

Studies on the Xanthine Oxidase Activity of Mammalian Cells

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*Xanthine oxidase in man is confined to but a few tissues and is absent from cultured cell strains. In rodents, however, the enzyme is more widely distributed among the tissues and can be demonstrated in most cell lines. Rodents possess the enzyme uricase and are therefore able to carry purine catabolism one step further than man. Preliminary results suggest that uricase is restricted to but a few rodent tissues and is absent from cultured rodent cells. Hence it may be that in each species only the final enzyme of purine catabolism is tissue restricted. In other experiments, mammalian cells were grown in the presence of compounds known to induce xanthine oxidase in a eukaryotic fungus (*Aspergillus nidulans*). These compounds did not induce the enzyme in mammalian cells.*

INTRODUCTION

In man and certain other primates, purine catabolism does not go beyond uric acid (Keilin, 1959; Christen *et al.*, 1970). Uricase, which converts uric acid to allantoin, is absent from these species, although it is present in all other mammalian species which have been studied. The enzyme which catalyzes the synthesis of uric acid (xanthine oxidase) is tissue restricted in man, being confined mainly to liver and gastrointestinal epithelium and absent (or present in negligible amounts) from most other tissues, including cultured human cells (Watts *et al.*, 1965). Early studies (Morgan, 1926;

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Westerfeld and Richert, 1949; Richert and Westerfeld, 1951) of xanthine oxidase activity suggested that it was more widely distributed among the tissues of lower mammals.

In the work to be described, we attempted to do four things. First, we sought to confirm the absence of xanthine oxidase activity in cultured human cells. Second, we inquired into the tissue distribution of xanthine oxidase in a nonprimate mammal and looked for the activity in a variety of lines of cultured rodent cells. The interest of these experiments is that if one can find a line of mammalian cells which contains xanthine oxidase, one may have a way of employing microbiological methods for the study of uric acid synthesis in mammals. In addition, we hoped to answer the question of whether the enzyme is more widely distributed among the tissues in those species where xanthine oxidase is *not* the final enzyme of purine catabolism. In other words, is it usually only the final enzyme of the catabolic sequence which is tissue restricted? Third, in connection with this latter question, we made preliminary observations on the distribution of uricase among the tissue of a rodent. Fourth, we tried to induce xanthine oxidase in lines of human, and other mammalian, cells using compounds known to induce the enzyme in a eukaryotic fungus. These experiments of course deal with the question of whether the known inducers of the enzyme in one eukaryotic cell are effective in another one.

Studies which are helpful in obtaining an answer to the last question have been published by Scazzochio and Darlington (1968). These authors found that xanthine oxidase is inducible in *Aspergillus nidulans*. In this organism, the cellular level of activity is greatly increased if hypoxanthine or uric acid is present in the medium. The effect of hypoxanthine, but not of uric acid, is blocked if the medium contains allopurinol—a competitive inhibitor of xanthine oxidase. On the basis of these and other observations, Scazzochio and Darlington (1968) concluded that uric acid was a more proximate inducer of xanthine oxidase than hypoxanthine. The induction of an enzyme by its own product can, in theory, result in a circumstance where a transient chemical stimulus of the cell causes a hereditary change in cellular phenotype (without a change in the DNA). Product induction is represented in one of the “circuits” proposed by Monod and Jacob (1961) as a possible molecular model for cell differentiation in higher organisms.

MATERIALS

Xanthine oxidase (from milk, 0.14 unit/mg protein), uricase (from hog liver, approximately 14 units/g protein), and other biochemicals were obtained from Sigma Chemical Company, St. Louis, Missouri. Predicted optical extinction coefficients for hypoxanthine (249 nm, pH 7.0) and uric acid (292 nm,

pH 7.0) were, respectively, 10.6 and 11.6 units of optical density per millimole per liter per centimeter of optical path. Weighed quantities of each compound exhibited 98–102% of the expected optical density. Uric acid-6-C¹⁴ was purchased from ICN Chemical and Isotope Division, Irvine, California. The purity of this preparation will be discussed in *Results*.

Cell Strains

Human fibroblasts were established from minced skin biopsies of a normal individual, strain CR, and of a patient, NAY, affected with the Lesch–Nyhan syndrome (Lesch and Nyhan, 1964). Strains EL and WI-38 (Hayflick and Moorhead, 1961) were developed from human embryonic lung tissue. S3 is a subline (Puck *et al.*, 1956) of the HeLa cell (Gey *et al.*, 1952). Strain CHA₂ was derived from the V-79 Chinese hamster strain isolated by Ford and Yerganian (1958). Two clonal sublines of this strain have been designated M-IA and U-4. Strain CHO (Kao and Puck, 1967) is also a Chinese hamster line. The RAM (Krooth *et al.*, 1964) and HR lines were developed from rat muscle tissue. Strain MH₁C₁ (kindly provided by Dr. A. H. Tashjian of the Harvard School of Dental Medicine) was obtained from a rat hepatoma and performs many liver-specific functions (Richardson *et al.*, 1969; Tashjian *et al.*, 1970). 3T3 is an established mouse line (Todaro and Green, 1963).

