the collection of samples is also greatly appreciated.

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Relative Insulinase Activity in the Liver and Kidney of the Rat* (33044)

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The term "insulinase" was proposed by Mirsky and Broh-Kahn, 1949 (1) for the enzyme or enzyme system which preferentially degrades insulin. These workers determined the relative insulinase activity in various rat tissues by measuring the blood sugar fall in rabbits injected with insulin solutions previously incubated with such tissue homogenates. They reported that in the rat the insulinase activity per gram of wet tissue was highest in the liver, second highest in the kidney, and much less in the other tissues (1). Subsequently this group has focused on rat liver insulinase activity (2,3).

Tomizawa, 1962 (4,5) investigated the degradation of insulin by utilizing insulin-^{131}I tracer in the substrate. Using beef liver as a source, Tomizawa concentrated "insulinase" activity and demonstrated that insulin degradation is initiated by the reductive cleavage of insulin into its A and B chains by glutathione insulin transhydrogenase. Using a modification of Tomizawa's procedure, preliminary investigations in our laboratory indicated that rat kidney insulinase activity per gram wet tissue was greater than that of the liver. Therefore, the relative insulinase activity of rat kidney and liver has been studied in more detail.

Materials and Methods. Insulinase activity was determined by a modification of Tomizawa's procedure (4,5). Male Sprague-Dawley rats weighing 205–270 gm that had been fed *ad libitum* on Purina chow were sacrificed by decapitation and as much blood as possible was drained from the carcass. The liver and kidneys were removed, blotted, and placed in chilled beakers until weighed. The intact liver was weighed and then a piece of approximately 3 gm was removed and weighed. This piece of liver was placed in a glass tube and homogenized in a volume of cold phosphate buffer containing 5 ×

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10^{-3} *M* EDTA (pH, 7.4) which was equal to 1.0 ml of buffer/100 mg of tissue. The homogenization was carried out for 1 min with a Sorvall Omni-Mixer equipped with a teflon pestle. An additional 1.0 ml of buffer/100 mg of tissue was added to the homogenate and the homogenate was stored in an ice bath. The kidneys from each animal were homogenized in a similar manner. Tissues from five animals were prepared on a single day.

The homogenates were separated into three portions for each animal and centrifuged at 0°C as follows: I. 600*g* for 10 min to remove large cellular fragments and nuclei; II. 15,000*g* for 30 min to remove mitochondria and lysosomes; and III. 100,000*g* for 60 min to remove microsomes.

A series of 13 × 100-mm glass tubes were appropriately numbered and chilled in an ice bath. One ml of supernatant was transferred to each tube, seven tubes for each supernatant of each homogenate. When all of the homogenates had been pipetted to their respective tubes, 1 ml of substrate containing 30 μg of insulin,[1] 1 ng of insulin-^{131}I,[2] and 10^{-2} *M* mercaptoethanol in phosphate buffer containing 5×10^{-3} *M* EDTA (pH 7.4) was transferred rapidly to each tube with a Cornwall syringe pipette. Immediately, 2 ml of cold 10% TCA was added to the "0" tube of each supernatant of each homogenate and the remaining tubes were placed in a 37°C water bath. At intervals of 5, 10, 15, 20, 25, 30 min, 2 ml of cold 10% TCA were added to one tube of each supernatant of each homogenate, and these tubes were removed from the water bath.

At the termination of incubation (30 min) all of the tubes were centrifuged at 2000 rpm for 20 min. The supernatants were discarded by a water-section pipette. The ^{131}I radioactivities of the precipitates were counted in a Packard Autogamma 410 spectrometer.

The cpm of ^{131}I in the "0" tube of each homogenate indicated the maximum amount of insulin-^{131}I precipitable by 5% TCA prior to incubation, and represented 100% of the initial substrate of 30 μg of insulin (6). The cpm of ^{131}I in each of the other tubes for each homogenate were divided by the cpm of ^{131}I in the "0" tube for that homogenate to determine the percentage of insulin not degraded; the difference from 100% was the percentage of insulin degraded. Each initial homogenate contained 5×10^{-2} gm of tissue/ml; therefore:

$$\frac{\text{micrograms of insulin degraded}}{5 \times 10^{-2}\ \text{gm of tissue}} =$$

micrograms of insulin degraded per gram of tissue.

