# DISTRIBUTION AND DEGRADATION STUDIES WITH INSULIN-$I^{131}$ †

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Diabetes mellitus is of obscure etiology, often presenting profound metabolic derangement with a sparsity of morphopathologic change. Insulin reverses many of the metabolic changes, yet its mechanism of action is poorly understood; furthermore, in most patients with diabetes mellitus, the fundamental causes of the insulin insufficiency are not known. An important approach to this challenging problem is the study of insulin metabolism. Such a study has been hampered by unsatisfactory assay techniques, and, as a result, very little is known even of the fate of insulin. The use of labeled hormone greatly facilitates such a study, and, in this report, the validity of such an approach is considered, and distribution and degradation studies using $I^{131}$-labeled insulin are described.

## METHODS AND MATERIALS

### *Insulin-$I^{131}$*

Crystalline zinc insulin [3] was labeled with $I^{131}$, either by the method of Ferrebee, Johnson, Mithoefer, and Gardella (1), or by a method of the Abbott laboratories. Specific activity ranged from 4 to 1008 $\mu$c per mg. The radioactivity of the insulin-$I^{131}$ stayed with the protein fraction in trichloracetic acid precipitation, in dialysis, and in chromatography.[4] It had the same mobility as unlabeled insulin in paper electrophoresis (2).

The potencies of three different lots of insulin-$I^{131}$, two prepared by the Ferrebee technique, and one by the Abbott method, were tested in the acceleration of the glucose uptake of rat diaphragm *in vitro*. Each determination measured the glucose uptake of eight quarter diaphragms as described by Groen, Kamminga, Willebrands, and Blickman (3). Samples and controls were run in duplicate. One dose level in the sensitive part of the dose response curve was used. In all instances the insulin-$I^{131}$ gave the predicted response, and no loss of activity was demonstrated.

In a control experiment, a sample of insulin, 27 units per mg., was treated with $I^{127}$ instead of $I^{131}$. In all other respects, the insulin was iodinated exactly as in the Abbott insulin-$I^{131}$ method. An *in vivo* bioassay was performed on this sample by the Lilly Research Laboratories. A standard mouse convulsion test was used initially to determine the potency of the insulin. Three assays were run, using 72 mice each time. The mouse assay was then checked on rabbits using a crossover technique. No definite loss of activity was demonstrated.[5] Further bioassays, using radioactive material, are planned.

### *Experimental animals*

Unless otherwise noted, all animal experiments were performed as follows. Male Sprague-Dawley rats were used. They were kept at 80° F., and fed a standard diet of Purina Fox Chow with vitamin supplements. To ensure standard conditions of carbohydrate metabolism, food and water were replaced by 10 per cent dextrose in 0.9 per cent saline (10 per cent D/S) 17 hours before each experiment. This solution was removed 2 hours before the experiment, and the animals fasted thereafter. The isotopic preparation (10 $\mu$c or less), was injected into the tail vein in 0.5 ml. of 0.9 per cent saline over a period of 15 seconds, with the animal under ether anesthesia. The doses were constant in any one experiment, and in all rat experiments were within the range, 0.2 to 0.8 units. Fifteen minutes later, again under anesthesia, the rat was exsanguinated by puncture of the dorsal aorta, the tissues were removed, aliquots weighed on a Roller-Smith balance, and their radioactivity determined.

### *Measurement of radioactivity*

Since the radioactivity of insulin-$I^{131}$ itself was at least 95 per cent precipitable with trichloracetic acid (TCA), it was assumed that non-precipitable radioactivity repre-

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[3] This preparation was kindly supplied by Eli Lilly and Co. It contained 26 or 27 units per mg., and only minimal amounts of hyperglycemic factor.

[4] Ascending paper partition chromatography was performed using the butanol phase of a system composed of n-butanol: glacial acetic acid: water, (78: 5: 17).

[5] In a previous publication (4), this bioassay was reported as showing some reduction in the biological activity of the labeled insulin, but subsequent information demonstrated that this was an error due to a misunderstanding regarding the amount of starting material.

sented breakdown products of the insulin-$I^{131}$ molecule. Accordingly, the radioactivity in all tissue and liquid samples was divided into two fractions, the TCA-precipitable, and the TCA supernatant fractions. In this procedure, the tissues were promptly minced and homogenized in 10 per cent TCA, and blood stirred in 20 per cent TCA. The supernatant was poured off after centrifugation, and the precipitate was washed once with 10 per cent TCA and again centrifuged. The supernatant fractions were pooled and diluted to 30 ml. with water. A second wash was found to remove less than 3 per cent of the radioactivity remaining in the precipitated fraction, and was therefore not used. The precipitated fraction, hereinafter noted as insulin *, was made up to 30 ml. with 30 per cent potassium hydroxide. Each specimen was then assayed for radioactivity in a well-type gamma counter.

