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ALTERATION OF QUATERNARY STRUCTURAL BEHAVIOUR OF AN HEPATIC OROTATE PHOSPHORIBOSYLTRANSFERASE-OROTIDINE-5′-PHOSPHATE DECARBOXYLASE COMPLEX IN RATS FOLLOWING ALLOPURINOL THERAPY

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Abstract—The administration of allopurinol to rats was found to interfere with *de novo* pyrimidine biosynthesis, resulting in increased urinary excretion of orotic acid and orotidine and elevated activities of the enzymes orotate phosphoribosyltransferase and orotidine-5′-phosphate decarboxylase in erythrocytes. Similar increases in the liver enzymes were also observed, though it was necessary to dialyse the liver homogenates to observe an increase in the phosphoribosyltransferase. On the basis of thermal inactivation and gel filtration studies, evidence is presented that both enzymic activities reside in a single protein moiety capable of association–dissociation phenomena. Allopurinol administration resulted in the formation of an inhibitor (or inhibitors) of these enzymes. The inhibitor appeared to stabilize the enzymes by promoting the formation of higher molecular weight forms.

STUDIES in this laboratory have been concerned with the enzymes affected in hereditary orotic aciduria, a rare anaemia in which *de novo* UMP synthesis is drastically impaired and which manifests with excessive excretion of orotic acid.[1] In most reported cases (Type 1) the subjects have been demonstrated to be deficient in two enzymes of the *de novo* pyrimidine biosynthetic pathway, orotate phosphoribosyltransferase (OPRTase; EC 2.4.2.10) and orotidine-5′-phosphate decarboxylase (ODCase; EC 4.1.1.23),[1] though a variant form (Type II)†, in which only the decarboxylase appeared to be absent has been described.[2] In the latter case, the subject also excreted significantly increased amounts of orotidine.‡

Recently, it was shown that allopurinol (4-hydroxypyrazolo-(3,4-d)-pyrimidine) a hypoxanthine analog used in the treatment of hyperuricaemia,[3] also interfered with pyrimidine biosynthesis in man.[4,5] The effect of allopurinol partially simulated the variant form of orotic aciduria, in that the major metabolite excreted was orotidine, though there was also increased excretion of orotic acid. Thus, the major metabolic block appeared to be at the level of ODCase and we have presented evidence that the orotidinuria is caused by a metabolite of allopurinol, oxipurinol ribonucleotide (4,6-dihydroxypyrazolo-(3,4-d)-pyrimidine ribonucleotide).[6]

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† Nomenclature suggested by Dr. L. H. Smith.

‡ R. M. Fox, M. H. Wood and W. J. O'Sullivan, unpublished observations.

Allopurinol administration in man also appeared to cause a substantial increase (2- to 5-fold) in the level of both OPRTase and ODCase in eyrthrocytes. The two activities remained coordinate over a wide range of activity, as has been reported for the enzymes from bovine erythrocytes,[7] thymus[8] and calf brain.[9] The apparent increase could be ascribed, at least partially, to a stabilization of both activities so that their rate of degradation in erythrocytes was substantially reduced. Supporting evidence has come from studies with inorganic phosphate, an inhibitor of ODCase; high concentrations of phosphate tended to cause aggregation of the protein complex with an apparent increase in specific activity.[10]

A shortcoming in the experiments with man is that although some work has been reported with fibroblasts,[11] enzyme measurements are largely confined to changes in erythrocytes, where the enzymes concerned are residual and of relatively low specific activity.[12] To overcome this, the present study was initiated to see if similar experiments could be carried out in rats. It would thus be possible to compare the enzymes in different tissues, including the liver, where presumably, most of the metabolic effects described would be occurring.

MATERIALS AND METHODS

A highly inbred strain of Wistar rats were used. Allopurinol, a gift from Burroughs Wellcome (Aust), was thoroughly mixed with standard laboratory chow using a Nauta-mix mixer. The average dose/rat/day was 15 mg, i.e., about 60 mg/kg/day. (The therapeutic dose in man is usually 5–10 mg/kg/day). Some experiments were also carried out with rats on an average dosage of 15 mg/kg/day.

