found in any parameter between treated and control offspring.

In summary, we found that in the absence of any overt sign, offspring from methylmercury-exposed mice behaved significantly different from controls when tested for subtle deviations during postnatal development. We believe there is need to examine subtle parameters when assessing the risks from methylmercury exposure. It seems likely that many people with minor symptoms or subclinical damage have gone undetected.

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9. Following the last period in the open field, mice were placed in a 3-inch wide by 11-inch long by 8-inch deep glass tank filled with water at room temperature, and their swimming behavior was observed for 10 minutes. Video tape recordings were made for subsequent evaluation and confirmation of findings.
10. Methods have been published for protein [O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951)].
11. For choline acetyltransferase assay, 20 μl of buffer substrate as originally described [R. E. McCaman and J. M. Hunt, *J. Neurochem.* **12**, 253 (1965)] containing 350 μM acetyl-1-[^{14}C]-coenzyme A was incubated with 2 μl (5 to 6 μg of protein) of brain homogenate prepared in distilled water. For cholinesterase assay, 20 μl of buffer-substrate (containing acetyl-1-[^{14}C]choline iodide) was incubated with 2 μl (0.4 to 0.5 mg of protein) of brain homogenate as originally described [M. N. McCaman, L. R. Tomery, R. E. McCaman, *Life Sci.* **7**, 233 (1968)]. In each case, after incubation, 250 μl of 3-heptanone containing tetraphenylboron (75 mg/ml) was added [F. Fonnum, *Biochem. J.* **115**, 465 (1969)]. The samples were mixed and then centrifuged for 10 minutes at 900*g* in the cold. A 200-μl portion of the 3-heptanone layer was placed in liquid scintillation vials for counting to estimate choline acetyltransferase; or the 3-heptanone layer was removed by aspiration and 10 μl of the aqueous layer was placed in vials for counting to estimate cholinesterase. One milliliter of hyamine hydroxide (0.3*M* in methanol) and 15 ml of toluene containing 5 g of diphenyloxazole and 0.1 g of 1,4-bis methyl-5-phenyloxazol-2-yl benzene per liter of toluene were added to each vial, and radioactivity was estimated in a Packard Tri-Carb liquid scintillation spectrometer. Mean activity in micromoles per brain per hour: choline acetyltransferase, controls = 4.08, MMD = 4.29; cholinesterase, controls = 254.7, MMD = 274.9.
12. We thank D. A. Spyker for assistance with the statistical analyses and J. J. Pollock for assistance with the neurochemical analyses. Supported by grants from the Minnesota Medical Foundation (FSW-14-71), the University of Minnesota graduate school, and NIH grants NS-08709 and ES-00034.

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Human Insulin: Facile Synthesis by Modification of Porcine Insulin

Abstract. *Human insulin differs from porcine insulin by a single amino acid—the carboxyl terminal residue of the B chain. By means of chemical and enzymatic treatment, it is possible to remove quantitatively and selectively the carboxyl terminal octapeptide from porcine insulin B chain. This fragment can be replaced by an analogous synthetic human octapeptide to give a protein which is identical to human insulin by a number of criteria. By this method, human insulin can be prepared on a large scale simply and inexpensively from porcine insulin. The method is also useful for preparing specifically labeled radioactive human insulin, as well as insulins with modified amino acid sequences, for research purposes.*

Insulin is a protein composed of two polypeptide chains linked together by two disulfide bridges. The A chain is composed of 21 amino acid residues, while the B chain contains 30, as shown in Fig. 1. It was the first protein for which chemical structure (*1*) and precise molecular weight (*2*) were determined. The complete de novo synthesis of insulin has been successfully accomplished in a number of laboratories (*3*). While these chemical syntheses represent major breakthroughs in synthetic protein chemistry, they are in no way practical for large-scale and inexpensive production of insulin. The de novo synthesis of a protein containing 51 amino acid residues is time-consuming and expensive. In addition, the yields of insulin are rather low at the stage where the A and B chains are linked together by disulfide bridges. In this regard, the method which I have devised has the special advantage that at no stage are the disulfide bridges between the A and B chains disrupted.

