PROPERTIES OF PROINSULIN AND RELATED POLYPEPTIDES

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INTRODUCTION

Historical Background

Although it was known that insulin consisted of two polypeptide chains connected by disulfide bonds,[1] the biosynthetic process by which this double chain is formed was not known prior to 1967. Previously it had been thought that the most likely sequence of events was independent synthesis of the two chains followed by oxidative combination of sulfhydryl groups.[2] In 1967, Steiner and co-workers showed clearly that insulin is formed from a larger molecular weight, single chain precursor which they named proinsulin.[3] This material is proteolytically cleaved in the beta cell to form the more active double chain insulin. It is of great interest that subsequent to this discovery several other polypeptide hormones have been shown to be formed from larger, biologically less active precursors by the process of limited intracellular proteolysis. Hormones of the pancreatic alpha cell,[4,5] parathyroid,[6] and pituitary[7] are apparently synthesized in this manner.

Since the demonstration of proinsulin as the precursor of insulin by Steiner and co-workers in 1967,[3,8] and the elucidation of its structure by Chance et al.,[9] more than 100 publications concerned with various aspects of this precursor have appeared. In addition, many reviews have been published regarding the physiological significance of this prohormone.[10-23] The purpose of this presentation is to review some of the more recent studies on proinsulin and related polypeptides and to examine in some detail the recent advances in the area related to biochemical properties of polypeptides derived from proinsulin. For this review the authors have drawn largely upon the investigations published since 1969, as well as from the work initiated in their laboratories on polypeptides. The latter work would not have been possible without the help of Drs. Chance and Steiner who generously supplied these proinsulin derivatives.

Definitions and Nomenclature

For the purpose of clarity, a working definition will be offered for all compounds which will be discussed. The amino acid sequence of some of

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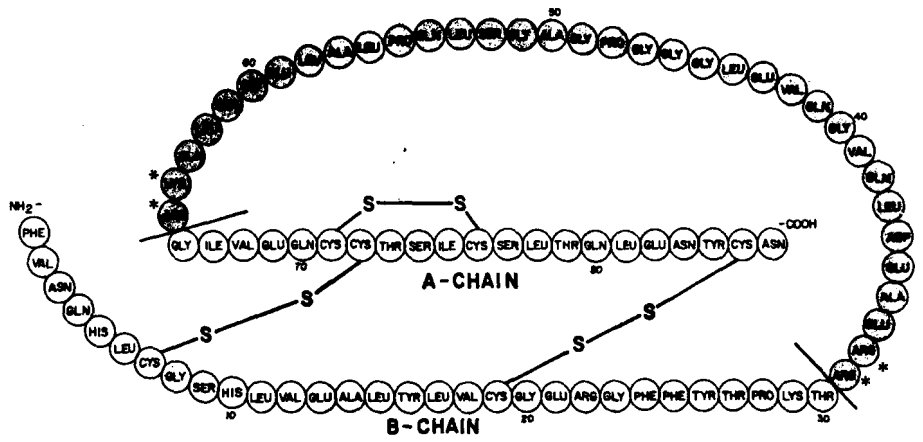


FIGURE 1. Amino acid sequence of human proinsulin as proposed by Oyer et al.[33] The identity of the residues designated with an * has not been established. The connecting peptide moiety is depicted by the shaded circles.

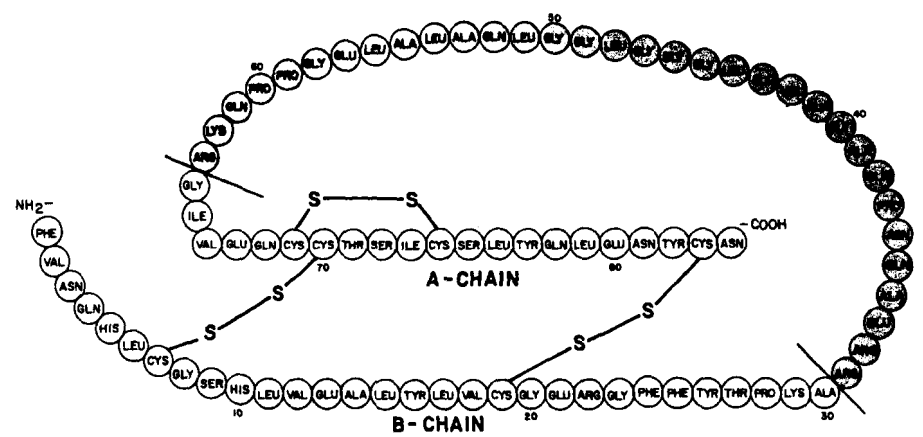
these derivatives will also be depicted in subsequent pages. Some of these intermediates have also been defined elsewhere in selected publications.[22,24,25]

1. **Proinsulin** – This is a minor component of both crystalline zinc insulin and acid ethanol extractable protein from pancreatic tissue which has now been shown to be the biosynthetic precursor of insulin in human,[3,26] rat,[27,28] pork,[9] beef,[29,30] angler fish,[31] cod,[32] monkey,[33] and rabbit.[34] Proinsulin consists of the A and B chains of insulin plus a polypeptide which connects the amino end of the A chain with the carboxy end of the B chain.

The amino acid sequences of proinsulin for human, porcine, and bovine are presented in Figures 1, 2, and 3, respectively.

2. **Porcine C-peptide** – This term refers to the 29-residue glutamyl peptide B_{33-61} which is liberated from intact porcine proinsulin by limited tryptic digestion.[24]

3. **Beef C-peptide** – This is a polypeptide liberated by proteolysis from beef proinsulin containing residues number 33-58. For the pur-



PORCINE PROINSULIN

FIGURE 2. Primary structures of porcine proinsulin (adapted from Chance et al.).[9] The connecting peptide moiety is depicted by the shaded circles.

Amino acid sequence of porcine proinsulin depicted in Figures 2, 5, 6, 7, and 8 all refer to the proinsulin structure proposed by Chance et al.[9] at which time position 35 was thought to be glutamine. Subsequently, position 35 was found to be glutamic acid as in human and bovine.

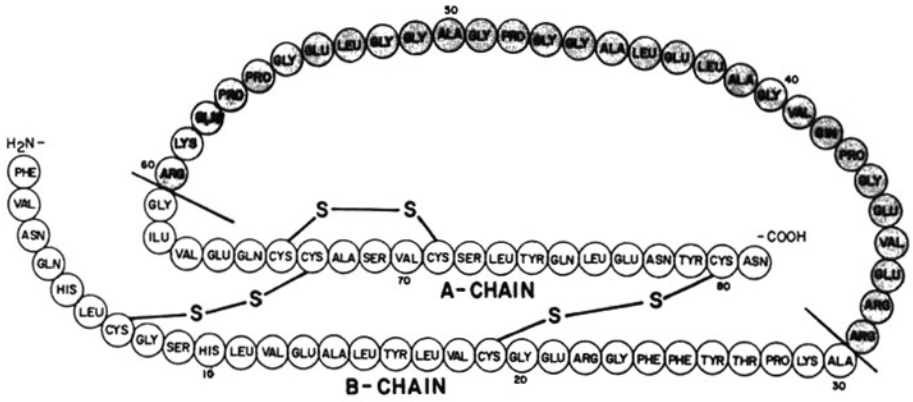


FIGURE 3. Amino acid composition and sequence of bovine proinsulin (adapted from Nolan et al.).[35] The connecting peptide moiety is depicted by the shaded circles.

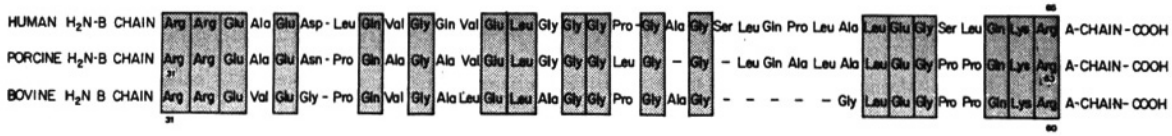


FIGURE 4. Amino acid composition of connecting peptide of human,[33] porcine,[9] and bovine.[35] Shaded areas designate similarities of amino acid composition in the three species.

pose of comparison, the amino acid sequences of connecting peptides of the human, porcine, and bovine are presented in Figure 4 where shaded areas denote amino acid similarity in the three species.

From the definition given above, and comparison of the amino acid sequence in Figure 4, it is clear that porcine C-peptide, by the definition given by Chance,[24] is devoid of B_{31-32} (Arg-Arg) residue.

4. C-peptide – This refers to the connecting peptide from which the terminal basic residues have been removed.

Comparison of Figures 1, 2, 3, and 4 also reveals that although the amino acid sequence of the insulin molecule in human does not vary for more than one or three amino acids from pork or beef, respectively, the C-peptide of human varies from pork by 10 amino acids and from bovine by 14 amino acids. Variations in amino acid composition of connecting peptides have also been noted for cod,[32] and angler fish[31] which, like pork, have 33 amino acids in contrast to 30 for bovine[35] and 35 for human and monkey.[33]

5. Split porcine proinsulin (cleaved Leu_{54}-Ala_{55}) (Figure 5) – This compound is obtained from proinsulin by chymotrypsin-like activity. This bond appears to be the most easily hydrolyzable by chymotrypsin.

6. Products of tryptic hydrolysis of split proinsulin – These compounds consist of diarginine, monoarginine, and desalanine insulin, as well as 22-residue glutamyl peptide (B_{33-54}). The polyacrylamide disc gel electrophoretic patterns both before and after tryptic digestion, as reported by Chance,[24] are shown in Figure 6.

7. Desdipeptide proinsulin (Lys_{62}-Arg_{63} missing) (Figure 7) – This is a compound which could conceivably be formed from proinsulin via the combined action of trypsin and carboxypeptidase B.[24] Tryptic hydrolysis of desdipeptide proinsulin yields diarginine, monoarginine, and desalanine insulins, as well as a 29-residue glutamyl peptide (B_{33-61}) analogous to the physiologic C-peptide which was defined by Clark et al.[36] Desdipeptide proinsulin was also isolated from commercial crystalline bovine insulin preparations by Steiner et al.,[22] and by Schmidt and Arens.[37]

8. Desnonapeptide proinsulin (B_{55-63} missing) (Figure 8) – This compound may be derived from proinsulin, either by chymotryptic hydrolysis of desdipeptide proinsulin, or by tryptic hydrolysis of the split proinsulin. Recent work of Chance,[24] however, suggests that the former action is more plausible.

Desnonapeptide proinsulin upon tryptic hydrolysis gives diarginine insulin ($Arg_{31,32}$ attached at Ala_{30}), monoarginine insulin (Arg_{31} attached

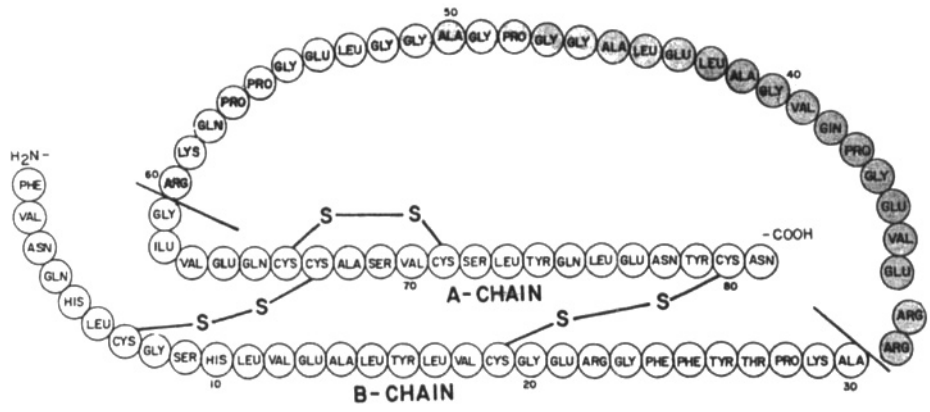


FIGURE 5. Primary structural sequences of split porcine proinsulin. Arrow designates where cleavage of the peptide has occurred. Shaded circles designate connecting peptide portion of proinsulin.

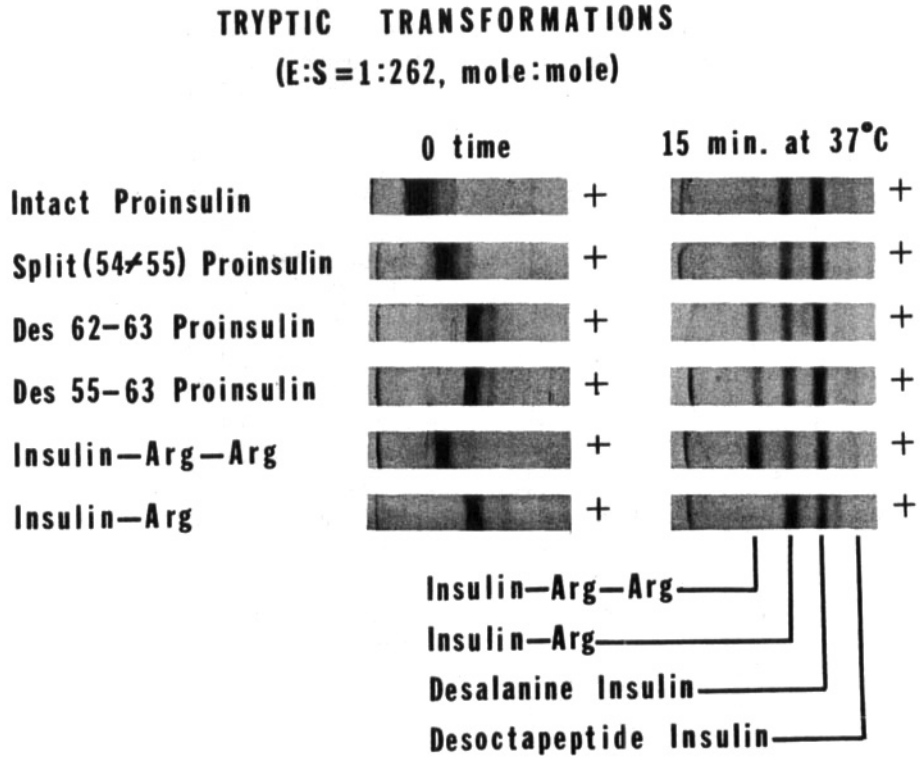


FIGURE 6. Polyacrylamide disc gel electrophoretic pattern before and after trypsin digestion. Conditions for electrophoresis and enzymatic hydrolysis were as described by Chance et al.[24] (Reproduced by permission from The Proceedings of the 7th International Congress of the International Diabetes Federation.)

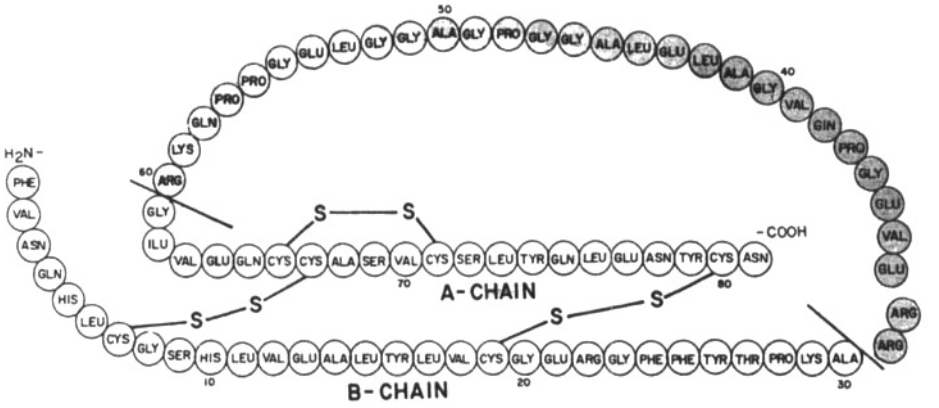


FIGURE 7. Primary structure of porcine desdipeptide proinsulin. The shaded circles are the connecting peptide portion of proinsulin.

