Distribution of Injected Insulin and Insulin-Antibody Complexes in Normal and Insulin-Immunized Animals*

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Summary. The distribution of insulin injected into normal rats and guinea pigs as the free hormone or as insulin-antibody complexes was studied by extraction of immunoreactive insulin and by following the fate of ^{125}I - or ^{131}I -labeled insulin in the plasma, liver, kidneys and spleens of the injected animals. Injected free hormone rapidly disappears from the plasma to accumulate transiently in the liver, and, to a greater extent, in the proximal convoluted tubules of the kidneys. When insulin-antibody complexes formed *in vitro* are injected, the hormone disappears more slowly from the plasma and concentrates in the liver and spleen where it can be

demonstrated by autoradiography in reticulo-endothelial cells; little or none is found in the kidneys. After injection into insulin-immunized guinea pigs, the hormone disappears very slowly from the plasma and accumulates almost exclusively in the liver with no evidence of concentration by any particular cell type; little or none is found in the kidneys or spleen.

Key words: Insulin, insulin-antibody complexes, rat, guinea pig, insulin-immunized guinea pigs, insulin distribution, reticulo-endothelial system.

Several tissues, and particularly the liver [9] and kidneys [16], play roles in the utilization of secreted or injected insulin in normal animals and man. Little is known, however, of how this normal process may be affected by the presence of insulin antibodies which appear in the plasma of all animals and human subjects treated with the hormone [6]. In the present study normal and insulin-treated animals were injected intravascularly with radio-iodinated or unlabeled insulin to show that such antibodies, whether homologous or heterologous, have a marked effect upon insulin utilization, an effect which still requires delineation and explanation.

Materials and Methods

Insulin

Unlabeled porcine insulin (Lot Nos. PJ5589, 21.1 U/mg, and PJ5632, 23.9 U/mg; Eli Lilly and Company, Indianapolis, Indiana) was stored at 0 to 4°C as a stock solution (100 U/ml) in dilute acetic acid (0.6%, w/v); these were crystalline zinc-insulin preparations. Solutions containing ^{125}I -labeled porcine insulin (110–120 mC/mg, 0.8–1.0 µg/ml; Cambridge Nuclear Corporation, Cambridge, Mass.) were obtained commercially, diluted upon receipt in 3% NGPS (see below) and stored in the frozen state (–10°C) for a maximum of one month; samples used for injections were used within 3 days of receipt without dilution and

storage. Stock solutions were appropriately diluted for injection or assay purposes immediately before use.

Guinea Pig Sera

Normal serum (NGPS) was obtained commercially (Hyland Laboratories, Los Angeles, California). **Anti-insulin sera** (GPAIS) were obtained from guinea pigs immunized with bovine (Lot No. 524, binding 1.5 U insulin/ml) or porcine (Lot No. 551, binding 1.5 U insulin/ml) insulin by methods described in detail elsewhere [22]. All sera were stored in the frozen state (–10°C) and thawed for dilution immediately before use.

Inocula

Labeled and/or unlabeled insulins in relatively small volumes of buffer (3% NGPS) or normal serum (NGPS) were added in appropriate amounts to NGPS or GPAIS and allowed to stand at room temperature (23 to 28°C) for 30 to 60 min. The resulting solutions (0.5 or 1.0 ml) contained sufficient TCA-precipitable radioactivity (ca 8.3×10^6 cpm/inoculum) to get conveniently measurable radioactivity in the plasma, livers and spleens after injection. Of the total radioactivity contained in these inocula, the TCA-precipitable portion accounted for 87% (rats) or 95% (guinea pigs); these figures closely approximate to the proportions of labeled material bound by an excess of GPAIS and are therefore assumed to reflect the immuno-reactive contents.

