Research, Development, Production, and Safety of Biosynthetic Human Insulin

RONALD E. CHANCE, PHD
BRUCE H. FRANK, PHD

This paper provides some historical aspects on the research and development of Humulin® (rDNA origin), the first human health-care product derived from rDNA technology more than a decade ago. Also referred to as biosynthetic human insulin, Humulin® is currently produced via the human proinsulin route, using an *Escherichia coli* fermentation process. The authenticity, high purity, and safety of BHI has been investigated and verified by a complex battery of analytical and physicochemical methods. The daily treatment of more than two million diabetic patients worldwide with this rDNA human insulin not only demonstrates the value of rDNA technology in providing an important medical product, it is assurance that diabetic patients will have unlimited supplies of this vital hormone as well as potential analogue refinements.

HISTORY— Humulin® was the first marketed human health-care product derived from rDNA technology. This important achievement in 1982 was the result of a vast network of basic and applied scientific advances that began in the 1950s with the classic structural studies on DNA by Watson and Crick (1) and on insulin by Sanger (2). Many of the new research developments in cell biology and rDNA technology of the early 1970s were discussed at the 16th Lilly Insulin Symposium in Indianapolis on 24–25 May 1976, which featured a central theme of insulin biosynthesis (3). At that time, a group of Lilly scientists was actively investigating several alternatives to glandular insulin. We were especially interested in the new biotechnology because it seemed to offer a viable way to provide a nonpancreatic source of insulin that could eliminate the predicted worldwide shortage expected by the end of the 20th century (4,5).

The subsequent race to synthesize the human insulin gene in the late 1970s was chronicled by Hall (3); a 10-year anniversary celebration commemorating its development was held 1 December 1988 at the Arnold and Mabel Beckman Center of the National Academies of Sciences and Engineering, Irvine, CA. Many of the scientists who helped "lay the foundation" for this occasion presented recollections of their research programs. A booklet of 20 previously published papers written by the speakers was compiled (1,4–22), and a videotape was made of the event (23). David Goeddel from Genentech indicated that the first rDNA human insulin was obtained on 24 August 1978 (3,23). A small but detectable amount was determined by RIA after combining A- and B-chains that were individually expressed in *E. coli* using chemically synthesized genes (17). Especially important for the timely success of this project was a newly developed method for rapid chemical synthesis of DNA (6,18,20,24). Also important was the emerging technology of RP-HPLC, which not only aided the purification and preparation of appropriate DNA fragments, but also the detection and characterization of the expressed proteins (16,17). RP-HPLC continues to play a vital role in the day-to-day manufacturing and analytical operations for Humulin® (25–31).

Soon after the first biosynthesis of human insulin chains in *E. coli*, Lilly entered into a contractual arrangement with Genentech, whereby Lilly would develop and commercialize the preparation of rDNA human insulin (unofficially referred to as recombinant human insulin or, more particularly, BHI) using the Genentech plasmids that contained the synthetic A- and B-chain genes (5). Subsequently, this arrangement included the gene for authentic human proinsulin. This was an enormous undertaking by Lilly because significant resources were required for the many phases of research, development, control, and clinical evaluation of this new product from rDNA technology (22). Likewise, many regulatory and fermentation containment issues had to be addressed (4,5,22,32–36). It also is noteworthy that new personnel were added to the Metabolic-Endocrine Division of the FDA in antic-

From the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana.

Address correspondence and reprint requests to Ronald E. Chance, PhD, Department MC797, (Mail Drop 1543), Lilly Corporate Center, Indianapolis, IN 46285.

BHI, biosynthetic human insulin; RIA, radioimmunoassay; RP-HPLC, reversed phase-high performance liquid chromatography; FDA, Food and Drug Administration; CNBr, cyanogen bromide; Trp LE', tryptophan synthetase protein; ECPs, *E. coli* proteins/peptides; S-SO_3, S-sulfonate; Met, methionine.

Table 1—*Chronological developments for BHI via* E. coli *fermentation*

24 August 1978	First rDNA human insulin (~20 ng) prepared by Goeddel et al. (working at City of Hope and Genentech), (3, 17, 23) using A- and B-chains expressed in *E. coli* with chemically prepared genes and an A+B-chain combination procedure
25 August 1978	Genentech and Lilly sign agreement to commercialize rDNA human insulin, announced 6 September (3, 5)
June/July 1979	A few milligrams of biologically active BHI (as demonstrated in rabbits) obtained from an improved chain combination procedure, (38) using A- and B-chains from multiple 10-L fermentations conducted at both Genentech and Lilly
5 October 1979	Lilly received permission from the Recombinant Advisory Committee for 150-L fermentations
7 December 1979	Zinc crystals obtained from first significant batch of BHI (Fig. 4); probably the first crystals of an rDNA product
April 1980	605 mg of BHI obtained in Indianapolis for pharmacology, analytical, and toxicology studies
May 1980	Team led by LeRoy Baker obtained 753 mg of BHI at Lilly Research Centre Limited, Erl Wood Manor, in England for clinical pharmacology in the U.K.
3–4 June 1980	Conference on Insulins, Growth Hormone, and Recombinant DNA Technology held at the National Institutes of Health (sponsored by the FDA's Bureau of Drugs) (32, 39, 71–73)
15 July 1980	First doses of BHI administered to normal human volunteer subjects at Guy's Hospital in London (Fig. 5; 41).
July/August 1980	Normal and diabetic subjects in Austria, Germany, Greece, and the U.K. received BHI in clinical pharmacology studies (74). BHI also supplied to numerous laboratories for in vitro testing and animal experimentation (75)
September 1980	Basic and clinical investigators meeting in Athens, Greece (76)
December 1980	Basic and clinical investigators meeting in Wiesbaden, Germany (76)
9 July 1981	First 40,000-L fermentation completed in Indianapolis (W. L. Muth, unpublished observations)
June 1982	Symposium on results of extensive clinical testing in Europe and the U.S. (78)
26 August 1982	Approval to market BHI in U.K.
13 October 1982	Approval to market BHI in Germany
28 October 1982	Approval to market BHI in the U.S.
10 April 1986	Approval to market BHI derived from human proinsulin in the U.S. Similar approval followed in other countries
1992	BHI registered and marketed in 65 countries

ipation of the many new drug applications involving rDNA technology (37).