Experiments were commenced after the treated animals had been on allopurinol therapy for 21 days. The presence of orotic acid-reacting substances in the urine was estimated by the colorimetric method of Rogers and Porter.[13] The relative amounts of orotic acid and orotidine present were determined by liquid chromatography by Varian Aerograph, Walnut Creek, California, U.S.A.[14]

Haemolysates for the measurement of OPRTase and ODCase activity were prepared by removing the plasma and buffy coat after centrifugation, washing in 0·155 M KCl and freezing in an ethanol–dry-ice mixture.[6] Liver homogenates were prepared at a concentration of 20 g/100 ml in 0·25 M sucrose, centrifuged at 20,000 *g* for 30 min and the supernatants used for the assays.

Assay of orotate phosphoribosyltransferase (OPRTase) and orotidine -5'-monophosphate decarboxylase (ODCase)

Both enzymes were assayed by the method of Smith *et al.*[15] with slight modifications as described previously.[6,16] For the OPRTase assays, the reaction mixture consisted of 0·22 mM carboxy-^{14}C orotic acid (0·13 mc/mmole), 0·25 mM phosphoribosylpyrophosphate (PRPP), 3 mM $MgCl_2$, and approximately 0·1 unit of partially purified yeast ODCase (OPRTase activity less than 1×10^{-4} units) in 0·05 M sodium phosphate buffer, pH 7·4, with 0·1 ml of red cell haemolysate or liver homogenate, in a final volume of 1·0 ml. The reaction mixture for the measurement of ODCase activity consisted of 0·16 mM carboxy-^{14}C orotidine-5'-monophosphate (OMP) (0·13 mc/mmole), in buffer as above, with 0·1 ml of haemolysate or homogenate, in a final volume of 1·0 ml. Normally, incubation times of 1 hr were used for haemoly-

sates and of 20 min for the liver homogenates. For the latter, ODCase activity was linear up to 1 hr but the OPRTase started to depart from linearity after 20 min.

Gel-filtration chromatography. The crude supernatant from the liver homogenate was further centrifuged at 100,000 *g* for 2 hr and the supernatant (3 ml) applied to a 90 × 4 cm Sephadex G-150 column equilibrated at 4° with 0·01 M potassium phosphate, pH 7·4. Elution was carried out with the same buffer and 5 ml fractions were collected. The column was calibrated with human haemoglobin (M.W. 65,000), rabbit muscle creatine kinase (M.W. 82,600) and rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (M.W. 140,000).

Thermostability experiments. The liver homogenates were heated in the assay mixture lacking either PRPP (for OPRTase measurements) or OMP (for ODCase measurements) for periods between 2–12 min at 55°. The samples were cooled in ice, re-equilibrated at 37° and the reaction initiated by the addition of PRPP or OMP, respectively.

RESULTS

Orotic acid and orotidine excretion

As had been previously observed,[4] the administration of allopurinol to rats resulted in excess excretion of orotic acid—reacting compounds (Fig. 1). The urinary concentration of "orotic acid" was estimated by the semiquantitative method of Rogers and

