Further Studies on an Abnormal Insulin of Diabetes Mellitus

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SUMMARY

Insulin isolated from normal and diabetic pancreas was isolated and injected intraperitoneally into rats together with C-14 glucose. Measurement of C-14 incorporation into the diaphragm showed a significantly lesser biologic activity for diabetic insulin.

These results are in accord with earlier work on the resistance of diabetic insulin to insulinase and on the inheritance of diabetes in terms of the abnormal insulin. The possibilities for a mutation site in the insulin molecule are discussed in relation to known molecular variations. DIABETES *16*:572-75, August, 1967.

Earlier work in this laboratory demonstrated a protective factor in the serum of children with diabetes[1] which inhibits the destruction in vitro of insulin in the presence of rat diaphragm. The findings in subsequent studies suggest that this inhibitor is an abnormal insulin in terms of its resistance to insulinase[2] and is inherited in a simple Mendelian fashion.[3]

The present study was undertaken to determine whether this abnormality of the serum insulin in juvenile diabetes was present also in diabetic adult pancreatic insulin and could be expressed in terms of impaired physiologic action. The rate of incorporation of C-14-labeled glucose into glycogen in the rat diaphragm following simultaneous intraperitoneal injection of C-14 glucose and insulin was used as the testing model.

METHODS

Insulin was prepared from human diabetic and normal pancreas, either by acid ethanol extraction, or by acid extraction, followed by immunological purification. After suitable standardization, it was mixed with C-14 glucose and injected intraperitoneally under standard conditions into groups of 200 gm. male Wistar rats. The amounts of the label incorporated into rat diaphragmatic glycogen were then compared for normal and diabetic insulin.

A. Extraction of insulin

Normal pancreas was removed as promptly as possible after death from two elderly nondiabetic males with no personal or family history of diabetes and from six elderly diabetic adults, four male and two female, three of whom had been controlled on small doses of insulin, two on sulfonylurea drugs, and one on diet. Pancreas from two additional cases of juvenile-onset diabetes contained no extractable insulin.

In five of the diabetic and in one control pancreas, the tissue was extracted for five minutes in a Waring blender with 6 ml. per gram of 1.5 per cent HCl and 75 per cent ethanol in water.[4] The suspension was left overnight at *p*H 1 to 1.5 at 4° C., centrifuged, re-extracted and the supernatants combined. The bulk of contaminating proteins was precipitated by adjusting to *p*H 7.5 with ammonia. The insulin was then precipitated in the cold by ethanol:diethyl ether and redissolved in 0.5 M acetic acid at *p*H 3.5, lyophilized, and stored at —18° C. For use, it was diluted in 5 per cent bovine serum albumin in *p*H 7.5, 0.1 M phosphate buffer at a concentration of around 1,000 μU. per milliliter.

B. Immunologic purification of insulin

One normal and one diabetic pancreas were separately extracted in a Waring blender with 1 M acetic acid *p*H 3.0 to 3.2. After centrifuging, the supernatant was gel-filtered on a 50 cm. × 3.5 cm. G 50 Sephadex column. The crude peptide fraction was then complexed with guinea pig anti-insulin serum[2] and gel-filtered again with a borate buffer at *p*H 8.4 to remove non-reacting peptide. Immunologically pure insulin was finally separated from the globulin complex by a final gel filtration in 1 M acetic acid. The insulin was thus separated and finally prepared for use as in the acid ethanol extraction.

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C. Test of biological activity

The insulin content of pancreatic extracts was assayed by a double antibody technic[5] and resuspended in buffered bovine serum albumin to give 800 to 1,200 μU. per milliliter. For each experiment, aliquots of the control and diabetic insulins were assayed three times in triplicate. From the mean assay, one or the other was then diluted to give identical insulin concentrations and the assay repeated. Immediately prior to each experiment, equal parts of insulin solution and of a 2 μc. per milliliter C-14 glucose (specific activity ≧ 2 μc. per micromole) in phosphate-buffered bovine serum albumin were mixed.