Animals

Normal rats (male, 180–225 g body wt) and **guinea pigs** (male, 800–1200 g body wt) were fed on standard

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diets and used in the fully fed state. *Insulin-immunized guinea pigs* had received 8 subcutaneous injections of bovine insulin over a period of six months; the method of immunization has been described [22]. One week before they were used, their sera bound 0.9 to 4.4 Units porcine insulin/ml (Mean, 2.1 ± 0.2 U/ml).

Experimental Procedures

Rats were each injected intravenously (tail vein) with the same volume (1.0 ml) of inoculum and killed at timed intervals up to 90 min later. All guinea pigs were first injected subcutaneously with an antihistamine (Chlorphenyramine maleate, Chlortrimeton, 10 mg; Schering Corporation, Bloomfield, New Jersey) and then lightly anesthetized with ether. Approximately 15 min later the inoculum (0.5 ml) was injected into the heart, the animals being killed by decapitation 10 or 30 min later. Blood was collected from the severed necks of all animals into heparinized beakers and the plasma separated rapidly by centrifugation. The livers, spleens and kidneys were removed rapidly and weighed portions or the whole organs examined as follows:

a. Radioactive Contents. Samples of *plasma* (0.1 ml) were diluted in 3% NGPS (0.4 ml) and weighed aliquots of the *tissues* (ca. 1.0 g) were homogenized (Polytron, Kinematica GMBH, Lucerne, Switzerland) in distilled water (10 ml). Aliquots (0.5 ml) of these diluted or homogenized samples were assayed for total gamma-radioactive content. TCA-precipitable radioactivity was determined after treatment of each aliquot (0.5 ml) with an equal volume of trichloroacetic acid (0.5 ml; 20%, w/v), washing of the precipitate with TCA (1.0 ml, 10%, w/v), and removal of the supernatant solution. The gamma-radioactive contents of these solutions, suspensions and precipitates were measured in a well-type scintillation counter (Series 300, Model 5052; Packard Instrument Company, LaGrange, Illinois), the periods of counting, except when negligible radioactivity was detectable, being sufficient to obtain at least 5000 counts per sample.

b. Immuno-reactive Insulin Contents. Plasma and weighed aliquots of liver and kidney from rats injected with inocula containing only unlabeled porcine insulin were extracted with acid-alcohol (10 vols, w or v/v) and the extracts neutralized with a saturated solution of sodium bicarbonate; details of the method have been described [21]. The insulin contents of these neutralized extracts were measured by immunoassay [11]. Briefly, all reagents used in the assay were dissolved in a buffered solution ($\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, 0.05 M, pH 7.0) containing sodium chloride (0.4%, w/v) and NGPS (3%, v/v); this buffered solution is here termed 3% NGPS. The neutralized tissue and plasma extracts (5 to 20 μL /0.1 ml 3% NGPS) and appropriate solutions of porcine insulin (0 to 120 μU /0.1 ml 3% NGPS) were each pre-incubated for 60 min at 36°C with GPAIS (20 μL Lot 524/0.2 ml 3% NGPS), and then incubated for 30 min at room temperature (ca. 25°C)

with a mixture of unlabeled (350 μU) and ^{125}I -labeled (ca. 10 μU) porcine insulin in 3% NGPS (0.1 ml). Control tubes containing an excess or no GPAIS were similarly treated. Antibody-bound labeled insulin was precipitated with alcohol (1.6 ml, 95%, v/v) and the precipitates washed once with more dilute alcohol (2.0 ml; 76%, v/v) before counting.

c. Autoradiography. Tissues (liver, kidney, spleen etc.) were rapidly removed after death from rats and guinea pigs injected with labeled insulin and fixed at room temperature for 24 h in 10% neutral buffered formalin. The fixed tissues were dehydrated and embedded in paraffin. Sections (thickness, 5 μ) were coated with Kodak NTD emulsion and stored at 4°C. Separate sets of slides were developed one, two and three weeks after coating or, in other words, 28, 35 and 42 days after the injections. The sections were then stained with hematoxylin and eosin for microscopic examination.

