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REVIEW PAPERS

Human Insulin: Basic Sciences to Therapeutic Uses

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INTRODUCTION

Insulin (Fig. 1) is the most important regulatory hormone in the control of glucose homeostasis. An epidemiologic study conducted in 1970s estimated that approximately 20 million persons worldwide suffer from insulin-dependent diabetes mellitus and require daily parenteral injections of insulin to stay healthy and live normally (1). A recent report indicated that there are more than 50 million people around the world who suffer from diabetes (World Health Organization, May–June/91 report).

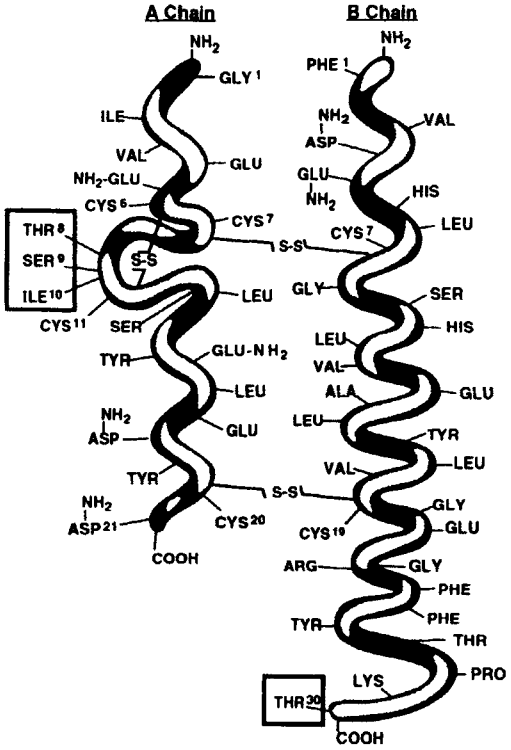
In 1977 the U.S. Food and Drug Administration (FDA) released a study, referred to as "FDA study 39," which questioned the adequacy of insulin supply in the United States within the next decades (2). Upon request from FDA, the National Diabetes Advisory Board (NDAB) established an "Ad Hoc Insulin Study Committee" to perform a comprehensive analysis of insulin supply and demand (3).

Following the analysis of the data on the multitude of factors affecting the supply of and demand for insulin, the committee estimated that the diabetic population will grow to 11.9–16.5 million by the year 2000 in the USA alone (Table 1); among them, 3.8 (± 0.6) million will be insulin- dependent diabetes (Table 2), and demand for insulin is projected to be 55.9 (± 8.6) billion units (Table 3). The National Diabetes Data Group performed an analysis of the data and projected that 1996 is the earliest year that demand for insulin could possibly exceed supply (3). However, the possibility was also recognized that new sources of insulin supply resulting from the advances in biotechnology and/or the improvement in forms of treatment, when achieved, could delay the potential shortage.

This article intends to provide an overview of the physiologic basis and historic development of insulin, the synthesis of insulin, and the pharmaceutics aspects of insulin preparations for glycemic control as well as the pharmacokinetics and pharmacodynamics of insulin in the treatment of diabetes.

PHYSIOLOGIC BASIS OF INSULIN

The connection between disturbance of the pancreas functions and occurrence of diabetes mellitus was noted as early as 1788 (4); then in 1889, diabetes mellitus was induced experimentally by disruption of the pancreas (5). The pancreatic substance responsible for the lowering of blood glucose level was discovered to be pro-



Species	A-Chain (21 amino acids) 8th	9th	10th	B-Chain (30 aa) 30th
Human	Thr	Ser	Ile	Thr
Pork	Thr	Ser	Ile	Ala
Dog	Thr	Ser	Ile	Ala
Sperm Whale	Thr	Ser	Ile	Ala
Rabbit	Thr	Ser	Ile	Ser
Sei Whale	Ala	Ser	Thr	Ala
Beef	Ala	Ser	Val	Ala
Sheep	Ala	Gly	Val	Ala
Horse	Thr	Gly	Ile	Ala

Ala: Alanine	Ser: Serine
Gly: Glycine	Thr: Threonine
Ile: Isoleucine	Val: Valine

Figure 1. Primary structure of human insulin and the species variation in the type of amino acid residues among some mammalian insulins.

duced in the Langerhans islet cells (6,7). This unknown substance was later named "insulin" by de Meyer (8).

Biosynthesis

In the healthy nondiabetic human, insulin is synthesized in the β cells of the Langerhans islets (6,7). The islets are scattered over the entire pancreas (they amount to only approx. ~1%). Investigations on the biosynthesis of insulin with labeled amino acids were made easier using the isolated islet tissue of fish, whose exocrine pancreas is physically separated from the endocrine pancreas. The isolation of insulin with high specific activity from other animal species was also made possible by

Table 1

Estimate of U.S. Diabetic Population: 1975-2000

Source of Statistics	Population (millions) 1975	1985	1995	2000
Nonprofit organizations				
National Center for Health Statistics	4.78	—	—	—
Food and Drug Administration[a]	4.78	8.38	11.98	13.78
Insulin Manufacturers				
Eli Lilly & Co.[b]	4.80	8.04	13.48	16.46
Armour Pharm. Co.[c]	4.70	6.82	9.91	11.94

[a]Based on a net increase of 360,000 diabetics per year.
[b]Based on an annual growth rate of 5.3%.
[c]Based on an annual growth rate of 3.8%.

Table 2

Estimate of U.S. Insulin-User Diabetic Population: 1975–2000

Source of Statistics	Population (millions) 1975	1985	1995	2000
Nonprofit Organizations				
National Center for Health Statistics[a]	1.08	1.89	2.71	3.11
Food and Drug Administration[b]	1.43	2.51	3.58	4.12
Insulin Manufacturers				
Eli Lilly & Co.[c]	1.30	2.18	3.65	4.46
Armour Pharm. Co.[d]	1.42	2.06	2.99	3.61
Mean	1.31	2.16	3.23	3.83
(± SD)	(0.16)	(0.26)	(0.46)	(0.59)

[a]Based on the rate of 22.6%.
[b]Based on the rate of 29.9%.
[c]Based on the rate of 27.1%.
[d]Based on the rate of 30.2%.

separating endocrine pancreas from exocrine pancrease (9,10).

The biosynthesis of insulin was noted to follow the general pattern of protein biosynthesis: it occurs in the endoplasmatic reticulum (by ribosomes), with the synthesis of the B chain beginning at the *N*-terminus (11). The separate biosynthesis of the insulin's A and B chains was demonstrated in doubly labeled insulin from fish islets. The biosynthesis of insulin in the ox pancreas was reportedly stimulated slightly by glucose, but glucose did not show any effect in fish islets (12,13).

A special problem in the biosynthesis of insulin is the linking of the A and B chains via disulfide bridges. The question is whether linkage of the two chains in vivo occurs spontaneously, or whether the process is catalyzed by an enzyme called "insulin zipase" (11). A glutathione insulin transhydrogenase, which is capable of splitting insulin molecule into its A and B chains by reducing its disulfide bridges, has been isolated from liver (14–16). The enzymes capable of carrying out a reductive cleavage of insulin appear to exist, not only in the liver, but in the pancreas as well.

Table 3

Estimate of U.S. Insulin Demand: 1975–2000

Source of Statistics	Insulin Usage[a] (billion units) 1975	1985	1995	2000
Nonprofit Organizations				
National Center for Health Statistics	15.77	27.59	39.57	45.41
Food and Drug Administration	20.88	36.65	52.27	60.15
Insulin Manufacturers				
Eli Lilly & Co.	18.98	31.83	53.29	65.12
Armour Pharm. Co.	20.73	30.08	43.65	52.71
Mean	19.09	31.54	47.20	55.85
(± SD)	(2.38)	(3.83)	(6.67)	(8.63)

[a]Based on a daily dose of 40 units, or 14,600 units per person per year.

Physiologically, it requires around 12 days to synthesize the amount of insulin normally present in the pancreas, which is about 250 IU (10 mg) in humans. This amount of insulin is 5–10 times the daily consumption of insulin for glucose homeostatis.

Secretion

After its biosynthesis in the endoplasmatic reticulum, insulin is stored in the secretory granules by forming a complex with other proteins; this complex constitutes about 10% of the proteins in the granules (17). The islet cells are known to contain high levels of zinc (18,19), which could be important to the storage of insulin.

The transport of insulin from the synthesis site (in the ribosomes) to the secretory granules and the process of secretion [via the fusion of the granule membrane with the cell membrane, with the simultaneous liberation of insulin into the capillaries (20)] appear to be similar to the secretion process in the exocrine pancreas (21). Insulin secretion is regulated mainly by the levels of both glucose and insulin in the circulation (22). A direct dependence of secretion rate on the glucose level has been observed (23,24). The secretion was reported to be inhibited by the addition of insulin to a pancreas preparation (25).

Mode of Action

Insulin is an anabolic hormone; that is, it promotes the synthesis of glycogen, fat, and protein.

There are a number of theories in which most actions of insulin have been attributed to one primary effect. The best known of these is the "theory" of glucose permeability (26), which postulates that the primary effect of insulin is to promote the entry of glucose into the cell. The increased oxidation of glucose, the facilitated synthesis of glycogen and fat, the decreased degradation of protein and fat, and the prevention of ketone formation can be easily explained on the basis of this primary effect. This theory is supported by numerous results obtained in vivo and in vitro (27,28).

Insulin has also been observed to promote the transport of some amino acids (29,30) and of K^+ ion (31), even in the absence of glucose. Similarly, insulin also inhibits the liberation of free fatty acids and of glycerol from the adipose tissue. It has been shown that all insulin effects on the adipose tissue can be imitated by phospholipase C (32), and the action of insulin on the plasma membrane is thus postulated to result from the modification of membrane lipids (32). This would expand the original "glucose permeability theory" into a more generalized "theory of membrane permeability." However, some of the insulin's effects, such as the stimulation of protein synthesis (33) and of glycogen synthetase (34), cannot be explained even with this expanded theory.

A promising approach in clarifying insulin's mode of action appears to be the investigation of its active center.

Metabolism

From the pancreatic vein, insulin molecules are first transported into the liver, where approximately half of the molecules are degraded and removed from hepatic circulation (35,36). The half-life of insulin is about 30 min in man (37). The liver and the kidneys are the two principal organs responsible for the metabolism of insulin. Insulinase (38), which is probably identical to glutathione insulin transhydrogenase (14–16, 38–41), already discussed under Biosynthesis, has also been reported to catalyze the reductive cleavage of the interchain disulfide bridges in the insulin molecule; the reduced chains are further hydrolyzed to some smaller fragments (42,43).

In addition to the liver, the pancreas (34,41,44,45), muscle tissue (44), and adipose tissue (46,47) have also been reported to have the ability to cleave the insulin molecule by reduction (40,41) or proteolysis (44–47).

HISTORY OF INSULIN DISCOVERY

Historically, the discovery of insulin was credited to Banting and Best, who extracted the active principle from the dog pancreas as early as 1921 and demonstrated its therapeutic effects in diabetic dogs and human subjects in 1922 (48). However, it should be pointed out that many researchers had paved the way to this momentous discovery; among the prominent events are the following.

As early as 1788 Cowley (4) observed a connection between disturbance of pancreas function and diabetes mellitus.

Von Mering and Minkowski (5), in 1889, together demonstrated that diabetes mellitus could be induced experimentally by disruption of the pancreas.

In 1900 Schulze (6) and Ssobolew (7) showed that the pancreatic substance responsible for lowering

of blood glucose level was formed in the Langerhans islet cells, which was thus named "insulin" by de Meyer (8) in 1909.

The extraction of insulin from the pancreas was achieved as early as in 1911, but it was not carried out consistently until 1922, by Banting and Best (49).

The historic development and chemistry of insulin was extensively reviewed by Klostermeyer and Humbel (50) in 1966 and by Galloway (51) in 1988.

There were two working hypotheses that could have contributed to Banting's successful approach: (i) the Langerhans islet is the tissue that secretes insulin, and (ii) the failure to isolate insulin is due to the proteolytic destruction of insulin by the pancreatic enzymes during the course of extraction. A simple approach was devised elegantly to circumvent the difficulty by tying the pancreatic ducts to degenerate the acinar tissue and leave the islet tissue undisturbed, and so the active principle could be extracted directly from the remaining islet (49). Further development has improved the process so that insulin can be extracted directly from the excised pancreas (fresh or deep-frozen) by using acidified alcohol (60–80% ethanol at pH 1–3); this acidic alcohol also inactivates the exocrine proteases in the pancreas and thus prevents the proteolytic destruction of insulin.

The discovery of insulin has been credited with having led to more advantages than its dramatic success in the glycemic control of diabetes mellitus. It has also triggered attention on the therapeutic uses of naturally occurring substances as replacements for what the body is incapable of supplying in quantities adequate to maintain normal health.

Two authoritatively researched books (52,53) have been published to present the details on and call attention to some of the remarkable elements contributing to insulin's discovery. While working in MacLeod's laboratory in Toronto, Banting and Best accomplished a feat that had eluded researchers for at least three decades. Banting's persistence convinced MacLeod and Collip, the two collaborators with scientific and technical expertise essential to the isolation of insulin, which was a critical factor in the overall success of the research program. A combination of these efforts was the first element for success in the discovery of insulin. A second element was luck, when Collip reclaimed his initial success in the isolation of the active principle, "insulin," from the pancreatic materials extracted by Banting and Best.

Another element of the success could be attributed to the interest of a pharmaceutical firm in developing a procedure to produce insulin in commercial quantity. In December of 1921 Eli Lilly & Co. offered its facilities and cooperation, through the University of Toronto, to Banting and Best, for the development and production of insulin. By April of 1922 the two groups were actively collaborating in a team effort to produce a purified insulin. The first batch of commercially available insulin was thus successfully produced in 1923 (51). In the following year, the production of insulin was also initiated by other companies in Australia, Denmark, Germany, the Netherlands, and the United Kingdom (54).

CONVENTIONAL METHODS OF INSULIN PRODUCTION

The consistent extraction of insulin from the pancreas made by Banting and Best (49) started the chemistry of insulin (55), which has since progressed through the isolation of pure insulin (56) in 1926, the assay of its constitution (57) in 1955, through its synthesis (58–62) in 1963–1964, and to the determination of its steric structure.

Using methods (62) developed from the pioneering work of Collip (63) and of Somogyi et al. (64), up to 5 mg of insulin could be isolated. The methods call for the extraction of insulin from a fresh or a deep-frozen gland using 60–80% ethanolic solution (at pH 1–3); this treatment inactivated the exocrine proteases of the pancreas. The extract was evaporated under vacuum (freed from fats); the insulin was then salted out, redissolved, and finally precipitated isoelectrically at pH 5.3–5.4. In a more recent process, the insulin was precipitated at pH 8, and residual enzymes were destroyed by brief heating in an acidic solution; the insulin was then adsorbed on ion-exchange resin and eluted. This method leads to a 20–30% improvement in yield (65). As much as 2000 IU of insulin could be produced from 1 kg of ox pancreas; from calf pancreas, up to 10,000 IU per kg could be obtained (since the pancreas' exocrine functions have not yet developed in the calf) (66).

