The Effect of Proinsulin on the Immunoassay of Insulin and its Possible Relation to States of Hyperinsulinemia*,† (34056)

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The measurement of insulin or insulin-like moieties by a variety of bioassay and immunologic procedures has resulted in the designation of such nonspecific varieties as typical and atypical insulin (1), bound and unbound insulin (2), suppressible and nonsuppressible insulin (3). Insulin determined by the radioactive immunoassay and referred to as immunoreactive insulin, IRI, has been judged by most to represent an accurate appraisal of absolute insulin levels in biologic fluids (4); as such, its biologic activity, although inferred, has never been entirely certain. In the immunoassay method, the immunospecificity of antigen to specific antibody is of necessity assumed. Accordingly, if the reaction between insulin-I^{131}, insulin antiserum, and endogenous insulin is specific, then serial dilution of serum containing IRI will yield comparable assay measurements over the range of dilutions studied.

In this laboratory where a modification of the Yalow and Berson chromatographic method (4) or the dextran-coated charcoal technique (5) is used to measure radioimmunoassayable insulin, certain sera have consistently failed to show such agreement (6), and have resulted in progressively higher than predicted IRI values with serial dilution of the serum sample. In the main these sera have come from patients in whom the diagnosis of insulinoma was being entertained where unusually high levels were anticipated so that initial measurements of IRI were made at several dilutions. To date, sera from five patients with so-called reactive hypoglycemia and many samples from a patient with organic hyperinsulinism, an insulinoma, have demonstrated this phenomenon of greater than 100% increase in IRI over several-fold dilutions of serum, appropriate dilution eventually resulting in linear agreement (Fig. 1). Furthermore, when present, this phenomenon appears to be greater when absolute IRI values are higher (Fig. 2).

It seemed probable that some other peptide immunologically cross-reactive with insulin could be responsible for this effect. In 1965 Steiner and Oyer (7) described the presence of a precursor of insulin in the cells of this particular islet cell tumor and subsequently in commercial insulin and in isolated islets of Langerhans (8). This protein,

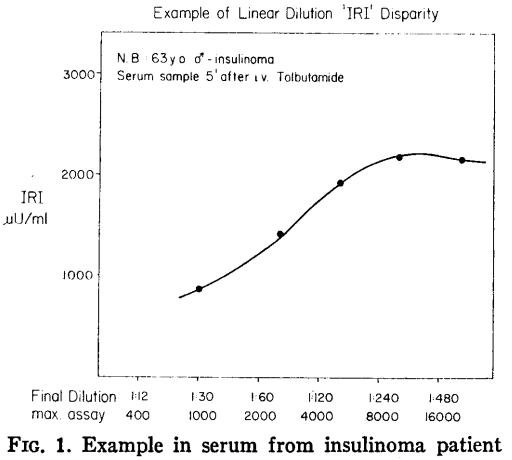

FIG. 1. Example in serum from insulinoma patient of progressive increase in IRI values from a final dilution of 1:12–1:240.

* A report of this work was presented at the annual jointly sponsored subspecialty meetings of the American Federation for Clinical Research and The Central Society, Chicago, Illinois, October 31, 1968.

† This work was supported by the Fisher Endocrine Research Fund and by Grants Hd-01290, AM 5170 and Clinical Research Center Grant FR 55 from the USPHS.

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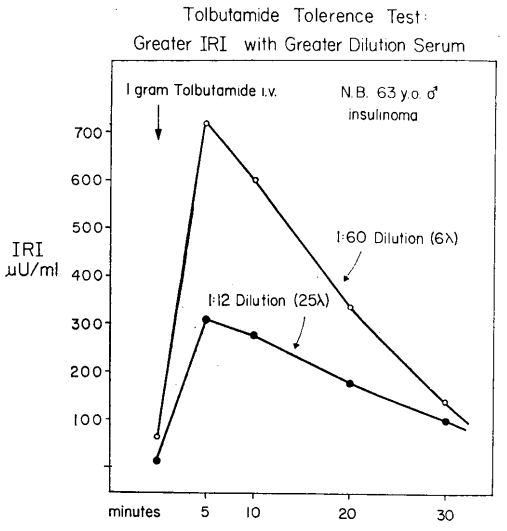

