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# EFFECT OF ALLOPURINOL AND ITS METABOLIC DERIVATIVES ON THE CONFIGURATION OF HUMAN OROTATE PHOSPHORIBOSYLTRANSFERASE AND OROTIDINE 5′-PHOSPHATE DECARBOXYLASE

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**Abstract**—The administration of allopurinol or oxipurinol leads to an apparent increase in the specific activity of orotate phosphoribosyltransferase and orotidine 5′-phosphate decarboxylase in man. In the present study, we have shown that human orotate phosphoribosyltransferase and orotidine 5′-phosphate decarboxylase exist in a complex as three different molecular species with molecular weights of 55,000, 80,000 and 113,000. The larger forms of the complex are more stable than the small form. In the presence of allopurinol ribonucleotide or oxipurinol-7-ribonucleotide, but not the free bases, allopurinol or oxipurinol, the largest, most stable species predominates. These findings lend support to the hypothesis that the increased activity of these two enzymes may be related in some manner to their stabilization. The possibility that the apparent increase in activity is due largely to stabilization of the complex during cell lysis and extraction rather than actual stabilization *in vivo* is most consistent with the data currently available.

Allopurinol and its major metabolic product, oxipurinol, are potent inhibitors of uridine monophosphate biosynthesis *de novo* [1, 2]. This effect is due to inhibition of orotidine 5-phosphate decarboxylase by the ribonucleotide derivatives of both allopurinol and oxipurinol [2–5]. In addition, the administration of these drugs is followed by an increase in the specific activity of both orotate phosphoribosyltransferase (OPRT) and orotidine 5′-phosphate decarboxylase (ODC) [1, 3, 6–8]. This effect has been attributed to enzyme stabilization *in vivo* [3] or enzyme "activation" [6]. This investigation of the effect of allopurinol and its derivatives on the molecular configuration of OPRT and ODC provides further insight into the nature of this effect.

## MATERIALS AND METHODS

Orotic acid-7-$^{14}$C (10·2 mCi/m-mole) and orotidine-5′-monophosphate-7-$^{14}$C (21·0 mCi/m-mole) were obtained from New England Nuclear Corp. The sodium salt of PP-ribose-P was purchased from Sigma Chemical Co. Purified powdered catalase (4800 units/mg) and purified powdered yeast alcohol dehydrogenase (342 units/mg) were obtained from Worthington Biochemical Corp. Bio-Gel-A 0·5 M, 200–400 mesh, 10% agarose content, was purchased from Bio-Rad Laboratories. Allopurinol, oxipurinol and allopurinol ribonucleotide were gifts from Dr. Gertrude B. Elion, Burroughs–Wellcome Co., Research Triangle Park, N.C. Oxipurinol-7-ribonucleotide was generously provided by Henning Chemie & Pharmawerk, Berlin, West Germany. The other chemicals and reagents used were of the highest quality commercially available.

Venous blood samples were collected in heparinized tubes and centrifuged at 600 *g* for 10 min at room temperature. The buffy coat and plasma were removed and the erythrocytes washed twice with cold 0·9% saline. The washed erythrocytes were lysed by freeze–thawing in an acetone–dry ice bath and dialyzed for 2 hr at 4° against 0·01 M Tris–Cl buffer, pH 7·4.

### *Enzyme assay*

*Orotidine 5′-phosphate decarboxylase.* This enzyme was assayed by following the liberation of $^{14}CO_2$ from OMP-7-$^{14}$C as described by Kelley and Beardmore [2]. For the determination of enzyme activity in fractions of sucrose density gradients, an incubation time of 2–3 hr was used. In addition, when inhibitors of the enzyme were present in the sucrose gradients, it was necessary to desalt each fraction, using a small Sephadex G-25 column as described previously [9].

