Specificity of Insulin Degradation Reaction

Neil J. Elgee, M.D., and Robert H. Williams, M.D.,† Seattle*

In a previous publication,[1] inferential evidence was presented indicating that the distribution of the radioactivity of injected insulin labeled with radioactive iodine (I^{131}) is probably representative of the distribution of unlabeled insulin. In the present report, further evidence of the specificity of insulin-I^{131} is presented in the demonstration that the degradation of a trace amount of insulin-I^{131} is competitively lessened by a load of either labeled or nonlabeled insulin, and not by other proteins of similar size. The distribution and degradation of ribonuclease, a nonhormonal protein of comparable molecular weight to insulin and labeled with I^{131}, is, however, shown to be very similar to that of insulin-I^{131}.

METHODS

The basic technics have been described previously.[1] Sprague-Dawley rats were prepared for use by replacing their food and water with 10 per cent dextrose in 0.9 per cent saline the night before use and withdrawing this one to two hours before the experiment. The labeled protein (in various preparations to be described below) was dissolved in 0.067 *M.* phosphate buffer,‡ *p*H 7.5, and injected into the tail vein. At a given time thereafter, the animal was sacrificed and the liver, kidney, muscle, and blood were removed. In some instances plasma was separated. Since the radioactivity of the I^{131}-labeled proteins was essentially completely precipitable with trichloracetic acid (TCA), degradation of the labeled protein was estimated by dividing the radioactivity of the tissues into a supernatant and a precipitate fraction by means of TCA precipitation and washing, as described previously.[1] The appearance of nonprecipitable radioactivity was taken to represent degradation. In some instances the concentration of radioactivity was expressed as [T]/[B], a value calculated by multiplying the tissue fraction radioactivity concentration, (the percentage of the total I^{131} dose, per gram of tissue) by the body weight/100, so that values greater than 1 represented concentrations of radioactivity greater than that in the body as a whole.

EXPERIMENTS AND RESULTS

1. Influence of an Insulin Load on the Distribution and Degradation of Insulin-I^{131}

One group of rats received 0.4 ml. (200 units) of concentrated crystalline insulin intravenously immediately before 0.5 ml. (0.5 units) of insulin-I^{131}* was similarly administered. A control group received insulin-I^{131} only. Tissue radioactivity was fractionated and assayed fifteen minutes later, as described in methods above.

The results are shown in figure 1. Relative distribution of total radioactivity in the various organs was not greatly altered. Precipitable radioactivity was increased in liver and blood and decreased in kidney in the loaded animals. The outstanding finding was a significant reduction in the supernatant fraction of all tissues in the animals which received a load of nonlabeled insulin. The apparent interpretation of this finding is that the degradation of insulin-I^{131} was competitively depressed by nonlabeled insulin.

2. Depression of Insulin-I^{131} Degradation by Labeled and Nonlabeled Insulin

Even though the above animals showed little adverse reaction during the short time interval they were permitted to live, the insulin load used was relatively enormous, and a second experiment, using smaller but increasing increments in dosage, was performed. Since it was felt to be probable that the degradation of the insulin-I^{131} substrate was diminished because of competition for the degradation system by the unlabeled insulin, the effects of adding increasing amounts of unlabeled insulin to a trace amount of labeled insulin were compared with the effects on degradation found when

From the Department of Medicine, University of Washington, Seattle.

Supported in part by grants-in-aid from the United States Public Health Service, the Atomic Energy Commission, Eli Lilly and Company, and Initiative #171 fund of the State of Washington.

*Public Health Service Research Fellow of the National Heart Institute.

†Professor and Executive Officer, Department of Medicine, School of Medicine, University of Washington, Seattle 5, Wash.

‡This is referred to simply as buffer in the remainder of the paper.

