

# Insulin Biosynthesis in Experimental Hereditary Diabetes

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## SUMMARY

Studies of proinsulin-insulin relationships were performed on serum and isolated pancreatic islets from normal (C57BL/Ks) and spontaneously diabetic (C57BL/Ks-*dbdb*) mice. The diabetic syndrome in these animals is inherited as an autosomal recessive trait with complete penetrance and is characterized by the early development of obesity and hyperinsulinemia, followed by progressive hyperglycemia.

Gel filtration chromatography of diabetic mouse serum revealed that proinsulin comprised a relatively small proportion (9.6 per cent) of the total circulating insulin immunoreactivity.

Although diabetic mouse islets were enlarged, insulin content per islet was diminished, while proinsulin content per islet was similar to normal. Islet proinsulin:insulin ratios correlated significantly with both blood glucose and serum insulin levels. This appeared to result from the fact that

sustained hyperglycemia caused mainly a depletion of islet insulin, whereas proinsulin content remained unchanged, thereby increasing proinsulin:insulin ratios.

In vitro labeling experiments with H-3-leucine demonstrated no significant difference in the rate or pattern of incorporation of radioactivity into proinsulin and insulin fractions of normal and diabetic islets after 90 and 180 minutes of incubation.

Therefore, the apparent biological ineffectiveness of the high circulating levels of immunoreactive insulin in this diabetic syndrome did not appear to be the result of a disproportionate release of biologically less potent proinsulin, nor did there appear to be a detectable abnormality in proinsulin-insulin biosynthesis as assessed by these methods. *DIABETES* 20:677-85, October, 1971.

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Several investigators have suggested that hereditary diabetes in laboratory animals and in man is attributable to a defect in insulin biosynthesis or secretion.<sup>1-7</sup> Recently, it has been established in man and in lower animals that insulin biosynthesis proceeds through proinsulin, a larger single chain polypeptide,<sup>8</sup> and that both molecules are secreted into the circulation.<sup>2,9</sup> Numerous investigations have demonstrated diminished biologic potency of proinsulin,<sup>10-16</sup> but its precise extrapancreatic role in metabolic regulation remains undefined. To date,

few studies have critically examined the hypothesis that abnormal biosynthesis or defective conversion of proinsulin to insulin, or disproportionate proinsulin secretion might accompany the onset of spontaneous diabetes mellitus.

These studies assess this hypothesis in diabetic mutant C57BL/Ks-*dbdb* mice. Diabetes in these animals involves the expression of an autosomal recessive gene (*db*) with complete penetrance. In the early stages, the syndrome is characterized by obesity, greatly elevated levels of serum immunoreactive insulin (IRI) and hyperglycemia.<sup>17,18</sup> It was postulated that increased amounts of proinsulin with high immunoreactivity but low biologic activity might explain, at least in part, these findings; therefore, gel filtration chromatography was employed to fractionate insulin and proinsulin in serum and pancreatic islet extracts from both normal and diabetic mice. In addition, proinsulin and insulin biosynthesis was assessed in both normal and diabetic isolated

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islets by radioactive leucine incorporation studies. The results of these experiments do not support the hypothesis that a defect in insulin biosynthesis or proinsulin secretion accompanies the diabetic syndrome in these mice.

#### METHODS

*Animal preparation.* Male and female diabetic mutant mice from the C57BL/Ks-*dbdb* strain and normal male and female mice from the C57BL/Ks strain were obtained either from the Production Department of The Jackson Laboratory, Bar Harbor, Maine, or from an inbred colony maintained in our laboratory. Animals were fed Old Guilford mouse pellets\* containing 7.5 per cent fat and allowed free access to water. Normal mice were fed ad libitum throughout life and were sacrificed by decapitation at fourteen to sixteen weeks of age. Diabetic mice were placed on a schedule of food restriction and refeeding in order to facilitate islet isolation by promoting islet enlargement and by retarding structural alterations in the islets.<sup>18</sup> This schedule consisted of food restriction three times weekly (eight hours on Monday, Wednesday and Friday) between the seventh and eleventh weeks of life, followed by return to ad libitum feeding until sacrifice at fourteen to sixteen weeks.