Expression of Results

The radioactive contents of plasma and of individual tissues are expressed as percentages (Mean $\pm$ SEM) of the TCA-precipitable radioactive contents of the inocula injected into rats and guinea pigs. To determine the radioactive contents of the plasma compartment, it was assumed that the plasma volume of rats is equal to the volume of distribution of radio-iodinated albumin one minute after its intravenous injection. The volume found during preliminary studies in a group of 4 rats (4.37 ± 0.15 ml/100 g body wt) was not significantly different from that (4.30 ± 0.13 ml/100 g body wt) reported previously in a much larger group ($n=14$) of normal rats two minutes after injection [23]. The plasma volume of guinea pigs was assumed to be 3.94 ml/100 g body wt [13]. Unless otherwise stated, the quoted radioactive contents of plasma and tissues refer to TCA-precipitable material found in them and presumed to represent unchanged insulin. The immuno-reactive insulin contents of livers and kidneys from rats injected with unlabeled insulin are expressed as percentages (Mean $\pm$ SEM) of the amount injected. All statistical analyses were carried out by methods described by Snedecor and Cochran [17].

Results

Fate of Labeled Insulin Injected into Normal Rats

In one study, normal fully fed conscious rats (Mean body wt, 198 ± 1.7 g) were injected intravenously with pre-incubated solutions (1.0 ml) containing ^{125}I -labeled (ca. 0.7 mU) and unlabeled (10.0 mU) porcine insulin in either (a) NGPS (0.90 ml) or (b) an excess of GPAIS (Lot No. 551, 0.90 ml). The animals were killed by decapitation at intervals up to 90 min after injection.

As shown in Fig. 1, TCA-precipitable radioactivity rapidly disappeared from the plasma in animals given inocula containing no antibodies (open circles); less

than 10% could be recovered after 10 min. Recovery from the livers of these animals never exceeded 10%, little or none being found after 15 min. By contrast, TCA-precipitable radioactivity in the plasma of rats injected with insulin which had previously been allowed to react with antibodies (closed circles) decreased more slowly; more than 10% of the inoculum was still present in the plasma after 90 min. Recovery of labeled material in the livers of these animals increased rapidly, more than 40% of the inoculum being recovered at all times between 10 and 70 min after injection. The livers and spleens of these animals were not examined for radioactive contents but they and the livers were examined by autoradiography (see below).

Fig. 1. Recovery in TCA-precipitable form of ^{125}I -labeled insulin (ca. 0.6 mU) mixed with unlabeled insulin (ca. 1.0 U) and incubated before injection with NGPS (open circles) or GPAIS (closed circles). Each point represents recovery from the liver or plasma of single animals killed 5 to 85 min after injection

Fate of Labeled Insulin Injected into Normal and Insulin-Immunized Guinea Pigs

All guinea pigs, whether normal (Mean body wt, 922 ± 28 g) or insulin-immunized (Mean body wt, 1022 ± 42 g), were injected with the same volume of preincubated solution (0.5 ml) containing the same mixture of ^{125}I -labeled (ca. 2.3 mU) and unlabeled (ca. 200 mU) porcine insulins and either (a) NGPS (0.38 ml), or (b) an excess of GPAIS (0.39 ml). The animals were killed by decapitation either 10 or 30 min after injection.

As shown in Table 1, little (9%) of the insulin injected in the absence of antibodies could be recovered from the plasma of *normal guinea pigs* after 10 min, more having by then accumulated in the livers (20%)

and kidneys (15%). Expressed in terms of concentration of radioactivity in these tissues, those found in the liver (124 cpm/g) and kidney (603 cpm/g) were higher than that present in plasma (54 cpm/ml). Almost no radioactive material was found in the spleen. When normal guinea pigs were killed 30 min after injection of insulin which had been pre-incubated with antibodies, much more of the inoculum (21%) was recovered in the plasma, most of the remainder being found in the liver (68%); very little appeared in the kidneys or spleen. In this case, the concentrations of radioactive material in the liver (444 cpm/g) and spleen (211 cpm/g) exceeded that in the plasma (108 cpm/ml) and little could be found in the kidneys (15 cpm/g).

