Table 1. Partial purification of antitumor agent in *Mercenaria* extracts on Sephadex-gel columns. Results obtained with Swiss mice implanted with Krebs 2 carcinoma.

Sephadex gel column	Antitumor activity in vivo	Units of activity injected in sample	Mean wt. of tumor (mg)
	Treated		
G-100	20	63	1742±125
G-75	25	63	1413±480
G-50	30	63	1382±83
G-25	67	43	1340±400
	Controls		
G-75, G-50, and G-25			2096±715
G-100			2088±150

eliminated by dialysis for 24 to 36 hours against distilled water at 4°C.

Preparation and partial purification of the crude extract was as follows. Fresh *Mercenaria* were removed from the valves and homogenized in a Waring blender after the addition of distilled water and precipitation with 20 percent ammonium sulfate solution. The homogenate was centrifuged at room temperature at 5000 rev/min for 15 minutes. The supernatant was then dialyzed for 24 to 36 hours against distilled water at 4°C. The crude material was concentrated to a powder by freeze-drying at −20°C and the powder was stored at −10°C until used.

For further, but incomplete, purification, 5-ml samples containing a predetermined number of mouse units of extract were reconstituted and introduced onto a Sephadex-gel column with a void volume of 45 ml. An appropriate buffer of neutral 0.1*M* NaCl was used as the elution fluid. Approximately 125 to 150 ml of the fractionated sample were collected at room temperature over a period of 2 to 3 hours. The total collection was lyophilyzed at −20°C, and then reconstituted with 0.1*M* NaCl and used to treat test animals for 7 days.

Of the Sephadex gels tried (G-100, G-75, G-50, and G-25), the fractions from the G-25 produced the greatest amount of active antitumor substance per milliliter of extract sample and per number of units introduced onto the column. This would seem to suggest fractionation in the molecular-weight range of less than 10,000 grams.

Partial purification to date seems to indicate almost pure inhibitor. There seems to be no great traces of promotor present to suppress the activitity of the inhibitor material. Further purification may indicate the presence of a growth promoter (*1, 2, 8*). Fortunately many of the difficulties encountered with mammalian tissues, in which the action of inhibitors is compensated by promotors, have been eliminated.

These studies seem to indicate a new agent widely distributed in nature, especially in marine fauna. During the summer months this agent can be found in concentrations eight to nine times as great as that extracted from *Mercenaria* during the remainder of the year. Antitumor activity may be due to some metabolic agent or agents present in *Mercenaria* that is active as an antitumor compound or compounds. These substances may be useful as therapeutic and prophylactic agents in the treatment of cancer.

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Intestinal Phosphatase Activity: Acceleration of Increase by Puromycin and Actinomycin

Abstract. *Alkaline phosphatase activity of the mouse duodenum normally increases 20-fold between 13 and 20 days. Activity at 15 days is raised two to three times above normal by administering actinomycin D, puromycin, or the aminonucleoside of puromycin at 13 or 14 days. Phosphatase activity in the jejunum and kidney are not similarly affected.*

In the duodenum of the young mouse, the specific activity of alkaline phosphatase, measured in whole homogenates, increases approximately 20-fold during the 3rd week of postnatal life (*1*). This increase, which is dependent on the secretion of adrenocorticoids (*2*), is accompanied by changes in the characteristics of the enzyme activity. One of the most striking changes is an increase in the rate at which phenyl phosphate (PhP) is hydrolyzed in comparison with the rate at which β-glycerophosphate (bGP) is hydrolyzed; the ratio of rates of activity on the two substrates (PhP/bGP) rises from less than 1.0 near the end of the 2nd week to more than 3.0 at the end of the 3rd week (*3*). These events are not due to loss of dissociable inhibitors or to gain of dissociable activators (*4*).

