"BIG INSULIN": A NEW COMPONENT OF PLASMA INSULIN DETECTED BY IMMUNOASSAY

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Since the advent of radioimmunoassay,[1] the concentration of insulin in plasma of normal and diabetic subjects in response to many stimuli has been studied extensively.[2] However, the chemical nature of the circulating hormone is not entirely clear. We find that endogenous plasma insulin has two components. One is very similar to pancreatic insulin. The other component is larger in size and immunologically less reactive than pancreatic insulin. With our antiserum, it accounts for up to 50 per cent of plasma insulin measured by immunoassay.

Materials and Methods.—Purified crystalline porcine and bovine insulin were gifts of Eli Lilly. Highly purified A-chain and B-chain sulfonates of bovine insulin were gifts of Dr. Arnold Marglin.[3] Cytochrome *c*, pancreatic ribonuclease, and porcine adrenocorticotropin (ACTH) were purchased from Mann, crystalline human serum albumin from Armour, rabbit fraction II and normal guinea pig serum from Pentex, guinea pig fraction II from Hyland, and NaI^{125} and NaI^{131} from Union Carbide. Guinea pig anti-porcine insulin serum was a gift of Drs. A. Kagan and S. M. Glick. Antibody to guinea pig gamma globulin was generated in sheep by repeated injection of guinea pig fraction II in complete Freund's adjuvant (Difco). Albumin and ACTH were labeled with I^{125} and insulin with I^{131} by the chloramine T method at specific activities of 5, 5, and 450 $\mu c/\mu g$, respectively.[4] The albumin-I^{125} was freed of radioactive contaminants by batch adsorption with Dowex 1X10. The ACTH-I^{125} was purified by adsorption to silica and elution with acetic acid and acetone.[5] The insulin-I^{131}, prepared fresh for each assay, was purified on a cellulose column.[1]

The normal subjects for the experiments were healthy male volunteers in their early twenties without a family history of diabetes. For the standard oral glucose tolerance tests 100 gm of glucose was administered in the morning after an overnight fast; for 3 days prior to the studies the subjects received a normal diet that included 300 gm of carbohydrate daily. For the starvation tests, food was withheld until the urine reacted strongly positive for acetone; the fast, about 36 hr in duration, was terminated by the administration of glucose, 100 gm orally. Patient MIT is an obese adult female with long-standing glucose intolerance, delayed hyperinsulinism following oral glucose, and retinal microaneurysms typical of diabetes. Plasma that was essentially free of insulin was obtained after a 72-hr fast from a patient who had had an islet-cell adenoma removed several years earlier. None of the subjects had ever been treated with insulin.

Blood was drawn into heparin, refrigerated, and centrifuged, and the plasma stored at $-20°$. Plasma, 1–3 ml, was enriched with albumin-I^{125} and iodide^{125} and applied to a 1×50-cm column of Sephadex G-50 fine (Pharmacia). Shorter columns or coarser grades of gel were unsatisfactory. Since the results of radioimmunoassay can be affected by variations in the salt content of the samples,[6] the column was equilibrated and developed in the same diluent that was used in the immunoassay (see below); also, none of the column fractions corresponding to the salt peak (marked by the iodide^{125}) were assayed. Fractions of 1–1.5 ml were collected, the radioactivity counted to localize the plasma protein and salt peaks, and the fractions stored at 4° until assayed.

Diluent for the insulin assays and for the gel filtration was veronal (0.05 *M*, pH 8.6) to which had been added human serum albumin (2.5 mg/ml), rabbit fraction II (0.1 mg/ml), and toluene as a preservative. For the assay[7] a series of tubes were prepared each of which contained, in a total volume of 1 ml, insulin-I^{131} (approx. 0.05 mμg), insulin

antibody (1:80,000), ethylenediaminetetraacetate (EDTA) (0.01 *M*), normal guinea pig serum (0.005 ml), and either crystalline porcine insulin (0–10 mμg), or plasma (0.1 ml), or an aliquot of the fractions from gel filtration (0.4–0.8 ml). After 3 days at 4°, sheep anti-guinea pig gamma globulin (0.1 ml) was added to precipitate the antibody-bound insulin-I^{131}.[8, 9] After another 18 hr at 4° the mixtures were centrifuged and the supernates were decanted. Both the precipitates and supernates were counted in a well-type scintillation counter that had been set to exclude I^{125} radioactivity. The *B/F* of insulin-I^{131} was expressed as a function of the concentration of unlabeled porcine insulin. The precipitates were not washed. However, in our experiments, in the absence of anti-insulin serum, radioactivity in the precipitates was uniformly 6% or less. Likewise, damage to the insulin-I^{131} during incubation in our assay was slight and quite similar in all samples of a given assay (see below).

