Effect of Puromycin on Hepatic Glycogen Phosphorylase Activity.* (30822)

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It has been shown previously that *in vivo* administration of puromycin induces a pronounced loss of glycogen from the liver of mice and rats(1). Although this response could be partially due to an inhibition of the synthesis of enzymes responsible for gluconeogenesis and glycogen storage, the rapidity of the effect and the observation that analogs of puromycin, which do not inhibit protein synthesis, also cause glycogen dissolution(2), suggest that the loss of glycogen is largely the result of stimulation of glycogen breakdown. The initial enhancement of blood sugar levels (1) and the extent of the glycogenolysis induced by puromycin raise the question concerning possible alterations of activities of enzymes involved in glucose and glycogen metabolism. It has been shown that puromycin injection has no effect on the activity of hepatic glucose-6-phosphatase(3,4), but does cause a rapid depression of liver amylase (3,5,6). Ferguson(7) showed that adrenocorticotropic stimulation of glycogen phosphorylase activity in steer adrenal slices was further enhanced by puromycin, although puromycin itself had no effect on the activity.

In this report the effect of puromycin on hepatic phosphorylase has been examined. Enhancement of the enzymatic activity has been demonstrated by injecting anesthetized mice with puromycin. In addition, rat liver slices incubated in the presence of puromycin have elevated phosphorylase activity as compared to control slices.

Materials and methods. For the *in vivo* studies to be reported, young adult female ICR mice were purchased from A. R. Schmidt and Co., Madison, Wisc. Rats (Holtzman Co., Madison, Wisc.) weighing 190 to 210 g were used for preparation of liver slices for *in vitro* incubations. All animals were fed Wayne Breeder Blocks *ad libitum*.

Puromycin was administered to mice in a solution of 1.39% $NaHCO_3$ adjusted to pH 7.5. Single intraperitoneal injections of puromycin or vehicle (0.1-0.2 ml) were used. Sodium pentobarbital was given intraperitoneally in a single injection of 82 mg/kg. Mice were sacrificed by decapitation, bled and a portion of the liver removed, blotted, weighed and either quickly homogenized in ice-cold 0.1 M NaF solution for enzyme assay, or dropped into 2 ml of 30% KOH for glycogen isolation(8) and determination of the alcohol-precipitated carbohydrate by the anthrone method(9).

Liver slices approximately 1 cm square, weighing 50 to 150 mg were prepared by free hand slicing at 0-4°C from rats which were stunned and decapitated. Precautions were taken to maintain the slices at this temperature to prevent inactivation of phosphorylase prior to incubation. The medium employed was the bicarbonate K = 110 medium of Cahill *et al*(10) which, at 37°C in the absence of sodium ion, permits the conversion of phosphorylase to inactive dephospho-phosphorylase. Duplicate slices were incubated with shaking at 37°C under 95% O_2-5% CO_2 in 5 ml of medium, with or without puromycin. The buffering capacity was such that no pH change could be detected by addition of the amount of puromycin dihydrochloride used in these studies. To terminate the incubations, slices were transferred rapidly to 10 ml of ice-cold 0.1 M NaF.

Phosphorylase activity was estimated essentially by the procedure of Sutherland(11). Ten per cent (fresh weight) liver homogenates were prepared in chilled 0.1 M NaF from blotted excised liver or slices. The unfractionated homogenates were incubated at 37°C and pH 6.1 in the presence of glycogen, glucose-1-phosphate, NaF, and adenosine-5′-

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TABLE I. Effect of Puromycin on Liver Phosphorylase Activity in Unanesthetized and Anesthetized Mice.

Treatment	Phosphorylase activity* Min after injection† 15	30	60
Unanesthetized			
$NaHCO_3$	605 ± 13 (4)	606 ± 38 (4)	576 ± 22 (3)
Puromycin	552 ± 11 (4)	621 ± 15 (4)	601 ± 19 (4)
Anesthetized‡			
$NaHCO_3$	353 ± 63 (4)	269 ± 37 (4)	280 ± 48 (4)
Puromycin	231 ± 17 (4)	495 ± 41 (4)	443 ± 42 (4)

* Activity is expressed as units/g of liver. Means ± standard error of the mean are shown with number of animals used in the parentheses.

