Extractable Insulin Measured by Immuno-Chemical Assay: Effect of Tolbutamide.*† (24845)

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One of the mechanisms of action postulated for the hypoglycemic effect of sulfonylureas is stimulation of pancreatic secretion of insulin. An increased content of insulin in the pancreatic vein(1), a decrease of insulin extractable from the pancreas(2), and elevated peripheral levels of insulin in plasma(3) have been reported following treatment with sulfonylureas. Interpretation of these results is complicated by the difficulties encountered with current methods for insulin assay. Bioassays of small quantities of insulin in biological fluids in general are based upon the effect of insulin on glucose metabolism(4-6). Although these methods are highly sensitive, the fact that they measure physiological effects of insulin, rather than level of hormone itself, makes them nonspecific. Since many hormones from the adrenal or adenohypophysis affect glucose metabolism, the results of these biological assays are therefore usually reported in terms of "insulin-like" activity. Methods for measuring insulin chemically by chromatography (7) and by fibril formation(8) have proved more specific, but lack the sensitivity required for most biological studies. Immuno-assay of insulin by classical hemagglutination technics has been used only for measurement of comparatively pure insulin solutions(9). We have demonstrated the highly specific nature of binding of insulin-I^{131} to antibodies in serum from diabetics requiring large quantities of insulin(10). This binding phenomenon, measured by the technic of hydrodynamic flow (11), forms the basis of a sensitive, specific assay for insulin. The use of this new method to investigate changes in extractable insulin from pancreas after administration of tolbutamide is the subject of this report. The fact that prolonged treatment with sulfonylureas results in insulin depletion in the pancreas (2) does not necessarily indicate a cause-effect relationship between the drug and the level of hormone. The concomitant long-term hypoglycemia resulting from treatment may, in itself, cause insulin depletion by preventing insulinogenesis(12). For this reason only the immediate effects of sulfonylurea were studied.

Methods. Crystalline beef-pork insulin, glucagon free, (Lot No. T-2842) was obtained from Eli Lilly and Co. Tolbutamide (Orinase®) was obtained from Upjohn Co. Insulin-I^{131} was prepared by the method described previously(10). Antisera were obtained from diabetic subjects who required more than 500 units of insulin/day. Pooled antiserum was diluted with 5% bovine serum albumin until .5 ml bound about 85% of 0.1 μg of insulin-I^{131}. Addition of unlabeled insulin in increasing quantities resulted in saturation of binding sites, reflected by decrease in percentage of insulin-I^{131} bound. This change in percentage of bound, labeled insulin with increasing concentrations of insulin forms the basis of our method of assay. To obtain a standard curve various amounts of nonradioactive insulin contained in 25 μl of 5% albumin were incubated with 0.1 ml of diluted antiserum, 10 μl (0.02 μg) of insulin-I^{131}, and 0.1 ml of 30% urea in 5% albumin‡ (pH adjusted to 8.6 with concentrated base) for 30 min at room temperature. Fifty μl of the incubation mixture was then subjected to hydrodynamic flow in barbital buffer at pH 8.6(10). In this system, free insulin stays

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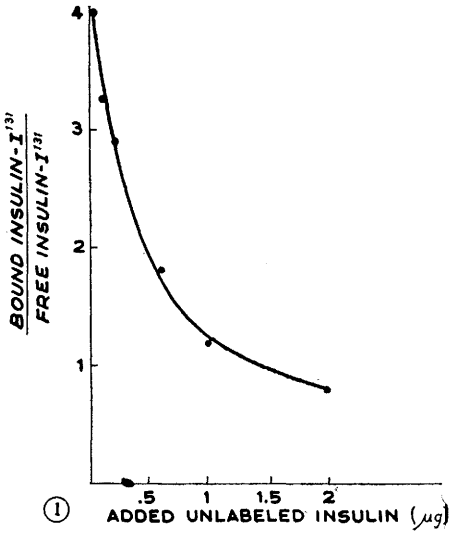
‡ Use of urea was found necessary to prevent precipitation of proteins in the acidic crude insulin solution, insoluble at higher pH. In the absence of urea about 50% of the insulin is lost during incubation by adsorption or co-precipitation with these insoluble proteins.



