Table 2. Effect of insulin on lipolysis induced by cyclic AMP and dibutyryl cyclic AMP. Values are means ± standard error of the mean; n, number of observations per datum.

Insulin conc. (molar)	Nanomoles of glycerol released per 10^5 cells per 1 hour*				
	Control ($n = 10$)	Cyclic AMP		Dibutyryl cyclic AMP	
		2.5 mM ($n = 6$)	5.0 mM ($n = 5$)	0.5 mM ($n = 8$)	1.0 mM ($n = 8$)
0	13.2 ± 1.5	27.6 ± 3.7	39.4 ± 2.8	48.6 ± 7.3	147.5 ± 16.1
1.4×10^{-11}	20.1 ± 3.4	4.2 ± 2.4	26.5 ± 6.6	28.3 ± 2.6	112.0 ± 13.2
1.4×10^{-10}	26.2 ± 3.2	8.8 ± 7.3	41.0 ± 5.4	26.2 ± 3.6	118.9 ± 16.0
6.6×10^{-9}	39.1 ± 6.0	0.8 ± 0.2	33.5 ± 7.2	74.2 ± 4.0	263.7 ± 10.5

* The condition of experiment is the same as in Table 1.

nucleotides further, the effect of low and high concentrations of insulin was tested. At low concentrations of cyclic AMP, low concentrations of insulin exert an antilipolytic effect (see Table 2). However, this effect, in most cases, is nullified at higher concentrations of cyclic and dibutyryl cyclic AMP. The endogenous lipolytic effect of both nucleotides was markedly reduced by 2 μunit ($1.4 \times 10^{-11} M$) of insulin, but not at high concentrations of insulin. This suggests a competitive mechanism of inhibition between insulin and both forms of cyclic AMP. Hepp *et al.* (*2*) reported that insulin had no effect on lipolysis induced by cyclic and dibutyryl cyclic AMP, but that their measurements were made only at high concentrations of insulin.

It has previously been assumed that the increased potency of dibutyryl cyclic AMP over cyclic AMP may be due to its more nonpolar nature, which permits easier penetration of the cell membrane in the biological system. The data presented here suggest that other explanations, in addition to solubility, must be offered for the divergent action of these two nucleotides, since the more nonpolar analog of the two cyclic nucleotides, dibutyryl cyclic AMP, showed no stimulatory effect on glucose oxidation, whereas the presumed more polar analog, cyclic AMP, showed significant stimulation of glucose oxidation and lipogenesis. The question may now be raised as to the nature and structure of the "second messengers" in biological systems. Apparently, profound differences are introduced by the addition of the two butyrate moieties on the cyclic AMP molecule. These data, furthermore, serve to emphasize the caution that needs to be exercised in assigning biological roles to derivatives of cyclic nucleotides that do not in fact possess the same structure.

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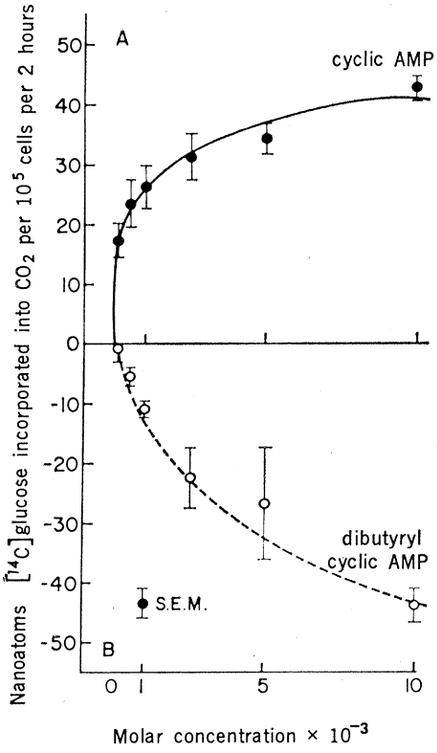

Fig. 1. Dose response curves for cyclic AMP (A) and dibutyryl AMP (B) as measured by glucose uptake and oxidation. The results are reported as nanoatoms of $^{14}CO_2$ released per 100,000 cells per 2 hours above or below the base line. The incubation system consists of 100,000 fat cells, 0.5 mM glucose in a Krebs-Ringer and bicarbonate buffer with 4 percent albumin (pH 7.4) in a total volume of 2 ml. Incubation was carried out under an atmosphere of 95 percent O_2 and 5 percent CO_2 at 37°C in the presence or absence of various concentrations of cyclic AMP or dibutyryl cyclic AMP. Each point is an average of five observations.

