PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 149, 900–902 (1975)

Growth Inhibitory and Arginase Activities in Liver and Hepatoma Extracts (38923)

J. ARLY NELSON, JEAN W. CARPENTER AND H. P. MORRIS

Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, Alabama 35205, and Department of Biochemistry, Howard University College of Medicine, Washington, D. C. 20001

While attempting to use an *in vitro* rat liver microsomal preparation to "activate" a chemotherapeutic agent, cyclophosphamide (1), we observed that marked growth-inhibitory activity toward tumor cells in culture could be derived from the microsomes themselves. Others have previously reported that arginase may be responsible for cytotoxicity of liver extracts (2–4) and that fast-growing hepatomas contain lower levels of arginase activity (5, 6). We report herein on the growth-inhibitory activities and arginase levels in liver extracts and in extracts of hepatomas with different growth rates.

Methods. Livers or hepatomas from Buffalo, Sprague–Dawley, or ACI rats 2.5–10 mo old were homogenized (20% w/v) in 0.25 *M* sucrose, 1 m*M* Tris pH 7.4 with a glass homogenizer equipped with a Teflon pestle. An aliquot of the homogenate was diluted to contain 50 mg tissue/ml with 50 m*M* phosphate buffer, pH 7.4. The remaining homogenate was fractionated to obtain the microsomal pellet, that which precipitates between 10,000 *g* for 20 min and 105,000*g* for 60 min. The microsomes were resuspended to 500 mg original tissue weight per milliliter using 50 m*M* phosphate buffer. Extracts of whole tissue or of microsomes were prepared by incubation at 37° for 1 hr followed by overnight freezing at −20°. Particulate matter was removed, after thawing, by centrifugation at 105,000*g* for 60 min. The extract thus prepared was sterilized by Millipore filtration and was stored at −20° until bioassayed.

Bioassay of the extracts was performed by a cloning technique using approximately 100 H. Ep. № 2 cells in 10 ml of SRI 14 medium (7). I_{50} values (the level of extract producing 50% decrease in cell viability) were determined from plots of relative cloning efficiency vs log extract concentration. All experiments were performed at least in duplicate throughout, and agreed within 20% of mean values shown.

Arginase activity was determined at pH 9.5 following activation with Mn^{2+} exactly as described elsewhere (8). Protein was determined by the method of Lowry *et al.* (9) using bovine serum albumin as a standard. DNA was measured by the diphenylamine reaction (10) using calf thymus DNA as a standard. Hepatomas were grown subcutaneously and were harvested when the tumor mass was approximately 3–5 *g*. Procedures for maintaining the tumors and characteristics of the hepatomas have been reported previously (11).

Sources of the enzymes used were as follows: bovine liver arginase, 25 units/mg, Sigma Chemical Co. St. Louis, MO; Pronase, B grade, 45 units/mg, Calbiochem, LaJolla, CA; ribonuclease A, 4500 units/mg, Worthington Biochemical Corp.; Freehold, NJ; and trypsin from bovine pancreas, 10,000 units/mg, Schwarz/Mann, Orangeburg, NY. Rabbit anti-rat serum (Lot 15) was obtained from Miles Laboratories, Inc., Kankakee, Ill.

Results and Discussion. Arginase and growth-inhibitory activities are lower in whole tissue or microsomal extracts of the fast-growing hepatomas, 7777, Novikoff, and 3924A, than in extracts of control rat livers (Table I). The data obtained from the livers of control, nontumor bearing rats of the strains used was combined since no striking differences were noted. Livers of host rats bearing the 7777 and 3924A hepatomas were about one-half as active as controls with respect to growth-inhibitory activities; however, host livers of the other tumor-bearing rats did not show such marked differences (data not shown). Since the growth inhibition produced by extracts of the 7777 and Novikoff hepatomas clearly