Initial fermentations were restricted to 10 L or less (35), which necessitated multiple *E. coli* K12 fermentations to obtain just a few milligrams of each insulin chain. By the end of 1979, larger-scale fermentations were allowed and a small, but significant, amount of BHI was prepared by an improved chain combination method (38). This rDNA human insulin was identical in all respects to a pancreatic human insulin standard (39–40), thus providing encouragement to scale-up and move into clinical evaluation quickly. In fact, the first rDNA human insulin was administered to normal volunteer subjects at Guy's Hospital, London, in July of 1980 (41). Table 1 summarizes several events in the development of BHI.

PREPARATION AND EVALUATION OF RDNA HUMAN INSULIN IN *E. COLI*— Lilly has prepared BHI by two different means—initially, by a chain combination procedure (21,38–40) and, since 1986, by transforming human proinsulin into human insulin (21,42–44). Both options are outlined in Fig. 1. The decision to begin manufacturing BHI using separate A- and B-chain fermentations was prompted by the ready availability of the appropriate A- and B-chain genes, the in-house expertise in chain combination chemistry, plus the added safeguard during the early rDNA era that only inactive protein products were being expressed in *E. coli*. Although the chain combination procedure worked quite well, the proinsulin approach required fewer processing steps (Fig. 2) and, consequently, superseded the chain method in 1986 (Table 1). Furthermore, the proinsulin route allows for the preparation of authentic human proinsulin, split forms of human proinsulin, as well as C-peptide for various research investigations (45–49). Also, the use of biosynthetic human proinsulin, which is chemically and structurally identical to endogenous proinsulin, distinguishes the production of Humulin® from a yeast-derived human insulin that is produced via a nonhuman proinsulin precursor protein (50–53).

Other changes also have occurred. Originally, Lilly used the Genentech plasmids that led to the expression of A- and B-chains fused to a large *E. coli* polypeptide (1004-residue form of β-galactosidase) via a Met linkage (21,40,54), which was conveniently cleaved with CNBr. Subsequently, better yields were obtained with the tryptophan promoter system (54,55) in which the resulting chimeric fusion protein consists of a 190 amino acid-tryptophan synthetase pro-

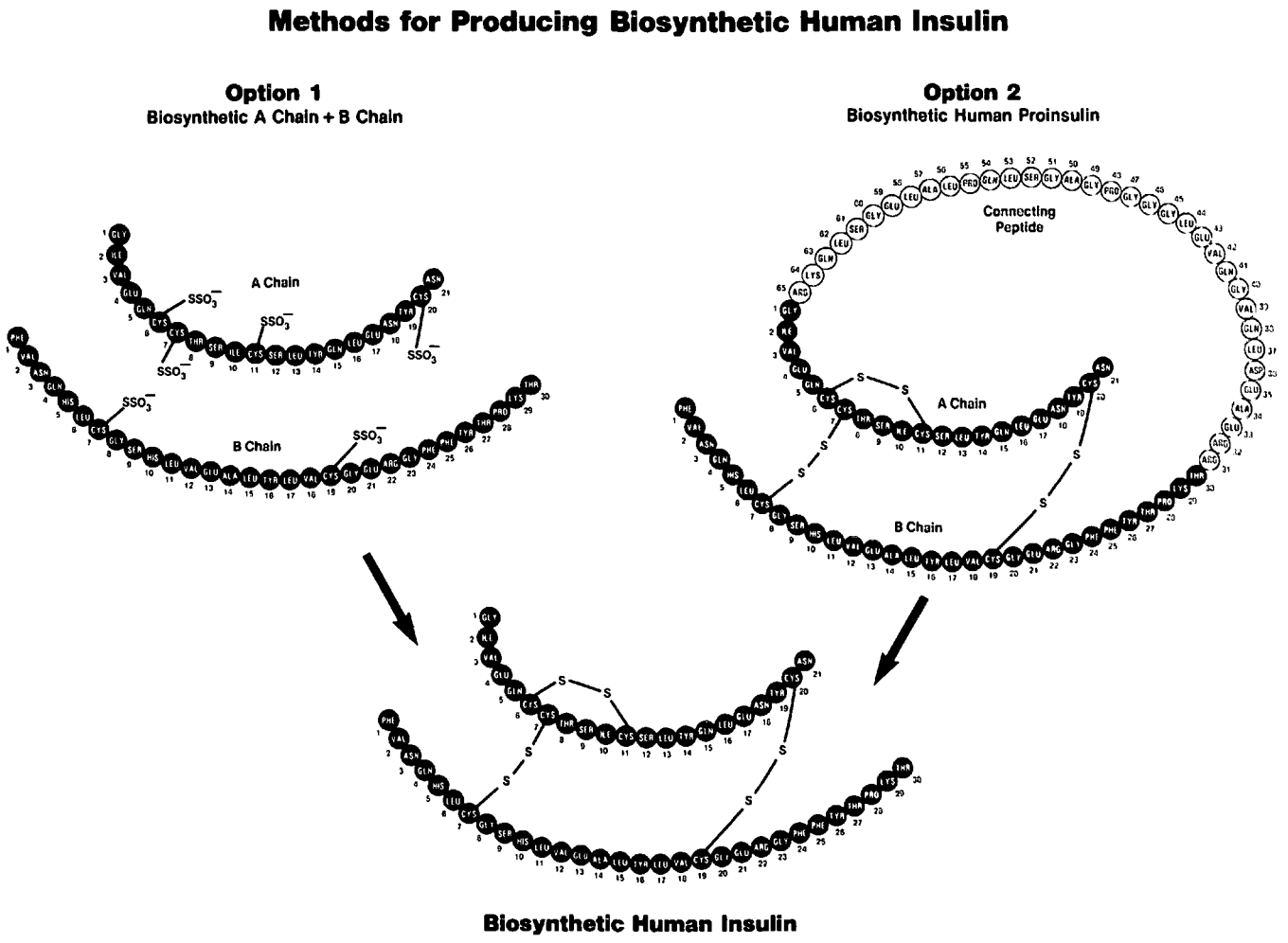

Figure 1—*Two pathways for producing BHI. From Frank and Chance (43). © by MMV Medizin Verlag, Munich.*

