[CANCER RESEARCH 46, 3838-3841, August 1986]

Decreased Concentration of Xanthine Dehydrogenase (EC 1.1.1.204) in Rat Hepatomas[1]

Tadashi Ikegami, Yutaka Natsumeda, and George Weber[2]

Laboratory for Experimental Oncology, Indiana University School of Medicine, Indianapolis, Indiana 46223

ABSTRACT

Xanthine dehydrogenase (EC 1.1.1.204), the rate-limiting enzyme of purine degradation, was purified 642-fold to homogeneity from liver of male Wistar rats. Antibody was generated to the purified enzyme in white rabbits and was partially purified. For the immunotitration a radioassay of high sensitivity was developed to determine low enzyme activities. Titration curves with the antibody showed that the xanthine dehydrogenase enzyme protein amounts in slowly growing hepatoma 20 and rapidly growing hepatoma 3924A were 34 and 4% of those of normal liver, which was in good agreement with the decrease in the activity of the enzyme to 33 and 2%, respectively. The contents of flavin adenine dinucleotide, the essential cofactor of the enzyme, in the immunoprecipitates in hepatomas 20 and 3924A were 27 and 4% of that of the normal liver. This is the first report to provide immunological evidence that a decreased enzyme activity in rat hepatomas, that of xanthine dehydrogenase, was due to a decrease in the enzyme protein amount. The markedly decreased xanthine dehydrogenase activity and amount have far-reaching biochemical and pharmacological implications for the tumors.

INTRODUCTION

The molecular correlation concept proposed that the metabolic imbalance in cancer cells, as revealed in the activities of key enzymes, was stringently linked with neoplastic transformation and progression and that this imbalance was interpreted as a manifestation of reprogramming of gene expression (1–3). This idea was supported by independent lines of evidence obtained in rat hepatomas by immunotitration which showed an increased amount of enzyme protein of the key enzymes, phosphofructokinase (EC 2.7.1.11) (4), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (5), amidophosphoribosyltransferase (EC 2.4.2.14) (6), CTP synthase (EC 6.3.4.2) (7), thymidine kinase (EC 2.7.1.21) (8), and carbamoyl-phosphate synthase II (EC 6.3.5.5) (9).

Previous studies in this laboratory showed that the activity of xanthine dehydrogenase (EC 1.1.1.204), the rate-limiting enzyme of purine degradation, decreased in all human, rat, and mouse tumors examined (3, 10–13). The purpose of the present study was to test whether the decrease in the activity of xanthine dehydrogenase in hepatomas was due to decreased enzyme protein amount.

MATERIALS AND METHODS

Materials. Xanthine, hypoxanthine, uric acid, Freund's complete adjuvant, and goat anti-rabbit IgG were obtained from Sigma Chemical Co., St. Louis, MO, and [8-^{14}C]hypoxanthine was purchased from Amersham Corp., Arlington Heights, IL. Hydroxylapatite (Bio-Gel HTP) was from Bio-Rad Laboratories, Rockville Center, NY, and Ultrogel ACA 22 was from LKB Instruments, Inc., Gaithersburg, MD. Immunodiffusion disc was from Miles Laboratories, Inc., Naperville, IL. All other chemicals were also of analytical grade.

Tissues. Chemically induced transplantable hepatomas were maintained as described previously (9). Hepatoma 20 was transplanted in male Buffalo strain rats, and hepatoma 3924A was carried in male ACI/N rats. Livers from Buffalo and ACI/N rats were used as controls. Hepatoma 3924A was homogenized with 3.3 volumes and hepatoma 20 and the livers were homogenized with 5 volumes of 50 mM potassium phosphate buffer, pH 7.4, containing 0.25 M sucrose and 0.3 mM EDTA, respectively. The homogenates were centrifuged at 100,000 × *g* for 30 min, and the clear supernatants were used for the enzyme assays and the immunotitration studies.