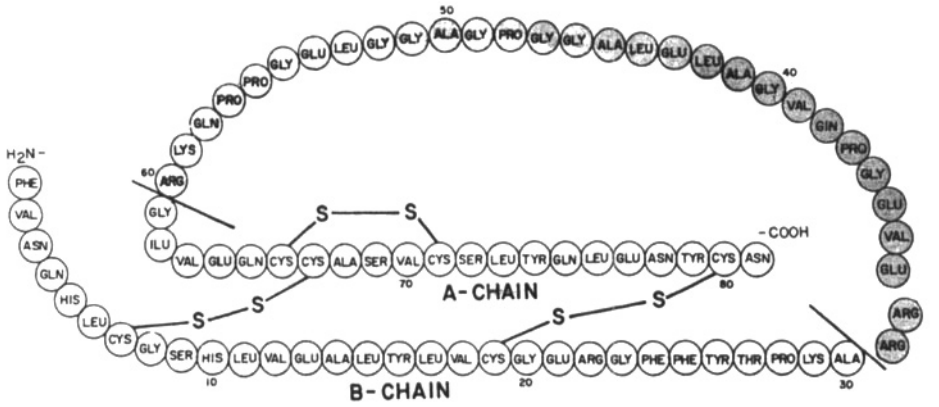


FIGURE 8. Primary structure of porcine desnonapeptide proinsulin according to Chance.[24]

at Ala_{30}), and desalanine insulin (Ala_{30} removed from insulin), plus a 22-residue glutamyl peptide (B_{33-54}).

9. Destridecapeptide proinsulin (B_{51-63} missing) (Figure 9) – This compound is derived from desnonapeptide proinsulin by treating the latter compound with carboxypeptidase (E:S=1:100 w/w) for 64 min at 37°C.[38]

10. "Single-component" insulin – This refers to the major purified protein from the pancreas which exhibits only one protein band on polyacrylamide disc gel electrophoreses.

11. Beef intermediate I – This refers to beef proinsulin lacking residues 59 and 60 (Lys-Arg) (Figure 10).[35]

12. Beef intermediate II – This refers to beef proinsulin lacking residues 31 and 32 (Arg-Arg) (Figure 11).[35]

PROINSULIN

Chemical and Immunological Properties

1. Chemical – Detailed discussions of the chemical and physical properties of proinsulin have recently been reported in several excellent reviews.[14,19,22,24] The reader is referred to these reviews, as well as to the original papers, for more detailed information. This discussion will be limited to material necessary for understanding the basic properties of proinsulin as related to its biological and immunological activity.

Proinsulin is a single polypeptide chain with a

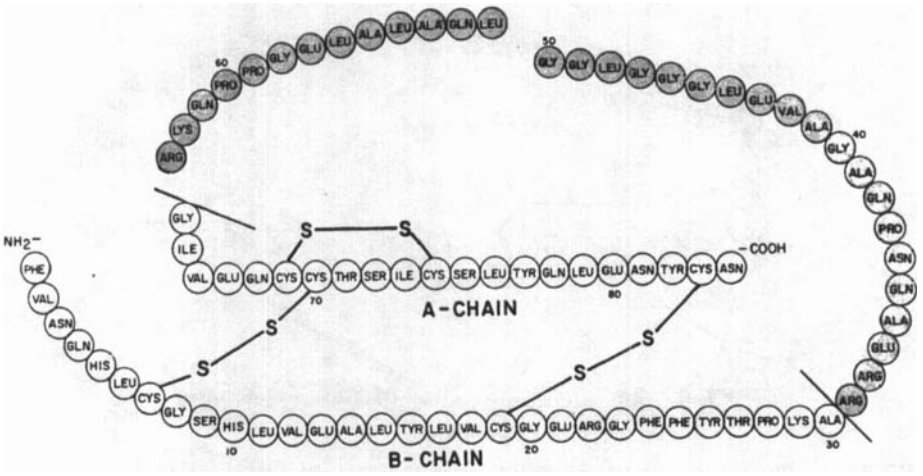


FIGURE 9. Primary structure of porcine destridecapeptide.[24] Connecting peptide portions are depicted in shaded circles.

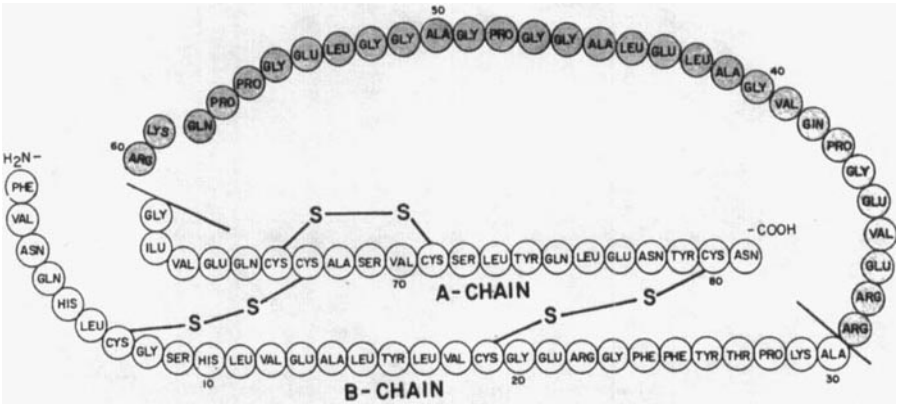


FIGURE 10. Primary structure of bovine intermediate I according to Nolan et al.[35] Shaded circles depict amino acid composition of the connecting peptide portion.

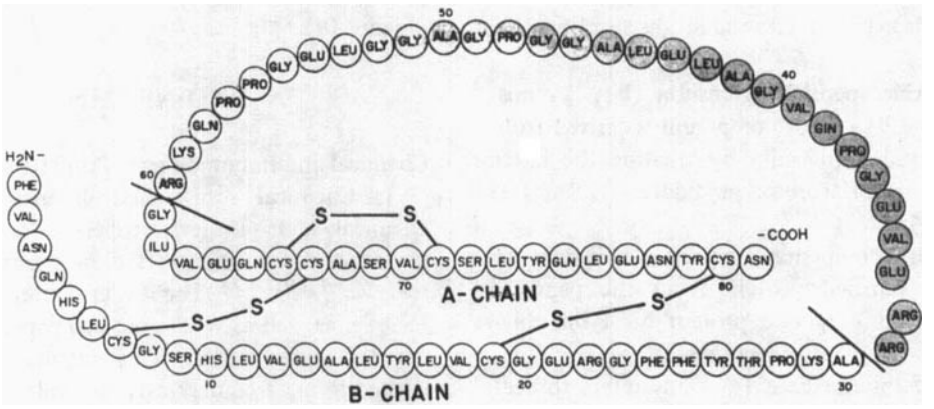


FIGURE 11. Primary structure of bovine intermediate II.[35] Shaded circles depict amino acid composition of the connecting peptide portion.

molecular weight of approximately 9000. The structures of human, porcine, and bovine proinsulin are shown in Figures 1, 2, and 3, respectively. After complete reduction of the proinsulin molecule in 8 M urea, reoxidation in dilute alkaline buffer results in a recovery of 70 to 80% of the original material. In contrast, reduction-reoxidation of insulin results in a recovery of only 1%.[39] This, along with other properties,[22] suggests that the primary function of proinsulin may be to allow the pairing of the cysteine residues to form the disulfide bonds necessary for the structure of insulin. The question as to whether or not the structure of proinsulin specifically allows this, or merely serves as a more efficient biosynthetic material, is not answered.[33] The great species variability in the structure of the connecting peptide segment of proinsulin (Figure 4) would suggest that the essential biosynthetic requirement is a single chain rather than a double chain structure. Analysis of the similarities among different species, however, reveals that there may be a specific necessity for a certain amino acid composition in the connecting peptide. The location of polar amino acids at the ends of the connecting segment, with relatively nonpolar ones in the middle, and the absence of aromatic residues, histidine and cysteine in the connecting segments of all species studied (Figure 4) indicate that the C-peptide plays an important and specific role in the folding of the peptide chain.[33]

Frank and Veros have proposed that the insulin moiety in proinsulin exists in the same conformation as insulin itself, based on optical rotary dispersion and circular dichroism data, and that the connecting peptide segment is in a random coil formation.[40] Other studies have indicated that there may be two short alpha helix areas in the connecting peptide.[41]

Frank and Veros have also shown that proinsulin can react with zinc to form a complex with a molecular weight of 55,000.[42] This complex corresponds to a hexamer of proinsulin, and requires two zinc ions per hexamer. This configuration is similar to that of insulin. Crystallization studies of proinsulin by Fullerton et al.[41] have shown that this material can be crystallized under a variety of conditions, both with and without zinc, although the crystals appear to be poorer in the presence of zinc.

Much of the interest in the chemical properties of proinsulin has been directed toward the problem of separating this material from insulin. Since proinsulin coprecipitates and cocrystallizes with insulin during its commercial preparation, the earlier method for isolation of proinsulin was by purification of commercially available insulin.[43] Chance has isolated proinsulin and various related proteins by the use of DEAE-cellulose chromatography and urea-containing buffer.[24]

Other methods available for the isolation of proinsulin from plasma and other tissues are by chromatography on Sephadex® G-50,[44] or Biogel P-30[45] after acid-ethanol extraction of tissue. Additional methods of chemical and physical isolation of proinsulin, such as paper chromatography, and starch and polyacrylamide gel electrophoresis[46] can be used, but none of these procedures will completely separate the various intermediate forms of proinsulin from proinsulin itself. The thin layer gel electrophoretic method[47] for the isolation of proinsulin has not been widely used.

2. **Immunological** – Work in the laboratories of various investigators,[17,24,48-51] as well as in the laboratories of the authors,[15,25,52] has demonstrated the immunological properties of proinsulin to be similar to insulin in various ways. For example, it has been shown that the insulin antiserum which is used as the first antibody in the routine immunoassay for insulin cross reacts with proinsulin. The extent of cross reactivity of proinsulin varies from 25 to 50%, or even more, depending on the particular antiserum used. The explanation for this lesser reactivity may be that some of the antibody binding sites on the insulin portion of the molecule are masked by the connecting peptide.[50] Since the amino acid compositions of insulin of different species vary only slightly, antiserum directed against the insulin of one species will usually cross react with insulin of other species, and since proinsulin of different species contains the same antigenic determinants (i.e., the insulin molecule), anti-insulin serum will generally cross react with heterologous proinsulin.[49,50] Hence, proinsulin of human, beef, and pork, as well as insulin of human, beef, and pork, will interact with porcine insulin antiserum. These relationships are depicted in Figures 12 and 13, where the immunoreactivity of insulin, proinsulin, and C-peptides from various species (Figure 12), and porcine polypeptides of insulin and proinsulin (Figure 13), are compared with porcine insulin antiserum. These figures demonstrate that (a) C-

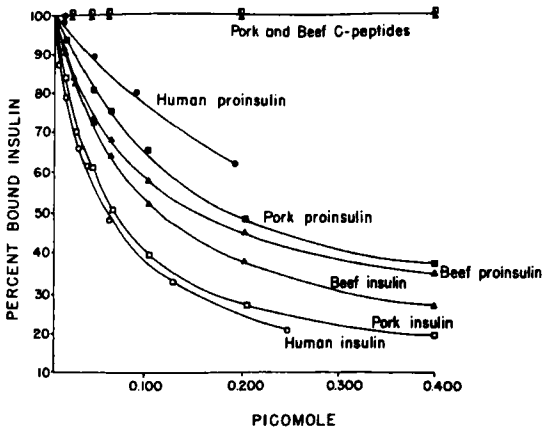


FIGURE 12. Immunoassay curves of human, pork, and beef insulin, and proinsulin, as well as pork and beef C-peptides, using antiserum to pork insulin and pork insulin I^{125}, by the double antibody immunoassay.[52]

peptides of beef and pork have no immunoreactivity against insulin antiserum; (b) insulin of various species interacts with the porcine insulin antiserum to more or less the same degree; (c) proinsulin of various species shows considerable immunoreactivity although to a lesser degree than respective insulin molecules; and (d) polypeptides of proinsulin show considerable variation in immunoreactivity, the extent of which is determined by the chemical structure of the polypeptide.

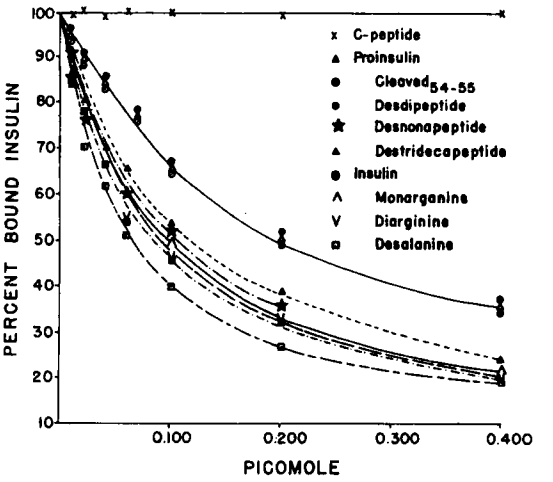


FIGURE 13. Immunoassay curves of porcine proinsulin, insulin, and their related polypeptides, as well as C-peptide to porcine insulin antiserum and porcine insulin I^{125} using the double antibody immunoassay.[52]

Proinsulin antiserum also shows cross reactivity with insulin and proinsulin of different species. The degree of cross reactivity varies with different forms of antisera and methods of assay.[18,19,50] The cross reactivity is presumably due to antibodies directed against antigenic determinants on the insulin portion of the molecule since immunoadsorbance with insulin-Sephadex preparations results in an antiserum highly specific for proinsulin and C-peptide.[53,54] This antiserum, unlike insulin antiserum, is species-specific and does not cross react with proinsulin of other species. Proinsulin antiserum which has not been adsorbed with insulin does react with the proinsulin of other species, as shown in Figure 14, in which porcine proinsulin antiserum (kindly supplied by Dr. M. Root) inhibits the biological activity of bovine proinsulin and insulin on the isolated fat cell.

The presence of antibodies against C-peptide in proinsulin antiserum has also been demonstrated by the presence of immunoreactivity against proinsulin, even after the addition of excessive amounts of insulin.[48]

Studies by Root as quoted by Chance[24] on the antigenic determinant of porcine connecting peptide suggested that since all of the proinsulin-like fragments containing sequence B_{33-54} reacted similarly with porcine proinsulin antiserum, the determinant resides in sequence B_{33-54}. It would appear that Leu_{54} is specifically important for immunoreactivity, since the removal of Leu_{54} caused a loss of cross reactivity.

Since proinsulin antiserum is directed against antigenic determinants on the connecting peptide sequence, the proinsulin immunoassay will cross react with C-peptide. This is not of much consequence in the commercial preparation of insulin, since C-peptide is separated from insulin and proinsulin, but in the peripheral circulation the presence of C-peptide[55] will interfere with the immunoassay of proinsulin unless column chromatography of the plasma extract has separated C-peptide from proinsulin prior to immunoassay.

The development of a specific anti-proinsulin antibody, i.e., one that does not react with either insulin or C-peptide, is theoretically possible. It would require that the antigenic determinant for the antibody include a portion of the insulin part of the molecule and a portion of the C-peptide segment. The antigenic determinant would also include the two basic residues which are present in the connecting peptide segment of proinsulin, but

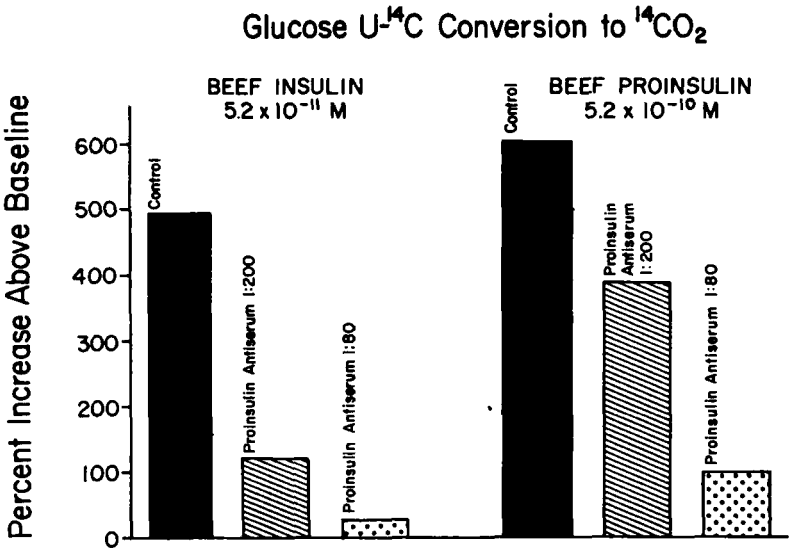


FIGURE 14. Effect of nonadsorbed porcine proinsulin antiserum on the insulin-like activity of beef insulin and proinsulin. Insulin-like activity is measured by glucose-U-^{14}C conversion to $^{14}CO_2$ in isolated fat cells of rats according to Kitabchi.[25]

not in the free C-peptide. An antibody such as this would be expected to be species-specific, and would probably be quite weak, requiring a high concentration to show immunoreactivity.