*Pancreatic islet preparation, incubation, and extraction.* Isolated islets were prepared similarly to the collagenase† method of Lacy and Kostianovsky.<sup>19</sup> Groups of twenty-five islets were incubated for either ninety or 180 minutes under continuous gassing with water saturated 95 per cent O<sub>2</sub>-5 per cent CO<sub>2</sub> in 150  $\mu$ l. Krebs-Ringer bicarbonate buffer supplemented with nineteen naturally occurring amino acids,‡ 100  $\mu$ M each (leucine excepted), 16.7 mM glucose,§ 0.029 mM bovine serum albumin,|| 1  $\mu$ g. per ml. soybean trypsin inhibitor,|| and 100  $\mu$ Ci per ml. 3-H-4, 5-l-leucine (SA 58 Ci per mmole).\*\*

Acid-ethanol extraction of islets plus incubation medium was accomplished following precipitation with 250  $\mu$ l. cold 10 per cent trichloroacetic acid containing 1 mM carrier l-leucine. The precipitate was washed twice with cold 5 per cent trichloroacetic acid containing 1 mM l-leucine and dissolved in 0.70 ml. cold acid-

ethanol.<sup>20</sup> The extract volume was adjusted to 1 ml. with 0.3 ml. 3 M acetic acid and stored at -20° C. prior to gel chromatography. Recovery experiments indicated 94 to 97 per cent recovery of added I-125-insulin by this extraction procedure.

*Gel chromatography.* A 0.5 ml. aliquot from each sample was fractionated by polyacrylamide gel chromatography,\* column dimensions 1.5 by 90 cm., at room temperature. Column standardization was accomplished by chromatography of 500  $\mu$ g. ovalbumin,† 150  $\mu$ g. porcine proinsulin,‡ and 400  $\mu$ g. purified mouse insulin. Protein was determined in effluent fractions, following lyophilization, by the method of Lowry<sup>21</sup> using porcine insulin as standard.

A pooled serum sample from diabetic mice was chromatographed on a column equilibrated with borate-albumin buffer, pH 8;<sup>22</sup> immunoassay was performed on 1 ml. effluent fractions. Acid-ethanol extracts of incubated isolated islets were chromatographed on columns equilibrated with 3 M acetic acid. Two ml. fractions were collected; 1 ml. was mixed with 2 ml. Bio-Solv Solubilizer BBS-3§ plus 10 ml. toluene liquid scintillation counting fluid and assayed for radioactivity in a Beckman LS-133 liquid scintillation spectrometer. The remaining 1 ml. was lyophilized, reconstituted in 1 ml. borate-albumin buffer<sup>22</sup> and immunoassayed for insulin. Recovery was calculated by dividing the sum of the immunoreactivity in each fraction of the chromatogram by the immunoreactivity in the 0.5 ml. aliquot applied to the column.

*Insulin immunoassay.* Samples were assayed for immunoreactivity by a modification<sup>22</sup> of the double antibody method of Morgan and Lazarow<sup>23</sup> using purified mouse insulin as standard. Mouse insulin was prepared by extracting 50 gm. of frozen mouse pancreas,|| according to the method of Davoren<sup>20</sup> and purified by polyacrylamide gel chromatography in 3 M acetic acid to a specific activity of 25 U. per mg., as determined by fat pad bioassay.<sup>24</sup> The insulin antiserum used reacted identically with porcine proinsulin and human insulin on an equimolar basis. Immunochemical comparison with mouse proinsulin has not been accomplished to date.

*Miscellaneous procedures.* Glucose was determined by

\* Emory Morse Company, Guilford, Connecticut.

† Collagenase, Lot CLS-8DA, Worthington Biochemical Corp., Freehold, N.J.

‡ Nutritional Biochemicals Corp., Cleveland, Ohio.

§ Fisher Chemical Co., Boston, Mass.

|| Sigma Chemical Co., St. Louis, Missouri.

\*\*Schwartz Bioresearch, Orangeburg, New York.

\*Bio-Gel P-30, 100-200 mesh, Bio-Rad Laboratories, Richmond, California.

† Sigma Chemical Co., St. Louis, Missouri.

‡ A gift from Eli Lilly and Company, Indianapolis, Indiana.

§ Beckman Instruments, Inc., Fullerton, California.

|| Pel-Freeze Biologicals, Inc., Rogers, Arkansas.

the method of Hoffman<sup>25</sup> adapted for the Technicon AutoAnalyzer. Statistical analyses were performed on Mathatron and Digital PDP-12 computers using standard programs for computation of mean, SEM, linear regression analysis, and unpaired *t* test.

RESULTS

Figure 1 summarizes the information at the time of sacrifice for the fourteen to sixteen week old mice used in the study. All animals were sacrificed between 10 and 11 a.m. and had no access to food for two hours prior to sacrifice. Mean body weight of the normal group was 26.5 gm., mean blood glucose 118 mg. per 100 ml., and mean immunoreactive insulin (IRI) 20  $\mu$ U. per ml. Values for diabetic animals exceeded normal values and were more variable. Mean body weight of the diabetic group was 56 gm., mean blood glucose 355 mg. per 100 ml., and mean IRI, 936  $\mu$ U. per ml.