*Orotate phosphoribosyltransferase.* This enzyme was assayed under similar conditions using orotic acid-7-$^{14}$C and an excess of partially purified human orotidine 5′-phosphate decarboxylase. Incubation mixtures contained 0·05 M Tris–Cl buffer, pH 7·4, 4·9 mM $MgCl_2$, 1·5 mM PP-ribose-P, 0·1 mM orotic acid-7-$^{14}$C and enzyme protein in a total volume of 0·1 ml. The reaction mixture was incubated in a water bath for 1–3 hr at 37° in tightly stoppered 16 × 100 mm glass tubes with a center well containing 0·2 ml of hydroxide of Hyamine. The orotate phosphoribosyltransferase

reaction was stopped by the injection, through airtight rubber caps, of 150 μl of 0·2 M EDTA, pH 7·4, containing an excess of partially purified human ODC. After an additional incubation time of 15 min at 37°, the reaction was terminated by injecting 0·2 ml of 4 M perchloric acid through the rubber caps. The tubes were shaken for another 30 min to trap all the $^{14}CO_2$ evolved. The plastic center wells were then removed from the tubes, wiped on the outside, suspended in 10 ml of Triton X-100 containing scintillation mixture and counted in a Packard Tri-Carb scintillation spectrometer with an efficiency of 73 per cent.

*Catalase.* The activity of catalase was assayed at 25° by following the disappearance of hydrogen peroxide at 240 nm with a Zeiss spectrophotometer as described by Beers and Sizer [10].

*Yeast alcohol dehydrogenase.* This enzyme was assayed at 25° by following the increase in absorbancy at 340 nm with a Zeiss spectrophotometer as described by Vallee and Hoch [11].

All assays were linear with regard to time and protein.

*Gel filtration*

Attempts to determine the molecular Stokes radius of OPRT and ODC in hemolysate were unsuccessful because of the viscosity of the sample. Therefore, a partially purified preparation had to be used. Hemolysate was prepared from 500 ml of fresh blood as described above and stirred mechanically with approximately 60 g DEAE-cellulose (exchange capacity of 0·75 mequiv./g), which was prepared according to the procedure of Hennessey *et al.* [12]. This and all subsequent steps were performed as 4°. After 2 hr, the mixture was placed on a Buchner funnel and the hemoglobin-rich filtrate discarded. The DEAE-cellulose was resuspended in 10 mM potassium phosphate buffer, pH 7·4, and poured into a glass column (40 × 2·4 cm). After this procedure, the DEAE was washed with 1–2 l. of 10 mM potassium phosphate buffer, pH 7·4, and a linear gradient (800 ml) from 10 mM to 0·5 M potassium phosphate buffer, pH 7·4, was applied to the column. OPRT and ODC eluted as a single peak. Fractions containing the peak activity of OPRT and ODC were pooled. For each liter of pooled eluate, 243 g crystalline ammonium sulfate was added and the resulting solution was stirred for 1 hr. The precipitate was removed by centrifugation at 30,000 *g* for 20 min. To each liter of the supernatant, an additional 132 g ammonium sulfate was added and the solution was stirred for 1 hr and centrifuged as above. The precipitate was resuspended in a minimal volume of 10 mM potassium phosphate buffer, pH 7·4, and the solution was clarified by centrifugation at 30,000 *g* for 20 min. A portion (1·5 ml) of the redissolved pellet from the ammonium sulfate precipitation was applied to a 10% agarose column (1·7 × 86·5 cm) equilibrated with 25 mM potassium phosphate buffer, pH 7·4, and containing 0·25 M sucrose. The column was calibrated with catalase (5 mg/ml), yeast alcohol dehydrogenase (5 mg/ml) and ovalbumin (5 mg/ml). The void volume was determined by using blue dextran (1·5 mg/ml). The molecular Stokes radius for the enzyme complex was calculated by the method of Ackers [13].

