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COMBINED ACTION OF 5-FLUOROURACIL AND OTHER CYTOTOXIC AGENTS WITH CRYSTALLINE RIBONUCLEASE ON MOUSE ASCITES TUMOR†

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(With the technical assistance of Zinaida Lashischenko)

As Brachet[1,2] has emphasized for many years, cytoplasmic ribonucleic acid (RNA) is of fundamental importance to life and is now generally believed to be the blueprint or template directly involved in the synthesis of the body's enzymes. The RNA somehow directs the correct number of the specific amino acids into the proper sequence to form the protein chain or chains of the enzyme. It, in turn, is probably constructed according to directions from the nuclear desoxyribonucleic acid (DNA). Although some believe that DNA (location of genes) is capable of duplicating itself, ribonuclease (RNase) treated protoplast cannot resynthesize DNA.[3] Roth[4] has detected an increase in desoxyribonuclease (DNase) activity in cells incubated with crystalline RNase, suggesting a degradative function of the latter enzyme. Nucleolar RNA is quantitatively greater in all malignant cells.[5] Taylor and Long[6] have noted up to 70 times greater RNA content of Grade III ovarian and endometrial neoplastic nucleolar cores as compared to Grade I.

Migliarese[7] reported an increase in RNase activity associated with the growth of certain tumors in man and animals. Other workers[8] have shown an elevation of serum RNase in 71% of patients with cirrhosis and 62% of leukemic patients, but in only about 30% of patients with carcinoma or Hodgkins disease. It is of great interest that ribonuclease has been reported to demonstrate strong anti-mitotic and growth-inhibiting effects on sea urchin eggs,[9,10] living amoebae,[11] onion root tips,[12-14] salamander eggs,[15] cells in tissue culture[16] and cells synthesizing tobacco mosaic virus,[17] as well as certain experimental tumors.[18-22] There is even a report of RNase causing a temporary remission in chronic myelogenous leukemia.[23]

Perhaps the most significant finding has been made by Ledoux who found that RNase was capable of producing regressions of spontaneous mammary tumors in C_3H mice,[20] which are unusually refractory to chemotherapy. However, other workers found the drug ineffective in these cases.[24] For the past six or seven years Ledoux has been emphasizing the importance of ribonuclease in tumor therapy. He has received some criticism because of the well known heterogenous nature of RNase, as well as the fact that the bovine pancreatic enzyme he worked with has a much smaller molecular weight than intracellular RNase's.

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†Drugs used in this study were obtained as follows: 5-Fluorouracil was supplied by Dr. William Wilson, formerly, Chief, Cancer Chermotherapy Section, Hahnemann Medical College and Hospital, Philadelphia, Pa. Glucose-1-Phosphate hydrazine and Polyphosphate were prepared and supplied by Dr. Cardenas. 9-B-D Psicofuranosyl adenine was supplied by Dr. James Lawson, Upjohn Co., Kalamazoo, Michigan.
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We have been extensively studying the action of RNase on experimental tumors. This communication records the effects on Ehrlich ascites tumor of RNase in combination with other agents reported to have a cancer chemotherapeutic effect. The drugs were: 5-Fluorouracil (5-FU);[25-30] Glucose-1-phosphate hydrazine (G-1-P-H);[31,32] 9-B-D psicofuranosyl adenine (psicofuranine);[33-35] or condensed polymerized phosphate chains.[36,37]

These drugs were selected for a variety of reasons. 5-FU is a fluorinated uracil and a pyrimidine antagonist which is incorporated into ribonucleic acids.[38] In man the drug's highest concentration is found in the RNA of tumors and intestinal mucosa.[30] It can prevent thymine synthesis by inhibiting thymidine synthetase[39] and blocks uracil phosphatase so that preformed uracil cannot be utilized in RNA synthesis.[40] G-1-P-H demonstrates a cytotoxic action presumably because of its hydrazine group. The metabolic itself has been reported to be taken up selectively by tumor cells.[41,83] Psicofuranine was recently developed as an antibiotic effective against M. aureus and E. coli and produced by a strain of actinomycete identified as *Streptomyces hygroscopicus* var. *decoyicus*.[42] It is unusual in that it is an abnormal ribonucleoside in which the sugar moiety (6 carbon sugar with pentose lactone linkage), rather than the heterocyclic base, has a structural defect. The drug inhibits the formation of guanylic from xanthanilic acid.[43] Polyphosphate chains inhibit several enzyme systems *in vitro* by competitive inhibition or by sequestrating cations (Ca^{++} and Mg^{++}).[37] They may interfere with high energy phosphate systems *in vivo*.

MATERIALS AND METHODS

Sarcoma 37 ascites tumors were maintained in adult female Swiss white mice averaging 25 grams in weight by weekly inoculation of 0.2 ml. of cell suspension. After such intraperitoneal (I. P.) injection of fresh ascites fluid, the mice regularly develop large amounts of milky ascites (5-15 ml.) in 6-12 days and die in 10-15 days. These transplantable cancers have 100% "takes" and rarely show spontaneous regressions.

The RNase used was 5x recrystallized bovine pancreatic ribonuclease purchased from Sigma Chemical Company. The mice were separated into several small groups (10 controls and 10 experimentals, each). All experimental animals received 2 mg. RNase (80 mg/kilo) I.P. daily for a week after the tumor had been established 24 hours (days 1-8). The other drugs were administered in a similar manner on the same days as separate injections. 5-FU was only given on days 1-6. Polyphosphates were prepared by dehydration of mixtures of monosodium and disodium orthophosphate.[44] The maximal chain length was 67 phosphorus atoms. Doses used were:

5-FU	0.5 mg/day	(20 mg/kilo)
G-1-P-H	2.0 mg/day	(80 mg/kilo)
psicofuranine	8.0 mg/day	(320 mg/kilo)
polyphosphate	1.0 mg/day	(40 mg/kilo)

All drugs were administered through a Swinney filter adapter to assure sterility. Antibiotics were not used. Diet was Purina Laboratory Chow and tap water supplied ad libitum. The mice were maintained in air conditioned quarters (74°F).

RESULTS

In previous studies,[45-47] we reported that 4-5 mg. crystalline RNase (Sigma) given intraperitoneally daily 24 hours after an ascites tumor was established, prolonged the survival time by a factor of two. While the effect of a 2 mg. dose was variable in this respect, it nevertheless produced the same marked alteration in 8 day tumor cytology seen with the larger doses. One mg. doses had no effect. Although combinations of total body x-irradiation plus I.P. RNase (i.e., 2 mg. RNase daily plus 600r, day 2) seemed to have a synergistic effect in that survival time was further prolonged, the tumor total packed cell volume was not inhibited to the degree seen with 4-5 mg. RNase alone. A finding of perhaps greater significance is an apparent radiation protective effect of the enzyme. Mice which had received doses of ionizing radiation which were 100% lethal to the controls, lived either long enough to die from their transplanted cancer, or even longer.

