The Ribonucleases of Mouse Ascites Tumors*

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SUMMARY

The ribonuclease activities of seven ascites tumors of mice have been studied. Homogenates of all tumors exhibited two apparent activity optima—one between pH 4.7 and 5.4, the other between pH 8.5 and 9.3. These two values are outside the range in which normal tissues have optimal activity.

In the presence of *p*-chloromercuribenzoate, these two optima disappeared and were replaced by a single peak at a pH of 7.0–7.3, suggesting that the two optima seen in the absence of *p*-chloromercuribenzoate result from the dissociation of an enzyme-inhibitor complex.

A ribonuclease inhibitor has been isolated from Ehrlich ascites tumor cells and shown to inhibit bovine pancreatic ribonuclease. An investigation of the effect of pH on the inhibition of the crystalline enzyme by the tumor cell inhibitor has provided data which support the premise that the two optima obtained with tumor cell homogenates in the normal assay system are due to dissociation of an enzyme-inhibitor complex.

In a previous report (1) the results of a study of the ribonuclease (RNase) activities of cells of the Ehrlich ascites tumor and of a number of normal mouse tissues were presented. It was shown that the tumor cells had apparent activity optima at pH 4.8 and 8.5—values well outside the pH range in which any normal tissue exhibited optimal RNase activity. However, in the presence of *p*-chloromercuribenzoate (*p*-CMB), a compound which has been shown to reverse the inhibition of RNase by a naturally occurring RNase inhibitor of rat liver (3), the acid and alkaline peaks disappeared and were replaced by a single maximum at pH 7.0 with a tenfold increase in activity at that point. It seemed apparent that the Ehrlich cells contained a potent inhibitor of RNase, and it was suggested that the two optima seen in the pH activity curve in the absence of *p*-CMB were due to dissociation of the enzyme-inhibitor complex at acid and alkaline pH's.

The results of an extension of these investigations to other mouse ascites tumors are reported here. In addition, data obtained from studies of the effects of pH on the inhibition of crystalline bovine pancreatic RNase by the Ehrlich cell RNase inhibitor are presented. These data provide support for the premise that the two optima—at abnormally low and high pH values—seen in the case of the Ehrlich, and other, ascites tumors are artifacts produced by dissociation of an enzyme-inhibitor complex.

* Supported by grants from the National Institutes of Health (C-4534 and 2G-142-C1), the American Cancer Society (E-89), and the Samuel S. Fels Fund.

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Received for publication July 18, 1960.

MATERIALS AND METHODS

Tumors and tumor cell homogenates.—The tumors examined were the Ehrlich tetraploid, the Lettré hyperdiploid line of the Ehrlich tumor, Sarcoma 37, Sarcoma 180, Krebs-2, TA3, and the Andervont ascites tumor. All were maintained by intraperitoneal passage in mice, random-bred Swiss being used for all tumors with the exception of the Andervont tumor, which was grown in C3H mice.

The tumor cells were harvested 7 days post-implantation, except in the case of the more slowly growing Lettré-Ehrlich and Andervont tumors, which were harvested after 9–10 and 15–17 days of growth, respectively. The cells were washed 5 times in Hanks balanced salt solution (2) and then suspended in a 0.25 per cent solution of sodium deoxycholate in distilled water to give 10 per cent

(v/v) suspensions. Homogenization for 5 minutes at 0° C. was performed in a Serval stainless steel omni-mixer. Deoxycholate was necessary to produce complete disintegration of the cells. It was established that essentially identical results were obtained when cells were disrupted in the absence of deoxycholate. Therefore, the results described in what follows cannot be ascribed to the presence of this agent.

RNase assay.—The assay method (1) depended on the formation, from yeast ribonucleic acid (RNA) (Schwartz Laboratories, Inc.), of perchloric acid-soluble substances absorbing at 260 mμ. Incubations were carried out at 37° C. in veronal-acetate buffers of constant ionic strength (0.06) at 15–20 pH values between 4.2 and 9.0. Blanks, in which the precipitant and substrate were added simultaneously after the incubation period (usually 30 minutes) were included, as were a complete set of reaction mixtures for precise pH measurements.