*Sucrose density ultracentrifugation*

Sucrose gradient ultracentrifugation was performed with a Spinco SW41 rotor in a Beckman model L2. Isokinetic gradients (10 to 28·2 per cent) of 11·8 ml were prepared according to the method of McCarty *et al.* [14]. The sucrose was dissolved in 0·01 M potassium phosphate buffer, pH 7·4, and other compounds were added as indicated in the text. Hemolysate was diluted 1:10 with 0·01 M potassium phosphate buffer, pH 7·4, centrifuged for 20 min at 10,300 *g* in order to remove cell membranes, and 200 μl of the supernatant was applied to the top of the gradient. After centrifugation for 30–40 hr at 40,000 rev/min at 4°, 235-μl fractions were collected with a Gilson microfractionator. $S_{20,w}$ values were calculated on the basis of the linear relationship of the sedimentation coefficient to the distance migrated in the isokinetic gradient. Hemoglobin, which was used as an internal standard, was detected by its absorbancy at 430 nm with a Zeiss spectrophotometer.

*Determination of molecular weight*

Molecular weights were calculated from the $S_{20,w}$ and Stokes radius by the following formula:

$$M = \frac{6n\pi\, Nas}{(1 - \bar{v}p)}$$

where $M$ = molecular weight, $a$ = Stokes radius (1 Å = $10^{-8}$ cm), $s$ = sedimentation coefficient (× $10^{-13}$), $\bar{v}$ = partial specific volume (a value of 0·725 $cm^3/g$ was assumed as representative of most proteins), $n$ = viscosity of medium (0·01), $p$ = density of the medium (1·0) and $N$ = Avogadro number (6·023 × $10^{23}$).

The molecular Stokes radius and/or the $S_{20,w}$ for catalase, alcohol dehydrogenase and ovalbumin were obtained from the literature [15].

## RESULTS

*Molecular heterogeneity of orotate phosphoribosyltransferase and orotidine 5′-phosphate decarboxylase*

The partially purified preparation of OPRT and ODC eluted with three distinct peaks of activity from 10% agarose (Fig. 1). The elution volume of each peak was the same for both enzymes. The molecular Stokes radii of the small, intermediate and large forms were 37·5, 47·5 and 54·7 Å respectively.

Sucrose gradient ultracentrifugation of the hemolysate consistently resulted in the demonstration of two peaks of ODC activity with mean $S_{20,w}$ values of 5·0 and 3·6 respectively, which correspond to the large and small form of the enzyme observed after gel filtration

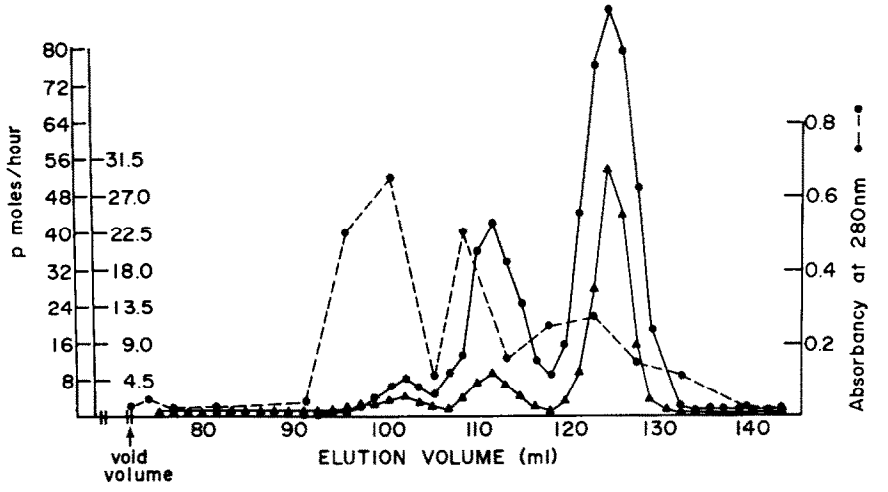


Fig. 1. Gel filtration of partially purified OPRT and ODC on 10% agarose. ODC, ●——● (outside scale); OPRT, ▲—— ▲ (inside scale); ●- - - -●, optical density, 280 nm. Final recovery is approximately 54 per cent.