Although there was quite a bit of variation in different trials using 2 mg. RNase alone, the average survival time of these animals in the various groups was 18.1 days (controls = 13.6 days). The results in the combination therapies are shown in Table I. Ribonuclease plus 5-FU gave the most interesting findings, with a prolongation of survival period to 2.5 times control (Fig. 1). Total body weight changes, which are felt to reflect tumor growth, are shown for the controls and for this group in Figs. 2 and 3.

There was some weight loss at these doses, but no deaths due to 5-FU toxicity. When therapy was stopped after the first week the weights returned to

Table I

SUMMARY OF COMBINATION THERAPY EFFECTS. 10 MICE IN EACH CONTROL AND EXPERIMENTAL GROUP. (DOSES: RNase, 2 mg/day; 5-FU, 0.5 mg; G-1-P-H, 2 mg; psicofuranine, 8 mg; polyphosphate, 1 mg)

Therapy (Daily injections, Days 1-8)	Average Survival Time (Treated/Control)	Average Weight Change/Day (T/C)
Ribonuclease alone	18.1/13.6 days	+0.15/+0.74 g
5-Fluorouracil + RNase	32.5/13.4	−0.01/+0.79
Glucose-1-Phosphate Hydrazine + RNase	20.2/13.3	+0.31/+0.83
Psicofuranine + RNase	16.5/13.9	+0.39/+0.81
Polyphosphate + RNase	14.5/13.6	+0.71/+0.91

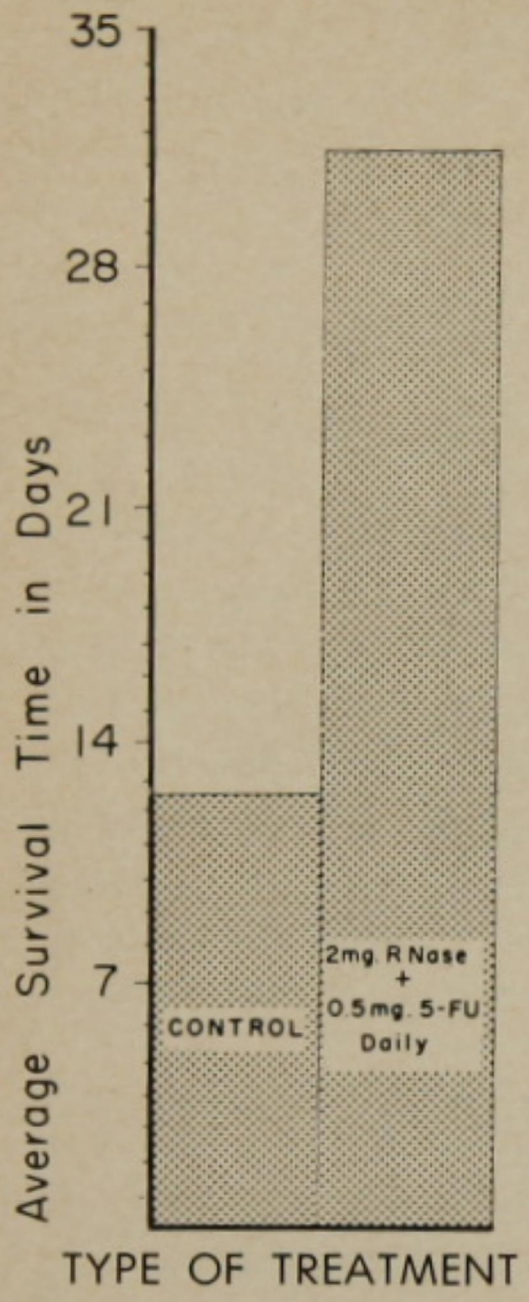

Figure 1

Prolongation of survival time by intraperitoneal therapy with ribonuclease plus 5-fluorouracil.

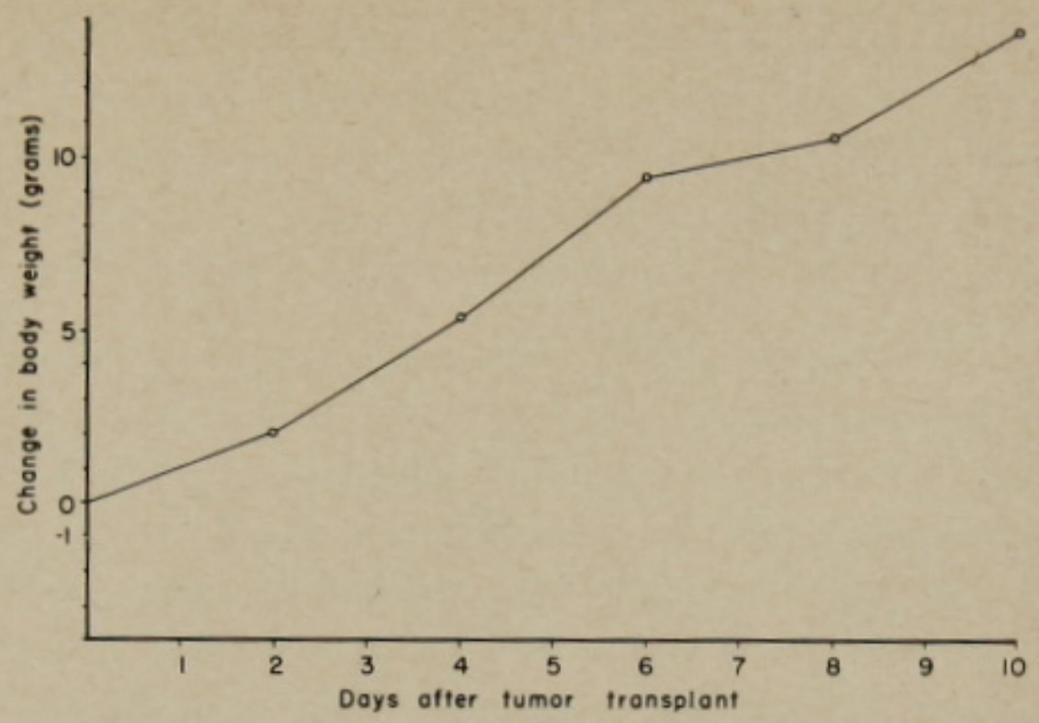

Figure 2

Average weight gain in control mice with ascites tumor.