In the presence of an excess of an anti-insulin serum, 85% or more of the radioactivity was in the precipitate. To calculate recoveries of plasma insulin following gel filtration, an aliquot of the plasma that had been applied to the column was assayed at the same time as the column fractions.

Cytochrome *c* was measured by its absorbance at 410 mμ, ribonuclease by its hydrolysis of denatured RNA,[10] and unlabeled ACTH by its fluorescence at 350 mμ.

Results.—When plasma was filtered on Sephadex G-50, the endogenous insulin was recovered in two peaks. One peak, quite discrete, appeared at 0.45 of the distance between the protein and salt peaks ("little insulin") (Fig. 1*A*). The other peak ("big insulin") was detected halfway between "little insulin" and the plasma protein peak and comprised from 0 to 50 per cent of the total plasma insulin (Fig. 1). When highly purified crystalline porcine insulin (pancreatic insulin) was added to insulin-free plasma, only a single peak was observed which corresponded to "little insulin" (Fig. 2). Likewise, when pancreatic insulin was added to plasma from patient MIT, in which "big insulin" constituted about one third of the endogenous insulin, the added pancreatic insulin appeared only with the "little insulin" peak (Fig. 1*B*).

To prepare large amounts of "big" and "little" insulin, 40 ml of plasma from patient MIT were filtered on Sephadex G-50 (4 × 54 cm) (Fig. 3*A*). The fractions corresponding to "big insulin" and to "little insulin" were pooled, dialyzed, lyophilized, and redissolved in 1 ml of diluent. Each fraction was filtered again on Sephadex G-50 (1 × 50 cm). Each of the two components retained its characteristic migration pattern and remained free of the other component (Fig. 3).

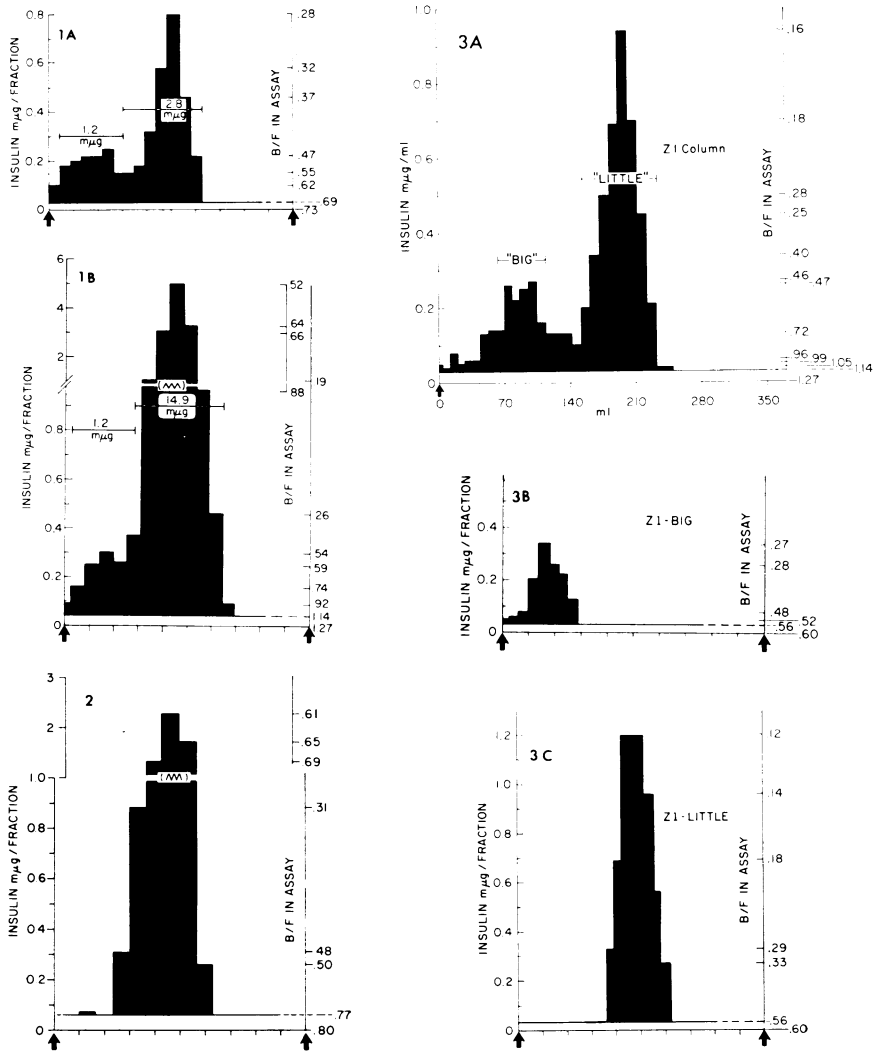
Purified human and porcine insulins differ by one amino acid. With our guinea pig antiporcine insulin serum, human insulin in plasma and purified porcine insulin reacted identically;[11] purified bovine insulin reacted less strongly (Fig. 4). The isolated A and B chains were essentially unreactive[12] (Fig. 4). "Little insulin," tested over a 20-fold range of dilutions, reacted identically with porcine insulin (Fig. 5). However, "big insulin," tested over a 40-fold range of dilution, reacted nonidentically (Fig. 6).