Crude insulin is most easily purified by repeated crystallization (67). This procedure gives products with consistent biological activity and, thus, the products were long thought to be absolutely pure. However, the use of more accurate analytical methods has shown that even the repeatedly recrystallized insulins are not homogeneous (68–70). Apart from glucagon, which is the

antagonistic hormone to insulin (71,72), the most frequently found impurity is deamidoinsulin (73), an insulinlike protein with little or no biological activity (74). Additionally, antigenic substances (75) have also been detected.

PHYSICOCHEMICAL PROPERTIES OF INSULIN

Having all of the structural features of a large protein molecule, insulin has been an ideal example for studying the structure, properties, and characteristics of proteins. Insulin has been one of the most extensively studied molecules in biochemistry (76).

Crystallization

Numerous crystalline insulin preparations are known, all containing crystallization water and also relatively small quantities of foreign ions. The insulin crystals are often externally similar, but they may differ if obtained from different animals (75–78).

When insulin was crystallized isoelectrically from a buffered solution at pH 5.5–5.6, zinc was often detected in these crystals. Furthermore, it was shown that bivalent metal ions, such as Zn^{2+}, Ni^{2+}, Co^{2+}, or Cd^{2+}, are essential to the crystallization of insulin (79); Cu^{2+}, Mn^{2+}, Fe^{2+}, etc., ions could do the same (73). It was found that half of the zinc in the insulin crystal can be replaced by magnesium or calcium (80).

The metal content in the insulin crystals is directly proportional to the atomic weight of the metal (81) (e.g., 0.52% for Zn). Crystals with lower metal contents, such as 0.33% Zn, are also known (76,77). The zinc content can be increased to as much as 5% (82,83), which forms a double salt of the type 2 M^{2+} insulin$^{4-} \cdot MX_2$ (76,84). The metal ions can be removed from these insulins without destroying the crystals (76,85); for example, zinc ion can be removed by dialysis or gel filtration (86), and this process was reportedly facilitated by the addition of glycine.

In addition to chloride, other anions (such as acetate, citrate, phosphate, or carbonate) may also be present for the crystallization of insulin (87). In acidic solutions (pH 2–2.5) with low ionic strength, insulin molecules become only slightly associated and crystallize as phosphate, chloride, acetate, and citrate even in the absence of metal (88–92). In addition, the crystalline salts of insulin with organic bases, free from zinc, also occur (92).

The acidic salts of insulin form orthorhombic crystals, which are probably built up from insulin dimers (93,94). The cubic "metal-free" form of insulin gives very small rhombododecahedral crystals, in which six insulin dimers appear to form an aggregate (95). The insulin molecules in the dimers exist in parallel position (93,94,97). The monoclinic crystals of zinc insulin, in which the unsymmetrical unit cell contains aggregates of six insulin molecules with two zinc ions, could be formed in a phenol-containing solution (95). The rhombohedral crystals of insulin, in which the unit cell contains two or four zinc ions in the insulin hexamer, are formed depending on the halide content (95,96).

Insulin crystals normally contain 30–51% of crystallization water, which shrinks in air to a water content of 10%. Part of the water (30 moles per mole of insulin) cannot be released even by drastic drying (98).

Structural Composition

Insulin was recognized as a protein molecule as early as 1928 (99), but the sequence of its 51 amino acids (Fig. 1) was not resolved until 1955 by the pioneering work of Ryle et al. (100). Its 17 different amino acids have been repeatedly found in its various hydrolysates. Insulin contains an unusually high content of sulfur (3.3%), all of which is present in the form of cystine (101,102). It has been concluded that active insulin must consist of at least two parallel peptide chains held together by disulfide bridges (103), which was confirmed by Sanger. In 1945 Sanger also detected phenylalanine (104) and glycine (105) as the *N*-terminal amino acid residues, which were present in the ratio of 1:1.

In addition, Sanger and his coworkers (106) established the overall structural formula of bovine insulin. They are the first research group to have determined the sequences of the 21 and 30 amino acids in the A and B chains (107–109), respectively, and then the positions of amide groups (110) and of three disulfide bridges (57). The molecular weight was found to be 5734.

Sanger (111,112) discovered differences in the amino acide composition of the A chains of ox, sheep, and pig insulin, which occurs in the $A_{8\text{-}10}$ region (Fig. 1). The primary structures are now known for insulins from more than 50 animal species (67,76,113–120). Among insulin's 51 amino acids, 10 in each chain are invariant residues and the others are variant residues (Fig. 2). The variation of insulin was observed to increase with increasing phylogenetic difference between the species. Even though the insulin molecule occurs in numerous

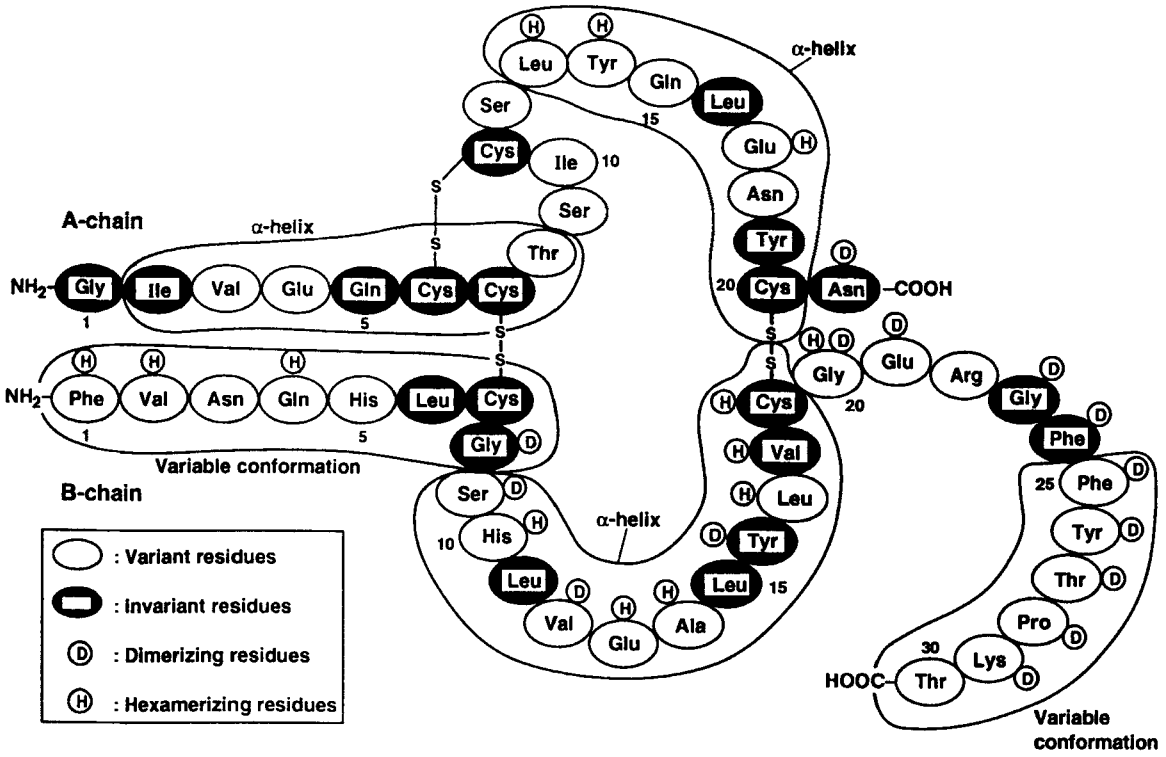


Figure 2. The amino acid sequence of human insulin and the conformations of its A and B chains. The variations in the types of amino acid residues and their potential participation in the dimer and/or hexamer formation are identified. (Modified from Brange and Langkjaer, 1993.)

varients, it is interesting to note that all have the same biological activity.

The 51 amino acids in the insulin molecule are arranged in two peptide chains, the A and B chains, which are connected by two interchain disulfide bridges (Fig. 2). In the A chain, the amino acid sequence in the segment connected by an intrachain disulfide linkage (primarily at A_8, A_9, and A_{10}) differs by species (Fig. 1). Mammalian insulins from human, pig, and dog have a similar amino acid sequence at these positions and differ only in the carboxyl terminus of the B chain. While pig and beef insulins have alanine at position B-30, human insulin has threonine instead. It should be recognized that the relative potencies of insulin isolated from the pancreas of different mammals are in the same order of magnitude when assayed in various mammals, even though some variations have occurred in the type of amino acids at these four positions (A_8, A_9, A_{10}, and B_{30}).

Using the highly purified A and B chains of insulin, studies indicated that these peptide chains have either a very low biological or immunological activity, or none at all (121).

A number of the amino acids in the insulin molecule have been shown to be nonessential for the biological activity of insulin. For instance, loss of alanine at the B-30 position by carboxypeptidase or elimination of the amide in asparagine at the A-21 position results in no diminution of biological activity. However, retainment of this asparagine is required for the full activity of insulin (122).

After splitting off the B23–B30 segment by enzymatic hydrolysis, bovine insulin was noted to have lost 85% of its biological activity, which indicated that this octapeptide, Gly-Phe-Phe-Tyr- Thr-Pro-Lys-Ala (Fig. 3), modifies the biological activity of insulin and is thus important to its hypoglycemic action (123).

Proinsulin (~9 kD), which is about 1.5 times the size of insulin (~6 kD) (124), has a molecular structure consisting of the A and B chains of insulin plus a C-peptide of 34 amino acids. It has biologic and immunologic activities that are approximately 10–20% that of

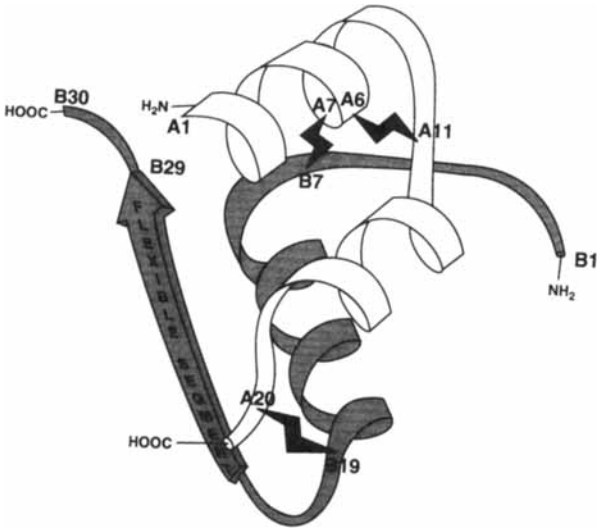


Figure 3. The conformation of human insulin by x-ray model showing its secondary and tertiary structure. (Adopted from Kline and Justice, 1990.)

insulin. The C-peptide is cleaved from the proinsulin molecule by carboxykinaselike enzyme or trypsin, with the removal of two basic amino acids from each end (Arg^{31} and Arg^{32}, and Lys^{64} and Arg^{65}).

Steric Structure

The three-dimensional arrangement of the atoms in insulin rhombohedral crystals was elucidated in the period 1969–1988 (125–127). The tertiary and quaternary structures of insulin molecule in its hexameric, dimeric, and monmeric states were revealed.

Primary Structure

The invariant amino acid residues in A and B chains (Fig. 2), which are common in all the insulins determined, are the residues responsible for the structural integrity of the insulin molecule and help to define the folding of the molecule (through the inter- and intrachain disulfide linkages between the cysteine residues). The variant amino acid residues, on the other hand, are the residues that interact in the folding of the molecule to form a three-dimensional structure (Fig. 3).

Secondary and Tertiary Structure

Despite the variations in the composition of the primary structure, the folding and packing of A and B chains into the three-dimensional conformation are essentially the same in all the insulin molecules isolated and studied. While the A chain forms two nearly antiparallel α helices (A_2 to A_8 and A_{13} to A_{20}), the B chain forms a single α helix (B_9 to B_{19}) followed by a turn and a β strand (B_{21} and B_{30}). The arrangement of the A and B chains, as shown in Fig. 3, buries the cysteine residues at the A-6 and A-11 positions as well as the aliphatic side chains of the residues at A-2, A-16, B-11, and B-15 in the nonpolar core. The surface of insulin monomer is thus covered by both polar and nonpolar residues (76).

Whereas the A chain and the B_9–B_{19} helix produce a stable structural unit, the B_{25}–B_{30} and the B_1–B_8 segments are variable in conformation.

The steric structure of insulin molecule is determined primarily by the positions of its disulfide bridges. It was found that insulin's steric structure depends on the van der Waals forces to a much greater extent than that of hemoglobin. The insulin molecule contains fewer hydrogen bonds in the solution state than in the solid state. The insulin chains have a helical content of only 50% (128), which scarcely exceeds 65% even in anhydrous media. This behavior must be due to the positions of disulfide bridges, since the B chain, when alone, forms almost completely a right-handed α helix under the same conditions (129). It has been found that removal of the *C*-terminal amino acid (Thr^{B30}) from the B chain has no effect on the conformation and the biological activity of insulin (130); if the *C*-terminal amino acid (Asn^{A21}) of the A chain is also removed, however, the insulin molecule changes its conformation and loses its biological activity. These observations suggested that a certain steric structure is essential to the hypoglycemic activity of insulin, in which the A and B chains are folded back on themselves (Fig. 3).

Quaternary Structure

Insulin molecules exist as a monomer only at low concentration (<0.1 μM or ~ 0.6 μg/ml). In physiological conditions, insulin is normally maintained at a concentration of less than 10^{-3} μM to ensure that it circulates and exerts its biological activities as a monomeric molecule (125,131–133).

At higher concentrations, which are often found in pharmaceutical preparations, insulin dimerizes (association dimer). In neutral solution (pH 4–8) and in the presence of Zn^{2+} ions, three dimers assemble further at concentrations greater than 10 μM to form a hexamer (125). Hexamers, with the shape of a slightly flattened

sphere, are the predominant association species of zinc insulin, at least down to a concentration of 10 μM. At a concentration of greater than 2 μM, the hexamer is formed at neutral pH even without the presence of Zn^{2+} ions (134). So, hexamer is the major association state found in the regular insulin preparation (U-100 or ~6 μM) in neutral solution (135). The hexamer found in the neutral solution has been believed to be the same as that observed in the insulin crystals (136).

It has been well established that insulin hexamers in solution exist in three different states (T_6, T_3R_3, and R_3) (137,138). The T_6 complex binds metallic ions such as Co^{2+} in an octahedral coordination. The addition and specific binding of phenol to insulin monomers causes the T_6 complex to transform into an R_6 complex (139), which binds Co^{2+} in a tetrahedral coordination instead. It was reported that by glucosylation of the Phe^{B1} in the insulin molecule, the formation of R_6 hexamer could be promoted in the presence of a high phenol concentration.

In the β cells of Langerhans islet, the pattern of assembly as "hexamer" facilitates the conversion of proinsulin to insulin as well as the subsequent crystallization and storage of insulin in the secretory granules. Only a few of the more than 50 animal insulins were noted to have impaired association properties and thus lack the ability to dimerize. These insulins were also found to have an altered tertiary structure and a reduced biological potency.

The dimer-forming surfaces are shown for human insulin in Fig. 4. This indicates that the dimer formation involves mainly β-chain residues and in several cases also includes the putative receptor-binding region of insulin molecule, which is thus essential, to a greater or lesser extent, for the biological potency of insulin (136).

