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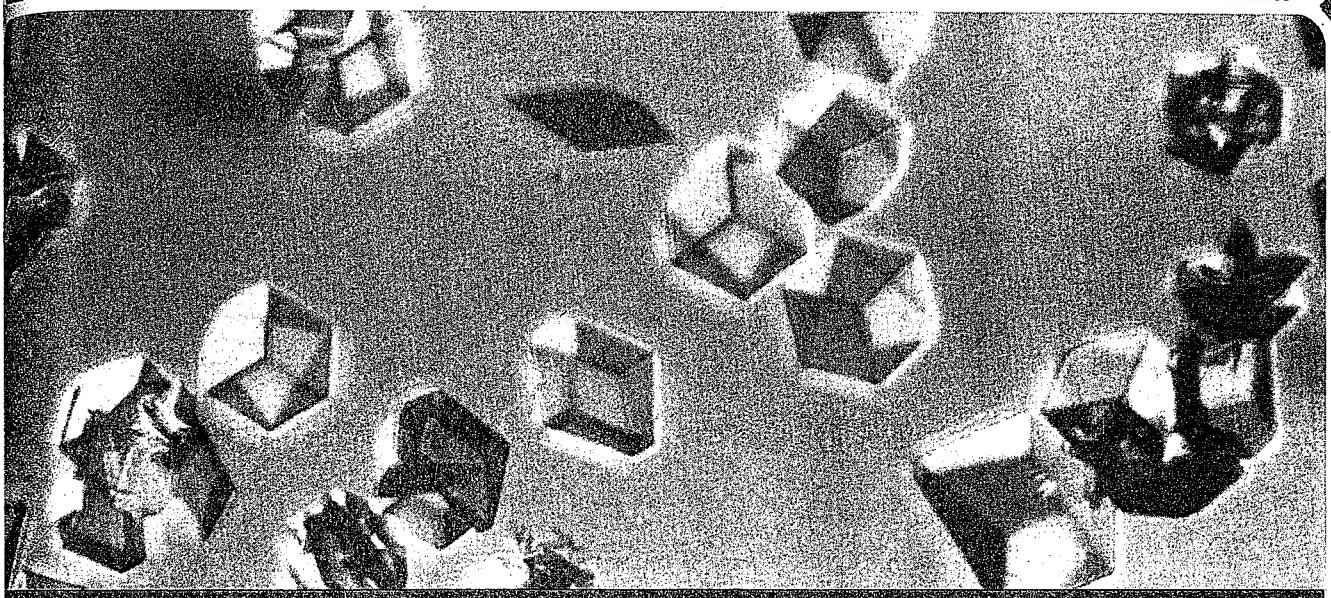

Fortschritte in der Insulin-Therapie

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Two Routes for Producing Human Insulin Utilizing Recombinant DNA Technology

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1. Human insulin synthesized in bacteria represents an important and safe source of highly purified insulin for the future treatment of the insulin-dependent diabetic.
2. Biosynthetic human insulin produced by recombinant DNA methods and prepared by either chain combination or conversion of proinsulin is chemically, physically, and biologically equivalent to pancreatic human insulin.

Zwei Wege zur gentechnologischen Gewinnung von Humaninsulin:
1. Die Synthese von Humaninsulin in Bakterien stellt eine wichtige und sichere Quelle zur Gewinnung von hochgereinigtem Insulin für die zukünftige Behandlung insulinabhängiger Diabetiker dar.
2. Biosynthetisches Humaninsulin ist chemisch, physikalisch und biologisch identisch mit pankreatischem Humaninsulin, gleich auf welchem Wege – entweder durch die Kombination getrennt hergestellter A- und B-Kette oder durch enzymatische Umwandlung von gentechnologisch hergestelltem Proinsulin – es gewonnen wird.

The preparation of human insulin utilizing recombinant DNA technology marks a significant accomplishment in the field of molecular biology and provides a secure source of insulin for the future treatment of the insulin-dependent diabetic. This manuscript will review some of the molecular biology that Eli Lilly has applied in order to accomplish this. Further, the procedures used to prepare and isolate highly purified human insulin are described. Finally, the results of analytical tests are presented to demonstrate that the human insulin produced using recombinant DNA technology is of high purity, is identical to the human insulin produced by the human pancreas, and is safe for use in humans.