This study was undertaken to investigate the possibility that the increase of activity is due to rapid synthesis of new enzyme molecules. The antibiotics puromycin dihydrochloride and actinomycin D are known to interfere with protein synthesis, puromycin by interrupting the formation of peptide chains (*5*), and actinomycin D by inhibiting the DNA-dependent synthesis of RNA (*6*). Both puromycin (*7, 8*) and actinomycin D (*8, 9*) prevent increases of certain enzyme activities in mature tissues, and puromycin has been shown to have the same effect during developmental stages (*8, 10*). The fact that the aminonucleoside of puromycin may interfere with enzyme-dependent processes without having a parallel effect on protein synthesis (*11*) led to the use of this compound as well (*12*).

Litters of nine young mice were used: three were killed at 13 or 14 days; three were injected subcutaneously with actinomycin D or aminonucleoside dissolved in saline or with puromycin dihydrochloride in 1.39 percent bicarbonate buffer at *p*H 7.3 (*11*);

and three were injected with equal amounts of saline or bicarbonate buffer. Actinomycin D was administered once (at 14 days) or twice (13 and 14 days); the other two agents were administered in several doses at approximately equal intervals, the last being given 6 hours before the animals were killed. The animals were examined and weighed twice daily before and during treatment. They were killed at 15 days, 30 hours after injections beginning at 14 days, or 54 hours after injections beginning at 13 days. Pieces of duodenum and jejunum (15 mm long) and the right kidney were removed, weighed, and placed in iced distilled water. After homogenization in a Ten Broeck grinder, protein concentration was determined (*13*) and the alkaline phosphatase activity was measured with phenyl phosphate and β-glycerophosphate under optimal conditions for each substrate (*3*). With kidney tissue, phenyl phosphate was used at a final concentration of 60 m*M*, *p*H 10.6, and β-glycerophosphate at 60 m*M*, *p*H 9.6.

It was anticipated that if the increase of activity in the duodenum were due to synthesis *de novo*, adequate dosage of puromycin and possibly actinomycin D would inhibit it; failure to obtain inhibition would indicate that synthesis of alkaline phosphatase was not involved. The unexpected result was that the three test substances accelerated the increase (Table 1), the degree of acceleration being greater when the drugs were allowed to act over a period of 54 hours. The protein content of the duodena of the treated mice was slightly (5 to 10 percent) below that of controls of the same age. The elevated alkaline phosphatase activity shows the normal enhanced preference for phenyl phosphate, expressed as PhP/bGP ratio (Table 1). In all litters except 4Cl the enzyme activities in all three of the 15-day experimental mice were higher than any of those in the control groups of three; these differences are significant ($<.05$) according to the Mann-Whitney *U* test (*14*).

To determine whether the observed effects are peculiar to the duodenum, the alkaline phosphatase activities of jejunum and kidney were also tested. In both these organs there is normally a small increase between 13 and 15 days, although the PhP/bGP ratio remains unaltered (about 0.8 for jejunum, 1.6 for kidney). Table 1 shows that when antibiotics were injected at 14 days, the increase was largely or completely prevented; in litter 4Cl there was severe depression of activity in two puromycin-treated animals that died in the last hour of the experiment, as well as in the third, which was moribund. Injection at 13 days brought about some increase of activity, though the PhP/bGP ratio did not change; the increase is probably to be attributed to the presence in the jejunum of a small amount of the duodenal phosphatase that hydrolyzes phenyl phosphate preferentially (*15*).

Table 1. Influence of actinomycin, puromycin, and the aminonucleoside of puromycin (PAN) on the alkaline phosphatase (AIP) activity in three organs of the young mouse. The dose of antibiotic is per gram of body weight. The numbers in parentheses are the number of injections. The activities given are those determined with phenyl phosphate as substrate, with activity in the three animals killed at time of initial injection being set at 100 percent. PhP/bGP is the ratio of the amount of P released from each substrate by the same quantity of tissue protein in 5 minutes, C is the control group, uninjected or injected with saline or buffer; E is the experimental group, injected with test substance; numbers under "group" refer to age of animals in days at the beginning and end of the experiment.

