and mast cell distributions did not follow the same patterns. If it is assumed that heparin is derived from mast cells, then our results indicate that the distribution of heparin through the body was modified by regional differences, either in the composition of the mast cell granula (proportion of heparin to hyaluronic acid and other mucopolysaccharides), or in the capacity of tissues to retain heparin after its release from the cells.

In the course of the present study, more heparin was found in the tissues of the rat than in the tissues of the rabbit. As the former species exhibits greater resistance to hyperlipemia and atherosclerosis, the observations agree with the theory that the heparin content of the tissues may be one of the factors which contribute to this resistance.

Summary. Tissue heparin contents and mast cell counts were determined on liver, lung, intestine, kidney, spleen and thymus of male rats and rabbits. In most instances, distribution of heparin and mast cells did not follow the same pattern. Rat organs showed higher heparin values, but lower mast cell numbers than the corresponding rabbit tissues.

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Physiological Response of "Insulinase" and Its Inhibitor in the Hypoinsulin State.* (22906)

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Mirsky *et al.*(1-6) have demonstrated a tissue activity in liver capable of inactivating insulin and have named this activity "Insulinase". They also have described the ability of filtrates from heated homogenates of the same livers to inhibit Insulinase. The response of this system of Insulinase and its inhibitor to the level of circulating insulin and its conceivable role in the homeostasis of blood sugar values in the frog and rat have been investigated.

Methods. Intact and depancreatized frogs (*Rana pipiens*) were divided into 2 groups: one group maintained at 23°C and the other at 2-5°C. Pancreatectomy in frogs was accomplished as follows. The frogs were anesthetized with ether and a mid-line abdominal incision was made exposing the pancreas. The major blood vessels of this organ were ligated and cut prior to removal of the pancreas, and the abdomen was then closed in the usual manner. 5-0 silk was used throughout the procedure. The following day the group of normal and operated animals kept at 23°C were force-fed (minced calves liver and bone meal dipped in cod liver oil). After 6-7 days, frogs of both groups were pithed, blood samples were taken by heart puncture for sugar determinations(7), and the livers then extirpated, chilled in ice-water and prepared as indicated below. Male rats of the Holtzman strain weighing 175-200 g were kept in individual wire-bottomed cages and fed a stock diet. Animals were fasted for 65 hours prior to subcutaneous injection of 15 mg of alloxan (Eastman) per 100 g body weight as a 3%

* The authors wish to acknowledge the assistance of Lois I. Prirster for nitrogen determinations.

solution, and subsequently kept on the mixed diet for 15 days. At this time they were transferred to metabolism cages and supplied with 10% sucrose in the drinking water as sole source of calories. Urine was collected for 18 hours and the glucose excretion determined(7) and recorded in Table III. The animals were grouped according to severity of their diabetes as judged from their glycosuria, and the livers of each group were pooled for assays. The blotted livers from rats or frogs were weighed and homogenized in Waring blender for 2 minutes with 4 volumes of M/15 phosphate buffer of pH 7.8. The homogenate was filtered through gauze and cotton and centrifuged at 2,000 r.p.m. for 20-30 minutes. The supernatant was adjusted to pH 7.8, if necessary, and again filtered. This supernatant was either used immediately or kept frozen at –20°C until assayed. Previous experiments have established that Insulinase and its inhibitor were stable at –20°C for periods of at least 4 months. *"Insulinase inhibitor"* was obtained from aliquot of the same supernatant by boiling for 5 minutes, filtering through gauze-cotton pad and adjusting to pH 7.8. Total nitrogen and non-protein nitrogen were determined on all fractions and dilutions of about equal nitrogen content were used for the assays. Assays for *Insulinase* and its inhibitor were essentially similar to those of Mirsky *et al.*(1-6). The substrate was crystallized insulin, (20-23 units /mg), containing approximately 1/1000th of its weight of I^{131} treated insulin. Contrary to Tomizawa *et al.*(8), no difference was observed in rate or extent of Insulinase action at substrate level of 50-800 γ/ml, if the preparation contained one part of iodinated insulin in any dilution from 1 to 100,000 parts of normal insulin. A typical example using 200 γ insulin substrate, shaken with 1 ml of standard rat liver Insulinase for 30 minutes at 38°C in a Dubnoff water bath, is illustrated in Table I. To 0.5 ml of insulin substrate (400 γ/ml of Sørensen's buffer) Insulinase and/or its inhibitor was added in 0.2, 0.5 or 1 ml quantities, and volumes were adjusted to 2 ml with Sørensen's M/15 pH 7.8 phosphate buffer. The mixture was incubated at 38°C for periods of 30 minutes to 2 hours in a Dubnoff water bath with shaking at 100 oscillations per minute. After incubation, the reaction was stopped with 2 ml 10% trichloroacetic acid and one hour later centrifuged for 10-20 minutes. The radioactivity of the whole mixture and of the TCA supernatant was