RNase inhibitor.—A slight modification of the method of Roth (4) was used for the isolation of inhibitor from Ehrlich (tetraploid) ascites tumor cells. In brief, this involved fractional precipitation with $(NH_4)_2SO_4$, followed by adsorption on, and elution from, calcium phosphate gel. The final solution of inhibitor (in 0.2 M phosphate buffer, pH 7.3) represented a fivefold concentration of the original 10 per cent cell homogenate. The inhibitor was assayed by a method similar to that used for the RNase estimations. The reaction mixtures consisted of 1.0 ml. veronal-acetate buffer, 0.5 ml. inhibitor solution, 0.3 ml. of a standard crystalline RNase solution, 0.2 ml. 10^{-3} M versene, and 1.0 ml. of 1 per cent yeast RNA.

Crystalline RNase (Worthington Biochemical Corp.) was made up at a concentration of 1 μg/ml in 0.1 per cent gelatin, and stored at −25° C. Immediately before use it was thawed and diluted to the required concentration with distilled water.

p-Chloromercuribenzoate (Mann Research Laboratories) was dissolved in a small volume of 1 N sodium hydroxide and diluted with distilled water to a concentration of 4×10^{-3} M. A slight insoluble residue was removed by filtration.

RESULTS

pH activity curves of ascites tumor RNase.—All seven ascites tumors examined showed similar patterns of ribonuclease activity in the normal assay system. All exhibited two apparent optima, one between pH 4.7 and 5.5, the other between pH 8.5 and 9.3. These values are, in all cases, outside the range in which normal mouse tissues have optimal activity (1). In Table 1, the apparent pH optima for each of the tumors are listed, as are estimated activities at those pH's. For purposes of comparison, the RNase activity of each of the tumors at pH 7.0 is indicated. The pH activity curve obtained with the Lettré-Ehrlich tumor employing the normal assay system is illustrated by the open circles of Chart 1.

The effect of adding *p*-CMB (final concentration = 4×10^{-4} M) to the assay system was the same with all seven tumors. In every case, the two apparent pH optima were abolished and were replaced by a single peak at a pH of 7.0–7.3. The position of this new optimum and the RNase activity at that pH are listed for each of the ascites tumors in Table 1. Graphical demonstration of the phenomenon is provided by Chart 1, which illus-

TABLE 1

THE RIBONUCLEASE ACTIVITIES OF MOUSE ASCITES TUMORS; EFFECT OF *p*-CHLOROMERCURIBENZOATE (*p*-CMB)

Tumor	Normal assay system (absence of *p*-CMB)					In presence of *p*-CMB	
	Acid pH optimum	Activity at acid optimum*	Alkaline pH optimum	Activity at alkaline optimum*	Activity at pH 7.0*	pH optimum	Activity at optimum*
Ehrlich	4.8	4.9	8.5	5.1	1.4	7.0	15.3
Andervont	5.4	2.1	8.6	4.5	0.6	7.2	4.5
Sarcoma 37	4.8	6.2	8.7	5.1	0.0	7.2	17.2
Sarcoma 180	4.8	1.3	8.5	1.8	0.0	7.3	8.5
Krebs-2	5.0	4.0	8.8	3.1	0.2	7.0	14.7
TA3	5.0	2.1	8.7	1.5	0.0	7.3	5.6
Lettré-Ehrlich	5.2	2.8	8.5	7.4	1.6	7.2	7.7

* Ribonuclease activity is expressed as the increase in optical density at 260 mμ of the acid-soluble supernatant produced by 1 gm. (wet wt.) of tissue in 30 min. under the conditions of assay. Values are the means of several estimations.

trates (closed circles) the pH activity curve obtained with homogenates of Lettré-Ehrlich cells in the presence of 4×10^{-4} M *p*-CMB.

