Biochemical Pharmacology, Vol. 22, pp. 3061–3075. Pergamon Press, 1973. Printed in Great Britain.

MECHANISM OF ANTIURIC ACTION OF 4-OXY- AND 4-THIOPYRAZOLOPYRIMIDINES*

PIERRE H. CARTIER and MICHÈLE HAMET

INSERM, Unité 75—Laboratoire de Biochimie CHU Necker,
156 rue de Vaugirard, Paris 15ème., France

(*Received* 1 *March* 1973; *accepted* 9 *May* 1973)

Abstract—Among the pyrazolopyrimidine derivatives used in hyperuricemia, thiopurinol [4-mercaptopyrazolo-(3,4-d) pyrimidine] exhibits different therapeutic effects than those of allopurinol [4-hydroxypyrazolo-(3,4-d) pyrimidine] and oxypurinol [4,6-dihydroxypyrazolo-(3,4-d) pyrimidine] which leads us to assume that its hypouricemic mechanism is different. The action of the three derivatives has been compared: (a) as xanthine oxydase inhibitors: thiopurinol activity is intermediate between that of the two others, (b) as subtrates of the phosphoribosyl transferase system: the nucleotide of thiopurinol has been isolated and identified, (c) as natural substrate HGPRT inhibitors (guanine and hypoxanthine). Finally, their action on PRPP consumption and on its intracellular level has been specified. In the absence of precise data on the concentrations realized *in vivo* with the therapeutic doses used, the interpretation of enzymatic kinetic data does not appear significant. It seems, however, that allopurinol and thiopurinol reduce *de novo* synthesis of purine ribonucleotides by depressing the PRPP intracellular level.

SEVERAL pyrazolopyrimidines are used in the treatment of hyperuricemia. Allopurinol [4-hydroxy-pyrazolo-(3,4-d) pyrimidine], a structural analogue of hypoxanthine, and its principal metabolic oxidation product, oxypurinol [4,6-dihydroxypyrazolo-3,4-d) pyrimidine], an analogue of xanthine, are substrates and potent competitive inhibitors of xanthine oxydase.[1–5] They decrease uric acid formation at the final steps of purine catabolism, namely the oxidation of hypoxanthine to xanthine and of xanthine to uric acid. They considerably reduce urinary elimination of uric acid and increase xanthinuria.

In fact, the mechanism of uric acid inhibition appears much more complex. The study of oxypurine elimination in treated hyperuricemic patients shows that the reduction of uric acid is not accompanied by a stoichiometric accumulation of xanthine and hypoxanthine, and that allopurinol also inhibits *de novo* purine synthesis. This metabolic effect has been confirmed by the reduction of ^{14}C-glycine incorporation into uric acid.[6] Allopurinol acts on purine biosynthesis only after transformation into its ribonucleotide[7] and this depends on the presence of HGPRT. Allopurinol is

* This work was supported by Research Grant ATP no. 1, from the Institut National de la Santé et de la Recherche Médicale.

Abbreviations: HGPRT, hypoxanthine-guanine-phosphoribosyltransferase (EC. 2.4.2.8); APRT, adenine-phosphoribosyltransferase (EC. 2.4.2.7); OPRT, orotate-phosphoribosyltransferase (EC. 2.4.2.10); ODC, orotidylate-decarboxylase (EC. 4.1.1.23); PRPP, 5-phosphoribosyl-1-pyrophosphate; Hx, hypoxanthine; G, guanine; HPP, allopurinol; diHPP, oxypurinol; MPP, thiopurinol; (HPP)RP, 1-allopurinol ribonucleotide; (MPP)RP, 1-thiopurinol ribonucleotide.

therefore without action in HGPRT deficient subjects. The inhibition is located at the first regulation step of *de novo* synthesis, phosphoribosylamine formation by PRPP-amidotransferase. These findings suggest that the metabolic effect of allopurinol is not dependent on inhibition of xanthine oxydase and may be partly attributed to (a) intracellular depletion of PRPP which is consumed in the phosphoribosyltransferase reaction and (b) formation of allopurinol ribotide, a potent inhibitor of amidotransferase.[8]

Another pyrazolopyrimidine derivative, thiopurinol [4-mercaptopyrazolo-(3,4-d) pyrimidine], also used in the therapy of hyperuricemia, shows a more notable difference between the uricodepressor effect and oxypurine accumulation in gouty patients. It appears to be as efficient as allopurinol in the reduction of uricemia and uricosuria and in the elimination of urate (tophi) deposits, but it does not produce xanthine accumulation.[9] Furthermore, in gouty patients with HGPRT deficiency[10] or in Lesch–Nyhan's disease,[11] allopurinol normally decreases hyperuricemia, but dangerously increases xanthinuria and xanthinemia. In these cases, only the inhibition of xanthine oxydase is evident. Thiopurinol, however, has none of these effects. According to Delbarre *et al.*,[9] thiopurinol would have little effect on xanthine oxydase and significant action on PRPP amidotransferase, after conversion into its ribonucleotide.

In the present study we have considered thiopurinol (MPP) activities as follows:

(a) as a xanthine oxydase inhibitor
(b) as a substrate of the phosphoribosyltransferase system which permits the direct synthesis of the corresponding ribonucleotide
(c) as an inhibitor of the natural substrates of HGPRT-guanine and hypoxanthine
(d) we have finally determined the influence of thiopurinol on PRPP at the intracellular level.

These activities have been compared under analogous conditions to those of allopurinol (HPP) and oxypurinol (diHPP).

EXPERIMENTAL PROCEDURE

Materials. Phosphoribosyl-1-pyrophosphate (Sigma sodium salt) concentration was measured according to Kornberg,[12] the degree of purity varies between 45 and 70 per cent. Allopurinol, oxypurinol and thiopurinol were synthesized according to Robins[13] or were provided by Miss Auscher. 8-^{14}C-Guanine (51·5 mCi/m-mole), 8-^{14}C-hypoxanthine (47·4 mCi/m-mole), 2-^{14}C-allopurinol (52·4 mCi/m-mole) and 2-^{14}C-thiopurinol (50 mCi/m-mole) were prepared by CEA (Saclay, France). The guanine and hypoxanthine are Sigma or Boehringer products. The purine and pyrazolopyrimidine solutions were prepared each day by dilution of concentrated stock solutions. The MPP solutions were prepared extemporaneously from the pure product. The concentrations were verified by spectrophotometry (Table 1). The other reagents are commercial products of the purest quality.