Diabetic mouse serum was pooled and 0.5 ml. fractionated by polyacrylamide gel chromatography to ascertain the proportion of proinsulin to insulin. The resulting chromatogram is shown in the second graph of figure 2, below the standardization data for the column. The pooled serum total IRI was 775  $\mu$ U./0.5 ml.; 75  $\mu$ U. was recovered in the area of distribution of porcine proinsulin and 700  $\mu$ U. was recovered in the insulin distribution area of the chromatogram, indicating a proinsulin:insulin ratio of 0.107. Normal mouse serum IRI was too low to allow fractionation for comparison; however, the results obtained were consistent with previously described serum proinsulin:insulin relationships in

normal humans<sup>9</sup> and indicated that the high level of circulating IRI in these mutant diabetic mice was not due to an excessive proportion of proinsulin.

In the next group of experiments pancreatic islet proinsulin-insulin content and biosynthesis were studied. A chromatogram from a representative experiment with twenty-five islets is illustrated in the third graph of figure 2. Four peaks of radioactivity were observed. The first coincided with the void volume of the column and probably represented albumin-bound H-3-leucine (see below). The second peak, found in all chromatograms, represented an unidentified protein synthesized by both normal and diabetic islets; the third and fourth peaks represented proinsulin and insulin respectively, and coincided with measured immunoreactivity.

Control experiments further supporting the above interpretation of the chromatograms are shown in the fourth and fifth graphs of figure 2. Control experiments with no islets resulted in no immunoreactivity and radioactivity only in the first peak, supporting the tentative interpretation that this peak may represent albumin-bound H-3-leucine. Although carrier leucine was added to the trichloroacetic acid precipitant, complete displacement of labeled amino acid did not occur. The control experiment with cycloheximide\* (250  $\mu$ g. per ml.), an inhibitor of protein synthesis, resulted in no incorporation of label into protein.

Immunoreactive data from ten experiments with normal islets and twelve experiments with diabetic islets

\* Sigma Chemical Company, St. Louis, Missouri.

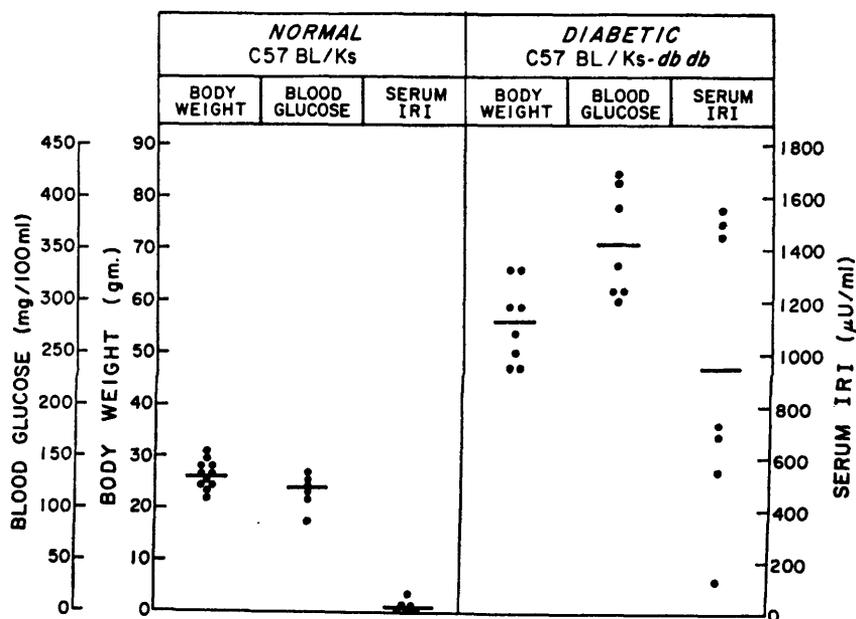


FIG. 1. Comparison of baseline data between normal and diabetic animals at time of sacrifice.