In the case of the Lettré-Ehrlich, Andervont, and TA3 tumors, there was some departure from the shapes of the pH activity curves obtained with the other four tumors. With the Ehrlich, Sarcoma 37, Sarcoma 180, and Krebs-2 tumors, the control

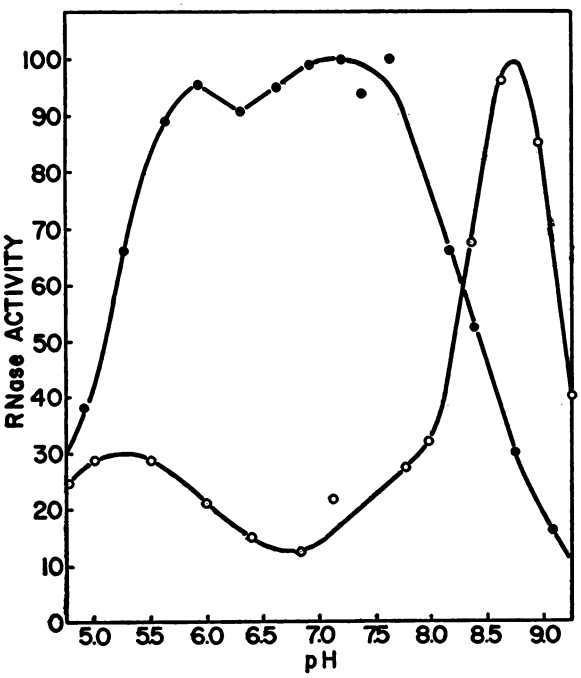

CHART 1.—pH activity curve of Lettré-Ehrlich ascites tumor cells measured: ○ in the normal assay system (*p*-CMB absent), and ● in the presence of 4×10^{-4} M *p*-CMB. RNase activity is expressed as the percentage of the activity at the optimum pH with inhibitor immobilized by *p*-CMB.

and *p*-CMB curves coincided, or very nearly coincided, at the extreme limits of the pH range. This observation has been described previously for the Ehrlich ascites tumor (1). With the other three tumors (see Chart 1), however, the alkaline peak in the control curve was higher than the *p*-CMB curve in the extreme alkaline range. The reason for this difference is not clear.

The general behavior pattern of the ascites tumors in this system suggested that they all contained significant levels of an RNase inhibitor—levels sufficiently high to effectively inhibit RNase activity in cell homogenates at physiological pH's. The effect of *p*-CMB is compatible with the premise that this compound releases RNase from an inactive enzyme-inhibitor complex between pH 5.0 and 8.5. In the absence of *p*-CMB, the dissociation of the enzyme-inhibitor complex at about these pH's would produce the apparent maxima seen in the control pH activity curves of all the ascites tumors.

Effect of Ehrlich cell RNase inhibitor on crystalline RNase.—To examine the above hypothesis, the effects of partially purified RNase inhibitor isolated from Ehrlich cells on the activity of crystalline bovine pancreatic RNase were investigated. A number of dilutions of inhibitor were mixed with a standard solution of the enzyme, and the activity of the mixtures was measured over a wide pH range. The results are shown in Chart 2. The pH activity curve of the uninhibited enzyme had a sharp optimum at pH 7.8. As the ratio of inhibitor:enzyme was increased, the activity at pH 7.8 decreased, and the position of

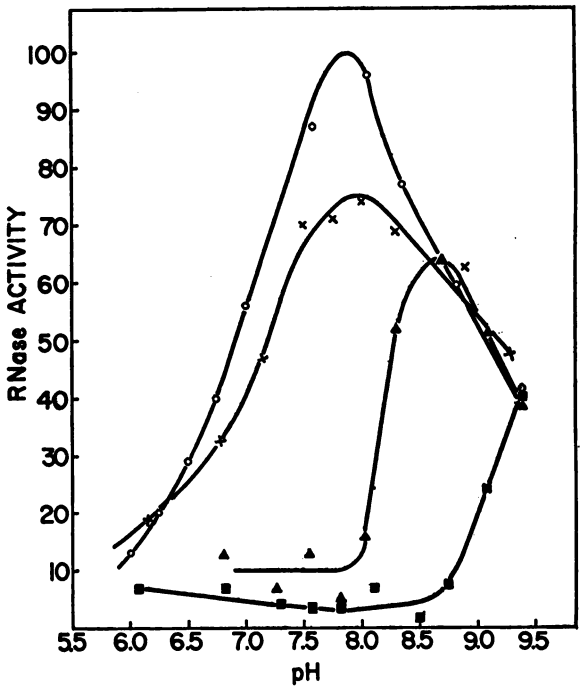

CHART 2.—Effect of pH on the inhibition of crystalline bovine pancreatic RNase by Ehrlich ascites tumor cell RNase inhibitor. Concentration of crystalline enzyme = 0.015 μg/ml. Activities are expressed as a percentage of the activity of the uninhibited enzyme at its pH optimum of 7.8.

