Studies of Human Insulin from Nondiabetic and Diabetic Pancreas

Joe R. Kimmel, M.D., Ph.D., and H. G. Pollock, M.S., Kansas City

SUMMARY

Insulin has been isolated from single human pancreases from diabetic and nondiabetic sources. Purification was accomplished primarily by gel filtration. The average yield of insulin from the nondiabetic pancreas was 8.6 mg. per 100 gm. of tissue, and from the diabetic pancreas 2.9 mg. per 100 gm. The amino acid composition of nondiabetic insulin was in agreement with the known structure of human insulin. The insulin isolated from the diabetic pancreas had the same amino acid composition as normal human insulin in all cases but one. Based upon the amino acid analysis, it is possible that a different amino acid sequence occurs in the insulin from this diabetic source. DIABETES *16*:687-94, October, 1967.

A genetic basis of diabetes has been well established, but the molecular abnormality responsible for the disease has not yet been clearly defined. The presence of insulin in the plasma of many diabetic patients in the face of an insulin deficiency syndrome suggests that this abnormality might involve insulin itself. Since insulin is a polypeptide whose structure is presumably under genetic control,[1,2] it is possible that a single gene defect could result in an amino acid replacement in insulin with consequent alteration of biologic activity. This possibility has been discussed by Conn,[3] Williams,[4] and by Schwartz and Hechter,[5] and recently Elliott, O'Brien and Roy[6] have presented experimental evidence for a structural difference in insulin from diabetic serum.

The minute amount of insulin present in human plasma precludes isolation and study of structure by even the most sensitive chemical technics. It should be possible, however, to isolate enough insulin from a single human pancreas to permit a limited amount of structural analysis. For example, Wrenshall, Bogoch, and Ritchie[7] found insulin activity equivalent to 8 to 12 mg. of insulin per 100 gm. in the nondiabetic human pancreas, and about 4 mg. per 100 gm. in the diabetic pancreas. It is the purpose of this report to describe amino acid analyses of insulin isolated from single human pancreases from diabetic and nondiabetic sources. In most instances, the insulin from the diabetic pancreas has the same amino acid composition as normal human insulin. In one case, it is possible that an abnormal amino acid sequence existed.

From the McIlvain Laboratories and the Departments of Biochemistry and Medicine, University of Kansas Medical Center, Kansas City, Kansas.

MATERIALS AND METHODS

Reagent grade chemicals and solvents were used throughout this study. Assays for insulin were performed by paper chromatography according to Light and Simpson[8] as applied by Fenson.[9,9a] This method was found to be rapid and generally reliable when compared to results of immunoassays. The method of Yalow and Berson[10] was used for immunoassay of insulin, and a sample of pork insulin (Lilly, assayed at 24.6 U. per milligram) was the reference standard.

For amino acid analyses, samples of insulin were hydrolyzed in vacuo at 110° in 3× distilled 6N HCl for twenty or forty hours.[11] The hydrolysates were concentrated to dryness several times on a flash evaporater and analyzed on a Spinco Amino Acid Analyzer.

Isolation of insulin

Specimens of pancreas were collected at autopsy, frozen at —20° and stored at this temperature until used. The time between death and actual freezing of the tissue varied from less than one hour to a maximum of twenty-four hours.

The frozen pancreas was sliced into small sections and excess fat dissected or cut away. It was then weighed, homogenized in a Waring Blendor, and extracted as described by Pettinga.[12] After centrifugation at 1,000 × g, the extract was filtered through fluted paper to remove suspended lipid, adjusted to *p*H5 with N NaOH, and poured into six volumes of ethanol:ether

(1:2 v/v). Precipitation was allowed to proceed overnight at 4°. The precipitate (Fraction 1) was collected by filtration, washed on the funnel with acetone followed by ether, and air-dried. The filtrate was discarded.

Fraction 1 was extracted for one to three hours at room temperature with 0.05 N HCL (one-seventh volume of crude extract) and centrifuged to remove moderate amounts of insoluble material. The supernatant solution was adjusted to *p*H 5.0 to 5.3 by addition of N NaOH, and solid NaCl (25 gm. per 100 ml.) was added to precipitate insulin. After sixteen hours at 4° the precipitate (Fraction 2) was collected by centrifugation. The supernatant solution was discarded.