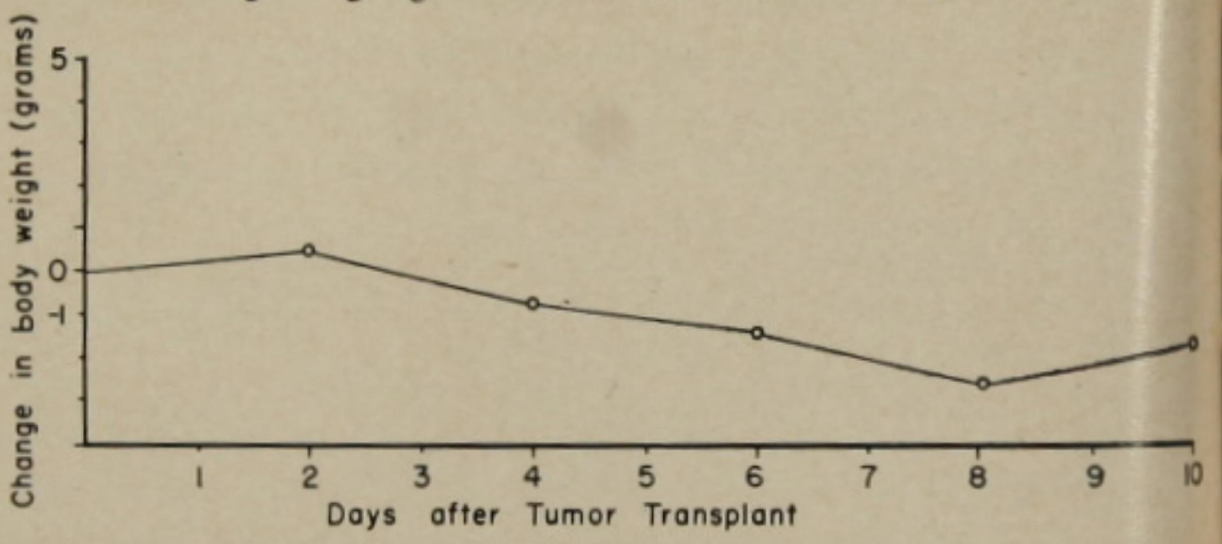

Figure 3

Average weight change in tumor-bearing mice treated with RNase plus 5-FU.

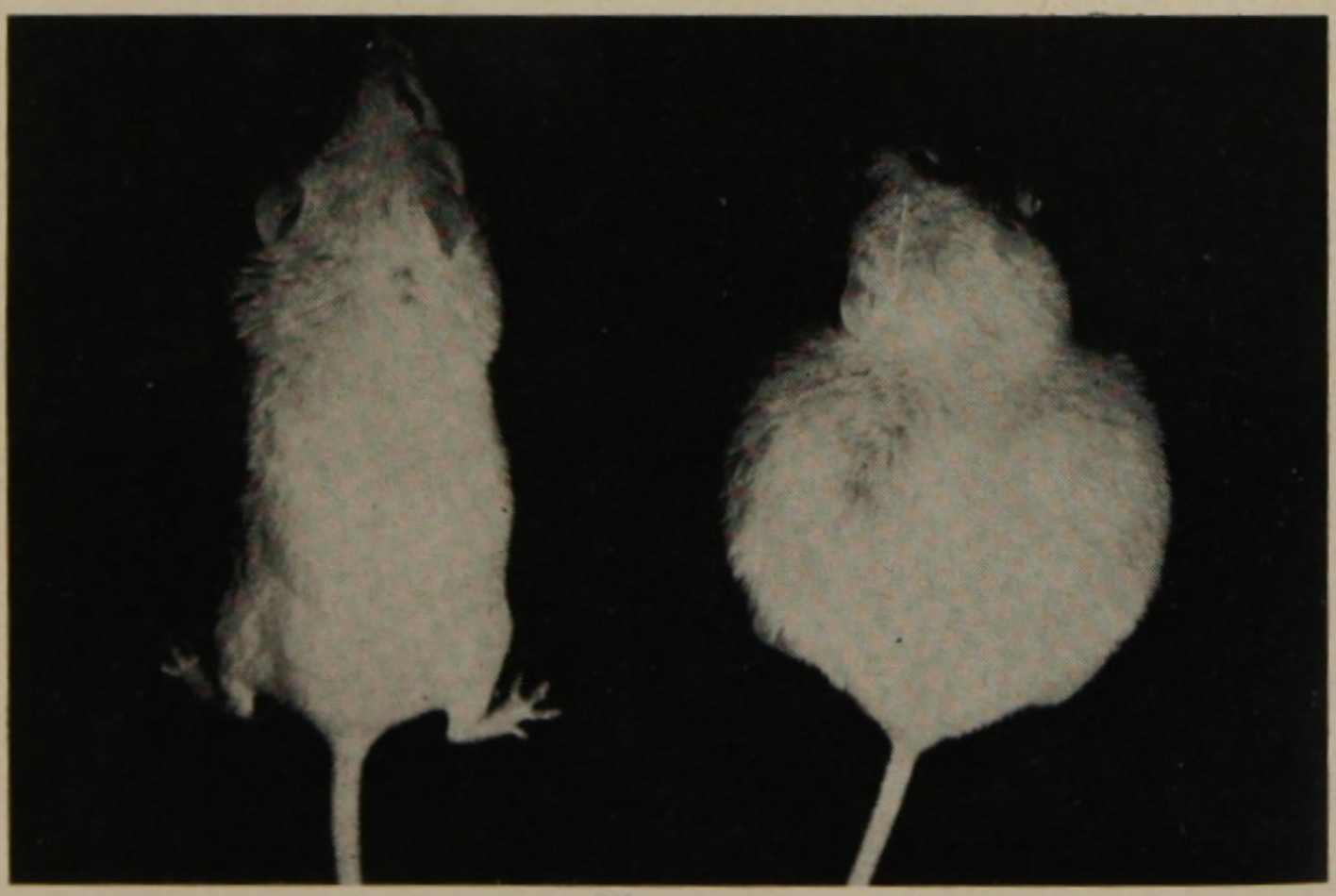

Figure 4

Control mouse (right) and one with identical tumor transplant, but treated with 2 mg. RNase plus 0.5 mg. 5-FU, daily. (13 days after tumor inoculation.)