The particle weight of insulin in solution depends on concentration, pH, ionic strength, temperature, and foreign ions. For example, zinc insulin, at low concentrations in acidic solutions (pH ≤ 2), should have a particle weight of 6000 (with hydration taken into account) (141,142); as insulin concentration increases and its solution becomes less acidic, the molecule becomes dimerized. Above pH 4, insulin–metal complexes are formed in the presence of suitable metal ion (e.g., Zn^{2+}); as pH increases to 4–7, these complexes precipitate as amorphous and highly aggregated products due to the reduction in aqueous solubility. In a solution with a high zinc content, insulin takes up an increasingly greater amount of Zn^{2+} as solution pH increases (e.g., 2.2% at pH 7.2). The solubility of insulin decreases sharply in the neutral range (pH 6–8) (143). The relative sizes of monomer, dimer, and hexamer of human insulin are compared in Fig. 5. The nature of the binding of zinc by insulin has not yet been elucidated. At pH 5.6–6.0, the amorphous precipitate was noted to slowly crystallize.

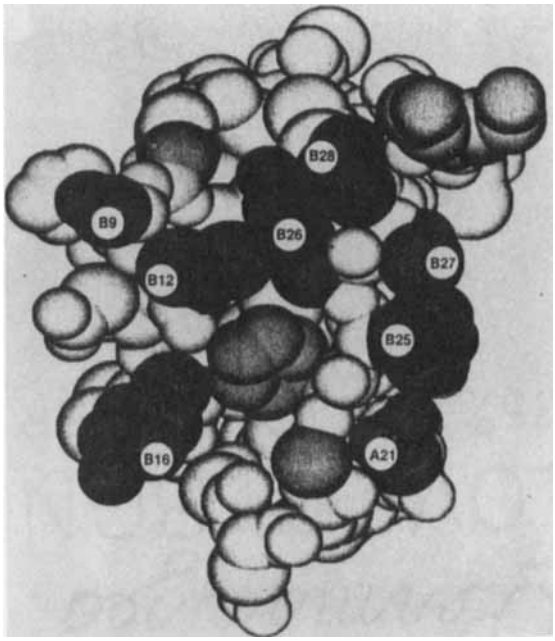


Figure 4. The human insulin monomer by space-filling model (van der Waal radii) showing the dimer-forming surface, in which the side chain atoms of residues with ≤ 4 Å contacts in the dimer are shown in dark color and those denoted with number of residue are for those amino acid residues that can be substituted to reduce dimer formation. (Adopted from Brange, et al., 1990.)

In solution at pH < 7, zinc insulin has a particle weight of 36,000 to 48,000 (144); that is, it exists either as a hexamer or as an octamer. In contrast, the "metal-free" insulin aggregate, under these conditions, dissociates to various extents into the monomer (depending upon its concentrations) (145). Zinc insulin becomes dissociated only at pH > 9 (146), which appears to yield a monomer with some changes in conformation (147), and the insulin molecule is inactivated (148). It has been reported that guanidinium chloride (149); urea (150); and some salts (135), organic solvents (151–153), and detergents (154,155) deaggregate zinc insulin.

In acidic solutions (at pH 2), insulin molecules form predominately the dimer at 20°C, but thixotropic gel at 80°–100°C. In the thixotropic gel, the micelles consist of fibrillar insulin (156). Fibrillar insulin is biologically

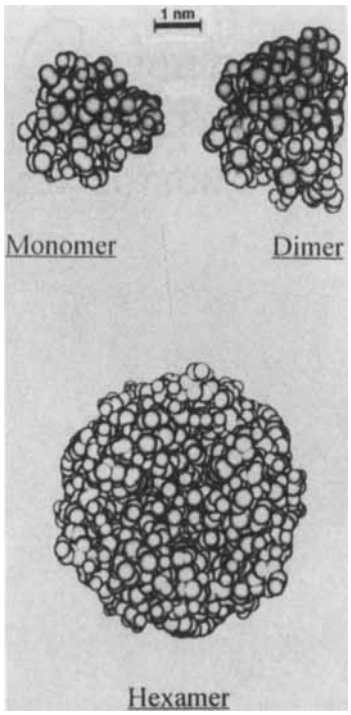


Figure 5. The human insulin by space-filling model (van der Waal radii) showing the relative sizes of monomer, dimer, and hexamer. (Adopted from Brange et al., 1990.) The hydrodynamic diameter for a hexamer is ~5.6 nm, while it is ~2.6 nm for a monomer. In contrast, the shape of a dimer is most likely described as an ellipsoid with dimensions of ~2.5 × 3.5 × 4.5 nm.

inactive but can be mostly converted into crystallizable, active insulin by treatment with alkali (pH 11–11.5) at 0°C (157,158), or by dissolution in cold 20% hydrochloric acid (159) or in phenol (160) or urea solutions (161). Each fibril has dimensions of <20,000 Å length and ~80 Å diameter (162–164), and appears to consist of four filaments with a diameter of 25–30 Å each and a period of 1200 Å (164); the diameter corresponds roughly to that of dimer.

At pH 1, the fibrils form spherical aggregates (165). Fibrils could also be formed from the acetylated or esterified insulin (166). The association must be due to nonpolar forces (167,168).

In addition to zinc ion, insulin forms mixed complexes also with protamine (169).

Recently, research has been undertaken to create insulins with reduced self-association and/or aggregation via glucosylation or computer-aided molecular modeling and genetic engineering (136,140). Several monomeric analogues of insulin have been synthesized by chemical synthesis or biotechnology process, in which some amino acid residues have been selectively glycosylated or replaced, to have a significant reduction in self-association and produce a more rapid absorption of insulin from the subcutis following subcutaneous administration (development of fast-acting analogues is discussed later under PK–PD Relationships).

STABILITY OF INSULIN

Physicochemical Stability

Pure insulin is known to be very stable and retains its activities for years in a sterile solution (pH 4) at 2°C. The biological activity of a crystalline insulin has been found to stay intact for 2 years at 0°C but shows a 20% loss after 1 year at 20–25°C (170).

In diluted acids, insulin is cleaved into several fragments (90) with hydrolysis occurring preferentially at its asparagine and glutamine residues (171,172). In alkaline solutions, insulin is rapidly decomposed, resulting from the hydrolysis of its amide linkages and the degradation of its cystine residues. In nonaqueous media, an N → O peptidyl shift also occurs in the serine and threonine residues (173–175). On acidic and basic ion exchangers, bonds at the carboxyl terminus of the B chain appear to be preferentially hydrolyzed (176). Concentrated urea causes carbamylation (165,177,178) of insulin without a reduction of its activity. Reducing agents, such as hydrogen sulfide and cysteine, rapidly inactivate insulin.

It has been reported that insulin is very sensitive to high-energy radiation; thus, sterilization with ^{60}Co γ rays has yielded a rapid inactivation of insulin as a result of alteration in its polar and aromatic groups (179–181). Ultraviolet light was found to break down the cystine residues; the tyrosine residues were also decomposed but at a slower rate (182). Photooxidation has led to a preferential breakdown of the imidazole group of histidine residue (183). Ultrasound has been reported to trigger a degradation of insulin but in a less specific manner.

Insulin can form a monomolecular layer at an interface (184); but unlike other globular proteins, this process does not result in a denaturation of insulin.

Nonenzymatic Degradation

The primary β-amide group in the *C*-terminal asparagine residue (A_{21}) is particularly sensitive to hydroly-

sis (185). Therefore, insulin could be partially degraded to deamido-insulin during the acidic extraction of the pancreas; but the deamido-insulin has reportedly retained almost fully the biological activity of insuliin (68–70).

Insulin was reported to lose its activity as soon as one of the three disulfide bridges was broken (186,187). Peroxyacids were found to break all three disulfide bridges in the insulin molecule. The "oxidized insulin" formed has no hypoglycemic activity. Even though the "oxidized insulin" is stable physicochemically, it is subjected to degradation by proteases (188).

It has been reported that insulin is reduced when treated with hydrogen sulfide or thioglycolic acid (189). The reduction occurs slowly in acidic media but rapidly in alkaline media, and it is accelerated by alcohols and heavy metal ions (190). Thiol (-SH) groups in the reduced cysteine residues are oxidized in the presence of air.

The two interchain disulfide bridges, which link the A and B chains, are more prone to reduction than the intrachain S–S bridge in the A chain (191–193). The intrachain (A_{6-11}) disulfide bridge appears to be more difficult to attack than the two interchain S–S bridges (A_7–B_7 and A_{20}–B_{19}).

Enzymatic Degradation

Native insulin is highly resistant toward hydrolyzing enyzmes in vitro, which appears to be particularly related to its zinc content (e.g., leucine aminopeptidase digests only the "metal-free" insulin) (194). Native insulin is cleaved only slowly by trypsin and chymotrypsin (195); as soon as one bond has been cleaved, however, the remainder of the hydrolysis proceeds rapidly (196). Insulin is attacked most strongly by subtilisin (197–198).

By careful degradation with carboxypeptidase (126), a fully active dealanine-insulin can be obtained by removing only this *C*-terminal alanine from the B chain (B_{30}) (199). On further digestion with the same peptidase, the amide linkage in the *C*-terminal asparagine (A_{21}) of the A chain is broken and the resultant dealanine-deamido-insulin (de-de-insulin) has lost one-third of insulin's activity (185,200,201). If the aspartic acid resi-

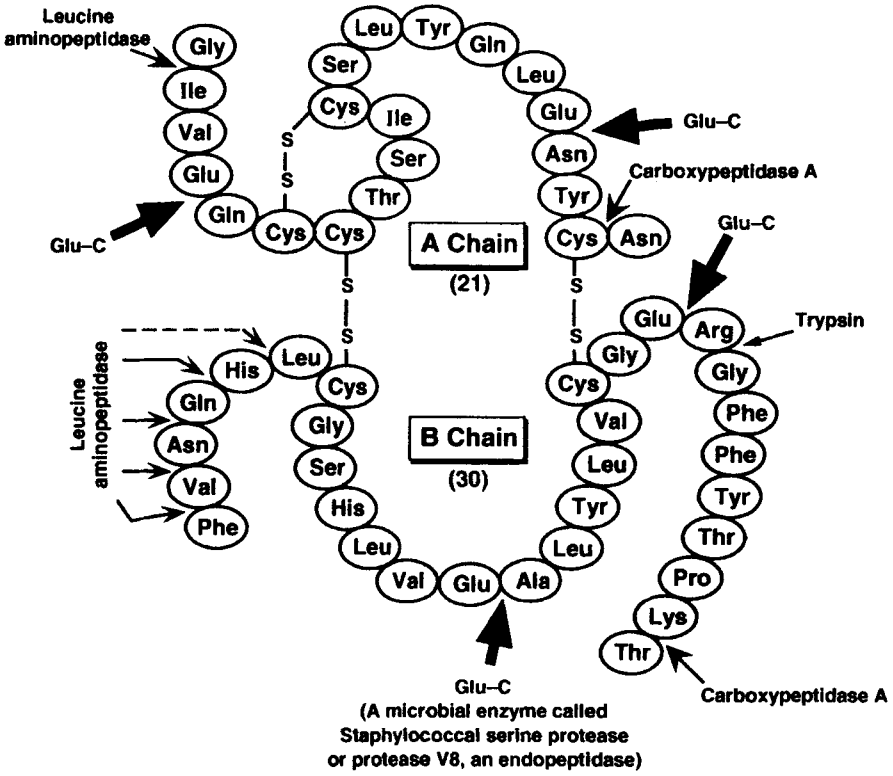


Figure 6. The primary structure of human insulin showing various locations in the A and B chains which are known to be potentially labile to the degradation by various proteolytic enzymes.

due so formed is removed, the de (Ala^{B30})-de (Asn^{A21})-insulin is a biologically inactive product (202,203).

Trypsin has been reported to cleave only the B chain (Fig. 6), slowly between B_{22} and B_{23} but rapidly between B_{29} and B_{30} (200). The resultant deoctapeptide (B_{23}-B_{30})-insulin is biologically inactive (201,202).

Leucine aminopeptidase was observed to rapidly break down the B chain from its lipophilic *N*-terminus (Fig. 6). The results indicated that the B_1-B_6 segment (Phe-Val-Asn-Gly-His-Leu) is not essential for the hypoglycemic action of insulin (192).

Enzymatic reduction of the disulfide bridges is apparently the first step in the in vivo degradation of insulin, even though it is insignificant under in vitro conditions.

SUBSTITUTION REACTIONS AND INSULIN ACTIVITIES

In 1927 Freudenberg began a series of systematic studies to investigate the effect of various reagents on the activity of insulin. Chemical modification studies over the years have demonstrated that the amide, phenolic hydroxyl, imidazole, and carboxyl groups are essential to the activity of insulin; whereas three amino groups, aliphatic hydroxyl groups, and the guanidine residue have been found not essential (182,183,203–209).

Substitution was also noted to cause a marked change in the isoelectric point of insulin and thus in its aqueous solubility: the isoelectric point was shifted from pH 5.6 to pH 13.0, and its solubility was altered with increasing degree of esterification. The alteration in the solubility of insulin should be accompanied by a parallel change in its biological activity (210).

The following two substitution reactions are of practical importance to insulin.

Sulfation

In concentrated sulfuric acid, a preferential esterification of the insulin occurs at the aliphatic hydroxyl groups (even at low temperatures), but less at the aromatic hydroxyl groups (211,212). Slightly esterified insulins were still hypoglycemically active and not very strongly antigenic, and also scarcely bound by antibodies (213); they have been produced for the treatment of insulin-resistant diabetics.

Iodination

The iodination of insulin was reported to occur at the position ortho to the hydroxyl groups in the four tyrosine residues. The ease of reaction varies from one tyrosine to another with the following rank order: $A_{19} > A_{14} > B_{16} > B_{26}$ (214–218). Theoretically, it is possible to obtain a total of 3^4-1 = 80 iodoinsulins, and more if additional reactions also occur at the histidine residues (219). No conditions are known as yet that lead exclusively to mono- or di-iodoinsulins. Highly iodinated insulins have been reported to be biologically inactive (220–222).

Insulins labeled with ^{131}I and ^{125}I are the most important tracers (223,224). Despite its wide use, it is not yet known whether iodinated insulin behaves in the organism in the same manner as native insulin.

DEVELOPMENT OF HUMAN INSULIN PRODUCTION

The desire to provide human insulin in economic quantities for the treatment of insulin-dependent diabetes mellitus has been reflected in the development of a variety of strategies and techniques for its production. The following techniques have been successfully developed:

Synthesis

Advances in peptide chemistry have stimulated several research groups to synthesize insulin by chemical processes. The main obstacle to a total synthesis of insulin appeared to be the linking of its A and B chains. The separate synthesis of A and B chains, on the basis of du Vigneaud's work (225), and the linkage of the SH groups in the resultant chains by oxidation appear to be the simplest route to completion of the insulin synthesis process.

Zahn and his coworkers (58) and Katsoyannis, in collaboration with Dixon (59–61), succeeded in the synthesis of sheep insulin at about the same time, in 1963. Later, Kung and his group in China completed the total synthesis of bovine insulin in 1965 (226–228).