*Crystalline insulin was kindly supplied by Eli Lilly and Company. It was iodinated with I^{131} by the Abbott Laboratories at Oak Ridge. The properties of insulin-I^{131} have been presented previously.[1]

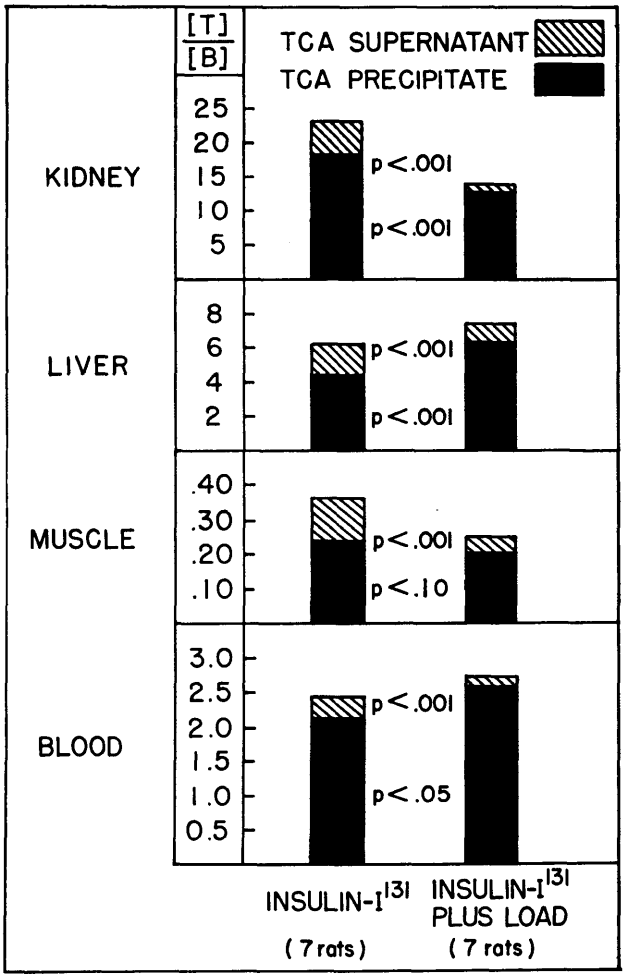

FIG. 1. Comparison of the concentrations, [T]/[B], of radioactivity in tissue fractions fifteen minutes after intravenous insulin-I^{131} was given to control rats and to rats which, immediately before the insulin-I^{131}, were given a load of 200 units of nonlabeled insulin intravenously. Tissue radioactivity was divided into a supernatant and a precipitate fraction by means of trichloracetic acid (TCA) precipitation. The figures between the columns are p values of the differences between the fractions in the two adjacent groups.

increasing loads of insulin-I^{131} alone were used. The experiment was therefore designed using a technic comparable to that of substrate competition, chiefly to see if the degradation system distinguished between labeled and unlabeled insulin in vivo.

Eleven female rats, weighing between 206 and 242 gm., were given doses of insulin-I^{131} ranging from 2 to 222 *ug*. Twelve female rats, weighing between 195 and 240 gm., were given 2 *ug*. of insulin-I^{131} in solution with doses of nonlabeled insulin varying from 1 to 122 *ug*. so that the total dose varied from 3 to 124 *ug*. of insulin. The nonlabeled insulin used was the same lot of crystalline insulin,* 27 units per mg., from which the insulin-I^{131} was prepared. Each rat received a particular dose of either insulin-I^{131} or a particular combination of insulin-I^{131} with nonlabeled insulin, each dose being dissolved in 1 ml. of buffer. The animals were sacrificed fifteen minutes later. One aliquot of blood was immediately precipitated in TCA. Plasma was similarly precipitated after it was separated from a second aliquot of blood.

The percentage of the total radioactivity per milliliter of blood or plasma that was in the TCA supernatant fraction is plotted against the total dose of insulin (labeled and nonlabeled) injected, in figures 2 and 3, respectively.