Preparation of Specific Antibody to Rat Liver Xanthine Oxidase. Xanthine oxidase (EC 1.1.3.22) was purified from livers of male Wistar rats as described elsewhere (14). Briefly, the method included 0 to 40% acetone fractionation, 40 to 60% $(NH_4)_2SO_4$ fractionation, hydroxylapatite column chromatography, Ultrogel ACA 22 column chromatography, and Sepharose 4B/folate affinity column chromatography. The enzyme was purified 642-fold and was homogeneous as judged by disc-gel electrophoresis. One mg of purified enzyme was injected intradermally at multiple sites on the back of male New Zealand white rabbits with an equal volume of Freund's complete adjuvant. The injection was repeated 3 times at 3-wk intervals. The increase in titer of antibody in serum was examined every 2 wk. Forty days after the last immunization when the titer was maintained at the highest level, the serum was collected and partially purified by repeated ammonium sulfate fractionation (0 to 50%). The antibody obtained was dialyzed against 0.1 M borate buffer, pH 8.4, and stored in liquid nitrogen.

Immunotitration Assay. Reaction mixture contained 200 μl of tissue extract, 100 μl of antibody with 0.9% NaCl solution, and 100 μl of anti-rabbit IgG with 0.9% NaCl solution in a total volume of 400 μl. The anti-rabbit IgG was added in excess to precipitate enzyme-antibody complex. Reaction tubes were incubated at 10°C for 4 h and at 4°C overnight and then centrifuged at 1000 × *g* for 10 min. The residual enzyme activity was determined in the supernatants. FAD[3] contents were measured by the method of Yagi (15) in the immunoprecipitates which were completely precipitated by excess titer of antibody and washed with 50 mM potassium phosphate buffer.

Microradioassay of Xanthine Dehydrogenase. Xanthine oxidase is now known to exist in the dehydrogenase form in freshly prepared extracts of many mammalian tissues, which requires NAD^+ as an electron acceptor for the enzymatic catalysis. The dehydrogenase form is readily converted to the oxidase form by incubation at 37°C, proteolysis with trypsin (16–18), or purification procedures such as ammonium sulfate fractionation (19). Thus, the assay mixture should include NAD^+ to obtain the total activity of dehydrogenase and oxidase forms. In the assay with NAD^+, the total dehydrogenase plus oxidase activity was not affected by the conversion of the dehydrogenase form to the oxidase form in the immunotitration study for rat liver and hepatomas. The standard assay mixture for xanthine dehydrogenase contained 0.6 mM NAD^+, 0.304 mM [^{14}C]hypoxanthine, 60 mM potassium phosphate buffer, pH 7.8, 0.12 mM EDTA, and enzyme solution in a total volume of 25 μl. The reaction was initiated by addition of 5 μl of enzyme into the preincubated assay mixture. The assay tubes were incubated at 37°C for 5 min unless stated otherwise, immersed into boiling water for 15 s to stop the reaction, and then placed on ice. The tubes were centrifuged at 1000 × *g* for 10 min, and 15 μl of the supernatant were carefully removed and spotted on Whatman 3 MM chromatography paper (27 x 92 cm). Sixty nmol each of authentic hypoxanthine, xanthine, and

Received 2/20/86; accepted 4/25/86.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by USPHS Grants CA-13526 and CA-05034 awarded by the National Cancer Institute, Department of Health and Human Services.

[2] To whom requests for reprints should be addressed, at Laboratory for Experimental Oncology, Indiana University School of Medicine, 702 Barnhill Drive, Indianapolis, IN 46223.

[3] The abbreviations used are: FAD, flavin adenine dinucleotide; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; PRPP, 5-phosphoribosyl 1-pyrophosphate.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/46/8/3838/2426869/cr0460083838.pdf by guest on 15 June 2024

uric acid and 120 nmol of allantoin were cospotted, and the reaction products were separated by high voltage electrophoresis, using Model FP-30A (Savant Instruments, Inc., Hicksville, NY) in 50 mM borate buffer, pH 9.0, at 3000 V for 90 min. The spots were detected under UV light and counted. To identify allantoin a clear yellow spot was produced by spraying *p*-dimethylaminobenzaldehyde:concentrated HCl:acetone (1 g:25 ml:100 ml). Radioactivities were corrected by subtraction of the values of 0-time control.

RESULTS AND DISCUSSION

Microradioassay of Xanthine Dehydrogenase. The migration distances for allantoin, xanthine, uric acid, and hypoxanthine in the paper electrophoresis were 22.1, 17, 14.4, and 12.2 cm, respectively. In the standard assay conditions, 100% recovery for radioactivity applied on Whatman 3 MM paper was observed in the sum of radioactivity in each spot of xanthine and hypoxanthine. Uric acid formation from xanthine catalyzed by the same enzyme was negligible in the standard assay for liver and hepatomas in which less than 5% of [^{14}C]hypoxanthine was consumed in the reaction mixture. As shown in Fig. 1 good proportionality of the enzymic activity with incubation time was achieved up to 10 min, and with the enzyme protein added from 3 to 27 μg (liver) and 20 to 79 μg (hepatoma).