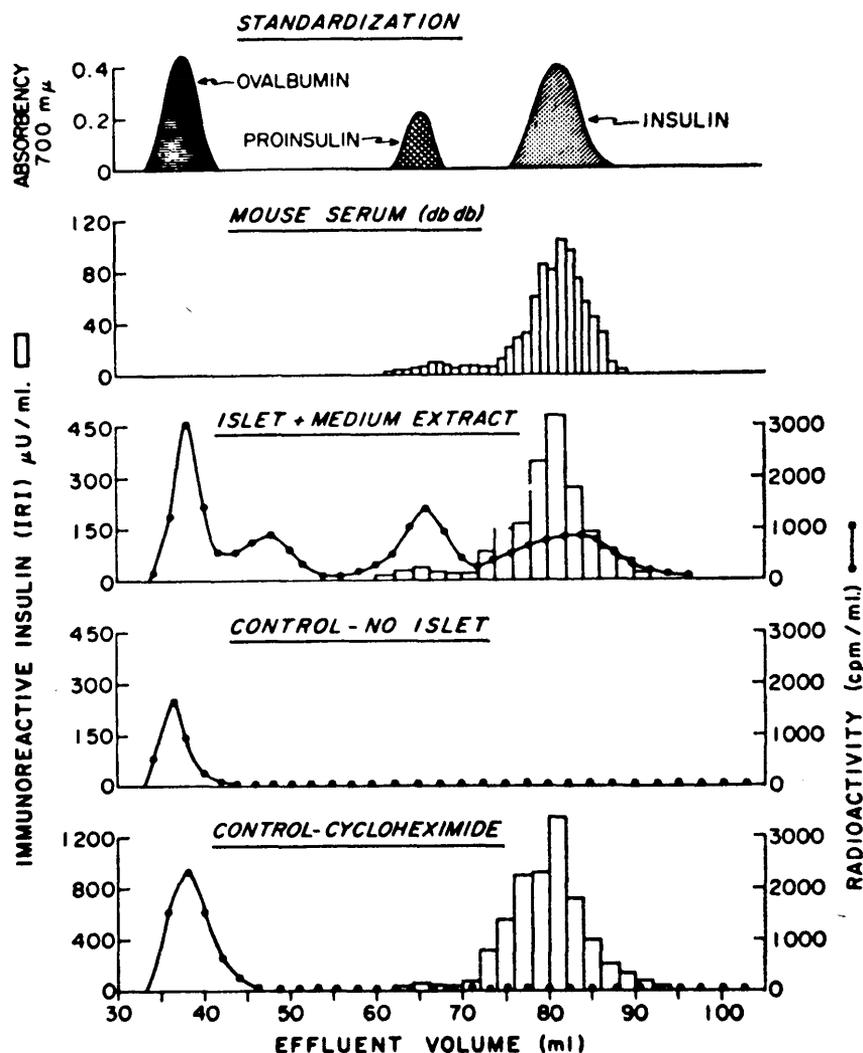


FIG. 2.

Polyacrylamide gel chromatography of diabetic mouse serum and pancreatic islet extracts. Immunochemical data resulting from chromatography of diabetic mouse serum is shown in the second graph. The third graph depicts representative immunochemical and radioactive data resulting from chromatography of extract of islets plus incubation medium. The fourth and fifth graphs illustrate chromatography of extracts from control experiments, one with no islets in the incubation medium, the other with cycloheximide present.

are summarized in figure 3. The mean recovery of immunoreactivity following chromatography in this series of experiments was  $77 \pm 2.9$  per cent for the normal group,  $78 \pm 7.3$  per cent for the diabetic group. Mean proinsulin content of normal islets was  $1.104 \pm 0.142$  mU./25 islets; mean proinsulin content of diabetic islets,  $1.248 \pm 0.190$  mU./25 islets. This difference was not significant. Mean insulin content of normal islets was  $36.980 \pm 2.980$  mU./25 islets, and  $15.900 \pm 2.516$  mU./25 islets for the diabetic group. This difference was highly significant ( $p < .001$ ); however, these data are not corrected for differences in mass between diabetic and normal islets. Diabetic islets are larger and possess more DNA than normal islets, indicating that they contain a greater number of cells.<sup>18</sup> If correction for differences in islet mass could be calculated, this might further serve to em-

phasize the significance of the difference between the insulin content of diabetic and normal islets, and might result in a significantly lower proinsulin content in diabetic islets.

Further analysis of these data revealed a higher proinsulin:insulin ratio in diabetic islets. The significance of this higher ratio in diabetics is illustrated in figure 4. Mean normal islet proinsulin:insulin ratio was 0.0305, compared to 0.0822 for diabetic islets ( $p < .001$ ). This difference was largely due to the lower stored insulin content in diabetic islets.