Isolation of thiopurinol ribotide. Two hundred and fifty μmoles of thiopurinol, 5 m-moles Tris–HCl, pH 7·4, 500 μmoles PRPP, 200 μmoles $MgCl_2$, 1 m-mole Na F, 500 μmoles 2-mercaptoethanol and purified HGPRT corresponding to 50 ml of red blood cells in a total volume 100 ml at pH = 8·4 were incubated for 20 hr at 37°, with agitation. The mixture was heated in a boiling water bath for 3 min, then rapidly

TABLE 1. PHYSICAL PROPERTIES OF PYRAZOLOPYRIMIDINES

	pH	λ_{max} (nm)	α_M (λ_{max})	pKa_2
Allopurinol	1	251	7350	9·34
	7	251	7340	
	13	258	6860	
Oxypurinol	1	252	5710	7·74
	7	243	6520	
	13	243	6180	
Thiopurinol	1	321	20,200	8·50
	7	321	19,420	
	13	310	13,280	

cooled in ice and centrifuged to eliminate the proteins. The residue was rinsed with 20 ml of distilled water and centrifuged. The combined supernatants were transferred to a Dowex 50-H^+ column (× 8, 100–200 mesh, 5 cm × 2·5 cm^2) to remove the excess of non-transformed thiopurinol. The effluent containing the non-adsorbed compounds was neutralized with dilute NH_4OH to pH 8 and chromatographed on Dowex 1-formate (× 8, 200–400 mesh, 20 cm × 0·5 cm^2) at a flow rate of 2–3 ml/min. The column was washed with 50 ml of 5 mM HCl and eluted by a linear gradient of lithium chloride (mixer: 5 mM HCl, 300 ml; reservoir: 200 mM LiCl + 5 mM HCl). The absorption at 320 nm was monitored with a Gilford 240 spectrophotometer. The nucleotide-containing fractions were pooled and concentrated under vacuum using a rotory evaporator, to a volume of about 5 ml. One hundred ml of a mixture of ethanol–acetone (1:4, v/v) and 1 ml of a 10 per cent barium acetate solution were added and kept at 0° for 2 hr. The precipitate was centrifuged, washed with 5 ml of ethanol–acetone, followed by 5 ml of ethanol and dried under vacuum. The preparation contained traces of two contaminants: the free base and the ribonucleoside. Electrophoresis on Whatman MM paper, at 2000 V, with a 50 mM sodium borate buffer, pH 9·0 separated the pure nucleotide which migrated more rapidly. The nucleotide, eluted by 0·1 N HCl, was evaporated to dryness and dissolved in 5 ml of double distilled water.

The spectrum of the nucleotide solution at pH 7·0 was identical to that of thiopurinol. The analysis provided the ratios MPP/ribose/phosphorus: 1/1·07/0·98. The nucleotide structure was established by enzymatic hydrolysis and characterization of products formed by electrophoretic separation. After alkaline phosphatase (EC 3.1.3.1; Boehringer no. 15 436) hydrolysis, a slow moving component was observed (thiopurinol–nucleoside). This ribonucleoside was degradated into thiopurinol by nucleoside phosphorylase (EC 2.4.2.1; Boehringer no. 15 356).

Enzymes. Xanthine oxydase from milk (Boehringer no. 15 347) has an activity of approx. 0·4 units mg^{-1}. It was diluted 1:50 with a 0·1 per cent bovine albumin solution before use.

Hypoxanthine-guanine-phosphoribosyl transferase (HGPRT) was purified from human red blood cells obtained from the "Centre National de Transfusion sanguine" according Hamet.[14]

After removal of hemoglobin by acidification at pH 5·2, neutralization and addition of DEAE-cellulose, the enzyme was fractionated by $(NH_2)SO_4$. The redissolved enzyme was dialysed, concentrated by ultrafiltration, and chromatographed on DEAE-cellulose.

Fractions with highest specific activity were pooled and concentrated by ultrafiltration. The faintly brownish solution was dialysed and stored at −20°.

The specific activity of the purified preparation (substrate = guanine) was about 85 μmoles hr^{-1} protein, which was between 300 and 400 times that of the initial hemolysate. The yield was about 40 per cent.

Enzyme assay. Xanthine oxydase activity was determined by recording at 25° the variation of absorbance at 295 nm of the following incubation medium: (for 3·3 ml) 75 mM glycine buffer, pH 8·0; xanthine, 11·2 or 45 μM; inhibitor, as indicated. The reaction was started by addition of 50 μl of diluted xanthine oxydase (4 milliunits). The initial velocity was calculated over the first 90 sec.[5]

The phosphoribosyl transferase activity was measured according to a previously described radioisotopic method[14] and expressed as pmoles of nucleotide formed per minute.

PRPP determination. The blood used in the PRPP consumption experiments was heparinized. The red blood cells, washed with cooled isotonic NaCl, were resuspended in an equal volume of Tris–HCl buffer pH 7·4 containing 5 mM $MgCl_2$ and 13 mM glucose and variable concentrations of orthophosphate buffer, pH 7·4. The final concentration of the medium was 154 mM. The suspension was incubated at 37° for 60 min. In the experiments with hemolysate, the red blood cells were frozen and thawed after addition of an equal volume of bidistilled water.

PRPP was measured by a modification of the method used by Sperling *et al.*,[16] the ^{14}C-AMP formed was separated from radioactive adenine by chromatography and counted in a scintillation spectrometer.[15]

RESULTS

The inhibitory action on xanthine oxydase

The inhibitory action of pyrazolopyrimidines on the oxidation of xanthine was determined with two xanthine concentrations, 11·2 and 45×10^{-6} M. The three pyrazolopyrimidines inhibit xanthine oxydase in a competitive manner and at various degrees; the K_i of thiopurinol ($2{\cdot}8 \times 10^{-6}$ M), is between that of allopurinol ($1{\cdot}0 \times 10^{-6}$ M), a strong inhibitor and that of oxypurinol ($7{\cdot}0 \times 10^{-6}$ M), a weaker inhibitor (Fig. 1).