Group	Alkaline phosphatase activity			
	Duodenum		Jejunum	Kidney
	Specific activity (%)	PhP/bGP	Specific activity (%)	Specific activity (%)
Litter 3X6, 0.39 μg of actinomycin D (1)				
14C	100	0.86	100	100
15C	111	1.06	106	104
15E	173*	1.47*	93*	54*
Litter 306, 0.40 μg of actinomycin D (2)				
13C	100	1.33	100	100
15C	221	2.04	110	120
15E	417*	3.15*	137*	54*
Litter 4Hl, 0.47 mg of puromycin (4)				
14C	100	1.91	100	100
15C	125	2.15	107	133
15E	201*	2.70*	74*	127
Litter 4Cl†, 0.81 mg of puromycin (5)				
14C	100	1.11	100	100
15C	147	1.16	157	139
15E	237	1.68	48*	89*
Litter 4Ml, 0.51 mg of puromycin (5)				
13C	100	0.88	100	100
15C	510	2.09	104	138
15E	1420*	3.64*	182*	113*
Litter 3R5, 1.05 mg of PAN (6)				
14C	100	1.29	100	100
15C	180	1.71	125	115
15E	249	2.08	107	90*
Litter 307, 0.75 mg of PAN (5)				
13C	100	1.04	100	100
15C	210	1.62	108	114
15E	624*	3.37*	169*	75*

* Values in these trios did not overlap those in control trios of same age. † Dose used for this litter was lethal (see text).

In the kidney alkaline phosphatase activity was reduced 54 percent by actinomycin D (Table 1). Puromycin and its aminonucleoside caused smaller but still significant depressions. The PhP/bGP ratio was unaffected. In the experimental animals the protein content of both jejunum and kidney was 5 to 10 percent below that of the controls at the same age. The wet weights of the kidneys were not affected.

By several criteria the observed effects are not due to morbidity or tissue damage. Histological preparations stained with hematoxylin and fast green show that the intestinal wall remains in good condition in all cases, although very few mitotic figures are found in the intestines of the injected animals. Kidney structure likewise was unchanged. Except in litter 4Cl, the young animals were in vigorous condition when they were killed, and even in the 4Cl litter the two animals that died in the last hour of the experiment had higher duodenal alkaline phosphatase values than the third, which was moribund. A number of mice that were raised after being treated with the antibiotics or the nucleoside at 13 days showed a transitory depression of growth starting on the day after injections were begun; but the animals soon recovered, grew normally, and reproduced at the usual age.

In the experiments reported here, actinomycin D and puromycin failed to inhibit the rise of alkaline phosphatase activity in the duodenum under conditions in which they interfere with the increase or maintenance of kidney alkaline phosphatase, retard body weight increase, at least after 24 hours, and cause severe reduction of spleen weight; in all experimental trios the weight of the spleens averaged less than 60 percent of that of the three control spleens at the same age. The quantities of antibiotics used, moreover, are comparable with those that inhibit increase of other enzyme activities in the rat and guinea pig (*7–10*). This indication that the upsurge of activity in the duodenum in the third postnatal week is not due to synthesis of a new kind of molecule is not altogether unexpected, for in some unpublished experiments, an antiserum to alkaline phosphatase from the 20- to 25-day duodenum, though it did not cross-react with jejunal alkaline phosphatase (*16*), precipitated enzyme prepared from the 12-day duodenum, despite the

differences in catalytic properties between preparations from the two stages (*3*). Evidently the upsurge of the 3rd week reflects some reorganization in one class of alkaline phosphatase molecules, with the result that their characteristics are altered and they become more active under appropriate circumstances. The fact that the increase takes place in duodena in which mitosis has been suppressed, as by the drugs used in this study, indicates that the reorganization may occur in cells that have already moved into place on the sides of the villi.