Biological Activity

1. **In vitro studies** – The biological activity of proinsulin has been investigated extensively. In vitro studies have been performed on fat, both whole fat pads[56-59] and isolated fat cells,[25,56,57,59-62] and on muscle using the rat hemidiaphragm.[58,60,63] been shown to have an effect on the toad skin in vitro,[64] and in the perfused rat liver.[65] In general, the biological assays have shown proinsulin to have activity qualitatively similar to insulin, but to be much less potent. Figure 15 demonstrates the results of our studies using pork and beef pro-

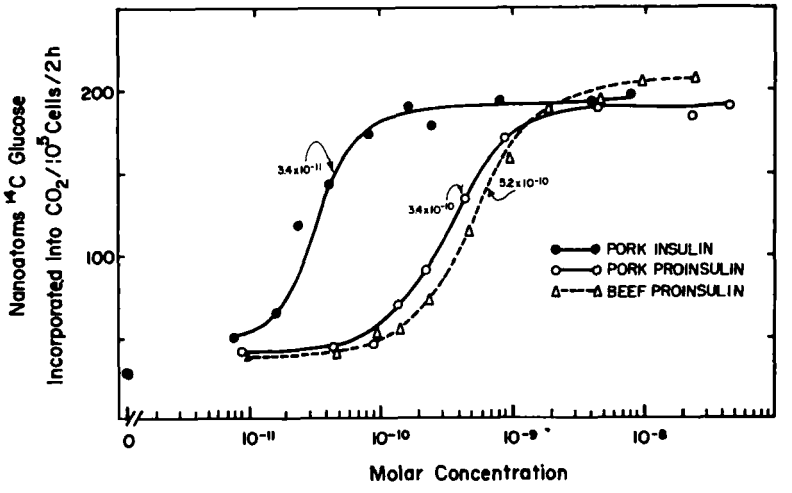


FIGURE 15. Concentration response curves for pork insulin, pork proinsulin, and beef proinsulin on isolated fat cells. Conditions of the experiments and the methods of assay are according to Kitabchi.[25]

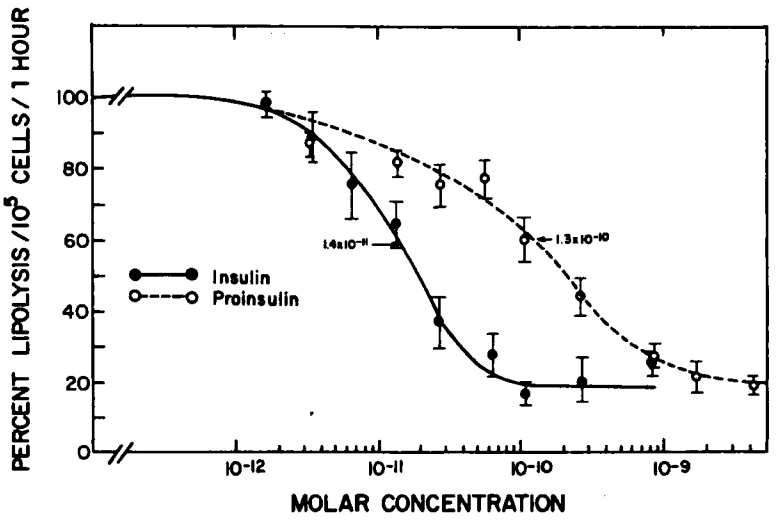


FIGURE 16. Inhibition of ACTH-induced lipolysis by beef insulin and proinsulin. The conditions and method of assay are according to Solomon et al.[61] (Adapted from Solomon et al.[61])

insulin to stimulate glucose conversion to CO_2 in the isolated fat cell. As can be seen, pork and beef proinsulin exhibits 1/10 and 1/15 the biologic activity of pork insulin, respectively. Figure 16 compares the antilipolytic properties of beef insulin and proinsulin. As can be seen, sigmoid dose response curves are obtained, but proinsulin has only 1/10 of the activity of insulin, calculated on the basis of half-maximum concentration.

Table 1 shows the comparable activities of insulin vs. proinsulin in the isolated fat cell, as reported by several different investigators. Reduced-reoxidized proinsulin has activity comparable to proinsulin,[57,62] in contrast to insulin, which has no activity after reduction-reoxidation. Connecting peptides have had no biological effect in any system tested.[25,57,61] Human proinsulin, extracted from fetal pancreas, has approximately 20% of the activity of insulin as measured on isolated fat cells.[66] Cod proinsulin had very little activity on rat epididymal fat pads, less than 0.2 units/mg.[32]

When using the fat pad assay, most investigators have found the activity of proinsulin, as compared to insulin, to be similar to that obtained with isolated fat cells, i.e., 1/10 to 1/20 as potent. Challoner and Yu,[56] however, presented data suggesting that proinsulin, although still much less potent than insulin, had relatively greater activity on fat pieces than on fat cells.

In studies on rat hemidiaphragm, proinsulin stimulated glucose uptake and glycogen in a manner similar to insulin, but with only 10 to 20% of the effectiveness of insulin on a molar basis.[58,60,63] The maximal effect of proinsulin was comparable to insulin. Studies on the in vitro effect of proinsulin on muscle have been, in general, much less complete than studies on fat.

In the perfused rat liver, proinsulin was found to have the same effects as insulin on glucose production, and on the metabolism of alanine and its incorporation into proteins.[65]

The possibility that the biological effect of proinsulin is due to its conversion in peripheral tissue to insulin was considered after Shaw and Chance[58] demonstrated that the stimulatory effect of proinsulin, on the conversion of labeled glucose to carbon dioxide in rat epididymal fat pads, could be blocked by Kunitz pancreatic trypsin inhibitor (KPTI). Two other protease inhibitors, Kazal and soybean trypsin inhibitors, did not produce this effect. A slight inhibition of glycogen formation by the hemidiaphragm in response to proinsulin was produced by the addition of KPTI, but this effect was only partial, and larger doses of KPTI resulted in no blockage of the proinsulin effect. A similar effect of KPTI on the proinsulin response of the rat hemidiaphragm was seen by Lazarus et al.[63]

Other investigators have subsequently studied the effects of KPTI, or Trasylol®, another protease inhibitor, on proinsulin-stimulated

TABLE 1

Relative Potencies of Insulin, Proinsulin and Related Peptides on the Isolated Fat Cells

Investigator	Parameter studied	Material used	Half-maximal dose
Challoner et al.[56]	glucose uptake	pork insulin	9.3×10^{-11} M
		pork proinsulin	2.15×10^{-9} M
	antilipolysis	pork insulin	5.6×10^{-11} M
		pork proinsulin	1.53×10^{-9} M
Gliemann et al.[57]	glucose uptake	beef insulin	3.3×10^{-11} M*
		beef proinsulin	1.65×10^{-9} M*
		beef intermediate	1.8×10^{-10} M*
		beef reduced-reoxidized proinsulin	0.9×10^{-9} M*
		beef C-peptide	–†
Kitabchi[25]	glucose uptake	pork insulin	3.4×10^{-11} M
		pork proinsulin	3.4×10^{-10} M
		beef insulin	4.3×10^{-11} M
		beef proinsulin	3.7×10^{-10} M
		beef intermediates	1.1×10^{-10} M
		pork C-peptide	–†
		beef C-peptide	–†
Lavis et al.[60]	glucose uptake	beef insulin	7.02×10^{-11} M
		pork proinsulin	7.8×10^{-10} M
	antilipolysis	beef insulin	2.96×10^{-11} M
		pork proinsulin	4.1×10^{-10} M
Solomon et al.[61]	antilipolysis	beef insulin	1.7×10^{-11} M
		beef proinsulin	2.7×10^{-10} M
		beef intermediates	3.5×10^{-11} M
		beef C-peptide	–†
Steele et al.[59]	antilipolysis	pork insulin	7.2×10^{-11} M
		pork proinsulin	6.0×10^{-9} M
Toomey[62]	glucose uptake	pork insulin	1.2×10^{-11} M
		pork proinsulin	5.5×10^{-9} M
		pork reduced-reoxidized proinsulin	6.5×10^{-9} M
	antilipolysis	pork insulin	3.2×10^{-11} M
		pork proinsulin	1.1×10^{-9} M
		pork reduced-reoxidized proinsulin	1.4×10^{-9} M

*Half-maximal dose calculated from the data.
†No activity.

glucose uptake and on antilipolysis in fat. No effect has been seen on proinsulin-induced antilipolysis in either isolated fat cells or in whole fat pads.[56,59,61,62] Similarly, KPTI failed to block proinsulin-stimulated glucose uptake in isolated fat cells.[25,56,62] In whole fat pads, however, a significant decrease in the glucose uptake produced by proinsulin was found after treatment with KPTI.[56] In addition, KPTI has been found to block the effect of proinsulin on the ventral toad skin in vitro.[64]

The failure of KPTI or Trasylol to block the biological activity of proinsulin on the isolated fat cell can be seen in Figures 17 and 18. Although both of these inhibitors prevent the trypsin-induced conversion of proinsulin to an insulin-like component,[25] neither had any effect on the biological activity of proinsulin. Figure 17 shows the maximum effect of the hormones, and Figure 18 shows the effect at submaximal concentrations.

Another indication that the biological activity of proinsulin does not depend on its conversion to insulin can be seen in Figure 19. This study shows that the CO_2 production, in response to proinsulin, is linear in time without a lag period. The lack of a lag period has also been seen in liver studies.[65] Insulin contamination of the proinsulin has been ruled out, both by polyacrylamide gel electrophoresis[25] and by experiments using reduced-reoxidized material.[57,62] All of these studies, therefore, indicate that proinsulin has significant intrinsic biological activity independent of its conversion to insulin. The explanation for the effect of KPTI on some tissues is not clear.

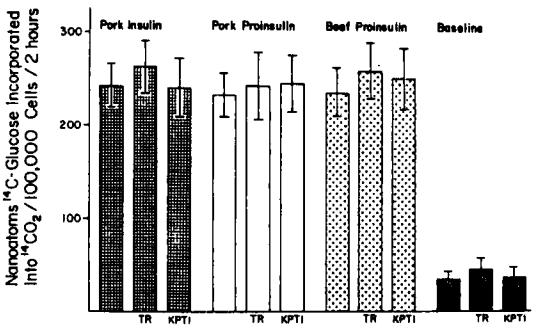


FIGURE 17. Effect of kallikrein (TR) and trypsin (KPTI) inhibitors on the biological activities of insulin and proinsulin at maximal concentrations of these hormones. The figure is drawn from data of Kitabchi.[25]

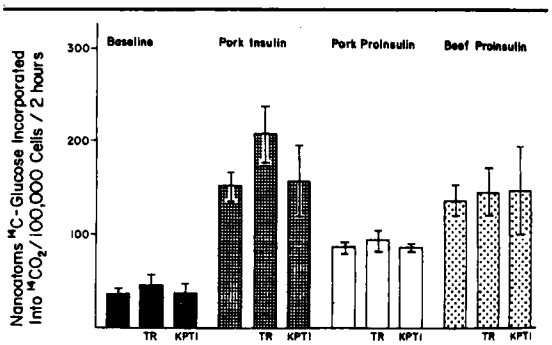


FIGURE 18. Effect of kallikrein (TR) and Kunitz pancreatic trypsin (KPTI) inhibitor on the biological activities of insulin and proinsulin at submaximal concentrations of these hormones. Data obtained from Kitabchi.[25]

2. **In vivo studies** – In vivo studies of the action of proinsulin have also indicated a direct action of proinsulin in tissues, but in contrast to the in vitro studies, some qualitative differences between insulin and proinsulin have been suggested.

The injection of proinsulin intravenously in animals or man results in a decrease in the blood glucose.[24,63,67-71] The relative potency of proinsulin is approximately 1/4 to 1/8 that of insulin, but in some studies the duration of the hypoglycemia is significantly greater than that induced by insulin in both animals and man.[24,67,71] Hypophysectomy or adrenalectomy potentiated the effect of the proinsulin in animals.[63] Eviscerated animals have had the same response to proinsulin as normal animals in some,[68,69] but not all, studies.[72] The in vivo administration of KPTI to rats had no effect on the hypoglycemia produced by proinsulin.[63]

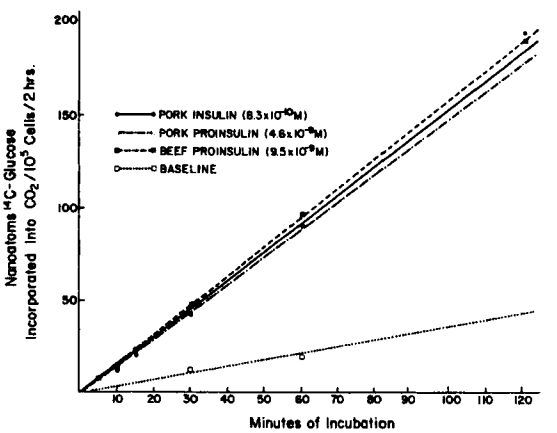


FIGURE 19. Time course of glucose-U-^{14}C conversion to $^{14}CO_2$ by the isolated fat cells of rats in the absence of insulin□, and the presence of pork insulin●, beef proinsulin■, and pork proinsulin○, at various times. (Reproduced by permission from *J. Clin. Invest.*[25])

When comparable doses of insulin and proinsulin (as determined by mouse convulsion assay) were given to baboons or swine, similar decreases in blood glucose were seen, but the hypoglycemia produced by proinsulin lasted significantly longer.[71] Calculation of the immunological halflife of the proinsulin[71] resulted in a figure of 18 to 20 min while the insulin halflife was 6 to 8 minutes.

Chance has also shown that the duration of the hypoglycemia seen after proinsulin injections is greater than that which is seen after insulin injections, and that the fall in glucose following proinsulin administration is somewhat comparable to that produced by NPH insulin.[24] The prolongation of hypoglycemia may be due to the longer halflife of proinsulin in the circulation. This question deserves additional clarification, as does the whole problem of the physiological role of proinsulin in the circulation.

An additional qualitative difference between proinsulin and insulin was suggested in the studies of Fineberg and Merimee,[73] in which a human forearm perfusion was used. They showed that the effect of proinsulin was less than that of insulin, but that proinsulin had a greater effect on adipose tissue than on muscle, while insulin's effect was more marked on muscle than on fat.[73,74]

Using a slow perfusion of proinsulin and insulin into dogs, Rees and Madison[70] found comparable effects of the two materials in hepatic glucose uptake, arterial glucose, and free fatty acid concentration. The infusion doses of the two hormones were similar; however, data on the maximum plasma concentration of insulin and proinsulin achieved during the infusion were not available.

Additional in vivo studies on the effect of proinsulin on fat and muscle metabolism have been done in rats in our laboratories.[75] Representative data are presented in Table 2. Using the technique of intraperitoneal injections[76] of insulin or proinsulin along with labeled glucose, and studying the uptake of the radioactive material in fat and muscle, proinsulin has been found to have 1/8 to 1/10 the potency of insulin on a molar basis. No marked differences in the effect on muscle vs. fat were found.