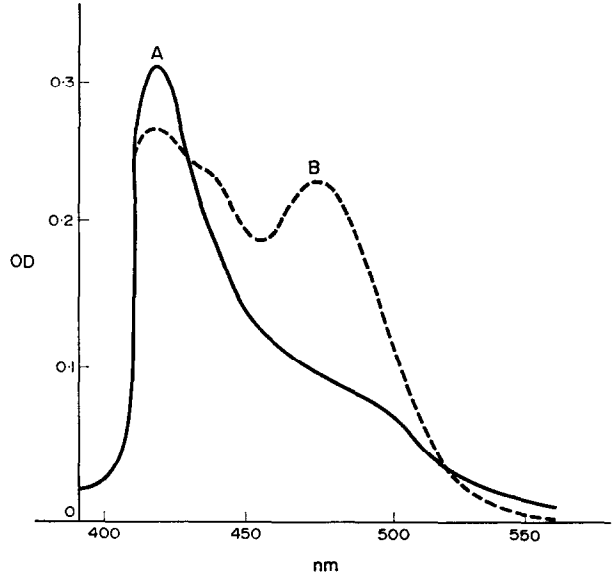

FIG. 1. Recorded spectra of urine treated according to the method of Rogers and Porter.[13] Curve A; urine from control rats; Curve B; urine from rats receiving allopurinol (15 mg/kg/day).

Porter[14] to be of the order of 30–60 μg/ml compared to less than 2 μg/ml for control animals. The very high background absorption with rat urine made it impossible to obtain a more accurate estimate. The magnitude of this background relative to the "orotic acid" peak also meant that the estimation of the relative amounts of orotic

acid and orotidine by extraction and subsequent thin layer chromatography, was impractical. However, analysis of a urine sample from a rat on 60 mg allopurinol/kg/day by high resolution liquid chromatography,[14] indicated that the predominant component was orotic acid although increased levels of orotidine were also present. The relationship of the relative amounts of orotic acid and orotidine excreted to the administered dose of allopurinol has not yet been established. As was observed in man, the results indicated inhibition of both OPRTase and ODCase.

Effect of allopurinol therapy on enzymic activities

Erythrocytes. An increase in the specific activity of both OPRTase and ODCase in erythrocytes of allopurinol treated rats in comparison to the control group was observed (Fig. 2). A co-ordinate relationship between the two activities was maintained

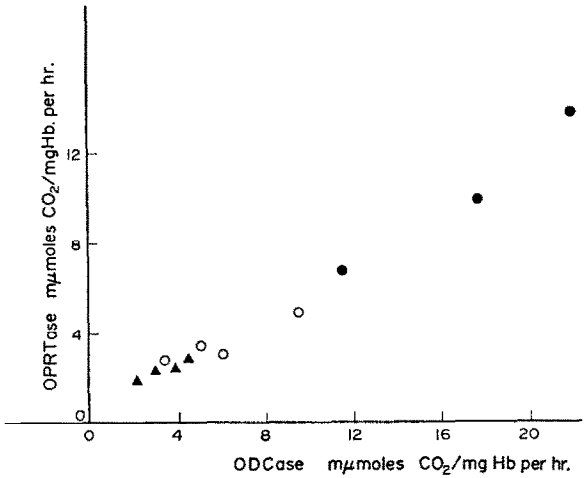

FIG. 2. Activity of OPRTase and ODCase in haemolysates from allopurinol treated rats, 15 mg/kg/day, ○; 60 mg/kg/day, (●) and the control group (▲).

over the whole range. The magnitude of the response appeared to be approximately related to the dosage of allopurinol. Significantly higher values were obtained for both enzymes with rats on 60 mg/kg/day compared to those on 15 mg/kg/day. Reticulocyte counts from control and treated groups of animals at the time of assay were identical.

Hepatic tissue. OPRTase and ODCase activities were assessed in liver homogenates from a group of control animals as well as rats receiving 60 mg allopurinol/kg/day. The specific activity of ODCase was considerably elevated in the treated animals, however the OPRTase activities did not differ significantly from the controls (Fig. 3). In view of this apparent contrast with the results obtained with erythrocytes the liver homogenates were dialysed against 0·01 M potassium phosphate buffer pH 7·4 (4 ml against 4 l. for 18 hr) and the specific activities of both OPRTase and ODCase redetermined. A substantial increase in OPRTase activity was observed following this dialysis, with a slight decrease in activity of ODCase (Fig. 3). This dialysis of rat liver homogenate led to the establishment of a co-ordinate elevation of both OPRTase and ODCase. The mean increase in hepatic tissue of treated animals was approximately