After injection of labeled insulin in the absence of antibodies into *insulin immunized guinea pigs* much of the inoculum was recovered in TCA-precipitable form from the plasma after 10 (59%) and 30 (43%) min. Meanwhile there was a corresponding increase in recovery from the liver between 10 (45%) and 30 (63%) min. Little radioactivity could be found in either the kidneys or spleen at either time of killing. Relative to the concentrations of radioactive materials found in the plasma after 10 (326 cpm/ml) and 30 (212 cpm/ml) minutes, that in the liver was equal after 10 min (301 cpm/g) and much greater after 30 min (405 cpm/g). Concentrations of radioactivity in the kidneys and spleens were much lower than that in the plasma at both times of killing.

Autoradiography of Tissues from Animals Injected with Labeled Insulin

In sections of tissues taken from *normal rats* and *guinea pigs* the same distributions of radioactive granules were observed. Thus after injection of labeled insulin in the absence of antibodies, radioactive granules were concentrated over the cytoplasm of cells lining the proximal convoluted tubules of the kidneys; a typical example is shown in Fig. 2A. No radioactive grains were seen to have concentrated over any particular cell type either in the liver (Fig. 2B) or spleen. After injection of labeled insulin which had been previously allowed to react with anti-insulin serum *in vitro*, radioactive granules were found to have localized over reticular macrophages in the liver (Fig. 2C) and spleen (Fig. 2D), but no specific localization was seen in the kidneys. After injection of labeled insulin in the absence of antibodies into *insulin-immunized guinea pigs*, some localization of radioactive granules was seen over reticular macrophages in the spleen. In the liver, however, the granules were diffusely distributed in the vascular sinusoidal spaces with no evidence of particular cellular localization (Fig. 2F). No radioactive granules were found in the kidneys.

Fate of Unlabeled Insulin Injected into Normal Rats

Groups of 4 to 6 normal rats (Mean body wt, 201 ± 2.7 g) were injected intravenously with pre-incubated solutions (1.0 ml) containing unlabeled porcine insulin

(ca. 1.0 Unit) in either (a) NGPS (0.90 ml) or (b) a slight excess of GPAIS (Lot No. 551; 0.90 ml). The animals were killed by decapitation at intervals up to 90 min later.

were consistently high (79 to 96 mU/g) while those found in the kidneys, with one exception after 10 min (107 mU/g), were low (5 to 13 mU/g). The spleens and plasma of these animals were not examined.

Table 1. Recovery of ^{125}I -labeled porcine insulin injected into normal and immune guinea pigs

Animal group	Inoculum	Time killed (min)	Tissue analyzed			
			Plasma	Liver	Kidneys	Spleen
(a) Recovered radioactivity (%)						
Normal	Ins	10	9.4±0.3	19.7±1.2	14.7±1.0	0.1
	Ins/Ab	30	20.5±1.3	68.0±2.0	0.4	0.9
Immune	Ins	10	59.4±9.8	44.7±3.2	0.6	0.3
	Ins	30	43.1±5.4	62.5±3.4	0.5	0.3
(b) Tissue radioactivity (Counts/g)						
Normal	Ins	10	54±3	124±8	603±47	28±3
	Ins/Ab	30	108±4	444±46	15±1	211±24
Immune	Ins	10	326±53	301±37	23±3	75±9
	Ins	30	212±18	405±18	16±1	59±10

Table 1. Groups (n = 5) of normal and insulin-immunized guinea pigs were injected (intracardiac) with a solution (0.5 ml) containing ^{125}I -labeled (ca 2.3 mU) and unlabeled (ca 200 mU) porcine insulin in NGPS (Ins) or in GPAIS (Ins/Ab). Shown in the Table are the mean values ($\pm$ SEM) for (a) the proportions (%) of injected radioactive material recovered in TCA-precipitable form from the plasma, liver, kidneys and spleen and (b) the tissue radioactivity (Counts/g tissue) in each tissue.