The concentration of radioactivity in each tissue and fluid was calculated as the per cent of the dose per gram or ml. This figure was multiplied by the $\frac{\text{body weight}}{100}$, a dilution factor, to allow comparison of data on animals of different size, and, in short time intervals before appreciable excretion had occurred, to afford comparison of the concentration in a specific tissue or fluid with that in the body as a whole. This ratio was referred to as $\frac{[T]}{[B]}$. Any value above 1 indicated that the isotope was more concentrated in the respective specimen than it was in the body as a whole.

Tissue for radioautography was obtained from an adult male rat 15 minutes after the intravenous injection of 200 $\mu$c of insulin-$I^{131}$. The tissue was fixed in Bouin's solution, imbedded in paraffin, and 10 $\mu$c sections were floated on Kodak lantern slide medium plates for 24-hour exposure. They were then developed in the conventional manner, and stained with hematoxylin and eosin.

*Distribution in humans*

Insulin-$I^{131}$ was administered to agonal patients in doses of 2.9 to 4.2 units, containing 30 to 40 $\mu$c of radioactivity. It was injected intravenously within one minute, and glucose solution was infused constantly thereafter. Aliquots of tissue were obtained at autopsy, weighed, and their total radioactivity determined in the well-type gamma counter. The results were expressed as per cent dose per gram $\times 10^{-5}$, without correction for body weight.

## EXPERIMENTAL PROCEDURES AND RESULTS

### *Distribution in rats*

The concentrations of radioactivity in rat tissues 15 minutes after intravenous insulin-$I^{131}$ are shown in Figure 1. Each bar represents the average concentration in that tissue from a group of at least 5 rats, weighing between 180 and 264 Gm. The gastrocnemius was used as a representative muscle, and tibia as a representative bone. Fat

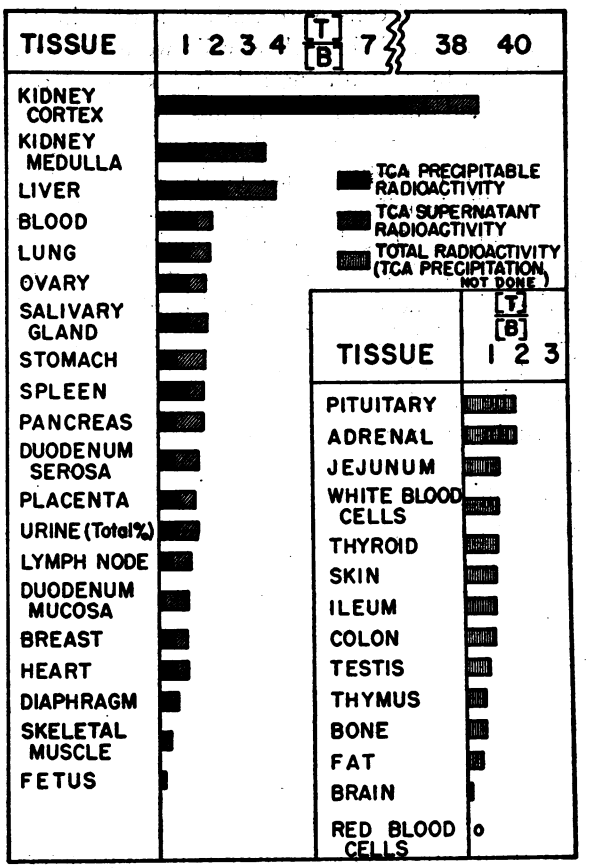


FIG. 1. CONCENTRATION OF RADIOACTIVITY IN RAT TISSUES 15 MINUTES AFTER I.V. INSULIN-$I^{131}$

Concentration, $\frac{[T]}{[B]}$, was calculated by multiplying the per cent of the dose of $I^{131}$ per gram of tissue, by the $\frac{\text{body weight}}{100}$, so that values greater than 1 represent concentrations of radioactivity greater than that in the body as a whole. Urine was not weighed, so is recorded as per cent of the dose.

