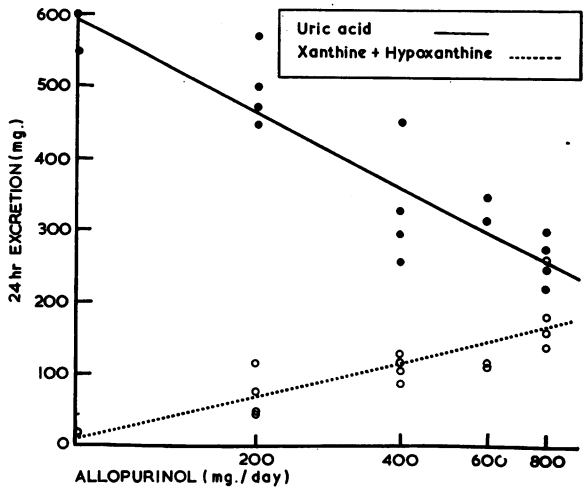

Fig. 4.—Dose : Response relationship of allopurinol.
Patient BBM was treated intermittently. Three of the values at 800 mg./day represent a single day's treatment; each other point represents one of two successive days' treatments. The values represent 24-hr excretions.

A plot of pretreatment purine excretion *v.* the decrease after therapy gave a scattergram (*cf.* Fig. 5, overleaf) which suggests a rough correlation between the initial degree of overproduction and the later "deficit" in purine end-product.

A more precise description of this effect is possibly to be found in dose-response studies in animals. In the rat, for example (Fig. 6), allantoin excretion decreased progressively with increased dosage; oxypurine excretion at first rose, but then levelled off

[5] The oxidation of 6-MP to TU was exceedingly sensitive to the inhibitor. Effects were observed with doses as small as 50-75 mg./day and extensive inhibition (60-90 per cent.) could be seen with doses of 200-300 mg./day (Rundles and others, 1963). The inhibition of urinary urate formation was about half as extensive. The greater sensitivity of 6-MP oxidation is probably to be attributed to the lower turn-over number of 6-MP as compared with hypoxanthine or xanthine as substrate, since the binding constants for the three substrates are comparable.

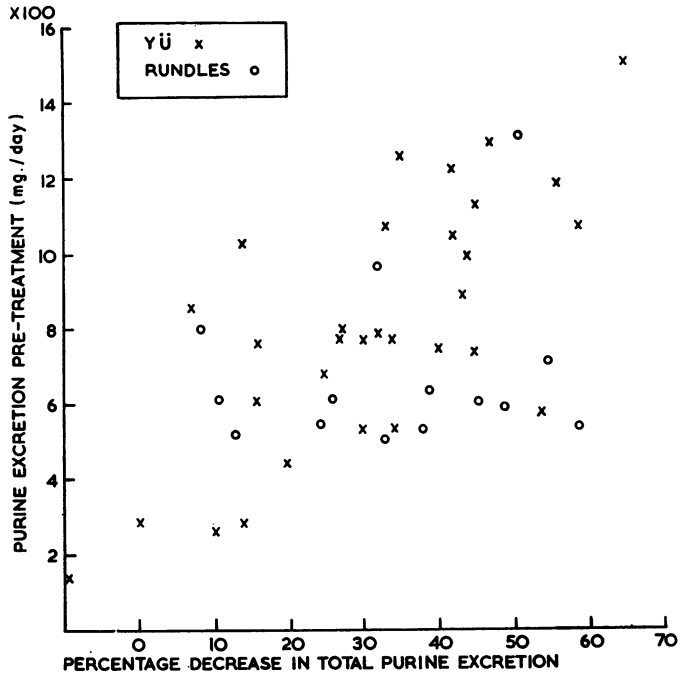

Fig. 5.—Decrease in purine excretion as a function of initial urinary urate.

$$\frac{\text{Initial Urate—(Oxypurine + Urate during Therapy)}}{\text{Initial Urate}} \times 100 = \text{Percentage Decrease in Total Purine Excretion.}$$

Values calculated from data of Yü and Gutman (1964) and Rundles and others (1963).

at the higher levels of allopurinol dosage. If one subtracts the sum of allantoin and oxypurines from the control value for allantoin, one obtains figures for the change in total purine end-product. The decrease in total purine end-product is seen to be a direct function of dosage, and rises from levels that would be inapparent (10-20 per cent.) at the lower dosage levels, to values in the neighbourhood of 50 per cent. at a dose of 50 mg./kg. It seems probable that there is no real disagreement among investigators with regard to the occurrence of a "deficit" in total purine end-product resulting from therapy. The differences reported reflect patient selection, dosage, and the complicating factor of tophi which, during early therapy, may contribute significantly to the urinary urate by their partial solubilization, thus obscuring part of the decrease in urate formation.

