Studies on Experimental Insulin Immunity

II. The Organ Distribution of Insulin in Immune Rabbits

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SUMMARY

The circulating half-life of injected insulin-I-125, and the uptake of this radioactive hormone by various tissues, have been compared in the normal and insulin-immune rabbit. In the normal animal the injected radioactive insulin is carried by albumin or an alpha-globulin, while in the immune animal it is carried in complex with antibody. The circulating half-life appears to have a fast and a slow component. In the normal rabbit the fast component was about 4.8 minutes and the slow component about forty-eight minutes, while in the immune animal the former was ten minutes and the latter about ninety minutes.

In the normal rabbit only about 17 per cent of the injected radioactive insulin remained as TCA-precipitable radioactivity (intact insulin) in blood and tissues one hour after injection, while in the immune animal about 75 per cent remained in this form at the end of one hour. It would appear, however, that some biologic effect was derived from the injection of radioactive insulin into immune animals, since some hypoglycemia occurred.

In both normal and immune animals most of the TCA-precipitable radioactivity one hour after injection of radioactive insulin was retained in serum, muscle, kidney and liver. However, the proportion of intact insulin was seventeen times greater in serum, 50 per cent greater in the heart, about 30 per cent greater in liver and muscle, about 16 per cent greater in lung and about the same in kidney of immune as compared with normal rabbits. DIABETES *16*: 402-08, June, 1967.

A previous report[1] has dealt with the physicochemical characteristics of free insulin antibody and of insulin-antibody complexes. Guinea pigs were used in these studies because they more often form precipitating as well as soluble antibody in complex with insulin, and therefore provide an opportunity for the study of both types of antibody. In the course of reporting these studies, the pertinent literature regarding insulin immunity in animals and in the human has been reviewed.

This initial investigation on induced insulin immunity provided information on the half-life of circulating insulin in normal and immune guinea pigs, and on the characteristics of the uncomplexed and precipitating antibodies as determined by various types of electrophoresis and Sephadex G-200 filtration; it also provided data permitting the estimation of the molecular weight equivalent of the immune complex. The purpose in obtaining such information relates to the suggestion that the development of the angiopathies of diabetes may be associated with the accumulation of insulin-antibody complexes in certain vascular tissue components.[2-4] The present study deals specifically with the organ distribution of insulin in rabbits, and a subsequent report will deal with the associated pathologic lesions. For the present studies rabbits have been used, since—like the human with whom comparisons should ultimately be made—they are less prone than the guinea pig to form precipitating antibody.

MATERIAL AND METHODS

Three 2.0 to 2.5-kg. male albino rabbits received a series of injections at three to four-week intervals of beef insulin and complete adjuvant prepared as previously described,[1] each consisting of 7.2 ml. given intramuscularly, 0.4 ml. into the foot pad, and 0.4 ml. given intradermally. Three rabbits of the same sex and weight range served as controls. Four to six weeks after the last immunization injections, the immunized and control rabbits were first fasted for twenty-four hours, but were provided with water containing a few drops of Lugol's solution to prevent the uptake of iodine-containing radioactive insulin by the thyroid; they then received 59 to 65 μc. per kilogram (equivalent to .30 to .49 U. per kilogram) of an insulin-I-125 solution* diluted with normal rabbit serum injected into a marginal ear vein. They were subsequently bled from the opposite ear vein at intervals up to twenty-four

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*Bovine insulin-I-125 (Specific activity 2.24 to 5.56 μc. per milligram) was purchased from Abbott Laboratories, Oak Ridge, Tennessee.

hours. Glucose concentration, radioactivity and specific activity of TCA-precipitable and TCA-nonprecipitable fractions were determined on each sample. One animal from each group was sacrificed by cardiac puncture one hour after the injection of insulin-I-125 and organs and tissues removed for study.

Differences between the two groups in three types of determinations were compared: (1) half-life ($T\frac{1}{2}$) of circulating insulin, (2) the characteristics of the insulin-binding proteins, and (3) radioactivity of a number of organs and tissues.