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Allopurinol: Alteration in Pyrimidine Metabolism in Man

Abstract. *In addition to its well-established inhibitory effect on uric acid synthesis, allopurinol appears to alter substantially pyrimidine metabolism, as evidenced by a striking increase in the urinary excretion of orotidine and orotic acid. Allopurinol ribonucleotide and xanthosine 5′-monophosphate are potent inhibitors of human erythrocyte orotidylic decarboxylase and provide a possible mechanism for this effect.*

Allopurinol [4-hydroxypyrazolo(3,4-*d*)pyrimidine] is widely used in the treatment of hyperuricemia and gout. This agent, an analog of hypoxanthine, is a potent inhibitor of xanthine oxidase, which catalyzes the conversion of hypoxanthine to xanthine and xanthine to uric acid (*1*). Accordingly, its administration in man leads to a prompt decrease in the formation of uric acid and an increase in the excretion of hypoxanthine and xanthine (*2*). In the present study, we demonstrate that the administration of allopurinol and its major metabolic product, oxipurinol, also produces a substantial inhibition of de novo pyrimidine biosynthesis.

The urinary excretion of purines and pyrimidines in six patients with gout, before and during allopurinol therapy, was assessed with an ultraviolet analyzer (*3*). With the use of this instrument, low molecular weight constituents in urine are separated by automated high-resolution, high-pressure, anion-exchange chromatography and those substrates exhibiting absorbance in the ultraviolet spectrum are detected with a

Table 1. Effect of allopurinol and oxipurinol on the urinary excretion of purines and pyrimidines. Urine was collected for 24-hour periods at room temperature with 3 ml of toluene as preservative. Uric acid content (in milligrams per 24 hours) was determined by a specific enzymatic assay (*12*). The excretion of hypoxanthine, xanthine, orotidine, and orotic acid (in milligrams per 24 hours) was calculated from estimation of peak areas on the ultraviolet chromatogram (*13*). The short-term studies were conducted after a period of equilibration on a purine-free diet. Duration of therapy is given in days (d) or years (y).

Subject	Drug	Dose (mg/day)	Duration of therapy	Urine volume (ml/24 hr)	Uric acid	Hypo-xanthine	Xanthine	Orotidine	Orotic acid
W.J.	None*			1900	304	9.7	8.6	8.3	< 1.9
	Allopurinol	400	4d	2000	135	19.5	58.7	52.0	29.8
	Oxipurinol	400	3d	1000	163	23.1	28.8	34.9	32.3
B.D.C.	None			1350	374	5.8	4.7	3.2	< 1.4
	Allopurinol	200	3d	1880	280	16.5		31.7	17.6
	Oxipurinol	200	3d	1480	250	18.1	26.8	59.3	21.7
E.D.B.	None			2020	368	7.7	5.1	10.0	< 2.0
	Allopurinol	400	4d	1320	162	5.6	22.0	24.6	8.2
	Oxipurinol	400	4d	1280	205	6.2	26.4	36.2	8.4
J.K.P.	None*			1680	164			10.2	< 1.7
	Allopurinol	800	3d	1750	72			121.0	11.4
	Oxipurinol	800	3d	1715	132			174.5	11.2
J.E., Jr.	Allopurinol*	300	6y	1516	393			40.0	
O.E.	Allopurinol*	400	6y	2000				28.9	

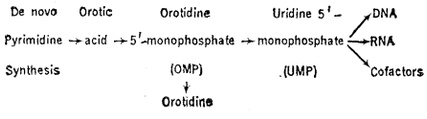
* Regular diet.

Beckman DB-G spectrophotometer. The expected decrease in excretion of uric acid and increase in excretion of hypoxanthine and xanthine was observed with institution of therapy (Table 1). In addition, allopurinol, as well as the several major products of its metabolism, oxipurinol and allopurinol ribonucleoside, was readily identified. However, two additional ultraviolet absorbing peaks were observed during treatment with allopurinol which could not be attributed to the known effects or metabolites of this drug.

The first unknown peak, eluting 25 ml after uric acid, was collected and identified as orotidine (> 95 percent purity) by several methods including (i) its ultraviolet spectrum at *p*H 12.0, 7.0, and 1.0; (ii) ascending chromatography in three different solvents; (iii) high-voltage electrophoresis in borate buffer, *p*H 9.0; and (iv) gas chromatography. The second unknown peak, eluting 123 ml after uric acid, was found to be identical with orotic acid (> 90 percent purity) by gas chromatography and mass spectroscopy. Authentic samples of orotidine and orotic acid were found to elute from the anion-exchange column at the same position as the first and second unknown peaks, respectively.