3. **Is proinsulin antagonistic to insulin?** – As soon as biochemical studies on proinsulin became available and its role in insulin biosynthesis was clarified, investigators attempted to assess the biological effect of this prohormone. Since the presence of proinsulin in human circulation was reported shortly after its discovery,[77] interest in its peripheral activity was increased. The concept of insulin antagonism was an attractive possibility to assign for proinsulin, and in a preliminary communication this was considered,[78] but as additional experiments were conducted for both porcine and bovine proinsulin in isolated fat cells, an antagonist role for proinsulin was disproved. These studies have been reported in detail elsewhere.[25] Table 3 shows the effects of beef insulin and proinsulin, separately and in combi-

TABLE 2

Uptake of Glucose ^{14}C by Rat Fat Pads and Diaphragms After Intraperitoneal Injection of Insulin or Proinsulin*

	Fat pad†	Muscle††
Control	2,308	196
Insulin 8.3×10^{-12} M	19,965	25,932
Proinsulin 4.3×10^{-11} M	17,088	21,306

*Method of assay is that of modified[75] method of Rafaelson.[76]
†Reported as cpm of $^{14}CO_2$ formed per g of tissue
††Reported as cpm of ^{14}C labeled glycogen per g of tissue

TABLE 3

Lack of Antagonist Effect of Beef Proinsulin on Biological Effect of Beef Insulin in the Isolated Fat Cells of Rat*

Preparation(s)	Concentration	Nanoatoms of Glucose-U^{14}C carbon converted to CO_2	Lipids
Control		42.5	83.3
Insulin	8.3×10^{-10} M	253.3	495.5
Insulin	4.7×10^{-11} M	174.0	355.0
Proinsulin	4.8×10^{-9} M	240.5	461.0
Proinsulin	4.5×10^{-10} M	177.7	363.5
Insulin plus Proinsulin	8.3×10^{-10} M 4.8×10^{-9} M	255.1	498.2
Insulin plus Proinsulin	4.7×10^{-11} M 4.5×10^{-10} M	226.0	428.8

*Data taken from Kitabchi.[25]

nation, on oxidation in the isolated fat cell. Table 4 summarizes similar data on antilipolysis using procine insulin and proinsulin. As can be seen from these tables, proinsulin, at 1/2 maximal concentration, has an additive effect on insulin but no inhibitory effect. Furthermore, at maximal concentrations of insulin, proinsulin exerts no inhibitory or stimulatory effect. To study the binding site of insulin and proinsulin in isolated fat cells, additional experiments which are reported in Table 5 were conducted in which insulin or proinsulin was first preincubated with the fat cells and then followed by the addition of the other material. These experiments gave essentially the same results as the experiments in Table 3, i.e., a lack of an inhibitory effect of proinsulin on insulin. In addition, these experiments demonstrated lack of pre-emptive binding by proinsulin or insulin to the fat cell receptor site. Lack of antagonistic effect of proinsulin on insulin has also been confirmed in vitro by Lavis and coworkers,[60] and in vivo by Kitabchi and coworkers.[75]

4. Mechanism of action of proinsulin on the target tissue – From the accumulated work so far reviewed, it appears that the action of proinsulin on various tissues in vivo and in vitro clearly parallels the action of insulin, since, so far, very few studies have indicated that proinsulin functions qualitatively differently from insulin, and even those differences may be due to the slower clearance of proinsulin. The primary difference appears to be the potency of the molecule which, as it was stated earlier, appears to be 5 to 20% as effective as insulin, depending on the type of tissue and species of proinsulin studied.

Therefore, it appears that the mechanism of action of proinsulin is similar to, or identical with that of insulin. Since the mechanism of action of insulin is, of course, unknown, a discussion of the various theories and the evidence for them are beyond the scope of this presentation. The

TABLE 4

Effect of Porcine C-Peptide on Corticotropin Induced Lipolysis of Isolated Fat Cell*

Compound	Concentration	Addition	Concentration	Lipolysis: nanomoles glycerole released/10^5 cells/hour
Baseline				
β1-24 corticotropin			0.15 μg/ml	10.4
+insulin			4×10^{-11} M	650.7
+insulin			1×10^{-9} M	215.9
+proinsulin			3×10^{-10} M	113.2
+proinsulin			1×10^{-8} M	257.9
+C-peptide			4×10^{-8} M	99.2
+C-peptide			4×10^{-7} M	612.8
				580.4
+insulin	4×10^{-11} M	proinsulin	3×10^{-10} M	
+insulin	1×10^{-9} M	proinsulin	1×10^{-8} M	112.3
+insulin	4×10^{-11} M	proinsulin	1×10^{-8} M	146.6
+insulin	4×10^{-11} M	C-peptide	4×10^{-7}	124.9
+proinsulin	3×10^{-10} M	C-peptide	4×10^{-7}	199.0
+proinsulin	1×10^{-8} M	C-peptide	4×10^{-7}	220.9
				133.9
Insulin			4×10^{-11}	
Insulin			1×10^{-9}	6.1
Proinsulin			3×10^{-10}	10.8
Proinsulin			1×10^{-8}	10.1
C-peptide			4×10^{-8}	13.5
C-peptide			4×10^{-7}	11.5
				7.4

*Experimental procedure according to Solomon et al.[61]

TABLE 5

Experiments on the Lack of Preemptive Binding by Proinsulin or Insulin on the Fat Cell Receptor Site

Addition	Nanoatoms of glucose-U-^{14}C converted into $CO_2/10^5$ cells/2 hr	
	experiment A†	experiment B‡
None (control)	63.9 ± 4.5	63.9 ± 4.5
Proinsulin (preincubate)* + insulin	376.1 ± 17.8	369.6 ± 2.7
Insulin (preincubate)* + proinsulin	304.7 ± 27.4	386.9 ± 31.7
Insulin + proinsulin	321.9 ± 22.5	370.3 ± 23.2
Insulin	271.5 ± 10.9	354.5 ± 24.6
Proinsulin	304.5 ± 7.7	368.4 ± 11.3

†Hormone concentrations used were at submaximal concentrations.
‡Hormone concentrations used were at maximal concentrations.
*Cells were preincubated for 5 min with the hormone designated prior to addition of the second hormone.

remarks below will be limited to factors which have been specifically shown to be applicable to proinsulin. As has been postulated for some time,[79,80] experimental data are now available indicating that the biological effects of insulin require only an interaction of insulin with the cell membrane. Work by Cuatrecasas, as well as by others, has shown that insulin, covalently bound to agarose beads, will produce biological effects in isolated fat cells,[81] isolated mammary cells,[82,83] and liver.[84] The large size of the insulin agarose derivatives clearly limits the contact of the insulin to the cell membrane, or at most a partial penetration of the cell by the insulin and, therefore, shows that interaction with superficial membrane structures alone may serve to initiate insulin-induced metabolic alterations.[85] Additional evidence that insulin reacts with the cell membrane has been shown by studies of the specific binding of insulin to, and the release from, cell membranes of fat cells[85,86] and liver cells.[87] The binding to fat cell membranes is saturable and reversible. No discernible change in the properties of the insulin occurs during the process of binding. Additional studies have shown that the tyrosyl, and possibly the histidyl residues, may be important in the binding.[88]

Related studies of proinsulin's interaction with the cell membrane are at present incomplete, but it has been shown that proinsulin will bind to the fat cell membranes approximately 1/20 as well as insulin. This degree of binding is consistent with the biological activity of proinsulin in isolated fat cells.[88] Thus, it appears that the decreased biological activity of proinsulin in comparison with insulin may be due to the decreased binding of the prohormone to the cell membranes of the hormonally sensitive tissue.[85,88]

Pancreatic Metabolism

Detailed reviews of the discovery of proinsulin and its role as the precursor of insulin are available.[14,22] The reader is referred to these reviews and to the original publications for comprehensive coverage of these topics. For the purpose of the present discussions, it will suffice to state that studies in vitro in human islet tumor tissue,[3,26] fetal rat pancreas,[28] and isolated islet of rat,[22] mice,[35] cod fish,[32] angler fish,[31] and calf pancreas[89] indicate that, with the use of radioactive amino acids in the incubation mixture, labeled proinsulin is the earliest radioactively-labeled material structurally related to insulin that can be detected. Glucose and mannose, but not fructose, stimulated the synthesis of labeled proinsulin.[90] No evidence has been found for the biosynthesis of peptide material with the exclusive characteristics of A or B chain.[35] Time studies indicate that endogenously labeled proinsulin is gradually converted via a double chain intermediate[89] to single component insulin. Proinsulin has been identified in the beta cells of the pancreas by fluorescein and peroxidase-labeled specific antibody.[91] In rats

which have two species of insulin[92] two forms of proinsulin have been found.[27]

It is, therefore, clear that in all species studied the double chain insulin molecule is formed from a single chain precursor, as was predicted on theoretical grounds several years before the actual discovery of proinsulin.[93,94] In the light of recent studies on the transformation of proinsulin to insulin, details pertaining to intrapancreatic events leading to insulin synthesis will be briefly discussed.

1. Intrapancreatic events leading to insulin biosynthesis – Although the elegant work of Steiner and co-workers has amply demonstrated that insulin is synthesized in the pancreas from a single chain precursor, the characterization and subcellular localization of the enzymes leading to the formation of insulin are not completely elucidated. It is, however, probable that the two processes, i.e., biosynthesis of insulin and intracellular transport, and release from the beta cells of the pancreas, are two entirely separate events, and are subject to different regulatory mechanisms. It appears that the site of synthesis of the newly formed insulin precursor (proinsulin) is on the ribosomes,[95] and that the newly formed precursor is transferred to cisternae of the rough endoplasmic reticulum.[96] The beta granules are then formed, either by the Golgi complex,[96] or by part of the endoplasmic reticulum which loses its ribosomes.[14] This pattern of secretory protein synthesis is very similar to the formation of the secretory protein of the acinar cells of the exocrine pancreas.[97,98] The converting enzyme which proteolytically cleaves proinsulin is located within either the Golgi elements or the secretory granules.[28] The products of proinsulin breakdown, insulin and C-peptide, are then stored in the beta granule until released into the circulation in equimolar concentration.[55] The release of these compounds in response to stimuli is achieved by the process of emiocytosis. Since the presence of proinsulin or proinsulin-like substance[44,77] has been demonstrated in circulation, it must be assumed that a certain amount of proinsulin is released from the pancreas by an as yet unknown mechanism. This assumption has been substantiated by the clear demonstration that proinsulin is secreted by the isolated rat islet in response to various stimuli,[99] and that proinsulin can be secreted by human tumor tissue.[26] Incubation of the islets with dibutyryl cyclic AMP(dc-AMP), theophylline, glucose, and tolbutamide all resulted in an increase in proinsulin in the incubation medium.[99] In addition, the percentage of proinsulin in the medium increased with incubation with various agents including dc-AMP. Further evidence that proinsulin is secreted by the pancreas was shown by Burr et al.[100] After 60 minutes' stimulation of a perifused rat pancreas with glucose, 18% of the total immunoreactive substance in the perifusate consisted of proinsulin while extracted rat pancreas had a proinsulin content of only 1 to 2%. In one experiment, proinsulin was detected in the perifusate after 30 to 40 minutes of glucose stimulation.

The difficulty in obtaining any appreciable amount of proinsulin from normal pancreas of rat, or freshly autopsied but unstimulated human pancreas, suggests that proinsulin does not constitute any major form of stored hormone in the pancreas. This has been demonstrated in human fetal pancreases in which proinsulin constituted 0.26 to 1.6% of their immunoreactive material.[66] The demonstration of the release of insulin and C-peptide in equimolar concentration[55] gives further support to this assumption.

In studies on spontaneously diabetic mice, the proinsulin content of the islets was shown to be similar to that of normal mice, although the insulin content was diminished. This resulted in an increase in the proinsulin:insulin ratio. No defect in the conversion of proinsulin to insulin could be detected.[101]

2. Proinsulin converting enzyme – Subcellular localization of a proinsulin converting enzyme has been reported by Sorenson et al.,[102] and Kemmler and Steiner.[103] Both studies suggested that the enzyme was membrane bound. The studies of Sorenson et al.[102] demonstrated that the secretory granule fraction of the rat islet was the most important fraction in converting proinsulin to the intermediate product and insulin. These authors estimated the time of conversion for proinsulin to the intermediate product to be about 10 minutes, and for the intermediate to insulin conversion to be about 30 minutes. Thus, these pulse-labeling studies suggested that the total time from synthesis of proinsulin until its final conversion was slightly less than one hour. The studies of Kemmler and Steiner,[103] also using rat islet with endogenously labeled proinsulin, demonstrated the presence of proinsulin converting enzyme in the secretory granules with

no conversion of labeled proinsulin in the microsomal or soluble protein fraction. Although the converting enzyme was not isolated and purified, studies on conversion of proinsulin to insulin showed that repeated freezing and thawing, as well as detergent treatment, strongly inhibited the reaction, whereas the addition of soy bean trypsin inhibitor did not alter the rate of transformation of the proinsulin. Additional studies into further fractionation of crude granules have not been successful; hence more precise localization of the converting enzyme will require better techniques for islet fractionations.

A partially purified enzyme which converts proinsulin to insulin from bovine pancreas has been reported by Yip.[104] Since this material was obtained from ammonium sulfate precipitate of pancreatic extract and did not contain insulin activity, the subcellular localization of the enzyme could not be determined. The enzyme was purified by ammonium sulfate precipitation, Sephadex G-50, Sephadex G-100, DEAE-cellulose and Sephadex G-50 chromatography. The purified enzyme exhibited a single band on polyacrylamide disc gel electrophoresis and had a molecular weight of about 70,000 with an isoelectric point at pH 4.82.

Further biochemical studies on the enzyme by Yip[104] suggested that the enzymatic conversion consisted of a two-step reaction – a fast step consisting of hydrolysis of proinsulin to an intermediate, followed by a slow cleavage of the intermediate to insulin. The connecting peptide was released intact since only arginine and no other amino acid was detected during hydrolysis.

Since free lysine was not found, this suggests that one of the products of this enzyme was C-peptide plus lysine or lysylarginine on the carboxyl end. This is not the form in which C-peptide is found in the pancreas;[22] therefore, it seems likely that different enzymes, or at least additional ones participate in the proinsulin to insulin conversion in vivo.[105]

The absence of carboxypeptidase B activity in the enzyme preparation, the demonstration of a single protein band by electrophoresis, the molecular size, and the data on the isoelectric point suggest that the converting enzyme studied by Yip[104] consists of a single enzyme and is distinguishable from pancreatic trypsin or trypsinogen. Because the enzyme was inhibited by KPTI, soybean, lima bean, and porcine pancreatic inhibitors, as well as diisopropylfluorophosphate, but not by TPCK, it suggests that the enzyme has tryptic characteristics.

In these same studies by Yip[104] it was suggested that although zinc inhibited the conversion of the intermediate to insulin, it had no effect on the conversion of proinsulin to intermediate. Furthermore, although incubation of proinsulin with the enzyme for 18 hours in the absence of zinc resulted in degradation of insulin to desoctapeptide insulin, the addition of zinc prevented such a degradation.

The mechanism by which zinc inhibits the conversion of intermediate to insulin and prevents degradation of insulin to desoctapeptide insulin is not known, but the combination of zinc with insulin may bring about conformational changes in the molecule to make insulin resistant to tryptic digestion. This hypothesis would be in accordance with the observations of Wang and Carpenter[106] on the hydrolysis of zinc-free insulin by trypsin, and of Frank and Veros[42] on the role of porcine proinsulin zinc complex in the conversion of proinsulin to insulin.

Furthermore, the stabilization of insulin molecule by a ligand (zinc) against proteolytic enzymes and the lack of zinc in proinsulin may favor the formation of insulin from its less active precursor, proinsulin.

Steiner and co-workers[22,35,43] have pointed out several times that proinsulin could theoretically be converted to insulin by a combination of carboxypeptidase B and trypsin. Recently his group demonstrated experimentally that under carefully controlled conditions in vitro these enzymes can produce a proinsulin to insulin conversion with the same by-products and apparently the same sequence of events as in vivo. They point out that much evidence has accumulated which argues against there being highly specific endodipeptidases in the pancreas specifically for the conversion of proinsulin to insulin.[105]

Extra Pancreatic Metabolism

1. Presence in plasma – The presence of a proinsulin-like material in human plasma was first described in 1968.[44,77] Proinsulin and proinsulin-like materials have also been identified in bovine and swine plasma.[54,107] Although prior separation by column chromatography is required, the ultimate determination and measurement of

proinsulin in plasma have been based on the radioimmunoassay.

Since the specificity of the immunoassay depends on a specific antiserum and an appropriate standard,[53] the measurement of proinsulin has been handicapped by the lack of human proinsulin. Most measurements of this material have been done by using antisera prepared by the injection of porcine insulin into the appropriate experimental animal and using porcine insulin on the standard. Even when porcine or bovine proinsulin has been used as the standard, the extrapolation to human proinsulin may not be valid because of the great species variability in the connecting peptide segment of proinsulin. Because of the variation in antisera and standards used by various groups of investigators, comparison of absolute levels of proinsulin obtained under various situations is difficult.