Immunotitration Study of Xanthine Dehydrogenase. To elucidate the differences in immunoreactivity of the dehydrogenase form and the oxidase form, we investigated the immunotitration curves of the freshly prepared liver extract and of the liver extract which was incubated at 37°C. In the fresh liver extract, more than 80% of the enzyme existed as the dehydrogenase form, whereas incubation at 37°C for 2 h converted it to the oxidase form (Table 1). As shown in Fig. 2, no difference of the slope of immunotitration curves was observed between the fresh extract and the incubated one. Double immunodiffusion studies also showed that the antibody reacted with both crude and purified xanthine dehydrogenase (oxidase) resulting in a single precipitin line (data not shown). Thus, we ensured that the antibody for the oxidase form can be used for immunotitration study in crude extract in which dehydrogenase and oxidase forms coexist.

Immunotitration curves for rat livers and hepatomas are shown in Fig. 3. Rat liver from ACI/N was the control for hepatoma 3924A, and that from Buffalo for hepatoma 20. As shown in this figure the titration curves of liver from ACI/N

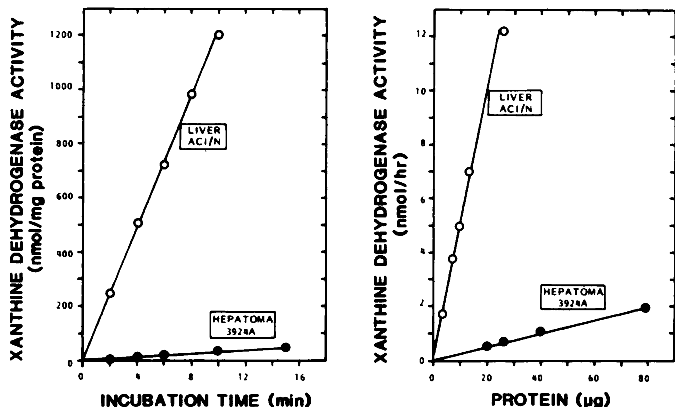

Fig. 1. Proportionality of xanthine dehydrogenase activity with elapsed incubation time and with amount of protein added in liver (○) and hepatoma 3924A (●). The standard assay described in "Materials and Methods" was used.

Table 1 *Conversion from dehydrogenase form to oxidase form by incubation*

	Activity, μmol/h/g tissue	
Enzyme forms[a]	Fresh extract	Incubated[b]
Dehydrogenase + oxidase	31.5 (100)[c]	32.2 (100)
Oxidase	5.4 (17)	31.5 (98)

[a] The conversion to oxidase form was measured spectrophotometrically by the method of Waud *et al.* (19). To measure total activity (dehydrogenase plus oxidase) the reaction mixture contained 50 mM potassium phosphate buffer, pH 7.8, 0.15 mM xanthine, and 0.5 mM NAD$^+$. Oxidase activity was measured by the reaction mixture without NAD$^+$.

[b] The freshly prepared extract of rat liver from ACI/N was incubated at 37°C for 2 h.

[c] Numbers in parentheses, percentage of total activity.

