A Comparative Study of the Insulin Content of Islet Tissue of Various Species Using Anti-Insulin Serum and the Epididymal Adipose Tissue Methods* (34052)

PADMAKAR K. DIXIT, CARL R. MORGAN[1], ARNOLD W. LINDALL, JR.[2], AND ARNOLD LAZAROW

Department of Anatomy, University of Minnesota School of Medicine, Minneapolis, Minnesota 55455

Insulin-like activity (ILA) of serum or serum protein fractions has been measured by using different immunoassay techniques and comparing the activity against fat pad assay method (1, 2). Goetz (3) attempted a comparison of insulin activity in plasma using different bio- and immunoassay techniques and has concluded that each method is valid only for the determination of relative rather than absolute amounts of insulin values. The data presented by him appeared to indicate that there was a greater similarity between the values obtained by mouse hypoglycemia, rat epididymal adipose tissue, and rat diaphragm technique while in general the immunoassay method gave considerably lower values.

Similarly it has been noted by us that the insulin values in the pancreas as determined by the fat pad technique are appreciably higher than those determined by the immunoassay technique. We have therefore, undertaken a study to compare the "insulin" content of the islet tissue obtained from rat and goosefish as determined by these methods. In addition, using the fat pad method we have also studied the effectiveness of insulin antibodies produced in guinea pigs (to beef insulin) in neutralizing these various islet insulin preparations.

Materials and Methods. The procedures for microdissecting the rat pancreatic islets, weighing, and subsequent insulin assay by the fat pad method were carried out as previously described (4); the immunoassay was carried out by the two-antibody method of Morgan and Lazarow (5, 6).

Approximately 20 microdissected rat islets (weighing between 0.42 and 1.37 μg) were suspended in 10 or 20 ml of a bicarbonate buffer medium (pH 7.4) containing 200 mg/100 ml gelatin and 100 mg/100 ml glucose. One-milliliter aliquots of these preparations were used in replicate determinations for assay by the two methods.

Samples of principal islets of the goosefish (*Lophius piscatorius*) were homogenized in 0.25 *M* sucrose solution and then fractionated by differential centrifugation as previously described (7). The various cell fractions were assayed for "insulin" content.

The insulin content of rat and goosefish islet preparations was determined also by the fat pad method after the addition of various amounts of normal guinea pig serum (NGPS) and anti-insulin (to beef-pork) guinea pig serum (AIS-GP) (8).

Results. Figure 1 illustrates a linear correlation between the insulin content of various rat islet preparations as determined by the epididymal fat pad and immunoassay technique, using crystalline beef insulin as the standard. The value for "insulin" content, as measured by the epididymal fat pad method

* This investigation was supported in part by Research Training Grants AM-5127 and AM-6517 from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, USPHS, and also in part by Research Grant H-06308, USPHS.

[1] Present address: Department of Anatomy, Indiana University Medical Center, Indianapolis, Indiana.

[2] Present address: Hennepin County General Hospital, Minneapolis, Minnesota 55415

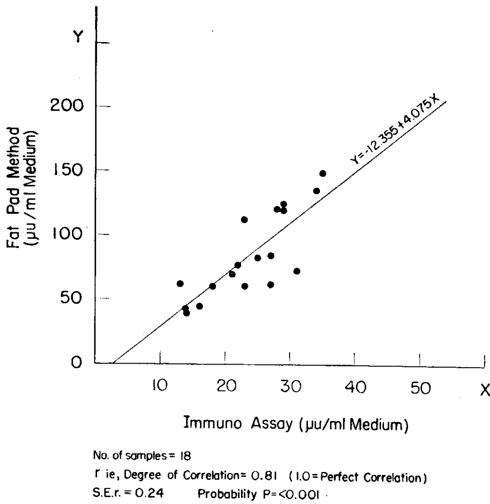

FIG. 1. Comparison of insulin content of rat islet as measured by fat pad and immunoassay techniques.

was approximately four times that obtained by immunoassay. The degree of correlation (r) between the two methods was 0.81 [1.0 being a perfect correlation (9); this result was highly significant ($p<.001$)].