The two components of the total radioactivity, the trichloracetic acid (TCA) precipitate and the TCA supernatant, were determined for most tissues. Insulin-$I^{131}$ is TCA precipitable, and supernatant radioactivity therefore consisted of breakdown products of the insulin-$I^{131}$ molecule. Total radioactivity only, was determined on the tissues in the insert.

Note that the renal cortical concentration was so high that the scale had to be interrupted. The ratio of TCA precipitate to supernatant is accurately represented however.

was obtained from the perirenal area. Urine was collected in one group of rats 15 minutes after the administration of insulin-$I^{131}$ by tying off the urethra and washing out the bladder with saline.

The red blood cells were washed 4 times in 0.9 per cent saline. White blood cells were obtained from blood by centrifuging a 1 : 5, dextran (1 per cent) : blood dilution for 5 minutes, and aspirating the material over the red cell layer. The aspirate was diluted 1 : 4 with Hanks' buffer and the white blood cells centrifuged down, removed, weighed, and counted. The blood was originally drawn from the rat 10 minutes after the insulin-$I^{131}$ injection, since the white cells could not be removed for at least 5 minutes thereafter, and it was desired to have a measure of concentration comparable to the 15 minute study in the other tissues.

Insulin-$I^{131}$ concentration in thyroid was determined on a group of rats which received 100 mg. of propylthiouracil by stomach tube 4 hours before, and 100 mg. of potassium thiocyanate subcutaneously 1 hour before the experiment. This procedure was used to prevent accumulation in the thyroid of radioactive iodide released from insulin (2, 4).

The contents of the gastro-intestinal tract were removed before the radioactivity of this organ was determined.

The ovaries were from non-pregnant, 193 Gm. female rats. Data on breast, placenta, and fetus were from a group of 4 pregnant female rats, averaging 310 Gm. in weight.

This distribution study showed (Figure 1) a marked concentration of radioactivity in the renal cortex, over 9 times greater than that in any other tissue. This finding was further investigated in studies reported in a later section of this paper.

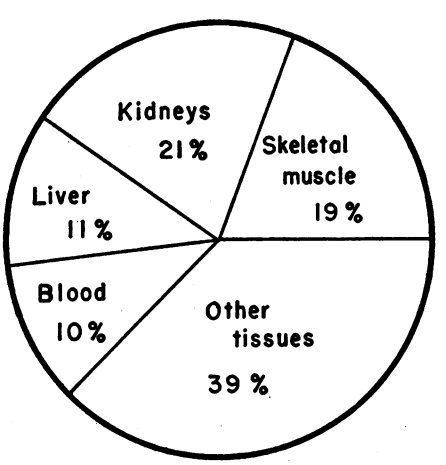


FIG. 2. TOTAL RADIOACTIVITY IN ENTIRE RAT TISSUES 15 MINUTES AFTER I.V. INSULIN-$I^{131}$

Liver had approximately twice the concentration of the blood. The concentration in the brain was very low and little was in the urine. Concentrations were not marked in the gastro-intestinal tract.[6] Radioactivity was found in white blood cells, but not in red blood cells. A higher concentration was found in ovary than in testis. The main tissues with concentrations less than the body as a whole, were bone, fat, and muscle. Heart and diaphragm had greater concentrations than skeletal muscle, the latter being relatively low. When total muscle mass was considered however,[7] and the total radioactivity in liver, kidney, muscle, and blood was plotted, as in Figure 2, muscle was seen to be a major site of localization.

*Time studies*

In the tissues of principal interest—liver, kidney, muscle, and blood—the concentration of radioactivity was determined at varying times after insulin-$I^{131}$ injection. Three or more rats were used in each time period. Their average weight was 195 Gm. Those sacrificed at 2, 5, and 15 minutes were prepared in the usual manner. Those sacrificed at 1, 2, 4, and 6 hours received 10 per cent D/S for 17 hours up to the time of the insulin-$I^{131}$ injection, and then were fasted. The insulin-$I^{131}$ was given intravenously and the animals sacrificed after the appropriate time had elapsed. To ascertain the rate of breakdown of the labeled hormone, trichloracetic acid (TCA) precipitation was used, and the precipitable and supernatant tissue and blood radioactivity separated as described above. Supernatant radioactivity was considered to consist of breakdown products of insulin-$I^{131}$.