Two milliliters of each insulin/glucose mixture were injected intraperitoneally into ten 200 ± 5-gm. Wistar rats with a blunt-tipped No. 21 needle.[6] All animals were killed by decapitation exactly two hours after injection. Fourteen control animals were injected with buffered bovine serum albumin only. The diaphragms were promptly removed over ice, washed momentarily in ice-cold 1 per cent sodium fluoride to remove contaminant activity, weighed and placed in 2 ml. of 30 per cent KOH at 93° C. The bowel was carefully inspected for perforation and the experiment discarded if such was found. Glycogen was separated by conventional ethanol precipitation.[6] The final glycogen precipitate was suspended in 0.5 ml. of water and washed into a vial with 3 × 5 ml. aliquots of aqueous scintillation fluid liquid in which Cab-o-sil had been added to form a thyxotropic gel and counted in a Packard Tricarb Liquid Scintillation Counter.

RESULTS

The results for the first part of the experiment with the acid ethanol extracts are shown in table 1. It is apparent that the influence of diabetic insulin on glucose incorporation into diaphragm glycogen was, in all cases, significantly less than for nondiabetic insulin. Nevertheless, it seemed important to exclude the possibilities that the results were a function either of other substances in the acid ethanol extract such as pressor amines, glucocorticoids, glucagon or the β chain of insulin. In addition, the effects of different types of insulin as well as small but critical differences in the amounts of insulin administered were examined.

To investigate these possibilities, 500 μc. of C-14 ring-labeled norepinephrine (mc./m-m.mol., 0.01 mc./ml.) and 250 μl. of H-3-labeled cortisol (2,000 mc./m.mol., 0.01 mc./ml.) solutions were added to separate acid ethanol extracts of 10 gm. of pancreas. Less than 0.1 per cent of activity was detected in the final insulin extract in both instances. The glucagon content of the final pancreatic extracts was kindly determined for us by Dr. Roger Unger. The normal pancreas extract contained no glucagon and values for the diabetic pancreas in μg. per milliliter were 15, 0, 18, 174 and 10, respectively.

TABLE 1

C-14 glucose incorporation into diaphragmatic glycogen with acid ethanol pancreas extracts

Extract no.	Dose injected C-14 glucose cpm × 10^6	Insulin μU.	n	$\bar{x}$	S.D.	p
D1	1.81	920	7	10.5	6.0	<0.01
C1	1.65	990	8	58.4	25.1	
D2†	1.85	890	10	14.5	6.2	<0.01
C2	1.91	1,065	9	31.6	12.1	
D3†	1.93	1,025	7	5.5	2.6	<0.01
C3	1.87	990	8	12.0	4.7	
D4†	1.41	840	8	6.5	3.4	<0.01
C4	1.38	770	10	26.6	10.7	
D5	1.65	1,150	9	10.0	6.2	<0.01
C5	1.62	890	10	21.9	8.8	
Controls*	1.70	0	14	1.2	1.1	—

D = diabetic; C = nondiabetic control; n= number of animals; $\bar{x}$ = mean counts per minute per mg. of wet diaphragm; p = significance of difference between diabetic and nondiabetic by Aspin-Welch test (Biometrika *43*: 203, 1956).

*Controls were sham injected with 2 ml. *p*H 7.5, 0.1 M phosphate buffer in 5 per cent bovine serum albumin not containing insulin.

†Pancreas from insulin-treated diabetic subjects.

In regard to the insulin, no effect of β chain added to purified normal human insulin was observed (table 2).[7] There is a possibility that the lower counts in the animals injected with diabetic insulin might have been due to a lower injected dose, but the figures in the second part of the table suggest that this was improbable. It also seemed possible that diabetic pancreas might be contaminated with beef insulin and account for differences in activity, but comparable activities were observed. Finally, because the results of the preliminary experiments seemed to confirm a difference between normal human insulin and insulin from a diabetic subject in its ability to incorporate C-14 glucose into glycogen in the rat diaphragm preparation, a final experiment was carried out using normal and diabetic pancreatic insulin that had been immunologically purified. The results, shown in the last section of table 2, once again confirm the significantly different results obtained from normal as opposed to diabetic insulin.