Table 2. Recovery of immunoreactive porcine insulin injected into normal rats

Tissue Nature		Recovered immunoreactive insulin (%)				
	Weight (g)	5 min	10 min	30 min	50 min	90 min
(a) Insulin in NGPS						
Liver	7.76±0.3	14.9±4.4	24.5±4.2	11.3±1.5	5.2±1.0	2.9±0.1
Kidneys	1.65±0.1	19.6±3.2	44.2±1.7	9.9±0.6	4.9±0.3	1.2±0.1
Animals	23	4	4	5	6	4
(b) Insulin in GPAIS						
Liver	8.36±0.3	66.5±1.7	80.2±2.3	66.9±3.6	70.9±4.9	43.1±4.9
Kidneys	1.70±0.1	0.8±0.4	18.3±1.3	1.8±0.6	2.2±0.3	0.6±0.3
Animals	23	4	4	5	6	4

Table 2. Groups of normal fed rats (4 to 6) were injected intravenously with unlabeled porcine insulin (ca 1.0 U) in the same volume (1.0 ml) of (a) NGPS and (b) GPAIS. Shown in the Table are the mean values ($\pm$ SEM) for the weights of the organs assayed and the proportions (%) of injected unlabeled insulin recovered in immunoreactive form from the livers and kidneys, the numbers of animals used under each experimental condition, and the times after injection (min) at which they were killed.

As shown in Table 2, insulin injected in the absence of antibodies was mainly recovered from the kidneys, a peak rate (44%) being observed after 10 min. Smaller amounts appeared in the liver during the first 10 min. The concentration of insulin found after 10 min in the kidneys (268 mU/g) was much greater than that found in the livers (32 mU/g). After neutralization with a slight excess of anti-insulin serum *in vitro* injected unlabeled insulin rapidly accumulated in the liver, more than 60% of the injected dose being recovered at periods from 5 to 50 min after injection. During this period, concentrations of insulin found in the livers

Discussion

The observations outlined above confirm previously published reports of the fate of radio-iodinated insulin injected into normal animals. Thus, as reported by others including Izzo *et al.* [9], labeled insulin rapidly disappears from the plasma of normal rats and guinea pigs to accumulate transiently in the livers and kidneys (Table 1, Fig. 1). Within the liver no particular type of cell appeared to concentrated labeled material in the present studies (Fig. 2B) and the reports of others are contradictory. Thus parenchymal cells [10], Kupffer

cells [1], both [20] and neither [18] of these hepatic cell types have been reported to accumulate injected labeled insulin in normal animals. In the kidneys, as uniformly reported elsewhere [1, 10, 20], labeled in-

derived from endogenous sources but it is clear from the results shown in Table 2 that unlabeled as well as labeled insulin concentrates transiently in both the livers and kidneys of normal rats. This pattern of in-

Fig. 2. Autoradiographs of tissue sections obtained from normal and immune guinea pigs injected with ^{125}I -labeled insulin in NGPS or GPAIS; experimental details are shown in Table 1 and in the text. Sections (X 200–400), which were stained with hematoxylin and eosin for microscopic examination, were obtained from (A) the kidney and (B) liver of a normal guinea pig injected with labeled insulin in NGPS and killed after 10 min; from (C) the liver, (D) spleen and (E) kidney of normal guinea pigs injected with labeled insulin in GPAIS and killed after 30 min; and from (F) the liver of an insulin-immunized guinea pig injected with labeled insulin in NGPS and killed after 30 min.

sulin concentrates in the lumen and cells lining the walls of the convoluted tubules (Fig. 2A). Less attention has been paid to the fate of unlabeled insulin in normal animals and part of the immuno-reactive insulin recovered in the present studies might have been

sulin utilization by the normal animal has yet to be defined in detail but it is grossly disturbed by the presence of antibodies to insulin.