TABLE I. ARGINASE AND GROWTH-INHIBITORY ACTIVITIES IN EXTRACTS OF VARIOUS TISSUES

Tissue	Arginase, nmolar units/mg		I_{50}, mg tissue/ml		I_{50}, Arginase units/ml		Protein, μg/mg tissue		DNA, μg/mg tissue	Dates of harvest months
	WTH[a]	MIC[b]	WTH[a]	MIC[b]	WTH[a]	MIC[b]	WTH[a]	MIC[b]		
Control rat liver	895 +130[c]	89 ±14[c]	0.24 ±.02[c]	2.4 ±0.2[c]	215	214	75	4.0	1.6	—
Novikoff hepatoma	21	2.4	2.5	18	52	43	49	2.8	1.6	½
7777 Hepatoma	—	2.2	2.8	12	—	26	31	2.7	2.3	½
3924A Hepatoma	—	—	3.1	12	—	—	43	3.8	1.6	½
7787 Hepatoma	62	11	2.8	10	174	110	96	6.0	1.6	2
7794A Hepatoma	—	92	—	3.3	—	304	—	4.3	—	2½–3
47C Hepatoma	210	71	0.60	3.0	126	213	60	3.9	1.5	7
Fetal rat liver	—	—	—	7.3	—	—	—	2.8	2.8	—

[a] Extract of whole tissue homogenate.
[b] Extract of microsomal fraction.
[c] Mean value ± SE, n = 8–15. Other values are means of two or more determinations.

occurred at lower levels of arginase activity (26–52 units/ml vs 215 units/ml in controls), factor(s) other than arginase may be responsible for the inhibition of growth in these extracts. Alternatively, there may be a unique property associated with the enzyme from these sources. The arginase from hepatoma 7777 appears to be immunologically distinct from that of normal rat liver (12). Microsomal extracts from the pooled livers of 36 fetuses (3 days before term) contained less inhibitory activity. The arginase level in fetal rat liver has been reported to be lower than in normal adult rats (see for example, 13). Similarly extracted microsomes from brain and kidney of adult rats contained less than one-third the growth-inhibitory activity of control livers (data not shown). The differences in activities of extracts given in Table I do not appear to relate to differences in amount of tissue used as reflected in the DNA determinations nor to be due to differences in extractable protein. The I_{50} for commercial bovine liver arginase was 223 units/ml, similar to that of the rat liver enzyme.

An approximate threefold purification of the growth inhibitory factor resulted in a similar degree of purification of arginase (Table II). A crude microsomal extract was treated with 1% streptomycin to remove the RNA and was then fractionated with ammonium sulfate at a saturation between 40 and 60%. The 60% ammonium sulfate precipitate was exhaustively dialyzed without loss of either activity. Growth-inhibitory activity in the 60% ammonium sulfate precipitate was associated with protein having a molecular weight in the range of 100,000–150,000 as determined by chromatography on Sephadex G-100 and G-200 gels. This roughly corresponds with the molecular weights previously reported for rat liver arginase (see 14).

TABLE II. PARTIAL PURIFICATION OF GROWTH-INHIBITORY FACTOR AND OF ARGINASE ACTIVITY FROM RAT LIVER

Preparation	Arginase nmolar units/μg protein	I_{50} μg protein/ml	I_{50} units/ml
Whole tissue extract	15	18	270
Crude microsomal extract	27	9.5	257
60% Ammonium sulfate fraction	48	5.3	254

Treatment of crude microsomal extracts from control rats with trypsin, pronase or heat reduced the arginase and growth-inhibitory activities (Table III). RNA was

TABLE III. EFFECTS OF VARIOUS PRETREATMENTS ON THE GROWTH-INHIBITORY AND ARGINASE ACTIVITIES OF LIVER MICROSOMAL EXTRACT

Pretreatment	Viability	Arginase
	% of control	
None, liver extract, 5 mg/ml[a]	0	100
Pronase, 4.5 units/ml, 1 hr at 37°	100	5
Ribonuclease, 450 units/ml, 1 hr at 37°	0	96
Trypsin, 1000 units/ml, 1 hr at 37°	75	39
Heat, 2 min at 100°	100	0
Rabbit antiserum to rat serum, 0.1 ml/ml	0	—

[a] The concentration of extract shown refers to the level in the bioassay. Pretreatments were performed at a level of 100 mg/ml.

present in the crude extracts; however, ribonuclease was without effect on growth-inhibitory activity. Although not shown in Table III, the inhibition of growth was also reversed by adding arginine or citrulline (1–5 m*M*) to the bioassay medium.