† Puromycin (300 mg/kg) or $NaHCO_3$ was given at zero time in a single intraperitoneal injection.

‡ 82 mg of pentobarbital/kg was administered 30 min prior to $NaHCO_3$ or puromycin injection.

monophosphate. Aliquots of the reaction mixture were removed at zero time and 10 minutes and pipetted into cold 8% trichloroacetic acid, which was found not to hydrolyze glucose-1-phosphate. Orthophosphate was measured by the method of Fiske and Subbarow (12). Kinetics of the reaction was determined to be zero order up to 11 μmoles of phosphate released (22%). Enzyme assays in this study were within that range. One unit corresponds to the liberation of 1 μmole of phosphate under the above conditions. Activity is expressed as units/g wet weight of liver.

Puromycin dihydrochloride and rabbit liver glycogen were obtained from Nutritional Biochemicals Corp. Dipotassium glucose-1-phosphate and adenosine-5′-monophosphate were purchased from Sigma Chemical Co. Pentobarbital (Nembutal 60 mg/ml, diluted with saline to 10 mg/ml) was acquired from Abbott Laboratories. Double distilled water and reagent chemicals were utilized for all solutions involved in incubations or enzyme assays.

Results. Using rabbit liver slices, Sutherland and Cori first demonstrated that phosphorylase activity could be rate limiting for the conversion of hepatic glycogen to glucose (13). It was therefore a primary interest to examine liver phosphorylase in puromycin-treated animals over a period during which the animals were in a state of rapid glycogenolysis. A dose of puromycin (300 mg/kg) known to induce rapid glycogen loss from the liver was given to mice and the phosphorylase activity examined at 15, 30, and 60 minutes after injection. Previously it had been shown that this dosage of puromycin causes marked inhibition of protein synthesis at 30 and 60 minutes and a loss of 20.0/mg of glycogen/g liver/hour(1). It can be seen in Table I that uniformly high phosphorylase activities were obtained for both puromycin-treated and control animals injected with $NaHCO_3$. Therefore, in spite of the inhibitory effect of puromycin on protein synthesis, phosphorylase activity was not depressed over the 1-hour period studied. It is apparent that an enzymatic pathway involving phosphorylase remains as a possible means for glycogen loss in puromycin-treated animals. On the other hand, lack of discernible phosphorylase activation by puromycin is not surprising in view of the findings of others who have attempted to demonstrate *in vivo* activation of hepatic phosphorylase. The suggestion has been made that phosphorylase may be maximally activated in rodent liver at the time of killing, thus masking any stimulation which may occur in the unhandled animal(14-16).

Since it is possible that nonspecific release of adrenergic compounds was responsible for stimulation of phosphorylase at time of sacrifice, mice were treated with saline or pentobarbital 30 minutes prior to killing to determine if anesthesia would result in a lowered control activity. The phosphorylase activity of 5 unanesthetized mice was found to be 542 ± 48 units/g of liver whereas a lower activity of 330 ± 96 units/g of liver was found in

4 unanesthetized mice. It was therefore felt that if a puromycin-activation of phosphorylase occurred *in vivo,* it might be possible to demonstrate this effect in pentobarbital-treated animals.

Puromycin or buffer was given to anesthetized mice and hepatic phosphorylase activity was measured up to 1 hour after injection. Table I shows that puromycin-injected mice killed at 15 minutes had depressed phosphorylase activity. In contrast, at 30 and 60 minutes after puromycin treatment phosphorylase activity had increased to 180 and 160% of the respective control values. Although the enzymatic activity following administration of puromycin was considerably greater than with $NaHCO_3$ treatment in these animals, maximal activation was not achieved as compared to unanesthetized animals.‡

If puromycin were activating phosphorylase *in vivo* it should be possible to demonstrate this action by giving puromycin followed by anesthesia to prevent nonspecific stimulation. In this way animals killed at each interval would be subjected to pentobarbital for the same time period. Puromycin was administered in a single injection and the animals sacrificed at intervals up to 3 hours. Thirty minutes before sacrificing, the mice were given pentobarbital. Consequently one group, which was killed at 20 minutes after puromycin treatment, received pentobarbital 10 minutes prior to the puromycin injection. As shown in Fig. 1, phosphorylase activity from livers of puromycin-treated animals was above the control levels, while glycogen diminished at a rate somewhat slower than was shown previously for unanesthetized animals (1). At 3 hours phosphorylase activity from puromycin-treated animals approached values observed in unanesthetized mice (460 units/g liver, wet-weight), while glycogen levels at this time were at the lowest value.