Up to this time, the only practical supply of insulin for treatment of diabetes mellitus has been from animal sources. The similarities in structure (*4*) and biological activity of these insulins to human insulin has made this possible. Unfortunately, immunologic intolerance to these nonhuman proteins develops in patients (*5*) and this intolerance to heterologous protein may be significant in bringing about the pathological changes that occur among diabetic patients maintained on insulin therapy for extended periods of time. It would therefore seem to be most desirable if insulin identical in structure to the human protein could be made available for such patients. To this purpose I have developed a method whereby readily available porcine insulin can be converted easily and inexpensively to human insulin.

An outline of the procedure is given below and is depicted schematically in Fig. 1:

1) Reversible blocking of all of the six carboxyl groups in porcine insulin; the four γ-carboxyl groups from glutamic acid residues and the two terminal carboxyl groups.

2) Digestion of blocked porcine insulin with trypsin. This generates a single free carboxyl group at the arginine residue in position 22 of the B chain.

3) Reversible blocking of all free amino groups with BOC (*6*) azide (*7*).

4) Coupling of the free carboxyl at position 22 to a synthetic octapeptide

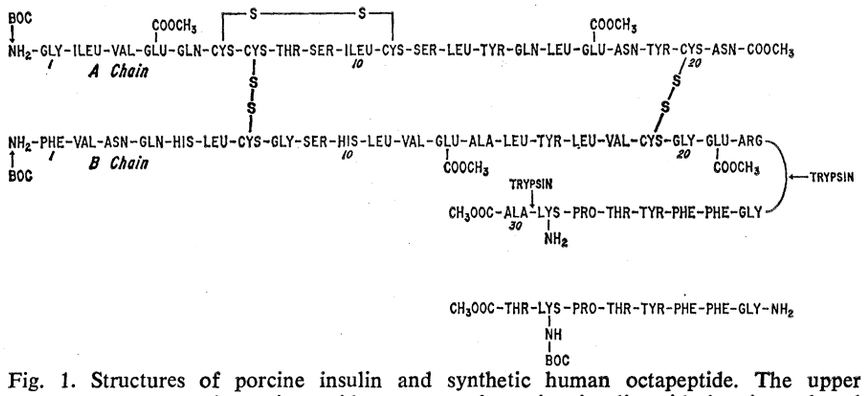

Fig. 1. Structures of porcine insulin and synthetic human octapeptide. The upper structure represents the amino acid sequence of porcine insulin with its six carboxyl groups esterified. Digestion with trypsin rapidly cleaves the molecule at the positions shown. After isolation of the resultant desoctapeptide insulin and reversible blocking of its two free amino groups with BOC azide as indicated by the arrows, the material can be coupled to blocked synthetic human octapeptide, the lower structure. Subsequent removal of protecting groups gives human insulin.

which corresponds to the sequence at the carboxyl end of the B chain in human insulin.

5) Removal of all protecting groups and final purification of the human insulin.

If the procedure is to have practical application, then steps 1 to 5 must be simple, reproducible, quantitative, and innocuous to the insulin protein at all stages. With these criteria in mind, I investigated a number of amino and carboxyl protecting groups. The most suitable were found to be the BOC group for amino protection and the methyl ester formed from diazomethane for reversible carboxyl blocking.

Porcine insulin was obtained from Elanco Products (lot No. 4VV73D). It was purified by chromatography on a column of carboxymethyl cellulose with a linear gradient of volatile pyridine acetate buffer. First buffer, 0.2*M* pyridine acetate, *p*H 5.00; second buffer, 4.0*M* pyridine acetate, *p*H 5.00. The major peak, containing pure porcine insulin as determined by amino acid analysis (Table 1), emerged at a molarity of 1.7. It was isolated by lyophilization.

In a typical experiment, 2.7 g of human insulin were prepared from 4.0 g of purified porcine insulin. This porcine insulin was first treated with diazomethane at *p*H 4.6 in a *p*H stat as described by Chibnall *et al.* (*8*). When a 30-fold excess of diazomethane was employed, the six carboxyl groups were quantitatively converted to the methyl esters, and there were no apparent side reactions, as indicated by a subsequent check of the amino acid composition. The extent of esterification was determined by microanalysis for methoxyl content, by using the Zeisel procedure (analysis performed by Huffman Laboratories). Calculated for insulin hexamethyl ester: OCH_3, 3.04; found: OCH_3, 3.2.