In spite of the difficulties discussed above, valuable information regarding the levels of proinsulin-like material in plasma has been obtained. First, it has been established that this material is present in normal sera. This was shown by fractionating plasma on a molecular sieve column, either Sephadex G-50 or Biogel P-30, into two immunoreactive components, a larger molecular weight one and a smaller molecular weight component. The latter of these corresponds to insulin. The larger molecular weight material has been shown to have many of the characteristics of proinsulin and is, therefore, considered to be proinsulin or proinsulin-like material. It has an elution pattern similar to ^{125}I-proinsulin, is converted to desalanine insulin by trypsin as is proinsulin, and has a biological activity similar to proinsulin.[108,109] It is not, however, absolutely certain that this larger molecular weight component always consists entirely of proinsulin. For example, a human intermediate compound resembling beef intermediate I or II[35] could not be separated from proinsulin by the above column fractionations.

The column fractionation technique followed by immunoassay of the fraction has also been used to demonstrate fluctuation in plasma proinsulin after various secretagogues have been administered. In normal young subjects it has been shown that the level of proinsulin is low shortly after the administration of oral glucose and rises later.[105,107] This results in an early, relatively low percentage of proinsulin, and then an increase in percentage after several hours, both due to the decrease in insulin levels and to the rise in proinsulin. In obese subjects, most of whom were glucose intolerant, the levels of proinsulin have been higher than in the normal subjects and there has been less proinsulin change after the oral glucose.[108,110] This resulted in a higher early percentage of proinsulin in obese subjects as compared to normal subjects which later became of equal magnitude in both groups of subjects. Acromegaly and starvation were also associated with some increases in proinsulin.[110] Patients with myotonic dystrophy had proinsulin levels indistinguishable from normal subjects.[111]

Stimulation with tolbutamide has shown the same type of pattern as with oral glucose, i.e., low levels early with a later rise.[110] The combination of glucose and tolbutamide has resulted in very high levels of proinsulin but there was no increase in percentage since the insulin levels also were very high.[112] The maximal percentage of proinsulin in normal or diabetic subjects, as determined by column chromatography has, in general, been about 20%.[113]

Insulin producing tumors have been associated with the highest levels of proinsulin.[110,112-115] In one patient with elevated immunoreactive insulin levels and an insulinoma, 77% of the material was proinsulin.[115] In a large series of patients with islet cell adenomas, 9 of 11 patients had elevations of proinsulin ranging from 28 to 89% of the total immunoreactive material.[112] Not all beta cell adenomas or carcinomas, however, had increased proinsulin levels. The relationship of the high proinsulin levels to the hypoglycemia in the patients is not clear since one patient treated with streptozotocin for a malignant islet cell tumor had an improvement in hypoglycemic symptoms associated with a decrease in proinsulin levels, although the total immunoreactive material level remained essentially unchanged.[116] This is of interest since elevated proinsulin levels have been found in two otherwise normal subjects who developed mild hypoglycemia during glucose tolerance tests.[117] The significance of this is not clear, since other hypoglycemic subjects have not had increases in their proinsulin levels. Recently, studies were reported on fasting levels of proinsulin-like materials in patients with elevated immunoreactive insulin values.[114] These authors found the proinsulin component to comprise 20% of the immunoreactive levels in subjects without

tumors, and from 26 to 79% in patients with islet cell tumors.

Although the determination of proinsulin by means of column fractionation followed by immunoassay of the fraction has yielded much important information, the difficulty of this method makes it impractical for studying large numbers of samples from many different patients. The ideal method for measuring proinsulin would be one which is specific for proinsulin and which does not require separation of proinsulin from other materials in plasma. Such a procedure is not available and at the moment does not seem practical. A specific immunoassay is unlikely, since plasma contains both insulin and C-peptide, and antisera to proinsulin would cross react with one of these materials. For the moment, therefore, it will continue to be necessary to immunoassay the proinsulin after separating it from insulin.

At present, the simplest method for separating proinsulin from insulin is by the enzymatic method established in our laboratory.[52] This method consists of degrading the insulin to nonimmunoassayable components by incubating the plasma with a proteolytic enzyme, insulin specific protease (ISP) (see below), which specifically degrades the insulin without affecting the proinsulin (Figure 20).[118] The details of the method, comparison of it to more complicated column methods, and validity of it under various physiologic methods have been described elsewhere.[52]

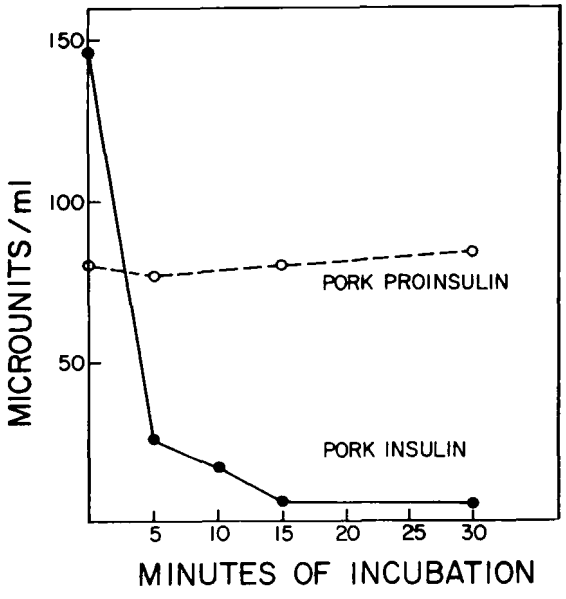


FIGURE 20. Comparison of the degradation of pork insulin and proinsulin by a purified preparation of insulin specific protease from rat skeletal muscle. Conduction and method of assay are according to Kitabchi et al.[52]

A similar type of approach to the question of abnormal circulating immunoreactive insulin-like material was made in 1965,[119] even before the discovery of proinsulin. These workers found a material circulating in plasma of juvenile diabetics which was poorly degraded by insulinase. This material subsequently has been postulated to be proinsulin.[120]

Since plasma is now known to contain two components, insulin and proinsulin, which react with the antisera to insulin, removal of the insulin by ISP allows proinsulin to be measured with insulin antisera. Proinsulin antiserum is not used since ISP does not degrade C-peptide and this material would, therefore, interfere. With this method the determination of proinsulin is no more difficult or time consuming than the measurement of the total immunoreactive material; therefore, large series of patients can be studied for small variations in the levels of proinsulin. Using this method, we have demonstrated nondegradable material (i.e., proinsulin-like material) in the plasma of normal subjects and have shown fluctuations in these levels after oral glucose.[52] We have also shown high levels of proinsulin in the plasma of a patient with an insulinoma and demonstrated that these levels fall after removal of the tumor. Availability of this method has allowed us to study proinsulin values of large numbers of subjects, including obese and lean diabetics. We have shown a correlation between the amount of proinsulin-like material and both the degree of carbohydrate intolerance and the degree of obesity.[121] The details of these studies will be reported elsewhere.[122]

2. Conversion of proinsulin to insulin – As discussed previously, the major site of conversion of proinsulin to insulin is in the pancreas.[21] It has been suggested that proinsulin may be converted to insulin outside the pancreas and that this may explain the biological activity of proinsulin.[58] One group has suggested that this conversion might occur in the liver, since eviscerated animals had no response to injected proinsulin.[72] Another group,[68] however, showed a good response to proinsulin in eviscerated animals. In support of the peripheral conversion of proinsulin, Mashiter and King[123] reported that incubation of proinsulin

with tissue homogenates resulted in a change in immunoreactivity suggestive of conversion to insulin followed by degradation of the insulin. Isolation and characterization of the conversion products were not reported. Our own studies do not show this pattern of proinsulin degradation by peripheral tissue, however, and we, as well as others, have demonstrated that proinsulin has intrinsic biological activity.

Other workers have shown that labeled proinsulin injected into rats can be recovered unchanged as long as three hours after injection. The recovered material has all the properties of proinsulin.[63] In addition, there was no evidence of conversion of proinsulin to insulin in isolated liver perfusion system.[65] On the basis of this information, the peripheral conversion of proinsulin to insulin must be considered unlikely, although the question is not resolved and deserves additional investigation.

3. **Proinsulin degradation** – The mechanism by which insulin is degraded to inactive substances has long been a matter of great interest. Two major systems have been identified which will inactivate this hormone. One of these is the insulinase system described by Mirsky.[124] This system was considered to be a group of enzymes which proteolytically degrade insulin with considerable specificity. Insulinase has been described in many different organs, with the liver system, perhaps, the most active and best characterized. The Km for the liver enzyme was found to be 0.06 μM, which is well within the physiological range.[124]

The other major insulin inactivating system has been an enzyme called glutathione insulin transhydrogenase (GIT).[125] This enzyme, which is found in the liver, splits insulin into A and B chains by the reductive cleavage of the disulfide bonds with transfer of hydrogen from glutathione to insulin.[126] GIT has been prepared by solubilizing with detergent[127] or acetone[128] and has been highly purified. It has a relatively high Km (43 μM) and is nonspecific since it will also reduce other disulfide bonds such as are present in vasopressin and bovine albumin.[126,129] For these reasons it has been felt that GIT may be less important physiologically than more specific insulin degrading enzymes.

Insulin degradation has also been shown in fat tissue and some specificity for this action has been described, although part of the degradation is by nonspecific peptidases.[130] Insulin destruction has been seen in both the lipid fraction[131] and in the particulate fraction[132] of fat tissue homogenates. The physiological significance of these different degradative systems in fat requires much additional clarification.

In 1968, Brush,[118] working with a partially purified rat skeletal muscle soluble enzyme, found that this enzyme specifically degrades insulin, but not proinsulin, ACTH, growth hormone, or albumin. Studies of biochemical properties of this enzyme demonstrated that the enzyme was located in the 100,000 x g supernatant fraction, had a physiologic pH optimum and a physiologic Km, and degraded insulin by proteolysis since the product of insulin interaction with the enzyme was ninhydrin-positive material.[118,133] Because of this high specificity for insulin degradation, the enzyme was named insulin specific protease (ISP). Since the major bulk of insulin is said to be metabolized by liver, Burghen et al.,[134,145] in our laboratories, investigated in detail the nature of degradation of insulin and proinsulin in the sucrose homogenate and various subcellular fractions of the liver, as well as in purified ISP. By fractional centrifugation of whole liver homogenate, the insulin degrading enzyme system of Mirsky was separated into four fractions (debris, mitochondrial, microsomal, and supernatant), and insulin and proinsulin degrading ability of the fractions was tested in the presence and absence of glutathione. Of the four fractions, the soluble portion was found to be responsible for 96% of the total insulin degrading activity. The only fraction which was stimulated by glutathione, and presumably contained GIT, was the microsomal fraction which, even after glutathione addition, has less than 2% of the total insulin degrading activity. The Km for purified liver ISP was found to be 0.1 μM, which is similar to the Km of Mirsky's insulinase of 0.06 μM and in marked contrast to that of liver GIT which has a Km of 43 μM.

In comparison with insulin, much less is known about the peripheral metabolism of proinsulin. Conversion of proinsulin to insulin outside the pancreas is thought to be unlikely for the reasons discussed above. The liver appears to be of little importance in the disposition of proinsulin, while approximately 50% of insulin injected into the portal system of humans is cleared by the liver in one circulation.[136] In isolated rat liver perfusion,

the immunological halflife of insulin was 17 minutes. In contrast, there was no clearance by this system of either proinsulin or C-peptide.[137]

The kidney may be of more importance in the disposition of proinsulin since it has been reported that the arteriovenous difference of proinsulin across the dog kidney was 23 to 50%.[19] In addition, both insulin and proinsulin are present in human urine.[77] Studies on the degradation of proinsulin in tissue are very limited. Muscle ISP has very little proinsulin degrading ability,[118] and proinsulin degradation by liver ISP occurs at less than 10% of the rate of insulin.[135] GIT will inactivate proinsulin, but at a slower rate than insulin. It is also of interest that GIT will reactivate "scrambled" proinsulin.[138]

Challoner,[139] studying insulin and proinsulin degradation in fat, found that insulin and proinsulin degradation to TCA soluble material was similar in fat cells after two hours of incubation. The possibility must be considered that the residual crude collogenase which is used in preparation of fat cells could have contributed to nonspecific protease activity and degradation of proinsulin in fat cell preparation. In whole fat pieces, however, insulin degradation was approximately twice as much as that of proinsulin even after two hours. Homogenization of the fat pieces increased degradation of both insulin and proinsulin, but homogenization of the fat cells had little effect. The mechanism for the significant difference in degradation of proinsulin in isolated fat cells vs. fat pieces needs further clarification.

In order to investigate the contribution of each major organ to the degradation of insulin and proinsulin, Stentz, in our laboratories, has studied the ability of various organ homogenates of rats to degrade insulin and proinsulin. The homogenates of liver, pancreas, brain, heart, diaphragm, spleen, lung, kidney, fat, and testes were fractionated into 100,000 x g supernatant and precipitate. Disappearance of proinsulin or insulin at 0, 2, 5, 10, and 15 minutes was measured by the immunoassay procedure, and the rate of degradation on the first order kinetics was calculated from this. Table 6 presents the summary of such data on the average of 4 experiments from 14 rats. These studies demonstrate that (a) greater than 95% of insulin degrading activity in all organs is located in the postmicrosomal fraction, (b) proinsulin degradation, with the exception of liver, is 1/10 as much as insulin, and (c) the most active organs for degradation of proinsulin are liver, kidney, testes, and spleen, respectively.

Thus it appears that an insulin specific protease system is present in all organs studied and may be the major enzyme system for degradation of insulin. Since the protease is a soluble enzyme, it suggests that the intracellular degradation of insulin and possibly proinsulin is the major route for degradation for these hormones. This agrees well with the investigation of Cuatrecasas et al.[140] of

TABLE 6

Degradation of Insulin and Proinsulin in Various Organ Homogenates of Rats

	Degradative activity†			
	Supernatant		Precipitate	
Tissues	Insulin	Proinsulin	Insulin	Proinsulin
Adipose tissue	1.7	0.2	0.02	0.0001
Brain	5.9	0.6	0.06	0.007
Muscle	8.0	1.0	0.28	0.011
Heart	11.5	1.0	0.12	0.008
Lung	13.0	1.6	0.32	0.013
Spleen	25.0	2.0	0.14	0.008
Kidney	26.6	5.0	0.28	0.014
Testes	26.6	2.0	0.18	0.010
Pancreas	27.2	5.0	0.34	0.016
Liver	53.8	5.0	0.28	0.012

†Expressed as picomoles of substrate (insulin or proinsulin) destroyed per g of tissue per min.

the insulin-receptor interaction of liver and cell membrane where insulin binding site (membrane) is not associated with the degradation of insulin.

C-PEPTIDE

As stated in the earlier part of this review, the C-peptide consists of the connecting peptide with one basic dipeptide removed from each end of the connecting peptide. C-peptides have been isolated from pancreatic tissues of most species and have also been isolated from crystalline proinsulin after hydrolytic action of trypsin. The amino acid sequences of bovine,[35] porcine,[9] and human C-peptide[33] have been elucidated and are presented in Figure 4.

1. Chemical properties – C-peptide is extracted by acid ethanol along with insulin, but unlike insulin it is soluble in 15% NaCl because of its more polar nature. Recently, Markussen, Sundby, Smyth, and Ko[141] have reported the isolation of C-peptide from human pancreas by the use of successive acetone precipitation, SE-Sephadex G-50 cation exchange chromatography, and ammonium acetate elution by which 90% of the protein was kept on the column, but C-peptide was eluted readily. The eluate of the cation exchange column was then placed on a column of QAE-Sephadex A-25 and eluted with a linear gradient in acetic acid from pH 5.0 to 4.0. The C-peptide was located in each fraction by measuring extinction at 276 mμ and by the Folin-Ciocalteu reaction. The lyophilized pooled fraction from this column was then placed on a Sephadex G-25 Column and eluted with 0.1M acetic acid. The C-peptide was eluted at approximately one half of the bed volume. After elution, it was then lyophilized, dissolved in water, and placed on SE-Sephadex G-50 cation exchange column for final purification. The final yield of C-peptide was 44.5 mg from 40 g of acetone precipitate which had been obtained from 50 kg of human pancreas. The ratio of Folin-Ciocalteu to UV-extinction increased from 0.14 (2nd column) to 2.3 (3rd column) and to 7.9 (4th column), whereas the amounts of peptide decreased by factors of 10, 14, 3, and 2 for the columns respectively. Beef and pork C-peptides were also obtained from beef and pork pancreas and purified by Sundby and Markussen.[142] These C-peptides were identical in amino acid structure to the C-peptides obtained by chemical cleavage of the respective proinsulins. The preparative procedure used for the beef and pork C-peptides was similar to that used for the human material.