FIG. 2. Example of greater IRI increase with serum dilution where absolute insulin values are higher as, for example, at 5 and 10 min after intravenous tolbutamide.

dubbed proinsulin, consists of a single peptide representing the A and B chains of insulin linked by a connecting peptide of 28 amino acids (8). Although possessing far less biologic activity (9, 10), proinsulin reacts significantly with anti-insulin serum (8). Sephadex fractionation of acid–alcohol extracts of this particular insulinoma serum can be shown to contain material of molecular weight comparable to that of proinsulin which is immunologically reactive with insulin antiserum. Thus it was reasoned early on that significant amounts of proinsulin in serum could account for nonlinear immunoassay results with serial dilution of certain serum samples. This study examines the effect of adding proinsulin to serum on the now widely used and previously assumed, highly specific, radioimmunoassay for serum insulin.

Methods and Results. Figure 3 illustrates the effect of adding a large amount of insulin to fasting serum, an equimolar quantity of porcine proinsulin[2] to fasting serum, and it compares absolute IRI values at varying serum dilutions with serum from the insulinoma patient (10 min after intravenous tolbutamide) and with serum from a normal volunteer 30 min after oral glucose. The addition of proinsulin to fasting serum yielded measurement of material which would previously have been assumed to be biologically active insulin, and additionally had the effect of yielding progressively higher IRI values with serial dilution of the serum sample containing proinsulin. A serum from the insulinoma patient obtained 10 min after intravenous tolbutamide showed a similar phenomenon. Although not shown here, acid–alcohol extraction of this same serum demonstrates identical findings. It can be seen that in normal serum with added proinsulin, final linear agreement corresponded quite well to the known amount of insulin present, although in the form of porcine proinsulin. Fasting serum with added single-component insulin and serum from a normal volunteer after oral glucose did not demonstrate discrepant assay results on serial dilution.

Since the addition of proinsulin to normal serum did cause a nonlinear effect with serial dilution, the avidity of the anti-insulin serum used in this assay for equimolar amounts of iodinated single component and labeled

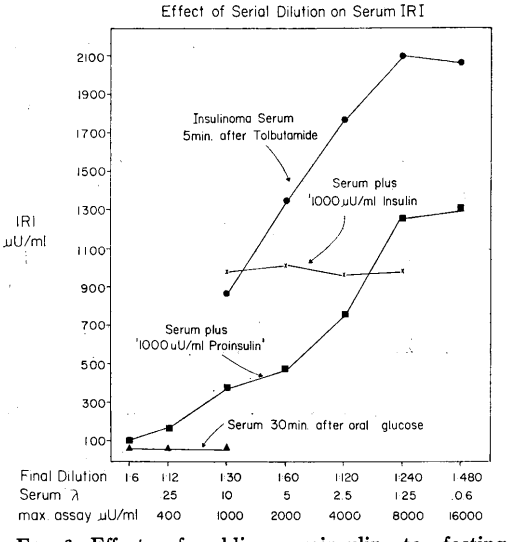

FIG. 3. Effect of adding proinsulin to fasting serum on insulin immunoassay results at serial dilutions.

[2] Generously donated by Dr. Ronald Chance of Lilly Laboratories, Indianapolis, Indiana.