It was postulated that insulin biosynthesis in mutant diabetic animals probably proceeds at an enhanced rate in order to maintain the high circulating levels observed. Comparative incorporation studies with H-3-leucine were performed to assess this possibility and are represented in figure 5. The proinsulin:insulin portion of gel chro-

Polyacrylamide gel chromatography

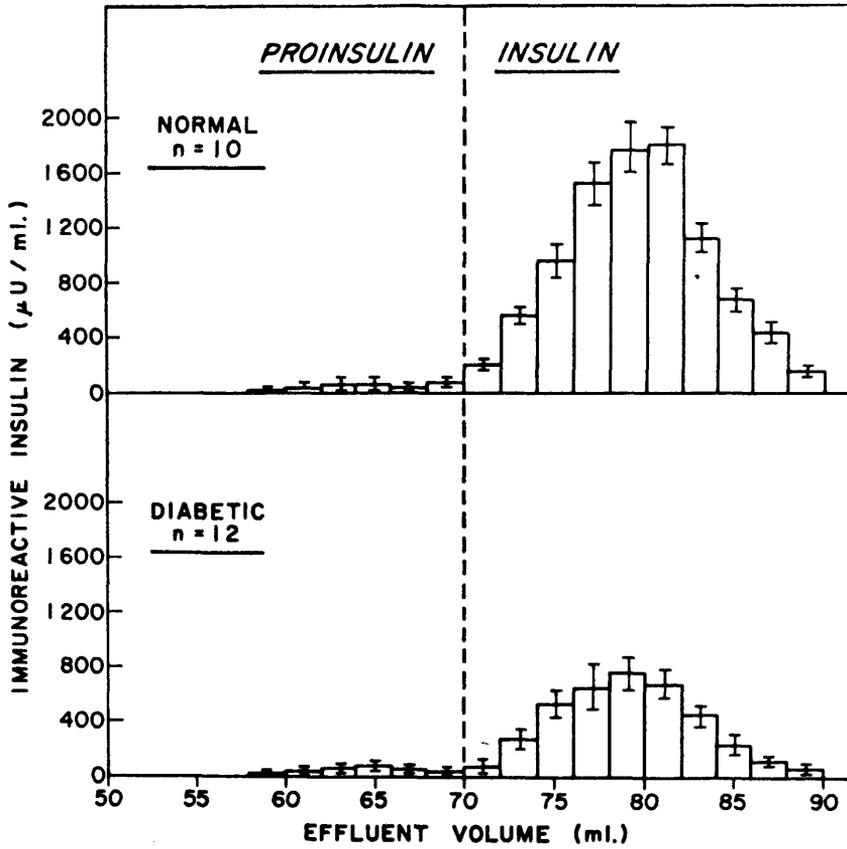


FIG. 3.

Immunoreactive proinsulin-insulin content of pancreatic islet extracts. Summary immunochemical data  $\pm$  SEM of polyacrylamide gel chromatograms are shown; "n" refers to the number of experiments.

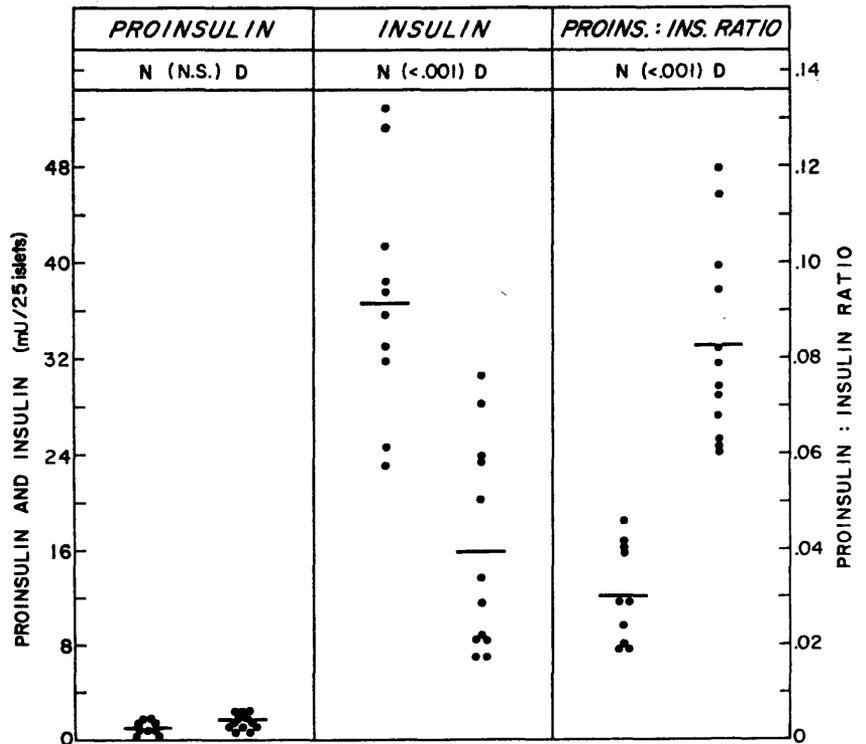


FIG. 4.