It is unlikely that actinomycin D and puromycin exert their accelerating effect simply by increasing the output of adrenocorticoids (*2*), for these drugs also enhance the alkaline phosphatase–stimulating action of exogenous corticoids administered before the pituitary-adrenal axis is functional (*17*). Rather it appears that the three substances more directly elicit, or help to elicit, a conversion reaction that has been repressed in the infant duodenum, though the mechanism by which they act is not evident. It is tempting to argue that they may act by blocking the production of a protein that inhibits the conversion of existing alkaline phosphatase molecules to an altered state; but this explanation is brought into question by the total ineffectiveness of the aminonucleoside of puromycin in inhibiting amino acid incorporation in vitro (*18*). In the liver of the intact mouse, however, 0.15 of nucleoside per gram of body weight administered in four hourly doses reduced the incorporation of glycine-2-C^{14} into liver protein by 19 percent (*11*); possibly the much larger doses used in this study, over a longer period, did act by lowering the rate of synthesis of some protein inhibitor of the conversion of alkaline phosphatase in the duodenum.

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Sialic Acid Concentrations in the Pituitary Glands of Normal and Ovariectomized Rats

Abstract. *The concentration of sialic acid in the anterior pituitary gland of young female rats is approximately 250 micromoles per 100 grams. After ovariectomy there is a marked and persistent rise in pituitary sialic acid; this increase is probably related to the known increase in the production of gonadotrophic hormones.*

The sialic acids are an important group of compounds that are widely distributed in tissues and body fluids; they may be considered as derivatives of the 9-carbon α-keto acid, neuraminic acid, and they are known to be constituents of a wide variety of mucopolysaccharides, mucolipids, and mucoproteins (*1*).

It was recently shown that purified follicle stimulating hormone (FSH) and luteinizing hormone (LH) (*2*) of ovine or human origin contain sialic acid (*3*). Furthermore, in the case of FSH, it has been demonstrated that the release of sialic acid from the hormone preparation by incubation with neuraminidase results in an almost total loss of biological activity of the hormone (*4*). In view of these facts, it was of interest to determine the normal concentration of sialic acid in homogenates of rat pituitary glands. In addition, we have followed the changes in pituitary sialic acid which follow bilateral ovariectomy. It is, of course, well known that gonadectomy in the rat leads to a marked increase in the concentration of gonadotrophic hormones in the pituitary gland (*5*).

We used the thiobarbituric acid method of assaying sialic acid as described by Warren (*6*). A reference standard of *N*-acetyl neuraminic acid (*7*) was used and for the tissue determinations optical density readings were made at 532 and 549 mμ. Calculations were based on the use of the correction equation recommended by Warren (*6*, equation 2). Values for the pituitary assays were based on the assumption that the sialic acid was present in the form of *N*-acetyl neuraminic acid.

The female rats used in this study were of the Holtzman strain (*8*). They were divided into groups of four to six animals each and both ovaries were removed from half of them at an age of 32 days. Animals were killed by decapitation and the pituitary gland was quickly exposed by lifting up the brain. The posterior lobe was discarded and the fresh anterior lobe was weighed on a torsion balance. Assays were carried out on groups of normal animals ranging in age from 24 days to 130 days. The means and standard errors for each group were calculated and subjected to statistical analysis.

The changes in the concentration of pituitary sialic acid with increasing age and with time after bilateral ovariectomy and shown in Fig. 1. In the normal animals a gradual decline in the concentration of sialic acid occurred during the first 45 days of the study period and the curve leveled off as the rats entered the period of sexual

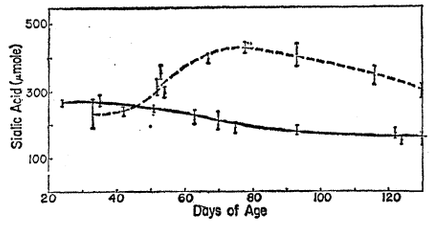

Fig. 1. Changes in the concentration of sialic acid in the pituitary gland with age and time after ovariectomy in the rat. The lower curve (solid line) shows the values for normal animals and the upper curve shows the values for ovariectomized animals. Ovaries were removed on day 32. Each point represents the mean of values from four to six animals and the vertical bars show standard errors of the means.