Media

Cells were grown in a medium previously described (Krooth, 1964) except that 12% fetal calf sera, rather than 12% human sera, were used in the present experiments. "Automedium" (Krooth, 1964) is a somewhat simpler medium and contains dialyzed rather than whole fetal calf sera. Both media are based on Eagle's (1959) Minimum Essential Medium.

Animals

Adult, white, male Sprague-Dawley rats were used.

METHODS

Cell Culture Methods

The methods employed for cell culture are given elsewhere (Krooth and Weinberg, 1961).

Preparation of Cells for Assay

Cells were washed twice with saline G (Puck *et al.*, 1958) and harvested by incubation at 37 C with 0.25% trypsin in tris-buffered saline (Martin, 1964).

The cells were then sedimented by centrifugation and resuspended in a small volume of ice-cold 0.1 M phosphate buffer, *pH* 7.8. Next the cells were disrupted by sonic energy (two pulses each of 30 sec duration), using a Milard probe sonicator. Clear samples were obtained after centrifugation at $15,000 \times g$ for 10 min, and were precipitated in an ice bucket for 30 min with 3 vol of a saturated $(\text{NH}_4)_2\text{SO}_4$ solution, *pH* 7.8. The precipitate was collected by centrifugation (30 min at $15,000 \times g$) and dissolved in a small volume of 0.1 M phosphate buffer, *pH* 7.8. This solution contained 70% of the xanthine oxidase activity initially present—a figure which was determined by the addition of purified enzyme to the cell suspension prior to sonication.

Preparation of Rat Organ Homogenates

Fresh tissue was excised and washed three times with saline to remove as much blood as possible. The specimen was then homogenized in a Waring blender (four cycles, each of 30 sec duration) in 0.01 M sodium phosphate buffer, *pH* 7.0. The homogenate was further dispersed by sonication (four pulses of 30 sec duration) at 4 C. Clumps were removed by centrifugation, and the supernatant was frozen and stored at -85 C. To determine the xanthine oxidase activity, the supernatant was thawed and the enzyme precipitated with $(\text{NH}_4)_2\text{SO}_4$, as described in the preceding paragraph.

Determination of Xanthine Oxidase Activity

Xanthine oxidase activity was measured colorimetrically by tetrazolium salt reduction as described by Fried (1966). The reaction mixture contained 1 mg nitro blue tetrazolium (Sigma, grade III), 0.01 mg phenazine methosulfate (Calbiochem), 0.1 μmole EDTA, and 0.2 mg Bacto-gelatine (Difco) in a total volume of 1.4 ml of 0.1 M sodium phosphate buffer, *pH* 7.8. One milliliter of cell protein solution was added to both sample and reference cuvettes (1 cm lightpath), and the optical density at 540 nm was continuously monitored in a Beckman DB spectrophotometer. After determining that the recorded optical density gave a straight baseline, 0.6 μmole of hypoxanthine in 0.3 ml sodium phosphate buffer (*pH* 7.8) was added to the sample (and 0.3 ml of phosphate buffer was added to the reference cell). The rate of tetrazolium reduction was found to be linear between 2 and (at least) 10 min after the addition of substrate. During the first 2 min the rate was slightly higher than subsequently.

Under these conditions, the rate of tetrazolium reduction is proportional to cell protein concentration for concentrations of up to 13 mg protein per milliliter of cell protein solution. On reducing weighed amounts of nitro blue tetrazolium nonenzymatically with NADH, it was found that (under conditions of the assay) 1.0 OD unit at 540 nm corresponds to the formation of

115 μg formazan per milliliter. Converting known amounts of hypoxanthine to uric acid, with the commercially obtained purified enzyme, it was calculated that 1.0 OD unit at 540 nm corresponds to the conversion of approximately 80 μmoles hypoxanthine per milliliter.

The specificity of each assay was verified by the addition to the reaction mixture of allopurinol [4-hydroxypyrazole (3,4-*d*)-pyrimidine], a known inhibitor of xanthine oxidase (Watts *et al.*, 1965). At a final concentration of 2.5×10^{-4} M, allopurinol completely inhibited enzyme activity in all preparations. The addition of purified enzyme to reaction mixtures which did not otherwise show xanthine oxidase activity resulted in a marked increase in OD_{540} . The increase was linear with time and with quantity of purified enzyme added. The catalytic activity of the solutions of cell and tissue protein was destroyed when the solutions were boiled for 10 min prior to the assay.