The time curves are shown in Figure 3. Insulin * (TCA precipitable radioactivity) concentration reached a peak in liver, kidney, and muscle, in from 5 to 15 minutes, and then fell rapidly, paralleling that in blood. After 1 hour, the fall in insulin * concentration was slight. Supernatant radioactivity appeared within the first few minutes after the insulin-$I^{131}$ injection and increased in concentration in the tissues and blood in the

[6] Preliminary observations (2, 4) of high concentrations of radioactivity in the stomach may have been due to the presence of free $I^{131}$ in early insulin-$I^{131}$ preparations.

[7] The blood volume of a 190 Gm. rat was estimated as 10 ml., and total muscle mass as 85 Gm.

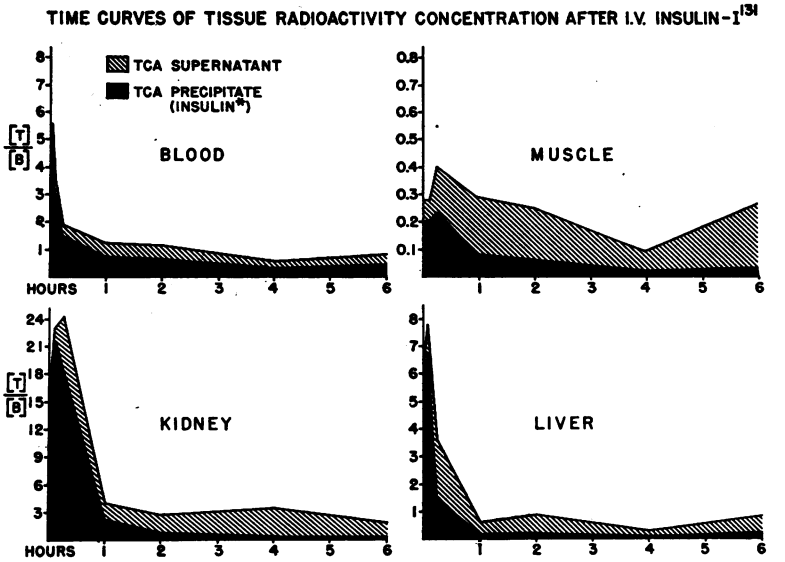


FIGURE 3

Note the rapid increase in concentration of supernatant radioactivity. Insulin-$I^{131}$ itself was essentially completely precipitable by trichloracetic acid (TCA). Supernatant radioactivity therefore represents insulin-$I^{131}$ degradation.

first 15 minutes. At later times, the concentrations of supernatant radioactivity varied without any consistent trend, and appreciable quantities of radioactivity were excreted in the urine in a non-precipitable form.

*Excretion studies*

Sixteen rats, averaging 211 Gm. in weight, were used. After the intravenous injection of insulin-$I^{131}$, from one to three rats were placed in each metabolic cage for the collection of urine and feces at various times. Two rats, in restraining cages, had total bile collections through polyethylene bile duct cannulae. For collection periods up to 6 hours in length the rats were given only 10 per cent D/S preceding, and nothing during, the experiment. When collections were continued for 24 or 48 hours, the rats were fed *ad libitum*. Feces were homogenized in the Waring blendor. All specimens were precipitated in 10 to 20 per cent TCA with dried human plasma added as carrier protein.

The results are shown in Figure 4. Each bar represents the average excretion of from two to four animals. The percentage excreted in the urine was many fold that in bile and stool. It is evident that the large part of the $I^{131}$ was not precipitated by TCA. At the end of 6 hours, only 0.44 per cent of the dose was present in the urine in a protein precipitable form; even less was in the bile. At 24 hours only 1.7 per cent could be precipitated from the urine, and 0.8 per cent from

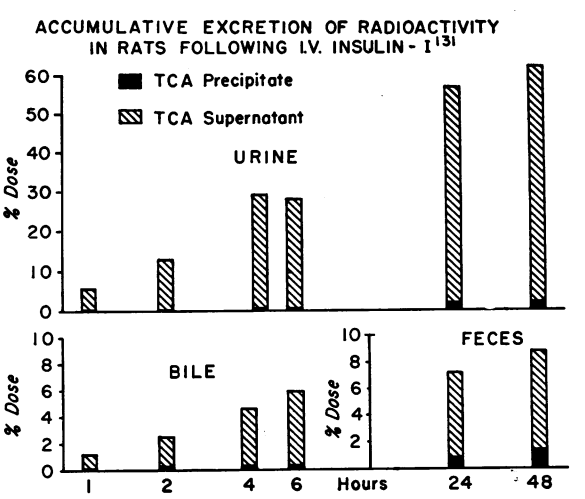


FIGURE 4

Bile and feces are plotted on the same scale, but urine is plotted on a smaller scale. Time is plotted logarithmically. Very little of the excreted radioactivity retained the property of insulin-$I^{131}$ of precipitation by TCA.