The functioning and genetics of the bacterium, Escherichia coli, has been studied for many years, and, as a result, most of the recombinant DNA research has been done with this microorganism. For safety and containment reasons, Eli Lilly has chosen the K-12 strain of E. coli to use in our recombinant DNA research and production, because this is a weakened strain of E. coli which cannot colonize the intestinal tract of humans or animals. The functioning of the protein synthesis apparatus of bacterial cells is obviously central for being able to produce human insulin in these cells. Although proteins are synthesized only on the ribosomes in the cytoplasm of the cell, the genetic code for production of proteins resides in both the chromosomal DNA and in the small rings of cytoplasmic DNA called plasmids. Both of these sources of DNA are transcribed into messenger RNA, which is subsequently translated into proteins. The basis of recombinant DNA technology is our ability to manipulate this bacterial plasmid DNA which is accomplished by isolating the plasmid DNA, cleaving with restriction enzymes and inserting the desired DNA. The desired DNA is obtained either by synthesis, isolation from natural sources, or by a combination of these procedures. In the human insulin work, the A- und B-chain genes were prepared by synthetic nucleotide chemistry, while the human proinsulin gene was semisynthetic – that is, the gene was constructed using a segment of the natural DNA which codes for proinsulin along with a fragment of synthetic DNA. The nucleotide synthesis was performed by Itakura and coworkers at the City of Hope, and by Goeddel and coworkers at the Genentech Corporation (5, 9). After the desired DNA is obtained and inserted, the plasmid DNA is rejoined using a ligase enzyme and then reintroduced into the host cell thru a process called transformation. The cell then is cloned, that is many copies are made, and, after verifying that the desired and correct gene is present, the material is stored in ampoules for future use in production. Thus each fermentation is started from the same seed pool which has been verified to have the correct gene present.

In order to maximize the production of the desired protein in the E. coli cells, the gene message that was inserted into the plasmid also contains a so-called promoter. This promoter determines the rate at which messenger RNA is formed; thus, if one uses a strong promoter more messenger RNA is formed, and consequently there is greater production by the cell of the desired gene product. For human insulin biosynthesis, two promoter systems have been used, originally Beta-galactosidase and now tryptophan synthetase, or Trp E. The Trp E promoter yields more of the desired product as compared to the Beta-galactosidase promoter system. When the E. coli cells are producing

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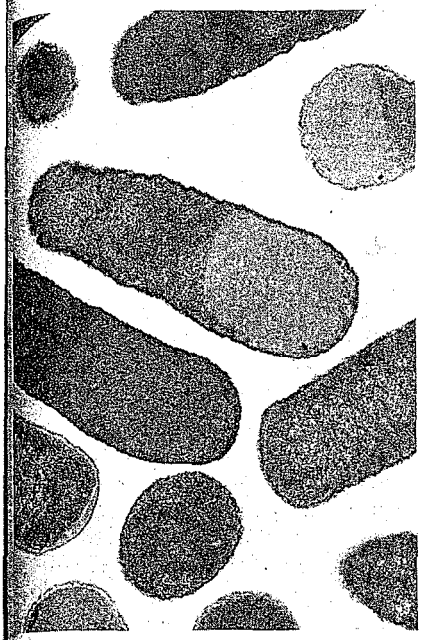

Figure 1: Transmission electron micrograph of inclusion bodies in E. coli cells of a culture producing Trp E-met-A-Chain chimeric protein. (Photograph kindly provided by Dr. D. C. Williams of the Lilly Research Laboratories).

the desired gene product, one can observe electron dense bodies (see Figure 1) which by immunocytochemical techniques (17) have been shown to be the promoter linked product (A chain or B chain or proinsulin) – called the chimeric protein. The chimeric product can be schematically represented as Trp E-Met-A Chain (or -B Chain or -Proinsulin). The methionine linkage provides a specific chemical cleavage site for release of the desired polypeptide from the promoter protein Trp-E.