Determination of Uricase Activity

Uricase was measured by the ability of a cell or tissue extract to effect the release of C^{14}O_2 from uric acid-6- C^{14} . The extract was incubated for 30 min in the presence of uric acid-6- C^{14} , 0.1 M glycine buffer, pH 9.2, and 0.04 M Na_2CO_3 . The final volume was 1.8 ml. Only 64% of the counts in the commercial preparation of uric acid were releasable by purified uricase. The number of counts in CO_2 , derived from uric acid, was computed indirectly. Each sonicate was incubated in the presence of uric acid-6- C^{14} and in the presence and absence of excess unlabeled uric acid. By the solution of simultaneous equations, the counts in CO_2 derived from uric acid could then be estimated. The final concentration of uric acid was varied over a range from 6.97×10^{-5} M to 2.88×10^{-3} M, and the specific radioactivity over a range from 0.077 to 3.188 $\mu\text{c}/\mu\text{mole}$. CO_2 was trapped by methods described previously (Howell *et al.*, 1967) except that in the present experiments we employed the liquid scintillation system of Jeffay (1963).

Protein Determinations

Protein determinations were performed according to the method of Lowry *et al.* (1951).

RESULTS

Xanthine Oxidase Activity in Cultured Mammalian Cells

Table I lists the cell strains which were tested for xanthine oxidase activity. As can be seen, none of the human cell lines had detectable enzymatic activity, whereas most of the rodent cell strains showed the presence of xanthine oxidase.

Table I. Xanthine Oxidase Activity in Cultured Mammalian Cells

Cell strain	Species of origin	Xanthine oxidase activity ^a (units of OD ₅₄₀ /min/mg cell protein) × 100	mg protein/ ml enzyme solution
CR	Human	0	6.08
NAY	Human	0	8.4
EL	Human	0	8.0
S3	Human	0	14.3
CHA ₂	Chinese hamster	2.75 (356)	14.8
M-IA	Chinese hamster	0.40 (51.8)	18.7
U-4	Chinese hamster	7.66 (993)	21.7
CHO	Chinese hamster	0	11.5
RAM	Rat	1.66 (215)	6.6
HR	Rat	0.87 (113)	4.4
MH ₁ C ₁	Rat	0	6.8
3T3	Mouse	0.33 (42.8)	3.1

^a Numbers in parentheses denote millimicromoles product formed per hour of incubation per milligram cell protein. The zero entries for specific xanthine oxidase activity of course denote the absence of detectable catalytic activity. The assay employed will reliably detect a change in optical density of 0.05 OD₅₄₀ unit per minute.

Xanthine Oxidase Activity of Rat Organs

Table II shows that all rat organs tested, except brain, indeed do contain xanthine oxidase activity. Thus it appears that xanthine oxidase does not have the sharp tissue restriction in rodents that it has in man (Watts *et al.*, 1965).

Inducibility of Xanthine Oxidase in Cultured Cells

Human diploid cells (strain CR) were grown for 6 days in automedium supplemented with one or all of the following ingredients: uric acid (2.5×10^{-3} M), allantoin (5×10^{-3} M), and allantoic acid (5×10^{-3} M). No xanthine oxidase activity was found in the cells. In addition, the enzyme could not be detected after growing CR cells in medium containing inosine (1×10^{-4} M), hypoxanthine (5×10^{-4} M), or xanthine (2.5×10^{-3} M). An attempt was made to induce xanthine oxidase in human embryonic cells (EL cells). Embryonic lung tissue was minced and then cultured in automedium supplemented with 1×10^{-3} M uric acid. After 4 weeks of culture, no xanthine oxidase activity was detected in the cells.

We have also inquired whether the enzyme might be inducible in cells which spontaneously contain significant levels of activity. Accordingly, CHA₂ cells were chosen for these experiments because of their high xanthine

Table II. Xanthine Oxidase Activity of Whole Rat Organs

Organ	Xanthine oxidase activity ^a (units of OD ₅₄₀ /min/mg protein) × 100	mg protein/ ml enzyme solution
Liver	2.12 (275)	9.25
Heart ^b	0.096 (12.4)	4.40
Muscle	0.157 (20.3)	5.40
Spleen	0.84 (109)	7.03
Kidney	0.55 (71.3)	6.67
Brain	0	2.30

^a Numbers in parentheses denote millimicromoles product formed per hour of incubation per milligram cell protein. The zero entry for specific xanthine oxidase activity of course denotes the absence of detectable catalytic activity. The assay employed will reliably detect a change in optical density of 0.05 OD₅₄₀ unit per minute.