tein (Trp LE') joined to the desired product through a CNBr-cleavable Met residue (Fig. 2). The transmission electron micrograph of *E. coli* shown in Fig. 3 illustrates the accumulation of Trp LE'-Met-Human Proinsulin into dense cytoplasmic inclusion bodies (56,57).

An impressive collaboration has been in effect throughout the Lilly organization as BHI has evolved from the microgram scale to mega-kilogram scale since 1978. Humulin® is rapidly becoming a dominant insulin for diabetic use. Today, more than two million patients worldwide use Humulin® to control diabetes. In the U.S, this rDNA human insulin is used in more patients than all other available insulins combined. We cannot begin to give appropriate credit to all the coworkers and collaborators who have contributed to the BHI project. However, we have listed most of the references for published papers in Table 2 to give an idea of the broad spectrum of effort required to develop and market this rDNA product.

Some of the analytical issues on this project necessitate further mention because extensive characterization was required to ensure that the diabetic patient received a safe and efficacious therapy. In fact, in becoming the first company to produce an rDNA product for human use, Lilly faced many new problems and challenges. The major challenge was to rigorously characterize BHI and determine its purity on a lot-by-lot basis to assure a safe and clinically effective material. Therefore, we established an extensive battery of analytical tests, many of which were quite complex and not routinely conducted in the conventional analytical laboratory. Initially, we used a number of physicochemical and analytical tests on reference standard materials to establish that we were making a human insulin identical to pancreatic human insulin (Table 3). These studies were conducted on BHI prepared by both the A- + B-chain and the proinsulin routes. We demonstrated that,

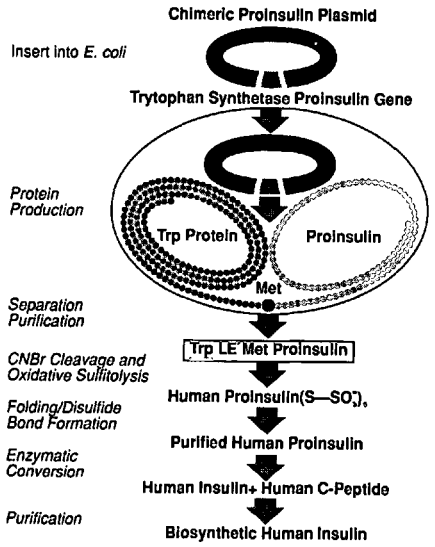

Figure 2—*General scheme for producing human insulin via the proinsulin route. From Galloway et al. (45) and Galloway (108) as adapted originally from Frank and Chance (44).*

though obviously of greater purity, biosynthetic Humulin® was identical chemically, physically, and biologically to human insulin extracted from the pancreas (5,21,38–40,42–44,58–61).

Defining the purity of Humulin® was a two-part challenge. First, we determined the nature and amount of impurities related to human insulin; second, we investigated the amount of impurities related to the host cell *E. coli*. In the case of the impurities related to human insulin, extensive efforts were made to prove that the folding and disulfide bonds of Humulin® were correct (21,22,38,43,60). Using RIAs, chemically related impurities, such as human proinsulin or C-peptide, were shown to be essentially absent from the product (44). Current batches of Humulin® consistently assay <1 ppm of proinsulin. Other chemical derivatives, such as A-21 desamido insulin, are controlled by carefully optimizing the isolation process for the product (60).

The major noninsulin contaminant issue we encountered in the development of Humulin® was that of host cell or ECPs. Considerable effort was first expended to develop an assay that would allow measurement of ECPs in our bulk insulin to prove its purity (21,62,63). As a further precaution, we undertook very large clinical studies to search for any possible immune response to these putative ECPs (60). In these studies, 18,500 serum samples from about 3,400 patients were assayed; 1,270 patients had used Humulin® for more than one year and 800 for at least two years. No defin-

Figure 3—*Transmission electron micrograph of* E. coli *cells fixed in late log phase of growth from a culture producing Trp LE'-Met-Human Proinsulin chimeric protein. Dense granulelike inclusion bodies represent expressed fused-gene protein (original magnification of × 89,000; reduced to 70% of original for publication). Photograph provided by Dan C. Williams of the Lilly Research Laboratories (56,57).*

Figure 4—*First zinc insulin crystals obtained on BHI (39,40; see 39 for a color photograph of these crystals). The crystallization experiment was conducted by James A. Hoffmann, and the photomicroscopy was provided by Fred P. Mertz (grandson of the first Lilly chemist) of Lilly Research Laboratories.*

able antibody response to ECPs was observed in patients treated with Humulin®.