The micrograms of insulin degraded per gram of tissue were divided by 6000 μgm/μmole to determine the micromoles of insulin degraded per gram of tissue; all values are presented as millimicromoles of insulin degraded per gram of tissue per unit of time at 37°C.

Results. Figure 1 illustrates the insulinase activity of rat liver. There was an appreciable loss of insulinase activity when the mito-

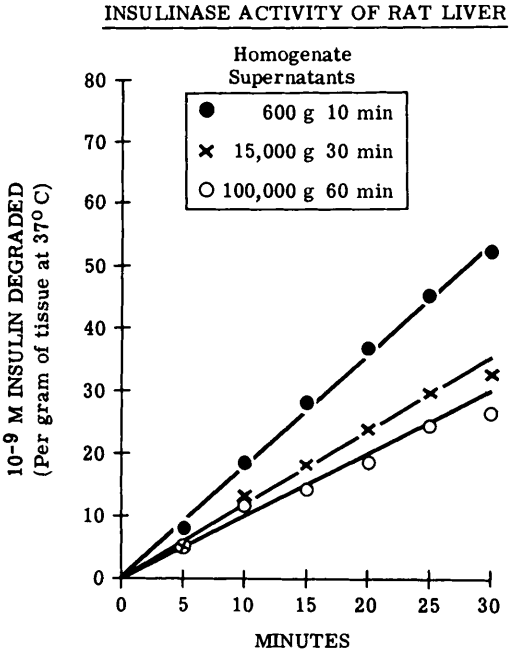

FIG. 1. Each point is the mean of 5 determinations.

[1] Pork insulin lot no. 818194 kindly donated by Eli Lilly Co., Indianapolis, Indiana.

[2] The insulin-^{131}I (pork) specific activity 100–200 mC/mg and containing less than one atom of iodine per molecule of insulin was purchased from Abbott Laboratories, North Chicago, Illinois.

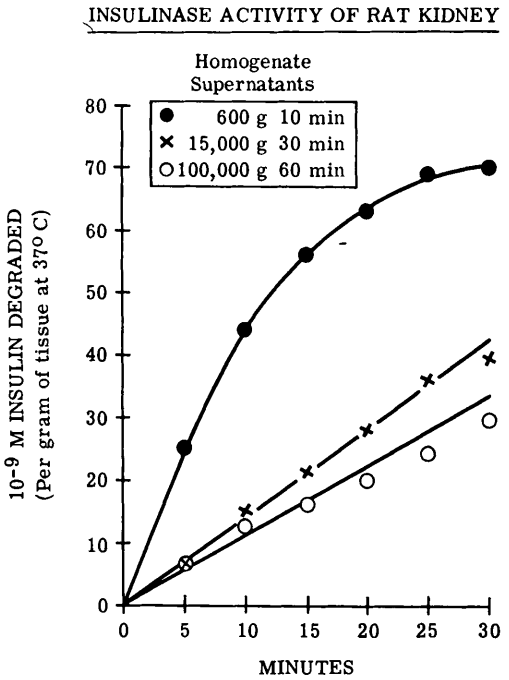

FIG. 2. Each point is the mean of 5 determinations.

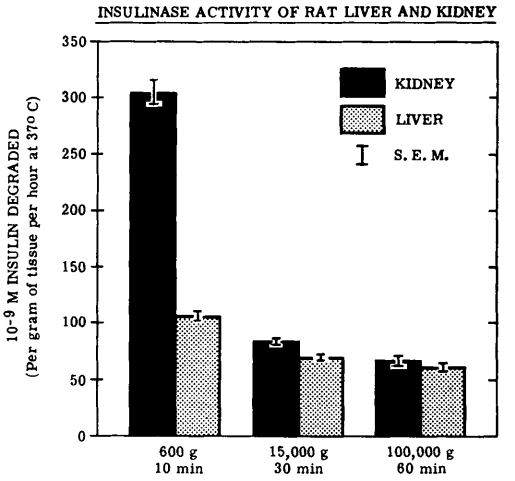

FIG. 3. The 600*g* kidney data was calculated from the 5-min interval data and is the mean of 5 determinations. All other bars represent the means of data from 6-time intervals with 5 determinations at each time interval. The differences between the kidney and liver values for each centrifugation fraction are highly significant, $p < .001$.

chondria-containing fraction was removed but very little additional loss of activity when the microsome fraction was removed.