pre-treatment levels and thereafter increased very little. Half of these animals showed no sign of ascites tumor or solid tumor. Some of these animals were alive 6-7 weeks after tumor transplantation, before starting to show some emaciation and succumbing to solid abdominal wall and peritoneal malignancies. The other 50% had a striking generalized carcinomatosis that was first seen at the third week in sacrificed animals. There were numerous discrete masses of tumor infiltrating the peritoneal tissues, especially the omentum and epiploons, as well as the chain of ganglia and lymph nodes. Parenchymatous organs such as the kidneys and liver were also often involved by single or multiple whitish gray nodules of a rubbery consistency and a small cystic surface on cut section. Cells were similar

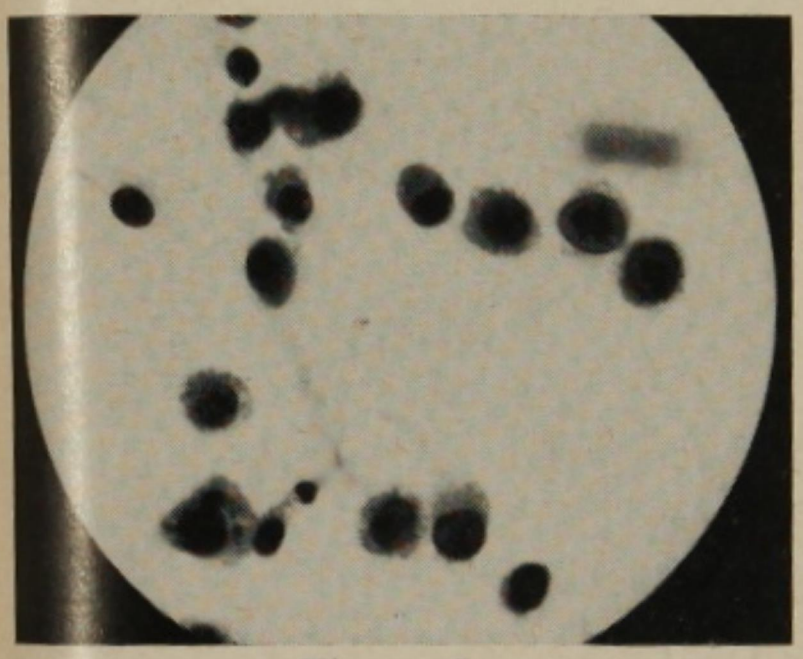

Figure 5a

Typical smear from a malignant ascites tumor.

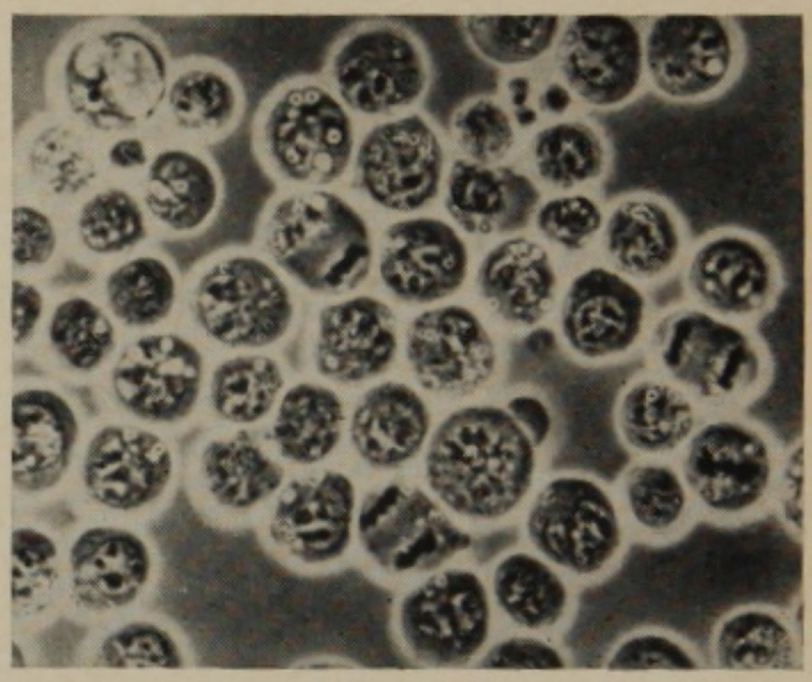

Figure 5b

Phase photomicrograph of a control smear, showing several cells in active division. (Courtesy of Dr. Jens Christensen)

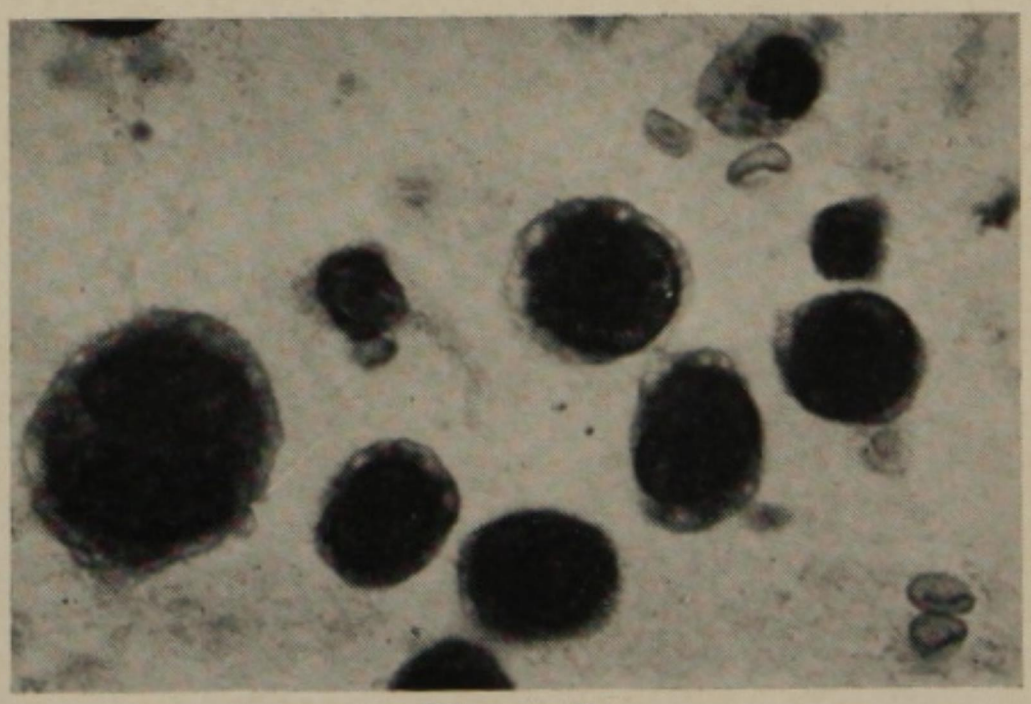

Figure 5c

1280 x enlargement of control smear. Several erythrocytes are also seen. Papanicalaou stain. (Courtesy of Dr. Irena Kowprowski)

to those of the original inoculum. Supra-diaphragmatic viscera showed a similar picture, as did lymph nodes (cervical, mediastinal and inguinal). Occasionally jaundice was seen when tumor involved the biliary tree. Several new solid tumors were detected in the subcutaneous tissues of the back.