Overall, no evidence suggests that any ECPs that might be present in Humulin® elicit an immune response. Thus, all of these studies clearly demonstrate that Humulin® is a very pure, homogeneous, native human insulin molecule that is clinically safe and effective. Equally important is Humulin®'s relative lack of immunogenicity when compared with animal insulins (64–66). The production of Humulin® via rDNA technology ensures unlimited supplies of highly purified human insulin products for the future (67). Certainly the preparation, development, and marketing of the first rDNA human insulin 11 years ago was an important accomplishment on the part of the Lilly organization and the hundreds of employees and collaborators throughout the world. It has also paved the way for an explosive development in biotechnology products (68,69). This effort, in a small way, rivals the incomparable efforts by Lilly during the early 1920s to make animal insulin available as a life-saving medicine (70). It is inter-

Table 2—***Pertinent references concerning the research, development, production, and control of BHI via* E. coli *fermentation***

Chemical DNA synthesis	Itakura et al. (6, 18, 20); Crea et al. (16); Riggs (79); Riggs et al. (19, 24, 80)
Molecular biology, cloning and expression in *E. coli*	Goeddel et al. (17); Riggs (79); Riggs et al. (19, 24); Burnett (54)
Chimeric fusion protein product	Kleid et al. (55); Williams et al. (56); Paul et al. (57); Burnett (54); Muth (34–36); Muth et al. (81); Patrick and Lagu (31); Prouty (82)
E. coli fermentation containment	Johnson (4, 5, 32, 33); Burnett and Marsh (83); Muth (34–36, 84, 85)
E. coli fermentation development, production and control	Rosteck and Hershberger (86); Hershberger and Rosteck (87, 88); Muth (34–36, 84, 85)
Large-scale isolation and purification	Johnson (89); Kroeff et al. (27); Prouty (82, 90–92); Riemen (93)
Characterization and authenticity	Johnson (5, 21); Galloway and Chance (22); Chance et al. (38–40); Frank and Chance (43, 44); Frank (60); Farid et al. (28)
X-ray diffraction studies	Chawdhury et al. (58, 61); Smith et al. (59)
Analytical development and control	ECPs—Baker et al. (62); Ross et al. (63); Frank et al. (94); Frank and Chance (44) Insulin— Kroeff and Chance (25); Frank and Chance (44); Smith et al. (26); Sittampalam et al. (95); Farid et al. (28); Frank (60); DiMarchi et al. (29); Patrick and Lagu (31); Atkins et al. (30); Nielsen et al. (96)
Product registration process	Galloway and Chance (22); Dinner and Fose (97, 98); Giss (99); Weissinger (100)
Formulation development	Massey and Sheliga (101); Blackshear et al. (102)
Clinical immunology	Fineberg et al. (64–66); Galloway et al. (103)
General and reviews	Johnson (4, 5); Galloway (46); Enzmann and Burnett (104); Ladisch and Kohlmann (105); Skyler et al. (106); Galloway et al. (107)

Figure 5—*First volunteers to receive injections of BHI on 15 July 1980 (41). Left to right: Del Evans, Brian Swann, Roger Harrison, Alan Glynne, William Ross, Alec Todd, Richard Marsden, and Gordon Snook. Photograph provided by Brian Swann of Lilly Research Centre Ltd., Erl Wood Manor, Surrey, England. Information from the archives about this study was provided by Richard Lucas, also of Lilly Research Centre, Ltd.*

esting to speculate about the nature and timing of the next major breakthrough for the treatment and/or prevention of diabetes. Hopefully, it will not take another 60 years. Perhaps recombinant technology, with its potential to provide human insulin analogues with more optimal and convenient time actions, and thus better patient compliance, is the key to the next discovery (this issue, Galloway, p. 16–23).

Table 3—***Tests used to evaluate BHI***

USP rabbit hypoglycemia assay (144 rabbits)
Insulin radioreceptorassay
Amino acid composition
Amino acid sequence
Gel electrophoresis
FAB/Mass spectroscopy
Disulfide bond verification
HPLC
Peptide mapping
Zinc crystallization
X-ray crystal structure
2D NMR
Limulus assay for bacterial endotoxin
Insulin receptor assays (in vitro)
USP rabbit pyrogen test
BP proteolytic activity assay
Proinsulin RIA
C-Peptide RIA
E. coli peptide RIA

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References

1. Watson JD, Crick FHC: A structure for deoxyribose nucleic acid. *Nature* 171: 737–38, 1953
2. Sanger F: Chemistry of insulin. *Science* 129:1340–44, 1959
3. Hall SS: *Invisible Frontiers. The Race to Synthesize a Human Gene.* Sidgwick and Jackson, London, 1988
4. Johnson IS: Pharmaceutical applications: microbial production of insulin. *Research with Recombinant DNA: An Academy Forum* Washington, DC, Natl. Acad. Sci., 1977, p. 156–78
5. Johnson IS: Human insulin from recombinant DNA technology. *Science* 219:632–37, 1983
6. Itakura K, Hirose T, Crea R, Riggs AD, Heyneker HL, Bolivar F, Boyer HW: Expression in *Escherichia coli* of a chemically synthesized gene for the hormone somatostatin. *Science* 198:1056–63, 1977
7. Nirenberg MW, Matthaei JH: The dependence of cell-free protein synthesis in *E. coli* upon naturally occurring or synthetic polyribonucleotides. *Proc Natl Acad Sci USA* 47:1588–602, 1961
8. Nirenberg M, Leder P, Bernfield R, Brimacombe R, Trupin J, Rottman F, O'Neal C: RNA codewords and protein synthesis, VII: on the general nature of the RNA code. *Proc Natl Acad Sci USA* 53:1161–68, 1965
9. Khorana HG: Total synthesis of a gene. *Science* 203:614–25, 1979
10. Subramani S, Berg P: Homologous and nonhomologous recombination in monkey cells. *Mol Cell Biol* 3:1040–52, 1983
11. Smith AJH, Berg P: Homologous recombination between defective neo genes in mouse 3T6 cells. *Cold Spring Symp Quant Biol* XLIX:171–81, 1984
12. Byrne G: A bit of science history is lost. *Science* 241:907, 1988
13. Morrow JF, Cohen SN, Chang ACY, Boyer HW, Goodman HM, Helling RB: Replication and transcription of eukaryotic DNA in *Escherichia coli*. *Proc Natl Acad Sci USA* 71:1743–47, 1974
14. Greene PJ, Poonian MS, Nussbaum AL, Tobias L, Garfin DE, Boyer HW, Goodman HM: Restriction and modification of a self-complementary octanucleotide containing the *Eco*RI substrate. *J Mol Biol* 99:237–61, 1975
15. Heyneker HL, Shine J, Goodman HM, Boyer HW, Rosenberg J, Dickerson RE, Narang SA, Itakura K, Lin S-y, Riggs AD: Synthetic *lac* operator DNA is functional in vivo. *Nature* 263:748–52, 1977