Steiner et al.[143] have also recently isolated purified C-peptide from beef pancreas. Their method of purification, however, differs from that of Markussen et al.[141] for the purification of human C-peptide. The acid ethanol extract of the beef pancreas was further treated with ethanol ether (pH 5.3), and the precipitate (dissolved in acetic acid) was chromatographed successively on Sephadex G-50, Biogel P-30, and CM-cellulose. These procedures were followed by desalting and electrophorising in 30% formic acid, and again in 1.2 M pyridine acetate. The final purification was obtained by cellulose column chromatography. The amino acid sequence of bovine C-peptide is presented in Figure 4.

2. Immunologic properties – Since C-peptide does not cross react with insulin antiserum, the C-peptide of pork, beef or human is not immunoreactive in the insulin immunoassay system (Figures 12 and 13), and therefore does not interfere with the routine determination of insulin in plasma or other tissues. Up to the present time, the only material in plasma which has been proven to interfere with the immunoassay of insulin as originally described[144] is proinsulin.[113] However, since C-peptide contains the same antigenic determinants as the proinsulin molecule, it does interfere with the proinsulin immunoassay using proinsulin antiserum with unfractionated plasma. Prior purification of plasma, therefore, on a column is necessary to separate proinsulin from C-peptide. To date, no specific method of immunoassay for C-peptide is available.

Melani and co-workers[45] have recently developed an immunoassay for human C-peptide which is not affected by insulin but which does react with proinsulin. They prepared antiserum in guinea pigs against human C-peptide which had been covalently bound to rabbit serum albumin using 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride. Human proinsulin and C-peptide competed with tyrosylated human C-peptide labeled with ^{131}I for binding to this antiserum. Human proinsulin was approximately two thirds as active as C-peptide with this assay on a molar basis. Human insulin, as well as pork and beef insulin, proinsulin, and C-peptide, did not bind to this antiserum. Since human proinsulin reacts with this antiserum, the use of this immuno-

assay for determining C-peptide in plasma requires separation of these materials by gel filtration prior to immunoassay. By following this procedure these workers confirmed that C-peptide and insulin were present in equimolar concentrations. This finding is consistent with the concept of proinsulin as a biosynthetic precursor of insulin.[20]

3. **Biological properties** – Although C-peptide appears to be a by-product of proinsulin degradation in the pancreas and is demonstrated in equimolar concentration with insulin in circulation, no definite biologic effect has been attributed to it other than its role in the biosynthesis of insulin. Kitabchi,[25] using isolated fat cells of rats, demonstrated the lack of a stimulatory effect of porcine or bovine C-peptide on glucose-U-^{14}C conversion to $^{14}CO_2$ or lipids. Similarly, Solomon et al.[61] demonstrated the lack of an antilipolytic effect by porcine and bovine C-peptide on ACTH or cyclic nucleotide-induced lipolysis in isolated fat cell.

Lack of biologic effect of C-peptide on the isolated fat cell was also confirmed by Gliemann and Sorensen.[57] Since the possibility existed that some C-peptides may regulate the activity of insulin and proinsulin, Kitabchi conducted a series of experiments where antagonistic or potentiating effect of C-peptide was studied in the isolated fat cells of rats in combination with beef insulin, proinsulin, or intermediates at submaximal and maximal concentrations. These studies[25] demonstrated conclusively that bovine C-peptide on the isolated fat cells of rats (a) has no insulin-like effect, (b) does not antagonize the effect of insulin, proinsulin or intermediates, and (c) does not potentiate or stimulate biologic effect of insulin, proinsulin, or intermediate at maximal or submaximal concentrations. However, due to a large sequence variation of C-peptide among various species, it has been implied that C-peptide might exhibit a regulatory function if homologous rather than heterologous tissue were used.[19] To investigate this possibility, Yu and Kitabchi studied the comparative effect of bovine (Table 7) and porcine (Table 8) C-peptides with insulin and proinsulin on adipose tissue of beef and pork, as compared to isolated fat cells of rat. Furthermore, the effect of the combination of C-peptide of beef or pork in combination with homologous and heterologous insulin and proinsulin was studied on porcine fat pieces, and compared to fat cells of rats (Table 9). Again, these studies clearly demonstrate that C-peptide has no regulatory mechanism on heterologous or homologous tissues and, furthermore, that C-peptide, in as much as ten times the concentration used for insulin, neither exhibits any insulin-like activity on the fat tissue of homologous or heterologous species, nor does it exhibit an antagonistic or synergistic effect on insulin or proinsulin. Similarly, the lack of an antilipolytic property of porcine C-peptide on the rat fat cell, alone or in combination with insulin

TABLE 7

Comparative Effect of Bovine Insulin, Proinsulin, and C-Peptide on Glucose-U-^{14}C Incorporation into $^{14}CO_2$ of Beef, Pork, and Rat Fat Tissues

Preparation	Concentration	Rat fat cell* nanoatom of $^{14}CO_2$ released/ 10^5 cells/2hr	Bovine fat pieces† nanoatom of $^{14}CO_2$ released/ g tissue/2 hr	Porcine fat pieces nanoatom of $^{14}CO_2$ released/ g tissue/2 hr
Baseline		40.1	420.5	631.5
Insulin	5×10^{-11} M	183.2		
Insulin	1.0×10^{-10} M	222.0	586.0	1152.4
Insulin	2.0×10^{-9} M	257.6	865.5	1502.6
Proinsulin	3×10^{-10} M	155.2		
Proinsulin	1×10^{-9} M	243.2	584.7	1070.9
Proinsulin	2×10^{-8} M	258.3	804.0	1571.0
C-peptide	4×10^{-8} M	40.2	430.5	681.9
C-peptide	4×10^{-7} M	40.2	420.0	657.9

*Data taken from Kitabchi.[25]

†Method of assay is similar to that of Bray.[154]

TABLE 8

Effect of Porcine Insulin, Proinsulin and C-Peptide on Glucose-U-^{14}C Conversion to $^{14}CO_2$ on Bovine, Porcine, and Rat Fat Tissues

Preparation	Concentration	Rat fat cells† nanoatom of $^{14}CO_2$ released/ 10^5 cells/2 hr	Bovine fat pieces* nanoatom of $^{14}CO_2$ released/ g tissue/2 hr	Porcine fat pieces* nanoatom of $^{14}CO_2$ released/ g tissue/2 hr
Control		40.1	420.5	631.5
Insulin	4×10^{-11} M	142.9		
Insulin	1×10^{-10} M	210.5	705.1	1312.1
Insulin	2×10^{-9} M	234.8	904.5	1555.3
Proinsulin	3×10^{-10} M	140.23	599.9	945.4
Proinsulin	1×10^{-9} M	227.3		
Proinsulin	2×10^{-8} M	242.4	754.3	1479.4
C-peptide	4×10^{-8} M	42.6	414.8	684.0
C-peptide	4×10^{-7} M	43.4	454.2	665.0

†Conditions and method of assay are according to Kitabchi.[25]
*Method of assay is similar to that of Bray.[154]

TABLE 9

Studies on the Effect of C-Peptide on the Biological Activities of Insulin and Proinsulin

Preparation	Concentration	Rat fat cells† nanoatoms of $^{14}CO_2$ released/ 10^5 cells/2 hr	Porcine fat pieces* nanoatoms of $^{14}CO_2$ released/ g tissue/2 hr
Baseline		42.5	631.5
Porcine C-peptide	4×10^{-7} M		665.0
Bovine C-peptide	8.3×10^{-10} M	39.3	
Porcine insulin	1×10^{-10} M		1312.1
Bovine insulin	8.3×10^{-10} M	253.3	
Porcine proinsulin	1×10^{-9} M		945.4
Bovine proinsulin	4.8×10^{-9} M	240.5	
Bovine C-peptide + bovine insulin		242.1	
Porcine C-peptide + porcine insulin			1303.2
Bovine C-peptide + bovine proinsulin		223.7	
Porcine C-peptide + porcine proinsulin			955.6

†Data taken from Kitabchi.[25]
*Method of assay is according to Bray.[154]

and proinsulin, was demonstrated and is presented in Table 4.

It would thus appear unlikely that C-peptide exerts any significant biologic effect on insulin sensitive tissue such as adipose tissue. Whether or not it exerts any effect on other tissues such as muscle and liver is not known, but appears unlikely.

In the light of the above findings, one is hard pressed to assign any physiologic role for C-peptide in circulation, although one interesting speculation has been advanced.[138] This is based on the observation that GIT reactivates "scrambled" insulin in the presence of C-peptide but not in its absence. "Scrambled" insulin is insulin which is reduced and then reoxidized and, therefore, contains randomized "incorrect" disulfide bands. The reactivation of "scrambled" insulin by GIT suggests that C-peptide might exert some stabilizing effect on the double chain insulin in circulation. This speculation would, of course, require much additional experimentation before its acceptance.

A practical clinical use for the measurement of C-peptide has also been described.[145] By the use of the C-peptide immunoassay, this material can be used as an index of circulating endogenous insulin in diabetic patients who receive insulin treatment. Since commercially prepared insulin does not contain C-peptide, the levels of this material reflect endogenous insulin release.

POLYPEPTIDES RELATED TO PROINSULIN

The fact that commercially available insulin of various species contains more than one protein component with biological activity was reported by Mirsky and Kawamura in 1966.[146] Using polyacrylamide gel electrophoresis, these investigators demonstrated heterogeneity of commercially available insulin from many pharmaceutical companies. Furthermore, they showed that the multiple fractions were not due to contamination with glucagon, nor to artifacts of the purification process. Bioassay of the bands of commercially prepared beef insulin showed all fractions to have biologic activity by their hypoglycemic action in rabbits when injected in amounts equivalent to 0.1 U/kg body weight as computed from the immunoassay. Because of the fact that each band was both biologically and immunologically active and the bands on further purification did not disappear, Mirsky and Kawamura suggested that these bands may reflect the presence of molecules of insulin in the pancreas with different primary or tertiary structures. With the discovery of proinsulin as the biosynthetic precursor of insulin,[3] it became quite plausible that the heterogeneity of commercial insulin preparation could be partially due to the presence of proinsulin and other minor intermediate components, since proinsulin and various intermediate forms have been reported to be present within the beta cells of the pancreas of numerous species.[3,30,32]

Although the presence of insulin, proinsulin and C-peptide has been demonstrated in circulation,[44,45,77] unequivocal proof for the existence of proinsulin intermediates in normal human circulation is lacking. Part of the reason for this may be that the proinsulin intermediates, if indeed they do exist in circulation, cannot be separated and distinguished from proinsulin by the procedures usually used for separation of insulin and proinsulin in plasma (i.e., molecular sieve chromatography) because of the similarity of molecular weight and ionic charges between proinsulin and intermediates.

Due to the potential physiological significance that these intermediates might have in pathological conditions, as well as their importance as a tool to study structure-function relationship in regard to insulin and its action, a brief discussion of these molecules will follow.

1. Bovine intermediates – The detailed procedure for the isolation and determination of the chemical properties of beef[35,43] and pork[24] intermediates is published elsewhere, and the reader should refer to these original articles for specific information. In general, purification of beef proinsulin intermediate was accomplished by chromatography of crude proinsulin on a column of CM-cellulose. The two fractions obtained were then each chromatographed on a column of DEAE-cellulose. Chromatographic fractions were then pooled, acidified with acetic acid to a concentration of 1 N, passed through a column of Sephadex G-25 in 1 N acetic acid, concentrated, and then diluted with water and lyophilized. This fraction, which is known as proinsulin "intermediates," consists of intermediate I (40%) and intermediate II (60%). Separation of these intermediates into two distinct isolated peaks (inter-

mediates I and II) by prior chemical treatment has now been accomplished.[147] The biological properties of these two intermediates (Figures 10 and 11) have been studied in our laboratories and will be reported below.

2. **Porcine intermediates** – The summary of the chemical properties of intermediate fractions of porcine proinsulin has been given earlier in the section under nomenclature. For detailed discussions of these properties the reader is referred to the original articles by Chance.[24]

3. **Immunological properties** – Immunological interrelation of bovine proinsulin and its related fractions is described in detail by Rubenstein et al.[48,49] All of these fractions appear to react with insulin antisera to varying degrees depending upon the molecular structure of the compound tested, as well as the different antisera used. In general, proinsulin and its convertible fraction reacted more strongly with antiserum to purified proinsulin. The order of reactivity was reversed in favor of insulin when an insulin antiserum was used.

By the use of guinea pig antiserum to porcine insulin we have compared immunoreactivity of insulin and proinsulin of human, porcine, and bovine. These results are depicted in Figure 12. As can be seen, insulin molecules of the three species have considerably higher reactivity with porcine insulin antisera than their corresponding proinsulin molecule.

Chance studied the immunologic reactivity of porcine proinsulin intermediates, as well as insulin and related polypeptides, to both porcine insulin antisera and purified porcine proinsulin antisera.[24] These results are summarized in Table 10. As can be seen, immunoreactivity of proinsulin to insulin antisera is 45% as compared to single component insulin, but this immunoreactivity is increased with desdipeptide and desnonapeptide proinsulin. Proinsulin and related polypeptides, however, all exhibit approximately the same degree of immunoreactivity against proinsulin antisera with the exception of desdipeptide, which shows 84% of the reactivity. The purity and specificity of proinsulin antisera in this study were demonstrated by the lack of cross reactivity of insulin, or insulin-like components, to porcine proinsulin antisera.

Immunologic studies in our laboratory with various fractions of porcine insulin, proinsulin, and related polypeptides against porcine insulin antiserum are depicted in Figure 13. As can be seen from this figure, the three closely related molecules of proinsulin, split proinsulin, and desdipeptide proinsulin exhibit similar degrees of immunoreactivity with insulin antisera, whereas desnonapeptide and destridecapeptide show greater degrees of immunoreactivity than the above mentioned compound. As expected, single component insulin and its related polypeptides show the highest immunoreactivity to porcine insulin anti-

TABLE 10

Biological and Immunological Properties of Polypeptides of Insulin and Proinsulin*

Compounds	Biological activity mouse convulsion assay (equimolar)	Immunological reactivity	
		to insulin antisera	to proinsulin antisera
Proinsulin-like			
Proinsulin	18%	45%	100%
Split proinsulin	20%	45%	107%
Desdipeptide	58%	61%	84%
Desnonapeptide	62%	61%	107%
Insulin-like			
Diarginine insulin	62%	80%	0%
Monoarginine insulin	66%	67%	0%
Single component insulin	100%	100%	0%
Monodesamido insulin	98%	80%	no data

*Data taken from Chance.[24]

serum. C-peptide shows no immunoreactivity to this antiserum.

4. Biological activity of intermediate fractions of insulin and proinsulin – Relatively little work has been done on the biological effect of the polypeptides of insulin and proinsulin. One reason for the paucity of data has been the difficulty in obtaining highly purified preparations. As stated earlier, however, the advantages of such studies would be twofold. First, since these intermediates may be obligatory components of the proinsulin to insulin conversion, it is possible that they could be present in the circulation. In such a case it would be of great interest to know the contribution of these materials to the biological activity of the circulating immunoreactive materials. Second, these compounds would also be useful as models to study structure-function relationships of insulin and proinsulin in a biologic system.

Chance,[24] in his review of the biological activity of proinsulin related polypeptides, examined the in vivo biological effect of proinsulin-like and insulin-like polypeptides by the mouse convulsion assay and concluded that in comparison to proinsulin or split proinsulin, desdipeptide and desnonapeptide have 3 times more biological activity than proinsulin, but exhibit only 60% as much biologic activity as that of single component insulin (Table 10). Similar results on a molar basis were apparently obtained by Toomey, as quoted by Chance,[24] for desnonapeptide on an isolated fat cell assay.

