TABLE II. Acenocoumarin Plasma Levels 1.5 Hours After Acenocoumarin. (25 mg/kg ip; 4 guinea pigs/group)

Pretreatment for 2 days	Acenocoumarin concentration (mg/liter) Average	Range
Saline (0.4 ml/day)	23.1	17.8–26.4
Glucagon (10 mg/kg per day)	24.3	18.8–34.1

may be altered by factors which either affect the physiologic disposition of the drug, or the site at which the drug acts. Clues as to the nature of the drug action may be obtained by studying the factors which influence sensitivity to the drugs without altering the fate of the drug.

The effect of Vitamin K in blocking the action of coumarin anticoagulants is well documented, and is not correlated with changes in the physiologic disposition of coumarins as reflected in plasma levels of the drug. In contrast, the effect of barbiturates is mediated through altered disposition of the coumarins (1,4). The tranquilizers chlorpromazine and reserpine exaggerate prothrombin response while not influencing plasma levels of the coumarin drug. This effect may be correlated with the action of these agents on NAD synthesis (5).

The mechanism by which starvation causes an exaggerated response to coumarins is not known. Elements of protein origin are probably involved (2). The finding of a similar effect by glucagon suggests that the metabolic fate of tyrosine and/or tryptophan may be important to prothrombin synthesis. The fact that saline diluted plasma demonstrates the glucagon effect at least as well as undiluted plasma indicates an exaggerated prothrombin deficiency rather than a superimposed heparin-like effect.

The observation that insulin has no detectable effect on sensitivity to acenocoumarin, but can block the sensitizing effect of glucagon may have clinical significance in helping to explain the "unpredictable" manner in which sensitivity to anticoagulants may be altered by an agent under one set of conditions, and not under another.

It remains to be determined whether the effect of starvation on sensitivity to coumarin anticoagulants is mediated through a stimulation of endogenous glucagon production during starvation.

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Liver Insulinase Activity in Insulin Deficient Rats* (32795)

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The term "insulinase" was proposed by Mirsky and Broh-Kahn, (1) for the enzyme or enzyme system which preferentially degrades insulin. Initially the *in vitro* insulinase activity of rat liver was determined by measuring the blood sugar fall in rabbits injected with insulin solutions previously incubated with rat liver homogenates under specified conditions. Using this method, Broh-Kahn and Mirsky (2) observed a marked reduction in liver insulinase activity in fasted rats.

Insulin-^{131}I provides a much more sensitive method for quantitating the degradation of insulin (3). The insulin-^{131}I and insulin are degraded equally well and competitively (4).

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Mirsky *et al.* (5) and Tomizawa *et al.* (6) utilized insulin-^{131}I in studying the *in vitro* insulinase activity of rat liver homogenates. Tomizawa (7, 8) presented evidence that beef liver insulinase degrades insulin by reductive cleavage of the disulfide bonds separating it into reduced A and B chains. Insulin is precipitated in cold 5% trichloroacetic acid (TCA); soluble ^{131}I indicates cleaved insulin.

The studies of Weber *et al.* (9) indicate that insulin is an inducer of key glycolytic enzymes in the rat liver. Our studies were initiated to test the hypothesis that insulin is an inducer of insulinase in rat liver. As a first approach to this problem we compared the insulinase activity of livers from normal and insulin deficient animals.

Materials and Methods. Male Sprague-Dawley strain rats of 180-220 gm were used in these experiments. Plasma insulin deficiency was produced by fasting or alloxan treatment. A 2% solution of alloxan was injected iv (40 mg/kg of body wt.). Ninety-six hours after initiation of the fast or alloxan treatment, rats were sacrificed by decapitation; as much blood as possible was drained from the carcass. The liver was quickly removed, blotted, and weighed. A piece of the liver weighing approximately 1 gm was excised and weighed. This piece of liver was homogenized in phosphate buffer containing 5×10^{-3} *M* disodium ethylenediaminetetraacetate (EDTA), pH 7.4, with a Sorvall Omni-Mixer equipped with a teflon pestle. The homogenate was chilled in an ice bath until centrifuged at 2400 rpm (1450*g*) for 30 min at 4°C in an International centrifuge.