16. Crea R, Kraszewski A, Hirose T, Itakura K: Chemical synthesis of genes for human insulin. *Proc Natl Acad Sci USA* 75:5765–69, 1978
17. Goeddel DV, Kleid DG, Bolivar F, Heyneker HL, Yansura DG, Crea R, Hirose T, Kraszewski A, Itakura K, Riggs AD: Expression in *Escherichia coli* of chemically synthesized genes for human insulin. *Proc Natl Acad Sci USA* 76:106–10, 1979
18. Itakura K, Riggs AD: Chemical DNA synthesis and recombinant DNA studies. *Science* 209:1401–405, 1980
19. Riggs AD, Itakura K, Crea R, Hirose T, Kraszewski A, Goeddel D, Kleid D, Yansura DG, Bolivar F, Heyneker HL: Synthesis, cloning, and expression of hormone genes in *Escherichia coli*. *Rec Prog Horm Res* 36:261–76, 1980
20. Itakura K, Rossi JJ, Wallace RB: Synthesis and use of synthetic oligonucleotides. *Ann Rev Biochem* 53:323–56, 1984
21. Johnson IS: Authenticity and purity of human insulin (recombinant DNA). *Diabetes Care* 5 (Suppl. 2):4–12, 1982
22. Galloway JA, Chance RE: Human insulin rDNA: from rDNA through the FDA. In *Proceedings of the 2nd World Conference on Clinical Pharmacology and Therapeutics*. Lemberger L, Reidenberg MM, Eds. Bethesda, MD, American Society for Pharmacology and Experimental Therapeutics, 1984, p. 503–20
23. *Genetically Engineered Insulin* Videotape. Dec. 1, 1988, meeting of the National Academy of Sciences and Beckman Research Institute of the City of Hope. Arnold O. Beckman and Eugene Roberts, Organizers. A copy of the videotaped proceedings is available in the History of Medicine Division of the National Library of Medicine, Bethesda, MD, and can be acquired via interlibrary loan through local medical, university, or public library, per personal communication with John Parascandola, Chief, History of Medicine Division
24. Riggs AD, Itakura K, Hirose T, Kraszewski A, Crea R, Goeddel D, Kleid D, Yansura D, Bolivar F, Heyneker H: Chemical DNA synthesis as an approach to peptide synthesis: the human insulin project. In *Peptides, Structure and Biological Function. Proceedings of the 6th American Peptide Symposium*. Gross E, Meienhofer J, Eds. Rockford, IL, Pierce Chemical Company, 1979, p. 985–92
25. Kroeff EP, Chance RE: Applications of high-performance liquid chromatography for analysis of insulins. In *Hormone Drugs, Proceedings of the FDA-USP Workshop on Drug and Reference Standards for Insulins, Somatropins, and Thyroid-Axis Hormones*. Rockville, MD, The United States Pharmacopeial Convention, 1982, p. 148–62
26. Smith HW Jr, Atkins LM, Binkley DA, Richardson WG, Miner DJ: A universal HPLC determination of insulin potency. *J Liq Chromatogr* 8:419–39, 1985
27. Kroeff EP, Owens RA, Campbell EL, Johnson RD, Marks HI: Production scale purification of biosynthetic human insulin by reversed-phase high-performance liquid chromatography. *J Chromatogr* 461:45–61, 1989
28. Farid NA, Atkins LM, Becker GW, Diner A, Heiney RE, Miner DJ, Riggin RM: Liquid chromatographic control of the identity, purity and "potency" of biomolecules used as drugs. *J Pharm Biomed Anal* 7:185–88, 1989
29. DiMarchi RD, Long HB, Kroeff EP, Chance RE: Utilization of analytical reversed-phase HPLC in biosynthetic insulin production. In *High Performance Liquid Chromatography in Biotechnology*. Hancock WS, Ed. New York, Wiley, 1990, p. 181–89
30. Atkins LM, Miner DJ, Sittampalam GS, Wentling CD: Recommendations for establishment of reference standards for recombinant-DNA-derived proteins and polypeptides. *J Assoc Off Anal Chem* 70:610–17, 1987
31. Patrick JS, Lagu AL: Determination of recombinant human proinsulin fusion protein produced in *Escherichia coli* using oxidative sulfitolysis and two-dimensional HPLC. *Anal Chem* 64:507–11, 1992
32. Johnson IS: Industrial recombinant DNA procedures: an overview of progress and limitations. In *Insulins, Growth Hormone, and Recombinant DNA Technology*. Gueriguian JL, Ed. New York, Raven, 1981, p. 183–89
33. Johnson IS: Role of the recombinant advisory committee. *Science* 224:5–8, 1984
34. Muth WL: Large scale fermentation of recombinant micro-organisms. In *Biotechnology: Applications and Research*. Cheremisinoff PN, Ouellette RP, Eds. Lancaster, PA, Technomic, 1985, p.156–64
35. Muth WL: Scale up biotechnology safely. *Chemtech* 15:356–61, 1985
36. Muth WL: The industrialization of *E. coli*. *SIM News* 37:12–14, 1987
37. Galloway JA, Chance RE, Su KSE: Human insulin and its modifications. In *The Clinical Pharmacology of Biotechnology Products*. Reidenberg MM, Ed. Amsterdam, Elsevier, 1991, p. 23–34 (ref. 5; personal communication between S. Sobel and J.A. Galloway in May 1988)
38. Chance RE, Hoffmann JA, Kroeff EP, Johnson MG, Schirmer EW, Bromer WW, Ross MJ, Wetzel R: The production of human insulin using recombinant DNA technology and a new chain combination procedure. In *Peptides: Synthesis-Structure-Function. Proceedings of the 7th American Peptide Symposium*. Rich DH, Gross E, Eds. Rockford, IL, Pierce Chemical Company, 1981, p. 721–28
39. Chance RE, Kroeff EP, Hoffmann JA: Chemical, physical, and biological properties of recombinant human insulin. In *Insulins, Growth Hormone, and Recombinant DNA Technology*. Gueriguian JL, Ed. New York, Raven, 1981, p. 71–86
40. Chance RE, Kroeff EP, Hoffmann JA, Frank BH: Chemical, physical, and biologic properties of biosynthetic human insulin. *Diabetes Care* 4:147–54, 1981
41. Keen H, Glynne A, Pickup JC, Viberti GC, Bilous RW, Jarrett RJ, Marsden R: Human insulin produced by recombinant DNA technology: safety and hypoglycaemic potency in healthy men. *Lancet* ii:398–401, 1980
42. Frank BH, Pettee JM, Zimmerman RE, Burck PJ: The production of human proinsulin and its transformation to hu-