The nonidentical cross-reactivity of "big insulin" accounts in part for the truncated appearance of its peak on gel filtration. When "big insulin" was measured against a "big insulin" standard, instead of the porcine insulin standard, the "big insulin" peak had a more usual appearance. Because "big insulin" and porcine insulin did not react identically, and since in all other experi-

ments we measured "big insulin" by reference to porcine insulin, we usually underestimated the fraction of total insulin that was "big insulin."

One other aspect of the cross-reactivity deserves comment. Although one of the two components of circulating insulin reacts with our antiserum nonidentically with porcine insulin, insulin in unfractionated human plasma was not distinguished, under our experimental conditions, from purified porcine insulin. An analogous result was obtained by mixing bovine with porcine insulin; they differ in their reactivities about as much as "big" and "little" insulin. Although the individual components were distinguished, mixtures of bovine and porcine, even up to 1:1, were barely distinguishable from pure porcine insulin (Fig. 4).

To obtain an approximate estimate of the size of the two components, markers



of known molecular weights were filtered on columns of Sephadex G-50. Cytochrome *c* (mol wt 12,400) and ribonuclease (mol wt 13,700) yielded peaks at about 0.05 and ACTH-I^{125} and unlabeled ACTH (mol wt 4,600) at about 0.50 of the distance between albumin and iodide. Thus, under our experimental conditions, "little insulin" with its peak at 0.45 corresponded to a molecular weight of 5,000–6,000, while "big insulin" with its peak at 0.22 corresponded to a protein with a molecular weight of 8,000–9,000.

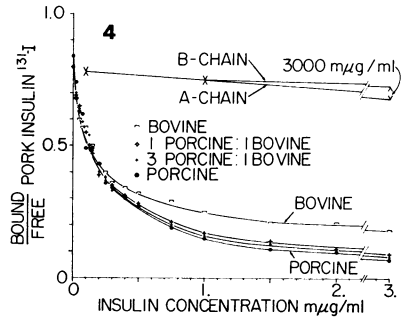


FIG. 4.—Immunological reactivity of purified hormone preparations. The *B/F* of porcine insulin-I^{131} is plotted as function of the concentration of unlabeled hormone or hormone derivative.

To show that the pancreas was the source of "big" as well as "little" insulin, a dog was infused with glucose, and blood was obtained from the pancreatic vein and from the femoral artery. At 10 minutes and at 25 minutes after completion of the glucose infusion, pancreatic venous plasma had a more than 50-fold greater total concentration of insulin. At both times the concentrations of "little" and of "big" insulin were distinctly higher in the pancreatic effluent than in the peripheral arterial plasma (Fig. 7).

The ingestion of glucose results in a prompt rise in plasma insulin that persists for an hour or more.[1] At early times after glucose ingestion by two normal subjects nearly all of the insulin was "little insulin." By one to two hours much more of the insulin was "big insulin," although plasma insulin concentrations and recoveries were about the same as in earlier specimens (Fig. 8). A similar effect was noted in one patient with typical maturity onset diabetes.