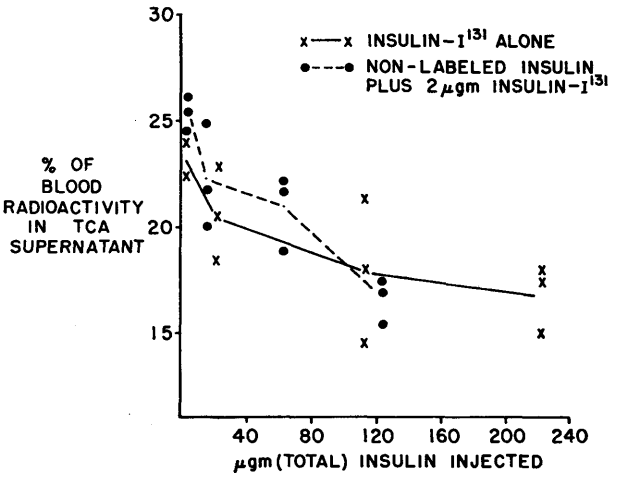

FIG. 2. Rats were injected intravenously with varying doses of insulin-I^{131}. Twelve received 2 ug. of insulin-I^{131} and varying doses of nonlabeled insulin in addition. All rats were sacrificed fifteen minutes later, and an aliquot of blood was precipitated with trichloracetic acid (TCA). The percentage of the blood radioactivity that was in the TCA supernatant is plotted against the total ug. of insulin (labeled and nonlabeled) injected. Each point represents one rat, and the lines indicate the mean.

As the dose was increased the percentage of radioactivity in the supernatant fraction was seen to fall. This was seen to be true, and to the same extent, whether the dose increase was made up of labeled or nonlabeled insulin. Either substrate was able to "load" the system and depress degradation, and the presence of an I^{131} label appeared to make no difference.

3. Effects of Other Proteins on the Degradation of Insulin-I^{131}

Proteins of molecular weights similar to insulin were

*Kindly supplied by Eli Lilly and Company.

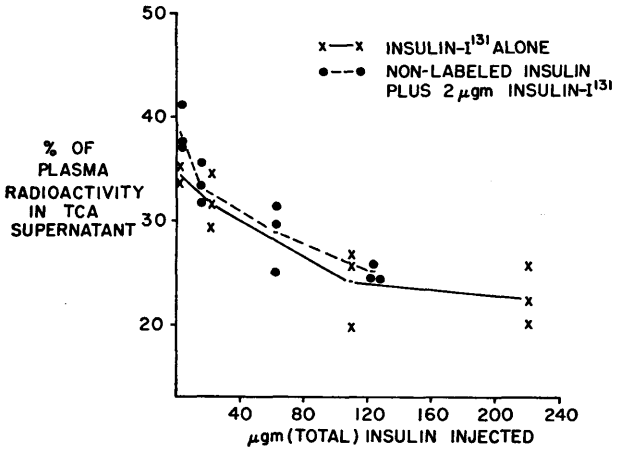

FIG. 3. Data obtained in similar fashion to that of figure 2 except that plasma was used instead of blood. Note that the curves are essentially superimposable — the effect of "load" on degradation was the same whether labeled or nonlabeled insulin was used.

tested to see if they competed in or inhibited the degradation of insulin-I^{131}. Using a technic similar to that in the preceding experiment, insulin-I^{131} was injected into rats in combinations with these other proteins, and blood radioactivity was determined fifteen minutes later. The addition of 150 *ug.* of corticotropin,* 150 *ug.* of ribonuclease, or 200 *ug.* of *a* lactalbumin to 4 *ug.* of insulin-I^{131} was found to have no effect on the percentage of radioactivity that appeared in the supernatant fraction of blood and plasma, it being the same as when the 4 *ug.* of insulin-I^{131} was injected alone. As noted before, however, the addition of 150 *ug.* of insulin to the insulin-I^{131} significantly diminished the percentage of radioactivity that appeared in the supernatant.

4. Distribution and Degradation of Ribonuclease-I^{131}

Ribonuclease was obtained from Montgomery Biochemicals and labeled by the Abbott Laboratories in North Chicago using I^{131} in the technique by which they label insulin. After dialysis overnight against buffer its radioactivity was found to be 98.2 per cent precipitable by TCA. Approximately 0.2 mg., 10 *uc.*, of dialyzed ribonuclease-I^{131} dissolved in 0.5 ml. of buffer was then given intravenously to each of fifteen female rats, weighing between 152 and 193 gm. Five rats were sacrificed at each of three time periods after the injection — five, thirty, and sixty minutes. The concentrations of radioactivity in the various tissue fractions are plotted against time in figure 4.