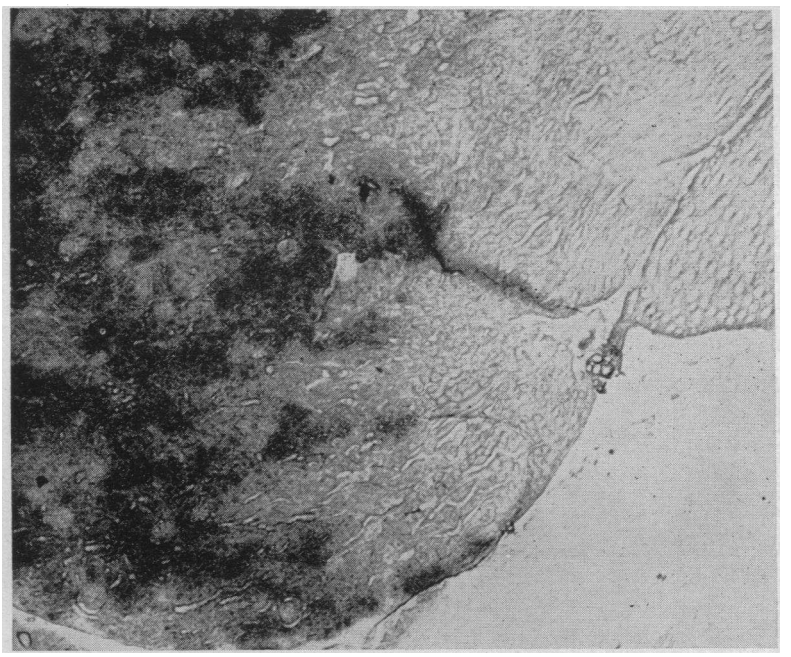

FIG. 5. RADIOAUTOGRAPH OF RAT KIDNEY AFTER I.V. INSULIN-$I^{131}$
Note the concentration of radioactivity in the proximal convoluted tubules.

the feces. The total radioactivity recovered in the urine and feces in 48 hours approximated 70 per cent of the administered dose.

*Renal radioactivity*

The high renal concentration of radioactivity with insulin-$I^{131}$ was investigated further. Radioautography (Figure 5) demonstrated that relatively little of the radioactivity was in the medulla and glomeruli, but that it was highly concentrated in the convoluted tubules, particularly the proximal tubules.

Perfusion of the kidney failed to remove appreciable quantities of radioactivity. In this experiment, six rats averaging 255 Gm. in weight were used. In three, the upper abdominal aorta and the right renal artery were tied off 15 minutes after the insulin-$I^{131}$ was given, and the left kidney, with its vein cut, was perfused with 20 ml. of normal saline, through the distal aorta. In the other three animals, the usual procedure was followed, no perfusion was done, but right and left kidneys were counted separately as a control for the previous experiment.

The data in Table I show that practically none (5.1 per cent) of the insulin * was removed from the kidney by perfusion. However, 38 per cent of the non-precipitable material was removed. The over-all reduction of radioactivity, then, was of the order of 13 per cent.

*Comparison of distribution of insulin-$I^{131}$ with $I^{131}$-labeled human serum albumin and $NaI^{131}$*

The possibilities that the distribution might have been related to (a) the $I^{131}$ label, or to (b) the fact that the insulin-$I^{131}$ was a foreign protein, were investigated by studying the distribution of (a) $NaI^{131}$ alone, and (b) $I^{131}$-labeled human serum albumin (HSA-$I^{131}$). Eleven rats, averaging 300 Gm. in weight, were treated in the same manner as that used to inject insulin-$I^{131}$. Four were given $NaI^{131}$ and seven received HSA-$I^{131}$, the latter in doses of 40 $\mu$g. The former were fasted, the latter not.

Rats were sacrificed at 5 and 15 minutes. In Figure 6, the data are plotted along with that of insulin-$I^{131}$. The renal concentration with insulin-$I^{131}$ was many fold that with either comparison substance. Hepatic concentration was also higher, although not as striking. Blood levels with $NaI^{131}$ and insulin-$I^{131}$ were comparable, but with HSA-$I^{131}$, were very much higher.