In 1970, Kitabchi[25] studied the comparative biologic effect of beef insulin, proinsulin and a mixture of intermediates I (40%) and II (60%) on glucose-U-^{14}C conversion to CO_2 and lipid in the isolated fat cell. These experiments demonstrated that (a) on a molar basis, proinsulin is 1/15 as effective as insulin, (b) the mixture of intermediates corresponding to long A chain and long B chain has biologic activity between insulin and proinsulin, (c) the intermediate fraction has no inhibitory effect on the biological effect of insulin or proinsulin, and (d) this fraction does not influence biologic inactivity of C-peptide. Based on these findings, he proposed that if proinsulin produces its insulin-like effect by combining with the same receptor as insulin, then it is evident that the presence of the connecting segment considerably weakens the binding of proinsulin in comparison with insulin.

Whether this effect was due to the blockage of the amino end of the A chain or the carboxy end of the B chain, or both, or was due to other factors such as steric hindrance, could not be unequivocally decided from the data presented, since the increased activity of the intermediate fractions in comparison with proinsulin could have been due either to the presence of intermediate I or II, or both. Availability of purified intermediate I and II of beef, as well as polypeptides of porcine proinsulin, prompted us to examine these possibilities by the following three parameters: (a) biological activities on the isolated fat cells of rats by measuring glucose-U-^{14}C conversion to $^{14}CO_2$ and lipid, as well as antilipolytic properties of these molecules against ACTH-induced lipolysis, (b) ability of these chemicals to act as substrates for insulin specific protease by measuring the diminution of immunoreactivity of these substrates at various time intervals using a modified[52] double antibody immunoassay method[148] with insulin antiserum, and (c) measurement of immunoreactivity of these compounds against 1:300,000 dilutions of porcine insulin antisera.

5. Effect of polypeptides on glucose oxidation – All glucose oxidation studies presented in Tables 11 and 12 are reported as nanoatoms of glucose carbon converted into CO_2 or lipids/100,000 cells/2 hr. Similar results were also obtained on the antilipolytic properties of these compounds but the details of these studies will be reported elsewhere. Summary of one series of studies on glucose oxidation is reported in Table 11. For calculations of the biological activity of each compound, at least ten different concentrations of that compound were used for each experiment and the results of three to four experiments were plotted on a semilogarithmic graph for each series. A sigmoid concentration response curve was obtained for each compound from which half-maximal response concentrations were calculated and compared to other compounds tested, with insulin response being taken as 100%.[149] As can be seen from Table 11, the results of in vitro studies on the isolated fat cells are qualitatively similar to those reported by Chance and summarized in Table 10, i.e., diarginine and monoarginine insulin both have diminished activity compared to insulin, but both are less active than is reported by Chance in the in vivo studies.[24]

Our studies on the polypeptides of porcine proinsulin suggest that desdipeptide and split proinsulin have slightly increased biological

TABLE 11

Biological Activity of Polypeptides of Porcine Insulin and Proinsulin in Isolated Fat Cells†

Compounds	Glucose conversion into CO_2		Glucose conversion into lipids	
	1/2 maximal concentration	% activity	1/2 maximal concentration	% activity
Insulin	3.50×10^{-11} M	100%	3.40×10^{-11} M	100%
Monoarginine insulin	9.00×10^{-11} M	38.9%	8.45×10^{-11} M	40.2%
Diarginine insulin	9.20×10^{-11} M	38.0%	8.70×10^{-11} M	39.0%
Desalanine insulin	3.65×10^{-11} M	95.9%	3.42×10^{-11} M	99.4%
Proinsulin	3.00×10^{-10} M	11.6%	3.30×10^{-10} M	10.3%
Split proinsulin	2.50×10^{-10} M	14.0%	2.70×10^{-10} M	12.6%
Desdipeptide proinsulin	2.45×10^{-10} M	14.3%	2.60×10^{-10} M	13.1%
Desnonapeptide proinsulin	1.48×10^{-10} M	23.7%	1.45×10^{-10} M	23.4%
Destridecapeptide proinsulin	0.95×10^{-10} M	36.8%	0.92×10^{-10} M	36.9%

†Method of assay is according to Kitabchi.[25]

activity as compared to proinsulin, but further removal of amino acid peptides from the amino terminus of the A chain renders the molecule more active than proinsulin. Similar results were also obtained for antilipolytic properties of these compounds (data not shown). Studies of beef polypeptides which are reported in Table 12 are of special interest since the two intermediates of bovine proinsulin, i.e., long A chain and long B chain, may represent the most likely intermediates of proinsulin found in the bovine circulation. The studies summarized in Table 12 indicate that the biological activity of intermediate I (long B chain) is much higher than that of intermediate II (long A chain), the latter resembling proinsulin closely. Addition of I and II in the proportion originally isolated as "intermediates" by Steiner exhibited an additive effect of individual components. These studies suggest that a partial explanation for the demonstration of decreased activity of proinsulin as compared to insulin may be the blocking of the amino terminus of the A chain by connecting

TABLE 12

Biological Activity of Beef Insulin, Proinsulin and Intermediates in Isolated Fat Cells†

Compounds	Glucose-U-^{14}C conversion into CO_2		Glucose-U-^{14}C conversion into lipids	
	1/2 maximal concentration	% activity	1/2 maximal concentration	% activity
Insulin	3.60×10^{-11} M	100%	3.45×10^{-11}	100%
Proinsulin	3.10×10^{-10} M	11.6%	3.20×10^{-10}	10.78%
Intermediate I	1.10×10^{-10} M	32.7%	1.18×10^{-10}	29.2%
IntermediateII	2.10×10^{-10} M	17.1%	2.25×10^{-10}	15.3%
Intermediate I & II (40% and 60%)	1.33×10^{-10} M	27.0%	1.50×10^{-10}	23.0%

† Method of assay is according to Kitabchi.[25]

TABLE 13

Degradation of Polypeptides of Insulin and Proinsulin by Insulin Specific Protease of Rat Skeletal Muscle†

Compound	% degradation*
Proinsulin	6.2
Cleaved (split)	21.5
Desdipeptide	28
Desnonapeptide	69
Destridecapeptide	79
Insulin	100
Desalanine	102
Monoarginine	98
Diarginine	98

†Method of assay is according to Kitabchi et al.[52]
*Insulin degradation (0.4 picomole/min) is taken as 100%.

peptide. The deblocking of A terminus only partially reactivated the molecule but did not completely reverse the inhibition, while removal of additional amino acids from C-peptide segments further activated the proinsulin molecules (Table 11). This suggests that additional factors such as steric hindrance and conformational structure of the molecule may also play important roles in the interaction of molecules with receptor site of fat cells.

6. Substrate studies for insulin specific protease – As stated earlier, reports by Brush[118] of a specific enzyme which proteolytically degrades insulin, but not proinsulin, gave impetus to the development of a method in our laboratories for direct measurement of proinsulin in plasma. Aside from its clinical usefulness, this enzyme with its high specificity for insulin offered us an opportunity to examine its biochemical properties and binding site using different polypeptides of proinsulin or insulin. Brush, in earlier studies, investigated the ability of various insulin derivatives to act as substrate and showed that the removal of both amino or carboxyl terminal residues of insulin elevated Km five times, and removal of an octapeptide from the B chain did not alter the Km, suggesting the importance of the A chain of insulin for conformational shape of insulin and recognition site of insulin for ISP. These findings correlate well with the three-dimensional structure of crystalline insulin as described by Adams et al.[150]

Our studies summarized in Table 13 give the results of the initial rate of degradation of polypeptides of porcine proinsulin with purified ISP calculated per mg of protein. As can be seen, the rate of disappearance of the substrate is increased progressively, i.e., proinsulin < split proinsulin < desdipeptide < desnonapeptide < destridecapeptides.[151] The derivatives of insulin (diarginine, monoarginine, and desalanine) do not appear to show significantly different rates of degradation from insulin with ISP. In Table 14, the results of degradation studies of bovine insulin and proinsulin are compared to those of intermediates I and II, as well as to the mixture of I and II intermediates. As in the studies on the isolated fat cells in which intermediate I has more biological activity than intermediate II, intermediate I appears to be a better substrate for ISP than intermediate II. This supports our assumption that deblocking of the A chain somehow

TABLE 14

Degradation of Beef Insulin, Proinsulin, and Intermediates by Insulin Specific Protease of Rat Skeletal Muscle†

Substrate(s)	Rate of distruction picomole/min	% activity
Insulin	.0370	100
Proinsulin	.0053	14.2
Intermediate I	.0078	21.0
Intermediate II	.0004	10.8
Intermediate I (40%) + intermediate II (60%)	.0063	16.9

†Method of assay is according to Kitabchi et al.[52]

makes the conformational structure of the molecule such that a binding site on the A chain of insulin becomes available to the active site of the enzyme. The blocking of the A chain, however, is not the only factor which causes the decreased affinity of ISP for proinsulin since intermediate I remains less degradable than insulin by ISP even though the amino terminal end of the A chain is free. It is of great interest that the biological activity of the various polypeptide derivatives of insulin and proinsulin correlates very well with their degradability by ISP. This suggests that both the receptor on the cell membrane and the active site on the enzyme may be recognizing the same active site or sites of the insulin molecule.

SUMMARY AND PROSPECTS

The purpose of the present review has been to focus on some of the recent advances in the area of proinsulin research since its discovery in 1967. Special emphasis has been placed on the biological properties of proinsulin and the more recently available polypeptides which have a chemically related structure. Although much less potent than insulin on a molar basis, it has been well established that proinsulin has insulin-like biological activity on numerous tissues both in vivo and in vitro. This activity, at least in certain tissues, such as the isolated fat cell, is intrinsic and does not require conversion to insulin.[25,63] The conflicting data on the intrinsic biological effect of proinsulin in other tissues have not been completely resolved and need further investigation.

The future area of investigation of proinsulin is as wide and as exhausting as the area of insulin research since, with all the recent advances made on molecular biology, a full understanding of the mechanism of insulin action, and consequently of proinsulin action, has not been attained. Much additional investigation on the binding of insulin to the cell membrane and the subsequent intracellular events will be required before these processes are completely understood and before the mechanism of insulin action is explained.

Although the recent development of an enzymatic assay for proinsulin[52] has provided a simple method by which to study large numbers of patients and to investigate the significance of this prohormone in the circulation, additional methods of proinsulin assay must be sought, since neither column chromatography nor the enzymatic method is specific enough if the assumption is correct that proinsulin intermediates are present in circulation. Although the presence of these intermediates has not been established in circulation, neither column chromatography nor the enzymatic method can detect the presence of these intermediates if they indeed exist in plasma. Specific methods for human proinsulin must, therefore, await the availability of large amounts of highly purified human proinsulin free of possible intermediates.

The significance of proinsulin outside the pancreas is not known. One theory of the role of circulating proinsulin, however, is that it provides a low level of anabolic insulin-like hormone which has a longer halflife but which is less potent with regard to glucose metabolism. The effect of proinsulin on protein synthesis and enzyme induction should be examined, as these are areas where the chronic effect of insulin might be found. No work in this area has been reported at the present time.

The role of C-peptide in the circulation is also unclear. This material has no biological activity in any system examined, yet it is released into the circulation in equimolar quantities with insulin.[55]

Although proinsulin polypeptides, unlike C-peptide, exhibit biological effects in hormonally sensitive tissues commensurate with their conformational structure, the physiologic significance of these chemically synthesized, but theoretically plausible, intermediates also remains to be elucidated.

In addition to the above areas of interest, two intriguing areas of research which remain at the embryonic stage must be investigated. These are the intrapancreatic and extrapancreatic metabolism of proinsulin. The former relates to the biosynthetic events leading to the production of insulin and the role of the converting enzyme, or enzyme system. Although this subject has been one of great interest, much additional investigation of the converting enzyme and factors influencing it (or them) will be necessary.

Very little is known about the extrapancreatic metabolism of proinsulin. It is possible that proinsulin can be converted to insulin outside the pancreas, but on the basis of the available information this appears unlikely.[63] If it is not converted to insulin, the question arises as to whether or not there is a specific mechanism for

the removal and/or degradation of proinsulin outside the pancreas. It would appear that, at least in the rat, no single organ exhibits any great ability to specifically degrade proinsulin instead of insulin. Virtually nothing is known about the metabolism of proinsulin in human tissue except that it can be excreted by the kidney.[77]

The subject of greatest practical interest is the role of proinsulin in conditions associated with glucose intolerance, including diabetes mellitus. It has been well established that proinsulin does not comprise a major portion of the increased levels of immunoreactivity seen in some forms of carbohydrate intolerance such as adult onset diabetes mellitus,[108,110,122] but complete elucidation of its role in various disorders must await studies of larger numbers of patients. Classification of patients by age, weight, genetic makeup, age of onset, and other factors known to influence insulin release may be of benefit in ascertaining the role of proinsulin. Studies on the easily releasable, and less easily releasable pools of insulin[151,152] should also include information about proinsulin, since the contribution of proinsulin to these pools is not known.

The whole area of juvenile diabetes and its relationship to proinsulin has been virtually untouched. Studies on synthesis, conversion, and release from the pancreas, as well as observations on peripheral metabolism and degradation, will be necessary before the role of this precursor in juvenile diabetes is understood.

Great strides have been made in the area of proinsulin research, but these new findings have only presented greater problems and raised additional important questions which need to be seriously considered if we are to understand and provide clues into the problems of diabetes mellitus.

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REFERENCES

1. **Ryle, A. P., Sanger, F., Smith, L. F., and Kitai, R.,** The disulphide bonds of insulin, *Biochem. J.*, 60, 541, 1955.
2. **Humble, R. E.,** Biosynthesis of the two chains of insulin, *Proc. Natl. Acad. Sci. USA*, 53, 853, 1965.
3. **Steiner, D. F. and Oyer, P. E.,** The biosynthesis of insulin and a probable precursor of insulin by a human islet cell adenoma, *Proc. Natl. Acad. Sci. USA*, 57, 473, 1967.
4. **Rigopoulou, D., Valverde, I., Marco, J., Faloona, G., and Unger, R. H.,** Large glucagon immunoreactivity in extracts of pancreas, *J. Biol. Chem.*, 245, 496, 1970.
5. **Noe, B. D. and Bauer, G. E.,** Evidence for glucagon biosynthesis involving a protein intermediate in islets of the angler fish (Lophius americanus), *Endocrinology*, 89, 642, 1971.
6. **Sherwood, L. M., Lundberg, W. B., Targovnik, J. H., Rodman, J. S., and Seyfer, A.,** Synthesis and secretion of parathyroid hormone in vitro, *Am. J. Med.*, 50, 658, 1971.
7. **Sachs, H., Fawcett, P., Takabatake, Y., and Portanova, R.,** Biosynthesis and release of vasopressin and neurophysin, *Recent Progr. Horm. Res.*, 25, 447, 1969.
8. **Steiner, D. F., Cunningham, D., Spigelman, L., and Aten, B.,** Insulin biosynthesis: Evidence for a precursor, *Science*, 157, 697, 1967.
9. **Chance, R. E., Ellis, R. M., and Bromer, W. W.,** Porcine proinsulin: Characterization and amino acid sequence, *Science*, 161, 165, 1968.