The synthesis of the A and B chains by the chemical process required, respectively, a total of 89 and 132 reaction steps. An additional 3 steps were needed for the linkage of the two chains. All reaction sequences had been so chosen that changes in the 48 asymmetric centers were practically impossible (229). But the yield was noted to be extremely low, and insulin content in the products obtained by various synthesis methods was often in the range of 0.2–1.0% (0.05–0.25 IU/mg).

Semisynthesis

By linking a synthetic chain (A or B chain) to an opposite (B or A) chain of native insulin, a "semisyn-

thetic insulin" has been produced. It was found that the synthetic A chain (59,230) gives a higher yield of insulin than the synthetic B chain (59,231). On removal of the final protective groups, however, the B chain was noted to degrade very rapidly (mainly at the B_{27} and B_{28} bond) (232).

By semisynthesis, a yield of 2.9% has been obtained for the protected A chain and a yield of 7.0% for the protected B chain, but the final product has an insulin content of only 0.25–0.55% (230,231). Similar yields were also probably attained in the American and Chinese approaches (226,228).

Chemical synthesis of the insulin molecule has been recognized to make the most important contribution to insulin chemistry, which has provided techniques crucial to the synthesis of insulin derivatives. Study of these derivatives, including the A and B chains and their modifications, has revealed much about the structure, function, and immunology of insulin (124). Information derived has also been useful in the development of bioengineered human insulin (233).

Because of the complexity of the process involved in the chemical synthesis and the success of recombinant DNA technology, chemical synthesis has not been successfully reduced in practice as a commercial source for human insulin.

Enzymatic Conversion

Enzymatic conversion is the technology developed to convert a natural occurring insulin isolated from animal pancreas into human-type insulin by a synthetic process involving enzymatic catalysis (234).

Shortly following the determination of the amino acid sequence in human insulin in 1960 (235), which identified the similarities and dissimilarities among various insulin molecules from humans and animals (Fig. 1), attempts began to substitute the B-30 alanine residue in porcine insulin with a threonine residue to form human insulin. In the first such attempt, porcine insulin was treated with a large excess of free threonine in the presence of either trypsin or carboxypeptidase A. The attempt, however, was not substantiated experimentally, even though a US patent was granted in 1966 (236).

Then, in 1978, the thermodynamic foundation became available for the synthesis of human insulin esters by coupling of porcine des (Ala^{B30})-insulin with a threonine ester, using trypsin as catalyst (237,238). The present form of transpeptidized human insulin has been successfully synthesized from porcine insulin via a single enzymatic step, called "transpeptidation," in a mixture of water and an organic solvent (239).

The process of transpeptidation is shown in Fig. 7. At first, porcine insulin associates with porcine trypsin to form a Michaelis complex. Next, an ester bond is formed between the carboxyl group of lysine at the B-29 of insulin and the hydroxyl group of the serine residue at the active site of trypsin to form the insulin trypsin ester (with the cleavage of B-30 alanine).

This insulin–trypsin ester is liable to reaction with nucleophiles, such as water and amino groups. In aqueous solution, the insulin–trypsin ester tends to be hydrolyzed to yield des (Ala^{B30})-insulin. By maintaining a high concentration of an inert organic solvent, on the other hand, the amino group of a threonine ester competes favorably with water for the insulin–trypsin ester; under such conditions, hydrolysis is virtually blocked and the aminolysis of insulin trypsin ester by the threonine ester becomes kinetically the predominant reaction, and human insulin ester is thus yielded. The transpeptidation of porcine insulin to human insulin ester has been optimized to have a yield as high as about 97%.

Trypsin in the reaction mixture is then removed by gel filtration at low pH, where trypsin is inactivated; and via anion-exchange chromatography, the human insulin ester is separated from any residual unconverted porcine insulin. The human insulin ester is then cleaved to yield human insulin, which is separated from any trace amounts of uncleaved human insulin ester by ion- exchange chromatography.

The most convincing single identity test for human monocomponent (MC) insulin is the comparison of x-ray diffractions of the insulin crystals (240), which proves identity of sequence, conformation, and state of aggregation.

The biologic potencies of 15 batches of human MC insulin were determined by mouse convulsion assay (241,242) and by blood glucose assay, using mice (241) or rabbits (241–243). The results indicated that, like the other monocomponent insulins, human MC insulin maintains a potency of 185 IU/mg N, which corresponds to 28.5 IU/mg dry insulin or 168×10^6 IU/mol insulin. Using the WHO s Fourth International Standard (244), on the other hand, a potency of 24.0 IU/mg, which corresponds to 154 IU/mg N, has been established.

Immunogenicity studies were performed in rabbits, and the results from 11 batches of human MC insulin and 7 batches of porcine MC insulin indicated that neither porcine nor human MC insulin resulted in the formation of antibodies, in any significant amounts, in the majority of rabbits, in both groups, only a few positive responses were seen (as shown in Fig. 12, rabbit insu-

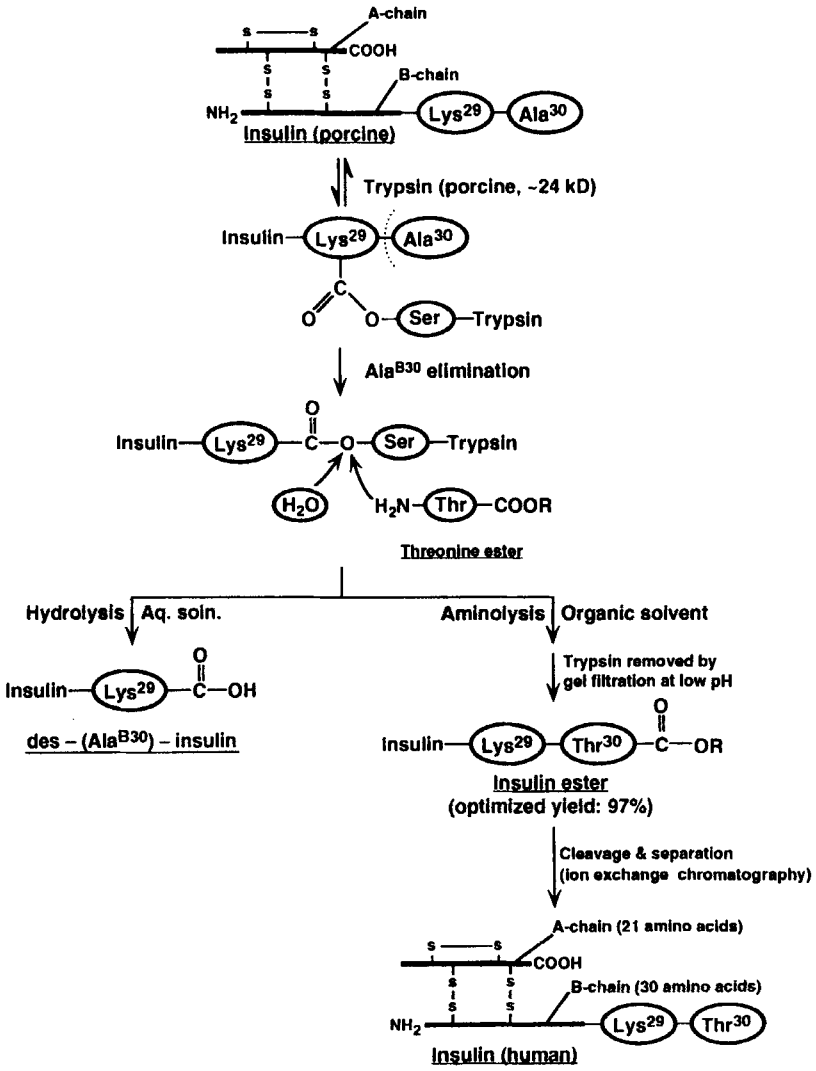


Figure 7. Procedure involved in the enzymatic conversion of human insulin from porcine insulin by transpeptidation process. (Based on Damgaard et al., 1983.)

lin differs in the B-30 amino acid residue from human and porcine insulins).

Bioengineering

As discussed at the beginning of this review, the US National Diabetes Advisory Board's 1978 report projected a potential shortage of insulin as early as in 1996, as a result of an increasing number of insulin-dependent diabetic patients and the declining supply of animal pancreas (3). It also emphasized that the successful application of recombinant DNA technology to the biosynthesis of human insulin is the most promising means of ensuring an abundant supply of insulin.

In Genentech, through a collaborative research program with Eli Lilly & Co., bacteria were genetically altered to produce human insulin (245). Genes for insulin A and B chains were synthesized separately and then joined individually, by recombinant methods, with the gene which codes for β-galactosidase (β-gal gene; later, tryptophan synthetase was used) (Fig. 8). The cloned plasmids, which contain the modified gene (for either the A or the B chain) were inserted individually into different strains of *Escherichia coli* (special K12 strain), which were then fermented separately to pro-

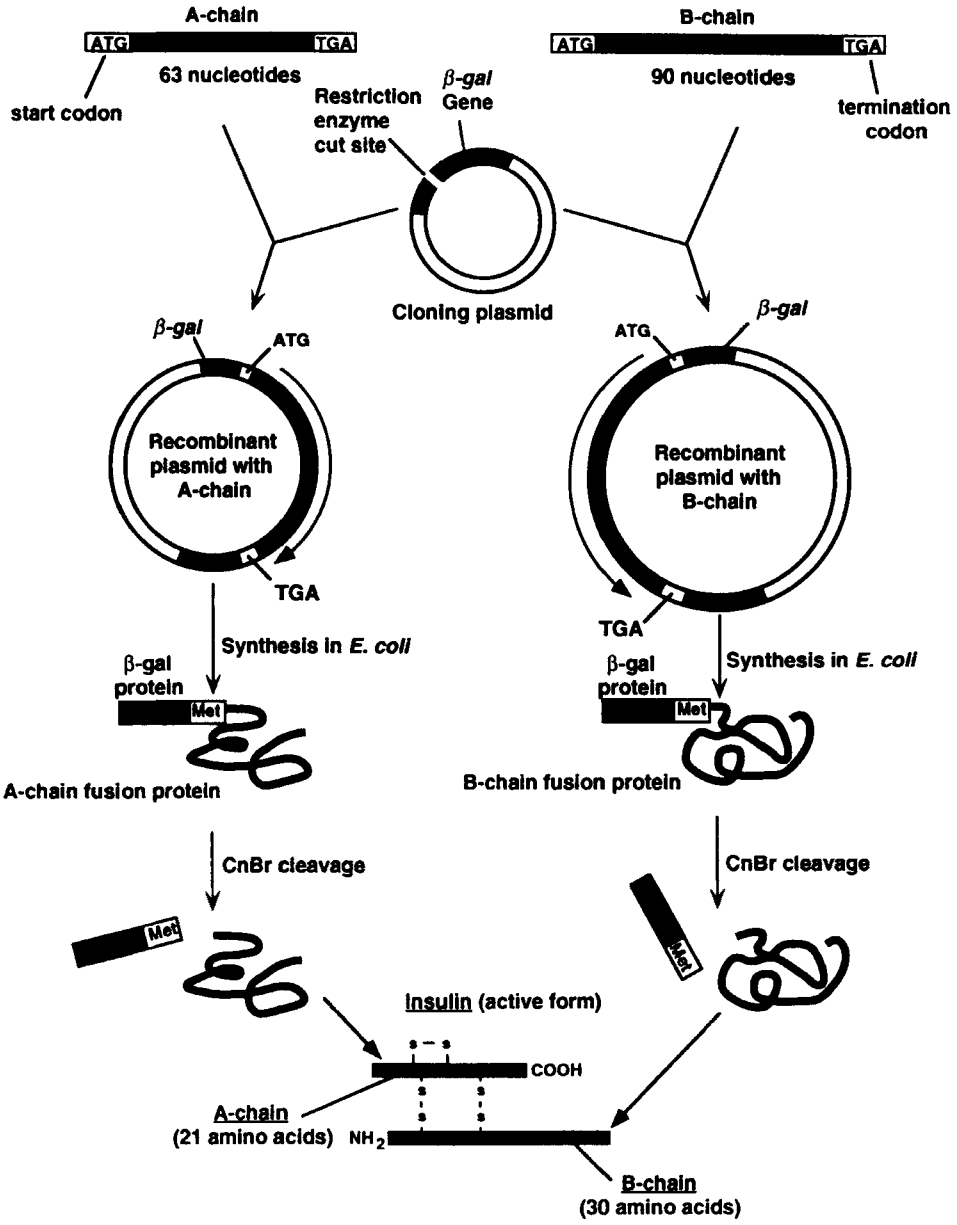


Figure 8. Procedure involved in the production of bioengineered human insulin in the special strains of *Escherichia coli* by recombinant DNA technology using synthetic genes for insulin's A and B chains (Method I). (Modified from Watson et al., 1983.)

duce insulin A or B chains (245–247). The A and B chains were cleaved from the bacterial proteins (β-gal protein) using cyanogen bromide (CnBr), purified, and then chemically combined.

Recombinant human insulin can also be produced by preparing the human proinsulin gene, in part synthesized chemically and in part derived from the natural DNA sequence found in human cells (248). Human proinsulin has been produced, in large quantities, by fermentation of the *E. coli* containing the proinsulin gene-carrying plasmids and then converted the proinsulin formed to insulin, using carboxypeptidase-B and trypsin to remove the C-peptide (248). This method is illustrated in Fig. 9.

Commercially available recombinant human insulin has proven indistinguishable from the insulin isolated from human pancreas in both chemical properties and biological activities (Fig. 10). Each milligram has a biologic potency of not less than 27.5 USP insulin (human) units.

The commercial process using the recombinant DNA technology to produce human insulin (rh-insulin) avoids the potential contamination with glucagon, somatostatin, and proinsulin, and has shown no sign of bacterial fermentation contaminants (247). The commercially available rh-insulin may contain only a trace (≤4 ppm) of immunoreactive *E. coli* polypeptides.