Determination of half-life

The intact injected radioactive insulin remaining at each time interval was calculated from the TCA-precipitable radioactivity. The concentration of insulin-I-125 in the circulation at zero time was extrapolated by a graphic technic.[5] The calculation and analysis were based on the initial concentration with blood volume estimated according to the method of Courpice.[6] Since the results gave nonlinear curves, the data were analyzed by the method of least squares and divided into two component systems, a fast and a slow component (lines B and C respectively, figures 1 and 2).

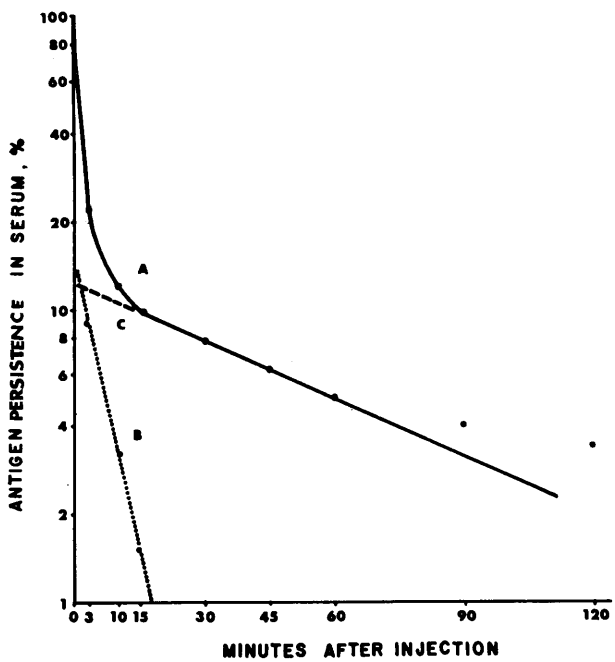

FIG. 1. Graphic analysis of curve of insulin persistence in the serum of a normal rabbit after the intravenous injection of radioactive insulin. Line A represents the original configuration of the fast component; lines B and C are on a logarithmic scale vs. time at which the sample was taken, according to the method of Courpice.[6] Line B represents the fast component and line C the slow component.

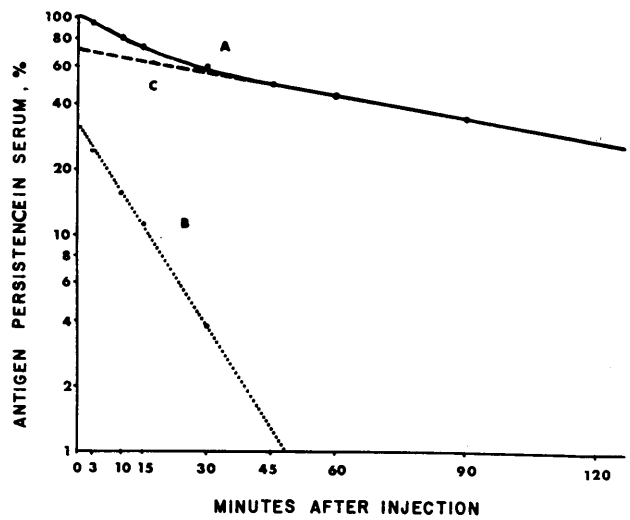

FIG. 2. Graphic analysis of the curve of insulin persistence in the serum of an insulin immune rabbit after the intravenous injection of radioactive insulin. The designation for the various lines are the same as for figure 1.