An interesting fact to note is that all of the E. coli cells contain product, while in contrast, only a small fraction of the cells of the pancreas contain insulin. Thus, one actually has lesser amounts of impurities to remove during the isolation of the biosynthetic human insulin (BHI) as compared to the pancreatic insulins.

Figure 2 indicates the two schemes we have explored for producing biosynthetic human insulin. The current method is to make the A and B chains in separate E. coli fermentations, while the second route is the production of proinsulin in a single E. coli fermentation and eventually to transform it to human insulin. As far as we have been able to determine, both methods yield equivalent preparations of biosynthetic human insulin (2, 8).

Figure 3 illustrates in more detail the current method used to produce biosynthetic human insulin. The chimeric protein, Trp E-Met-Chain, is produced in the E. coli cells in separate fermentations. Methionine is used as a cleavage point since it is sensitive to the chemical cyanogen bromide, or CNBr, and because methionine does not occur either in the A or B-chains or proinsulin. After the cyanogen bromide cleavage, the A and B chains are converted to the stable S-sulfonate derivatives, purified and chemically combined to yield insulin. The insulin is then purified by modern gel-filtration and ion-exchange chromatographic procedures. At this point it should be emphasized that all of the biosynthetic human insulin presently being produced by Eli Lilly is derived from this chain combination procedure and that *all* clinical studies have been conducted with such insulin.

Before reviewing the characterization of the insulin produced by this process, a description of the chain combination procedure developed at the Eli Lilly Research Laboratories is of interest (2). This procedure consistently gives higher yields of insulin than ever reported for this reaction (see Figure 4). Optimal yields of human insulin are obtained using a 2:1 weight ratio of A chain to B chain in a 0.1 M glycine buffer at pH 10.5 and at 4°C. The S-sulfonate derivatives are reduced to sulfhydryl derivatives by use of nearly equivalent amounts of the thiol reducing agent, dithiothreitol, or DTT. The resulting solution is stirred for 24 hours at 4°C in an open vessel to permit the proper disulfide bonds to form as a result of air oxidation reactions. As shown, the insulin yield is approximately 60 percent relative to the limiting B chain. The biosynthetic human insulin is purified and isolated by column chromatography and crystallization. The excess chain materials and by-products are then recycled. This chain combination procedure is an extension of studies from several laboratories (7, 12, 13, 15) that were conducted during the 1960's when chemically synthesized chains of insulin were combined to yield synthe-

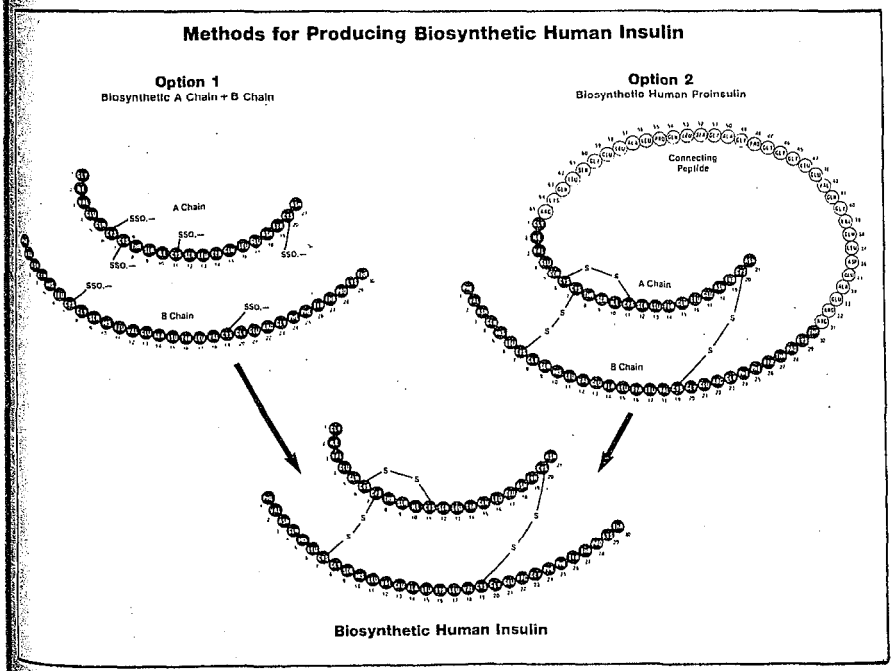

Figure 2: Two pathways to producing biosynthetic human insulin.