Pyrazolopyrimidines, as phosphoribosyl transferase substrates

HGPRT transforms allopurinol into its nucleotide (1-phosphoribosyl) but has no action on its oxidated derivative, oxypurinol. Thiopurinol is also transformed into 1-phosphoribosyl-thiopurinol and the reaction is catalysed by HGPRT: the purified adenine phosphoribosyl transferase (APRT)[18] and OPRT have no effect.

Kinetics. The quantity of nucleotide formed from allopurinol and thiopurinol is proportional to the quantity of enzyme, in so far as the percentage of transformation remains less than 30 per cent. It is also proportional to the incubation time. One unit of enzyme catalyses the transformation of 1 pmole of substrate per minute.

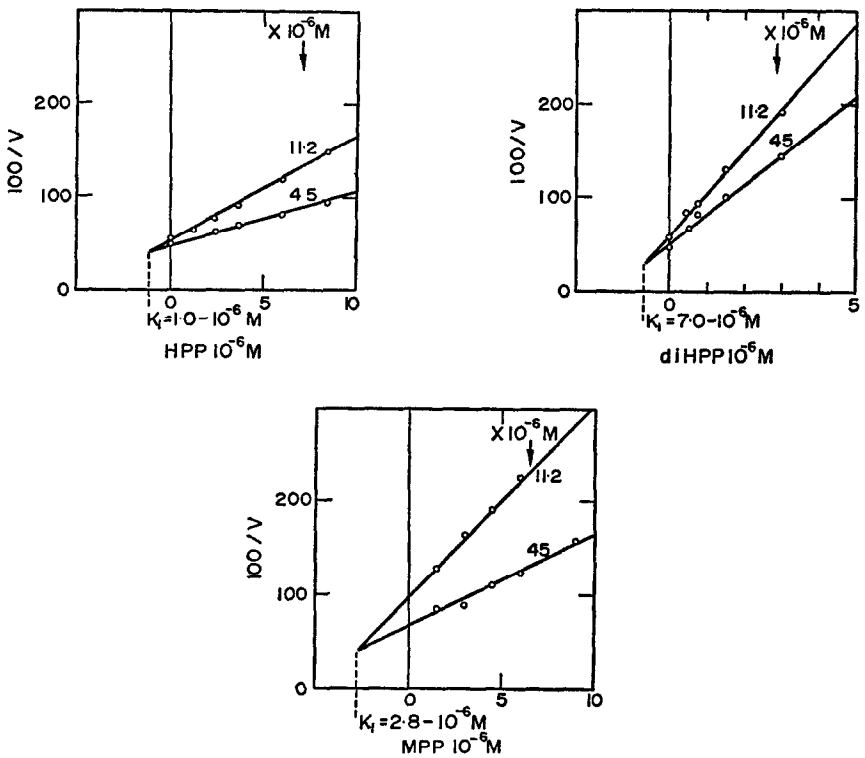


FIG. 1. Xanthine oxydase inhibition by HPP, diHPP and MPP. Dixon plot of inhibitor concentration against reciprocals of initial reaction velocities.

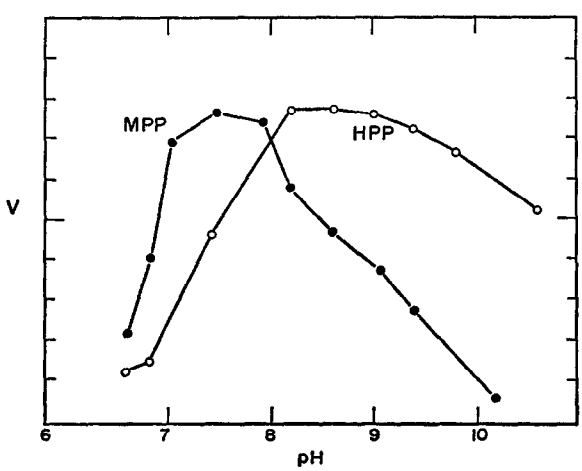


FIG. 2. Effect of pH on the nucleotide formation.

The optimum pH (Fig. 2) is between 8·2 and 8·6 for allopurinol and around 7·5 for thiopurinol. The pH modification, reported by Miller and Bieber for 8-azaguanine,[19] corresponds to a difference in p*Ka*. We have determined the pKa_2 of thiopurinol by the spectrophotometric method of Shugar and Fox:[20] it is less (8·50) than that of allopurinol (9·34).[21]

The initial velocity reaction was determined as a function of the pyrazolopyrimidine and PRPP concentrations. We report only the curves obtained with HPP, because the limited quantities of ^{14}C-MPP at our disposal did not permit us to analyze the reaction mechanism thoroughly: however, the results that we obtained showed that thiopurinol behaved like allopurinol and only the kinetic constants were different. The representation by double inverse plots of the initial velocity as a function of the PRPP and pyrazolopyrimidine concentrations for a series of fixed concentrations of the other substrate is presented in Fig. 3. The increase in the concentration of the second substrate modifies the slope of the curve but due to the weak affinity of the enzyme for the pyrazolopyrimidines the point of convergence of the lines is difficult to determine. With a HPP saturation concentration, the pyrophosphates inhibit the reaction in a non-competitive manner, while, the isotopic exchange between ^{14}C-HPP and the nucleotide exists only in the presence of pyrophosphates (Table 2): these various results illustrate a mechanism of an orderly sequential reaction, the same as for guanine, hypoxanthine[22] and adenine.[18]

Michaelis constants have been deduced graphically by the secondary representation of the reciprocal of molar concentrations ($1/S$) of allopurinol and PRPP. For an infinite concentration of the other substrate, the K_m values are indicated in Table 3.