Nothing unusual was noted with the other drugs, except that glucose-1-phosphate hydrazine had a similar or slightly synergistic effect with RNase. Psicofuranine or polyphosphate had no effect or inhibited RNase action.

Inoculation of experimental animals with malignant cells in which RNA content has been reduced by storage at 4°C is much less effective in producing tumor "takes" than inoculation with fresh cells.[48] Simultaneous administration of RNA causes an increased number of tumors.[49] When normal rat liver cells are treated with RNA from malignant liver cells and then inoculated I.P. into rats, 20% develop tumors whereas none did when normal liver RNA was added.[50] However, Niu[51,52] found RNA effect to be related to dose. In physiologic concentration it could produce differentiation of young urodele gastrula, while very large doses actually prevented further growth of the embryo or transplantation I.M. of ascites cells (incubated with normal mouse liver RNA).[51]

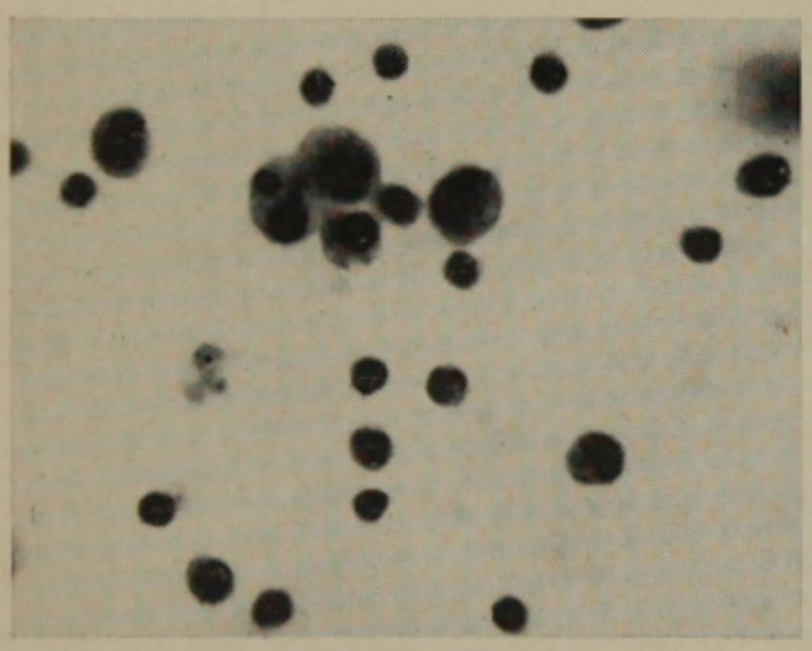

Figure 6a

Smear from animal treated with RNase plus 5-FU. Many of the cells are smaller than usual. Effect is similar to that seen with RNase alone.

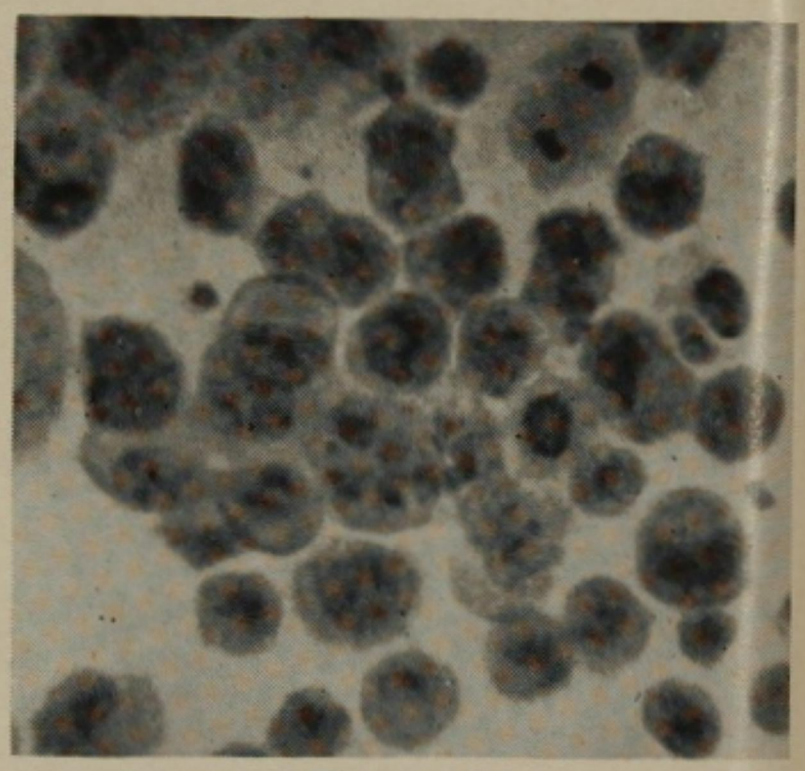

Figure 6b

Smear from animal treated with RNase plus polyphosphate. No great morphological alteration.