Alternatively, Fraction 1 was extracted as above and dialyzed for twenty-four hours at 4° against 0.05 N HCL in 18/32 Visking dialysis tubing.[13] The protein solution was freeze-dried (Fraction 2a). Either salt precipitation or dialysis was necessary to remove large amounts of inorganic salts and other low molecular weight material which interfered with subsequent gel filtration. Dialysis was chosen in preparation of diabetic insulin because of possible failure of sodium chloride to precipitate an abnormal insulin.

Further purification was accomplished by gel filtration as described by Epstein and Anfinsen.[14] For this purpose Fraction 2, or 2a was redissolved in a minimum amount of 0.2M NH_4HCO_3 solution, clarified by centrifugation, and applied to a column of Sephadex G-50, 2.2 × 85 cm. Elution was performed with 0.2 M NH_4HCO_3 at room temperature at a flow rate of 10 to 20 ml. per hour. Insulin containing fractions were pooled (Fraction 3) and lyophilized to remove water and NH_4HCO_3.

Fraction 3 was again subjected to gel filtration but this time on a column of Sephadex G-50, 1.2 × 100 cm., eluted with 1 N acetic acid. A major, symmetrical insulin-containing peak was obtained with this system. This fraction (Fraction 4) was concentrated to dryness in vacuo and insulin was crystallized from it at *p*H 6 in the presence of zinc acetate.[14]

Evaluation of isolation procedure

The selection of a method for isolation of insulin from a single human pancreas was based upon the considerations below. Minor differences in the structure of insulin can have a profound effect upon chromatographic behavior[15-17] by virtue of changes in charge, hydrophobicity, etc.; but these same changes do not, for the most part, alter molecular weight. Since the possibility existed that an abnormal insulin might not be detectable by standard assay methods, it was elected to isolate insulin by gel filtration because this technic accomplishes fractionation on the basis of molecular size. Therefore, all insulins could be expected to elute from columns of an appropriate dextran gel in a characteristic volume and, potentially, it would be possible to find an abnormal insulin in this position even though it did not react in an assay system. Gel filtration also offered the advantage that it could be used with crude extracts, thereby eliminating several purification steps and possible handling losses. Of the several gel filtration methods available, the procedures of Anfinsen and Epstein[14] and of Davoren[18] were chosen over that of Mirsky and Perisutti[19] because volatile buffers are used for elution. This minimizes handling in the final isolation of salt-free product.

The purification scheme described consists basically of extraction, dialysis (or salt precipitation), gel filtration, and crystallization.

Acid-alcohol was chosen for extraction because this medium has been used successfully for the extraction of insulin from the pancreas of many species, including man; apparently, the amino acid sequence differences among these types of insulin do not have a marked effect upon the extractability of the hormone. On the basis of this experience, it was assumed that an abnormal human insulin exhibiting a single amino acid replacement would be extractable with this solvent, although it should be noted that this assumption might not be justified if an amino acid replacement involved one of the half-cystine residues, and the linkage of the A and B chains was affected. The insulin content of the extracts was estimated by immunoassay and these values were used to evaluate recovery of insulin. No insulin could be detected in the supernatant solution after precipitation of protein and salts from the extracts with alcohol and ether.

The extraction of Fraction 1 with dilute HCL effectively dissolved all of the insulin but satisfactory gel filtration of this extract was not possible without first removing inorganic salts. This could be accomplished by precipitation of protein with NaCl or by dialysis. The former procedure was most convenient and generally resulted in a more highly purified product after gel filtration. Ten to 15 per cent of the insulin was not precipitated, however, under the conditions used. Because of this loss and the risk of incomplete fractionation of normal and abnormal insulin, dialysis instead of salt precipitation was performed with all diabetic samples.