Characterization of serum antibodies

The sera from both groups obtained one hour after the injection of insulin-I-125 were fractionated with cold ethanol into three fractions.[7] The fractions were dissolved in veronal buffer (*p*H: 8.2, ionic strength: 0.05) at a concentration of 10 mg. of protein per milliliter. Radioactive insulin (1.4 μc., 0.008 U.) was added to 5-ml. aliquots of each fraction, the mixtures stored at 8° C. overnight, and then subjected to electrophoresis.[8] Agar electrophoretic separation without the addition of radioactive insulin was also carried out on selected samples; the electrophoretograms were stained with amido black, dried and the strips scanned with an automatic strip counter (Picker, model no. 2978). Column electrophoresis of these fractions was also carried out as previously described.[1,9]

Radioactivity of organs and tissues

After each animal was killed, total muscle mass of carcass, and several organs were immediately removed, weighed, and homogenized in cold 0.9 per cent NaCl. Aliquots of homogenates were precipitated in 10 per cent TCA solution and centrifuged at 2,000 rpm at 8° C. for twenty minutes. The supernates were saved and the precipitates washed twice in 5 per cent TCA. Radioactivity of an aliquot of pooled supernates and of each TCA precipitate was determined with a well-type gamma counter (Picker, model no. 2204); these were within a 5 per cent error and corrected with a standard insulin-I-125 solution. Total radioactivity of each organ was calculated and protein determinations

carried out.[10] Total radioactivity was also calculated.[6] Total volume of urine was collected from the bladder and radioactivity also determined.

RESULTS

Half-life of injected radioactive insulin

The half-life (fast component) of injected insulin-I-125 was 3.5 to 5.5 minutes in normal and 10 to 15.5 minutes in immune animals, while the slow component was 34.5 to 46.0 minutes in normal and 82 to 165 minutes in immune rabbits (table 1, figures 1 and 2). The data in table 2 indicate that most of the hypoglycemic effect of the injected insulin was exerted during the fast component. In normal rabbits hypoglycemia was marked and persisted for a longer time than in immune animals. In the latter, despite an abundance of circulating antibodies, there was also a significant hypoglycemic effect during the first thirty to forty-five minutes after injection; 30 per cent of the intact radioactive insulin was still present in the circulation of immune rabbits at two hours after injection.

Characteristics of serum antibodies

There was a significant difference between normal and immune rabbits in respect to radioactivity of the TCA-precipitable fraction. In normal animals only 8.2 per cent remained one hour after injection of radioactive insulin as compared with 59.9 per cent in immune rabbits. In both groups the TCA precipitates of urine showed negligible radioactivity. On the other hand, insulin radioactivity of the supernates of blood and urine was higher in normal than in immune rabbits; in supernates of blood it was 8 as compared with 6 per cent, and of urine 22.7 as compared with 8.3 per cent (table 3). In the normal animal there was a progressive increase in nonprecipitable radioactivity up to about two hours and a concomitant decrease in precipitable radioactivity (figure 3). On the other hand, in the immune rabbit, while the TCA-precipitable radioactivity also fell, there was an insignificant increase in nonprecipitable radioactivity (figure 4).

The results of studies with column electrophoresis are shown in figures 5 (normal) and 6 (immune). In the sera of normal animals the peaks of radioactivity were in the albumin and alpha globulin areas, but extended beyond the latter, while in immune animals the radioactivity appeared as a complex corresponding to the gamma globulin peak.

A time course study was carried out on immune sera obtained 3, 15, 30, 45, 60 and 90 min. after the injection of insulin-I-125, as well as 2, 4, 8 and 12 hrs. In the immune animal the radioactivity appeared at the gamma globulin area throughout the entire period.