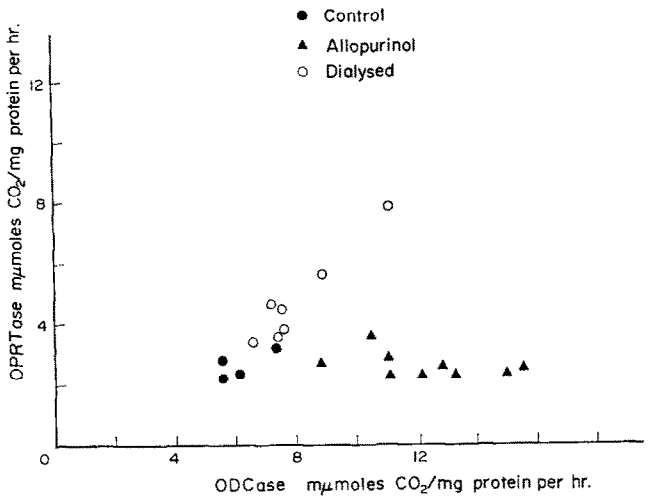

FIG. 3. Activity of OPRTase and ODCase in rat liver homogenates. Control group (●); allopurinol treated rats, before dialysis (▲), after dialysis (○).

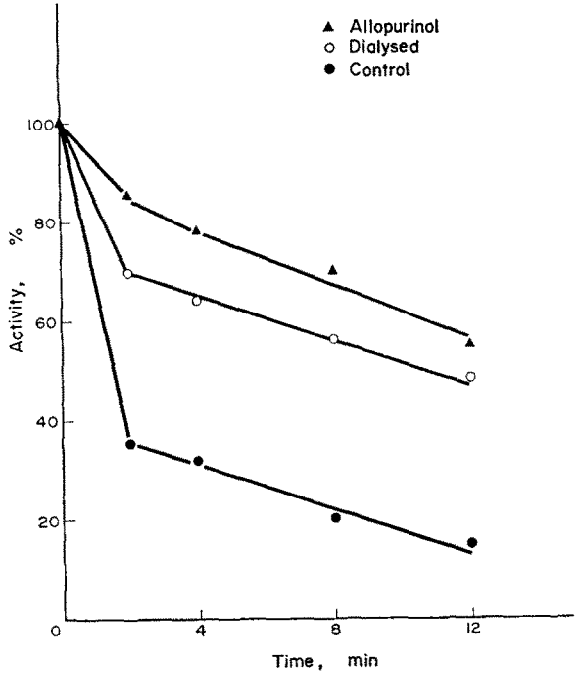

FIG. 4. Heat stability of ODCase in rat liver homogenates. Control group (●); allopurinol treated rats before dialysis (▲), after dialysis (○). (Initial relative activities were 100, 140 and 80 per cent respectively).

2-fold, compared to a 5-fold increase of the enzymes in the erythrocytes of these animals.

Thermostability of enzyme activities. In Fig. 4, the effect of incubation at 55° on the activity of ODCase under different conditions is illustrated. The activity of the enzyme was significantly stabilised in liver homogenates from the allopurinol treated rats and this stabilizing effect declined only slightly after dialysis as above. Experiments with OPRTase gave a similar pattern, though in all cases, ODCase activity was more stable to heating than OPRTase activity.

Gel filtration. Filtration of the liver homogenates on Sephadex G-150 essentially separated the enzyme activities into two peaks (Fig. 5). The peaks of activity in the eluant corresponded to molecular weights of approximately 90,000 and 240,000 respectively. Again, the phenomenon was better defined with ODCase than with OPRTase. Much of the latter activity was lost during the separation procedure, as has been observed for the human erythrocyte enzyme.[10]