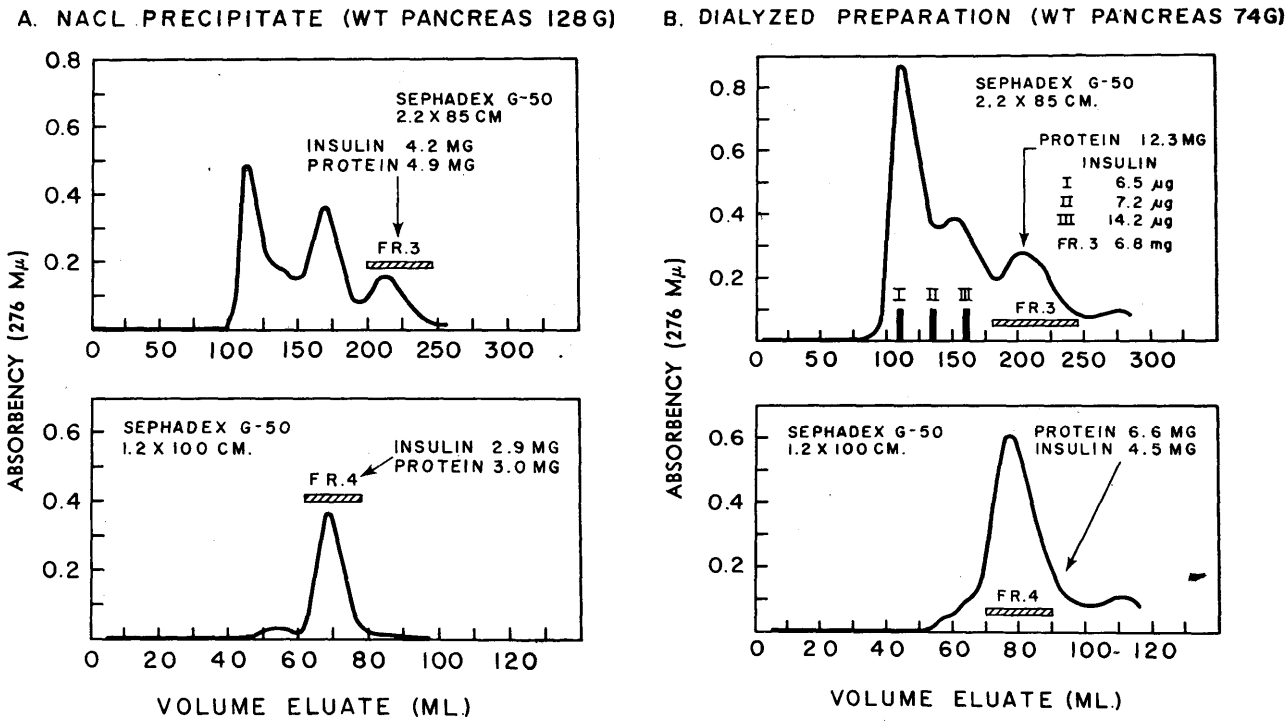

FIG. 1. Elution patterns from gel filtration of nondiabetic human pancreatic extracts. The upper diagram in each case was obtained by elution with 0.2M NH_4HCO_3.[14] Fraction 3 was pooled as indicated, lyophilized and redissolved in a few milliliters of 1 N acetic acid. Gel filtration was repeated, using 1 N acetic acid as eluant to give the pattern in the lower diagram.

Representative gel filtration patterns are shown in figure 1. As indicated above, when salt precipitation was used in the isolation (figure 1A), Fraction 4 from gel filtration in 1 N acetic acid was nearly pure as judged by immunoassay, paper chromatography, and amino acid analysis. When dialysis was substituted for salt fractionation (figure 1B), at least one crystallization was required to render the insulin better than 95 per cent pure by the criteria above. Immunoassay of representative areas of each peak from the NH_4HCO_3 pattern (figure 1B) demonstrated that significant amounts of insulin were present only in Fraction 3.

TABLE 1

Isolation of insulin from human pancreas

Representative data for nondiabetic tissue giving amount present in crude extracts, degree of purity of final gel filtration product (Fraction 4) for both salt precipitated and dialyzed preparations, and over-all recovery from crude extracts. Part A gives results with NaCl precipitates; Part B gives results with dialyzed preparations.

Preparation	Weight of pancreas (gm.)	Crude extract insulin* mg.	Fraction 4 Total protein† (mg.)	Fraction 4 Insulin content (mg.)	Fraction 4 Insulin content per cent	Overall recovery (per cent)
A 5-152	43	6.6	5.4	5.2‡	96	82
5-157	128	5.0	3.0	2.9‡	98	60
5-172	41	8.1	5.5	4.9*	89	68
B 5-177	31	3.9	3.3	3.2*	99	85
6-22	74	5.4	6.6	4.5*‡	68	83
6-75	72	9.0	6.2	4.6‡	74	51

*Determined by immunoassay.
†Estimated spectrophotometrically. An absorbence of 1.10 (1 cm.; 276 mμ.) corresponds to an insulin concentration of 1 mg. per ml. (calculated from amino acid analyses of solutions of human insulin).
‡Estimated from amino acid analysis.