Normal animals such as mice [2] and dogs [24] have been injected with radioiodinated insulin which has

previously been allowed to react *in vitro* with antibodies to insulin produced by the guinea pig. Under these conditions the labeled hormone did not accumulate in the kidneys but concentrated rapidly in the liver, observations confirmed and extended in the present studies. In both normal rats (Fig. 1) and guinea pigs (Table 1), radio-iodinated insulin which had previously been neutralized *in vitro* with an excess of guinea pig anti-insulin serum did not accumulate in the kidneys but did concentrate in the liver in TCA-precipitable form; its disappearance from the plasma was also retarded. When unlabeled insulin was injected into normal rats after neutralization with a slight excess of guinea pig anti-insulin serum, a significant proportion of injected immunoreactive hormone (18.3%) was recovered from the kidney at only one time (10 min), more than half of the injected dose accumulating in the liver within 5 min and persisting for over 50 min (Table 2). Part of this recovered immunoreactive insulin could have been derived from the injected rat's pancreas but the proportion was probably only small since most of the injected antibodies had been neutralized with exogenous insulin before injection. It should also be noted (Table 1) that after injection into normal guinea pigs, complexes formed with homologous antibodies by labeled insulin concentrate in the spleen to levels higher than those found simultaneously in the plasma and comparable with hepatic concentrations; this has also been observed (unpublished) in rats injected with the same (heterologous) labeled complexes. By autoradiography it was further shown that in these animals the labeled material had concentrated in macrophages of the liver and spleen (Fig. 2C and D), a finding which strongly suggests that injected insulin/antibody complexes formed *in vitro* are, in part at least, removed from the circulation by cells of the reticulo-endothelial system. The fate of insulin injected into insulin-immunized animals is less definite.

It has long been known, and is confirmed here (Table 1), that labeled insulin disappears at an abnormally slow rate from the plasma of human subjects [7, 19] and guinea pigs [8] which have previously been treated or immunized with insulin. The present studies further show that the injected labeled hormone does not accumulate in the kidney but concentrates progressively in the liver (Table 1); no accumulation was ever observed in the spleen. On autoradiography and in complete contrast to what was found in normal animals injected with complexes formed *in vitro*, concentration of labeled material was not demonstrated within any particular cell type in the liver. This discrepancy has yet to be explained but it is clear that whether the antibodies are homologous or heterologous, they do radically alter the fate of exogenous insulin.

At this stage of our knowledge of reactions between insulin and its antibodies, it is not possible to give an adequate explanation of these experimental phenomena. Other antigens which form insoluble antigen/antibody complexes are rapidly removed from the

circulation of injected animals by reticulo-endothelial cells [3] and there is now evidence to suggest that some soluble complexes may be removed rapidly if the ratio of antibody to antigen in those complexes exceeds 2:1 [12]. Although Berson and Yalow have long claimed that insulin only forms soluble complexes and is a monovalent antigen [5], there is well documented evidence that it can form insoluble complexes *in vitro* with anti-insulin sera from a variety of species including the guinea pig [15]. It has also been established that insulin can exist in solution in a dimeric form [14] and so even if it is monovalent in the monomeric form it could form complexes in solution which have high ratios of antibody to antigen. There are therefore some reasonable theoretical grounds for believing that insulin, like other antigens, could be metabolized by some well recognized immunological mechanism when antibodies are present and that the present observations could be explained on this basis. There is also the possibility that this abnormal process could in some way be responsible for the extreme resistance to insulin therapy which is seen in a small proportion of resistant diabetics. As Berson and Yalow [4] suggested many years ago, "owing to its degradation with antibody, a large fraction of the bound (injected) insulin may never become available to the tissues" in these patients. Until more is known of the compositions and properties of insulin/antibody complexes and of their disposal in immune species, observations such as those presented here can provide only suggestive evidence in support of such hypotheses.

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