TABLE I

*Renal perfusion study 15 minutes after i.v. insulin-$I^{131}$*

(a) Comparison of radioactivity in right and left kidneys
(Neither kidney was perfused)

| Animal No. | TCA precipitate | | | TCA supernatant | | | Total radioactivity | | |
|---|---|---|---|---|---|---|---|---|---|
| | CPM* right kidney | CPM left kidney | Per cent difference | CPM right kidney | CPM left kidney | Per cent difference | CPM right kidney | CPM left kidney | Per cent difference |
| 1 | 18,610 | 19,502 | 4.7 | 4,451 | 4,451 | 0 | 23,061 | 23,953 | 3.7 |
| 2 | 17,088 | 16,732 | 2.1 | 3,959 | 3,942 | 0.5 | 21,047 | 20,674 | 1.8 |
| 3 | 17,002 | 16,200 | 4.7 | 5,192 | 5,192 | 0 | 22,194 | 21,392 | 3.6 |

(b) Left kidney perfused; right control

| Animal No. | TCA precipitate | | | | TCA supernatant | | | | Total radioactivity | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CPM right kidney | CPM left kidney | per cent difference | Average per cent diff. | CPM right kidney | CPM left kidney | per cent difference | Average per cent diff. | CPM right kidney | CPM left kidney | per cent difference | Average per cent diff. |
| 4 | 20,088 | 18,350 | 8.7 | | 5,829 | 3,311 | 43 | | 25,917 | 21,661 | 16.4 | |
| 5 | 13,062 | 12,440 | 4.8 | 5.1 | 4,119 | 2,532 | 39 | 38.0 | 17,181 | 14,972 | 12.8 | 13.3 |
| 6 | 12,326 | 12,108 | 1.8 | | 4,836 | 3,231 | 33 | | 17,162 | 15,339 | 10.6 | |

* CPM = Counts per minute.

*Distribution in humans*

The data are plotted in Figure 7. In four instances the patients were terminal and the interval from insulin-$I^{131}$ injection to death is recorded, although the tissues were not actually obtained until autopsy some hours later. Tissues were obtained at operation in one case: insulin-$I^{131}$ was given intravenously during a gastrectomy and biopsy specimens for radioactivity were taken 15 minutes later. The distribution pattern in the human showed many resemblances to that in the rat, and data from the rat are included for com-

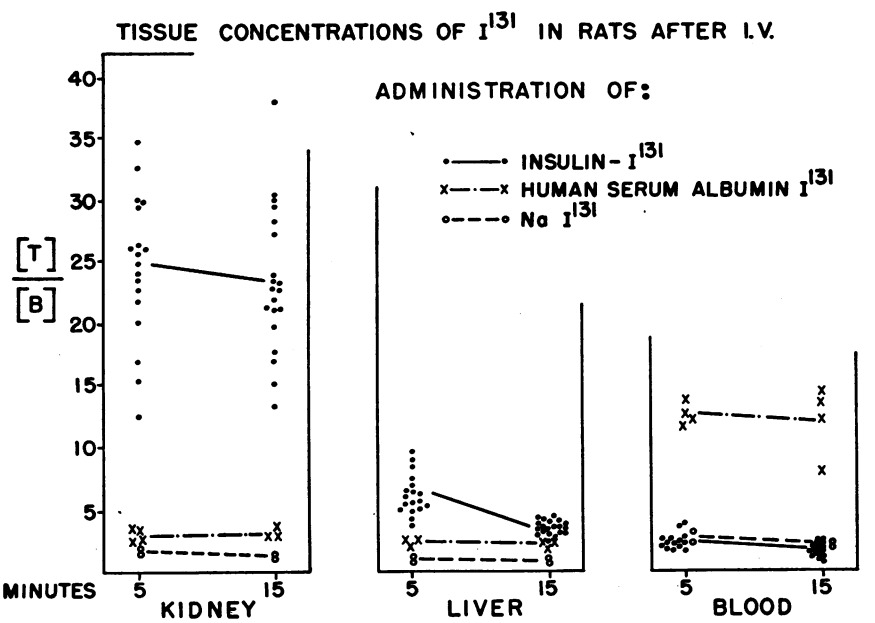


FIGURE 6

Each point represents the total radioactivity in the tissue of one rat. Note that insulin-$I^{131}$ is concentrated markedly in the kidney and to some extent in the liver.