Figure 2 illustrates the insulinase activity of rat kidney. There was a tremendous loss of insulinase activity with the removal of the mitochondria-containing fraction but very little additional loss of activity with the removal of the microsome fraction. Figures 1 and 2 are graphs to the same scale; comparison demonstrates that the insulinase activity of rat kidney is greater than that of rat liver.

When insulinase activity is expressed as millimicromoles of insulin degraded per gram of tissue per hour at 37°C (Fig. 3), the greater insulinase activity of rat kidney as compared to rat liver is readily apparent. Figure 3 also emphasizes that the liver insulinase activity of the 15,000*g* and 100,000*g* supernatants were 67 and 57%, respectively of that of the 600*g* supernatant. The kidney insulinase activity of the 15,000*g* supernatant was 28% that of the 600*g* supernatant; whereas, that of the 100,000*g* was 22%.

Discussion. Our results clearly demonstrate that the insulin degrading capacity (insulinase activity) of rat kidney exceeds that of the rat liver when comparison is made on the basis of gram wet tissue.

Mirsky reported that insulinase activity of rat liver homogenates was confined "principally to the supernatant after removal of cellular debris and mitochondria" (7). Brey reported similar results for insulinase activity of the livers of KL mice (8). However, our results indicate that the "mitochondria-free" and "microsome-free" supernatants of rat liver homogenates contain 30–40% less insulinase activity than the "nucleus + debris-free" supernatants. Even more striking was the 70% less insulinase activity in "mitochondria-free" fractions of rat kidney homogenates as compared to the "nucleus + debris-free" fractions. These results strongly suggest that mitochondrial enzymes play an important role in the reductive cleavage of insulin.

Summary. The insulinase activities of rat liver and kidney were compared by determining the amounts of insulin-^{131}I degraded by homogenates and subcellular fractions of these organs. The insulinase activity of rat kidney was greater than that of rat liver when

compared on the basis of gram wet tissue. "Mitochondria-free" fractions of rat liver had 67% of the insulinase activity of the "nucleus + debris-free" fractions; whereas, "mitochondria-free" fractions of rat kidney had 28%.

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Effect of Growth Hormone and Glucose on ^{14}C Incorporation by the Perfused Dog Pancreas* (33045)

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The feasibility of using the isolated, recirculated perfusion of the dog pancreas to study the biosynthesis of insulin *in vitro* has been demonstrated (1). Recent reports have indicated that pituitary growth hormone (GH) is necessary for or augments the release of immunoreactive insulin (IRI) (2–6), and stimulates the incorporation of tritiated leucine into insulin in rat pancreas slices (7). These observations suggested that an effect of GH on the incorporation of ^{14}C labeled amino acids into insulin might be demonstrated in the perfused dog pancreas.

Methods. Dog pancreata were perfused with a medium containing ^{14}C labeled amino acids as described previously (1). One series of perfusions was carried out at low glucose levels (approximately 50 mg/100 ml) with the *in vitro* addition of one or three units of porcine GH.[2] Another series was carried out at glucose levels of 250–350 mg/100 ml with or without the *in vitro* addition of GH. In addition, two dogs were pretreated with GH at a dosage of 3 mg/day given in equal subcutaneous injections morning and evening for 5 days prior to taking the pancreas. The last GH injection was given approximately 18 hours before the pancreas was removed. These pancreata were perfused at the high glucose level with the same amount of amino acids-^{14}C as those described above (1).

All perfusions were terminated at the end of 2 hours. Each pancreas was extracted for insulin as previously described (1) and the immunoreactive insulin (IRI) determined by method C of Hales and Randle (8)[3] and the ^{14}C content of the insulin containing fraction was determined. As described in the earlier report (1), the major portion of the IRI eluted in peak 2 from the Sephadex G-50 column, and since this peak contained the major portion of the radioactivity, it was assumed that the incorporation of ^{14}C into this fraction represented the biosynthesis of insulin.

Results. The results of the extractions of the perfused pancreata are shown in Fig. 1. As previously reported, the pancreas extract typically eluted from the Sephadex columns in 3 peaks. All three peaks from each fractionation were assayed for IRI and the ^{14}C content was determined. It may be seen in Fig. 1 that virtually all of the IRI and with one exception the major portion of the ^{14}C

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[2] Porcine GH was purchased from General Biochemicals, Chagrin Falls, Ohio 44022.

[3] The guinea pig anti-insulin serum and the crystalline porcine insulin used as standards were generously supplied by Dr. Mary Root of the Eli Lilly Research Laboratories.