Insulin content of the various subcellular fractions of goosefish islet homogenates determined by the immunoassay and fat pad methods is shown in Fig. 2. In a sucrose homoge-

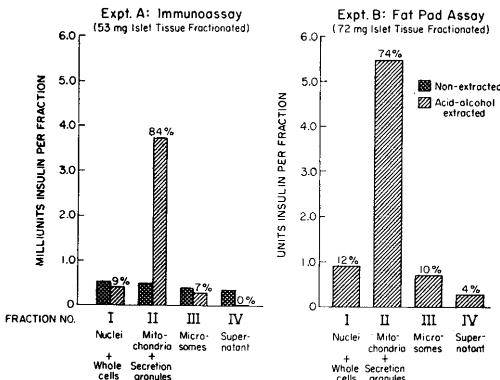

FIG. 2. "Insulin" content of goosefish islet subcellular fractions with or without acid–alcohol extraction.

nate of 53 mg of islet, only 1.73 mU of insulin (or 0.033 units/g) was determined by the immunoassay method. This was distributed evenly in the four fractions (Fig. 2A). When the fractions were extracted with acid–alcohol, fraction II which consisted predominantly of mitochondria and secretion granules, showed an eightfold increase in insulin, while other fractions showed lower quantities (Fig. 2A). Approximately 84% of the total insulin was found to be present in fraction II. After the acid–alcohol extraction, the insulin content of the four fractions isolated from 53 mg of islets totaled 4.4 mU (or 0.083 units/g of islets).

In experiment B, the fractions isolated from 72 mg of islet tissue were extracted with acid–alcohol and the insulin was determined by the fat pad bioassay. A total of 7.4 U of insulin were recovered which is equivalent to 103 U/g of islet tissue. Approximately 74% of the total insulin was found in fraction II (Fig. 2B). When the immunoassay method was used to determine the insulin content of these fractions (after acid–alcohol extraction) about 3.1 mU of insulin were recovered that is equivalent to 0.043 units/g of islet tissue. (These values are not shown in the figure). Using goosefish islets, the epididymal fat pad method measures approximately 1600-fold more insulin activity than does the immunoassay technique. Despite this thousand-fold difference in absolute amount of insulin the relative distribution of insulin in these subcellular fractions was similar irrespective of the assay method used.

We then compared the capacity of anti (beef-pork) insulin serum (produced in guinea pigs) to neutralize beef, rat, and goosefish insulin preparations. Varying quantities of the AIS-GP and/or NGPS were added to the media containing the different insulin preparations and the amount of unbound insulin remaining was determined by the fat pad method. As shown in Fig. 3, a medium containing 500 μU of beef insulin plus 10 μl of NGPS produced a 25% increase in glucose oxidation (by the epididymal fat pad) as compared to medium containing only insulin. By substituting 0.3 μl of AIS-GP for equal amounts of NGPS, the enhancing effect of NGPS was abolished and instead there was a 64% decrease in glucose utilization (as compared with the medium containing 500 μU of insulin plus NGPS).

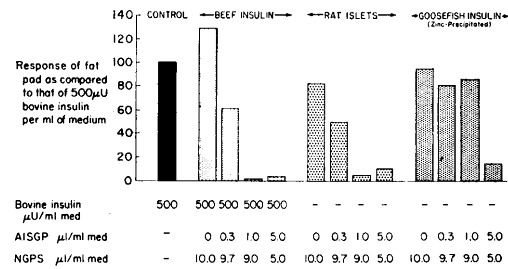

FIG. 3. *In vitro* neutralization by anti-insulin serum. (Comparison of bovine, rat, and goosefish insulin as measured by epididymal fat pad method).

One microliter of AIS-GP completely blocked the effect of 500 μU of beef insulin.

Using a rat (islet) insulin preparation, three-tenths, and 1 μl of antiserum neutralized 40 and 94% respectively of the insulin effect. By contrast, only 8% of the goosefish insulin activity was neutralized by 1.0 μl of AIS-GP; when 5.0 μl of the antiserum was used, approximately 96% of the goosefish insulin activity was neutralized. Thus more than five times the amount of antiserum is required to neutralize equivalent amounts of goosefish insulin (as compared to rat and bovine insulin).

Discussion. In these studies, the insulin content of rat islets, as determined by the fat pad assay, was four times greater than that determined by immunoassay. A similar discrepancy between these two methods has been noted by others (10) using various mammalian insulin preparations. Since the intraperitoneal injection of insulin antiserum (to beef-pork insulin) into rats produces severe diabetic symptoms, the antiserum reacts with biologically active endogenous rat insulin (8, 11). As demonstrated in Fig. 3, this antiserum can also effectively react with and thus neutralize the rat insulin *in vitro*.

The fat pad assay method, which is based on the evolution and determination of ^{14}C-labeled carbon-dioxide, is not specific for insulin, since many other compounds are known to similarly affect the fat pad (12–16). Further, we have observed that the epididymal fat pad tissue of rat responds to insulin obtained from different species of animals; namely, beef, sheep, pork, dog, and human to the same extent (not shown here). Further, the fact that in depancreatized diabetic dogs considerable amounts of insulin can be detected in the plasma using the fat pad method (17–19), also indicates lack of specificity of this method for insulin assay.