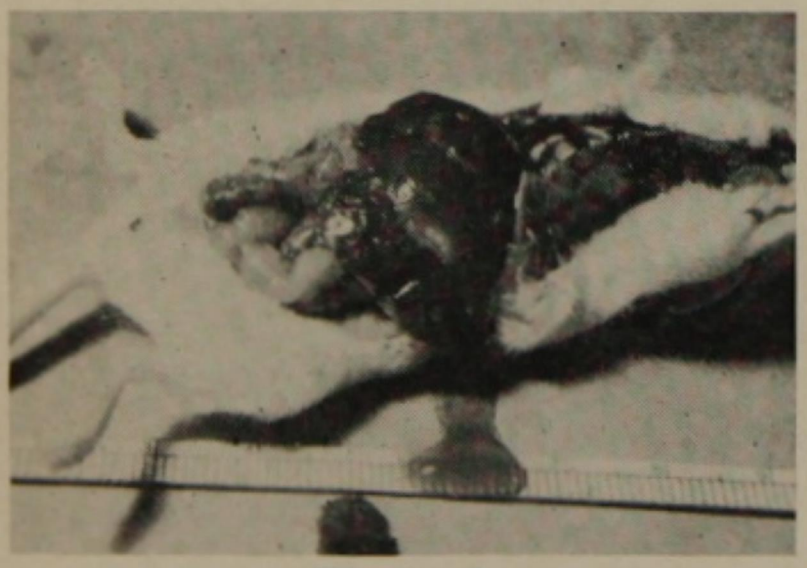

Figure 7

Animal sacrificed 28 days after Sarcoma 37 injection, and treated for one week with RNase and 5-FU. Note minimal amount of viscous ascites. Spleen is riddled with small solid tumors and several were present in the mesentery.

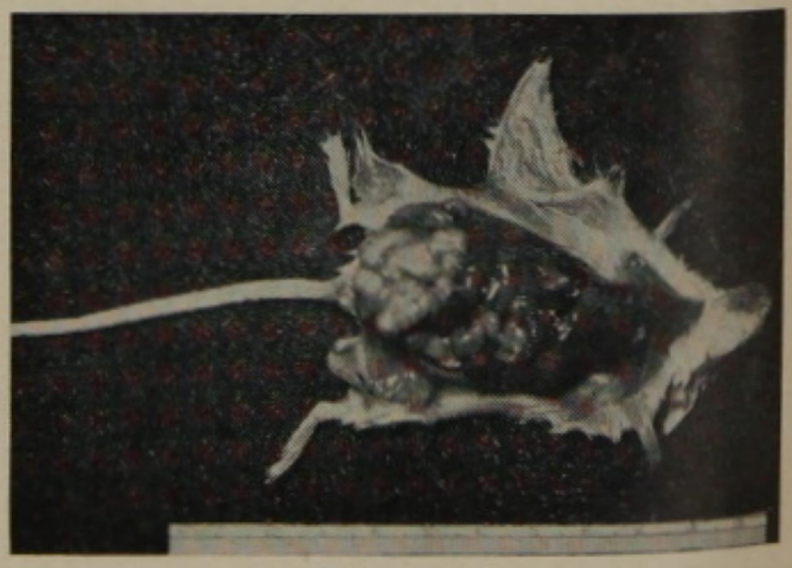

Figure 8

Animal sacrificed 28 days after tumor inoculation, treated with RNase plus 5-FU, showing large solid tumor filling lower abdomen and matting together much of the viscera. This tumor, as well as all of the animal's subcutaneous tissues and sclerae, was intensely icteric.

DISCUSSION

DeCarvalho and Rand[50] have reported a modification of rat Novikoff hepatoma growth by treating the cells with RNA from other hepatoma cells prior to transplantation. A much more widely disseminated neoplastic picture with unusual widespread metastases was seen, although prior treatment of the controls with normal rat liver RNA caused no such alteration. Despite the more malignant growth pattern of the former, some regressions were seen, although none were seen with the controls. Their description is quite similar to the RNase + 5-FU effect on some mouse ascites tumors. While it is conceivable that one or two of the solid tumors may have developed spontaneously, it is highly doubtful that all of them did.

Although RNase and G-1-P-H have a more beneficial effect when used together, it is not an additive one. While we feel that the action is due to a cytotoxic effect of the hydrazide, it is quite possible that the compound is a specific antagonist. Thus, D-glucosamine, a competitive inhibitor of glucose for hexokinase, inhibits both Sarcoma 37 and human epidermoid carcinoma in tissue culture.[53,54]

Extensive studies with polyphosphates on solid and ascites mouse tumors showed that they were ineffective in these animals, perhaps due to a species specific enzyme. The loss of the usual RNase effect when the drugs were used together may have been due to inactivation of the enzyme by phosphorylation which occurs even with a single phosphorus atom.[82] There has been at least one definite temporary

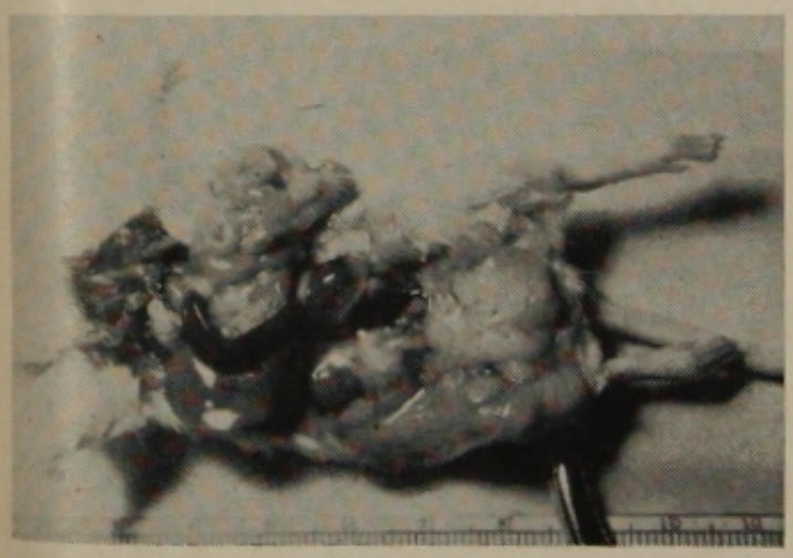

Figure 9

Autopsy 35 days after tumor transplant and treatment (RNase plus 5-FU), showing solid mesenteric tumor with isolated hepatoma. Latter has a smaller neoplasm near it.