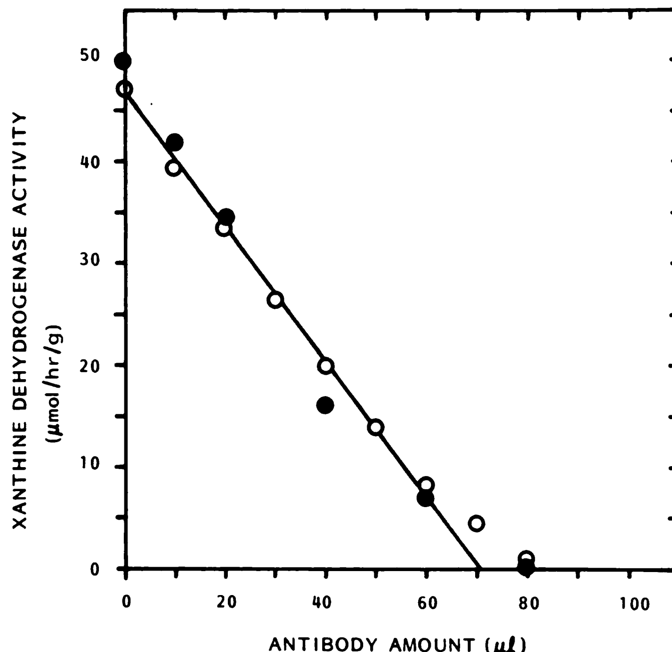

Fig. 2. Immunotitration curves for dehydrogenase form (○) and oxidase form (●). The freshly prepared extract of rat liver from ACI/N was used as dehydrogenase form. The extract incubated at 37°C for 2 h was used as oxidase form. Immunotitration and enzyme assay were as described in "Materials and Methods."

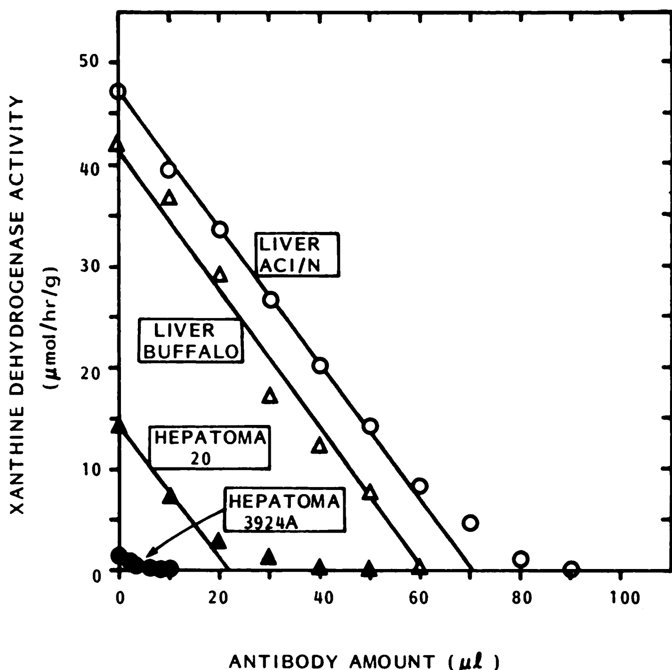

Fig. 3. Immunotitration curves for livers and hepatomas. Livers from ACI/N (○) and Buffalo (△) strain rats were used as controls for hepatoma 3924A (●) and hepatoma 20 (▲), respectively. Immunotitration and enzyme assay were described in "Materials and Methods."

Downloaded from http://aacrjournals.org/cancerres/article-pdf/46/8/3838/2426869/cr0460083838.pdf by guest on 15 June 2024

strain and hepatoma 3924A and liver from Buffalo strain and hepatoma 20 were parallel to each other. Xanthine dehydrogenase from both livers and hepatomas was completely neutralized by the antibody to liver xanthine oxidase. The relationship between enzyme activities and antibody was summarized in Table 2. Xanthine dehydrogenase activity in hepatoma 20 was 33%, and the volume of antibody required to complete neutralization was 34% of that of liver. Similarly, activity in hepatoma 3924A was 2% of that of the control liver, and the volume of antibody was 4%. These results suggest that xanthine dehydrogenases in liver and hepatoma were immunologically similar or identical, and the decreased enzyme activity in hepatoma reflected a decrease in the enzyme protein amount.

It was also relevant to measure flavin content in the immunoprecipitate. The flavin contents in the immunoprecipitates from hepatoma 20 and hepatoma 3924A extracts were 27 and 4% of that of control livers. Since one enzyme molecule of xanthine dehydrogenase from rat liver contains two FAD molecules (19), the decrease of flavin contents in hepatoma immunoprecipitates is direct evidence of decreased enzyme protein in neoplastic transformation.