TABLE 2. ISOTOPE EXCHANGE BETWEEN ALLOPURINOL OR THIOPURINOL AND RIBONUCLEOTIDES

	^{14}C-nucleotide formed (nmoles)
1. Allopurinol 2-^{14}C-35 μM (60 min at 37°)	
+ IMP (70 μM	0
+ IMP 70 μM + PP 1 mM	0·64
+ (HPP)RP 70 μM	0
+ (HPP)RP 70 μM + PP 1 mM	0·72
2. Thiopurinol 2-^{14}C-34 μM (90 min at 37°)	
+ IMP 70 μM	0·02
+ IMP 70 μM + PP 1 mM	0·21
+ (MPP)RP 70 μM	0·04
+ (MPP)RP 70 μM + PP 1 mM	0·19

TABLE 3. MICHAELIS CONSTANT OF PHOSPHORIBOSYL TRANSFERASE REACTION WITH ALLOPURINOL AND THIOPURINOL

Substrate variable	K_M (μM)	Substrate Fixed
HPP	645	PRPP
PRPP	240	HPP
MPP	870	PRPP
PRPP	320	MPP

The velocity was compared by measuring the nucleotide formation in the presence of a diluted hemolysate of human red blood cells in the presence of 1 mM PRPP and of 500 μM bases. Expressed in nmoles/hr and per mg of hemoglobin, they are respectively: guanine = 160, hypoxanthine = 99, allopurinol = 2·5, thiopurinol = 2·7.

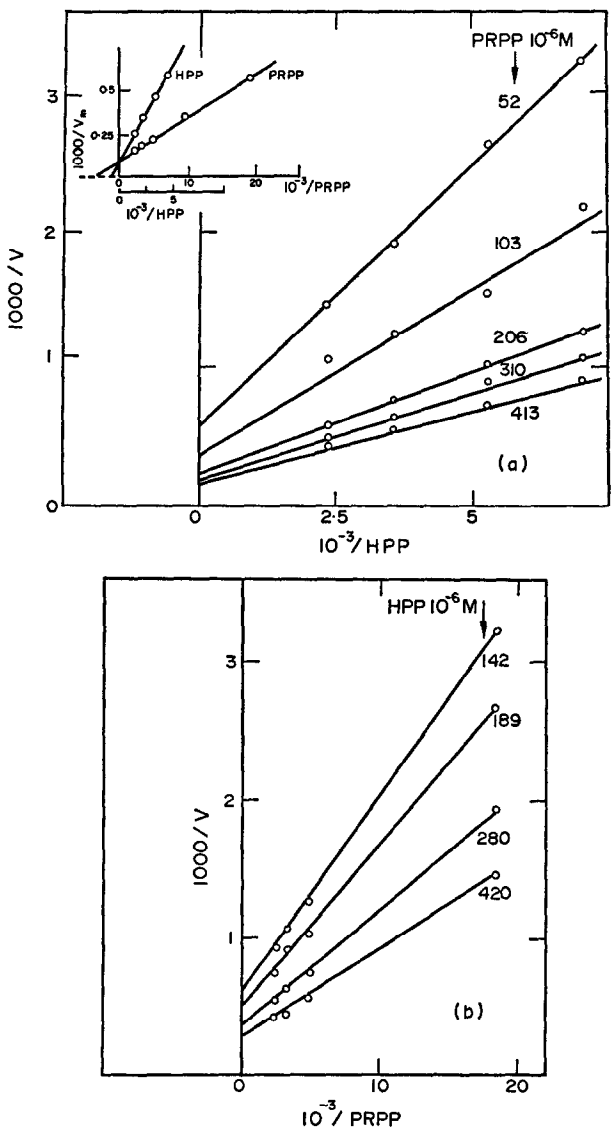


FIG. 3. (a) Double reciprocal plot of initial velocity against HPP concentration, PRPP concentrations as indicated. Inset shows a secondary plot of reciprocals of maximal reaction velocities (obtained from the ordinates of (a) against concentrations of HPP and PRPP. (b) Double reciprocal plot of initial velocity against PRPP concentration, HPP concentrations as indicated.

The inhibition of hypoxanthine-guanine-phosphoribosyl transferase by the pyrazolopyrimidines

The initial transformation velocity of ^{14}C-guanine into ^{14}C-GMP and of ^{14}C-hypoxanthine into ^{14}C-IMP was measured in the presence of increasing concentrations of HPP, diHPP and MPP with a saturating concentration of PRPP (2 mM). In the absence of an inhibitor the K_m of HGPRT for guanine (K_m (G) = 1·52 × 10^{-6} M) and hypoxanthine (K_m (Hx) = 2·06 × 10^{-6} M) were analogous to those published previously.[23] With the purified preparation of HGPRT we could not show any significant inhibition by oxypurinol. In contrast, allopurinol and thiopurinol inhibit the formation of IMP and GMP (Fig. 4). *It* acts as a non-competitive inhibitor for the second substrate PRPP (Fig. 5). The constants of K_i inhibition were determined graphically according to the Dixon representation [$1/v = f(1)$] and the values obtained are shown in Table 4.

Pyrazolopyrimidine effects on the erythrocyte concentration of PRPP in vitro

As Hershko *et al.*[24] have reported, the erythrocyte concentration of PRPP varies *in vitro* with the orthophosphate concentration of the medium (Fig. 6a). We can