On the other hand, when the immunoassay method is used, some of the biologically inactive insulin may be immunologically reactive and conversely some biologically active insulin may not react with insulin antibody and thus give false values by the immunoassay method. Likewise, the variable cross reactivity of insulin from different species (20, 21) precludes the assignment of absolute insulin values when the samples are measured by the immunoassay procedure unless the corresponding species-specific insulin was used in setting up the standard curve. Since quantitative recovery of added beef insulin from rat plasma has been reported (22), the immunological differences between beef insulin and extracted rat insulin observed by Karam and Grodsky (23) need further investigation. It should be emphasized, however, that the high degree of correlation between the two assay methods over a considerable concentration range, suggests that either method should be useful in measuring relative differences in the insulin content of rat tissues provided that the analyses are carried out under carefully controlled conditions.

Using the goosefish secretion granule fraction, acid–alcohol extraction markedly increased the apparent insulin content as measured by the immunoassay method. This suggested that the insulin contained in the secretion granules was not available to react with insulin antibodies; presumably the acid–alcohol extraction either modifies the secretion granule membrane to make the insulin more soluble or it activates the insulin molecule by releasing it from a complex within the secretion granule.

Steinke *et al.* (19) have observed that extracted serum obtained from depancreatized dogs was ineffective on the rat diaphragm, whereas after acid–alcohol extraction this serum has significant insulin-like activity, thus indicating that acid–alcohol extraction alters endogenous insulin.

By contrast there was no increase in the

apparent insulin content when the fat pad assay method was used after acid–alcohol extraction of goosefish islet fraction II (consisting of mitochondria and secretion granules). (These results are not shown in the text.) We have also found that the insulin released *in vitro* from toadfish islets (24) reacted directly with rat epididymal fat pad without prior acid–alcohol extractions. Thus, the fat pad may release insulin from the secretion granule, or it may activate the native form of insulin which is contained in the secretion granule. The immunoassay method, on the other hand, may be dependent upon the presence of free insulin.

The 1600-fold difference which we observed in the apparent insulin content of goosefish islet tissue fractions (when analyzed by the two methods), is considerably greater than that observed by other investigators (10). There are marked immunological differences between certain fish (*e.g.*, cod, pollack, and catfish) and mammalian insulins (25). Thus 40 times the amount of antibody (horse anti-bovine insulin serum) was required to neutralize cod insulin as compared to bovine, horse, or whale insulins (26). It is of interest, however, that despite the more than 1000-fold difference in absolute value, the relative amounts of insulin detected in the various subcellular goosefish islet fractions was the same when determined by either immunoassay or the epididymal fat pad methods.

In using the fat pad assay method we found that, the addition of 10/μl of NGPS augmented the effect of 500 μU of bovine insulin. Power and co-workers (27) also demonstrated that the addition of 2 mg of globulin (isolated from normal human serum) similarly augmented the effect of crystalline insulin on the fat pad. Assuming a globulin concentration of approximately 4 g/100 ml the 10 μl of the NGPS added would then contain 0.4 mg of the globulin. We do not know whether this amount of globulin would account for the 30% increase in insulin activity observed in Fig. 3. Whatever the cause of this potentiation, 1 μl of AIS-GP almost completely neutralized the effect of bovine and rat insulin (Fig. 3). Since at least five times the quantity of anti-insulin serum was required to block the effect of goosefish insulin, the anti-(beef-pork) insulin serum does not appear to react so readily with goosefish insulin as it does with beef or rat insulin. Furthermore, the goosefish insulin is almost ineffective in displacing beef ^{131}I insulin from its combination with the antibody, and hence the assay of goosefish insulin by the immunoassay method gives considerably lower values.

In assaying the insulin content of rat tissues by the immunoassay method anti-beef insulin serum may be used but it is advisable to use rat insulin for the standard curve. On the other hand, in analyzing goosefish tissues by the immunoassay method, it is necessary to prepare an antiserum to highly purified goosefish insulin in addition to using a goosefish insulin for the standard curve.

Summary. The insulin content of microdissected rat islets when measured by the two-antibody immunoassay method was approximately one-fourth that obtained when the epididymal fat pad method was used. Insulin content of the subcellular goosefish islet fractions after acid–alcohol extraction was approximately 103 U/g when measured by the fat pad method, as compared to 0.063 U/g when measured by the immunoassay method. Thus there is a 1600-fold difference in the values obtained using these two methods.