Quantitative comparison of proinsulin and insulin content between normal and diabetic pancreatic islets. The data are derived from immunochemical analysis of gel chromatograms of incubated pancreatic islet extracts.

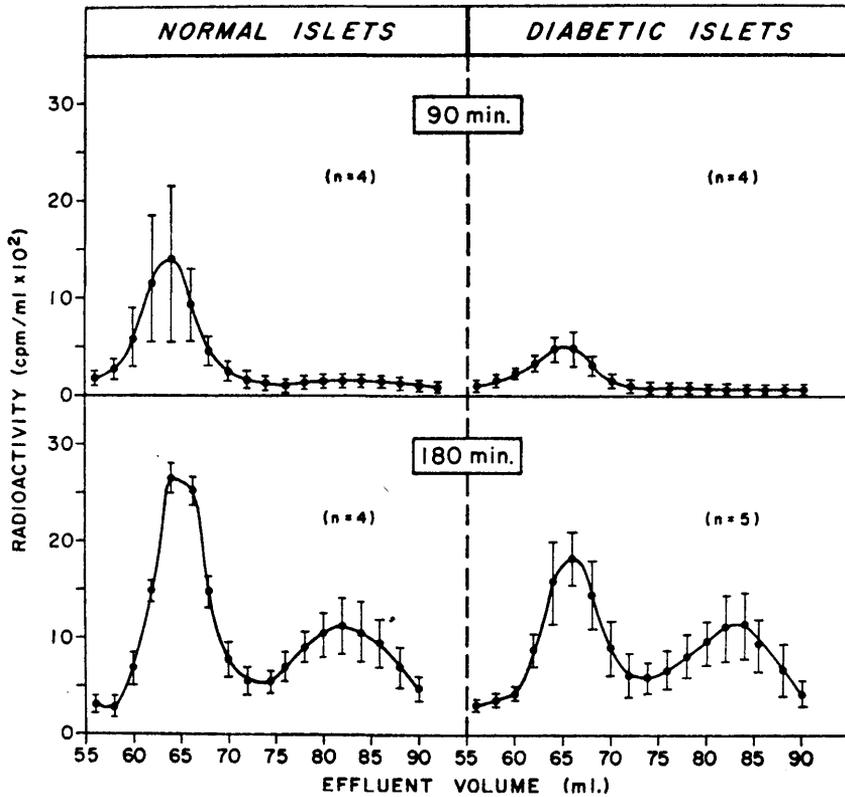


FIG. 5.

Incorporation of H-3-leucine into proinsulin-insulin fractions of normal and diabetic pancreatic islets at 90 and 180 min. of incubation. Summary radioactive data  $\pm$  SEM of polyacrylamide gel chromatograms are shown; "n" refers to the number of experiments. Incorporation into the proinsulin fraction is represented by the first peak in each chromatogram; incorporation into insulin is depicted by the second peak.

matograms from seventeen experiments is shown. Normal islets compared to diabetic islets appeared to incorporate more label into proinsulin at both 90 and 180 minutes of incubation; however, these modest increases did not achieve statistical significance ( $p < 0.05$ ). Furthermore, the appearance of label in the insu-

lin fraction of both groups at 180 minutes was nearly identical. These incorporation studies suggested that diabetic islets possessed a potential for proinsulin-insulin biosynthesis that was comparable to normal islets in this in vitro system.