man insulin and C-peptide. In *Peptides: Synthesis-Structure-Function. Proceedings of the 7th American Peptide Symposium*. Rich DH, Gross E, Eds. Rockford, IL, Pierce Chemical Company, 1981, p. 729–38
43. Frank BH, Chance RE: Two routes for producing human insulin utilizing recombinant DNA technology. *Münch med Wschr* 125 (Suppl. 1):S14–20, 1983
44. Frank BH, Chance RE: The preparation and characterization of human insulin of recombinant DNA origin. In *Therapeutic Agents Produced by Genetic Engineering "Quo Vadis?"* Joyeaux A, Leygue G, Morre M, Roncucci R, Schmelck PH, Eds. Toulouse-Labege, Symposium Sanofi Group, Paris MEDSI, 1985, p. 137–46
45. Galloway JA, Hooper SA, Spradlin CT, Howey DC, Frank BH, Bowsher RR, Anderson JH: Biosynthetic human proinsulin: review of chemistry, in vitro and in vivo receptor binding, animal and human pharmacology studies, and clinical trial experience. *Diabetes Care* 15:666–92, 1992
47. Polonsky KS, Licinio-Paixao J, Given BD, Pugh W, Rue P, Galloway J, Karrison T, Frank B: Use of biosynthetic human C-peptide in the measurement of insulin secretion rates in normal volunteers and Type I diabetic patients. *J Clin Invest* 77:98–105, 1986
48. Clark PM, Levy JC, Cox L, Burnett M, Turner RC, Hales CN: Immunoradiometric assay of insulin, intact proinsulin and 32–33 split proinsulin and radioimmunoassay of insulin in diet-treated type 2 (non-insulin-dependent) diabetic subjects. *Diabetologia* 35:469–74, 1992
49. Johansson B-L, Sjöberg S, Wahren J: The influence of human C-peptide on renal function and glucose utilization in Type 1 (insulin-dependent) diabetic patients. *Diabetologia* 35:121–28, 1992
50. Thim L, Hansen MT, Norris K, Hoegh I, Boel E, Forstrom J, Ammerer G, Fiil NP: Secretion and processing of insulin precursors in yeast. *Proc Natl Acad Sci USA* 83:6766–70, 1986
51. Diers IV, Rasmussen E, Larsen PH, Kjaersig I-L: Yeast fermentation processes for insulin production. In *Drug Biotechnology Regulation*. Chiu Y-yH, Gueriguian JL, Eds. New York, Marcel Dekker, 1991, p. 166–76
52. Christensen T, Dalbøge H, Snel L: Post-biosynthesis modification: human growth hormone and insulin precursors. In *Drug Biotechnology Regulation*. Chiu Y-yH, Gueriguian JL, Eds. New York, Marcel Dekker, 1991, p. 206–21
53. Raskin P, Clements RS Jr: The use of human insulin derived from baker's yeast by recombinant DNA technology. *Clin Therap* 13:569–78, 1991
54. Burnett JP: Commercial production of recombinant DNA-derived products. In *Experimental Manipulation of Gene Expression*. Inouye M, Ed. New York, Academic, 1983, p. 259–77
55. Kleid DG, Yansura D, Small B, Dowbenko D, Moore DM, Grubman MJ, McKercher PD, Morgan DO, Robertson BH, Bachrach HL: Cloned viral protein vaccine for foot-and-mouth disease: responses in cattle and swine. *Science* 214:1125–29, 1981
56. Williams DC, Van Frank RM, Muth WL, Burnett JP: Cytoplasmic inclusion bodies in *Escherichia coli* producing biosynthetic human insulin proteins. *Science* 215:687–89, 1982
57. Paul DC, Van Frank RM, Muth WL, Ross JW, Williams DC: Immunocytochemical demonstration of human proinsulin chimeric polypeptide within cytoplasmic inclusion bodies of *Escherichia coli*. *Eur J Cell Biol* 31:171–74, 1983
58. Chawdhury SA, Dodson EJ, Dodson GG, Reynolds CD, Tolley S, Cleasby A, Wood SP, Pitts JE: The crystal structure of two-zinc human insulin. In *Hormone Drugs, Proceedings of the FDA-USP Workshop on Drug and Reference Standards for Insulins, Somatropins, and Thyroid-Axis Hormones*. Rockville, MD, The United States Pharmacopeial Convention, 1982, p. 106–15
59. Smith GD, Swenson DC, Dodson EJ, Dodson GG, Reynolds CD: Structural stability in the 4-zinc human insulin hexamer. *Proc Natl Acad Sci USA* 81: 7093–97, 1984
60. Frank BH: The validity of preclinical studies for prediction of human response to insulin. In *Excerpts From a Workshop on Preclinical Evaluation of Peptides and Recombinant Proteins*. Sundwall A, Ekman L, Johansson H-E, Sjöberg B, Sjöholm I, Eds. Malmö, Skogs Grafiska AB, 1990, p. 61–67
61. Chawdhury SA, Dodson EJ, Dodson GG, Reynolds CD, Tolley SP, Blundell TL, Cleasby A, Pitts JE, Tickle IJ, Wood SP: The crystal structures of three non-pancreatic human insulins. *Diabetologia* 25:460–64, 1983
62. Baker RS, Schmidtke JR, Ross JW, Smith WC: Preliminary studies on the immunogenicity and amount of *Escherichia coli* polypeptides in biosynthetic human insulin produced by recombinant DNA technology. *Lancet* ii:1139–41, 1981
63. Ross JW, Baker RS, Hooker CS, Johnson IS, Schmidtke JR, Smith WC: Procedure for detection of potential *E. coli* peptides (ECPs) in biosynthetic human insulin (BHI), antibodies to ECPs in patients treated with BHI, and measurement of bacterial endotoxins in BHI. In *Hormone Drugs, Proceedings of the FDA-USP Workshop on Drug and Reference Standards for Insulins, Somatropins, and Thyroid-Axis Hormones*. Rockville, MD, The United States Pharmacopeial Convention, 1982, p. 127–38
64. Fineberg SE, Galloway JA, Fineberg NS, Rathbun MJ: Immunologic improvement resulting from the transfer of animal insulin-treated diabetic subjects to human insulin (recombinant DNA). *Diabetes Care* 5 (Suppl. 2):107–13, 1982
65. Fineberg SE, Galloway JA, Fineberg NS, Rathbun MJ, Hufferd S: Immunogenicity of recombinant DNA human insulin. *Diabetologia* 25:465–69, 1983
66. Fineberg SE, Fineberg NS, Galloway JA: Human insulin: should it replace other insulins? In *Diabetes Mellitus: Achievements and Skepticism*. L'Etang H-JC, Ed. London, Royal Society of Medicine International Congress and Symposium, Series No. 77, 1984, p. 65–72
67. Skyler JS: Human insulin of recombinant DNA origin: clinical potential. *Diabetes Care* 5 (Suppl. 2):181–86, 1982