Blood and muscle levels were virtually identical to those found previously with insulin-I^{131}. Renal concentrations were very high, in fact several fold higher than those with insulin-I^{131}, and hepatic concentrations were somewhat lower. The renal concentration was found to be high in cortex and low in medulla. In all tissues the supernatant fraction of radioactivity increased with time, while the precipitable fraction rapidly fell. Similar experiments were done in male rats with essentially the same findings.

*The corticotropin (HP Acthar) was kindly supplied by Armour Laboratories.

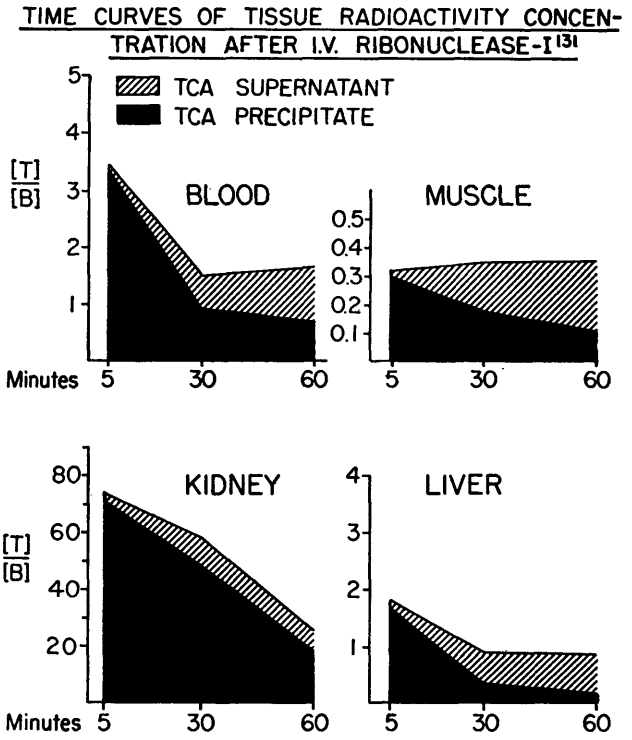

FIG. 4. The distribution of radioactivity in the TCA precipitate and supernatant fractions of tissues at various times after the intravenous injection of I^{131}-labeled ribonuclease into rats. The concentration of radioactivity is expressed as [T]/[B], the ratio of the concentration in a given tissue fraction to that initially in the body as a whole. A value of [T]/[B] greater than 1 indicates that the concentration of radioactivity exceeds that of the body as a whole.

DISCUSSION

The degradation of insulin-I^{131} by liver extracts in vitro, as measured by the loss of TCA precipitability, has been shown to proceed with essentially the same dynamics as the process of inactivation[2-4] of nonlabeled insulin, perhaps because these processes represent cause and effect. The dynamics have been found to be those of an enzyme system.[2-4] In experiment 1 of this report it is shown that excess crystalline insulin depresses the rate of degradation of insulin-I^{131} in vivo. In experiment 2 it is shown that this depression increases as the dose is increased, and

is the same whether labeled or nonlabeled insulin is used as excess substrate. These data are consistent with the view that the process of degradation in vivo is also enzymatic.

The substrate competition data demonstrate that the degradation system does not distinguish between I^{131}-labeled insulin and nonlabeled insulin. Further, since nonlabeled insulin can compete for or inhibit the degradation system it is most unlikely that the measure of degradation, that is, the appearance of radioactivity in the TCA supernatant, represents cleavage of free I^{131} from the protein molecule. This is consistent with the in vitro findings of others[2] in which nonprotein nitrogen appeared concurrently with supernatant radioactivity in insulin-I^{131} degradation.