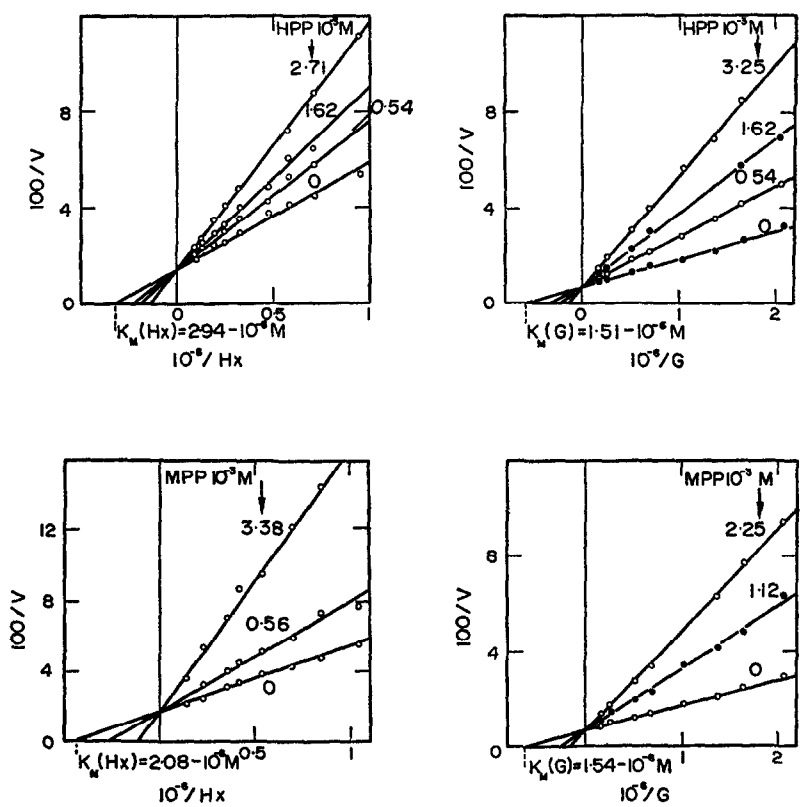


FIG. 4. Inhibition of IMP and GMP formation by HPP and MPP. Double reciprocal plot of initial velocity against hypoxanthine or guanine concentration. The concentrations of HPP and MPP are indicated. PRPP concentration = 1 mM.

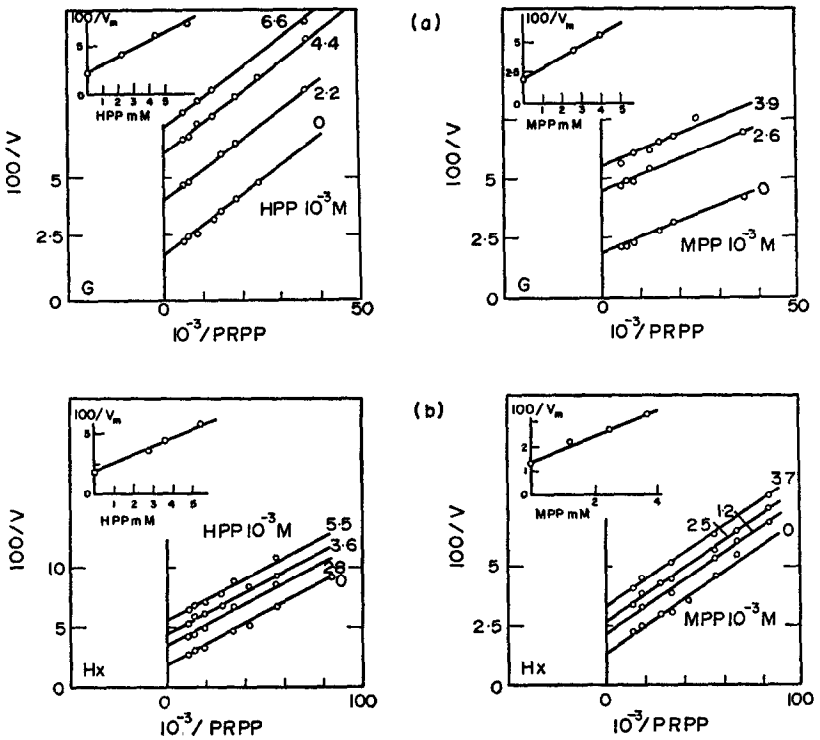


FIG. 5. (a) Inhibition of GMP formation by HPP and MPP. Double reciprocal plot of initial velocity against PRPP concentration. The concentrations of HPP and MPP are indicated. Guanine concentration = $2{\cdot}5 \times 10^{-6}$ M. (b) Inhibition of IMP formations by HPP and MPP. Double reciprocal plot of initial velocity against PRPP concentration. The concentrations of HPP and MPP are indicated. Hypoxanthine concentration = $2{\cdot}5 \times 10^{-6}$ M. Inset shows a plot of inhibitor concentration against reciprocals of maximal velocities [obtained from the ordinates of (a) and (b)].

TABLE 4. INHIBITION CONSTANT OF ALLOPURINOL AND THIOPURINOL UPON GMP OR IMP FORMATION

Inhibitor	Inhibition constants K_i (variable substrate/fixed substrate) (mM)			
	G/PRPP	PRPP/G	Hx/PRPP	PRPP/Hx
HPP	1·1 (comp)	2·7 (incomp)	1·7 (comp)	2·8 (incomp)
MPP	0·77 (comp)	2·1 (incomp)	1·35 (comp)	2·4 (incomp)

therefore study, the effects of variable PRPP concentrations on ribonucleotide formation from pyrazolopyrimidines and the effect of pyrazolopyrimidines on the intracellular PRPP level.

(1) Ribonucleotide formation is at the same time a function of the pyrazolopyrimidine concentration of the incubation medium and of the PRPP intracellular level (Fig. 6b). The phosphoribosyl transferase activity varies according to the substrate

and declines in the order Hx > G > HPP > MPP (Fig. 7). This order does not correspond to that of the velocity determined with the hemolysate: the stronger formation of IMP in relation to GMP is probably due to a faster rate of entry of hypoxanthine into the red blood cell.

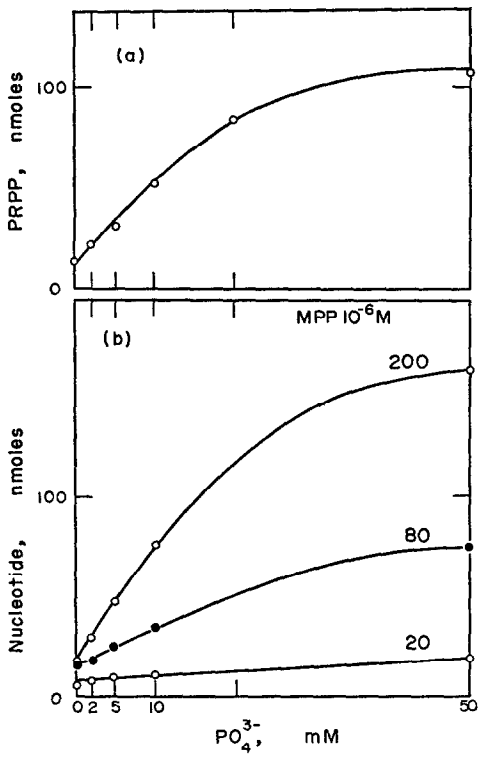


FIG. 6. (a) Effect of Pi on PRPP formation in intact erythrocytes (60 min/37°). (b) Nucleotide formation in intact erythrocytes at various levels of Pi (60 min/37°).