TABLE 1

Persistence of insulin-I-125 in the serum following intravenous injection of the radioactive hormone

Rabbit	Injected insulin-I-125 (U./kg.)	Half-life: Fast component (minutes)	Half-life: Slow component (minutes)
Normal 1	0.49	4.8	46.0
Normal 2	0.30	3.5	34.5
Normal 3	0.46	5.5	44.0
Immune 1	0.49	10.0	82.0
Immune 2	0.30	12.5	87.0
Immune 3	0.46	15.5	165.0

TABLE 3

TCA-precipitable and TCA-nonprecipitable radioactivity in serum and urine of normal and immune rabbits one hour after intravenous injection of insulin-I-125

Rabbit	CPM × 10^5: Precipitate	CPM × 10^5: Supernate	Per cent of dosage: Precipitate	Per cent of dosage: Supernate
Normal				
Blood	328.0	321.2	8.2	8.0
Urine	1.0	886.9	0	22.7
Immune				
Blood	2,886.7	289.6	59.9	6.0
Urine	0	182.1	0	3.8

TABLE 2

Hypoglycemic effect of radioactive insulin on fasted immunized* and normal rabbits

Rabbit	Body weight (kg.)	Insulin (U./kg.)	Blood sugar milligrams per 100 ml. Pre-injection		15′	30′	45′	60′	90′	120′	240′
Normal 1	2.28	0.43	87	85	58	56	60	56	73	79	88
Normal 2	2.6	0.49	111	106	52	56	70	74	77	95	108
Normal 3	3.4	0.46	109	107	53	49	58	71	78	92	97
Immunized* 1	3.87	0.43	92	95	94	83	87	101	94	99	101
Immunized 2	3.4	0.49	97	97	83	83	76	81	81	83	103
Immunized 3	3.45	0.46	111	108	78	70	72	87	95	99	92

*The rabbits were immunized with beef insulin, injected with bovine insulin-I-125.
†Blood sugar determinations were made by the method of Hagedorn-Jensen.

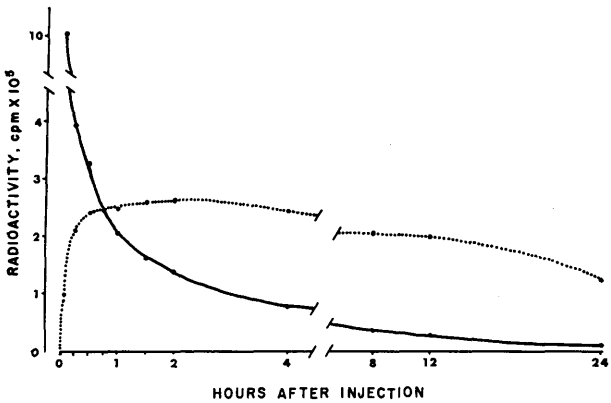

FIG. 3. Concentrations of TCA-precipitable and TCA-nonprecipitable radioactivity in the serum of a normal rabbit after the intravenous injection of the radioactive insulin. Solid line represents TCA-precipitable radioactivity and dotted line TCA-nonprecipitable radioactivity.

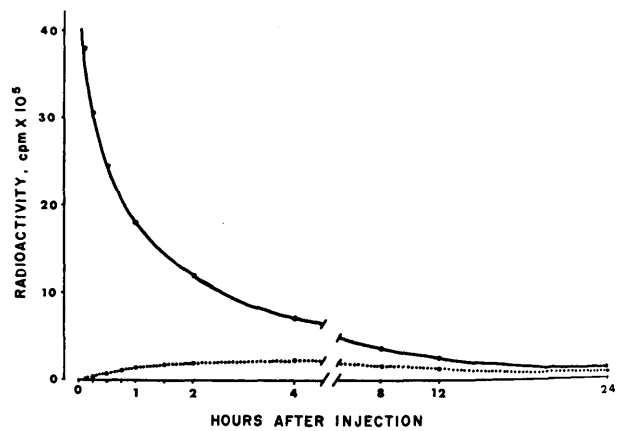

FIG. 4. Concentrations of TCA-precipitable and TCA-nonprecipitable radioactivity in the serum of an insulin immune rabbit after the intravenous injection of radioactive insulin. Solid line represents TCA-precipitable and dotted line TCA-nonprecipitable radioactivity.