Orotidine and orotic acid were not detectable (< 2.0 μg/ml) in urine obtained from six nongouty subjects and only small quantities of orotidine were noted in urine from four gouty patients prior to the institution of allopurinol therapy (3.2 to 10.0 mg/day) (Table 1) (*4*). Treatment with allopurinol in usual therapeutic doses ranging from 200 to 800 mg/day produced a striking increase in the excretion of orotidine (24.6 to 121.0 mg/day) in these four patients and a substantial increase in the excretion of orotic acid (8.2 to 29.8 mg/day). Two patients who had been treated continuously with allopurinol for 6 years excreted quantities of orotidine that were similar to those observed in three of the four patients given the drug for 3 to 4 days. The substitution of oxipurinol for allopurinol in four patients resulted in a further increase in orotidine excretion in three of the four. The magnitude of the increase in excretion of orotidine during allopurinol and oxipurinol therapy was approximately the same as the increase in xanthine excretion and substantially greater than the increase in hypoxanthine excretion resulting from the administration of these drugs.

The only known source of orotidine in man is from the catabolism of orotidylic acid. Orotic acid is the immediate precursor of orotidylic acid in the pyrimidine biosynthetic pathway. This relationship is indicated below.



The finding of an increased urinary excretion of both orotidine and orotic acid suggests that the conversion of orotidine 5′-monophosphate (OMP) to uridine 5′-monophosphate (UMP) has been inhibited. This reaction, which is essential for the de novo synthesis of pyrimidines, is catalyzed by orotidylic decarboxylase.

Orotidylic decarboxylase was assayed in dialyzed human erythrocyte lysates essentially as described by Appel (*5*). The liberation of $^{14}CO_2$ was proportional to time and concentration

Table 2. Inhibition of orotidylic decarboxylase activity. Incubation mixtures contained 1.03 nmole of [^{14}C]carboxyl orotidine 5′-monophosphate (21 mc/mmole; New England Nuclear Corporation; purity, > 98 percent), 1.8 μmole of tris buffer (*p*H 7.4), and 1.7 to 2.0 mg of protein from a dialyzed human erythrocyte hemolysate in a final volume of 200 μl. Inhibitors were present at a final concentration ranging from 1×10^{-5} to $1 \times 10^{-3}M$. The reaction mixture was incubated at 37°C for 15 minutes in a water bath in tightly stoppered 15-ml polyethylene tubes with a center well containing 0.2 ml of hydroxide of hyamine and was terminated by the injection of 0.2 ml of 4*M* perchloric acid through air-tight rubber caps. The tubes were shaken for an additional 60 minutes to trap all the $^{14}CO_2$ evolved. The plastic center wells were removed from the tubes, wiped on the outside, suspended in 10 ml of scintillation mixture and counted in a Packard Tri-Carb scintillation spectrometer with an efficiency of 81 percent. Control activity was 0.44 nanomole per milligram of protein per hour (11,343 count/min).

Inhibitor	Concentration	Relative activity (%)
None		100
Hypoxanthine	1×10^{-3}	98
Xanthine	1×10^{-3}	98
Allopurinol	1×10^{-3}	100
Oxipurinol	1×10^{-3}	108
Inosine	1×10^{-3}	107
AMP	1×10^{-3}	81
IMP	1×10^{-3}	112
GMP	1×10^{-3}	132
UMP	1×10^{-3}	34
XMP	1×10^{-3}	1.2
	1×10^{-4}	7.3
	1×10^{-5}	40
Allopurinol ribonucleotide	1×10^{-3}	1.7
	1×10^{-4}	4.9
	5×10^{-5}	15
	1×10^{-5}	56