Representative data from several preparations are given in table 1. It is apparent that despite efforts to minimize handling losses, recovery of insulin was incomplete. The data given represent maximum and minimum values. The average recovery of insulin in our hands was 75 per cent, based on insulin content of crude extract. It is apparent that the level of purity of insulin following the second gel filtration (Fraction 4) may be lower in dialyzed preparations than in salt-precipitated material, and final purification was accomplished by crystallization. For example, Preparations 6-22, and 6-75 (table 1) after crystallization were found to contain 23.3 and 24.1 U. per milligram, respectively, by immunoassay.

RESULTS

Nondiabetic tissue

Insulin was isolated from pancreases of twenty-nine individual nondiabetics. These persons ranged in age from twenty-five to seventy-seven at time of death and represented approximately equal numbers of each sex. The absence of diabetes was deduced from the clinical record and from pathologic examination. Both accidental and natural causes of death are represented in this group.

The yield of insulin from these tissues varied from 0.4 to 18.7 mg. per 100 gm. of pancreas (figure 2A). The average was 8.6 mg. per 100 gm. This figure is in reasonable agreement with the data of Wrenshall et al.[7] who found 8 to 12 mg. of insulin per 100 gm. of human pancreas, as measured by mouse convulsion assay of the crude acid-alcohol extract.

Our experience, although less extensive than that of Wrenshall et al., fails to show any correlation between yield of insulin and length of time between death and autopsy (figure 2B). Such a correlation is well known from industrial experience.

The results of amino acid analyses of eleven individual samples of insulin are shown in table 2. Since the amounts of insulin isolated were seldom sufficient for more than one analysis by the technic available, it was the policy to analyze a single forty-hour hydrolysate of each sample of insulin. This made it impossible to correct for destruction of threonine and serine during acid hydrolysis unless a standard correction was employed. It was elected to utilize the data without any corrections, since identical conditions were employed for both diabetic and nondiabetic insulin.

The data in table 2 show that average values for the amino acid composition of the hydrolysates of human in-

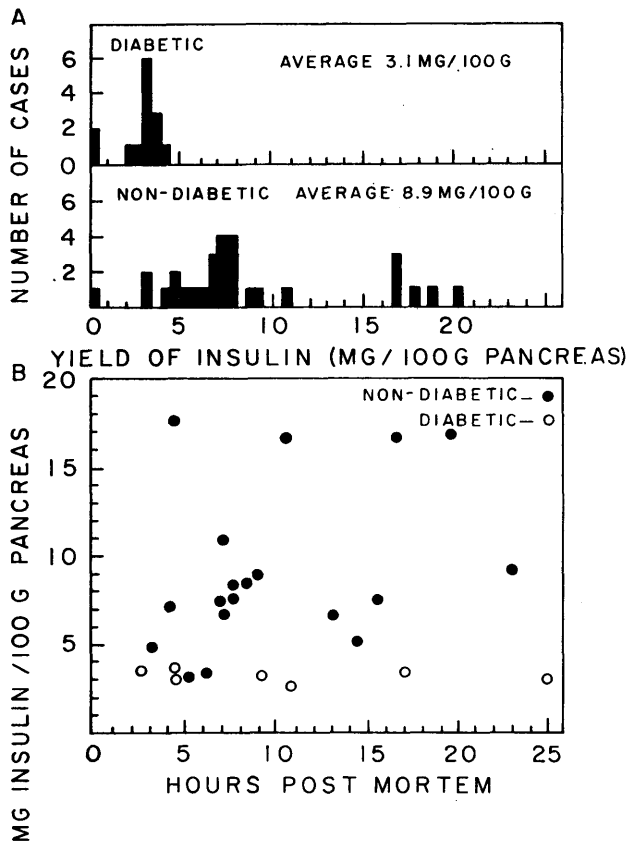

FIG. 2. Insulin yield from diabetic and nondiabetic pancreas. (A) Comparison of amount of immunoreactive insulin extractable from diabetic and nondiabetic pancreas. (B) Data from above plotted to show relationship between yield of insulin and hours postmortem.

sulin are consistent with the known composition of human insulin.[20] Some estimate of the precision of the data is provided from the standard deviations about the means. The amount of insulin applied to each column of the amino acid analyzer varied from 0.035 μ moles to 0.130 μ moles. Analyses performed at the lower level were less precise because this was near the lower limit of sensitivity of the amino acid analyzer available for this work.