TABLE I. Hydrolysis of Insulin.

Insulin I^{131} : Insulin	% hydrolysis by stand. "Insulinase"
1 : 1	25
1 : 3	21
1 : 600	24
1 : 6,000	28
1 : 12,000	26
1 : 123,000	25

TABLE II. Normal and Depancreatized Frogs.

	Blood glucose, mg %*	Insulinase hydrolysis of insulin after 2 hr	Heated Insulinase: Hydrolysis of insulin after 2 hr	Heated Insulinase: Inhibition of homologous unheated Insulinase
		%		
Hibernated (2–5°C)				
Depancreatized	3– 27	17–18	3	50
Normals	0	16–18	0	50
Active (20–22°C)				
Depancreatized	83–124	24–27	0	18–57
Normals	0– 20	22–27	4	15–26

* Blood sugars were unusually low in all cases though there is a significant difference between normals and depancreatized animals. In the case of the hibernating animal this is probably merely a reflection of the decreased metabolic state. Depancreatized animals, both hibernated and active, were obviously ill but hibernated animals appeared to fare worse than active animals. It may be that in hibernation even a small increase in blood sugar may be physiologically untenable in relation to other decreased metabolic functions.

TABLE III. Normal and Alloxanized Rats.

A		B	Heated Insulinase C	D
Avg glucose excreted/18 hr, mg	No. of animals	Insulinase hydrolysis of insulin after 2 hr	Hydrolysis of insulin after 2 hr	Inhibition of homologous unheated Insulinase
		%		
21.8 (Controls)	5	36 to 51	25	17
120.5 (Mildly diabetic)	3	34 to 56	26	9
690 (" ")	2	26 to 45	23	16
2,235 (Diabetic)	2	33 to 59	36	8
4,845 (")	4	40 to 51	25	13
5,942 (")	4	24 to 45	16	12

measured in a well-type scintillation counter and the counts corrected for background. Percent hydrolysis, as recorded in the Tables, was calculated by dividing the counts per minute per ml in the TCA supernatants by the counts per minute per ml of the whole mixture. Corrections were made for the small amount of radioactivity found in the TCA supernatants prepared at zero time. In all assays a "standard Insulinase" preparation from rat liver was run simultaneously to assure proper functioning of the assay technic.

Results. The results obtained are summarized in Tables II and III and the variations indicated in the Tables refer to results obtained in 3 different experiments on the same samples.

From the data it is apparent that there is no significant influence of the availability of insulin on either Insulinase or "Insulinase inhibitor". Under the conditions of these experiments the Insulinase system could not have influenced the blood sugar balance by an effect on either internally or externally provided insulin.

The data also shed some light on the nature of the Insulinase system. From columns C & D in Table III, it is apparent that there is substantial hydrolysis of insulin by heated Insulinase preparations. This has been observed by us regularly in rat and beef livers. We were also unable to obtain a graded response to varying amounts of Insulinase preparation using a wide variety of substrate concentrations (50 to 1600 γ) or incubation periods (15 minutes to 4 hours). Extensive separation and purification of the various enzymatic and non-enzymatic entities involved in the degradation of insulin by liver will be necessary to clarify the nature of the observed effects.

Summary. No evidence has been found that the "Insulinase"-"Insulinase inhibitor" system responds *in vivo* (rat and frog) to the absence of circulating insulin.

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