(Fig. 2). A third peak with an $S_{20,w}$ of 4·1, which corresponded to the intermediate peak observed after gel filtration, was observed on rare occasion under these conditions. Under the conditions of this study, OPRT activity was observed as a single peak with a mean $S_{20,w}$ of 3·8. In most cases, when OPRT and ODC were assayed in the same gradients, the peak activity of each enzyme was separated by one tube.

Based on the Stokes radius and sedimentation coefficient, the molecular weight of the three forms of ODC are 55,000, 80,000 and 113,000.

*Stability of orotate phosphoribosyltransferase and orotidine 5'-phosphate decarboxylase*

The three forms of OPRT and ODC obtained by gel filtration demonstrate different stability to storage at 4° (Table 1). While the larger forms of both enzymes are relatively stable, the small form of both enzymes loses activity rapidly at 4°. Examination of the different forms of ODC obtained from sucrose gradient ultracentrifugation revealed essentially the same results.

*Change in configuration produced by allopurinol ribonucleotide and oxipurinol ribonucleotide*

The effect of allopurinol ribonucleotide on the configuration of ODC is shown in Fig. 3. When hemolysate was incubated at 37° for 1 hr with $1 \times 10^{-3}$ M allopurinol ribonucleotide and then run in a gradient that contained the same concentration of this compound, there was a marked change in the enzyme profile. Under these conditions, a single peak of ODC activity which had an $S_{20,w}$ of 5·1 was observed; this cor-

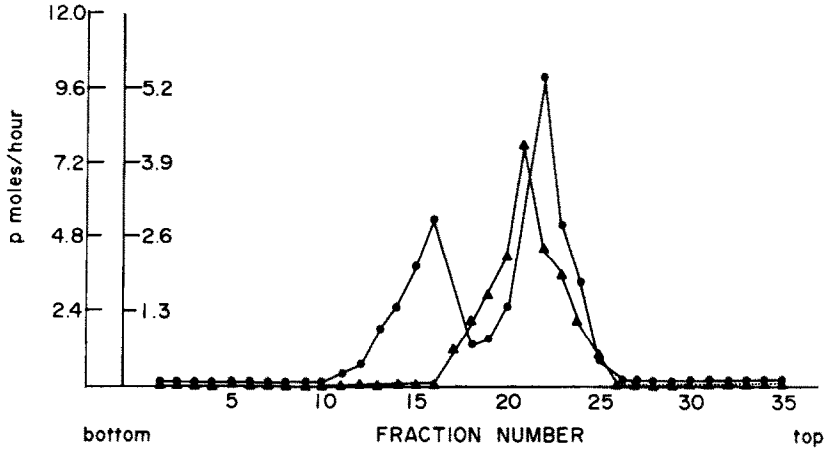


Fig. 2. Sucrose density gradient centrifugation of OPRT and ODC in hemolysate. ODC, ●——● (outside scale); OPRT, ▲——▲ (inside scale).

Table 1. Stability of the different molecular forms of orotate phosphoribosyltransferase and orotidine 5'-phosphate decarboxylase at 4°

| Molecular species | Specific activity (nmoles/mg/hr) | Enzyme activity (% of initial activity) Control | 2 days | 4 days | 6 days | 11 days |
|---|---|---|---|---|---|---|
| Orotidine 5'-phosphate decarboxylase | | | | | | |
| Small form | 11·6 | 100 | 77 | 50 | 32 | |
| Intermediate form | 4·5 | 100 | 108 | 99 | 96 | 70 |
| Large form | 0·5 | 100 | 100 | 107 | 113 | 110 |
| Orotate phosphoribosyltransferase | | | | | | |
| Small form | 8·3 | 100 | | 9 | | |
| Intermediate form | 1·1 | 100 | | 70 | | |
| Large form | 0·3 | 100 | | 100 | | |