Biochemical and Pharmacological Implications of the Decreased Xanthine Dehydrogenase Amount in Cancer Cells. Novel aspects and significant implications of the present study include: (*a*) purification of xanthine oxidase to apparent homogeneity from rat liver and production of monovalent polyclonal antibody to the enzyme; (*b*) demonstration of the equivalent immunoreactivity of xanthine oxidase and xanthine dehydrogenase against the antiserum; (*c*) generation of immunotitration curves for xanthine dehydrogenase in liver and 2 hepatomas of different growth rates; and (*d*) first demonstration of decreased flavin content in the immunoprecipitates of xanthine dehydrogenase in hepatomas. Although recent work suggested the existence of the inactive enzyme in rat liver (14), demonstration of the decreased flavin content in the hepatoma immunoprecipitates provides a firm conclusion for the decrease in xanthine dehydrogenase amount. (*e*) The demonstration of decreased xanthine dehydrogenase amount completes the chain of evidence showing that all the enzymes of purine degradation, including 5′-nucleotidase, inosine phosphorylase, and uricase, are decreased in activity (3). This indicates that the genetic control in cancer cells involves a coordinated decline in the production of all 4 enzymes of purine degradation. (*f*) Several examples showed that the increases in the activities of key enzymes in purine and pyrimidine metabolism represented an increase in the amount of these enzymes (3). However, this is the first demonstration that the decrease in the activity of a key enzyme represented a decrease in the amount of an enzyme, in this case, xanthine dehydrogenase. (*g*) With the increase demonstrated earlier in the amount of the rate-limiting purine synthetic enzyme, amidophosphoribosyltransferase (6), and the decreased amount of the rate-limiting purine catabolic enzyme, xanthine dehydrogenase, new evidence is provided that the reciprocal regulation of purine synthesis and degradation involves production of an increased amount of the synthetic and a decreased amount of the catabolic enzymes. This contributes to the amplified capacity of the cancer cell to make IMP and the lower capacity to degrade it. (*h*) With the maintenance in the hepatomas of HGPRT activity that utilizes hypoxanthine for salvage synthesis of IMP (20) the decrease in xanthine dehydrogenase amount yields a rise in the ratio of the activity of HGPRT to xanthine dehydrogenase; this imbalance significantly contributes to the increased capacity for salvage of hypoxanthine to produce IMP in the tumors. (*i*) The high HGPRT/xanthine dehydrogenase ratio steps up the capacity to convert 6-mercaptopurine into mercapto-IMP. This yields an enzymic imbalance in cancer cells that is similar to that obtained in cancer patients treated with allopurinol, which powerfully inhibits xanthine dehydrogenase. In humans this results in an increased conversion of the drug to mercapto-IMP, and the imbalance decreases the dose required for 6-mercaptopurine. Thus, rat hepatoma may be more sensitive to this drug than other tissues. (*j*) In purine metabolism, the increased capacity for PRPP production through increased PRPP synthase activity (21), the elevated PRPP concentration in hepatoma 3924A (20, 22), and the increased amidophosphoribosyltransferase/xanthine dehydrogenase (10) and HGPRT/xanthine dehydrogenase activity ratios should increase the imbalance towards an elevated capacity for IMP production.

Table 2 *Activity and immunotitration of xanthine dehydrogenase in rat livers and hepatomas*

	Xanthine dehydrogenase activity		100% neutralization titer		Flavin content	
Tissues	μmol/h/g	% of control	Antibody (μl)[a]	% of control	pmol/g	% of control
Liver (Buffalo)	42.0	100	61	100	1000	100
Hepatoma 20	13.7	33	21	34	265	27
Liver (ACI/N)	47.3	100	71	100	1400	100
Hepatoma 3924A	1.1	2	3	4	55	4

[a] Extrapolation of the linear portion of the enzyme activity *versus* antiserum quantity plot to 100% inhibition was taken as the amount of antibody required for complete neutralization.

ACKNOWLEDGMENTS

The authors thank Dr. Takeshi Nishino, Department of Biochemistry, Yokohama City University School of Medicine, Yokohama, Japan, for helpful discussions.