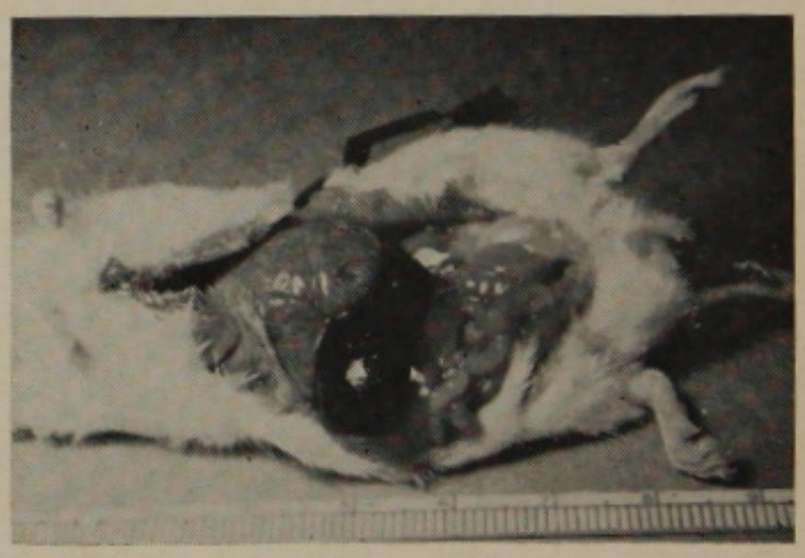

Figure 10

This animal represented another clinical "cure" but showed a small amount of bright yellow ascites plus multiple liver metastases when it died. A solitary left renal tumor was also present.

remission, both clinically and hemotologically, in a patient with leukemia on polyphosphate.[31] A tendency to hypocalcemia and even tetany is a significant toxic effect. At Henry Ford Hospital three terminal cancer patients were given polyphosphate in daily intravenous drips for 60 minutes at doses up to 600 mg. (8 mg/kilo).[55] No effect, including any change in serum calcium, was seen.

We were able to confirm the lack of chemotherapeutic action of psicofuranine on the Ehrlich tumor. When used with RNase, the biological effect was similar to that obtained only with the latter treatment. Because of its unique structure, psicofuranine has had extensive clinical trials, but antitumor activity at non-toxic

doses was negligible. An unusual syndrome produced is polyserositis, especially pericarditis.[56-58,68] Nine of 12 patients at this institution developed the fullblown picture of fever, mental confusion, moderate leucocytosis and severe chest pain with audible pericardial friction rub.[57] Symptoms subsided when the drug was discontinued and returned upon readministration. In some the picture resembled the Periodic Disease originally described by Reimann,[59-61] later referred to as Familial Mediterranean Fever[62] and recently renamed by Nixon and Priest[63-65] as Familial Recurring Polyserositis.

Ledoux[20] estimated that the Armour beef pancreas RNase he used in retarding C_3H mice spontaneous mammary tumor growth contained 25-30% of fully active

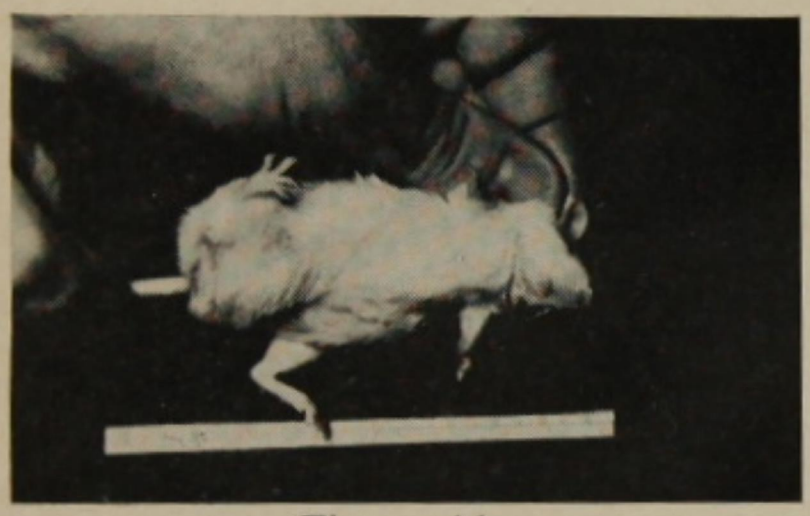

Figure 11a

28 day tumor treated with RNase plus 5-FU for one week after tumor transplant. Solid tumor in lower abdomen.

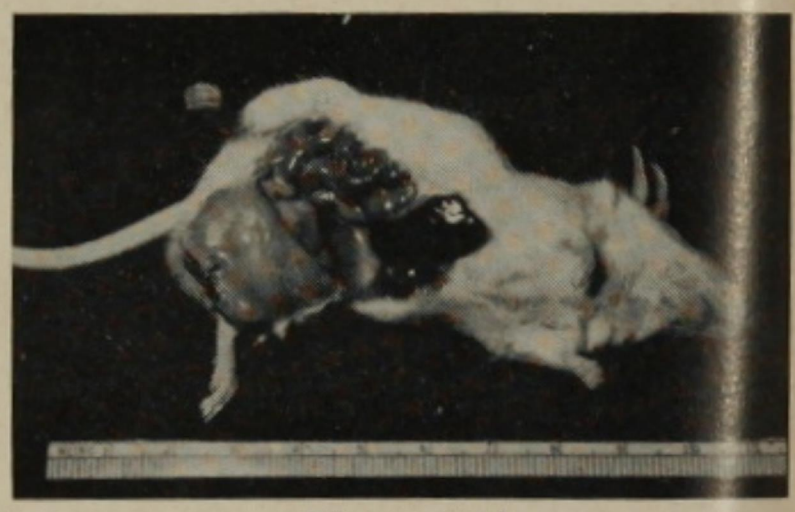

Figure 11b

Mass uncovered. It involves the ventral peritoneum. No ascites was present.

enzyme and 70-75% of inactive low molecular weight RNase. There was an impression that the inactive portion could inhibit the active one. He found that two intraperitoneal injections (days 7 and 8) of 2 mg. Armour RNase purified by chromatography (peak "D", the largest peak) *plus* 0.1 mg. nucleotide almost tripled the survival time of a group of ascites tumors.[19] Other doses when given without nucleotide or even 10-20 mg. doses of unpurified enzyme, had a less beneficial effect. Extensive studies[21,66,67] have led him to believe that the cancerocidal

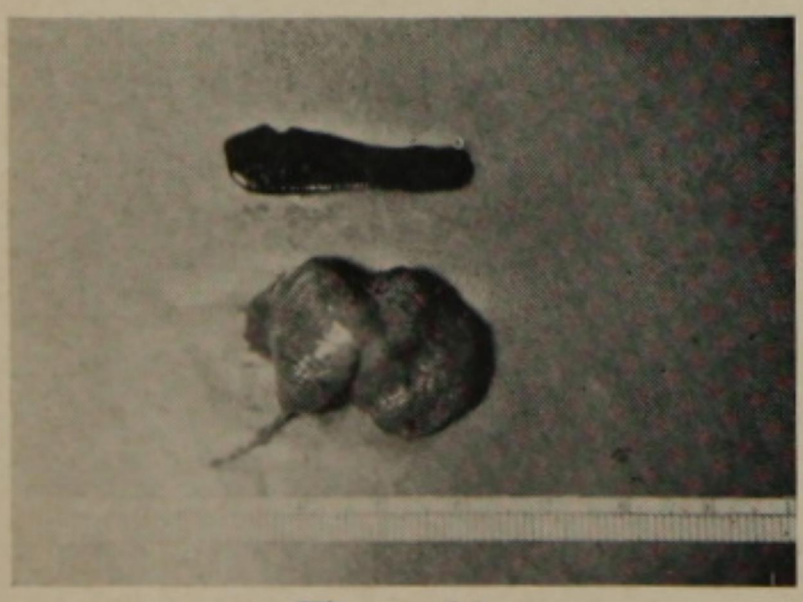

Figure 11c

The tumor dissected out. Above is the animal's spleen, which was larger than normal.