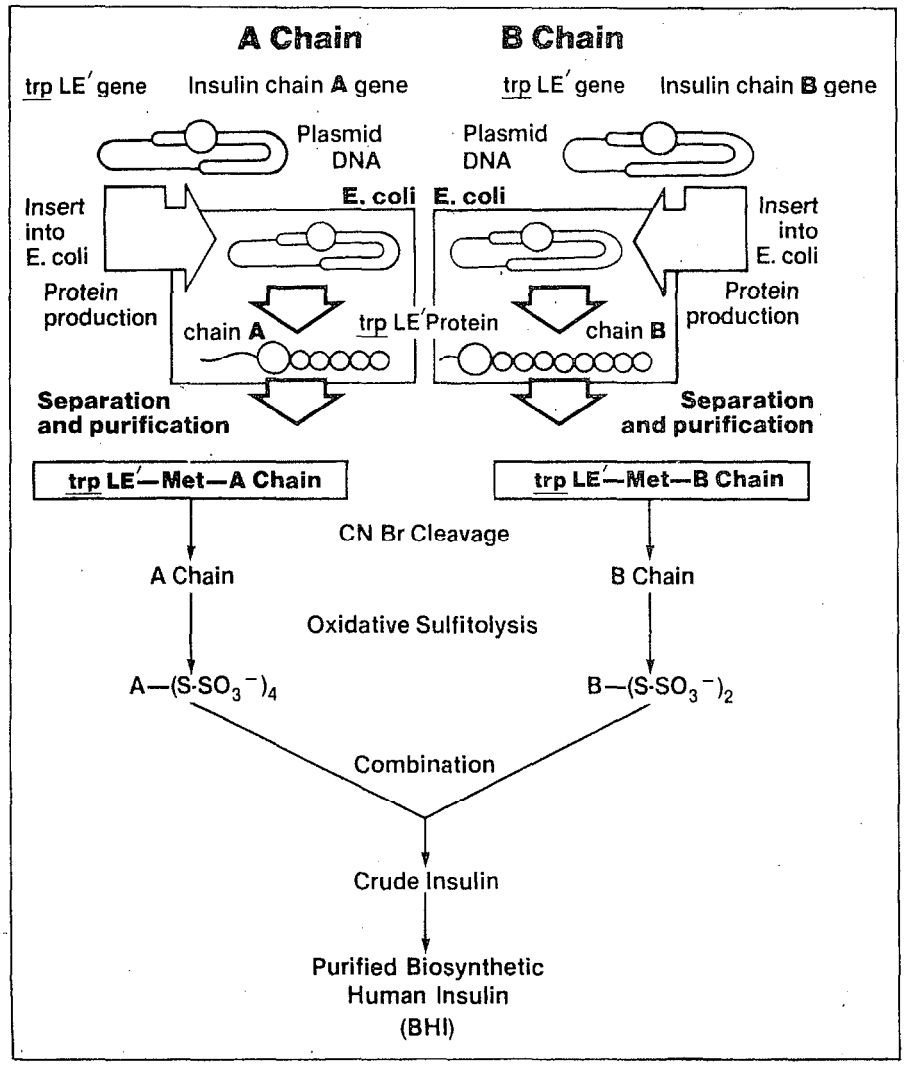

Figure 3: General biosynthetic and chemical modification process leading to the production of biosynthetic human insulin.

tic insulin preparations. However, the yields are significantly better than in the earlier studies due in part to the availability of modern analytical techniques such as high performance liquid chromatography (HPLC). The availability of this technique allowed the examination and optimization of the many variables of this complex series of reactions, and thus the achievement of excellent yields of human insulin.