68. Mossinghoff GJ: Biotechnology medicines in development. In *Survey Report*. Washington, DC, Pharmaceutical Manufacturer's Association, August 2, 1991
69. Fradd RB: Biotechnology medicines in development. In *Genetic Engineering News*. New York, Mary Ann Liebert, 1992, p. 27–28
70. Bliss M: *The Discovery of Insulin*. Toronto, McClelland and Stewart Limited, 1982
71. Ross MJ: Production of medically important polypeptides using recombinant DNA technology. In *Insulins, Growth Hormone, and Recombinant DNA Technology*. Gueriguian JL, Ed. New York, Raven, 1981, p. 33–48
72. Chiu Y-yH, Kertesz DJ, Kumkumian CS: The U.S. Food and Drug regulatory process: chemical and manufacturing controls. In *Insulins, Growth Hormone, and Recombinant DNA Technology*. Gueriguian JL, Ed. New York, Raven, 1981, p. 207–13
73. Miller HI, Gueriguian JL, Troendle G, Sobel S: Medical aspects of the drug regulatory process: recombinant DNA technology. In *Insulins, Growth Hormone, and Recombinant DNA Technology*. Gueriguian JL, Ed. New York, Raven, 1981, p. 215–22
74. Skyler JS, Pfeiffer EF, Raptis S, Viberti GC: Biosynthetic human insulin: progress and prospects. *Diabetes Care* 4:140–43, 1981
75. DeMeyts P, Halban P, Hepp KD: In vitro studies on biosynthetic human insulin: an overview. *Diabetes Care* 4:144–46, 1981
76. Chance RE, Enzmann FH, Galloway JA, Glynne A, Marsden JH: Introduction. Symposium on biosynthetic human insulin. *Diabetes Care* 4:139, 1981
78. Skyler JS: Symposium on human insulin of recombinant DNA origin. *Diabetes Care* 5 (Suppl. 2):1–186, 1982
79. Riggs AD: Bacterial production of human insulin. *Diabetes Care* 4:64–68, 1981
80. Riggs AD, Itakura K, Boyer HW: From somatostatin to human insulin. In *Recombinant DNA Products: Insulin, Interferon and Growth Hormone*. Bollon AP, Ed. Boca Raton, CRC Press, 1984, p. 38–45
81. Muth WL, Hershberger CL, Zavela DA, Rosteck PR Jr, Elliott DD: Maximizing biosynthesis of human insulin fusion proteins by controlling cell mass and gene expression in *Escherichia coli*. *Abstr Pap Am Chem Soc* 188:74, 1984
82. Prouty WF: Production-scale purification processes. In *Drug Biotechnology Regulation*. Chiu Y-yH, Gueriguian JL, Eds. New York, Marcel Dekker, 1991, p. 221–62
83. Burnett JP Jr, Marsh M: Recombinant DNA technology: tool of the 1980s. *Pharm Tech* 4:42–6, 1980
84. Muth WL: Fermenters for prokaryotic cells. In *Drug Biotechnology Regulation*. Chiu Y-yH, Gueriguian JL, Eds. New York, Marcel Dekker, 1991, p. 103–11
85. Muth WL: Fermentation processes for prokaryotic cells. In *Drug Biotechnology Regulation*. Chiu Y-yH, Gueriguian JL, Eds. New York, Marcel Dekker, 1991, p. 155–65
86. Rosteck PR Jr, Hershberger CL: Selective retention of recombinant plasmids coding for human insulin. *Gene* 25:29–38, 1983
87. Hershberger CL, Rosteck PR Jr: Stability of recombinant plasmids for production of heterologous proteins in *Escherichia coli*. In *Drug Biotechnology Regulation*. Chiu Y-yH, Gueriguian JL, Eds. New York, Marcel Dekker, 1991, p. 3–16
88. Hersberger CL, Rosteck PR Jr: Genetic and molecular studies to certify seed pools and production fermentations of industrial *Escherichia coli* strains. In *Drug Biotechnology Regulation*. Chiu Y-yH, Gueriguian JL, Eds. New York, Marcel Dekker, 1991, p. 86–101
89. Johnson RD: The processing of biomacromolecules: a challenge for the eighties. *Fluid Phase Equilibria* 29:109–23, 1986
90. Prouty WF: Lessons from production of recombinant insulin. *Biotech USA* 1989, p. 224–30
91. Prouty WF: How to recover recombinant protein products. *Chemtech* 22: 608–15, 1992
92. Prouty WF: Process chromatography in production of recombinant products. In *Chromatography in Biotechnology*. Horvath C, Ettre LS, Eds. Washington, DC, 1993, American Chemical Society Symposium, Series 529, p. 43–58
93. Riemen MW: Biosynthetic human insulin: manufacturing a pharmaceutical peptide. In *Peptides, Chemistry and Biology. Proceedings of the 12th American Peptide Symposium*. Smith JA, Rivier JE, Eds. Leiden, Netherlands, ESCOM, 1992, p. 669–71
94. Frank BH, Bromer WW, Chance RE, Scheetz ME, Smith WC, Ross JW: A radioimmunoassay (RIA) for a "marker" *E. coli* peptide to monitor the purity of biosynthetic human insulin (BHI). In *Diabetes Research and Clinical Practice*. XII Congress of the International Diabetes Federation 1985 (Suppl. 1), Amsterdam, Elsevier Science, p. S176
95. Sittampalam GS, Ellis RM, Miner DJ, Rickard EC, Clodfelter DK: Evaluation of amino acid analysis as reference method to quantitate highly purified proteins. *J Assoc Off Anal Chem* 71:833–38, 1988
96. Nielsen RG, Sittampalam GS, Rickard EC: Capillary zone electrophoresis of insulin and growth hormone. *Anal Biochem* 177:20–26, 1989
97. Dinner A, Fose J: Effectively dealing with the special chemical and pharmaceutical considerations in the U.S. and EEC for biotechnology products—part 2. *Drug Inf J* 23:501–509, 1989
98. Dinner A, Fose JM: Industry's experience with worldwide regulation of biotechnology products. In *Drug Biotechnology Regulation*. Chiu Y-yH, Gueriguian JL, Eds. New York, Marcel Dekker, 1991, p. 371–82
99. Giss HE: Critical analysis of the guidelines of the USA, EEC and Japan. In *Excerpts From a Workshop on Preclinical Evaluation of Peptides and Recombinant Proteins*. Sundwall A, Ekman L, Johansson H-E, Sjöberg B, Sjöholm I, Eds. Malmö, Skogs Grafiska AB, 1990, p. 117–128
100. Weissinger J: Non-clinical studies conducted in the development of biotechnology products. In *Excerpts From a Workshop on Preclinical Evaluation of Peptides and Recombinant Proteins*.