responded to the large form of ODC noted in the absence of allopurinol ribonucleotide. A similar effect was noted at concentrations of allopurinol ribonucleotide as low as $1 \times 10^{-4}$ M ($S_{20,w}$ of 5·3) and with oxipurinol-7-ribonucleotide at concentrations as low as $1 \times 10^{-6}$ M ($S_{20,w}$ of 5·2). When hemolysate was incubated at 37° with $1 \times 10^{-3}$ M allopurinol ribonucleotide and then run in a gradient that did not contain allopurinol ribonucleotide, an intermediate form with a sedimentation coefficient of 4·0 was found. In addition, a shoulder consisting of faster sedimenting enzyme activity was observed. This suggests that the largest form of the enzyme dissociates spontaneously in the absence of allopurinol ribonucleotide. OPRT

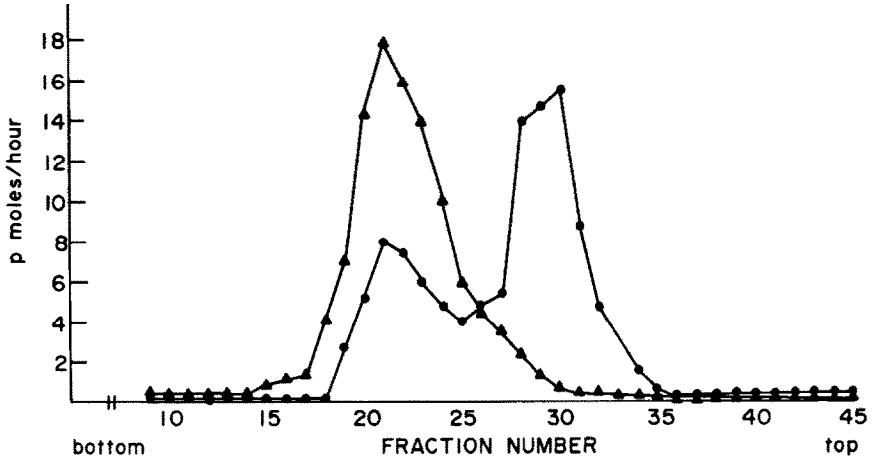


Fig. 3. Effect of allopurinol ribonucleotide on the configuration of ODC in hemolysate. Control, ●——●; allopurinol ribonucleotide ($1 \times 10^{-3}$ M), ▲- - ▲.

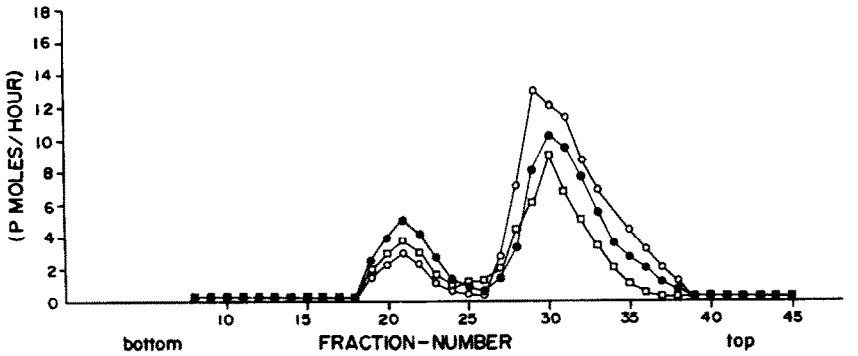


Fig. 4. Effect of allopurinol and oxipurinol on the configuration of OPRT and ODC in hemolysate. Control, ●——●; allopurinol, □——□ ($1 \times 10^{-3}$ M); oxipurinol, ○——○ ($1 \times 10^{-3}$ M).