The average yield of half-cystine was 4.7 residues per mole of insulin and individual values ranged from 2.0 to 5.2 residues per mole (6.0 is the known value). Destruction of half-cystine is often extensive during acid hydrolysis[11] and better estimates of half-cystine content can be made by analysis of hydrolysates of the performic acid oxidized protein for cysteic acid. This was not done in these studies because of the limited amount of sample available.

TABLE 2

Results of amino acid analyses of human insulin

The data below were obtained from analysis of single forty-hour hydrolysates of samples of human insulin isolated from eleven individual pancreases by the procedure described in the text. Some estimate of precision is provided in the values of standard deviation of the mean. The data are expressed as amino acid residues per molecule of insulin.

Amino acid	Residues per molecule Mean	2 S.D.	Theory	Amino acid	Residues per molecule Mean	2 S.D.	Theory
Asp.	3.03	0.21	3	Ileu.	1.89	0.16	2
Thr.	2.48	0.38	3	Leu.	5.83	0.51	6
Ser.	2.01	0.74	3	Tyr.	3.34	0.52	4
Glu.	6.94	0.35	7	Phe.	2.88	0.22	3
Pro.	1.06	0.14	1	Lys.	1.02	0.09	1
Gly.	4.00	0.32	4	His.	1.86	0.23	2
Ala.	1.11	0.20	1	Arg.	1.00	0.09	1
Val.	3.95	0.36	4	Half-cys.	4.70	—	—

TABLE 3

Insulin content of diabetic pancreas

The data presented below were obtained by immunoassay and/or paper chromatography of fractions obtained during isolation of insulin. Dialysis was used in each case prior to gel filtration. The sample numbers correspond to those in table 4.

Preparation	Weight of pancreas (gm.)	Crude extract insulin† (mg.)	Fraction 4 Crystallization Total protein* (mg.)	Insulin (per cent)	Crystals (mg.)	Mother liquor (mg.)	Specific activity of crystals† (units/mg.)	Over-all recovery‡ (per cent)
3	90	6.3	4.1	70	3.7	0.4	24.1	59
4	98	2.8	1.7	76	1.4	0.2	25.4	50
5	96	2.7	2.1	85	1.6	0.2	28.0	59
9	85	1.7	4.1	27	0.9	0.2	27.7	53

*Determined spectrophotometrically. An absorbence of 1.10 (1 cm.; 276 mμ.) corresponds to an insulin concentration of 1 mg. per ml.
†Estimated by immunoassay.
‡Calculated as follows: mg. of crystalline insulin/mg. of insulin in crude extract.

Diabetic tissue

Insulin was isolated from thirteen pancreases obtained from diabetic sources. All of these were assumed to be cases of adult-onset diabetes, but it was not possible to classify them in more detail nor was genetic information available. Ages ranged from fifty-six to eighty-nine years.

The diabetic tissue exhibited no gross differences from normal tissue during the insulin isolation. Over-all recovery, as estimated by immunoassay and amino acid analysis, was lower than for the nondiabetic pancreas (table 3), but it should be noted that insulin in the mother liquor from crystallization is not included in the calculation of recovery. Furthermore, the smaller quantities of insulin present in the extracts made handling losses more significant. Some representative gel filtration elution patterns are shown in figure 3 and it is apparent that these have the same general characteristics as those from nondiabetic pancreatic extracts. Furthermore, immunoassays were performed on all fractions of many of these eluates and significant amounts of insulin could be demonstrated only in the expected position (figure 3).

The yield of insulin from the diabetic pancreas ranged from 0.4 to 4.3 mg. per 100 gm. of pancreas, averaged 2.9 mg. per 100 gm. (figure 2A), and as with nondiabetic pancreas, did not appear to be affected by time between death and autopsy (figure 2B). This value is slightly lower than that found by Wrenshall et al.[7] for this age group and is significantly reduced from the yield from nondiabetic pancreas.