Sundwall A, Ekman L, Johansson H-E, Sjöberg B, Sjöholm I, Eds, Malmö, Skogs Grafiska AB, 1990, p. 129–30
101. Massey EH, Sheliga TA: Stability and properties of insulin for pumps. In *Continuous Insulin Infusion Therapy. Experience from One Decade*. Hepp KD, Renner R, Eds. New York, Schattauer, 1985, p. 27–32
102. Blackshear PJ, Robbins DC, Rohde TD, Langer RS, Moses AC, Massey EH: Insulin replacement: current concepts: synopsis of a panel discussion. *ASAIO* 9:646–55, 1986
103. Galloway JA, Fireman P, Fineberg SE: Complications of insulin therapy: a brief overview of four years of experience with human insulin (rDNA). In *Diabetes Mellitus: Achievements and Skepticism*. L'Etang H-JC, Ed. London, Royal Society of Medicine International Congress and Symposium, Series No. 77, 1984, p. 55–64
104. Enzmann FH, Burnett JP: Implications of recombinant DNA technology for the future of medicine. In *Diabetes Mellitus: Achievements and Skepticism*. L'Etang H-JC, Ed. London, Royal Society of Medicine International Congress and Symposium Series No. 77, 1984, p. 17–25
105. Ladisch MR, Kohlmann KL: Recombinant human insulin. *Biotechnol Prog* 8:469–78, 1992
106. Skyler JS, Galloway JA, Chance RE: Human insulin produced by recombinant DNA technology. In *Diabetes Mellitus*. Skyler JS, Cahill GF Eds. New York, Yorke Medical Books, 1981, p. 146–51
107. Galloway JA, Fineberg SE, Root MA, Spradlin TC: Human insulin (rDNA): a summary of clinical studies in the USA. In *Current and Future Therapies with Insulin*, Sakamoto N, Alberti KGMM, Eds. Amsterdam, Excerpta Medica International Congress, Series 607, 1983, p. 80–89
108. Galloway JA: Chemistry and clinical use of insulin. In *Diabetes Mellitus*. 9th ed. Galloway JA, Potwin JH, Shuman CR, Eds. Indianapolis, Lilly, 1988, p. 106–37