About 84% of the total insulin contained in goosefish islets (estimated by the immunoassay method, after acid–alcohol extraction) was recovered in the mitochondria plus secretion granule fraction. Using the fat pad, 74% of the total insulin was found in this subcellular fraction. Acid–alcohol extraction of the secretion granule plus mitochondria, produced an eightfold increase in apparent insulin content when measured by the immunoassay method; thus most of the insulin contained in the secretion granule was not available to react with the insulin–antibody until it was extracted or modified by the acid–alcohol extracted and unextracted subcellular fraction.

One microliter of antiserum (to beef-pork insulin) was able to completely neutralize the effect of 500 μU of either bovine or rat

(islet) insulin as measured by the fat pad method. However, five times this amount of antiserum was needed to neutralize the same quantity of goosefish insulin. The observed 1600-fold difference in insulin content of goosefish islet tissue when measured by the fat pad and immunoassay methods indicates that goosefish insulin is hardly able to displace beef ^{131}I insulin from its combination with the (beef–pork) antibody, whereas in the absence of a competing insulin only five times the quantity of antiserum is required to neutralize the effects of goosefish insulin.

Excellent technical assistance was provided by Mrs. Skaidra Ogrins.

1. Samols, E. and Bilkus, D. Proc. Soc. Exptl. Biol. Med. **115**, 67, (1963).

2. Kuzuya, T. and E. Samols, Fifth Congress of International Diabetes Federation, Toronto, Canada, 46, (1964).

3. Goetz, F. C., *in* "On the Nature and Treatment of Diabetes" (B. S. Leibel and G. A. Wrenshall, Eds.) p. 185. Excerpta Med. Foundation, (1965).

4. Dixit, P. K., Lowe, I. P., Heggestad, C. B., and Lazarow, A., Diabetes **13**, 71 (1964).

5. Morgan, C. R. and Lazarow, A., Proc. Soc. Exptl. Biol. Med. **110**, 29 (1962).

6. Morgan, C. R. and Lazarow, A., Diabetes **13**, 1 (1964).

7. Lindall, A. W., Jr., Bauer, G. E., Dixit, P. K., and Lazarow, A., J. Cell. Biol. **19**, 317 (1963).

8. Dixit, P. K., Morgan, C. R., Lowe, I. P., and Lazarow, A., Metabolism **12**, 642 (1963).

9. Bancroft, A., "Introduction to Biostatistics," Harper (Hoeber), New York (1962).

10. Berson, S. A. and Yalow, R. S., Am. J. Med. **31**, 874 (1961).

11. Armin, J., Grant, R. T., and Wright, P. H., J. Physiol. **153**, 146 (1960).

12. Lynn, W. S., MacLeod, R. M., and Brown, R. H., J. Biol. Chem. **235**, 1904, (1960).

13. Mirsky, I. A. and Perisutti, G., Endocrinology **71**, 158 (1962).

14. Dole, V. P., J. Biol. Chem. **237**, 2758 (1962).

15. Dixit, P. K. and Patel, N. G., Nature **202**, 189 (1964).

16. Dixit, P. K. and Lazarow, A., Am. J. Physiol. **213**, 849 (1967).

17. Leonards, J. R., Federation, Proc. **18**, 272, (1959).

18. Goldberg, H. L. and Egdahl, R. H., Federation Proc. **20**, 190 (1961).

19. Steinke, J., Sirek, A., Lauris, V., Lukens, F. D. W., and Renold, A. E., J. Clin. Invest. **41**, 1699, (1962).

20. Yalow, R. S. and Berson, S. A., Am. J. Med. **31**, 882 (1961).

21. Prout, T. E., Metabolism **12**, 673, (1963).

22. Morgan, C. R. and Lazarow, A., Diabetes **12**, 115 (1963).

23. Karam, J. M. and Grodsky, G. M., Proc. Soc. Exptl. Biol. Med. **109**, 451 (1962).

24. Watkins, D., Cooperstein, S. J., Dixit, P. K., and Lazarow, A., Science **162**, 283, (1968).

25. Wilson, S. and Dixon, G. H., Nature **191**, 876 (1961).

26. Moloney, P. J. and Aprile, M. A., Can. J. Biochem. Physiol. **37**, 793 (1959).

27. Power, L., Lucas, C., and Conn, J. W., Metabolism **14**, 104 (1965).

Received March 20, 1969. P.S.E.B.M., 1969, Vol. 131.