The porcine insulin methyl ester was next subjected to digestion with chymotrypsin-free trypsin at *p*H 7.5 for 20 hours, essentially as described by Young and Carpenter (*9*). The digestion by trypsin was quantitative, and when monitored in the *p*H stat, was found to reach completion in 45 minutes. This rapid rate of digestion, as compared to the rates observed by Young and Carpenter (*9*) and Carpenter and Baum (*10*), might be a consequence of the absence of the negatively charged group normally present at the glutamic acid adjacent to arginine in the B chain, together with the absence of the negative charge at carboxyl terminal alanine adjacent to lysine, both as a result of the esterification.

The entire material from the tryptic digestion was applied to a 4 by 100 cm column of Sephadex G-75 and eluted with 0.2*M* acetic acid. The desoctapeptide insulin pentamethyl ester emerged as a single peak (designated peak I). Amino acid analysis of this material is given in Table 1. A second peak (designated peak II) contained the heptapeptide Gly-Phe-Phe-Tyr-Thr-Pro-Lys, as well as alanine methyl ester. These were separated from each other on Sephadex G-10 in 0.2*M* acetic acid, and their composition was confirmed by amino acid analysis. The material in peak I was isolated by lyophilization and then treated with BOC azide as described by Levy and Carpenter (*7*). The BOC azide reacted quantitatively with the two free amino groups in the molecule. From the point of view of peptide coupling reactions, the only functional group on the molecule at this stage was the terminal carboxyl group at arginine on the B chain, and therefore this material was suitable for coupling to the amino terminal group on synthetic human octapeptide, which I prepared as described below.

Human octapeptide was prepared by a straightforward application of well-established synthetic methods. Threonine methyl ester was synthesized according to the procedure described by Poduska and Rudinger (*11*) and was coupled to ε-BOC-α-CBZ-Lys *N*-hydroxysuccinimide ester, prepared as described by Ruttenberg (*12*). The CBZ group was subsequently removed by catalytic hydrogenation in methanol by using palladium catalyst on charcoal, and the resultant dipeptide was coupled to CBZ-Pro *N*-hydroxysuccinimide ester. Again, the CBZ group was removed by catalytic hydrogenation, this time to give the tripeptide Pro-ε-BOC-Lys-Thr methyl ester, which crystallized from methanol. The pentapeptide CBZ-Gly-Phe-Phe-OBz-Tyr-OBz-Thr hydrazide was prepared by means of the solid phase method (*13*). The appropriate BOC amino acids (obtained from Sigma Biochemicals) were employed for each step, and the pentapeptide hydrazide was obtained by treatment of the pentapeptidyl resin with hydrazine as described by Ohno and Anfinsen (*14*). This pentapeptide hydrazide was treated with nitrous acid to form the azide, which was then combined with the free tripeptide Pro-ε-BOC-Lys-Thr methyl ester. I assumed that the coupling occurred without racemization, which should be the case with the azide method. The optical purity of the synthetic octapeptide has not been determined. Hydrogenation of the octapeptide with palladium catalyst on charcoal gave NH_2-Gly-Phe-Phe-Tyr-Thr-Pro-ε-BOC-Lys-Thr methyl ester, the carboxyl terminal sequence of human insulin B chain.

The synthetic octapeptide was then coupled to the di-BOC-desoctapeptide insulin pentamethyl ester, by using dicyclohexylcarbodiimide in tetrahydrofuran at 0°C, with the addition of one equivalent of *N*-hydroxysuccinimide. According to Zimmerman and Anderson (*15*), this method gives peptide coupling with virtually no racemization. Such was found to be the case; the

Table 1. Amino acid composition of peptides used in this study. Quantitative amino acid analysis was performed on acid hydrolysates with a Beckman-Spinco model 120B amino acid analyzer according to the procedure of Spackman *et al.* (*18*). Values found are given as ratios with respect to lysine or arginine. They are in good agreement with the expected values.

Amino acid	Chromatographically pure porcine insulin		Desoctapeptide porcine insulin		Synthetic human octapeptide		Chromatographically pure partially synthetic human insulin	
	Expected	Found	Expected	Found	Expected	Found	Expected	Found
Lysine	1	1.0	0	0.1	1	1.0	1	1.0
Histidine	2	1.8	2	1.9			2	1.8
Arginine	1	1.0	1	1.0			1	1.0
Aspartic acid	3	2.8	3	2.8			3	2.7
Threonine	2	1.9	1	0.9	2	1.9	3	2.7
Serine	3	2.7	3	2.8			3	2.8
Glutamic acid	7	6.7	7	7.1			7	6.8
Proline	1	1.0	0	0.0	1	1.0	1	1.0
Glycine	4	4.1	3	3.2	1	1.0	4	4.2
Alanine	2	2.1	1	1.1			1	1.1
Half-cystine	6	*	6	*			6	*
Valine	4	3.6	4	3.6			4	3.6
Isoleucine	2	1.7	2	1.7			2	1.8
Leucine	6	5.7	6	5.7			6	5.7
Tyrosine	4	3.9	3	3.1	1	0.9	4	3.8
Phenylalanine	3	3.1	1	0.9	2	2.1	3	3.1

* Not determined.

purified product was subjected to tryptic digestion, after removal of the BOC groups, and quantitative cleavage occurred at the newly formed arginylglycine bond.