To investigate the action of puromycin on phosphorylase activity in the absence of possible hormonal factors, the *in vitro* system described by Cahill *et al*(10) was employed. Phosphorylase is inactivated in this system which contains a high concentration of potassium and no sodium ion. Rat liver slices were incubated without additions, or in the presence of 4.6×10^{-5} M puromycin.§ Phosphorylase activity was measured after 20, 40, and 60 minutes and is expressed both in units and as per cent of the control value at each time. Table II shows that the initial control activity had fallen appreciably by 20 minutes. By 60 minutes a consistent increase in control activity was observed. Following 20 and 40 minutes of incubation in the medium containing puromycin, the phosphorylase activity of the liver slices was 118 and 133% of those incubated without puromycin. The largest effect of puromycin was most consistently observed after 40 minutes of incubation. Addition of 10^{-3} and 10^{-4} M puromycin directly to the phosphorylase assay mixture in lieu of adenosine-5′-monophosphate caused an inhibition of activity of 8.1 and 3.6% respectively. This finding indicates that the activation observed in the slices could not be due to residual puromycin.

TABLE II. Effect of Puromycin on Phosphorylase Activity in Rat Liver Slices.*

Min of incubation	Phosphorylase activity† Control	Puromycin	% of control
0	265		
20	100	118	118
40	106	141	133
60	135	140	104

* Each flask contained 2 liver slices and 5 ml of liver medium either without (control), or with puromycin (4.6×10^{-5} M). Flasks were gassed with 95% O_2–5% CO_2 and incubated at 37°C. At the times indicated slices were removed, placed in ice-cold 0.1 M NaF, and homogenized for phosphorylase assay.

† Mean activity is shown expressed in units/g of liver.

Discussion. In these studies it has been demonstrated that a dose of puromycin which causes marked inhibition of protein synthesis and glycogenolysis in the liver does not depress hepatic phosphorylase activity up to a period of 3 hours. The absence of a diminu-

‡ It should be noted that pretreatment of mice with pentobarbital inhibits glycogen loss measured at 1 hour following puromycin injection. This finding in view of the effect on phosphorylase is treated in the discussion.

§ This concentration was determined to be critical to obtain the maximal response(3).

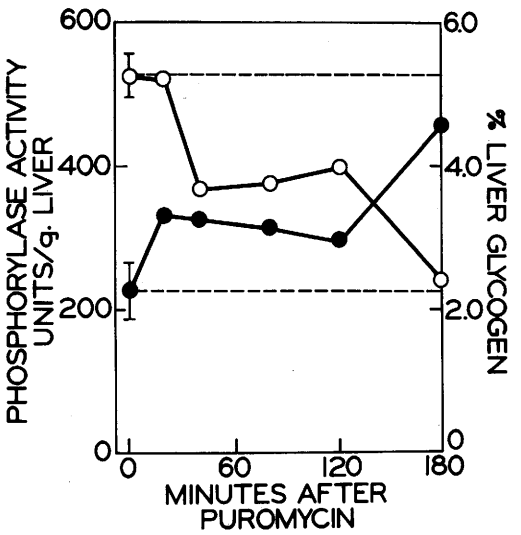

FIG. 1. Effect of puromycin and pentobarbital on mouse liver glycogen and phosphorylase activity. Each animal received $NaHCO_3$ or 300 mg/kg of puromycin at zero time and 82 mg/kg of pentobarbital 30 min prior to sacrificing. Mean values ± standard error are shown for 5 control mice. Three animals were used at each interval with phosphorylase activity ●, and liver glycogen ○, shown.

tion of phosphorylase and glucose-6-phosphatase(3,4) activities following puromycin administration, and the indication that puromycin does not inhibit phosphoglucomutase activity(7), shows that these enzymes are available to mediate glycogen loss from the liver. On the other hand hepatic amylase activity is greatly depressed by puromycin injection(3,5,6), indicating that this enzyme is not a means for the characteristic glycogenolysis observed after puromycin treatment.