TABLE 2

The effects of β chain, insulin dose, beef insulin and immunologically purified human insulin on the incorporation of C-14 glucose in diaphragmatic glycogen

Extract	Dose injected C-14 glucose cpm × 10^6	Insulin μU.	n	$\bar{x}$	S.D.	*t*	p
Immunologically purified normal human insulin	1.36	955	9	30.8	10.9		
Immunologically purified normal human insulin plus 0.064 mg. crystalline beef β chain	1.39	965	8	34.4	13.6	0.601	<0.60>0.50
Immunologically purified normal human insulin	1.42	695	10	9.2	2.7		
						4.41	<0.001
Immunologically purified normal human insulin	1.38	1,075	10	19.3	6.4		
						0.28	<0.80>0.70
Immunologically purified normal human insulin	1.48	1,390	10	20.7	7.3		
Immunologically purified normal human insulin	1.75	940	10	66.2	19.0		
						1.55	>0.10
Crystalline beef insulin	1.68	1,000	9	50.9	21.6		
Immunologically purified normal human pancreatic insulin*	1.70	1,112	25	42.2	21.9		
						5.34	<.01
Immunologically purified diabetic human pancreatic insulin	1.70	1,082	25	24.4	15.3		

p = significance of difference using the Student *t* test in conjunction with Snedecor's F test.
*Mean of results of three experiments.

DISCUSSION

The present data show that if a similar affinity of both insulins for guinea pig anti-insulin is assumed, diabetic insulin has a significantly diminished activity as compared to normal insulin in at least one important biologic activity. This not only confirms the previous studies on rate of breakdown by insulinase[2] in showing that there is, indeed, an abnormal insulin present in diabetes mellitus, but might indicate a logical association between this and the breakdown in carbohydrate metabolism in clinical diabetes.

Of course, it is possible that these results represent an increased affinity for anti-insulin as a result of which the sample assay reflects a relatively lower molar concentration for diabetic insulin. This point was not investigated further for lack of both a sufficiently discriminant alternative anti-insulin, and a crystalline sample of diabetic insulin whose specific activity could be measured. It seems improbable, however, that a steric change in the diabetic insulin would result in an increased, rather than a decreased, affinity for antibody. It is, in any case, immaterial to the argument that there is a difference between normal and diabetic insulin, whether the primary impact is immunologic, biologic, or both.

Since this difference is heritable,[3] the present findings are compatible with the concept of a geometric alteration in the molecule secondary to a change of amino acid sequence in one or other peptide chain. No affirmative information is available as yet on this point, primarily on account of the technical difficulties of isolating a pure insulin from diabetic pancreas in sufficient quantities for conventional sequencing technics. A significant change in amino acid composition would be a strong confirmation of this theory. Such information as is available[8] shows a similar hydrolysate pattern in most instances, although, in one case, there was a possible isoleucine-lysine interchange. For a number of reasons this tentative evidence for a similar amino acid composition does not necessarily disprove the hypothesis of an altered structure in diabetic insulin. To begin with, it is possible, though unlikely, that the diabetic insulin is not recovered at some stage in purification and final crystallization, and that the assay would reflect only normal insulin. Also, the variance in the over-all hydrolysis procedure is such that one or more point mutations would be undetectable if the molecular population of insulin in adult-onset diabetic pancreas is predominantly normal and the change is in only one of a group of different peptides or aggregates[9,10] with normally similar biologic activity. Moreover, further refinements would be called for to distinguish a change in glutamic or aspartic acids to their amines or vice versa and sequencing

would be required to detect a transposition of two amino acids.

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Yams and Liver Necrosis

Yams are used extensively as a primary source of food, especially among the lower income groups in many parts of the world. That this food may produce necrotic lesions in the livers of rats has been reported by C. Gilbert and J. Gillman (Nature *198*:196, 1963). They indicate that farmers in Ghana recognize that certain varieties of yams may prove fatal when eaten (species not stated). Apparently toxic compounds have been isolated from some of these yams.

From their brief report, it appears that Gilbert and Gillman fed weanling rats nothing but yams. None of the animals survived beyond 100 days. When autopsied, ten of thirteen rats receiving one variety of yam showed macroscopic evidence of acute necrosis. Microscopic examination showed typical massive necrosis and accompanying cholangiofibrosis and nodular hyperplasia. This ration was obviously deficient in many nutrients. Some of these might have been responsible for the symptoms seen. The most obvious that comes to mind would be a deficiency of choline or of methionine.

Until more careful work is done, it is impossible to evaluate the significance of these observations, but they do suggest that additional work is necessary to evaluate the effects produced when large amounts of yams are fed to animals.

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