Figure 6 depicts the linear relationship found between

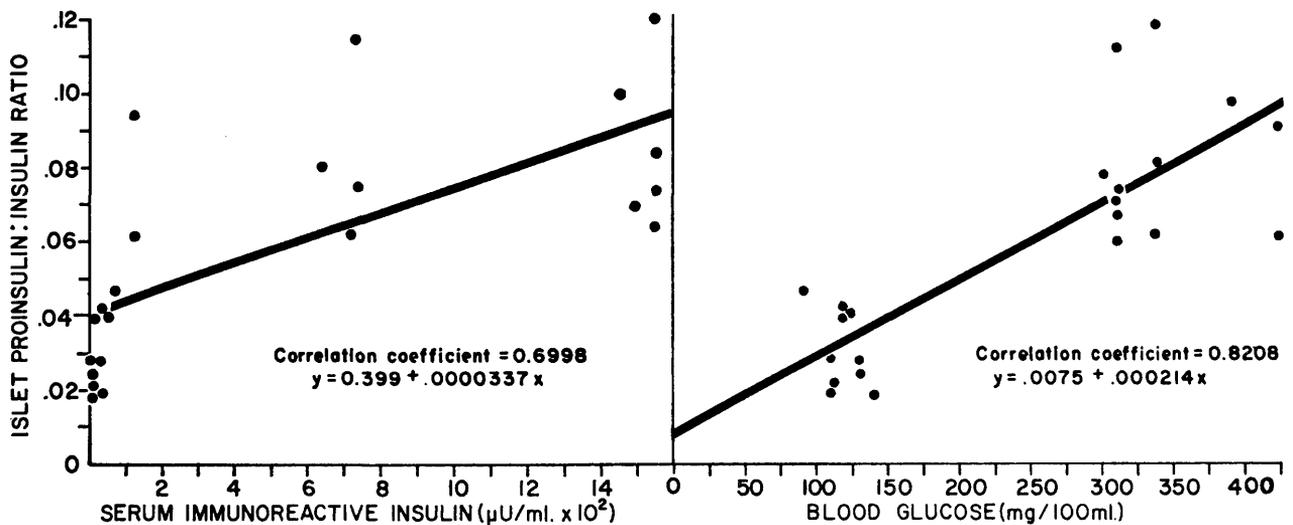


FIG. 6. Linear regression analyses of pancreatic islet proinsulin:insulin ratios to serum immunoreactive insulin or blood glucose are shown.

pancreatic islet proinsulin:insulin ratios and blood glucose ( $r = 0.8208$ ,  $p < .001$ ) or serum IRI ( $r = 0.6998$ ,  $p < 0.001$ ) in these mice. Interpreted in the context of both serum and pancreatic islet fractionation studies, these correlations inferred that the presence of sustained hyperglycemia, or the maintenance of high serum IRI, resulted mainly in depletion of islet insulin content. Islet proinsulin content apparently remained unchanged in these animals.

#### DISCUSSION

Since the discovery of the diabetic mutant mouse (C57 BL/Ks-*dbdb*) in 1965,<sup>26</sup> several aspects of the syndrome have been studied in detail.<sup>17,18,27-30</sup> A number of these features, including resemblances to human diabetes, make this animal a particularly useful model for examining possible pathologic alterations in proinsulin-insulin biosynthesis.

The development of the syndrome in these animals is characterized by early obesity and elevated levels of plasma immunoreactive insulin (three to four weeks of age), followed (at five to six weeks of age) by hyperglycemia, more marked hyperinsulinemia, and rapidly progressive adiposity. Subsequently, serum insulin levels fall, hyperglycemia becomes more pronounced, and weight loss eventually ensues prior to death at five to seven months of age.

We have delayed the onset of severe progressive hyperglycemia and fall in serum immunoreactivity in these animals by utilizing a restricted feeding schedule from seven to eleven weeks of age. Our findings of marked hyperinsulinemia (mean IRI of 936  $\mu\text{U./ml.}$ ) and hyperglycemia (mean blood glucose of 355 mg/100 ml.) during the period following this restricted feeding schedule confirms previous observations in this laboratory.<sup>18</sup> Two possible explanations for this persistent hyperglycemia despite markedly elevated levels of serum immunoreactive insulin are: (1) that enhanced biosynthesis and release of proinsulin occurs, or (2) that tissue resistance to insulin action exists.

Our results do not support the first possibility. Gel chromatography of a diabetic mouse serum pool containing 1,550  $\mu\text{U./ml.}$  IRI showed proinsulin comprised 9.6 per cent of the total; consequently, inordinately high circulating levels of proinsulin do not appear to accompany the diabetic state or explain the apparent biological ineffectiveness of the high serum IRI levels. It is alternately possible that defective coding of amino acids or subtle conformational alterations might also result in reduced biological potency; however, evidence for this at present in other species is scanty.<sup>31</sup>

Fractionation of normal and diabetic islet insulin immunoreactivity corroborated the serum findings. Summary data on a total of 250 islets from eleven normal animals and 300 islets from eight diabetic animals, studied in groups of twenty-five islets, showed that proinsulin comprised 2.9 and 9.4 per cent of the total immunoreactivity in normal and diabetic islets, respectively. The higher per cent of proinsulin in diabetic islets was due mainly to decreased insulin content; whereas, proinsulin content was relatively unchanged. The normal mouse proinsulin:insulin ratio is similar to previously reported findings in other mammalian species.<sup>8</sup>

The finding of decreased insulin content in diabetic islets is consistent with the degranulation observed histologically in previous studies at this stage of the diabetic syndrome in these animals.<sup>28</sup> In addition, the significant linear correlation between islet proinsulin:insulin ratios and serum IRI or glucose levels emphasizes the consistency of this finding, and supports the interpretation that persistent hyperglycemia promotes mainly depletion of islet insulin content.