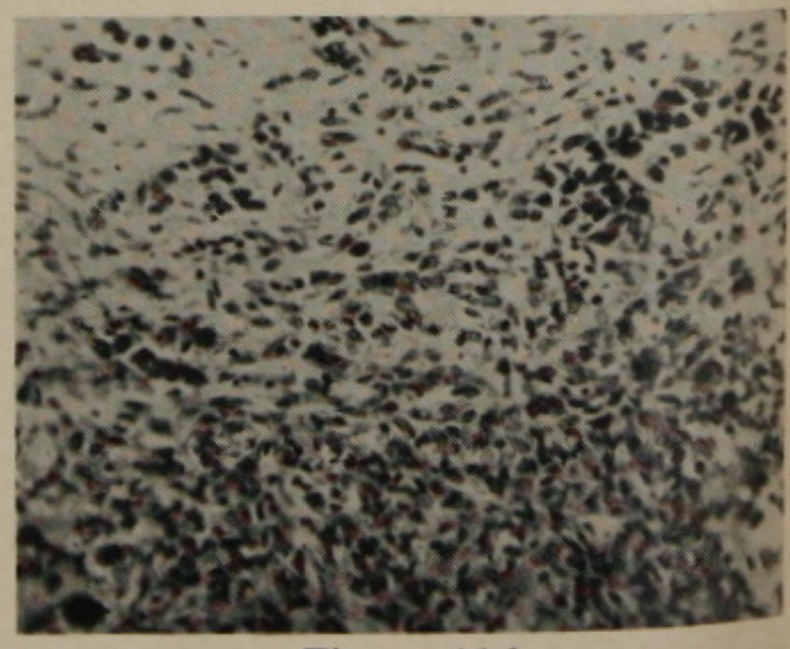

Figure 11d

Section of the tumor (hematoxylin and eosin, 175x). It is composed of closely packed cells of a type similar to those of the original inoculation.

effect of RNase is due to the rapid synthesis of a new and abnormal cellular RNA, rich in pyrimidine bases, which somehow initiates cellular alterations and a steady decrease in RNA.

RNase is frequently reported to be present in the cytoplasmic mitochondria.[69] Recent workers have shown that it is actually located in cytoplasmic particles called "lysosomes",[70] which may contaminate mitochondral fractions. It has been shown that rat liver RNase has two peaks of activity, one at acid pH and another at alkaline pH.[71,72] Roth has noted that an RNase inhibitor is normally present in the rat liver,[73] and has recently described a third rat liver RNase which is bound to this inhibitor and found in the supernatant fraction.[74] The optimum activity of the enzyme occurs at the same pH, whether in solution or adsorbed to a surface.[75]

An important report made earlier this year concerns a ribonuclease inhibitor which has been isolated from Ehrlich ascites tumor cells.[76] This has been shown to inhibit bovine pancreatic RNase and could possibly play a role in these chemotherapeutic trials. Bivalent ions such as Mg^{++}, Mn^{++} and Cu^{++} all inhibit RNase attack on rat microsomal ribonucleoprotein.[77] Several polyionic compounds, especially polymethacrylic acid, reverse RNase inhibition of protein synthesis.[78] Many workers have uncoiled the chain of 124 amino acids that make up RNase by breaking disulfide bridges but not the chain itself, so that the enzyme is inactivated. This process is reversible and thought to be a mere "uncoiling" rather than true denaturation because reactivation of RNase can be brought about by proper conditions. Thus the compound refolds under the influence of RNA, if there are polyvalent anions in the substrate, even in the presence of strong agents like 8 M urea or 1 to 3 M solutions of guanidium ions.[79] Activity of reduced and modified RNase is also restored by the presence of molecular oxygen and suitable alkalinity.[80,81]

SUMMARY

Ribonucleic acid is directly involved in the synthesis of most of the important enzymes and proteins, under physiological conditions. There is gorwing opinion that the riddle of cancer may lie in some abnormality of nucleoprotein metabolism, especially of RNA. Important work has been performed and frequently reported by Ledoux on ribonuclease action against various experimental tumors. We have confirmed some of these studies and extended the work. Other workers have had difficulty in reproducing his results, although Ledoux himself has reported a variation of biological effect with different sources and lots of crystalline bovine pancreatic RNase.

In the present experiments crystalline RNase was used in combination with several other drugs in the mouse Sarcoma 37 ascites tumor. Two of the agents, glucose-1-phosphate hydrazine and polyphosphate, were prepared in our own laboratories. The other drugs were an abnormal nucleoside, 9-B-D psicofuranosyl adenine, and 5-fluorouracil. Intraperitoneal injection of 2 mg. RNase daily (days 1-8) and 0.5 mg. 5-FU daily (days 1-6) resulted in a prolongation of average survival time 2.5 times that of the controls (32.5 compared to 13.4 days). Furthermore, steady weight gain as a manifestation of tumor growth was not seen in the treated mice. Half of them never developed any ascites tumor but died within six weeks

with large solid abdominal wall and peritoneal tumors. The other 50% had an unusual widespread carcinomatosis that was first detected three weeks after original transplant. Solid tumor involved all of the peritoneal and omental tissues, as well as liver, kidneys, heart, cervical, mediastinal and inguinal lymph nodes, and subcutaneous tissues of the back. Jaundice was sometimes seen. Peritoneal smear six days after tumor inoculation showed numerous small round cells with minimal cytoplasm and some nuclear pycnosis. Occasional typical ascites tumor cells were seen.

Glucose-1-phosphate hydrazine plus RNase produced a survival time of 20.2 days, which was slightly greater than with the enzyme alone. Psicofuranine had no additive effect and polyphosphate seemed to reduce the usual RNase effect. Ascitic smears from an 8 day animal of the latter group failed to show any unusual cytological changes.

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