The preparation of human insulin employing human proinsulin is shown in Figure 5. The chimeric protein produced by the E. coli cells is Trp E-Met-Proinsulin. As in the A and B chain case, the chimeric protein is cleaved using cyanogen bromide. The proinsulin is subsequently converted to its S-sulfonate derivative by oxidative sulfitolysis and then isolated. Then the proinsulin-S-sulfonate is treated with a thiol reagent, beta-mercaptoethanol, which allows the proinsulin molecule to fold and form the proper disulfide bonds. Yields as high as 70 percent are achieved in this process, which was also developed in The Lilly Research Laboratories (8). The proinsulin is then purified by ion-exchange chromatographic methods and the by-products of the folding reaction are recycled. The proinsulin is then converted in greater than 95 percent yield to insulin using a combination of the enzymes trypsin and carboxypeptidase B (see Figure 6). This process is a modification of the procedure originally developed by Kemmler and coworkers (14). The biosynthetic human insulin is subsequently purified by gel-filtration and ion-exchange chromatography, and by crystallization.

As indicated earlier, the biosynthetic human insulin produced via this scheme is identical to the insulin made by the chain combination method. Before turning to a discussion of the characterization of biosynthetic human insulin, we should point out that one of the exciting aspects of the proinsulin scheme is that we are now able to produce human proinsulin and C-peptide which may have interesting activities of their own and which can be investigated clinically for their potential usefulness in the treatment of diabetes.

The Characterization of Biosynthetic Human Insulin

A wide variety of evaluative tests have been used to exhaustively examine biosynthetic human insulin (see Table 1). The results of all of these tests can be best summarized by saying that biosynthetic human insulin has been shown to be chemically, biologically, immunologically, and physically identical to a native pancreatic human insulin standard (3). Tests in a variety of *in vivo* assays, as well as in many different insulin receptor assays, have demonstrated that biosynthetic human insulin exhibits equivalent biological activity to pancreatic human insulin. The same conclusion has been reached based on the data from the insulin radioimmunoassays as well as from the results of studies in the rabbit hypoglycemia test – it is obvious that biosynthetic human insulin is equivalent biologically to both pancreatic human insulin and porcine insulin.

The amino acid compositions of biosynthetic human insulin and pancreatic human insulin are identical to

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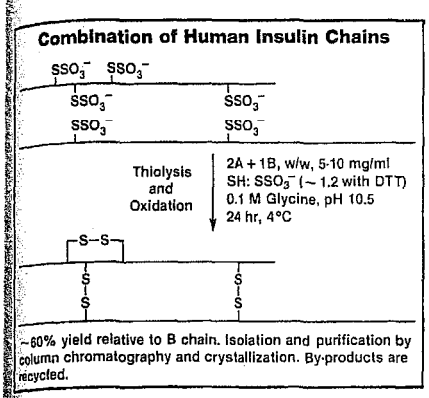

Figure 4: Summary of insulin chain combination method.

within experimental error (see Table 2). The results of conventional pH 8.5 polyacrylamide gel electrophoresis demonstrate that biosynthetic human insulin is of high purity and electrophoretically equivalent to the pancreatic human and pork insulin standard preparations. The same conclusion is apparent from the results of studies with isoelectric focusing in polyacrylamide gels.

One of the most useful tests for evaluating biosynthetic human insulin is high performance liquid chromatography, or HPLC, whose general principles can be described in the following manner. HPLC is an extension of liquid chromatography in which higher resolution, higher sensitivity, and more rapid elution times are achieved. Figure 7 is an illustration of the physical structure of an HPLC column. The column is tightly packed with small particles or resin (called the stationary phase) which is coated with hydrocarbon. In order to obtain flow thru the column and to elute the compounds applied to the column, the solvent (mobile phase) must be pumped thru the column under pressure. The basic principle of the separation is that each protein has a different affinity for the resin particles and the solvent. Thus, as the mixture of proteins flows thru the column, separation occurs because each protein spends different amounts of time interacting with the resin particles or with the solvent. Thus, as illustrated here for a mixture of beef, sheep, human and pork insulins, at the bottom of the column these four insulins have been resolved from one another. This example has been chosen because reverse-phase HPLC today is the most sensitive method available for distinguishing minor structural differences in closely related molecules such as these different species of insulin. Single amino acid differences in structure can be determined by different elution times as shown in Figure 8. This HPLC chromatogram shows baseline separation of a mixture of beef, sheep, human, and pork insulins as is routinely achieved in our laboratories. These separations are truly remarkable when one considers that the amino acid sequences of these four insulins are nearly identical, particularly the human and pork insulins. The single difference between human and pork insulin is at position 30 in the B chain where threonine resides in human insulin