The fact demonstrated in experiment 3 — that the degradation of insulin-I^{131} is not depressed by proteins other than insulin — is evidence of the specificity of this system, and adds weight to the view that measurement of insulin-I^{131} degradation provides information of value in the study of insulin metabolism.

That the type of distribution and degradation found with insulin-I^{131} is not unique, however, is shown by the ribonuclease data. Early in our work a very high renal concentration of radioactivity was noted in rat kidney shortly after the injection of insulin-I^{131}. Sonenberg[5] had found similar high renal concentrations of radioactivity after giving corticotropin-I^{131}, although more active preparations showed lesser concentrations. We found no comparable renal concentration with I^{131}-labeled human serum albumin,[1] and were interested in the possibility that the molecular weight of the protein might be a factor relating to the distribution pattern, the two hormones noted above both being quite small. Accordingly we have studied the distribution and degradation of an I^{131}-labeled, nonhormonal protein, of small molecular weight, ribonuclease-I^{131}.

In experiment 4, ribonuclease-I^{131} was found to have a distribution very similar to that of insulin-I^{131}. This is evidence that molecular size may have a bearing on the distribution pattern of these substances. Since ribonuclease is not a hormone, the similarity of its distribution to that of insulin-I^{131} and corticotropin-I^{131} makes it unlikely that the distributions of the latter two are related solely to their hormonal character. It is not surprising, then, that ribonuclease-I^{131}, like insulin-I^{131}, is rapidly degraded in the body. This suggests that degradation of insulin-I^{131} is probably not dependent on its hormonal character or action. It is, however, compatible with the previously expressed postulate that the degradation represents inactivation.[1]

SUMMARY

1. The degradation of insulin labeled with radioactive iodine (I^{131}) in vivo as measured by the appearance of trichloracetic acid supernatant radioactivity in tissues in rats, is depressed by excess nonlabeled insulin.

2. Degradation of insulin-I^{131} is depressed, and to the same extent, with increasing loads of either insulin-I^{131} or nonlabeled insulin.

3. Degradation of insulin-I^{131} is not depressed by the several proteins tested, other than insulin.

It is concluded that the degradation system has a certain degree of specificity, does not distinguish between labeled and nonlabeled insulin, and that, therefore, measurement of the degradation of insulin-I^{131} probably is representative of the degradation of nonlabeled insulin.

4. That the type of distribution and degradation pattern found with insulin-I^{131} is not a property solely of hormones, is shown in the demonstration that a very similar pattern is found with I^{131}-labeled ribonuclease.

REFERENCES

[1] Elgee, N. J., Williams, R. H., and Lee, N. D.: Distribution and degradation studies with insulin-I^{131}. J. Clin. Investigation *33*:1252-60, Sept. 1954.

[2] Tomizawa, H. H., Nutley, M. L., Narahara, H. T., and Williams, R. H.: Inactivation of insulin by rat liver extracts. J. Am. Chem. Soc. *76*:3357, June 1954. A detailed article is to be published in the J. Biol. Chem.

[3] Mirsky, I. A., and Broh-Kahn, R. H.: Inactivation of insulin by tissue extracts. 1. The distribution and properties of insulin inactivating extracts. Arch. Biochem. *20*:1-9, Jan. 1949.

[4] Mirsky, I. A., Perisutti, G., and Dixon, F. J.: Destruction of I^{131} labeled insulin by liver slices. Proc. Soc. Exper. Biol. & Med. *86*:228-30, June 1954.

[5] Sonenberg, M.: Studies with radioactively labeled anterior pituitary preparations. Ciba Foundation Colloquia on Endocrinology *4*:229-41, 1952.

SUMMARIO IN INTERLINGUA

Specificitate del Degradation de Insulina

1. In experimentos con rattos il esseva demonstrate que le injection de un excesso de nonetiquettate insulina deprime le degradation de dosages previemente administrate de insulina etiquettate con iodo radioactive (I^{131}) in tanto que ille degradation es mesurabile per le apparition de supernatante radioactivitate de acido trichloracetic in le texitos del animales.