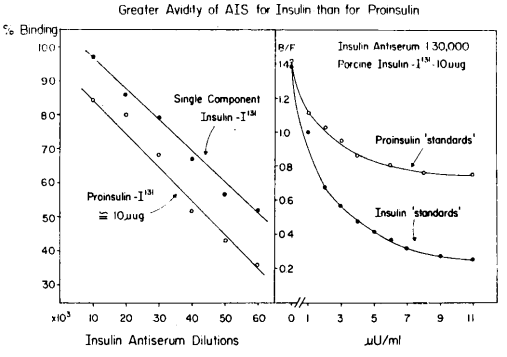

FIG. 4. Demonstration that porcine proinsulin is less effective in displacing insulin-I^{131} from insulin antiserum resulting in less sensitive assay standard curve.

proinsulin was also examined. In Fig. 4 it can be seen that although proinsulin–I^{131} is immunologically cross-reactive with anti-insulin serum in this system, it is not so avidly bound as is insulin–I^{131} at any antiserum dilution examined. It was not surprising, therefore, that equimolar amounts of proinsulin were less effective than insulin in displacing insulin–I^{131} from insulin antiserum, a factor which results in a flatter and less sensitive assay curve when proinsulin standards are substituted for insulin in the assay system (Fig. 4).

Using the radioimmunoassay system, the recovery of human insulin added to the insulinoma patient's serum was compared with recovery from serum containing proinsulin and from normal serum. In Fig. 5 it can be seen that recovery of insulin from normal serum in the presumed absence of proinsulin is high and varies between 90 and 120%. The addition of 2–10 μU/ml of insulin to the insulinoma serum failed to significantly displace radioactive insulin-I^{131} from insulin antiserum yielding less than 50% recovery above 2.5 μU/ml of added insulin. Finally, recovery from the insulin–proinsulin mixture was also poor and varied between 40–60% with higher recoveries of added insulin at lower total insulin concentration. Above 2 μU/ml there was progressive decline in recovery of insulin because of the obvious flattening of the recovery curve.

The frequency with which serial dilution of serum results in progressively higher IRI assay measurements is uncertain. Preliminary screening (Table I) suggests it is indeed uncommon in normal serum, very frequent in our group of so-called reactive hypoglycemics and in our one insulinoma patient studied. The six normal sera which demonstrated greater than 50% increase on successive dilutions were fasting samples in which IRI values were low; *i.e.*, 4–20 μU/ml and accordingly on the steepest portion of the standard curve where small differences in bound/free ratios yield large differences in absolute measurement.

Discussion. Proinsulin is immunologically reactive with the insulin antiserum used in this assay, although less so than insulin. It is more than likely that insulin antiserum used by different laboratories differ in their avidity for proinsulin. Thus, if the phenomenon of progressively higher IRI estimates with a range of serial dilutions of serum is truly related to the presence of proinsulin, then its effect will be demonstrated to a greater or lesser degree by other investigators. Since the addition of proinsulin to an insulin standard curve produced a distorted, flat, and insensitive curve and markedly interfered with recovery of added insulin, its presence may result in false estimates of biologically active insulin. At usual dilutions, the presence of proinsulin in our assay system would result in falsely low total measurable insulin and its presence may therefore account for the frequency with which insulinoma patients display so-called normal IRI values despite profound hypoglycemia (11).

Although exogenously administered proin-

TABLE I. Relative Frequency of Increased IRI Values at Serine Dilutions in Sera of Selected Patients.

Type[a]	No. sera analyzed at different dilutions	% >50–100% ↑ IRI with > dilution
Normal (10)	80	7
Reactive hypoglycemia (7)	53	71
Insulinoma (1)	50	78

[a] Figures in parentheses represent number of patients in group.