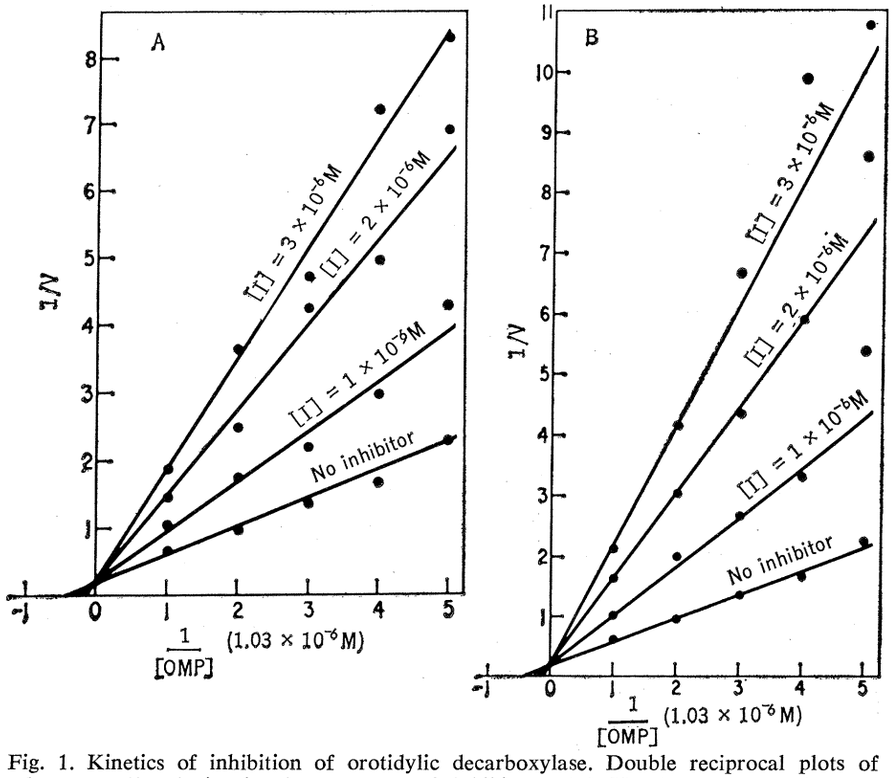


Fig. 1. Kinetics of inhibition of orotidylic decarboxylase. Double reciprocal plots of substrate and velocity in the presence of inhibitors. (A) Varying concentrations of allopurinol ribonucleotide; (B) varying concentrations of xanthosine 5′-monophosphate. [*I*] is concentration of inhibitor; *V*, velocity (nanomoles per milligram of protein per hour).

of protein. Several determinations of the Michaelis constant (K_m) for OMP averaged $1.5 \times 10^{-6}M$, which is similar to the values observed for this enzyme obtained from calf thymus ($2 \times 10^{-6}M$) (*6*), rat liver ($4.5 \times 10^{-6}M$) (*7*), cow brain ($3 \times 10^{-6}M$) (*5*), and yeast (7 to $8 \times 10^{-6}M$) (*8*). Allopurinol, oxipurinol, hypoxanthine, xanthine, inosine, inosine 5′-monophosphate, adenosine 5′-monophosphate, and guanosine 5′-monophosphate exhibited little if any inhibition at a concentration of $1 \times 10^{-3}M$ (Table 2). Uridine 5′-monophosphate, the product of this reaction, had a modest inhibitory effect at $1 \times 10^{-3}M$ which was similar to that reported in other mammalian tissues (*5*, *7*). Allopurinol ribonucleotide and xanthosine 5′-monophosphate (XMP), however, were potent inhibitors of this enzyme.

Preliminary studies indicated that the inhibitory effect of both compounds was competitive with respect to OMP (Fig. 1, A and B). The inhibition constant (K_i), determined by secondary plot of the slopes as a function of inhibitor concentration, was $8 \times 10^{-7}M$ for allopurinol ribonucleotide and $7 \times 10^{-7}M$ for XMP. Both of these purine nucleotides can be synthesized directly from their free bases, allopurinol and xanthine, in the presence of 5-phosphoribosyl-1-pyrophosphate and the enzyme, hypoxanthineguanine phosphoribosyltransferase, which is present in man (*9*). These observations, therefore, provide two possible mechanisms to account for the orotidinuria and orotic aciduria observed after treatment with allopurinol.

The increased excretion of orotidine and orotic acid following the administration of allopurinol or oxipurinol is quantitatively similar to that noted after the administration of a comparable dose of the pyrimidine analog, 6-azauridine (*10*). This latter compound is converted to its ribonucleotide, 6-azauridine-5-monophosphate, which is a potent inhibitor of orotidylic decarboxylase (*8*).

The possible consequences of this inhibitory effect of allopurinol on de novo pyrimidine biosynthesis in patients being treated with the drug are not clear. Patients who are homozygous for a deficiency of both orotate phosphoribosyltransferase and orotidylic decarboxylase, as well as orotidylic decarboxylase alone, usually have orotic acid calculi, megaloblastic anemia, and serious developmental retardation (*11*). Heterozygotes for this disorder, who have moderately decreased levels of these two enzymes and are clinically normal, excrete somewhat increased quantities of orotic acid (4 to 6 mg/day), although they exhibit no increase in the excretion of orotidine (*4*, *11*). The administration of uridine circumvents the metabolic block in the mutant homozygotes and leads to a reversal of most of the consequences of the block. It seems likely, therefore, that if side effects do result from the inhibition of de novo pyrimidine synthesis by allopurinol they should be reversible with uridine.

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