Table 2. Effect of allopurinol and its metabolic derivatives on the sedimentation coefficient of ODC

| | Concn (M) | Sedimentation coefficient ($S_{20,w}$) | |
|---|---|---|---|
| Control (14)* | | 3·6 ± 0·2† | 5·0 ± 0·2† |
| Allopurinol | $10^{-3}$ | 3·6 | 5·1 |
| Oxipurinol | $10^{-3}$ | 3·8 | 5·0 |
| Allopurinol ribonucleotide | $10^{-4}$ | ND‡ | 5·3 |
| | $10^{-3}$ | ND | 5·1 |
| Oxipurinol 7-ribonucleotide | $10^{-6}$ | ND | 5·2 |

* Number in parentheses indicates number of determinations.

† ± One standard deviation.

‡ ND = not detected.

also aggregated to the large and intermediate molecular size in the presence of allopurinol ribonucleotide. The activity of OPRT was very low, however, when compared to that of ODC. A change in the configuration of ODC was not observed in the presence of allopurinol ribonucleotide when an enzyme preparation partially purified by DEAE-cellulose chromatography was used. When hemolysate was incubated at 37° for 1 hr with either allopurinol or oxipurinol at a final concentration of $1 \times 10^{-3}$ M and then run in a gradient which also contained these compounds, no change in the molecular size was noted as compared to the control (Fig. 4). These data are summarized in Table 2.

## DISCUSSION

The mechanism by which the administration of allopurinol leads to an apparent increase in the activity of OPRT and ODC in man has remained controversial. Fox *et al.* [3] attributed the increased activity of OPRT and ODC after allopurinol therapy to stabilization of these two enzymes *in vivo*. This conclusion was based on a prolongation of their apparent half-life in circulating erythrocytes after administration of allopurinol. The finding that ODC from rat liver was more stable to thermal inactivation after allopurinol administration was consistent with this hypothesis [7]. Beardmore *et al.* [6] noted, however, that when the activity of OPRT and ODC was followed serially after initiation of allopurinol therapy in man, there was no apparent change in the half-life of either enzyme in circulating erythrocytes. In addition, these investigators [6] found that incubation of allopurinol or oxipurinol with intact mature erythrocytes from untreated patients led to an increase in the activity of OPRT within 1 hr, even though no enzyme synthesis was occurring; no increase was observed if the erythrocytes were obtained from a patient treated with allopurinol or if the cells were lysed prior to addition of the drug. Finally, the apparent increase in OPRT and ODC activity in circulating erythrocytes and leukocytes was not associated with a detectable decrease in the allopurinol-mediated inhibition of ODC *in vivo* [6]. These observations were inconsistent with the proposal that OPRT and ODC were stabilized to degradation *in vivo*. Since increased enzyme synthesis and decreased enzyme degradation could not account for the increased activity of OPRT and ODC observed in circulating erythrocytes *in vivo*, Beardmore *et al.* [6], suggested that enzyme "activation" in its broadest sense must be occurring.

More recently there have been several studies which suggest that a different molecular species of OPRT and ODC predominates *in vivo* and in cell culture after the addition of allopurinol or oxipurinol [7, 8]. Brown *et al.* [7], observed two forms of OPRT and ODC in rat liver homogenate which differed in molecular size. The larger form was more stable to thermal inactivation and was noted to be more prominent in liver homogenate prepared from a rat receiving allopurinol. Becker *et al.* [8], noted a single peak of ODC activity by gel filtration in extracts prepared from human lymphoblasts. After addition of oxipurinol to the lymphoblasts in culture, a single peak of ODC activity was again observed which appeared, by gel filtration, to have a molecular weight approximately three times higher (108,000 vs 41,000) than that observed in the absence of the drug. In both of these studies, the authors suggested that metabolites of allopurinol stabilized OPRT and ODC by shifting the complex into a larger, more stable form. However, in neither study could the authors exclude the possibility that the appearance of this larger species was due to conversion from an inactive form (e.g. membrane bound or proenzyme) in the cell.