Table 1: Evaluative tests on BHI (3)

1. USP rabbit hypoglycemia assay	27.5 ± 1.7 U/mg* ($P \leq 0.05$)
2. Insulin radioreceptor assay	Relative potency 98 ± 7% of purified pork insulin ($P \leq 0.05$)
3. Insulin radioimmunoassay	Relative immunopotency 98 ± 22% of pancreatic human insulin ($P \leq 0.05$)
4. Amino acid composition	Comparable to pancreatic human insulin (see Table II)
5. Quantitative NH_2 terminal analysis by Edman degradation	PTH-Gly and PTH-Phe equivalent to purified pork insulin
6. Amino acid sequence of A- and B-chains used to make insulin	Correct sequences verified
7. Absorption and circular dichroic spectra	UV and CD spectra identical to purified pork insulin
8. Zinc insulin crystallization	See Figure 11
9. HPLC	Peak retention identical to pancreatic human insulin
10. Polyacrylamide gel electrophoresis	Electrophoretic migration identical with pancreatic human insulin and purified pork insulin
11. Limulus amebocyte lysate test for pyrogenic bacterial endotoxin	< 0.6 ng/mg
12. USP rabbit pyrogen test	Satisfactory

* Volatiles-free potency based upon $O.D._{276\,nm}$ measurement.

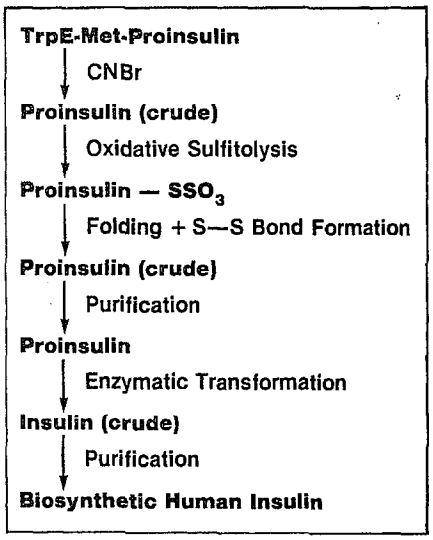

Figure 5: General biosynthetic and chemical modification process via human proinsulin, leading to the production of biosynthetic human insulin.

Table 2: Comparison of amino acid composition of biosynthetic human insulin and pancreatic human insulin[a]

Amino acid residue	Biosynthetic human insulin	Pancreatic human insulin
Aspartic acid	3.00 (3)	3.00 (3)
Threonine	2.77 (3)	2.77 (3)
Serine	2.56 (3)	2.63 (3)
Glutamic acid	7.11 (7)	7.10 (7)
Proline	1.03 (1)	0.99 (1)
Glycine	3.98 (4)	3.98 (4)
Alanine	0.97 (1)	0.99 (1)
Half-cystine	5.31 (6)	5.43 (6)
Valine	3.76 (4)	3.71 (4)
Isoleucine	1.66 (2)	1.61 (2)
Leucine	6.16 (6)	6.14 (6)
Tyrosine	3.91 (4)	3.90 (4)
Phenylalanine	2.99 (3)	2.91 (3)
Histidine	1.97 (2)	1.99 (2)
Lysine	0.97 (1)	0.97 (1)
Ammonia	6.89	6.95
Arginine	1.00 (1)	1.00 (1)

[a]Molar amino acid ratios calcuated using aspartic acid as unity, which was 160 nmoles/mg for biosynthetic human insulin and 156 nmoles/mg for pancreatic human insulin. Each value is the average of three determinations. Theoretical values are listed in parentheses.

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