The product from the coupling reaction was isolated by chromatography on Sephadex G-75, following removal of the BOC groups. The methyl esters were saponified as described by Chibnall *et al.* (*8*). The resultant human insulin was chromatographed on carboxymethyl cellulose as described above for porcine insulin. It was essentially homogeneous, and, as with the porcine insulin, the major peak emerged at a pyridine acetate molarity of 1.7. Homogeneity was further confirmed by countercurrent distribution as described by Harfenist and Craig (*2*), with a 200-tube automatic countercurrent apparatus. The purified material was found to have the correct amino acid composition for human insulin, as indicated in Table 1. Assay for biological activity was performed by the mouse convulsion technique as described by Katsoyannis and Tometsko (*16*). By means of a blind design assay, the chromatographed human insulin was found to have an activity of 25 I.U./mg which was equal to that of the chromatographically purified porcine insulin used as starting material for this synthesis.

The criteria, then, upon which I based my conclusion that the final product was in fact human insulin were: (i) the elution position and homogeneity of the product on carboxymethyl cellulose chromatography and the homogeneity on countercurrent distribution; (ii) the amino acid composition of the purified product, which was correct for human insulin (Table 1); (iii) digestion of the product with trypsin, which removed quantitatively the synthetic octapeptide, thereby confirming its attachment without racemization at position 22 of the B chain; (iv) the biological activity of the product, which was found to be equal to that of porcine insulin. Determination of the zinc content in the various insulin fractions was not made.

While the above criteria suggest that the synthetic human insulin is homogeneous, the possibility that there may be small amounts of impurities cannot be ignored. Such trace impurities might elicit an antibody response in human subjects. One source of contamination would be the persistence of methyl groups at sites other than carboxyls, as a result of the diazomethane treatment. This is unlikely because of the low *p*H at which the methylation reaction is performed. Such was also the conclusion of Chibnall *et al.* (*8*) in their studies on the diazomethane reaction with insulin. Another source of contamination would be racemized amino acids in the synthetic octapeptide. Appreciable racemization is not likely under the conditions which were used for synthesis; nevertheless, the optical purity of the synthetic peptide would have to be confirmed before the human insulin would be suitable for use with human subjects. Trace impurities in the synthetic peptide could be removed relatively easily by countercurrent distribution in a suitable solvent system.

The heptapeptide (peak II from Sephadex G-75) (*17*) need not be discarded, but can be collected and used as a natural precursor for the synthesis of human octapeptide. By treatment with carboxypeptidase B, it is possible to remove the carboxyl terminal residue of lysine from the heptapeptide. This was done, and the resultant hexapeptide was treated with carbobenzoxychloride to give the protected hexapeptide CBZ-Gly-Phe-Phe-Tyr-Thr-Pro-COOH. This was then coupled to ε-BOC-Lys-Thr methyl ester with the use of dicyclohexylcarbodiimide at 0°C in tetrahydrofuran in the presence of an equivalent of *N*-hydroxysuccinimide to yield the protected human octapeptide. Hydrogenation with palladium on charcoal in methanol gave NH_2-Gly-Phe-Phe-Tyr-Thr-Pro-ε-BOC-Lys-Thr methyl ester, identical to synthetic human octapeptide.

Aside from its major utility in providing a practical source of human insulin, the method which I have described can be used for the preparation of radioactively labeled human insulin. This is accomplished by employing labeled amino acids in the synthesis of the octapeptide. The method also permits the facile synthesis of insulin analogs, since any amino acid sequence may be attached to the carboxyl group on di-BOC-desoctapeptide insulin pentamethyl ester.