^b The homogenate prepared from rat heart may contain an inhibitor. Addition of heart homogenate to purified xanthine oxidase results in a 30% inhibition of the enzymatic activity.

Table III. Inducibility of Xanthine Oxidase in Chinese Hamster Cells

Supplement	Xanthine oxidase activity ^a (units of OD ₅₄₀ /min/mg protein) × 100	mg protein/ ml enzyme solution
None	2.75 (356)	14.80
Uric acid (1 × 10 ⁻³ M)	2.77 (359)	9.45
Hypoxanthine (5 × 10 ⁻⁴ M)	1.50 (194)	9.90
Allopurinol (5 × 10 ⁻⁵ M)	2.70 (350)	12.82

^a Numbers in parentheses denote millimicromoles product formed per hour of incubation per milligram cell protein.

oxidase level (see Table I). However, as Table III shows, no evidence of induction of enzymatic activity by any of the compounds tested was found.

Preliminary Observations on Uricase Activity in Rat Organs and in Cultured Mammalian Cells

It was found that rat liver homogenate converts approximately 16 μ moles of uric acid/min/mg protein to allantoin. Rat spleen homogenate also exhibited uricase activity, although less than $\frac{1}{200}$ of the value found for liver. However, since the labeled uric acid used in these experiments could be shown to contain radiolabeled impurities (*cf. Methods*), the reported values for uricase

activity represent only an approximation, and the trace activity found in spleen is especially open to question. There was no uricase activity detectable in homogenates from rat heart, muscle, or brain. In addition, no uricase could be found in human diploid cells (WI-38), HeLa cells, S3 cells, 3T3 cells, and cultured cells derived from rat muscle (RAM), rat embryo, Chinese hamster (CHA₂ and NCTC 4206), Syrian hamster, Walker rat carcinoma, Schmidt-Ruppin carcinoma, and cottontail rabbit.

DISCUSSION

We have confirmed the earlier observation (Watts *et al.*, 1965) that xanthine oxidase is absent from cultured human fibroblasts. Indeed this activity was undetectable in epithelioid—as well as fibroblastic—human lines. However, the activity is readily detected in most rodent lines. As noted earlier, rodents have the enzyme uricase and can therefore carry purine catabolism one step further than man and a number of other primates. Yet uricase activity has not been found in lines of cultured rodent cells. Thus most lines of rodent cells are, in a sense, analogous to a whole human being, for the cells of these lines can presumably catabolize purines to uric acid, but no further. Such cells may therefore be useful in experiments aimed at generating models for purine catabolism in man.

The presence of xanthine oxidase activity in the rodent lines and its absence in human ones is of interest in other respects. Xanthine oxidase is presumably repressed in some way in the cultured human cells. Will rodent-human somatic cell hybrids contain xanthine oxidase activity? Previous somatic cell crosses involving tissue-restricted functions have been obtained by hybridizing a cell line which performs such a function with a cell line that does not (Davidson, 1970). The function in every case has been thought to be tissue restricted in *both* parental species. No studies have been reported on functions which are normally tissue restricted in one parental species and are normally *not* tissue restricted in the other. Conceivably, through the application of the technique of somatic cell hybridization, it may eventually be possible to isolate clones of cells which synthesize human, as well as rodent, xanthine oxidase (Krooth and Sell, 1970; Klebe *et al.*, 1970; Weiss and Chaplain, 1971). Such clones, if they could be obtained, might make it feasible to study the xanthine oxidase of patients with various forms of gout—without having to biopsy internal organs. Lastly, one may ask whether the presence of xanthine oxidase in the cultured cells of a species that can carry uric acid catabolism one step further than man means that the enzyme is not tissue restricted in that species. Our results indicate that xanthine oxidase is not tissue restricted in rodents, and our preliminary results suggest that in the rat uricase (the final enzyme of the catabolic sequence in rodents) is confined

to but one or two organs. Hence it may be that only the *final* enzyme of the catabolic sequence, in each species, is tissue restricted. We are aware, however, that to prove this generalization further experiments are necessary. We have not of course exhausted all the ways of trying to demonstrate xanthine oxidase in the tissues and cells from which it appears to be absent. Moreover, we have measured the enzyme by its catalytic activity rather than by, say, its antigenic activity or some other index which might be less sensitive to experimental conditions. In addition, the generalization predicts that in forms which can catabolize purines one step further than rodents (as a result of allantoinase activity), uricase will be widely distributed among the tissues, and allantoinase only narrowly. We have not tested that prediction. Finally, our assay for uricase is still imperfect, because of a contaminant which we are unable to eliminate in one of our reagents. Despite these reservations the experimental predictions thus far tested make one suspect that the generalization holds.

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