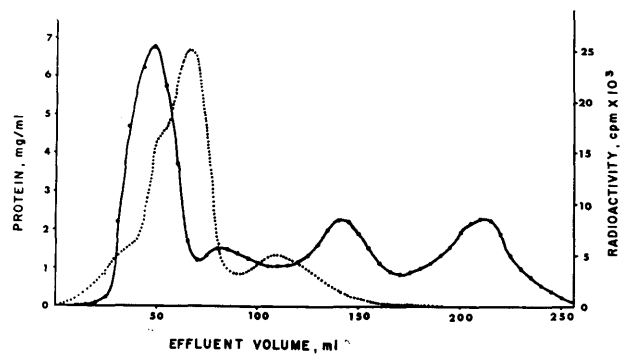

FIG. 5. Distribution of protein fractions and radioactivity in the circulating serum of a normal rabbit following column electrophoresis. Solid line represents protein concentration and dotted line radioactivity in cpm.

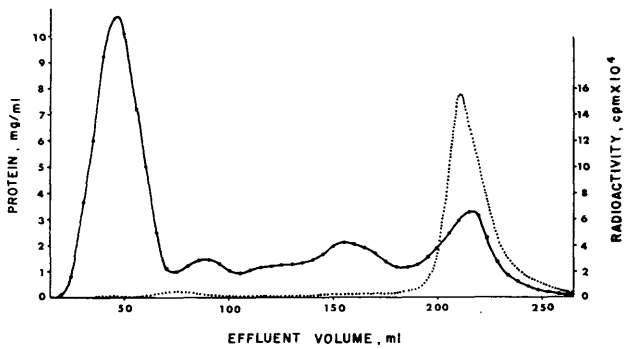

FIG. 6. Distribution of protein fractions and radioactivity in the circulating serum of an immune rabbit following column electrophoresis. Solid line represents protein concentration and dotted line radioactivity in cpm.

Analysis by cold ethanol fraction of serum of normal and immune animals obtained sixty minutes after the injection of radioactive insulin showed that Fraction B consisting mostly of beta globulin with a small amount of gamma$_1$ and gamma$_2$ globulins contained the highest concentration of radioactivity, although the levels of the latter were always higher in all fractions in immune than in normal animals (table 4). In some instances the gamma globulin fractions obtained by cold ethanol extraction of immune sera revealed a high insulin binding capacity on agar electrophoresis, while normal rabbit serum failed to show any binding capacity of the gamma globulin fraction. Thus the insulin binding components of the sera of immune rabbits were similar to those obtained in the guinea pig.[1]

TABLE 4

Specific activity of serum fractionated with cold ethanol one hour after intravenous injection of insulin I-125*

Rabbit	Original serum (CPM × 10³)	Fraction A (CPM × 10³)	Fraction B (CPM × 10³)	Fraction C (CPM × 10³)
Normal	2.4	0.7	2.4	0.2
Immune	24.5	5.5	145.0	53.8

*Dose of injection: 65 μc. per kilogram equivalent to 0.4 U. per kilogram.

Distribution of radioactivity in tissues and organs

In toto, normal animals retained only about 17 per cent of the injected radioactive insulin in the TCA-precipitable fractions of serum and tissues, as compared with about 75 per cent in immune animals (tables 5 and 6); or conversely, in the normal animal about 83 per cent of the radioactive insulin was metabolized in the first hour as compared with only 25 per cent in the immune rabbit. The last columns in tables 5 and 6 show

TABLE 5

Distribution of radioactivity in tissues of normal rabbit one hour after intravenous injection of insulin-I-125