PHARMACEUTICAL DEVELOPMENT OF INSULINS

Three milestones have been recognized to mark the pharmaceutical development of insulin: (i) crystallization

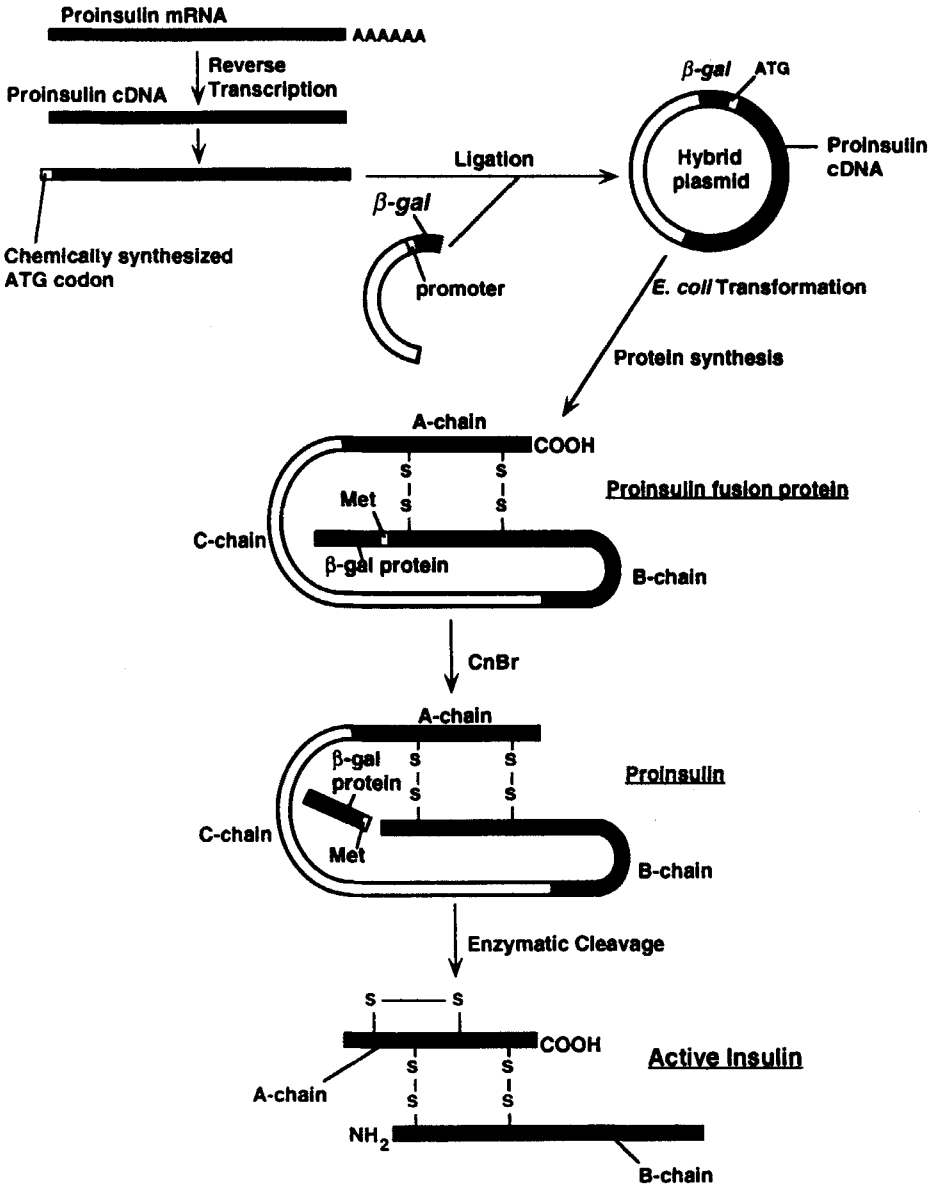


Figure 9. Procedure involved in the production of bioengineered human insulin in the special strains of *Escherichia coli* by recombinant DNA technology using semisynthetic proinsulin gene (Method II). (From Watson et al., 1983 with modifications.)

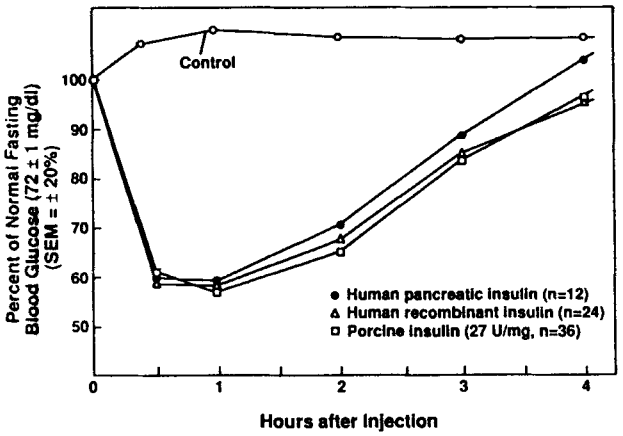


Figure 10. Comparative hypoglycemic activities between the bioengineered human insulin and the native pancreatic insulin from human and swine (rabbit hypoglycemia test). (From Dolan-Heitlinger and Antle, 1983, with modifications.)

of insulin in 1926 (249); (ii) development of modified insulins, which began in the late 1930s; and (iii) introduction of protein purification procedures in the late 1960s (250).

The following insulin preparations have been developed and are available commercially for the glycemic control in insulin-dependent diabetic patients.

Regular Insulin Preparations

This parenteral preparation is a sterile aqueous solution of fast-acting human insulin produced from either genetic engineering or the enzymatic conversion process:

1. Humulin® BR: formulated by dissolving zinc insulin crystals prepared from precipitation of bioengineered human insulin with Zn^{2+} in phosphate buffer (pH 7.0–7.8). The formulation contains 100 USP units/ml of human insulin, zinc (10–40 μg/ml), glycerol (1.4–1.8%), and cresol (0.225–0.275%).
2. Novolin®: formulated by dissolving zinc insulin crystals prepared from precipitation of transpeptidized human insulin with Zn^{2+} in phosphate buffer (pH 7.2–7.5). The formulation contains 100 units/ml of human insulin, zinc (10–20 μg/ml), and cresol (0.3%) [or phenol (0.17–0.23%)].

Both Humulin BR and Novolin R preparations are formulated from regular insulin. The hypoglycemic action of regular insulin following the subcutaneous injection is rapid; it has an onset time of 0.5–1.0 hr and reaches its maximal activity within 2-3 hr, but is short in duration (4–8 hr). Therefore, two to four injections daily of these preparations are required for a proper glycemic control of the diabetes.

Extended-Action Insulin Preparations

The demands for development of insulin preparations with extended action have become increasingly recognized in the long-term therapy of insulin-dependent diabetic patients. For instance, it was discovered that in the treatment of juvenile diabetes with regular insulin, the majority of these growing children have difficulty in waking up at night for injections. So, they often show marked hyperglycemia and urinary loss of nitrogen, which have placed them at risk for the diabetic dwarfism syndrome (250).

The following preparations are representative extended-action insulin preparations that have been developed from bioengineered or transpeptidized human insulin and are available commercially for the long-acting glycemic control of insulin-dependent diabetic mellitus:

1. Protamine-zinc-insulin (PZI): This insulin preparation is the first commercially available extended-action insulin preparation, developed in the 1930s by combining insulin with an excess of protamine and zinc (251). Unfortunately PZI, which is amorphous insulin, has the disadvantages of producing too little hypoglycemic effect in the daytime but too much nighttime effect (252). To overcome these disadvantages, regular insulin preparation has been often added to PZI preparations in amounts exceeding the binding capacity of the excess protamine.
2. Neutral protamine Hagedorn (NPH) insulin: This extended-action insulin preparation, which is a crystalline combination of insulin with protamine and zinc (in a stoichiometric ratio), was introduced in 1946 by Nordisk Insulinlaboratorium (253). It contains protamine at a concentration which is only 1/10 of that in the PZI preparation. NPH insulin, when given as a single dose in the morning, usually displays greater hypoglycemic activity during the day than at night. As for the PZI preparation, NPH insulin preparations can also be combined with regular insulin preparations to formulate an insulin preparation that produces more effective glycemic control for the personalized treatment of diabetic

patients than either NPH or regular insulin preparation administered alone.

3. Lente® insulin: Concern about the potential antigenicity of protamine (254), which is a protein extracted from the fish testes, and a need for broader selection of modified insulin preparations prompted the development of the Lente series insulin in 956 at Novo Industri A/S (255). Three types of Lente insulins were developed by varying the zinc content and the manufacturing conditions used: (i) microcrystalline Ultralente® insulin, which is long-acting insulin; (ii) amorphous Semilente® insulin, which has a normoglycemic action twice longer than that of regular insulin; and (iii) Lente insulin, a 70:30 mixture of Ultralente and Semilente insulins, which is an intermediate-acting insulin.

The times for the onset and the duration of hypoglycemic activities of these modified insulin preparations are compared with other insulin preparations in Table 4. Similar to the regular human insulin injection, the Lente-type insulin preparations can also be prepared from either bioengineered or transpeptidized human insulin. For example, the intermediate-acting Lente insulin preparations have the following two types:

1. Humulin L: formulated by suspending a mixture of microcrystalline (Ultralente) and amorphous (Semilente) bioengineered human insulin, at the ratio of 70:30, in the water-for-injection (pH adjusted to 7.0–7.8). This sterile preparation is a milky suspension containing a mixture of characteristic crystals (with maximal dimension of predominantly 10–40 μm) and amorphous particles (with maximal dimension of <2 μm). For every 100 USP units of bioengineered insulin, the preparation also contains zinc (120–250 μg/ml), ZnO (0.016%), sodium acetate (0.16%), NaCl (0.7%), and methyl paraben (0.1%).
2. Novolin L: formulated by suspending a mixture of crystalline and amorphous transpeptidized human insulin, also at the ratio of 70:30, in the water-for-injection (pH adjusted to 7.2 7.5). This sterile preparation is also a milky suspension containing a mixture of rhombohedral crystals (with maximal dimension of predominantly

Table 4

Hypoglycemic Activity and Duration of Some Commercial Insulin Products

Insulin Preparations	Hypoglycemic Activity[a]		
	Onset (hr)	Peak (hr)	Duration (hr)
Purified animal insulin			
Regular[b]	~0.5	2.5–5	~8
Semilente[c]	~1.5	5–10	~16
Lente[d]	~2.5	7–15	~24
Ultralente[e]	~4.0	10–30	~36
NPH insulin[f]	~1.5	4–12	~24
Human insulin[g]			
Humulin R/Novolin R	~0.5	2.5–5	~8
Humulin L/Novolin L	~2.5	7–15	~22
Humulin N/Novolin N	~1.5	4–12	<24
Humulin (70/30)/Novolin (70/30)[h]	~0.5	2–12	<24

Source. Compiled from *Physician's Desk Reference*, 47th ed., Medical Economics Co., Oradell, NJ, 1993.

[a]The time at which activity is evident.

[b]Solution of porcine (and/or bovine) insulin.

[c]Suspension of amorphous porcine (and/or bovine) insulin–Zn complex.

[d]Suspension of 70% crystalline and 30% amorphous porcine (or bovine) insulin–Zn complex.

[e]Suspension of crystalline porcine (and/or bovine) insulin–Zn complex.

[f]Suspension of protamine–Zn–porcine (or bovine) insulin complex.

[g]Human insulin is a product produced by recombinant DNA, which is synthesized in a special laboratory strain of *E. coli* that has been genetically altered by the addition of human gene for insulin production.

[h]Combination of human insulin isophane suspension and regular human insulin injection (70:30).

10–40 μm) and amorphous particles (with maximal dimension of <2 μm). For every 100 USP units of transpeptidized insulin, the preparation also contains zinc (~150 μg/ml), sodium acetate (0.14%), NaCl (0.7%), and methyl paraben (0.09–0.11%).

Unlike the PZI and NPH preparations, Lente series insulin preparations cannot be combined with a regular insulin preparation. This incompatibility is attributed to the presence of excess zinc in the Lente preparations, which tends to bind the insulin in the regular insulin preparation, if added, and blunts its rapid hypoglycemic action.

Similar to human insulin, porcine insulin can crystallize with 2 or 4 Zn^{2+} ions to form a rhombohedral hexamer crystal, which is isomorphically indistinguishable from human insulin. It is therefore possible to make a preparation from porcine insulin with extended action equivalent to the one prepared from the human insulin crystals. In fact, all the extended-action insulin preparations discussed above were first developed using porcine (and/or bovine) insulin until the successful production of human insulin by genetic engineering or the enzymatic conversion process.

PK-PD RELATIONSHIPS OF INSULINS

Insulin therapy is the only medication which permits self-injection with a self-adjusted regimen. Using ^{131}I-insulin, the absorption kinetics of insulin, regular and modified, was detailed in diabetic patients (256). Pharmacokinetic (PK) studies of serum insulin profiles and pharmacodynamic (PD) studies of blood glucose responses were conducted in normal, fasted volunteers (257,258). Techniques for studying the pharmacodynamics of regular and modified insulins have been introduced (259) and interested readers can consult various publications (260,261).

The PK–PD relationship of insulin following its parenteral administration and various factors affecting this relationship are discussed in the following sections.

PK-PD Relationship Following Parenteral Delivery

At physiologic concentrations (35–105 μU/ml) (261), the disposition of insulin was noted to follow a linear kinetics, with the rate of disposition proportional to the serum concentrations of insulin. But the clearance of systemic insulin was reportedly reduced at serum concentrations above the physiologic levels; the observed reduction in clearance apparently resulted from the saturation of the liver's capacity to metabolize insulin in high levels.

Intravenous Administration

Following an intravenous injection, insulin was observed to disappear from the systemic circulation following nonlinear kinetics (262). By intravenous infusion, insulin clearance in the nondiabetic subjects was found to be constant when the rate of infusion was maintained in the range of 2–12 IU/hr.

Following intravenous administration of ^{125}I-insulin in normal volunteers, the radioactivity was found to be localized in the heart, liver, and kidneys within 5–7 min after the injection. In these organs, insulin was reported to be rapidly metabolized (Fig. 11): the hepatic extraction of insulin could be as high as 70% on a single passage (261), while the kidney could account for 10–40%

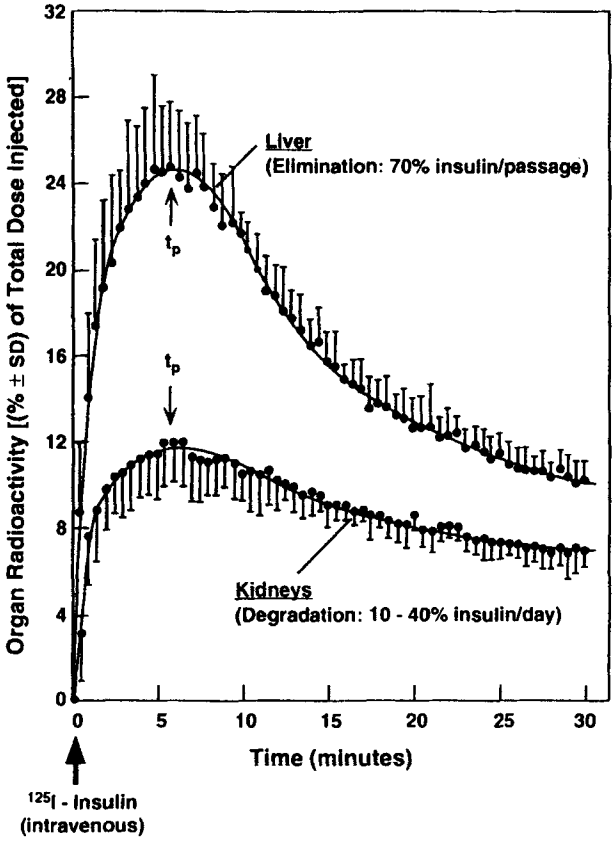


Figure 11. Tissue pharmacokinetic profiles of insulin in liver and kidneys following the intravenous injection of ^{125}I-insulin in nondiabetic subjects (n = 4) as detected by scintillation camera. (From Sodoyez et al., 1983, with modifications.)

of daily insulin degradation; thus, only less than 2% of the insulin dose filtered by the kidney was excreted in its intact form into the urine.