2. Le degradation de insulina a I^{131} es deprimite al mesme grado per augmentate cargas de insulina o eti-

quettate con I^{131} o nonetiquettate.

3. Le degradation de insulina a I^{131} non es deprimite per le serie de altere proteinas examinate in iste reaction.

Nos conclude que le systema de degradation possede un certe grado de specificitate, que illo non distingue inter insulina etiquettate e nonetiquettate e que ergo le mesuration del degradation de insulina a I^{131} es probabilemente un mesuration indirecte del degradation de insulina nonetiquettate.

4. Le typo de distribution e degradation observate con insulina a I^{131} non es un proprietate exclusive del hormones. Isto es demonstrate per le facto que un simile typo de distribution e degradation es observate con ribonuclease etiquettate a I^{131}.

Classification of Insulin Reactions

It is suggested that the symptoms commonly seen in insulin reactions be divided into two main types, (1) "adrenalin-like" and (2) "central-nervous-system-type," regardless of whether or not both sets of symptoms may be produced by a single mechanism. This separation of symptoms is not only logical but also permits a clearer visualization of what is occurring in any particular reaction. Even more important is the increased ability to detect atypical reactions and the better appreciation of the danger of the apparently mild central nervous system reactions that have become so common.

"Adrenalin-like" Reaction: The symptoms of the "adrenalin-like" reactions are thought to result from an excess of circulating epinephrine. The entire mechanism by which the adrenal medulla is stimulated to secrete epinephrine following a fall in blood sugar remains unknown. In general, reactions of this type are associated with rapid falls in blood sugar, and for this reason they most commonly follow the injection of fast-acting insulin, sudden and excessive physical activity, or the missing of a meal combined with ordinary physical activity. Occasionally, mild symptoms of this type may be observed in persons with a rapid fall in blood sugar even though a hypoglycemic level is not reached. Since early experience with insulin was limited to the fast-acting regular insulin, almost all hypoglycemic states due to insulin reactions were associated with symptoms of the "adrenalin-like" type. For this reason there is little wonder that it was this group of symptoms that became synonymous with the term hypoglycemia, and it is equally not surprising that insulin reactions in which the "adrenalin-like" symptoms are minimal or absent are still overlooked or misinterpreted.

Central-Nervous-System-Type Reactions: The "central-nervous-system-type" reactions are the result of reduced activity of the central nervous system. The exact mechanics of this situation remain poorly understood; however, it would appear that if the nerve cells were deprived of their single source of energy for any considerable period of time serious dysfunction would be bound to result. Should this deprivation continue for a prolonged period, irreparable damage would be expected to occur. Such are the clinical facts. It is also not only possible but probable that during periods of low cerebral activity, as during sleep or relative inactivity, the requirements of the central nervous system for glucose are greatly reduced. For this reason a slowly developing hypoglycemia could remain obscure. The slowly developing hypoglycemia that is produced by the slow-acting insulins does not cause an increase in circulating epinephrine. The absence of the epinephrine explains the absence of the "adrenalin-like" symptoms as well as the failure of the blood sugar to rise and correct the hypoglycemic state. Finally, as a result of a continuing deficiency of blood glucose, there is inadequate cerebral function and the appearance of the "central-nervous-system-type" of insulin reaction.

Mixed Reactions: Frequently both the "adrenalin-like" and "central-nervous-system-type" symptoms occur in a single reaction, although one or the other generally predominates. A reaction of this type may be visualized as occurring in several ways. In spite of an increase in circulating epinephrine, the blood sugar may fail to rise because of inadequate glycogen stores or an overwhelmingly large amount of insulin. It is also easy to conceive of a slowly developing hypoglycemia with mild central nervous system symptoms which bring on an increase of physical activity, with a consequent rapid drop in the blood sugar. The rapid fall would then stimulate an increase in circulating epinephrine and the development of "adrenalin-like" symptoms.

From "Insulin Reactions" by Robert K. Maddock, M.D., and Leo P. Krall, M.D., in the A.M.A. *Archives of Internal Medicine,* June 1953.