Source	Weight (grams)	Radioactivity (CPM × 10^{-5}) Total	TCA-precipitable	TCA-nonprecipitable (supernate)	Specific activity*	Percentage of dosage in TCA precipitate
Serum	140.5†	699.5	328.1	321.2	3.9	8.2
Urine	5.5†	903.4	1.0	886.9	0	23.0‡
Liver	66.0	74.0	45.1	28.9	0.4	1.1
Kidney	9.7	123.9	83.0	40.9	9.7	2.1
Heart	5.0	5.7	3.1	2.6	1.1	0.1
Lung	9.2	12.6	8.8	3.8	1.9	0.2
Thyroid	0.6	3.9	3.1	0.7	8.5	0.1
Spleen	1.1	1.3	0.6	0.5	0.8	0
Adrenals	1.0	0.6	0.2	0.5	0.7	0
Pancreas	1.0	0.7	0.2	0.5	0.5	0
Testis	5.4	4.5	2.0	2.4	0.6	0
Brain	6.0	1.4	0.3	1.2	0.1	0
Muscle	1,050.0	581.7	225.0	356.0	0.2	5.6

*CPM × 10^3 per milligram of protein.
†Total volume estimated by method of Courpice.[6]
‡TCA-nonprecipitable radioactivity, since there was no precipitable activity in urine.

TABLE 6

Distribution of radioactivity in tissues of immune rabbit one hour after intravenous injection of insulin-I-125

Source	Weight (grams)	Radioactivity (CPM × 10^5) Total	TCA-precipitable	TCA nonprecipitable (supernate)	Specific activity*	Percentage of dosage in TCA precipitate
Serum	168.5†	3,384.0	2,886.7	289.6	24.8	59.9
Urine	11.0†	182.2	0	182.1	0	3.8†
Liver	61.0	499.7	390.6	109.1	4.9	8.1
Kidney	16.5	72.6	49.3	23.3	4.4	1.0
Heart	6.4	12.1	10.2	1.9	2.3	0.2
Lung	10.5	19.4	15.7	3.8	3.1	0.3
Thyroid	0.7	3.0	2.7	0.3	6.7	0
Spleen	1.4	1.8	1.4	0.4	2.8	0
Adrenals	1.1	1.4	1.0	0.4	1.7	0
Pancreas	1.4	1.1	0.7	0.4	1.0	0
Testis	5.2	4.2	3.2	1.0	1.1	0
Brain	8.2	1.7	1.0	0.7	0.2	0
Muscle	1,200.0	333.0	173.2	155.4	0.1	3.6

*CPM × 10^3 per milligram of protein.
†Total volume estimated by method of Courpice.[6]
‡TCA-nonprecipitable radioactivity, since there was no precipitable activity in urine.

the percentage of dosage of radioactive insulin remaining in the serum and various tissues and organs after one hour. In the normal rabbit serum was highest, followed by muscle, kidney and liver, with small amounts in heart, lung and thyroid. In the immune animal percentage of injected dose was about seven times higher in the serum and liver than in the normal rabbit, was slightly lower in muscle and kidney, and about the same in heart and lung. No radioactivity was found in the thyroid of the immune rabbit.

On the basis that the proportion of TCA-precipitable radioactivity remaining after one hour represents intact injected insulin, it can be seen from table 7 that in the normal rabbit about 70 per cent was still present in lung, over 60 per cent in liver and kidney, about 55 and 40 per cent in heart and muscle respectively, with a small percentage in serum and thyroid. In the immune animal about 80 per cent was still present as intact insulin in serum, heart, lung and liver, with about 70 per cent in kidney and about 50 per cent in muscle. Thus, in the immune animal the proportion present as intact insulin is about seventeen times greater in the serum, about 50 per cent greater in the heart, about 30 per cent greater in liver and muscle and about 16 per

TABLE 7

Per cent intact insulin one hour after intravenous injection of insulin-I-125*

	Normal	Immune
Liver	61	78
Kidney	67	68
Heart	55	84
Lung	70	81
Muscle	39	52
Thyroid	0.8	0
Serum	4.7	82

$$*\frac{\text{TCA precipitable radioactivity}}{\text{Total radioactivity}} \times 100$$

cent greater in lung; the proportion present as intact insulin in the kidney was about the same in the immune as in the normal rabbit.