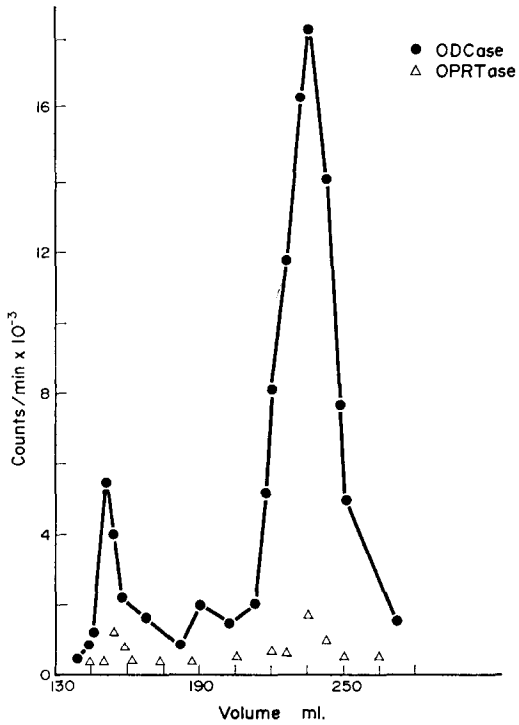

FIG. 5. Gel filtration chromatography of ODCase and OPRTase from control rat liver homogenates; ODCase (●), OPRTase (△).

In untreated animals, most of the enzyme activity was located in the lower molecular weight peak. It was noted that the OPRTase activity, however, appeared to be relatively stabilized in the higher molecular weight form as the OPRTase:ODCase ratio was considerably higher. Further, both activities in the higher molecular weight peak demonstrated greater stability to thermal inactivation (Fig. 6).

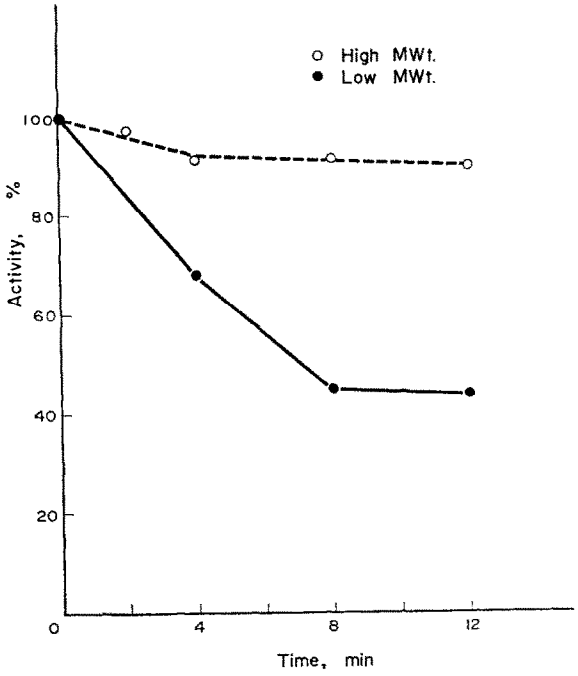

FIG. 6. Heat stability of ODCase in high and low molecular weight forms from control rat liver homogenate; high molecular weight form (○), low molecular weight form (●).

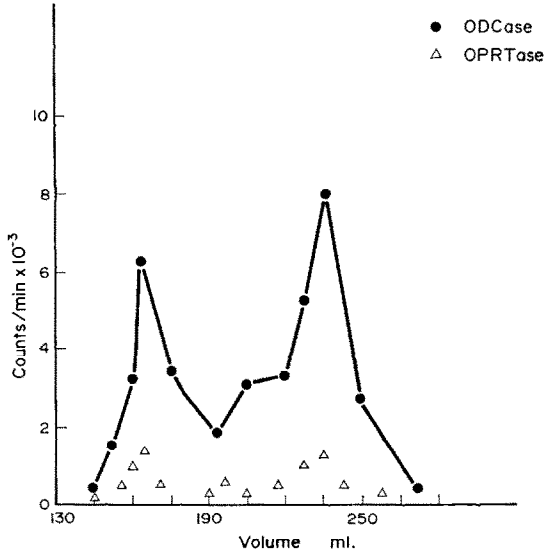

FIG. 7. Gel filtration chromatography of ODCase and OPRTase from allopurinol treated rat liver homogenates; ODCase (●), OPRTase (△).