Amino acid analyses were performed on all samples of diabetic insulin where sufficient material was available. These analyses are given in table 4. The amino acid composition of Preparations 2, 3, 5, and 9 does not differ from that of normal insulin when compared to the data of table 2. In all other samples, except Preparation 4, there are slight deviations from the normal composition but, since no consistent pattern is obvious, the significance of these deviations is doubtful. In Preparation 6, the deviations are associated with a low specific immunoreactivity and, therefore, are probably the result of impurities.

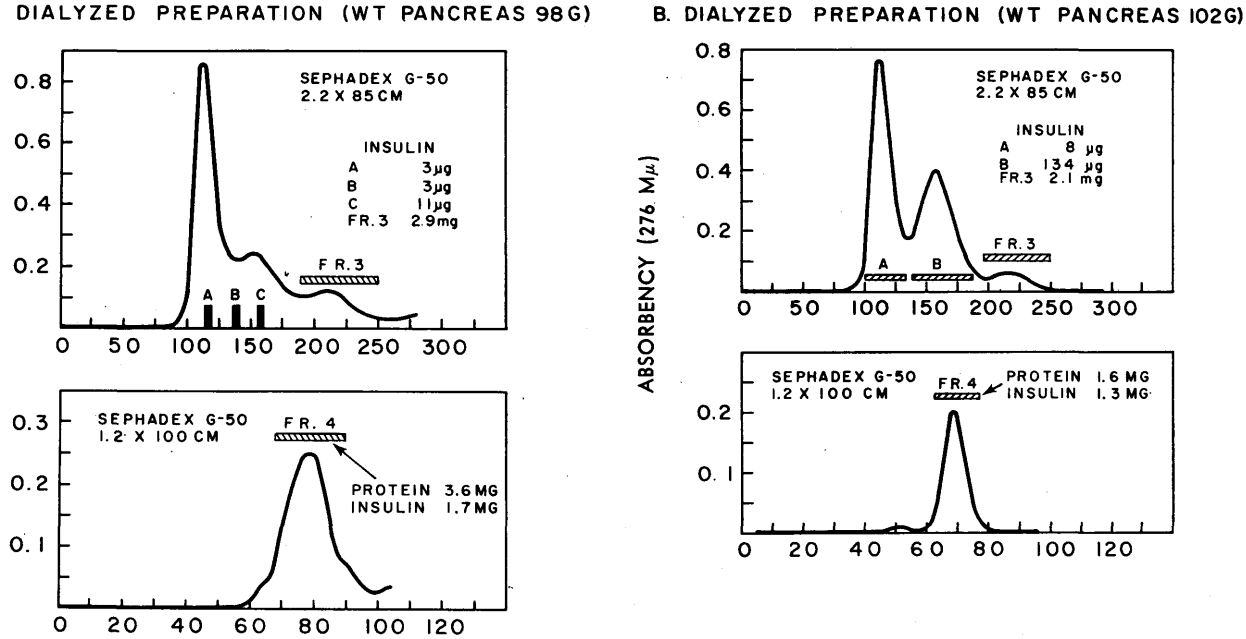

FIG. 3. Elution patterns from gel filtration of diabetic human pancreatic extracts. The conditions of gel filtration were the same as those given in figure 1. Note the similarity of these patterns to those in figure 1 and the virtual absence of immunoreactive-reactive insulin in all fractions except Fraction 3.

TABLE 4

Amino acid analyses of diabetic insulins

The data given were obtained from analysis of single forty-hour hydrolysates of samples of crystallized human insulin, isolated by the procedure described, from individual human pancreases each from a diabetic source. The results should be compared to the values in table 2 and are expressed as residues per molecule of insulin.