DISCUSSION

The half-life of insulin depends on a number of factors, including quantity of hormone injected, the binding characteristics of carrier proteins, the rate of uptake and utilization by the tissues, the rate of excretion, and possibly other factors. The half-life has a biphasic character, the initial phase (fast component) of which has generally been attributed to the rate of distribution of hormone in the body water compartment, and a second phase (slow component) attributed to the rate of hormone degradation. The duration of the initial phase may thus depend on vascular volume and permeability, available extracellular space, and possibly other factors, and the difference in the fast component between normal and immune animals probably reflects differences in the rate of exit from the vascular compartment of free insulin, insulin bound to normal carrier protein, and insulin in complex with antibody. Differences between normal and immune animals in respect to the slow component may reflect differences in the rate of release of hormone from the tissues for recirculation, and in the rate of degradation and release of split products into the circulation.

The shorter half-life of the initial phase in the normal rabbit in the present study as compared with others[11,12] may be attributed to the use here of Lugol's solution to minimize the uptake of iodine-containing radioactive insulin by the thyroid. The hypoglycemic effect of this fast component was demonstrable in both normal and immune animals, although it was about twice as long in the latter, and the hypoglycemia was not as marked.

In the normal rabbit the injected radioactive insulin appeared to be carried by albumin and an alpha globulin. The lack of complete correspondence of the protein and radioactivity curves may be due to radioactivity of either free or degraded insulin. There was also some variance, at different times, in the relative quantities of hormone associated with each protein component. Insulin and carrier proteins behave differently in different tissues in respect to degradation by insulinase,[13-16] as well as uptake,[17-19] and this may be reflected in the state of hormone in the circulation. In the over-all, Prout and Evans[17] found that in normal animals 86 per cent of the injected radioactive insulin is degraded in eighty minutes, and this is comparable to the present finding of 82 per cent in sixty minutes.

While the antibody does not release the hormone as readily as the nonantibody proteins, and while it has been reported that insulin in complex with antibody possesses no biologic activity on rat diaphragm assay,[20] the possibility nevertheless exists that in vivo some biologic activity of the slow component may occur. Diabetic patients with circulating insulin antibody have a prolonged half-life,[11,21,22] but only rarely exhibit a significant insulin resistance attributable to antibodies. This may be due to a slow dissociation of insulin and antibody, or to the possibility that the biologically active site on the hormone molecule and the antibody binding site are not at the same location.

Moreover, the half-life of antigens is not always prolonged in immune animals.[23,24] A shortened half-life in immune states has been explained on the basis of the fact that when the immune complex is a precipitating one, the precipitate is rapidly lysed by phagocytosis. While in the rabbit[25] as well as in the human[11,26-29] soluble complex predominates, in the rabbit, at least, some precipitating complex may occur.[25]

Finally, at one hour after the injection of radioactive insulin, the immune rabbits retained about eight times as much of the TCA-precipitable radioactivity (intact insulin) as the normal animals. On the other hand normal animals had over three times as much radioactivity in the nonprecipitable fraction in urine and blood as compared with immune animals. Normal rabbits retained only 9.2 per cent of the injected radioactive insulin in the TCA-precipitable form in the tissues after one hour as compared with 13.2 per cent in immune rabbits; in both most of this radioactivity resided in the liver, muscle and kidney. In the immune rabbit the ratio of intact radioactive insulin to total radioactivity was seventeen times greater than in the normal animal in the serum, about 50 per cent greater in the heart, about 30 per cent greater in liver and muscle, about 16 per cent greater in the lung, and in about the same proportion in the kidney.

ACKNOWLEDGMENT

This study was supported by Grant No. AM-07150 of the National Institute of Arthritis and Metabolic Diseases, and by the Nadah Fund Club of St. Louis, Missouri.

The insulin used in this series of studies was Lilly crystalline beef insulin, Lot No. 719106 (25.2 U. per milligram), and was kindly supplied by Dr. W. R. Kirtley of the Eli Lilly Co. Research Laboratories, Indianapolis, Indiana.

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