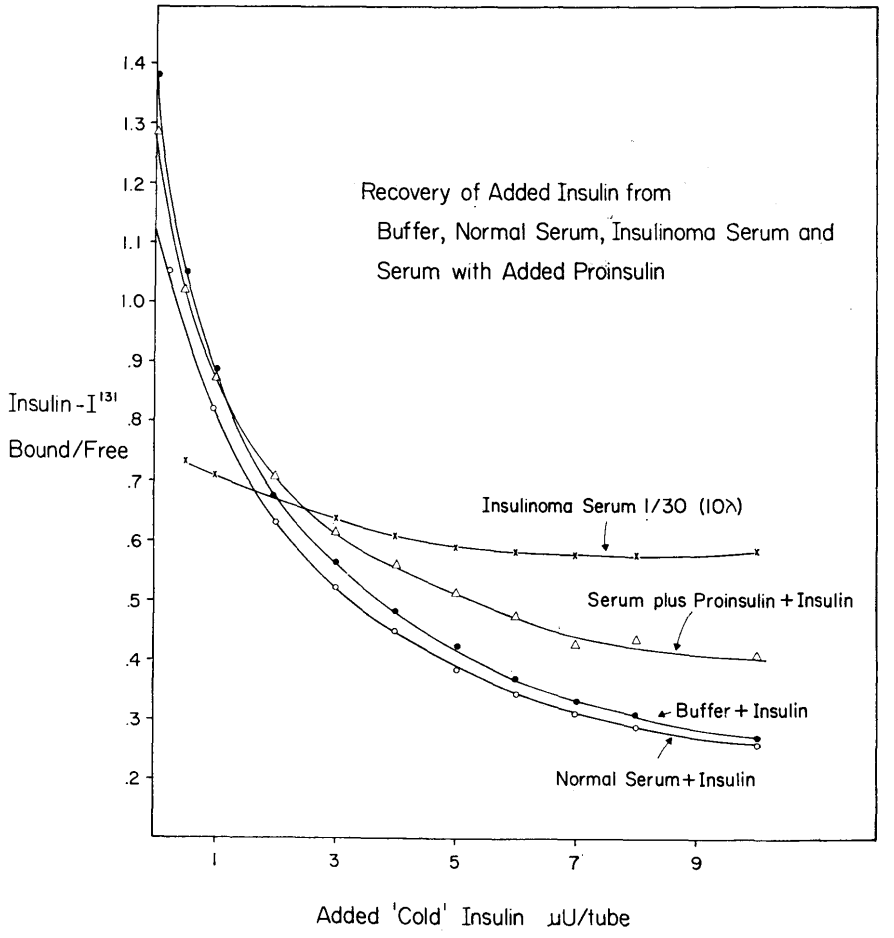

FIG. 5. Recovery of total immunoassayable insulin in presumed absence of and in presence of proinsulin with demonstration of lessened recovery of insulin in presence of proinsulin.

sulin has been reported to have substantially less biologic activity than does insulin (8), it is nevertheless likely that further work in this area will assign a physiologic activity to this insulin precursor and that its presence in large amounts may account for symptoms of hypoglycemia in certain individuals by mechanisms not yet understood. It is hoped that until more discriminatory methodology for the measurement of insulin and proinsulin are available that routine measurement of IRI at several dilutions will help to identify those patients in whom, by inference, the presence of proinsulin could be suspected.

1. Samann, N., Fraser, R., and Depster, W. J., Diabetes **12**, 339 (1963).

2. Antoniades, H. N., Bougas, J. A., and Pyle, H. M., New Eng. J. Med. **267**, 218 (1962).

3. Froesch, E. R., Burgi, H., Ramseier, E. B., Bally, P., and Labhart, A., J. Clin. Invest. **42**, 1816 (1963).

4. Yalow, R. S. and Berson, S. A., J. Clin. Invest. **39**, 1157 (1960).

5. Herbert, V., Lau, K. S., Gottlieb, C. W., and Bleicher, S. J., J. Clin. Endocrinol. **25**, 1375 (1965).

6. Lawrence, A. M. and Kirsteins, L. J., Clin. Lab. Med. **72**, 988 (1968).

7. Steiner, D. F. and Oyer, P., Proc. Natl. Acad. Sci. U. S. **57**, 473 (1967).

8. Steiner, D. F., Clark, J. L., Nolan, C., Rubenstein, A. H., Margoliash, E., Aten, B., and Oyer, P. E., Recent Progr. Hormone Res. 1969 (in press).

9. Steiner, D. F., Hallund, O., Rubenstein, A., Cho, S., and Bayliss, C., Diabetes **18**, 725 (1968).

10. Shaw, W. N. and Chance, R. E., Diabetes **17**, 737 (1968).

11. Samols, E. and Marks, V., Brit. Med. J. **1**, 507 (1963).

Received March 27, 1969. P.S.E.B.M., 1969. Vol. 131.