○ Uninhibited enzyme.
■ 1:10 dilution of inhibitor.
▲ 1:32 dilution of inhibitor.
× 1:64 dilution of inhibitor.

the optimum shifted to the right. With the highest level of inhibitor, a curve resembling the alkaline end of the ascites tumor pH activity curves was obtained with an optimum at approximately pH 9.0.

As would be expected, no peaking was seen at the acid end of the pH activity curves of the mixtures, since the uninhibited enzyme at the con-

centration used here has no measurable activity at pH's less than 5.5.

The quantitative relationship between the inhibitor and crystalline enzyme was found to be the same as that described by Roth (4). For a fixed amount of inhibitor, the logarithm of the percentage inhibition decreased linearly as the amount of crystalline enzyme employed was increased. This relationship held between 10 and 95 per cent inhibition. With a wide range of dilutions of inhibitor, a series of parallel lines relating logarithm of per cent inhibition to concentration of enzyme was obtained.

The stability of the inhibitor was estimated by assays performed over the course of 8 days. It was found to have a half-life of 48 hours at 0° C., in 0.2 M phosphate buffer.

DISCUSSION

The data obtained from investigations of the Ehrlich cell RNase inhibitor-crystalline RNase system provide evidence in support of our premise regarding the unique pH activity curves obtained with ascites tumor cell homogenates. They can readily be explained on the basis of a high level of intracellular RNase inhibitor which is strongly bound to RNase at neutral pH's but not at pH's below 5.4 and above 8.0. The apparent activity optima seen in the pH activity curves at unexpectedly high and low pH's are most satisfactorily explained as being due to dissociation of an enzyme-inhibitor complex at those points.

The foregoing studies emphasize the hazard in estimating optimum pH's for RNase activity in tissue homogenates. Since the position of the optimum pH clearly depends on the relative concentrations of inhibitor and enzyme, it would seem advisable to make such estimations in the presence and absence of *p*-CMB. This would also provide some estimate of the amount of inhibitor present in the homogenate.

There is no obvious explanation for the somewhat different behavior of the Andervont, Lettré-Ehrlich, and TA3 ascites tumors compared with the other tumors examined. It is possible that it is a purely quantitative phenomenon and might be explained by assuming that *p*-CMB not only releases the RNase from inhibitor, but also slightly inhibits the enzyme itself. It is possible that these three tumors contain two enzymes capable of degrading RNA to perchloric acid-soluble fragments, one of which is inhibited by *p*-CMB. It should be noted that in this work no effort has been made to define the specificity of the tumor cell enzymes. The method employed would measure the activity of all enzymes which hydrolyze RNA to acid-soluble derivatives, not merely that of the enzyme which splits the pyrimidine-3′-phosphate linkage specifically.

To suggest some physiological role for the tumor cell RNase inhibitor is difficult, particularly since no clear function has been demonstrated for intracellular RNase. If one assumed that these enzymes played a part in the normal balance between synthesis and degradation of RNA in resting cells, then a reasonable extension of this line of thought would be that a high level of inhibitor could disturb this balance and permit increased RNA and protein synthesis and thus growth. However, even in the presence of *p*-CMB, the RNase activity of these tumors is still very low in relation to that of other tissues. No comparison has yet been made between the amount of inhibitor present in the tumors and in normal tissues. It would seem desirable to extend these investigations to compare other rapidly growing tissues (regenerating liver, embryonic tissues, cultured cells, and other tumor types) with those in stationary phase. Such studies may reveal whether any correlation exists between RNase or RNase-inhibitor levels, or in the balance between them, and the state of cell growth. It is possible that the presence of the RNase inhibitor in homogenates is merely a fortuitous event.

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