FIG. 1.—Endogenous insulin in plasma and pancreatic insulin added to plasma filtered on Sephadex G-50. (*A*) Two ml of plasma, obtained from patient MIT 2 hr after oral glucose, was filtered on Sephadex G-50. (*B*) Highly purified porcine insulin (pancreatic insulin) was added to 2 ml of the same plasma and filtered on Sephadex G-50. The numbers over the brackets indicate the total insulin recovered in the corresponding column fractions. The arrows denote the location of the albumin and iodide peaks. *Vertical scale at the left:* millimicrograms of insulin in each column fraction. *Vertical scale at the right:* the actual bound-to-free ratios (*B/F*) observed in the particular assay for the corresponding column. Where there are two scales representing *B/F* in assay, column fractions were assayed at greater dilution to increase the precision of the assay. The *B/F* corresponding to 0 insulin represents the mean of the 0 points in the assay. The *B/F* opposite the dashed line represents the limits of sensitivity of the assay. Where the base line is solid, the corresponding columns fractions were assayed and were equal to or less than the sensitivity of the assay. Where the base line is broken, the fractions were not assayed.

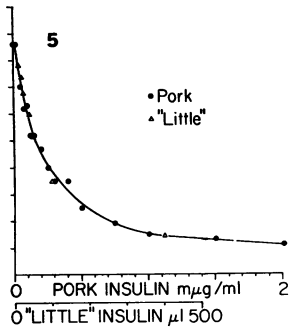
FIG. 2.—Highly purified porcine insulin added to insulin-free plasma. Highly purified porcine insulin was added to 2 ml of insulin-free plasma and filtered on Sephadex G-50. The notation is the same as in Fig. 1.

FIG. 3.—Separation of "big" and "little" insulin.

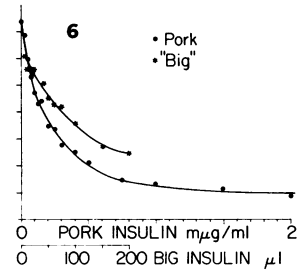
(*A*) Plasma (40 ml) from patient MIT was filtered on Sephadex G-50 (4 × 54 cm). The fractions enclosed by the brackets were pooled, dialyzed against 0.02 *M* $(NH_4)_2CO_3$, lyophilized, and redissolved in 1 ml of diluent.

(*B*) The concentrate of "big" was filtered on Sephadex G-50 (1 × 50 cm).

(*C*) The concentrate of "little" was filtered on Sephadex G-50 (1 × 50 cm). The notations on the graph are the same as described in the legend to Fig. 1.



(*Left*) FIG. 5.—Immunological reactivity of "little insulin." A salt-free concentrate of "little insulin" was prepared from 40 ml of plasma (see text and legend to Fig. 3) and redissolved in diluent. Multiple aliquots of this solution were assayed. *B/F* of porcine insulin-I^{131} is plotted as a function of the concentration of unlabeled porcine insulin and as function of the volume of the "little insulin" concentrate.



(*Right*) FIG. 6.—Immunological reactivity of "big insulin." *B/F* of porcine insulin-I^{131} is plotted as function of the concentration of porcine insulin and of the volume of "big insulin" concentrate. The scale of "big insulin" has been adjusted so that the upper part of the "big insulin" curve is coincident with the porcine insulin curve. If the scale is adjusted so that middle points of the "big insulin" curve are coincident with the porcine insulin curve, then the six upper points of "big insulin" will all lie to the left of the porcine curve and the six lower points will all lie to the right, confirming the nonidentical reactivity.

"Big insulin" reproducibly comprised one third to one half of her plasma insulin at two and three hours after oral glucose (Fig. 1).

Normal subjects who have fasted for 24–48 hours typically have intolerance to oral glucose,[13] so-called "starvation diabetes;" total plasma insulin rises somewhat slowly but to a higher level than that observed without the fast.[14] Under these conditions the concentration of "big insulin" was again much greater at 2 hours than at 15 minutes and also much greater than at the corresponding time during the standard glucose-tolerance test (Fig. 8).