The possibility that arginase may play a role in the regulation of cell growth has been discussed (2). It is also of interest that this enzyme was once considered to be involved in the process of hepatoma generation by certain azodyes (2); however, it was subsequently found not to be the principal binding protein for these carcinogens (15). A recent report reviews the effects of arginase on cells in tissue culture (16).

Summary. The growth-inhibitory activity against H.Ep. # 2 cells observed in phosphate buffer extracts of rat liver appears to be due to the enzyme, arginase. This conclusion is based on the findings that (1) extracts of hepatomas containing lower levels of the enzyme also contain less inhibitory activity; (2) partial purification of the inhibitory factor results in a similar purification of the enzyme; (3) molecular weights of the enzyme and of the inhibitory factor are similar; and (4) various treatments alter the enzyme and inhibitory activities similarly.

Supported in part by USPHS Grant CA 10729. The authors acknowledge the valuable technical support of Mrs. C. M. Jackson, Ms. Louise Lawson, and Ms. Tommie Lou Barker.

1. Hill, D. L., Laster, W. R., Jr., and Struck, R. F., *Cancer Res.* **32,** 658 (1972).
2. Sorof, S., Young, E. M., Luongo, L., Kish, V. M., and Freed, J. J., *in* "Growth Regulating Substances for Animal Cells in Culture" (V. Defendi and M. Stoker, eds.), p. 25. Winstar Institute Symposium Monograph No. 7, Wistar Institute Press. Philadelphia (1967).
3. Lieberman, I., and Ove, P., *Biochim. Biophys. Acta* **38,** 153 (1960).
4. Holley, R. W., *Biochim. Biophys. Acta* **145,** 525 (1967).
5. Richardson, U. I., Snodgrass, P. J., Nuzum, C. T., and Tashjian, A. H., Jr., *J. Cell Physiol.* **83,** 141 (1974).
6. Greenstein, J. P., and Leuthardt, F. M., *J. Nat. Cancer Inst.* **6,** 211 (1946).
7. Wheeler, G. P., Bowdon, B. J., Adamson, D. J., and Vail, M. H., *Cancer Res.* **30,** 100 (1970).
8. Worthington Enzyme Manual, p. 149. Worthington Biochemical Corp., Freehold, NJ (1972).
9. Lowry, O. H., Rosebrough, N. D., Farr, A. L., and Randall, R. J., *J. Biol. Chem.* **193,** 265 (1951).
10. Burton, K., *Biochem. J.* **62,** 315 (1956).
11. Morris, H. P., and Meranze, D. R., Induction and some characteristics of "Minimal Deviation and Other Transplantable Rat Hepatomas." *in* "Recent Results in Cancer Res., Special Topics in Carcinogenesis" (E. Grundmann, ed.). Springer-Verlag Berlin, Heidelberg, N. Y. (publishers) (1974).
12. Glass, R. D., and Knox, W. E., *J. Biol. Chem.* **248,** 5785 (1973).
13. Bradley, M. O. *Develop. Biol.* **33,** 1 (1973).
14. Hirsch-Kolb, H., and Greenberg, D. M., *J. Biol. Chem.* **243,** 6123 (1968).
14. Hirsch-Kolb, H., and Greenberg, D. M., *J. Biol. Chem.* **243,** 6123 (1968).
15. Sorof, S., and Kish, V. M., *Cancer Res.* **29,** 261 (1969).
16. Storr, J. M., and Burton, A. F., *Brit. J. Cancer* **30,** 50 (1974).

Received February 26, 1975. P.S.E.B.M., 1975, Vol. 149.