(2) Ribonucleotide synthesis brings about a consumption of intracellular PRPP. In Fig. 8, we have compared the rate of ribonucleotide formation of HPP and MPP and the erythrocytic PRPP level at the end of incubation. The ratio between the two is approximately stoichiometric. The hemolysate also forms PRPP, but as Hershko *et al.*[24] have reported using the same incubation conditions, this synthesis is less. In the presence of HPP or MPP, the PRPP level in the medium declines.

This decrease of PRPP noted in the red blood cell or the hemolysate in the presence of pyrazolopyrimidines is not due to an inhibition of the PRPP synthetase system. Fox *et al.*[25] have already shown that the decrease in PRPP only takes place in the presence of HGPRT and that blood cells deficient in HGPRT (subjects with Lesch–Nyhan's disease) maintain a normal PRPP level when they are incubated with allopurinol. We have confirmed this observation and have noted that a 5×10^{-4} M concentration of allopurinol and thiopurinol does not modify *in vitro* the activity of the red blood cell PRPP synthetase, purified according to Fox and Kelley.[26]

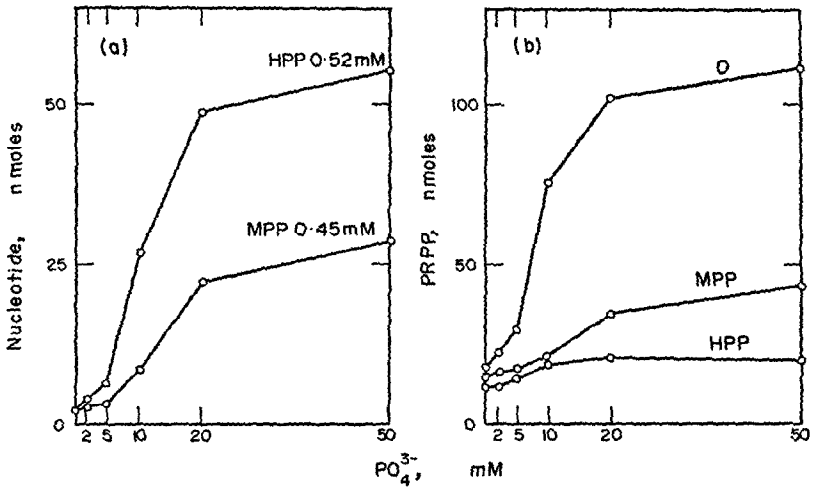


FIG. 7. Comparative nucleotide formation in intact erythrocytes with purines and pyrazolopyrimidines. (Purines and pyrazolopyrimidines concentration 0·5 mM; incubation 60 min/37°.)

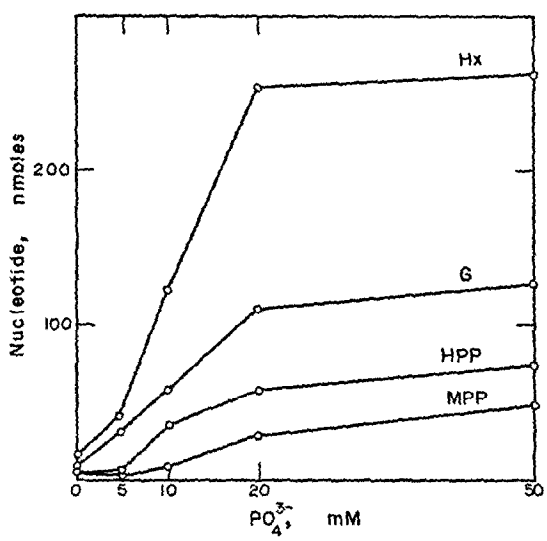


FIG. 8. Nucleotide formation and intracellular PRPP concentration in intact erythrocytes at different levels of Pi (60 min/37°).

DISCUSSION

Purine structural analogues, the pyrazolopyrimides, in the free base form, are potent xanthine oxydase inhibitors. This action, already demonstrated for allopurinol and oxypurinol, has been shown for the 4-thio derivative, thiopurinol. Its inhibitory activity (expressed by its K_i for xanthine oxidation) is intermediate between that of allopurinol and that of oxypurinol.

Delbarre[11] explains this weaker inhibitory action of thiopurinol by a decreased ability for electronic transfer when the —OH of C_4 is replaced by —SH. However,

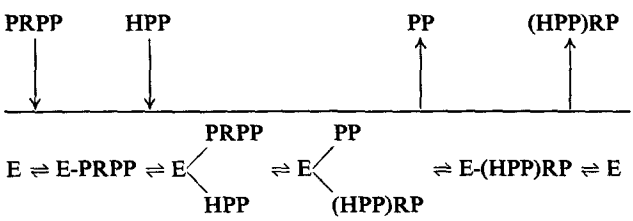
the inhibition constant for xanthine oxydase is not really different enough from that of allopurinol to explain the differences of activity demonstrated *in vivo*.

The pyrazolopyrimidines also interfere with the phosphoribosyl transferase systems either as substrates or as inhibitors. The behavior of oxypurinol differs from that of allopurinol and that of thiopurinol.

Oxypurinol, the oxidation product of allopurinol by xanthine oxydase, is not a substrate of HGPRT. This was reported by Krenitsky *et al.*[27] and, with our purified erythrocytic HGPRT preparation, we could not show ribonucleotide oxypurinol formation. Moreover, oxypurinol did not inhibit the enzyme.

Furthermore, Fox *et al.*[25] have verified that oxypurinol does not induce a measurable depletion of intraerythrocytic PRPP, *in vitro* as well as *in vivo*; our incubation experiments confirmed these results.

Allopurinol and thiopurinol are substrates of HGPRT. The kinetics of the reaction as a function of the concentration of base and phosphoribosyl pyrophosphate, the non-competitive inhibiting action of pyrophosphate and the isotopic exchange between the marked base and the nucleotide, indicate a similar reaction mechanism to that which has been demonstrated for the natural enzyme substrates, guanine and hypoxanthine, namely an orderly sequential reaction:



The affinity of the enzyme for the second substrate, PRPP, is not altered by the first substrate: the K_m (PRPP) values are of the same magnitude as the base, either guanine, hypoxanthine, allopurinol or thiopurinol. However, the affinity of HGPRT for pyrazolopyrimidines is much weaker: the K_m values which are less than 5×10^{-6} M for natural purines (Hx and G) or substitutes (6-mercapto-purine, 6-thioguanine) reach 5×10^{-4} M for the analogue derivatives with a pyrazolopyrimidine ring.