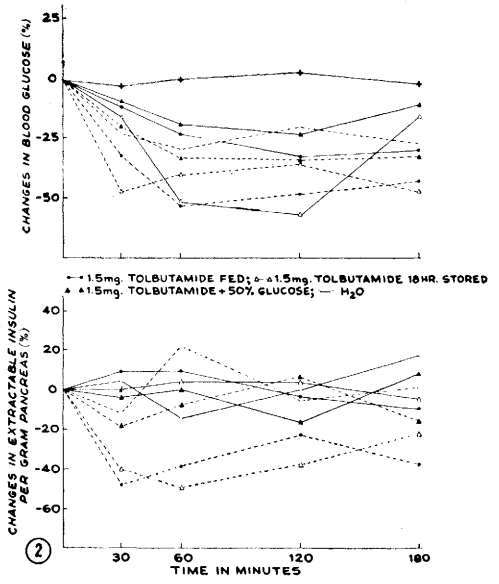


FIG. 1. Effect of concentration of unlabeled insulin on binding of insulin-I^{131} to antibodies obtained from insulin-resistant diabetics.

FIG. 2. Effect of tolbutamide on blood sugar and extractable insulin from pancreas. Solid lines, initial experiments; broken lines, duplicate experiments.

at the origin and bound insulin travels as a single peak with serum proteins. After 1 hr of flow the papers were dried, and distribution of I^{131} activity was determined by dividing the strips into 1 cm segments and counting them in a well-type scintillation counter. To obtain the standard curve, total activity in the bound fraction divided by total activity in the free fraction (B/F ratio) was plotted against concentration of added unlabeled insulin (Fig. 1). Since B/F ratio did not diminish linearly with, or as a logarithmic function of, insulin concentration, it was necessary to obtain at least 5 points corresponding to 5 different concentrations of unlabeled insulin in the incubation mixtures for determining the standard curve. The unknown crude insulin solutions were assayed simultaneously by substituting 25 μl portions of them for nonradioactive insulin-albumin solution used in the standards. Incubation and hydrodynamic flow were carried out as described above. The B/F ratio found for the unknown sample was read against the standard curve to obtain its insulin content. *Animal experiments.* 350 male mice, aged 6-8 weeks and weighing 15-18 g, either fed or starved 18 hr, were used. Only animals varying less than 10% in weight were used on a given day. The mice were given by intubation 1.5 mg tolbutamide, either in 0.2 ml water or 50% glucose solution, and were sacrificed in groups of 4 at intervals during ensuing 3 hr. An equal number of control animals, given either water or 50% glucose solution by intubation, were killed at same intervals. Mice in each group were stunned, then partially decapitated. From each animal, 0.1 ml of jugular vein blood was taken and pooled for blood sugar determination (13). The tail of the pancreas, which contains most of the islet tissue (14) and is compact and comparatively free of fat, was removed and placed in chilled saline. The excised organs from each group of 4 animals were pooled, washed for 5 min in chilled saline, blotted dry and weighed. To precipitate the protein, the pancreas (350-400 mg wet weight) was added to 4 ml of (v/v) chilled saline-10% trichloroacetic acid. After homogenization and centrifugation, the insulin was extracted from the precipitated protein with acidic alcohol (15 ml 12 N HCl diluted to 1 L with 75% ethanol) and further purified by the method of Grodsky and Tarver (7). The final precipitate of crude insulin was dissolved in 0.01 N HCl to give a volume of 0.5 ml. Other organs besides pancreas were treated in similar manner. In assaying, 25 μl of this un-

TABLE I. Assay of Extractable Insulin from Various Tissues of the Mouse.

Tissue	No. of determinations	Extractable insulin, μg/g tissue
Pancreas (tail)	12	25–42
Liver	4	.0
" + $\frac{100\ \mu g\ insulin}{g\ tissue}$	8*	85.7 (S.D. = 8.2%)
Spleen, brain, kidney, lung, heart, intestine	2 each	.0

* Refers to No. of repeated determinations of a single sample.

known insulin solution was incubated with antisera, insulin I^{131} and urea solution as described above. After B/F ratio was determined and amount of insulin in the 25 μl sample was obtained from the standard curve, insulin concentration/g of organ was equal to:

$$\frac{\text{insulin conc. of aliquot x 20 x 1000}}{\text{mg tissue}}.$$

Results. Table I records values obtained for extractable insulin from various tissues of the fed mouse. Of tissues investigated, only the pancreas contained detectable levels of insulin (25-42 μg/g tissue). The efficiency of the acid-alcohol extraction procedure is not known. When insulin-I^{131} was added to pancreas and the mixture extracted in the described fashion, only 45-55% of the label was recovered; the greatest loss occurred during first step of extraction procedure. Higher yields (74%) were obtained by Bornstein-Downie method(15), but this method was unsatisfactory because the extracted proteins were insoluble at neutral and alkaline pH's required for assay.