The failure to demonstrate activation of phosphorylase by puromycin above the control values in unanesthetized mice is most likely due to maximal activation of the enzyme both in control and treated animals. This phenomenon may be a result of catecholamine release during the killing procedure (14-16). Since a reduction of the control phosphorylase activity was obtained by pentobarbital injection, it was possible to detect a puromycin-induced activation of phosphorylase using anesthetized mice.

Although enhancement of phosphorylase activity following puromycin treatment of anesthetized mice was demonstrated in this report, it should be noted that pretreatment of mice with pentobarbital inhibited glycogen loss measured at 1 hour following puromycin administration(2). In addition, pentobarbital treatment for a period of 30 minutes prior to sacrificing puromycin-injected mice (Fig. 1) diminished the apparent rate of glycogen loss as compared to animals receiving only puromycin(1). This attenuation of the puromycin-induced glycogenolysis by pentobarbital treatment in view of the ostensible activation of phosphorylase by puromycin could be accounted for by the following hypothesis. If the effect of puromycin were to potentiate the activation of hepatic phosphorylase by endogenous catecholamines (or glucagon), then under conditions of minimal release of catecholamines, such as might occur in the anesthetized state, no effect of puromycin would be seen. Thus, because of a depressed level of adrenergic amines in the pentobarbitalized-puromycin-treated animals, the *in vivo* phosphorylase activity might in fact be suppressed to a level insufficient to cause glycogenolysis. Furthermore, puromycin-induction of glycogenolysis observed in the unanesthetized state is accordant with a potentiation of the action of endogenous catechols by puromycin, an effect that possibly would maintain phosphorylase in the active form. On the other hand, at the time of sacrificing the anesthetized mice, a release of some adrenergic amines may occur(15), which alone may cause no decisive stimulation of phosphorylase, but which, in the presence of puromycin, would result in an augmentation of phosphorylase activity. Therefore, an activation of phosphorylase by puromycin in anesthetized mice would be apparent only because of the release of a minimal amount of epinephrine-like compounds at the time of killing. It would be expected that the inhibition of catecholamine release would diminish following pentobarbital treatment, thus affording a possible explanation for the latent activation of phosphorylase by puromycin seen in anesthetized mice (Table I).

Consistent with this postulated role for puromycin, it was shown that the pentobarbital-inhibition of glycogenolysis induced by the puromycin analog, 6-dimethylaminopurine, is fully overcome by simultaneous adminis-

tration of epinephrine(3). In addition, in this report, puromycin-effected enhancement of phosphorylase activity could be detected in rat liver slices under conditions that permitted the initial control level to fall, and where, after a latent period, a small but consistent activation was observed in control slices.

Of interest are the findings of Ferguson (7), in which puromycin alone had no effect on phosphorylase in steer adrenal slices, but enhanced the stimulation observed in the presence of adrenocorticotropic hormone. A plausible explanation for the potentiation of the effect of hormonal agents on phosphorylase would be an inhibition of phosphorylase phosphatase or cyclic-3′,5′-adenosine monophosphate phosphodiesterase as has been suggested(1). Further studies will be needed to elucidate the mechanism of the puromycin-stimulation of hepatic phosphorylase activity, and to evaluate the role of this effect in glycogenolysis.

Summary. Liver glycogen phosphorylase activity was studied in mice at early times subsequent to *in vivo* administration of puromycin, and following puromycin addition to incubations of rat liver slices. No effect of puromycin on phosphorylase could be detected following injection of unanesthetized mice. Puromycin treatment of pentobarbital-anesthetized mice, however, resulted in an enhancement of the phosphorylase activity as compared to control values. Phosphorylase activity in rat liver slices incubated with puromycin was greater than those incubated without puromycin. These results indicate that puromycin can elevate liver phosphorylase activity, a finding consistent with the hepatic glycogenolytic action of the antibiotic *in vivo*.

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Erythrocyte Inorganic Pyrophosphatase Activity in the Newborn Infant.* (30823)

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The activity of several enzymes is different in young *vs* older erythrocytes, and in the blood of newborn *vs* adult individuals. Inorganic pyrophosphatase activity has been

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