By intravenous infusion of insulin, the circulatory insulin concentrations were observed to increase proportionally with the rate of infusion. However, its hypoglycemic effect in the diabetic patients was found to be less than that observed in nondiabetic humans: the rates of reduction in peripheral glucose in the diabetic patients were only about two-thirds of that attained in the nondiabetic subjects. The observations could be attributed to development of a significant resistance (or diminished sensitivity) to insulin in the peripheral tissues of diabetic patients.

Subcutaneous Administration

The serum profiles of insulin in nondiabetic humans following the subcutaneous administration of regular insulin preparation are shown in Fig. 12, which indicates that after the attainment of peak concentrations within 1–2 hr, insulin is eliminated from the systemic circulation by a first-order kinetic process. The serum levels increase proportionally with the doses administered subcutaneously. It should be pointed out that the absorption of insulin from the subcutis, following a subcutaneous injection, could involve a complex series of events influenced by many variables.

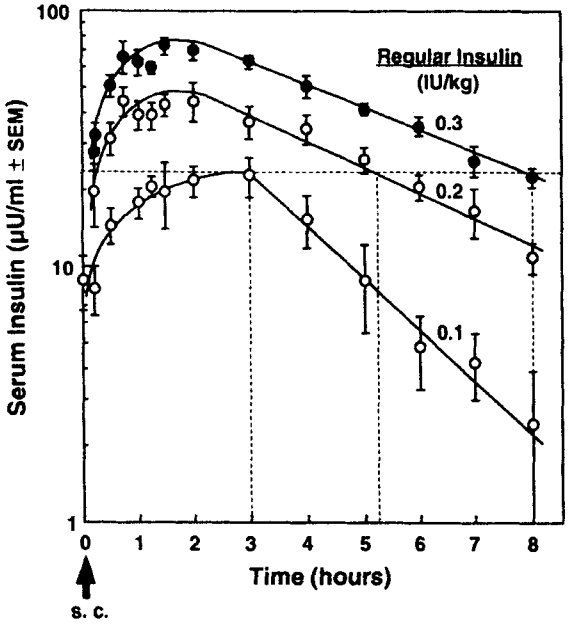


Figure 12. Serum pharmacokinetics profiles of insulin following the subcutaneous injection of regular insulin preparation, at three dosage levels, in nondiabetic subjects at fasting state. (From Galloway et al., 1973, with modifications.)

Following the subcutaneous injection of a regular insulin preparation, the subcutaneous pharmacokinetic profiles in Fig. 12 suggests that there exists an initial lag phase with a slowly increasing rate of absorption (263–268). This phenomenon has been hypothesized as due to a local vasoconstrictor effect of insulin or the distribution of insulin from the subcutis to the microvasculature by local diffusion (264,269). This initial delay in the subcutaneous absorption of insulin was reportedly shortened or even disappeared with reducing concentration of insulin or decreasing volume of injection (264,270,271).

In addition to the influence of pharmaceutical formulation, insulin absorption from subcutaneous tissue is, to a large extent, controlled by local blood flow (264,272–274). Therefore, factors known to influence blood flow (e.g., site and depth of injection) also play a role in the rate of insulin absorption from the subcutis. The influence of blood flow on the subcutaneous absorption of insulin depends on the rate of blood flow. In the region with low blood flow, the main factors are: (i) the recruitment of capillaries, (ii) the length of diffusion path, and (iii) the concentration gradient from interstitial space to blood circulation. In the region with high blood flow, on the other hand, factors limiting the absorption rate of insulin (275) include: (i) interstitial transport to the capillaries by diffusion and (ii) restriction for permeation through the capillary membrane (total area and permeability). The effect of these factors is governed primarily by the molecular size of the insulin molecule to be transported, as shown schematically in Fig. 13.

The hexamer of insulin, the prevailing association unit of insulin molecules in a neutral solution, has a hydrodynamic diameter of ~5.6 nm (125,126), while it is ~2.6 nm for insulin monomer (276,277). Figure 13 illustrates that following the injection, the hexameric insulin is first dissociated in the subcutis and transported to the microvasculature region by diffusion and then adsorbed by the capillaries in the smaller dimeric or monomeric form (see Fig. 5 for size comparison) (260,278,279). For this process to proceed, the removal of Zn^{2+} ions and a substantial dilution of the insulin depot are required, which would result in the delay of systemic absorption from the subcutis (278). Following the residual radioactivity of radiolabeled insulin in the subcutis indicated that human insulin disappears from the insulin depot according a triphasic first-order kinetics process (Fig. 14). This kinetic process coincides with

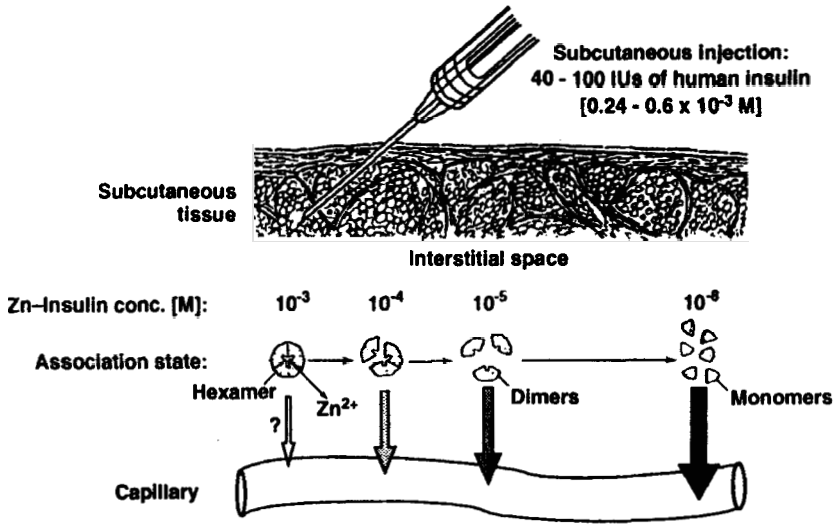


Figure 13. Schematic illustration of various putative events in the subcutis following a subcutaneous injection of regular human insulin preparation (at a dose of 40–100 IU/ml or 0.24–0.6 × 10^{-3} M). Hexameric insulin, the predominant association state in the preparation, disintegrates in the interstitial space into smaller units and dissociates first into mainly dimeric insulin, by 50- to 100-fold dilution, and then into monomeric insulin by further 1000-fold dilution. The uptake of hexamers, dimers, and monomers by capillary membrane is shown with increasing extent as the size reducing from hexamer, to dimer, and to monomer (as shown in Fig. 5). (From Brange et al., 1990, with modifications.)

the transformation of hexamer, first, to dimer and, then, to monomer (136).

The regular human insulin preparation has reportedly been absorbed at a rate which is slightly faster, resulting in higher plasma insulin levels than those achieved from regular porcine insulin preparation in both healthy subjects and diabetic patients (263,280–287). As discussed earlier, the mechanism for the more rapid absorption of human insulin is attributed to its hydrophilic character (288).

As shown in Fig. 13, the concentration of insulin is a main determinant of its association state. Furthermore,

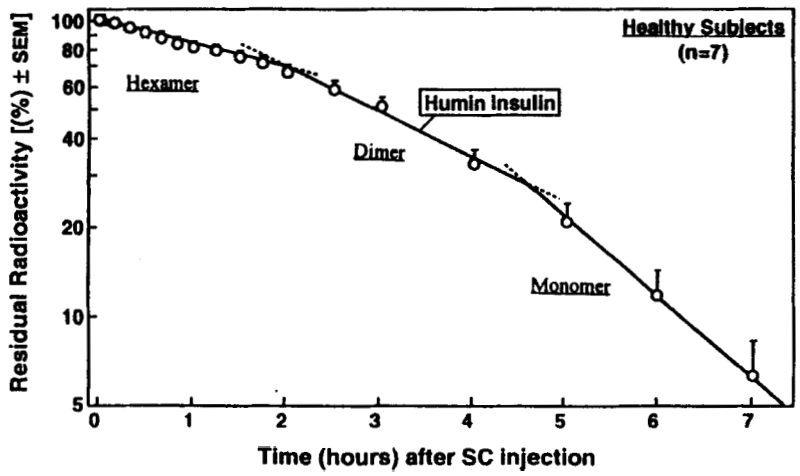


Figure 14. Semilogarithmic plot of the residual radioactivity at the injection site following the subcutaneous administration of regular human insulin preparation (0.6 nmol/kg). By linear regression analysis, the disappearance profile is observed to follow a triphasic first-order kinetic with respective subcutaneous absorption rate of 20.1%/hr for phase I (0–2 hr), 35.9%/hr for phase II (2–4 hr), and 60.5%/hr for phase III (5–8 hr). (From Brange et al., 1990, with modifications.)

insulin species may also affect the disassociation of hexamer on dilution. Temperature, in addition to its effect on blood flow, has a direct influence on insulin association; a shift from ambient to physiological temperatures has been noted to lead to an increase in the dissociation of hexamer.

Several factors have been reported to influence the absorption of insulin administered subcutaneously and/or its bioavailability (256,257,260). These are summarized below:

Site of injection Following the subcutaneous injection of regular insulin, the time required to reach the peak serum concentrations varies from one anatomic site to another and follows the rank order: abdomen ≤ deltoid < thigh ≤ buttock.

Rate of absorption Blood flow from the site of subcutaneous injection has been reported to augment the rate of insulin absorption. On the other hand, absorption of insulin was reportedly increased by massaging the injection site or exercising the limb after injection. This result was attributed to greater distribution (not due to increased blood flow) (257).

Manner of injection Injection into the cutaneous microcirculation network was noted to yield a more rapid absorption than injection into the subcutaneous fat tissue. Intramuscular injection was found to produce a slightly faster absorption than subcutaneous injection (256). As long as insulin is deposited at the same depth, however, there is no difference between injection at perpendicular direction and that at an angle (289).

Concentration of insulin No effect on the bioavailability of insulin was observed when insulin was administered subcutaneously in the concentration range of 40–100 IU/ml. However, insulin was found to be more rapidly absorbed when administered in a diluted solution (10 IU/ml) than in a concentrated solution (500 IU/ml) (257).

Dose of insulin The duration of hypoglycemic action following the subcutaneous administration of fast-acting regular insulin preparation was observed to increase with increasing dose of insulin. Subcutaneous injections of insulin in fasted, nondiabetic subjects showed that for each 0.1 IU/kg increase in dose, the duration of serum insulin level is extended by approximately 2-1/2 hr (Fig. 12).

On the other hand, the serum profiles of insulin in response to the variation in the administered dose of extended-action insulin preparations were nonlinear: tripling the dose of ^{125}I-NPH insulin, an intermediate-action insulin preparation, in patients was observed to produce only a twofold increase in insulin bioavailability in the first 24 hr after the injection (290).

Source of insulin The bioavailability of insulin from the extended-action insulin preparations was reported to be slightly varied between human and porcine insulins, and also among the human insulins from different manufacturers (259). For example, the subcutaneous injection of human insulin, in regular and NPH insulin preparations, was noted to produce a peak serum concentration which occurs sooner in time and/or is higher in magnitude than that attained by similar preparations prepared from a purified porcine insulin (258).

Furthermore, a modest but definite deterioration in hypoglycemic control was noted to occur when diabetic patients were switched from an animal insulin to either bioengineered or transpeptidized human insulin (291). Since the interaction with the insulin-binding antibodies was not altered in these patients, the observed deterioration was ascribed to the differences in pharmacokinetics between human and animal insulins. It should be pointed out that the presence of threonine at the *N*-terminus of the B chain (B-30) makes human insulin more hydrophilic and therefore more rapidly absorbed than porcine insulin, which has alanine at the B-30 position (292).

Another possibility is the difference in pharmacodynamics between human and animal insulins and the patient's reduced ability to sense the onset of hypoglycemia when transferred from animal to human insulin (293).

Insulin-binding antibodies Insulin-binding antibodies may affect the pharmacokinetics of insulin. Beef insulin, which is known to induce the formation of antibodies, has been observed to maintain a longer plasma half-life and thus produce a prolonged duration of hypoglycemic action than the bioengineered human insulin (293,294). Insulin-binding antibodies have been demonstrated to blunt the serum peak of insulin following the parenteral administration of a regular insulin preparation (295) and also delay the recovery from a hypoglycemia (296).

In contrast to the diabetic patients with a low antibody titer, the free-insulin concentrations in patients with moderate antibody titers were noted to rise slowly, following the subcutaneous administration of either human or beef insulin, and thus higher blood glucose levels were observed during the first 2-hr period. When

bound to the antibodies, insulin loses its ability to promptly reduce the postprandial blood levels of glucose. By acting as a reservoir (296), the antibodies slowly release the bound insulin, and thereby delay the recovery from hypoglycemia. In addition, insulin-binding antibodies could impair the counterregulatory response of type I diabetic patients to hypoglycemia.

Studies in nondiabetic humans and diabetic patients demonstrated that the pharmacokinetic profiles of insulin following either intravenous or subcutaneous administration are altered by interaction with the insulin-binding antibodies (293). A typical case was illustrated by the subcutaneous administration of insulin to diabetic patients with binding of greater than 10%. In these patients, the rate of increase in free-insulin concentrations was noted to be diminished, the time to the peak serum concentration was delayed, and the time for returning to baseline was longer than that observed in the nondiabetic subjects. Additionally, a ninefold increase in the volume of distribution (904 vs. 109 ml/kg) was obtained and a concurrent increase in the clearance rate of insulin (28.5 vs. 17.3 ml/kg/min) was noted. On the contrary, the pharmacokinetic profiles in patients with binding of less than 10% were found to be normal. Thus, the distortion of insulin pharmacokinetics by antibody binding of greater than 10% can undercut the efficacy of intensive insulin treatment programs to achieve normoglycemia in the insulin-dependent diabetic patients (295). Furthermore, insulin-binding antibodies may also contribute to the development of complications.

Glycemic level The circulatory concentrations of glucose can affect the time of peak action and the duration of hypoglycemic activity of insulin administered parenterally. In type I diabetic patients, hyperglycemia was reported to be delayed, and the hypoglycemic action of a regular insulin preparation was extended.

PK-PD of Insulin Combination Treatment

Because a single injection of an extended-action insulin preparation rarely provides adequate glycemic control for most insulin-dependent diabetic patients, supplementation with a fast-action insulin, usually the regular insulin preparation, is thus needed. Regular insulin preparation, which takes effect rapidly and has a relatively short duration of hypoglycemic activity, is ordinarily used to offset hyperglycemia after meals. It is important that the coadministration of an extended-action insulin preparation does not alter its fast-acting hypoglycemic effects.

It has been reported that the hypoglycemic activities of NPH and regular insulin preparations, coadministered in any proportions, are the same as if the two were administered separately (297,298). On the other hand, Lente insulin preparations were reported to blunt the peak hypoglycemic action of regular insulin preparation when administered simultaneously (299). The incompatibility was attributed to the interaction of the excess zinc in the Lente preparation with the insulin in the regular insulin preparation. Ultralente preparation was also found to reduce the rapid hypoglycemic action of regular human insulin preparation (300,301).

Insulin Degradation at the Injection Site

As much as 20–50% of the insulin administered subcutaneously was reported to be degraded at the tissue site of injection. Most of the evidence suggest that degradation is a real, albeit variable and inconsistent, phenomenon which may contribute significantly to the day-to-day intrapatient variation observed in insulin absorption and hypoglycemic action.