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Insulin Insensitivity of Large Fat Cells

Abstract. *Large insulin-insensitive adipocytes from adult rats have normal binding capacities and affinities for insulin. Diminished insulin-like responses to spermine and reduced rates of glucose oxidation are also evident in these cells. The results indicate that the defect responsible for this insulin-resistant state exists in a step subsequent to insulin binding, possibly in transmission of the insulin-receptor "signal" since insensitivity occurs under conditions where glucose transport and oxidative processes are not apparently impaired.*

Adipose tissue from obese humans is less sensitive to the action of insulin on glucose metabolism than fat tissue obtained from normal individuals (*1*, *2*). Similarly, the magnitude of response elicited by insulin in isolated fat tissue from adult rats is below that in tissue from young rats (*3*, *4*). In both instances the insulin insensitivity appears to be related primarily to increased cell size (*2*, *4*) and not to other factors such as age (*5*). The relative resistance of large adipocytes to insulin may explain the abnormally high circulating levels of insulin observed in obesity during the fed and fasting state and may contribute significantly to the glucose intolerance of maturity-onset diabetes mellitus (*6*). Little is known concerning the molecular site(s) responsible for insulin insensitivity in large adipocytes.

Insulin elicits many and possibly all of its biological effects by interacting with specific "receptor" sites located on the surface of the plasma membrane (*7*). In turn, this interaction modulates various cellular systems involved in the mediation of insulin action; thus, an alteration of any step in this sequence may be responsible for the insulin-resistant state. The possibility that insulin-cell association is involved in insulin insensitivity was examined by studies utilizing the insulin-like action of spermine (*8*) and by determining the extent of [^{125}I]insulin binding (*9*) in large (mean volume, 28 μmole of triglyceride per microgram of DNA) and small (mean volume, 9 μmole/μg) rat adipocytes. In addition, the capacity of both cell types to metabolize glucose in the absence of insulin was compared to assess the influence of glucose transport and oxidation on insulin resistance.

Insulin sensitivity in large and small adipocytes was determined by studies of the dose-response relationships for the effect of insulin on glucose oxidation (Fig. 1A). Although basal glucose oxidation was similar in both cell types, the maximal insulin response achieved by large cells was approximately one-half that of small cells. Similar insulin concentrations stimulated one-half maximal glucose oxidation in both cell types, suggesting that affinity of large cell receptors for insulin was not diminished.

Spermine and certain other polyamines mimic the action of insulin on fat cells by apparently acting at membrane sites separate from insulin receptors but which share a common pathway with insulin mediated responses (*8*). By virtue of its site of action, spermine would be expected to stimulate greater glucose oxidation in large cells than insulin if insulin resistance resulted from defective insulin-receptor interaction. However, as shown in Fig. 1B, the maximal spermine response of large cells was similar to the insulin response and was also one-half that of small cells.

More conclusive evidence that alterations of the association between insulin and large fat cells are not involved in their insulin resistance is given in Table 1. With saturating concentrations of [^{125}I]insulin, the total insulin-binding capacities of large and small cells were equal. Furthermore, the affinity between insulin and insulin-binding sites was not diminished in large insulin-insensitive cells since specific binding of [^{125}I]insulin at concentrations below saturation was similar in both groups of cells.

An assessment of the glucose transport and oxidizing capacities of large and small adipocytes was made by studying the effect of glucose concentration on glucose oxidation (Fig. 2A). The rates of CO_2 production by both cell types were essentially identical when

Table 1. Specific binding of [^{125}I]insulin to large and small adipocytes. Fat cells, prepared as described in the legend of Fig. 1, were suspended (6.1 and 8.6 μg of cell DNA for large and small cells, respectively) in 0.5 ml of Krebs-Ringer-bicarbonate buffer containing 1 percent (w/v) albumin, and the indicated concentration of [^{125}I]insulin (920 mc/μmole). After incubation for 50 minutes at 24°C, specific binding of insulin was determined by Millipore membrane filtrations as described previously (*9*). These data, from a single experiment, are representative of six separate experiments which gave similar results. The effects of insulin on the conversion of [^{14}C]glucose to $^{14}CO_2$ by the same cells used in this experiment were similar to those described in Fig. 1; the maximal oxidative response in small cells was twice that of large cells.

Concentration of [^{125}I]insulin ($10^{-12}M$)	Specific [^{125}I]insulin bound (count/min per μg DNA)	
	Small cells	Large cells
16	130	14,700
48	440	170
140	1,410	520
470	4,140	1,490
1400	10,400	4,370
3300	14,100	11,500