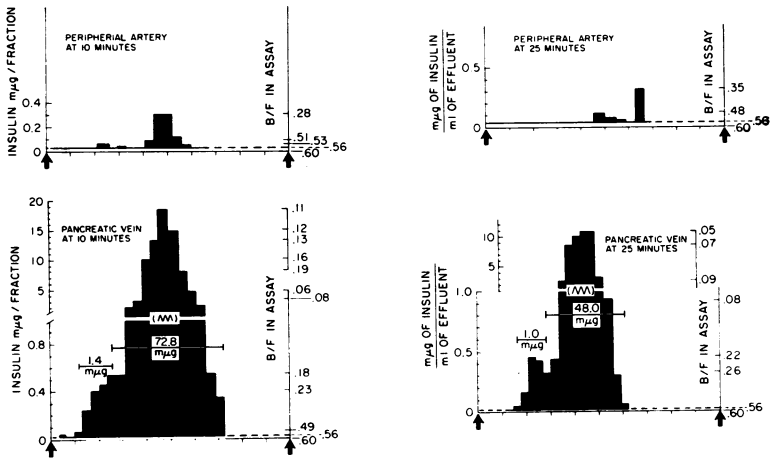


FIG. 7.—Arteriovenous difference of insulin across the pancreas. An anaesthetized dog was infused with glucose, 0.5 gm/kg body weight. Ten and 25 min later blood was collected from the pancreatic vein and femoral artery. Because of variation in the volume of the effluent fractions with the 25-min samples, the insulin was expressed as mμg/ml of effluent/2 ml of plasma applied. The other notations are the same as in the legend to Fig. 1.

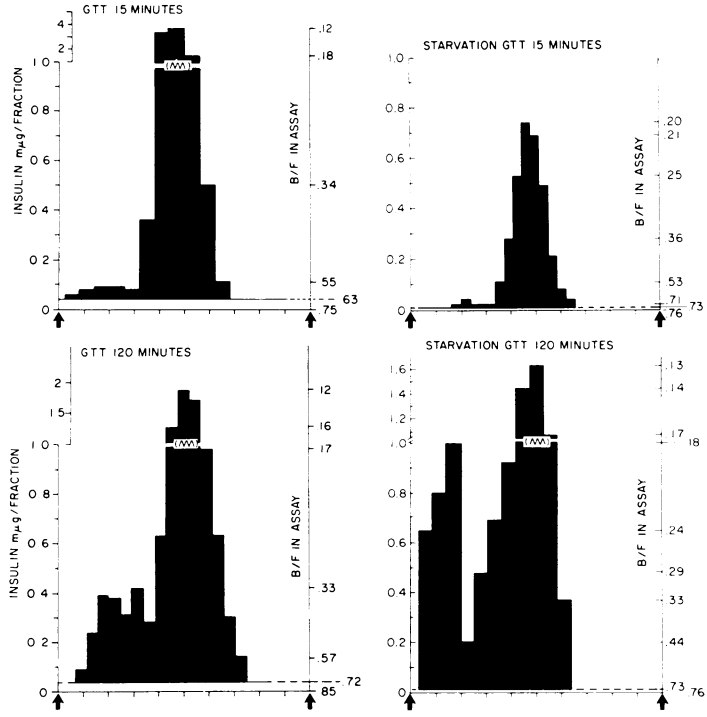


FIG. 8.—"Big" and "little" insulin in normal subject RUS following oral glucose under standard conditions and after starvation. Two ml of plasma were filtered on Sephadex G-50. *Upper left:* standard oral tolerance test at 15 min. *Lower left:* standard test at 120 min. *Upper right:* starvation test at 15 min. *Lower right:* starvation test at 120 min.

The fraction of total plasma insulin represented by "big insulin" was largely independent of the volume of plasma analyzed, buffer, storage of the plasma up to six months, completeness of recovery of plasma insulin, or the anticoagulant. When specimens were drawn from patient MIT, all at the same time, serum as well as plasma prepared in EDTA gave the same results as plasma that had been drawn into heparin.

To exclude the possibility that depressions in *B/F* were caused by something other than unlabeled insulin, e.g., damaged insulin-I^{131} or interference by plasma components with the precipitation of the guinea pig gamma globulin, the following experiment was performed. Two ml of heparinized plasma from patient MIT were filtered on Sephadex G-50 as usual. In the immunoassay, duplicate sets of tubes containing 0.4-ml aliquots were set up for each column fraction. After three days, just prior to the addition of sheep anti-guinea pig gamma globulin to the tubes of both sets, an excess of guinea pig antiporcine insulin was added to the tubes of one set. About 90 per cent of the radioactive insulin was precipitated in all of the tubes of this set, which indicated that the insulin-I^{131} was immunologically intact at the completion of the assay, and nothing was present to interfere with the antibody precipitation step. When this experiment

was repeated with plasma from patient MIT that had been drawn at the same time but into EDTA instead of heparin, identical results were obtained.