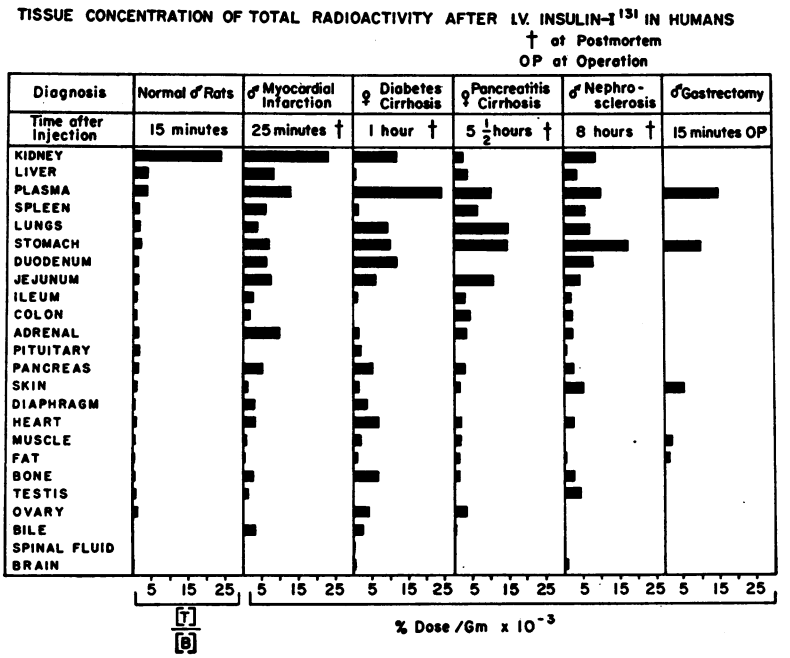


FIGURE 7

Data on rats are included for comparison.

parison. The resemblance was most apparent in the tissues of a man whose demise of a myocardial infarction occurred 25 minutes after the insulin-$I^{131}$ was given. The high concentration in kidney was striking. The gastro-intestinal tract in all instances seemed to have a higher proportion than seen in the rat, but of course, the interval permitted for distribution and breakdown was longer. Muscle, in general, had a low concentration. The liver contained considerable activity except in the patients with cirrhosis, in one of whom it was conspicuously low. Of special note was the low concentration in brain, cerebrospinal fluid, and bile.

## DISCUSSION

Iodine is not a normal constituent of insulin. In being labeled, the insulin binds iodide (one atom in 500 of which is radioactive) presumably on the tyrosine and/or histidine groups. Strictly, then, insulin-$I^{131}$ is an insulin derivative, and the data must be treated with this reservation in mind. However, such would be the case with any radioactive preparation since, of course, insulin normally does not contain radioactivity. Although heavy iodination has been demonstrated to inactivate insulin (5), in our experiments there was at the most one iodine atom for each insulin molecule.[8] Bioassay has not shown that this relatively light iodination effected any impairment of physiological activity.

The $I^{131}$ stayed with the protein fraction during chromatographic, electrophoretic, and TCA precipitation experiments, so that in certain physical, as well as physiological properties, insulin-$I^{131}$ behaved as insulin. In tissue, however, there was a rapid increase in the amount of radioactivity in the non-TCA-precipitable fraction. Presumably, as the insulin-$I^{131}$ molecule was broken down, some of the $I^{131}$ was associated with peptide fragments, or amino acids, or was liberated as iodide, and thereby was no longer precipitated by TCA. Accordingly, the relatively short time interval of 15 minutes was used in many of the distribution studies in an effort to allow time for adequate mixing

[8] This figure represents an approximation derived from the data of Ferrebee, Johnson, Mithoefer and Gardella (1). They calculated "two iodine molecules per insulin molecule" (molecular weight 40,000). Actual iodine assays of three of our Abbott preparations have shown one iodine atom per 20 to 40 insulin molecules (molecular weight 20,000).

and yet minimize the overlay of redistribution of labeled breakdown products.