Guanine inhibits the incorporation of ^{14}C-Hx into ^{14}C-IMP and hypoxanthine, that of ^{14}C-G into ^{14}C-GMP. This competitive type inhibition is found with allopurinol and thiopurinol. As in the cases of purines where the K_i inhibition constant is very near K_m, the K_i of the two pyrazolopyrimidines are elevated, as well *vis-à-vis* the first substrate, Hx or G (competitive inhibition) as the second substrate PRPP (non-competitive inhibition).

If, as Hill has done for an important G and Hx structural series of analogues, one compares the values of the constants of inhibition to those of K_m of the two natural substrates of HGPRT,[20] the ratio K_i/K_m represents an expression for the inhibiting effect: a powerful inhibitor has a ratio much less than 1. In the cases of allopurinol and thiopurinol, this ratio is very high and these derivatives are practically without action on the phosphoribosyl transferase reaction under physiological conditions.

In subjects deficient in HGPRT the pyrazolopyrimidines are without action.[29] only the ribonucleotide form acts on purine synthesis. It is admitted that the regula-

tion of *de novo* synthesis is assured at the level of the first reaction, phosphoribosylamine formation. The intensity of this reaction is controlled by two important factors: (1) the availability of substrates, glutamine and phosphoribosyl pyrophosphate, and (2) the activity of the responsible enzyme regulator, PRPP-amidotransferase, allosterically inhibited by numerous purine ribonucleotides.

(a) Of the two substrates of the amidotransferase reaction, glutamine hardly influences phosphoribosylamine formation, because its concentration is relatively higher. PRPP appears to be the limiting factor. Its intracellular concentration is only known in erythrocytes[15,30,31] and in fibroblast cultures:[32] it is very low (erythrocytes: 3–6 $\times$ 10^{-6} M; fibroblasts: 13 $\times$ 10^{-6} M) and we can suppose that it is of the same level in other cell types.

Actually these concentrations do not reflect the PRPP synthetase activity which functions under its maximum capacity. It is especially dependent on the low concentration of ribose phosphate and on the presence of an elevated level of certain inhibitors (2,3-diphosphoglycerate in the erythrocyte).

PRPP is the indispensable substrate for multiple synthesis reactions and in particular, for purine, pyrimidine or pyridine ribonucleotide formation which uses the same phosphoribosyl transferase pathway and consumes PRPP.[30] The low PRPP intracellular concentration must satisfy these multiple needs of synthesis.

The affinity for the PRPP of various enzymes which catalyse these reactions is very variable and generally weak (0·02–0·5 mM). Nevertheless, amidotransferase has the highest K_m (PRPP) (0·20–0·40 mM)[22] its affinity for example is 10–20 times less than that of APRT[33] and OPRT.[34] As the K_m *vis-à-vis* the PRPP is very elevated in relation to the intracellular concentrations, the PRPP-consuming reactions occur according to a first order kinetic and their velocity is very sensitive to little variations in PRPP concentration.

For each reaction which uses PRPP we must take into account its K_m *vis-à-vis* the PRPP and at the same time the concentration of the second substrate. Under physiological conditions, among all these reactions, the *de novo* purine ribonucleotide biosynthesis represents the most active pathway. The others are reactions of weak intensity and they function well under the phosphoribosyl transferase capacities, because the intracellular concentration of their second substrate is very low and for some even not measurable.

The available PRPP concentration constitutes the most important factor which in man limits *de novo* purine synthesis.[35] On the other hand, besides amidotransferase, HGPRT represents the principal enzyme which uses PRPP under physiological conditions and a deficiency in HGPRT is accompanied by a PRPP elevation in the erythrocytes[31] and the fibroblasts,[36] without having a modification of the intensity of the synthesis.

The fact of supplying a substrate for the HGPRT, natural or not, brings about a PRPP consumption and a reduction especially in *de novo* purine nucleotide synthesis. This action has been demonstrated *in vivo* in man with orotic acid, as well as on fibroblast cultures[30,38] and with hypoxanthine which reduces *in vitro* the purine fibroblast synthesis.[39] Likewise, allopurinol lowers the amount of PRPP in erythrocytes *in vivo* and *in vitro*[25] and inhibits purine fibroblast synthesis.[39]

Our results also show an erythrocyte PRPP consumption *in vitro* with allopurinol and thiopurinol, all the more important since PRPP synthesis is more intense.

(b) Allopurinol ribonucleotide inhibits amidotransferase ($K_i = 0{\cdot}6 \times 10^{-3}$ M). One can also suppose that thiopurinol nucleotide possesses a similar action and that, associated with AMP or GMP, the synergistic effect would be particularly intense. We have verified this possibility, but it does not appear to be responsible for the hypouricemic action found *in vivo*. The quantities of (HPP)RP and (MPP)RP formed remain small and one can calculate, like Krenitsky *et al.*[27] from the kinetic constants V_{max} and K_m that with the supposed concentrations *in vivo*, hypoxanthine forms 1000 times more nucleotide than allopurinol or thiopurinol.

One can also suppose that the inhibitory effect on xanthine oxydase induces hypoxanthine accumulation which HGPRT transforms into IMP: from where we get PRPP consumption and amido transferase inhibition. This explanation is not satisfactory because allopurinol inhibits *de novo* synthesis in fibroblast cultures, deprived of xanthine oxydase.

Murray[35] found it difficult to understand the role of the means of purine salvage and more particularly the transformation of hypoxanthine into IMP in the erythrocyte, because PRPP concentrations are much smaller than the value of K_m (PRPP) of HGPRT. However, this way pathway is functional. The same question is asked for allopurinol and thiopurinol which consume large quantities of PRPP, although the kinetic constants of HGPRT for these two substrates and PRPP are not favorable.