REFERENCES

1. Weber, G. Enzymology of cancer cells, Part 1. N. Engl. J. Med., *296:* 486–493, 1977.
2. Weber, G. Enzymology of cancer cells, Part 2. N. Engl. J. Med., *296:* 541–551, 1977.
3. Weber, G. Biochemical strategy of cancer cells and the design of chemotherapy: G. H. A. Clowes Memorial Lecture. Cancer Res., *43:* 3466–3492, 1983.
4. Dunaway, G. A., Jr., Morris, H. P., and Weber, G. Increased phosphofructokinase concentration in hepatoma 3924A: enzymic and immunological evidence. Life Sci., *11* (Part II): 909–918, 1972.
5. Selmeci, L. E., and Weber, G. Increased glucose 6-phosphate dehydrogenase concentration in hepatoma 3924A: enzymic and immunological evidence. FEBS Lett., *61:* 63–67, 1976.
6. Tsuda, M., Katunuma, N., Morris, H. P., and Weber, G. Purification, properties, and immunotitration of hepatoma glutamine phosphoribosylpyrophosphate amidotransferase (amidophosphoribosyltransferase, EC 2.4.2.14). Cancer Res., *39:* 305–311, 1979.
7. Tzeng, D. Y., Sakiyama, S., Kizaki, H., and Weber, G. Increased concentration of CTP synthetase in hepatoma 3924A: immunological evidence. Life Sci., *28:* 2537–2543, 1981.
8. Lai, M.-H. T., and Weber, G. Increased concentration of thymidine kinase in rat hepatomas. Biochem. Biophys. Res. Commun., *111:* 280–287, 1983.
9. Reardon, M. A., and Weber, G. Increased carbamoyl-phosphate synthetase II concentration in rat hepatomas: immunological evidence. Cancer Res., *45:* 4412–4415, 1985.
10. Prajda, N., Morris, H. P., and Weber, G. Imbalance of purine metabolism in hepatomas of different growth rates as expressed in behavior of xanthine oxidase (EC 1.2.3.2). Cancer Res., *36:* 4639–4646, 1976.
11. Weber, G., Kizaki, H., Tzeng, D., Shiotani, T., and Olah, E. Colon tumor: enzymology of the neoplastic program. Life Sci., *23:* 729–736, 1978.
12. Prajda, N., Donohue, J. P., and Weber, G. Increased amidophosphoribosyltransferase and decreased xanthine oxidase activity in human and rat renal cell carcinoma. Life Sci., *29:* 853–860, 1981.
13. Weber, G., Burt, M. E., Jackson, R. C., Prajda, N., Lui, M. S., and Takeda, E. Purine and pyrimidine enzymic programs and nucleotide pattern in sarcoma. Cancer Res., *43:* 1019–1023, 1983.
14. Ikegami, T., and Nishino, T. The presence of desulfo xanthine dehydrogenase

Downloaded from http://aacrjournals.org/cancerres/article-pdf/46/8/3838/2426869/cr0460083838.pdf by guest on 15 June 2024

in the purified and crude enzyme preparations from rat liver. Arch. Biochem. Biophys., in press, 1986.

15. Yagi, K. Simultaneous microdetermination of riboflavin, FMN, and FAD in animal tissues. Methods Enzymol., *8* (Part B): 290–296, 1971.
16. Stirpe, F., and Della Corte, E. The regulation of rat liver xanthine oxidase: conversion *in vitro* of the enzyme activity from dehydrogenase (type D) to oxidase (type O). J. Biol. Chem., *244:* 3855–3863, 1969.
17. Della Corte, E., and Stirpe, F. The regulation of rat liver xanthine oxidase: involvement of thiol groups in the conversion of the enzyme activity from dehydrogenase (type D) into oxidase (type O) and purification of the enzyme. Biochem. J., *126:* 739–745, 1972.
18. Krenitsky, T. A., and Tuttle, J. V. Xanthine oxidase activities: evidence for two catalytically different types. Arch. Biochem. Biophys., *185:* 370–375, 1978.
19. Waud, W. R., and Rajagopalan, K. V. Purification and properties of the NAD^+-dependent (type D) and O_2-dependent (type O) forms of rat liver xanthine dehydrogenase. Arch. Biochem. Biophys., *172:* 354–364, 1976.
20. Natsumeda, Y., Prajda, N., Donohue, J. P., Glover, J. L., and Weber, G. Enzymic capacities of purine *de novo* and salvage pathways for nucleotide synthesis in normal and neoplastic tissues. Cancer Res., *44:* 2475–2479, 1984.
21. Baló-Banga, J. M., and Weber, G. Increased 5-phospho-α-D-ribose-1-diphosphate synthetase (ribosephosphate pyrophosphokinase, EC 2.7.6.1) activity in rat hepatomas. Cancer Res., *44:* 5004–5009, 1984.
22. Weber, G., Natsumeda, Y., Lui, M. S., Faderan, M. A., Liepnieks, J. J., and Elliott, W. L. Control of enzymic programs and nucleotide pattern in cancer cells by acivicin and tiazofurin. Adv. Enzyme Regul., *22:* 69–93, 1984.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/46/8/3838/2426869/cr0460083838.pdf by guest on 15 June 2024