One-ml portions of the supernatant were pipetted to chilled 13 × 100 mm glass tubes. When all of the homogenate-supernatants for a particular experiment were pipetted, 1 ml of substrate was quickly added to each tube with a Cornwall syringe pipette. Immediately 2 ml of cold 10% TCA was added to the "0" tubes and the remaining tubes were transferred to a 37°C water bath and incubated for 5, 10, 15, 20, and 25 min. At the end of each time interval, 2 ml of 10% TCA was added to the appropriate tubes, and they were removed from the water bath. Then all of the tubes of the experiment were centrifuged at 2000 rpm for 20 min. The supernatants were withdrawn with water-pump suction connected to a Pasteur bent tip pipette. The precipitates were counted in a Packard Auto-gamma spectrometer.

The "0" tubes contained the maximum amount of TCA precipitable ^{131}I and represented 100% of the substrate insulin. The TCA precipitable ^{131}I in all other tubes represented the substrate that had not been degraded and the insulin degraded was calculated:

$$100 - \left(\frac{\text{cpm in "X" tube}}{\text{cpm in "0" tube}}\right) 100 = \%\ \text{insulin degraded}$$

(% insulin degraded) (micrograms insulin substrate) = micrograms insulin degraded

$$\frac{\text{micrograms insulin degraded}}{6000\ \text{gm/mole}} = \text{millimicromoles insulin degraded}$$

The insulin substrate used in these experiments consisted of U-40 Iletin,[1] 1 mμg of insulin-^{131}I (pork),[2] 10^{-2} *M* mercaptoethanol, and 1% bovine serum albumin (BSA) in 0.1 *M* phosphate buffer containing 10^{-3} *M* EDTA, pH 7.4.

Blood glucose was determined by the potassium ferricyanide method as adapted to the Technicon AutoAnalyzer. Plasma insulin was determined by a two-antibody immunoassay procedure (10).

Results. Plasma insulin levels of 96-hour fasted and 96-hour postalloxan rats were less than 1/5 the levels in nontreated control rats.

Liver insulinase activity was markedly reduced in both groups of animals, Table I.

Discussion. The reduced insulinase activity in both the fasted and alloxan treated animals correlates with the low plasma insulin levels of these two groups of animals. The blood glucose levels of the fasted rats was less than that of the normal fed rats; whereas, the blood glucose levels were much higher than normal in the alloxan treated rats.

[1] Kindly donated by Eli Lilly Co., Indianapolis, Indiana.

[2] Purchased from Abbott Laboratories, North Chicago, Illinois. This insulin-^{131}I had a specific activity of 100–200 mc/mg and contained less than one atom of iodine per molecule of insulin.

TABLE I. Rat Liver Insulinase; Effect of Insulin Deficiency.[a]

Rats	Body wt. (gm)	Liver wt. (gm)	Insulinase activity[b]	Insulinase activity (% normal)	Blood glucose (mg/100 ml)	Plasma insulin[c] (% normal)
Normal	215 ± 2	9.13 ± 0.23	785 ± 36	100 ± 5	131 ± 5	100 ± 5
96-hour fasted	141 ± 2	3.86 ± 0.27	289 ± 25	37 ± 3	80 ± 5	12 ± 4
Normal	213 ± 2	8.47 ± 0.17	797 ± 49	100 ± 6	127 ± 6	100 ± 8
96-hour postalloxan	160 ± 2	6.47 ± 0.24	490 ± 54	61 ± 7	390 ±47	17 ± 3

[a] Each value is the mean of determinations on 5 animals ± SE of the mean.

[b] Insulinase activity expressed as 10^{-9} *M* insulin degraded per liver per hour at 37°C.

[c] Insulin values determined by immunoassay with a beef insulin standard; therefore values are expressed as per cent of normal.

Liver shrinkage was much greater in the fasted rats than in the alloxan treated animals. Weber *et al.* (9, 11) have demonstrated that liver shrinkage during fasting and alloxan diabetes is not due to a decrease in liver cellularity. Lazarow and Palay (12) have reported foci of necrosis in livers of alloxan treated rats. It may be that the reduced insulinase activity of the alloxan treated rats is due in part to direct damage of liver cells; however, the reduction in insulinase activity is greater than the reduction in liver size and apparent liver cell damage.

The markedly lowered insulin levels in the fasted and alloxan treated rats is a common factor associable with reduced levels of liver insulinase activity. The hypothesis that insulin is an inducer for insulinase is tenable on the basis of this evidence and is being investigated more thoroughly.

Summary. Rat liver insulinase activity is greatly reduced in fasted and alloxan treated animals. This reduced insulinase activity is associable with low levels of plasma insulin.

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