Intrasubject Variation in Insulin Pharmacokinetics

Repeated injections in the same subject have reportedly produced a coefficient of variation as much as 20–30% in either the peak concentrations or the areas under the serum concentration (AUCs) of insulin for both fast-action and extended-action insulin preparations (302). This variability in pharmacokinetics is associated with a comparable variability in the pharmacodynamics of blood glucose responses. The only reliable method that could be used to minimize the observed intrapatient variation is to reduce the dose administered at any one time. When diabetic patients were treated with insulin administered by a continuous subcutaneous infusion, the variability of the peak serum insulin concentrations and AUCs was found to be 50% less than that observed in nondiabetic subjects who received insulin by a conventional parenteral administration (302).

TREATMENT OF DIABETES MELLITUS

Physiologic Considerations

A regimen that produces a serum insulin profile simulating that of healthy nondiabetic humans is the best

(303). Normal pancreatic secretion of insulin in a healthy nondiabetic human, which is shown in Fig. 15, is characterized by two modes: basal and meal-stimulated secretion (303). The function of basal insulin secretion is to restrain the output of glucose from the liver at the fasting state (i.e., overnight and between meals). The function of meal-stimulated insulin secretion is to promote the utilization of ingested nutrients, mainly glucose, by the peripheral tissues, principally the muscle and the adipose tissue. The serum insulin concentrations needed to promote the tissue utilization of absorbed glucose are substantially higher than those required to reduce hepatic glucose production (Fig. 16).

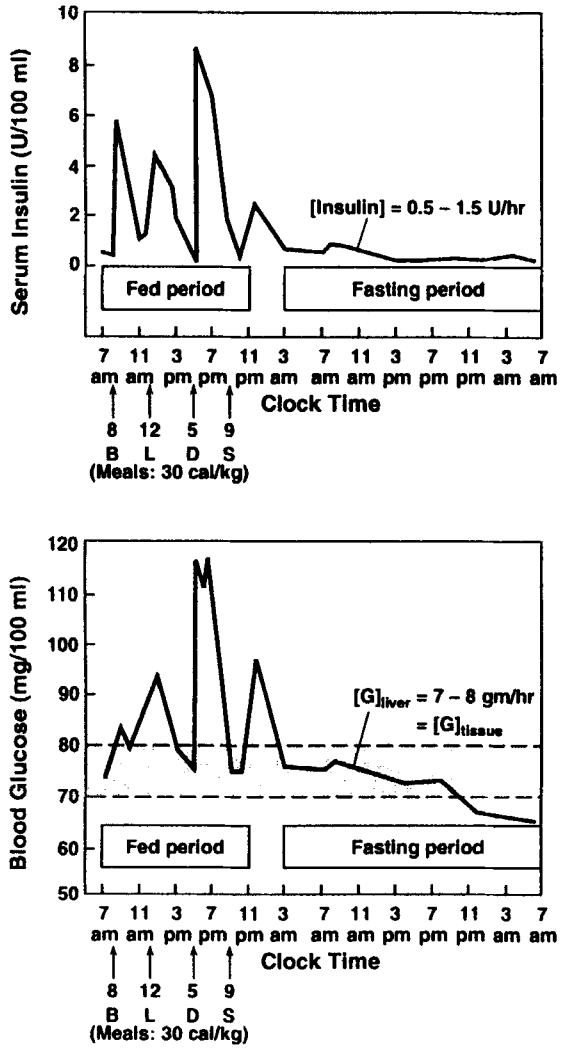


Figure 15. Diurnal insulin and glucose profiles in the systemic circulation of normal nondiabetic humans (6 normal men) taking a fixed meal of 30 cal/kg. Key: B = breakfast, L = lunch, and D = dinner. In the fast period, liver releases glucose at a rate of 7–8 g/hr to match the glucose utilization of various tissues, and pancreas secretes insulin at a rate of 0.5–1.5 units/hr to maintain the homeostatis of glucose.

Simulation of Normal Insulin Secretion

Subcutaneous controlled delivery of insulin by the continuous subcutaneous insulin infusion (CSII) system (Fig. 17) has reportedly produced a plasma insulin profile that simulates fairly well the physiological secretion pattern of insulin in the nondiabetic human (Fig. 18). Use of CSII for programmable insulin delivery in the treatment of diabetic patients thus has several pharmacokinetic and physiologic advantages over conventional insulin treatment programs (302,304). The only exception is the treatment regimens that use multiple daily insulin injections, which are as effective as CSII system (305).

The Holman Turner regimen has been shown to be capable of producing a serum insulin profile that most closely mimics the physiologic pattern of insulin secretion. This regimen calls for daily administration of Ultralente insulin preparation, which provides the basal insulin level needed, and supplementary injections of regular insulin preparation before mealtimes, which control the prandial hyperglycemia following the meal (303). This treatment regimen produces a diurnal plasma glucose profile (306) that compares favorably with the most widely used treatment regimen, which consists of one subcutaneous injection of an intermediate-action insulin preparation along with two injections of regular insulin preparation (one before breakfast and another before supper) (307).

Goals for Glycemic Control

The selection of an insulin treatment program depends on the goals set for glycemic control. For example, the Diabetes Control and Complications Trial (308) set the following goals as the targets: fasting and preprandial blood glucose levels of 70–120 mg/dl (with 3:00 a.m. values of ≥65 mg/dl), postprandial levels of <180 mg/dl (in the 90- to 120-min period), and glycohemoglobin levels of ≤6.05.

General Treatment Regimens

Two general programs have been established for wide applications in the treatment of insulin-dependent diabetes mellitus:

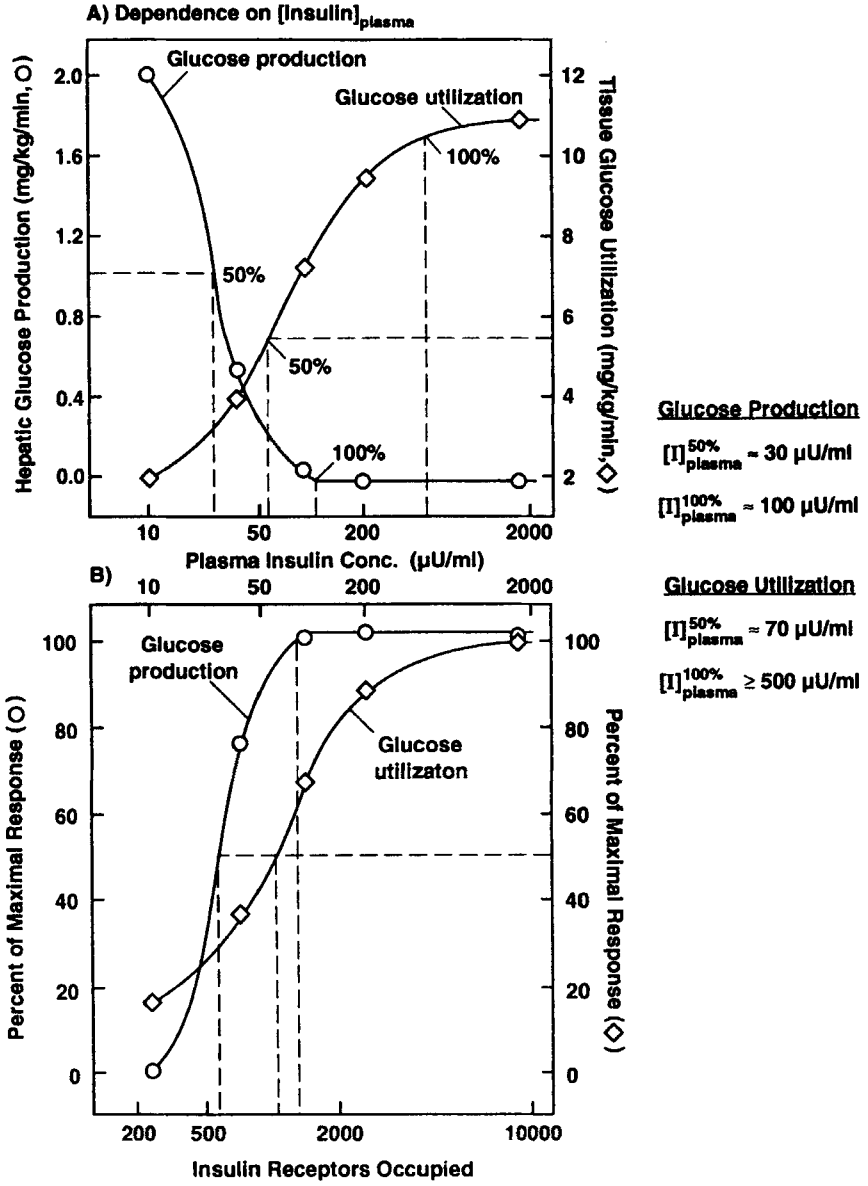


Figure 16. (A) Dependence of hepatic glucose production and tissue glucose utilization on plasma concentrations of insulin. (B) Relationship of the percent of maximal responses (for hepatic glucose production and for tissue glucose utilization) with the number of insulin receptors occupied. (Modified from Gerich, 1984.)

Program A

This program starts with a single daily subcutaneous injection of an intermediate-action insulin preparation (NPH or Lente) and then progresses to two doses of intermediate-action insulin preparation daily, one in the morning and another one in the evening, plus regular insulin preparations as needed.

In this regimen, the morning dose is determined by the fasting blood glucose level and the evening dose is varied according to the blood glucose concentration before supper. The variation of insulin dosage depends on the degree of hypoglycemia or hyperglycemia being corrected. The use of fast-action regular insulin preparation, in combination with the intermediate-action insulin preparation, depends on the severity of the diabetes

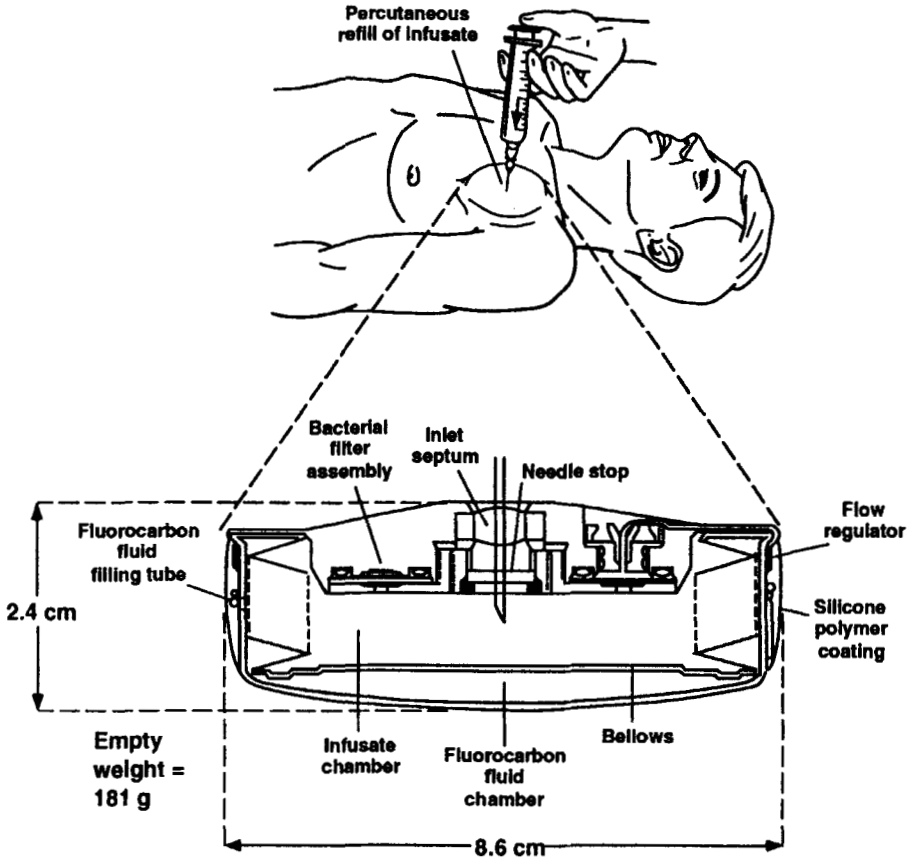


Figure 17. Schematic illustration of the in-situ percutaneous refilling of implantable insulin infusion pump and the cross-section view of this implantable vapor pressure-activated drug delivery device showing various major structural components. (Redrawn from Galloway et al., 1988, with modifications.)

and magnitude of the desire for near-normal glycemia. In general, the preprandial dose of insulin is increased or decreased by 1 unit for every 8–12 mg/dl deviation from the glycemic goal. While the dose of regular insulin administered may be changed from meal to meal, it is advisable to identify a trend over a day or two before instituting a change.

Program B

This program, which is also called the Holman Turner (303) program, uses Ultralente insulin preparation to provide the basal supply of insulin, with injection of regular insulin preparations to cover the prandial hyperglycemia. This regimen aims to mimic the normal pattern of pancreatic insulin secretion. Ultralente is highly effective in controlling basal hyperglycemia, which is essential to the normalization of glycemia for the ensuing 24-hr period (309). As discussed earlier, this regimen has produced a diurnal plasma glucose profile in insulin-dependent diabetic patients that compares favorably with that produced by the treatment regimen outlined in Program A. Because of the introduction of pen-type insulin injection devices (e.g., Novopen® and Novolinpen®), which have made multiple daily insulin injections more convenient and less painful, this treatment regimen has gained increasing popularity.

Factors to Be Considered

Because of its more rapid absorption from the injection site and reduced immunogenicity, human Ultralente has a slightly shorter duration of hypoglycemic activity than its bovine or porcine counterpart.

To function as an effective "basal" insulin, Ultralente insulin preparation is frequently administered either in

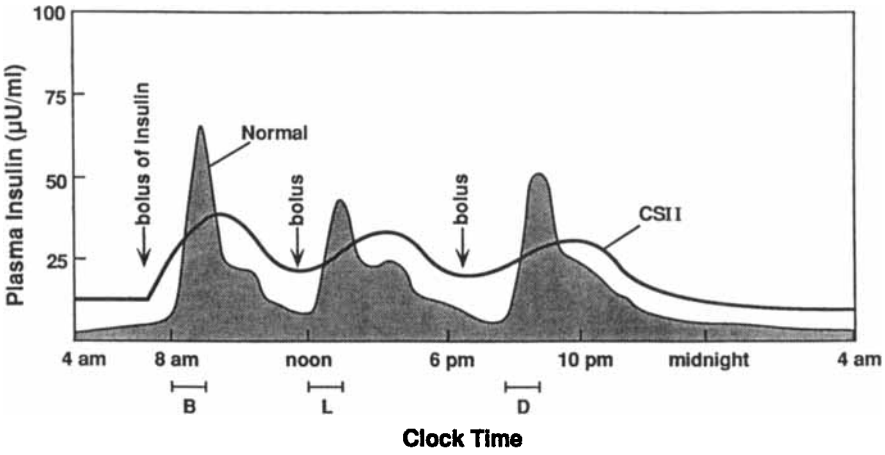


Figure 18. Simulation of the diurnal circulatory concentration profiles of insulin in normal nondiabetic humans by the subcutaneous controlled delivery of insulin by continuous subcutraneous insulin infusion (CSII) from the vapor pressure activated insulin delivery device. (Replotted from Galloway et al., 1988.)

the evening or twice daily. The latter is advisable in regions with hot climate, where increased cutaneous perfusion can shorten the duration of its hypoglycemic action to an average of only 15–20 hr, instead of the expected duration of 24 hr (303).