Discussion.—Both components of circulating insulin appear after glucose administration and are present at much higher concentrations in pancreatic venous than in peripheral arterial blood. "Big insulin," which is less retarded on Sephadex G-50, probably has a higher molecular weight. However, we have not excluded other differences that might cause greater exclusion from the gel, e.g., greater asymmetry of the molecule or a higher carbohydrate content.

From the degree of immunological reactivity of "big insulin" we suspect that it contains a major portion of the insulin structure intact within it. Guinea pig antibodies to porcine or to bovine insulin react strongly only with substances that have structures very similar to intact forms of these insulins.[12, 15] Guinea pig and piscine insulins, which retain the general configuration of porcine and bovine insulins but which differ greatly in amino acid composition, react quite poorly.[15-18] Isolated A chain with an intact intrachain disulfide bond may retain slight reactivity,[15, 19] whereas isolated A and B chains that have substituents on all of their sulfur atoms are essentially unreactive.[12] (Fig. 4).

"Big" and "little" insulin are not readily interconvertible in plasma because (1) concentrates of each component, when refiltered, do not yield significant amounts of the other. (2) Plasma that contained about the same concentrations of endogenous insulin, e.g., drawn 15 minutes and 2 hours after oral glucose administration, reproducibly had different fractions of the two components. (3) Pancreatic insulin, presumably the same as or very similar to "little insulin," added even in very large quantities to insulin-free plasma or to a plasma that had a substantial fraction of "big insulin," failed to produce "big insulin." Thus it is very unlikely that "big insulin" represents "little insulin" bound to a plasma constituent. The above observations, as well as the fact that "big insulin" was observed even at very low concentrations of insulin (10^{-8} to 10^{-10} M), exclude the possibility that "big insulin" represents the well-described polymerization of insulin. Thus it appears that "big insulin" in plasma is secreted in that form and that a major part of the insulin structure is linked very strongly within it. Whether this linkage is covalent or not is as yet undetermined.

The most extensive studies on the nature of immunoreactive insulin in plasma have been performed by Berson and Yalow.[15, 20] During ultracentrifugation they recovered essentially all of the endogenous plasma insulin above the albumin boundary.[20] This technique, however, was not sufficiently sensitive to separate "big insulin" from "little insulin." On starch gel electrophoresis endogenous insulin in plasma was recovered in the same region as crystalline insulin added to plasma.[15] "Big insulin," if it had been present in the samples, may have been undetected if it eluted poorly from the gel, if it migrated cathodally, or if its region of migration was quite close to that of "little insulin." Parenthetically, it should be noted that the various insulin-like activities in plasma that lack immunological reactivity are not pertinent to this study.[2]

Steiner and co-workers[21, 22] have shown that insulin in the pancreas is formed from a single-chain polypeptide precursor ("proinsulin"), which is less retarded than insulin on Sephadex G-50, is estimated to have a molecular weight of about 10,000, and reacts strongly with insulin antibody. At present we have insuffi-

cient data on "big insulin" with which to compare it with "proinsulin." Likewise we are as yet unable to relate our work to studies by others on the heterogeneity of pancreatic insulin.[23, 24]

The biological activity of "big insulin," its chemical nature, and its physiological significance are unclear. However, our findings indicate that the physiology of insulin secretion must be re-examined completely, especially in those conditions in man characterized by glucose intolerance with hyperinsulinism such as starvation, obesity, and maturity onset diabetes mellitus.

Summary.—Endogenous circulating insulin in man has two components, both of which are secreted by the pancreas. One component ("little insulin") is not distinguished from purified porcine (pancreatic) insulin by gel filtration or by its reaction with a guinea pig antiporcine insulin serum. The second component ("big insulin") is less retarded on gels and reacts somewhat differently with this anti-insulin serum; its molecular weight is estimated to be 8,000–9,000. Shortly after glucose is fed, most of the insulin in plasma is "little insulin," whereas one to two hours after glucose, up to 50 per cent of plasma insulin in this immunoassay is "big insulin." A prolonged fast prior to glucose administration (starvation diabetes) appears to enhance the output of "big insulin." It is as yet unclear whether "proinsulin," the presumed intrapancreatic precursor of insulin, is related to the "big insulin" in plasma.

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