In Fig. 7, comparative results with liver homogenates from the allopurinol treated animals are shown. Although the enzyme activities eluted in two peaks with elution volumes corresponding to those of the untreated animals the distribution of the two activities was markedly altered. The greater portion of both OPRTase and ODCase activity, eluted in a volume corresponding to the higher molecular weight form.

DISCUSSION

The results reported in this paper demonstrate that administration of the purine analogue, allopurinol, leads to significant interference in the pyrimidine biosynthetic pathway in the rat.[6] It was found that the effects seen in man, manifesting as increased excretion of orotic acid and orotidine and apparent increase in erythrocyte OPRTase and ODCase activities could be reproduced in rats. Further, similar changes in these two enzymes could be demonstrated in hepatic tissue.

Evidence has been presented elsewhere[6] that the most likely *in vivo* inhibitor in man giving rise to the orotidinuria, is oxipurinol ribonucleotide. Allopurinol is converted to oxipurinol by xanthine oxidase.[17] It appears that sufficient oxipurinol can be converted to its ribonucleotide, possibly by OPRTase, as it is an analogue of orotic acid. As oxipurinol ribonucleotide is an inhibitor of ODCase, accumulation of the former would be expected to lead to increased formation and excretion of orotidine.

This mechanism would also appear to be operating in the rat, though it is insufficient to account for all of the experimental observations. Thus, while ODCase activity in liver homogenates was increased, it was necessary to remove a small molecular weight compound by dialysis before an increase in OPRTase could be demonstrated. It is most likely that such a small molecular weight compound would be in the base form, probably either allopurinol or oxipurinol, presumably competing with orotic acid in the OPRTase reaction and causing an apparent reduction in its activity. Inhibition of the OPRTase reaction would give rise to the increased excretion of orotic acid. It should be noted that the results may not completely parallel the studies in man because of the much higher doses used; *viz.*, 60 mg/kg/day in rats compared to 5 mg/kg/day in man. In the former case, the relevant enzyme(s) could be saturated so that the base rather than the ribonucleotide was the predominant form. This is also consistent with orotic acid, rather than orotidine, being the major pyrimidine compound being excreted, i.e., the principal blockage is at the OPRTase. Studies to elucidate this are currently in progress.

The apparent increase in activity of the two enzymes appears to be partially due to stabilization by the formation of an aggregated state. The results illustrated in Figs. 5 and 7 were most consistent with the formation of a trimer of the original enzyme complex, though accurate determination of molecular weight from this type of experiment is somewhat hazardous. The aggregated state(s) of the enzyme appeared to be substantially more stable to heat treatment than the monomer forms. It was particularly noteworthy that removal of the presumed low molecular weight "stabilizer" by dialysis, only produced a slight decrease in activity and heat stability. If the stabilization observed is due to the formation of aggregated forms of the enzyme, it would appear that the aggregating effect is only partially reversed on removal of the low molecular weight compound. (This presumes that the "stabilizer" is not so firmly bound that it was only partially removed by the dialysis procedure, though this would be unlikely because of the increase in activity of the OPRTase after dialysis (Fig. 3).)

We conclude that the increased levels of OPRTase and ODCase in both erythrocytes and hepatic tissue, reported in this paper are partly explained as being due to stabilization of the enzymic activity following aggregation of the relevant protein(s). It has not yet been possible to ascertain as to whether any induced *de novo* synthesis of enzyme had also taken place. An increase in the concentration of OPRTase–ODCase could well lead to a greater proportion of the enzymic activity in higher molecular weight form.

In keeping with other studies on mammalian systems,[6–9] the gel filtration experiments and the observed co-ordinate activities of the two enzymes are consistent with the hypothesis that both OPRTase and ODCase activities are on the same protein. Furthermore, this study offers a novel mechanism for the alteration in tissue activities of an enzyme following drug administration.

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