Despite the above evidence that insulin-$I^{131}$ behaved as natural insulin, the premise that the distribution of radioactivity represented non-radioactive insulin or its breakdown products, can only be inferred, pending further biological and biochemical investigation. Present evidence supports this premise however: (a) The distribution of radioactivity was quite different from that following the injection of the label, $NaI^{131}$, alone. (b) The distribution was unlike that of $I^{131}$-labeled human serum albumin. This argues against speculation that the distribution represented simply that of any iodinated foreign protein. (c) It is pertinent to compare our observations of the distribution of $I^{131}$-labeled insulin with similar studies of another low molecular weight protein hormone, ACTH, labeled with $I^{131}$. With at least three such preparations, Sonenberg (6) has shown notably high concentrations of radioactivity in rat kidney. The physiological counterpart of this is striking in the work of Richards and Sayers (7), in which, giving rat ACTH to rats, and measuring the adrenal ascorbic acid depleting factor extractable from tissues, they found that the kidney had the highest concentration (15 per cent in 15 minutes) of any tissue. This shows a similarity of label and bioassay data in the case of ACTH. As regards bioassay data for insulin, there have been many reports of the extraction of insulin activity from organs other than the pancreas. Best, Jephcott, and Scott (8), however, were unable to duplicate the results and the search has apparently not been pursued. There is, then, no report of a physiological counterpart to the present study, and its need becomes apparent. The ACTH data, nevertheless, tend to support the concept that the label on these hormones does not affect their specificity. It is of interest that radioautographs of renal ACTH-$I^{131}$ (9) closely resemble those of insulin-$I^{131}$.

There are a number of similarities of tissue distribution between that of insulin-$I^{131}$ and that of other protein hormones. This is striking in the case of $I^{131}$ labeled prolactin as reported by Sonenberg, Money, Keston, Fitzgerald, and Godwin (10), in which the concentrations at 15 minutes in the rat kidney, liver, muscle, plasma and other tissues are almost identical to the findings with insulin-$I^{131}$. This suggests the possibility of common metabolic pathways apart from the individually specific pathways in target gland sites.

The rapid loss of insulin * from the blood and from all tissues, with the appearance of non-TCA-precipitable $I^{131}$ in rapidly increasing amounts, undoubtedly represent degradation of the insulin-$I^{131}$ molecule. It may be that the rapid breakdown of insulin-$I^{131}$ reflects the transient biological activity of insulin seen clinically. This breakdown may also explain the lack of insulin activity found in the bioassay of extrapancreatic tissues, as noted above. Measurement of the rate of breakdown of insulin-$I^{131}$ offers a valuable tool in the study of insulin metabolism; identification of the breakdown products may enable the degradation and metabolism to be further characterized.

The principal sites of action of insulin have classically included muscle and liver (11). Stadie, Haugaard, and Vaughan have shown insulin "binding" by these tissues, measuring, in the case of muscle, both physiological activity and uptake of labeled insulin (12). This is in accord with the demonstration of our studies that large amounts of injected insulin * go to these tissues (Figure 2). The lack of concentration in red blood cells is in harmony with the lack of effect of insulin on the glycolytic system of blood (13). The absence of appreciable insulin * from the brain is compatible with present evidence that the brain does not require insulin (14). A very small fraction, 1.7 per cent, of the insulin * injected, appeared in the urine in 24 hours. Similarly, bioassay for injected insulin has shown that only about 0.1 per cent of a dose came out in the urine in 24 hours, in man (15).

A ready correlation of the high concentration of renal tubular insulin * to renal physiology is not apparent. An insulinase system in liver, muscle, and kidney has been described by Mirsky and Broh-Kahn (16). The analogy of the renal insulin-$I^{131}$ to renal ACTH-$I^{131}$ has been noted above. It may be pertinent that ACTH is inactivated by kidney slices and homogenates (17).

The data on distribution in humans were obtained in terminal states, and were, in most instances, obtained hours after the insulin-$I^{131}$ was given. Accordingly, there was an overlay of re-

distribution of breakdown products. Nevertheless, the general pattern seems to resemble that in the rat.

SUMMARY

1. Studies using $I^{131}$-labeled insulin are reported and discussed.

2. Distribution studies in rats showed concentrations of radioactivity to be high in kidney and liver, low in red blood cells and brain, and intermediate in other tissues, including muscle. The distribution pattern was different from that of $NaI^{131}$ and $I^{131}$-labeled human serum albumin.

3. Perfusion and radioautographic studies showed that the renal radioactivity was bound in the convoluted tubules.

4. The concentrations of total and protein precipitable radioactivity in rat blood and tissues were maximal within 15 minutes after intravenous insulin-$I^{131}$ injection, and thereafter fell, in general at rapid rates. Non-protein-precipitable radioactivity, initially negligible, rapidly increased, as the insulin-$I^{131}$ was broken down. Fifty per cent of the $I^{131}$ was in the urine in 24 hours, but only 1.7 per cent was protein precipitable.

5. Distribution studies in the human showed many similarities to those in the rat.

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