Recovery of insulin added to extract of liver by binding method of assay was 85.7%. The precision of the method was indicated by the S.D. of 8.2% obtained after repeated assays of a single sample.

Fig. 2 shows the effect of 1.5 mg of tolbutamide on blood sugars and extractable insulin measured in 140 fed and fasted mice. In each experiment (20 animals), blood sugars decreased 25-50% within 60 min. Addition of 50% glucose during intubation increased actual glucose levels but did not affect percentage change after administration of tolbutamide. Intubation of water alone did not affect blood sugar levels.

Changes in amount of extractable insulin after administration of tolbutamide did not correlate with the drop in blood sugar. In 5 experiments involving 100 fed or starved mice (not counting controls), insulin levels remained constant despite severe hypoglycemic response. On 2 separate occasions, once in fed and once in starved mice, a decrease in extractable insulin was observed. The associated hypoglycemia, however, was less, or no more severe, in these animals than in similarly treated mice whose insulin levels remained unchanged.

It has been suggested(16) that the effect of tolbutamide on insulin secretion can be enhanced by simultaneously giving glucose to stimulate pancreatic insulin production. Our data show that intubation of 50% glucose did not modify the effect of tolbutamide on levels of pancreatic insulin.

Discussion. Binding of insulin-I^{131} to antisera forms the basis of an assay method for extractable insulin which is chemically specific for the protein molecule, rather than being dependent on physiological action of the hormone. Steroids, growth hormone, tolbutamide and glucagon do not interfere with the assay(10). Drawbacks of the method are its complexity and the necessity for assuming that mouse insulin and the crystalline beef-pork insulin used in making the standards have the same capacity for binding to the antiserum. Nevertheless, the values of 25-42 μg of insulin/g of mouse pancreas, found in this study, are comparable to those obtained with other methods(unpublished). The actual insulin content of pancreatic tissue may be considerably higher than the reported values, since studies with insulin-I^{131} show only a 50% recovery after extraction. This recovery value cannot be used to correct for total insulin content of pancreas, however, since reportedly crystalline insulin may not have the same extraction characteristics as endogenous or crude insulin(17).

Our studies on immediate effect of tolbutamide indicate that depletion of extractable insulin in mouse pancreas is not consistently seen in conjunction with appearance of hypo-

glycemia. These findings are in line with reports that degranulation of islet cells(18) or deposition of glycogen into dorsal fat(16) does not occur after short-term treatment with sulfonylureas. Contrary to hypothesis in the latter report, the pancreatic insulin level remained unchanged when glucose was administered together with tolbutamide.

The failure to observe depletion of insulin does not necessarily exclude the possibility that tolbutamide stimulates secretion of small quantities of insulin. Because of the limitation of the method a depletion of 15-25% of total insulin content of the pancreas would not be detectable; however, such an amount represents sufficient secretion of insulin (0.7-1.0 μg) to effect a severe hypoglycemia in the mouse. Also, if secretion of insulin were followed by rapid insulinogenesis, a net change in pancreatic insulin might not occur. On the other hand, the data are compatible with the concept of an extrapancreatic action of tolbutamide. Possibly the pancreas is producing an unknown hormone with chemical structure different from that of insulin, capable of exerting a hypoglycemic effect. Tolbutamide may act on the pancreas to stimulate secretion of this hormone. The existence of such a substance, although speculative, would explain why tolbutamide activity reportedly requires a functioning pancreas but does not elicit consistent changes in content of chemically measurable insulin.

Summary. The new method for immunochemical assay of extractable insulin from the pancreas described in this report is based on the change in binding of insulin-I^{131} to insulin antibodies in the presence of varying quantities of unlabeled insulin. The method was used to study changes in extractable pancreatic insulin after administration of tolbutamide to normal mice. Although tolbutamide caused a sharp decline in blood sugar within 2 hours of administration, a consistent decrease of insulin in the pancreas was not observed.

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Interference Between Coxsackie Viruses in Mice. (24846)

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Multiple infections with enteric viruses are of considerable interest and possibly of importance to the ecology of enteric virus diseases(1). The interfering effect of Group B Coxsackie virus infection on poliomyelitis has been extensively studied. More recently, it was found that combined infection with certain Group A viruses and an attenuated polio-