The concentration that is obtained *in vivo* with pyrazolopyrimidines in therapeutic doses is uncertain. We can estimate it to be 10 μM, in other words on a level corresponding to 1/50th K_m concentration for which the phosphoribosyl transferase reaction velocity is low.[27] However, allopurinol inhibits *de novo* purine ribonucleotide synthesis in fibroblast cultures at very low concentrations, hardly different from those which exist in the extracellular fluids of treated subjects.[39]

Of all the hypotheses which have been postulated to account for the action of allopurinol on purine synthesis, the competitive effect on PRPP consumption between the phosphoribosyl transferases and amidotransferase appears to furnish the most satisfying explanation of the *de novo* purine synthesis inhibition, but we must realize that there is a considerable difference between the interpretation that we obtain from the study of phosphoribosyl transferase kinetics for allopurinol or thiopurinol and the recorded effects *in vitro* or *in vivo*.

Acknowledgements—We wish to acknowledge Miss C. Auscher and the excellent technical assistance of Mrs Floriane Garreau and Miss Anne-Marie Boughezal.

REFERENCES

1. R. W. RUNDLES, H. R. SILBERMAN, G. H. HITCHINGS and G. B. ELION, *Ann. intern. Med.* **60,** 717 (1964).
2. T. F. YU and A. B. GUTMAN, *Am. J. Med.* **37,** 885 (1964).
3. J. R. KLINENBER, S. E. GOLDFINGER and J. E. SEEGMILLER, *Ann. intern. Med.* **62,** 639 (1965).
4. P. FEIGELSON, J. D. DAVIDSON and R. K. ROBINS, *J. biol. Chem.* **226,** 993 (1957).
5. G. B. ELION, *Ann. Rheum. Dis.* **25,** 608 (1966).
6. B. T. EMMERSON, *Ann. Rheum. Dis.* **25** (suppl. 6), 621 (1966).
7. R. W. BROCKMAN, *Cancer Res.* **25,** 1596 (1965).
8. R. J. MCCOLLISTER, W. R. GILBERT, D. M. ASHTON and J. B. WYNGAARDEN, *J. biol. Chem.* **239,** 1560 (1964).
9. F. DELBARRE, C. AUSCHER, A. DE GERY, H. BROUILHET and J. L. OLIVIER, *Presse méd.* **76,** 2329 (1968).
10. F. DELBARRE, P. CARTIER, C. AUSCHER, A. DE GERY and M. HAMET, *Presse méd.* **78,** 729 (1970).
11. F. DELBARRE, C. AUSCHER, A. DE GERY and T. KHA VANG, *Biochimie* **54,** 709 (1972).

12. A. KORNBERG, I. LIEBERMAN and E. SIMMS, *J. biol. Chem.* **215,** 389 (1955).
13. R. K. ROBINS, *J. Am. chem. Soc.* **78,** 784 (1956).
14. M. HAMET, Thèse Doctorat Sciences, Paris (1973).
15. P. CARTIER and M. HAMET, *Clin. Chim. Acta* **20,** 205 (1968).
16. O. SPERLING, G. EILAM, S. PERSKY-BROSH and A. DE VRIES, *J. Lab. clin. Med.* **79,** 1021 (1972).
17. T. D. BEARDMORE and W. N. KELLEY, *J. Lab. clin. Med.* **78,** 696 (1971).
18. S. SRIVASTAVA and E. BEUTLER, *Archs. Biochim. Biophys.* **142,** 426, (1971).
19. R. L. MILLER and A. L. BIEBER, *Biochemestry* **8,** 603 (1969).
20. D. SHUGAR and J. J. FOX, *Biochim. biophys. Acta* **9,** 199 (1952).
21. T. A. KRENITSKY, G. B. ELION, R. A. STRELITZ and G. H. HITCHING, *J. biol. Chem.* **242,** 2675 (1967).
22. J. F. HENDERSON, L. W. BROX, W. N. KELLEY, F. M. ROSENBLOOM and J. E. SEEGMILLER, *J. biol. Chem.* **243,** 2514 (1968).
23. K. O. RAIVIO and J. E. SEEGMILLER, *Curr. Topics Cell. Reg.* **2,** 201 (1970).
24. A. HERSHKO, A. RAZIN and J. MAGER, *Biochim. biophys. Acta* **184,** 64 (1969).
25. J. H. FOX, J. B. WYNGAARDEN and W. N. KELLEY, *New Engl. J. Med.* **283,** 1177 (1970).
26. I. H. Fox and W. N. KELLEY, *J. biol. Chem.* **246,** 5739 (1971).
27. T. A. KRENITSKY, R. PAPAIOANNOU and G. B. ELION, *J. biol. Chem.* **244,** 1263 (1969).
28. D. L. HILL, *Biochem. Pharmac.* **19,** 545 (1970).
29. W. N. KELLEY, F. M. ROSENBLOOM, J. MILLER and J. E. SEEGMILLER, *New. Engl. J. Med.* **278,** 287 (1968).
30. I. H. FOX and W. N. KELLEY, *Ann. intern. Med.* **74,** 424 (1971).
31. M. L. GREENE and J. E. SEEGMILLER, *J. clin. Invest.* **48,** 32a (1969).
32. W. N. KELLEY, I. H. FOX and J. B. WYNGAARDEN, *Clin. Res.* **18,** 457 (1970).
33. M. HORI and J. F. HENDERSON, *J. biol. Chem.* **241,** 1406 (1966).
34. I. LIEBERMAN, A. KORNBERG and E. S. SIMMS, *J. biol. Chem.* **215,** 403 (1955).
35. A. W. MURRAY, *Ann. Rev. Biochem.* **40,** 811 (1971).
36. F. M. ROSENBLOOM, J. F. HENDERSON, I. C. CALDWELL, W. N. KELLEY and J. E. SEEGMILLER, *J. biol. Chem.* **243,** 1166 (1968).
37. S. RAJALAKSHMI and R. E. HANDSCHUMACHER, *Biochim. biophys. Acta* **155,** 317 (1968).
38. W. N. KELLEY, I. H. FOX and J. B. WYNGAARDEN, *Biochim. biophys. Acta* **215,** 512 (1970).
39. W. N. KELLEY and J. B. WYNGAARDEN, *J. clin. Invest.* **49,** 602, (1970).