The question of defective proinsulin-insulin biosynthesis at this stage of the syndrome in these animals is more difficult to answer. Certainly in later stages, which are characterized by a fall in circulating IRI,<sup>18</sup> markedly diminished beta cell replication,<sup>18,28,29</sup> and nearly complete beta cell degranulation,<sup>17,27-29</sup> beta cell biosynthetic capacity for insulin must be reduced compared to earlier stages of the syndrome. In the period following the restricted feeding schedule used in these experiments, however, several observations suggest enhanced insulin biosynthesis.

It is therefore surprising that the labeling experiments performed in these studies with H-3-leucine failed to detect a significant difference in the appearance of label, either in proinsulin or insulin, between normal and diabetic islets. One possibility is that diabetic beta cells produce, in addition to proinsulin and insulin, another polypeptide in excess to account for, at least in part, the electron microscopic findings of increased rough endoplasmic reticulum. Indeed, repeated gel fractionations of islet extracts showed a labeled component distributed between the column void volume and proinsulin. Although the nature of this acid-alcohol soluble protein remains in question, no consistent differences were noted between diabetic and normal islet extract chromatograms.

Another possible explanation concerns the effect of glucose on proinsulin-insulin biosynthesis. Permutt and Kipnis,<sup>32</sup> and Tanese, Lazarus, Devim and Recant<sup>33</sup> have demonstrated glucose augmentation of insulin biosyn-

thesis by isolated rat islets with increasing glucose concentration in the incubation medium. Therefore, it is conceivable that in our experiments normal mouse islet insulin biosynthesis was enhanced, whereas diabetic islet insulin biosynthesis was not in the presence of 16.7 mM glucose. Numerous experiments comparing normal and diabetic islets at varying glucose concentration are necessary before this question can be resolved.

Lastly, differences in the rates of leucine translocation or in intracellular leucine pool size between normal and diabetic beta cells could influence specific activity of the precursor amino acid pool, and interfere with interpretation of labeling experiments. Unfortunately, there are few available data that relate to this problem and apparently no information exists on mouse pancreas or isolated islet amino acid transport and pool size. Eagle, Piez and Levy<sup>34</sup> assessed growth rates of various human cells in tissue culture as a function of concentration of intracellular amino acid and found a critical threshold of 10 to 50  $\mu$ M necessary to initiate growth. Maximum growth was achieved at concentrations only two to four times greater than threshold levels. Another pertinent observation relating intracellular amino acid pool size to protein synthesis are the kinetic analyses of Kipnis, Reiss and Helmreich<sup>35</sup> on rat muscle, and Rosenberg, Berman and Segal<sup>36</sup> on rat kidney cortex. These workers demonstrated that the rate of incorporation of amino acid into protein was independent of the rate at which an amino acid enters the intracellular pool. Finally, Wool has found intracellular leucine content of rat heart muscle to vary from 300 to 400  $\mu$ M.<sup>37</sup> These important observations, although not directly applicable, may significantly influence final interpretation of the labeling experiments with our isolated mouse islet preparation. Amino acid content of the medium for nineteen of the amino acids at the onset of incubation in these experiments was 100  $\mu$ M; however, leucine concentration was only 1.7  $\mu$ M (tracer alone added). Final interpretation must await analysis of amino acid translocation and measurement of pool sizes in normal and diabetic mouse isolated islets, or preferably in beta cells. The resolution of this problem is, of course, technically most difficult.

The failure to find significant differences in labeling of normal and diabetic islets raises the question of the mechanism of maintenance of elevated serum IRI in diabetic animals. Attempts to relate a decrease in insulin degradative mechanisms to alteration in circulating insulin levels in both animals<sup>38</sup> and man<sup>39</sup> have been largely unrewarding, and it presently appears that pancreatic release of insulin primarily determines circulating levels. Insulin disappearance rates have not been

performed in this mutant mouse to date. Therefore, until decreased degradative rates for mouse insulin have been shown, it seems reasonable to assume that insulin secretion probably proceeds at an accelerated rate in vivo, at least during the early development of the syndrome.

In summary, the present evidence does not support a defect in proinsulin-insulin biosynthesis or disproportionate proinsulin release as a cause of the disease in this model of experimental hereditary diabetes.

#### ACKNOWLEDGMENT

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