Two hypotheses have been put forward to explain the diminished purine turnover. The first suggestion was that allopurinol is converted into a nucleotide that inhibits glutamine-PRPP amidotransferase (Fig. 2) (McCollister, Gilbert, Ashton, and Wyngaarden, 1964). However, no trace of allopurinol could be found in the nucleotide pool, nor was there a detectable incorporation of the drug into the nucleic acids (Elion, Kovensky, Hitchings, Metz, and Rundles, 1966a). A more plausible explanation

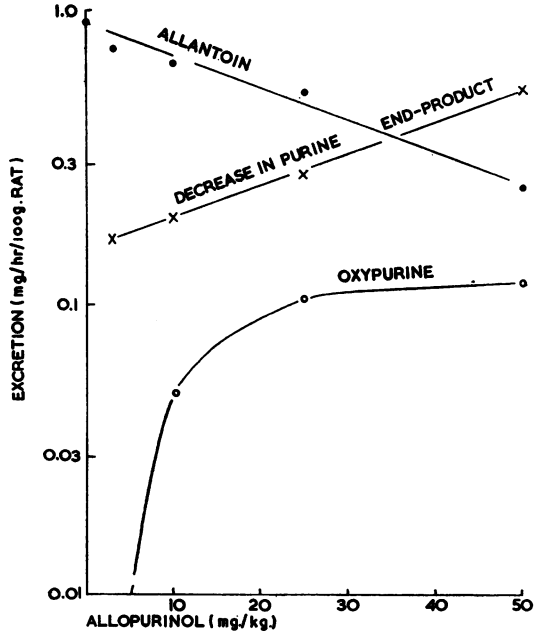

Fig. 6.—Dose : Response to allopurinol in the rat.
Each point represents the average of individual determinations on four male and four female rats. The decrease in total purine end-product was calculated by subtracting the sum of allantoin and oxypurine values from the control allantoin value of 0·92 mg./hr/100 g. rat.

derives from the effects of allopurinol on the protection of hypoxanthine and xanthine from oxidative destruction. When either of these oxypurines is given alone, it is converted almost entirely to urinary end-products, and little or none is utilized for nucleic acid synthesis. However, when allopurinol is administered with labelled hypoxanthine or xanthine, a substantial portion of the purine administered is found in nucleic acid adenine and guanine (Pomales, Bieber, Friedman, and Hitchings, 1963; Pomales, Elion, and Hitchings, 1965). Similarly, when Rundles administered inosine to a patient, 73 per cent. was recovered as uric acid. When allopurinol was added to the regimen, the recovery as urate was < 10 per cent, as oxypurine *c.* 35 per cent., leaving a large balance unaccounted for (Rundles and others, 1966b). Both types of experiment suggest that when oxypurines are protected from catabolic destruction, a considerable degree of re-utilization occurs. Such re-utilization tends to increase the pools of adenylic and guanylic acids, and these natural nucleotides control the initial phosphoribosylpyrophosphate amidotransferase reaction of purine biosynthesis (Wyngaarden and Ashton, 1959). This re-utilization, in turn, diminishes the total filtered load of oxypurines.

Experiments of this type may also provide clues to the nature of the uric acid overproduction that occurs in a quarter or more of gouty subjects. Such subjects convert isotopically labelled glycine into urate at an accelerated rate, giving rise to the suggestion that there exists an alternative "shunt" pathway from glycine to urate (Benedict, Roche, Yü, Bien, Gutman, and Stetten, 1952). Without attempting a detailed analysis of all the factors involved, it is possible to put forward an alternative interpretation. If purine biosynthesis is controlled primarily by negative feedback on phosphoribosylpyrophosphate amidotransferase by adenine and guanine ribonucleotides, a lower than normal rate of synthesis of one or both of these from inosinate would account for the excessive purine biosynthesis. A slightly defective xanthylate aminase, for example, would be able to maintain normal guanylate concentrations only when the xanthylate pool was increased. Since the reaction involving xanthylate pyrophosphorylase and xanthine is an equilibrium, increased concentrations of xanthylate would expose larger amounts of xanthine to catabolic oxidation. A displacement of the xanthylate $\rightleftharpoons$ xanthine equilibrium toward xanthine would have a similar effect. Excessive activity of xanthine oxidase also would drain the xanthine–xanthylate pool. In any of these aberrations xanthine would be converted to urate at a greater than normal rate giving the appearance of a "shunt" pathway. The excessive biosynthesis, in turn, would drain the glutamine pool (two molecules of which are required for every purine molecule synthesized) and account for the excessive conversion of glycine nitrogen to glutamine amide nitrogen observed by Gutman and Yü (1965) and possibly for the deficient renal production of ammonium ion from glutamine also observed in urate "hyper-secretors" (Gutman and Yü, 1965). The protection of xanthine from oxidative catabolism by means of allopurinol therapy would raise the levels of xanthine and xanthylate and in turn facilitate the formation of guanylate, permitting normal control of *de novo* synthesis.

Summary

Allopurinol and its metabolic product, alloxanthine, by inhibiting xanthine oxidase *in vivo*, reduce the formation of uric acid and its analogues from mono- and disubstituted purines. Purine end-product is excreted as a mixture of uric acid, hypoxanthine, and xanthine. Plasma levels of hypoxanthine and xanthine remain low because of their high renal clearance rates. Total purine end-product excretion is usually reduced, especially in the "hyper-excretor", probably as the result of re-utilization of hypoxanthine and xanthine in purine anabolism.

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