In the present study, we have examined the effect of allopurinol, oxipurinol, allopurinol ribonucleotide and oxipurinol 7-ribonucleotide on the molecular configuration of OPRT and ODC *in vitro*. Human OPRT and ODC exist in an apparent complex of three different molecular sizes; estimation of molecular weight by gel filtration and sedimentation velocity gave values of 55,000, 80,000 and 113,000. The large and intermediate forms are more stable than the small form. Although allopurinol and oxipurinol have no apparent effect on the configuration of OPRT and ODC, the presence of allopurinol ribonucleotide or oxipurinol 7-ribonucleotide results in a predominance of the large, most stable form of the enzyme complex. These studies suggest that the larger form of OPRT and ODC present after incubation of intact cells with allopurinol and oxipurinol [7, 8] could be due to a direct effect of allopurinol ribonucleotide or oxipurinol 7-ribonucleotide, respectively, on this complex. It seems likely that the oxipurinol 1-ribonucleotide would be capable of a similar effect. It is clear from our studies that this larger form of the complex need not represent the appearance in the cytoplasm of a new molecular species from a zymogen, an organelle, or a component of the membrane.

Our studies do not define the exact molecular basis for this change in configuration. The effect of allopurinol ribonucleotide could be due to a conversion of the small form of the complex to the large form. The fact that the molecular weight of the large form is twice that of the small form and that the appearance of activity in the large form is associated with a decrease in the activity present in the small form would be consistent with a dimerization process. However, incubation of the small form of the enzyme complex, isolated by gel filtration, with allopurinol ribonucleotide did not result in the formation of the large form; thus, dimerization could not be demonstrated directly. This result does not exclude such a model, since aggregation may depend on other factors which could not be exactly controlled. An alternative possibility is that allopurinol ribonucleotide stabilizes the large form of the enzyme but fails to protect the smaller forms of the enzyme from inactivation. Our data are equally consistent with this hypothesis.

Based on the data currently available, it seems likely that the mechanism by which allopurinol and oxipurinol increase the activity of OPRT and ODC is related in some manner to stabilization of the complex. The possibility that the increase in activity or apparent "activation" is due to stabilization of the complex during cell lysis and extraction is most consistent with the data currently available.

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## REFERENCES

1. R. M. Fox, D. Royse-Smith and W. J. O'Sullivan, *Science, Wash.* **168,** 861 (1970).
2. W. N. Kelley and T. D. Beardmore, *Science, Wash.* **169,** 388 (1970).
3. R. M. Fox, M. H. Wood and W. J. O'Sullivan, *J. clin. Invest.* **50,** 1050 (1971).
4. T. D. Beardmore and W. N. Kelley, *J. Lab. clin. Med.* **78,** 696 (1971).
5. J. A. Tyfe, R. L. Miller and T. A. Krenitsky, *J. biol. Chem.* **248,** 3801 (1973).
6. T. D. Beardmore, J. S. Cashman and W. N. Kelley, *J. clin. Invest.* **51,** 1823 (1972).
7. G. K. Brown, R. M. Fox and W. J. O'Sullivan, *Biochem. Pharmac.* **21,** 2469 (1972).
8. M. A. Becker, K. F. Argubright, R. M. Fox and J. E. Seegmiller, *Molec. Pharmac.*, in press.
9. T. E. Worthy and W. N. Kelley, *Proc. natn. Acad. Sci. U.S.A.*, **71,** 3031 (1974).
10. R. F. Beers, Jr. and I. W. Sizer, *J. biol. Chem.* **195,** 133 (1952).
11. B. L. Vallee and F. L. Hoch, *Proc. natn. Acad. Sci. U.S.A.* **41,** 327 (1955).
12. M. A. Hennessey, A. M. Waltersdorph, F. M., Huennekens and B. W. Gabrio, *J. clin. Invest.* **41,** 1257 (1962).
13. G. K. Ackers, *J. biol. Chem.* **242,** 3237 (1967).
14. K. S. McCarty, D. Stafford and O. Brown, *Analyt. Biochem.* **24,** 314 (1968).
15. M. H. Smith, in *Handbook of Biochemistry* (Ed. H. A. Sober), pp. CC-10–17. The Chemical Rubber Co., Cleveland, Ohio (1973).