Amino acid	Preparation 2	3	4	5	6	7	8	9
Asp.	3.0	3.1	3.4	3.1	3.4	3.4	3.3	3.1
Thr.	2.7	2.5	2.0	2.6	2.2	2.5	2.4	2.6
Ser.	2.7	1.9	1.5	2.4	1.7	1.9	2.2	2.1
Glu.	7.1	6.9	7.1	6.9	7.2	7.5	7.2	7.1
Pro.	1.2	1.1	1.1	1.0	1.1	0.9	1.0	1.0
Gly.	4.1	4.1	4.1	4.0	4.1	4.1	4.0	4.2
Ala.	1.2	1.1	1.3	1.2	1.5	1.0	1.4	1.1
Val.	4.1	4.1	3.7	3.9	3.8	4.1	3.9	4.2
Ileu.	1.7	2.0	1.2	1.9	1.9	2.0	1.8	2.0
Leu.	5.8	5.9	5.3	5.9	5.6	6.0	5.5	6.0
Tyr.	3.5	3.6	3.1	3.6	3.2	3.6	3.3	3.9
Phe.	2.9	2.9	2.5	2.8	2.6	2.7	3.0	2.9
Lys.	1.1	1.1	1.4	1.1	—	1.1	—	1.0
His.	1.8	1.9	1.8	1.9	—	1.9	—	2.0
Arg.	1.0	1.0	1.1	1.0	—	1.0	—	1.0
Half-cys.	4.9	5.4	4.6	4.5	4.5	4.7	4.9	5.2
Immunoassay units/mg.	—	24.1	25.4	28.0	20.9	—	—	27.7

Preparation 4 presents a different type of deviation and could possibly represent an "abnormal" amino acid composition. There are slight excesses of aspartic acid and alanine as in some of the other samples but the content of isoleucine and lysine differs significantly from the expected values. The low yield of isoleucine could be explained by incomplete hydrolysis since the isoleucine-valine sequence at positions 2 and 3 of the A chain is very resistant to acid hydrolysis.[14] However, if this is the cause, a low yield of isoleucine

should be accompanied by an equivalent deficiency of valine. This is not the case here since the valine value is within the expected range. The high lysine content is well outside analytical variation. Neither the excess of lysine nor the deficiency of isoleucine amounts to one residue per mole of insulin. These findings could result from a mixture of two insulins, one containing two residues of isoleucine, and the other containing a single residue of isoleucine, the second residue having been substituted by a residue of lysine.

DISCUSSION

The results of the work described here indicate that the amount of insulin extractable from diabetic human pancreas is less than that obtainable from nondiabetic pancreas. This is in agreement with the findings of Wrenshall et al.[7] and supplements their data by showing that the biologic activity which they observed can be accounted for by a substance identical in composition to human insulin. Unfortunately, we have been unable to obtain biologic activity measurements on our preparations, but immunoassays have shown them to be immunoreactive at the correct level (table 4).

Comparison of the amino acid composition of normal and diabetic insulins shows that most of our preparations have the expected composition. Therefore, it can be concluded that many adult-onset diabetics are capable of making insulin of normal composition and immunoreactivity. On the other hand, a case can be made for an abnormal insulin in one sample. The possible abnormality involves an isoleucine-lysine interchange, which could occur at either position 2 or 10 in the A chain. Position 10 is the site of many interchanges among species and basic amino acids are known to occur in this position without abolishment of biologic activity.[21] Position 2 is less variable.

Early in this work, a sample of diabetic insulin was isolated by a slightly different technic. This material was oxidized and the oxidized A and B chains isolated by paper electrophoresis. Analysis of the individual chains from this preparation showed the B chain to have a normal amino acid composition, and the A chain to be deficient in isoleucine. This was the only unusual feature among the neutral and acidic amino acids. The analysis for the basic amino acids was lost due to equipment failure.

From a genetic standpoint the amino acid composition of Preparation 4 (table 4) would indicate that both an abnormal and a normal insulin were present and suggests that the individual was heterozygous for a gene involved in insulin synthesis.

The use of 100 gm. of pancreas as a basis for comparison of insulin yield is somewhat misleading, since this amount of tissue was seldom available. Most isolations were performed with 30 to 50 gm. of tissue, and in the case of diabetic tissue, this seldom resulted in sufficient insulin for optimum precision. For this reason, it was not possible to study our insulin samples in more detail. Work is currently in progress using analytical methods of much greater sensitivity and a "fingerprint" technic for more detailed structural analysis of the isolated insulin.

It should be added that attempts were made to isolate insulin from two specimens of pancreas from sources judged to be the juvenile type of diabetes. Both individuals had severe diabetes of long standing and died at ages twenty-seven and forty-two, respectively. At autopsy both demonstrated pathologic findings consistent with the clinical diagnosis. No insulin could be isolated from either pancreas and the gel filtration patterns were quite different from those shown.

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