As a result of increased blood flow at the injection site, mild hypoglycemia could occur when one is exposed to excessive heat. The subcutaneous delivery of Ultralente insulin was reportedly increased when the patient had a fever (303).

Subcutaneous administration of insulin has been a common practice in the treatment of diabetes since the introduction of insulin in the 1920s (310). But is has not succeeded in normalizing glycemic control despite the efforts devoted to improvement in insulin preparations and treatment regimens.

Normalization of glycemic levels requires normalization of circulating insulin profile, with an appropriate elevation in plasma insulin during mealtimes, to prevent unphysiological postprandial hyperglycemia. Numerous investigations have focused on factors that might affect the rate of absorption of insulin from the subcutaneous injection site.

Clinical Control of Postprandial Hyperglycemia

Nondiabetic Humans

In healthy nondiabetic humans, blood glucose concentrations are maintained within a narrow physiological range (Fig. 19) by a highly efficient homeostatic mechanism.

Figure 19 illustrates the blood glucose and serum insulin profiles during the pre- and postprandial state in healthy humans. Serum immunoreactive insulin levels indicate a low basal level during the fasting state and a rapid increase in response to carbohydrate ingestion. Peak insulin levels are achieved within 0.5–1.0 hr from the onset of eating, returning to basal levels within 2–3 hr after meals.

Diabetic Patients

In non-insulin-dependent diabetes mellitus (NIDDM), an increasing deficit was observed in early-phase insulin secretion with deteriorating glycemic control (Fig. 19). This shows that inadequacies in the temporal and quantitative relationship between carbohydrate intake and insulin availability can seriously compromise the homeostatis of glucose. In insulin-dependent diabetes mellitus (IDDM), deficient insulin secretion from β cells is a common observation.

There is increasingly convincing evidence that microvascular complications are associated with a poor control of blood glucose levels. Therefore, the diabetologist is committed to strive for normoglycemia while trying to avoid the dangers of hypoglycemia (311,312).

The pharmacokinetics of insulin following the subcutaneous administration of short-, intermediate-, and long-acting insulin preparations makes it virtually impossible to achieve normoglycemia (313–320). Attempts to achieve normoglycemia in diabetic patients have therefore involved a multiplicity of insulin preparations, regi-

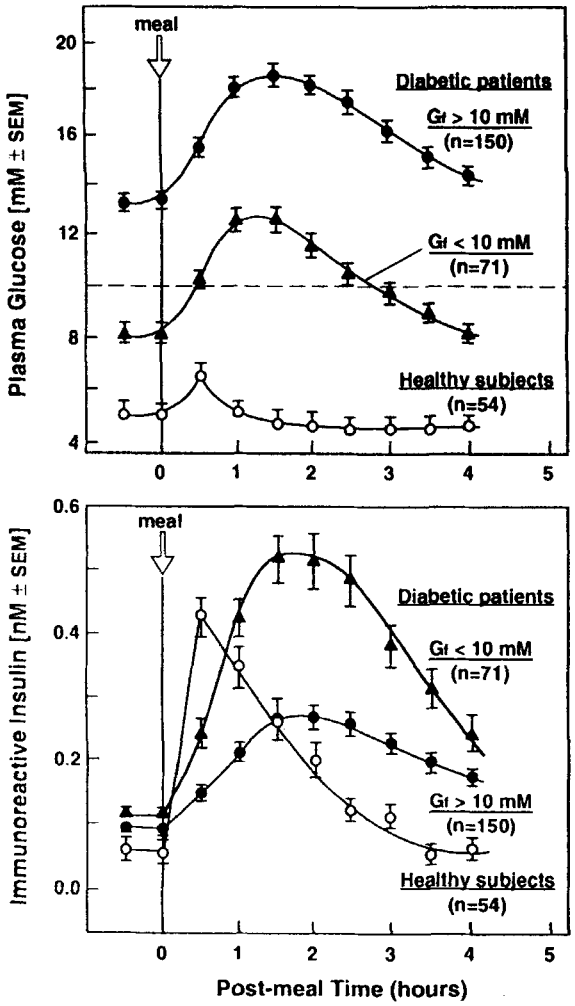


Figure 19. Comparison in the plasma profiles of glucose level (PD) and free-insulin (PK) between healthy subjects and previously untreated type II diabetic patients with fasting plasma glucose levels (Gf) either higher or lower than 10 mM. (Modified from Brange et al., 1990.)

mens, and delivery systems to meet both basal and meal-related insulin requirements (321–323).

As discussed earlier (under Intrasubject Variation in the section on PK–PD relationships), the most common subcutaneous insulin regimen involves twice-daily injection of mixtures of short- and intermediate-acting insulin preparations (307,322,324,325). Additional efforts have resulted in the introduction of continuous subcutaneous insulin infusion using a portable infusion device (Fig. 17) with adjustable rates (326–330). Comparable glycemic control can be achieved with intensively applied conventional treatment involving a subcutaneous administration of long-acting Ultralente insulin plus multiple preprandial injections of fast-action regular insulin preparation (331,332).

The use of multicomponent insulin regimens has demonstrated the value of providing a more physiological insulin therapy to meet the basal and meal-related requirements of glycemic control (325,331,333–338).

After subcutaneous injection of a regular insulin preparation into the femoral region, however, it takes 1–2 hr for the insulin to be absorbed at maximal rate (339). This slow rise to peak insulin concentration, as shown in Fig. 12, is likely to account for much of the post prandial hyperglycemia observed. Because the circulating insulin concentration falls slowly after the peak, the extended duration of insulin concentration at the elevated level results in a tendency toward late hypoglycemia (313). The peak effect may even persist for several hours if there are insulin-binding antibodies present in the circulation. The circulatory insulin patterns and glucose profiles in the type II diabetic patients bear no resemblance to those in the healthy subjects in response to a meal (Fig. 19).

Variation in subcutaneous absorption among different insulin preparations, dosage levels, administration sites, and injection techniques as well as the influence of exercise, massage, and ambient temperature have been extensively reviewed (316–319,339). Measures to alter these factors, aiming to enhance the absorption rate of insulin from the subcutaneous tissue, are either impractical, inconvenient, or unsafe for use by the insulin-dependent diabetic patient on a day-to-day basis.

Development of Fast-Acting Insulin Analogues

As discussed earlier, the self-association of insulin into dimers, hexamers, high-molecular-weight aggregates, and insoluble fibrils (at therapeutic concentrations) has been recognized as a problem in the treatment of diabetes mellitus. Active research programs have been initiated to resolve this problem by developing insulin analogues with reduced self-association. Several approaches have been taken; the following two approaches have shown promise:

Bioengineering Approach

By computer-aided molecular modeling and genetic engineering, several monomeric analogues of insulin with one or more amino acid residues in the dimer-forming surfaces replaced have been synthesized by the biotechnology process (136). These monomeric insulin analogues were observed to have a reduced self-associa-

tion and thus a more rapid absorption from the subcutis than native insulin (Fig. 20), following the subcutaneous injection. The disappearance profile of Asp^{B9}-Glu^{B27}-insulin analogue follows a monophasic first-order kinetic process (66.2%/hr) as compared to a triphasic pattern for human insulin (20.1%, 35.9%, and 60.5%/hr). The results in Fig. 20 also indicate that this monomeric insulin analogue yields a prompt rise in the plasma concentration of free-insulin and a greater reduction in plasma glucose level than native insulin.

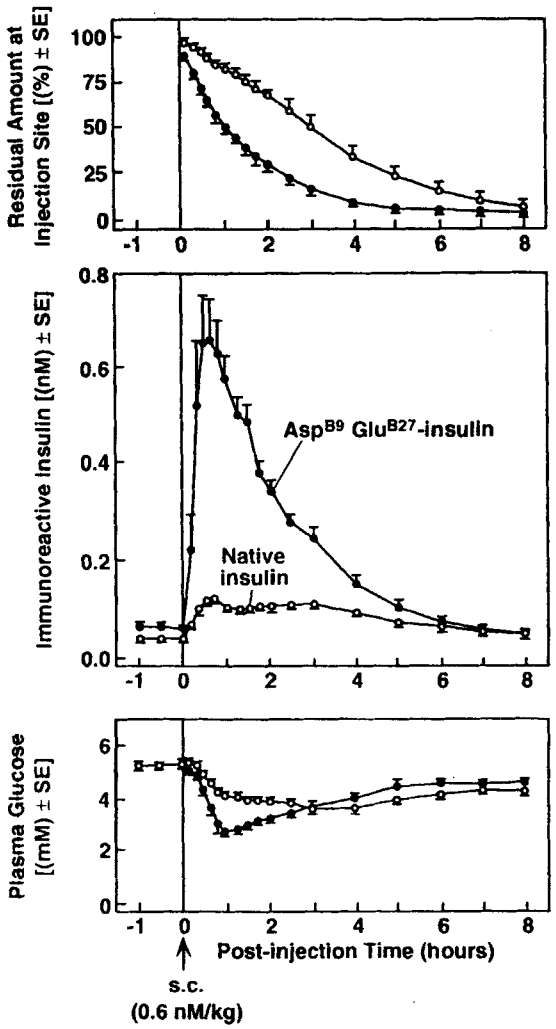


Figure 20. Comparison in the disappearance profiles at injection site, free-insulin profile (PK), and plasma glucose profile (PD) in healthy subjects (n = 7) following the subcutaneous administration (0.6 nM/kg) of native human insulin and its Asp^{B9}-Glu^{B27} analogue. (Modified from Brange et al., 1990.)

In the insulin-treated diabetic patient, the subcutaneous administration of this Asp^{B9}-Glu^{B27}-insulin analogue was also observed to produce a more rapid rise of the plasma free-insulin concentrations and a better control of the postprandial hyperglycemic level than that attained by human insulin (Fig. 21).

Chemical Approaches

Human insulin can also be modified by the covalent attachment of monosaccharide moieties to some of its amino group(s) to alter its self-association behavior and aggregation tendency, which leads to some improvement of both its pharmaceutical stability and biological activity.

The glycosylation of insulin's available amino groups at three amino acid residues (Gly^{A1}, Phe^{B1}, and Lys^{B29})

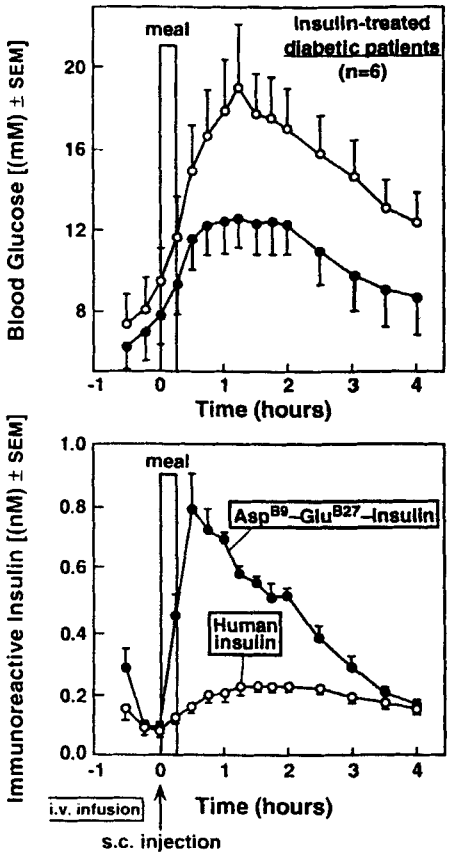


Figure 21. Comparison in the blood glucose profiles (PD) and plasma free-insulin profiles (PK) in insulin-treated patients (n = 6) following the subcutaneous injection of insulin and its Asp^{B9}-Glu^{B27} analogues (equimolar dose of 10 IU). A test meal of 500 kcal was taken at time zero. (Modified from Brange et al., 1990.)

with p-succinamidophenylglucopyranoside has resulted in seven possible glucosylated insulin derivatives [3 mono-, 3 di-, and 1 trisubstituted (340)]. While nearly all the derivatives were reported to retain in vivo biological activity comparable to insulin, self-association behavior was most suppressed with the disubstituted derivative, especially those substituted on Phe^{B1}, and the trisubstituted derivative (140). It was also observed that as the number of glucosyl moieties attached to insulin molecule increased, physical stability in solution dramatically improved. The glycosylation at the Phe^{B1} site has the most significant impact on insulin stability, which is most influential in suppressing the association of monomers and/or dimers into hexamers and thus the hexamerization of insulin derivatives.

Immunochemistry

The antigenic nature of insulin was established by Banting et al., who showed that the antibodies produced in guinea pig neutralize the insulins from pig, bovine, sheep, mouse, and rabbit (341,342). Insulin is a weak antigen and the existence of specific antibodies for insulin has been established (343).

^{131}I-insulin in the serum of nondiabetic humans was reported to have a electrophoretic behavior similar to that of albumin, due to its binding to albumin molecule. On the other hand, ^{131}I-insulin in the serum of diabetic patients who had been treated with insulin was found to be bound to the γ globulins (37).

The protein-binding capacity and the neutralizing antibodies of insulin exhibit no pronounced species specificity. Thus, the antibodies for bovine and porcine insulins react not only with the insulins of bovine and porcine origins, but also with the insulins from mouse, dog, rabbit, sheep, monkey, and human (37,342,344–346). However, quantitative differences do exist and these differences increase with increasing differences in the primary structures of the insulin molecules (347,348). In general, insulin antibodies do not appear to react with the circulating insulin of the test animal that produces the antibodies (249).

It has been postulated that a special form of endogenous insulin exists, differing from the insulin extracted from the pancreas or from serum and used for immunization (349). Antibodies for the extracted bovine insulin were detected in cattle following injection with bovine insulin (222). Antibodies for the extracted porcine insulin were also reportedly observed in swine (350,351).

Antisera have been developed that could distinguish the insulins extracted from sperm whale and pig, even though the amino acid sequences of these two insulins are thought to be identical (352). It has thus been concluded that the tertiary structures of the insulins from different mammalians can differ even when their amino acid sequences are the same (352).

The groups in the insulin molecule that are involved in the binding with the insulin-binding antibodies are probably neither the *N*-terminal amino acids (353) nor the *C*-terminal sequence $B_{23\text{-}30}$ (354). The possibility that ^{131}I-insulin and uniodinated insulin have different immunological behavior has been discussed (353), since the iodination of insulin takes place mainly at the tyrosine residues of the A chain (i.e., A_{14} and A_{19}) (212,355). These tyrosine residues are probably important to insulin's antigenic activity, since the immunologic specificity of insulin has been shown to be determined primarily by the A chain (356).

Insulin-binding antibodies were observed to react with intact insulin molecules, but only weakly with the B chain and not at all with the A chain. Based on these observations, it was concluded that the insulin molecule could contain several (three?) antigenic regions.

These insulin-binding antibodies were frequently detected in the serum of diabetics who had been treated with large does of insulin. It is interesting to note that these antibodies also appear to protect the insulin molecule from degradation (357).

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