10. Bromer, W. W., Proinsulin, *Bioscience*, 20, 701, 1970.
11. Chance, R. E. and Ellis, R. M., Proinsulin, *Arch. Intern. Med.*, 123, 229,.1969.
12. Chance, R. E., Ellis, R. M., and Bromer, W. W., Proinsulin:structure and physiologic significance, *Panminerva Med.*, 12, 216, 1970.
13. Gorden, P. and Roth, J., Circulating insulin, "big" and "little," *Arch. Intern. Med.*, 123, 237, 1969.
14. Grant, P. T. and Coombs, T. L., Proinsulin, a biosynthetic precursor of insulin, *Essays Biochem.*, 6, 69, 1970.
15. Kitabchi, A. E., Clinical significance of proinsulin in human plasma, *South. Med. J.*, in press.
16. Renold, A. E., Insulin biosynthesis and secretion – a still unsettled topic, *N. Engl. J. Med.*, 282, 173, 1970.
17. Rubenstein, A. H., Melani, F., Pilkis, S., and Steiner, D. F., Proinsulin: Secretion, metabolism, immunological and biological properties, *Postgrad. Med. J.*, 45, 476, 1968.
18. Rubenstein, A. H. and Steiner, D. F., Human proinsulin: Some consideration in the development of a specific immunoassay, *Adv. Metab. Disord.*, 1, 159, 1970.
19. Rubenstein, A. H. and Steiner, D. F., Proinsulin, *Annu. Rev. Med.*, 22, 1, 1971.
20. Rubenstein, A. H. and Steiner, D. F., Proinsulin – the single chain precursor of insulin, *Med. Clin. North Am.*, 54, 191, 1970.
21. Steiner, D. F., Proinsulin and the biosynthesis of insulin, *N. Engl. J. Med.*, 280, 1106, 1969.
22. Steiner, D. F., Clark, J. L., Nolan, C., Rubenstein, A. H., Margoliash, E., Aten, B., and Oyer, P. E., Proinsulin and the biosynthesis of insulin, *Recent Progr. Horm. Res.*, 25, 207, 1969.
23. Touber, J. L., Proinsulin, *Folia Med. Neerl.*, 13, 171, 1970.
24. Chance, R. E., Chemical, physical, biological, and immunological studies on porcine proinsulin and related polypeptides, in the Proceedings of the Seventh Congress of the International Diabetes Federations, Buenos Aires, *Excerpta Med.*, 119, 1970.
25. Kitabchi, A. E., The biological and immunological properties of pork and beef insulin, proinsulin, and connecting peptides, *J. Clin. Invest.*, 49, 979, 1970.
26. Lazarus, N. R., Tanese, T., Gutman, R., and Recant, L., Synthesis and release of proinsulin and insulin by human insulinoma tissue, *J. Clin. Endocrinol. Metab.*, 30, 273, 1970.
27. Clark, J. L., Steiner, D. F., Insulin biosynthesis in the rat: Demonstration of two proinsulin, *Proc. Natl. Acad. Sci. USA*, 62, 278, 1969.
28. Orci, L., Lambert, A. E., Kanazawa, Y., Amherdt, M., Rouiller, C., and Renold, A. E., Morphological and biochemical studies of B cells of fetal rat endocrine pancreas in organ culture, *J. Cell. Biol.*, 50, 565, 1971.
29. Yip, C. C. and Lin, B. J., Amino acid composition of bovine proinsulin, *Biochem. Biophys. Res. Commun.*, 29, 382, 1967.
30. Tung, A. K. and Yip, C. C., The biosynthesis of insulin and proinsulin in fetal bovine pancreas, *Diabetologia*, 4, 68, 1968.
31. Trakatellis, A. C. and Schwartz, G. P., Biosynthesis of insulin in angler fish islets, *Nature*, 225, 548, 1970.
32. Grant, P. T. and Reid, K. B., Biosynthesis of an insulin precursor by islet tissue of cod, *Biochem. J.*, 110, 281, 1968.
33. Oyer, P. E., Cho, S., Peterson, J. D., and Steiner, D. F., Studies on human proinsulin isolation and amino acid sequence of the human pancreatic C-peptide, *J. Biol. Chem.*, 246, 1375, 1971.
34. Boder, G. B., Root, M. A., Chance, R. E., and Johnson, I. S., Extended production of insulin by isolated rabbit pancreatic islets: Evidence for biosynthesis of insulin, *Proc. Soc. Exp. Biol. Med.*, 131, 507, 1969.
35. Nolan, C., Margoliash, E., Peterson, J. D., and Steiner, D. F., The structure of bovine proinsulin, *J. Biol. Chem.*, 246, 2780, 1971.
36. Clark, J. L., Cho, S., Rubenstein, A. H., and Steiner, D. F., Isolation of a proinsulin connecting peptide fragment from bovine and human pancreas, *Biochem. Biophys. Res. Commun.*, 35, 456, 1969.
37. Schmidt, D. D. and Arens, A., Proinsulin Von Rind: Isolierung, Eigenschaften and seine Aktivierung darch trypsin, *Hoppe Seylers Z. Physiol. Chem.*, 349, 1157, 1968.
38. Chance, R., Personal communication.
39. Steiner, D. F. and Clark, J. L., The spontaneous reoxidation of reduced beef and rat proinsulins, *Proc. Natl. Acad. Sci. USA*, 60, 622, 1968.
40. Frank, B. H. and Veros, A. J., Physical studies on proinsulin association behavior and conformation in solution, *Biochem. Biophys. Res. Commun.*, 32, 155, 1968.
41. Fullerton, W. W., Potter, R., and Low, B. W., Proinsulin: Crystallization and preliminary x-ray diffraction studies, *Proc. Natl. Acad. Sci. USA*, 66, 1213, 1970.
42. Frank, B. H. and Veros, A. J., Interaction of zinc with proinsulin, *Biochem. Biophys. Res. Commun.*, 38, 284, 1970.
43. Steiner, D. F., Hallund, O., Rubenstein, A. H., Cho, S., and Bayliss, C., Isolation and properties of proinsulin, intermediate forms, and other minor components from crystalline bovine insulin, *Diabetes*, 17, 725, 1968.
44. Roth, J., Gorden, P., and Pastan, I., "Big insulin": A new component of plasma insulin detected by immunoassay, *Proc. Natl. Acad. Sci. USA*, 61, 138, 1968.
45. Melani, F., Rubenstein, A. H., Oyer, P. E., and Steiner, D. F., Identification of proinsulin and C-peptide in human serum by a specific immunoassay, *Proc. Natl. Acad. Sci. USA*, 67, 148, 1970.
46. Lazarus, N. R., Gutman, R. A., and Recant, L., A method for electrophoretic characterization on polyacrylamide gel of circulating insulin immunoreactive substances, *Anal. Biochem.*, 40, 241, 1971.

47. **Ryan, W. G. and Robbins, P.**, Thin layer Sephadex chromatography of insulin and proinsulin, *Clin. Res.*, 17, 394, 1969.
48. **Rubenstein, A. H., Steiner, D. F., Cho, S., Lawrence, A. M., and Kirsteins, L.**, Immunological properties of bovine proinsulin and related fractions, *Diabetes*, 18, 598, 1969.
49. **Rubenstein, A. H., Welbourne, W. P., Mako, M., Melani, F., and Steiner, D. F.**, Comparative immunology of bovine, porcine, and human proinsulin and C-peptides, *Diabetes*, 19, 546, 1970.
50. **Wright, P. H. and Makulu, D. R.**, Reactions of proinsulin and its derivatives with antibodies to insulin, *Proc. Soc. Exp. Biol. Med.*, 134, 1165, 1970.
51. **Lawrence, A. M. and Kirsteins, L.**, The effect of proinsulin on the immunoassay of insulin and its possible relation to states of hyperinsulinemia, *Proc. Soc. Exp. Biol. Med.*, 131, 1142, 1969.
52. **Kitabchi, A. E., Duckworth, W. C., Brush, J. S., and Heinemann, M.**, Direct measurement of proinsulin in human plasma by the use of an insulin-degrading enzyme, *J. Clin. Invest.*, 50, 1792, 1971.
53. **Root, M. A., Ellis, R. M., Chance, R. E., and Galloway, J. A.**, Preparation and use of a purified porcine proinsulin antiserum, *Diabetes*, 19S-1, 359, 1970.
54. **Yip, C. C. and Logothetopoulos, J.**, A specific anti-proinsulin serum and the presence of proinsulin in calf serum, *Proc. Natl. Acad. Sci. USA*, 62, 415, 1969.
55. **Rubenstein, A. H., Clark, J. L., Melani, F., and Steiner, D. F.**, Secretion of proinsulin C-peptide by pancreatic β cells and its circulation in blood, *Nature*, 224, 697, 1969.
56. **Challoner, D. R. and Yu, P.**, Differential effect of porcine proinsulin on rat epididymal fat cells and fat pieces, *Diabetes*, 19, 289, 1970.
57. **Gliemann, J. and Sorensen, H. H.**, Assay of insulin-like activity by the isolated fat cell method. IV. The biological activity of proinsulin, *Diabetologia*, 6, 499, 1970.
58. **Shaw, W. N. and Chance, R. E.**, Effect of porcine proinsulin *in vitro* on adipose tissue and diaphragm of the normal rat, *Diabetes*, 17, 737, 1968.
59. **Steele, A. A., Brown, J. D., and Stone, D. B.**, Antilipolytic effect of porcine proinsulin, *Diabetes*, 19, 91, 1970.
60. **Lavis, V. R., Ensinck, J. W., and Williams, R. H.**, Effects of insulin and proinsulin on isolated fat cells and hemidiaphragms from rats, *Endocrinology*, 87, 135, 1970.
61. **Solomon, S. S., Brush, J. S., and Kitabchi, A. E.**, Antilipolytic activity of insulin and proinsulin on ACTH and cyclic nucleotide-induced lipolysis in the isolated adipose cell of rat, *Biochim. Biophys. Acta*, 218, 167, 1970.
62. **Toomey, R. E., Shaw, W. N., Reid, L. R., and Young, W. K.**, Comparative study of the effects of porcine proinsulin and insulin on lipolysis and glucose oxidation in rat adipocytes, *Diabetes*, 19, 209, 1970.
63. **Lazarus, N. R., Penhos, J. C., Tanese, T., Michaels, L., Gutman, R., and Recant, L.**, Studies on the biological activity of porcine proinsulin, *J. Clin. Invest.*, 49, 487, 1970.
64. **Konsek, J. P., Rubenstein, A. H., and Ehrlich, E. N.**, Biologic action of proinsulin on ventral toad skin *in vitro*, *Endocrinology*, 89, 847, 1971.
65. **Rudorff, K., Albrecht, G., and Staib, W.**, Effect of insulin and proinsulin on the metabolism of alanine in the rat liver, *Hormone Metab. Res.*, 2, 49, 1970.
66. **Rastogi, G. K., Letarte, J., and Fraser, T. R.**, Proinsulin content of pancreas in human fetuses of healthy mothers, *Lancet*, 1, 7, 1970.
67. **Galloway, J. A., Root, M. A., Chance, R. E., Rathmacher, R. P., Challoner, D. R., and Shaw, W. N.**, *In vivo* studies of the hypoglycemic activity of porcine proinsulin, *Diabetes*, 18-5-1, 341, 1969.
68. **Penhos, J. C., Voyles, N., Lazarus, N., Tanese, T., Gutman, R., and Recant, L.**, Hypoglycemic action of porcine proinsulin in eviscerated, functionally hepatectomized rats, *Hormone Metab. Res.*, 2, 43, 1970.
69. **Puls, W. and Kroneberg, G.**, Experimentelle Untersuchungen uber die blutzuckersenKende Wirtung Von Rinder – Proinsulin, *Diabetologia*, 5, 325, 1969.
70. **Rees, K. O. and Madison, L. L.**, The effect of proinsulin on hepatic glucose output, arterial glucose, and free fatty acid concentrations in dogs, *Diabetes*, 18-5-1, 341, 1969.
71. **Stoll, R. W., Touber, J. L., Winterscheid, L. C., Ensinck, J. W., and Williams, R. H.**, Hypoglycemic activity and immunological half-life of porcine insulin and proinsulin in baboons and swine, *Endocrinology*, 88, 714, 1971.
72. **Willms, B., Appels, A., Soling, H. D., and Creutzfeldt, W.**, Lack of hypoglycemic effect of bovine proinsulin in eviscerated, hepatectomized rats, *Hormone Metab. Res.*, 1, 199, 1969.
73. **Fineberg, S. E. and Merimee, T. J.**, Proinsulin: Metabolic effects in the human forearm, *Science*, 167, 998, 1970.
74. **Fineberg, S. E. and Merimee, T. J.**, Proinsulin and single component insulin in the human forearm: A comparative study, *Diabetes*, 20-5-1, 333, 1971.
75. **Kitabchi, A. E., Duckworth, W. C., and Benson, B.**, *In vivo* effects of insulin and proinsulin on diaphragm and epididymal fat pads in rats, submitted.
76. **Rafaelson, O., Lauris, V., and Renold, A. E.**, Localized intraperitoneal action of insulin on rat diaphragm and epididymal adipose tissue *in vivo*, *Diabetes*, 14, 19, 1965.
77. **Rubenstein, A. H., Cho, S., and Steiner, D. F.**, Evidence for proinsulin in human urine and serum, *Lancet*, 1, 1353, 1968.
78. **Kitabchi, A. E., Benson, B., Davis, R., and Heinemann, M.**, Proinsulin: A possible insulin inhibitor, *Clin. Res.*, 17, 386, 1969.

79. Levine, R., Cell membrane as a primary site of insulin action, *Fed. Proc.*, 24, 1071, 1965.
80. Levine, R. and Haft, D. E., Carbohydrate homeostasis, *N. Engl. J. Med.*, 283, 175, 1970.
81. Cuatrecasas, P., Interaction of insulin with the cell membrane: The primary action of insulin, *Proc. Natl. Acad. Sci.*, 63, 450, 1969.
82. Turkington, R. W., Stimulation of RNA synthesis in isolated mammary cells by insulin and prolactin bound to Sepharose, *Biochem. Biophys. Res. Commun.*, 41, 1362, 1970.
83. Oka, T. and Topper, Y. J., Insulin-Sepharose and the dynamics of insulin action, *Proc. Natl. Acad. Sci. USA*, 68, 2066, 1971.
84. Blatt, L. M. and Kim, K. H., Regulation of hepatic glycogen synthetase. Stimulation of glycogen synthetase in an *in vitro* liver system by insulin bound to Sepharose, *J. Biol. Chem.*, 246, 4895, 1971.
85. Cuatrecasas, P., The insulin receptor in Proceedings of Eli Lilly Symposium on Insulin, *Diabetes suppl.*, in press.
86. Cuatrecasas, P., Insulin receptor interaction in adipose tissue cells: Direct measurement and properties, *Proc. Natl. Acad. Sci. USA*, 68, 1264, 1971.
87. Freychet, P., Roth, J., and Neville, D. M., Insulin receptors in the liver: Specific binding of ^{125}I-insulin to the plasma membrane and its relation to insulin bioactivity, *Proc. Natl. Acad. Sci. USA*, 68, 1833, 1971.
88. Cuatrecasas, P., Properties of the insulin receptor of isolated fat cell membranes, *J. Biol. Chem.*, 246, 7265, 1971.
89. Tung, A. K. and Yip, C. C., Biosynthesis of insulin in bovine fetal pancreatic slices: The incorporation of tritiated leucine into a single-chain proinsulin, a double-chain intermediate, and insulin in subcellular fractions, *Proc. Natl. Acad. Sci. USA*, 63, 442, 1969.
90. Lin, B. J. and Haist, R. E., Insulin biosynthesis: Effects of carbohydrates and related compounds, *Can. J. Physiol. Pharmacol.*, 47, 791, 1969.
91. Logothetopoulos, J., Yip, C. C., and Coburn, M. E., Proinsulin in β cells of bovine islets demonstrated by fluorescein or peroxidase-labeled specific antibody, *Diabetes*, 19, 539, 1970.
92. Smith, L. F., Species variations in the amino acid sequence of insulin, *Am. J. Med.*, 40, 662, 1966.
93. Markus, G., Electrolytic reduction of the disulfide bonds of insulin, *J. Biol. Chem.*, 239, 4163, 1964.
94. Givol, D., DeLorenzo, F., Goldberger, R. F., and Anfinsen, C. B., Disulfide interchange and the three-dimensional structure of proteins, *Proc. Natl. Acad. Sci. USA*, 53, 676, 1965.
95. Bauer, G. E., Studies on insulin biosynthesis: Subcellular distribution of leucine-H^3 radioactivity during incubation, *J. Cell. Biol.*, 28, 413, 1966.
96. Howell, S. L., Kostionovsky, M., and Lacy, P. E., Beta granule formation in isolated islets of Langerhans, *J. Cell. Biol.*, 42, 695, 1969.
97. Jamieson, J. D. and Palade, G. E., Intracellular transport of secretory proteins in the pancreatic exocrine cell. I. Role of peripheral elements of Golgi complex, *J. Cell. Biol.*, 34, 577, 1967.
98. Jamieson, J. D. and Palade, G. E., Intracellular transport of secretory proteins in pancreatic exocrine cell. II. Transport to condensing vacuoles and zymogen granules, *J. Cell. Biol.*, 34, 597, 1967.
99. Tanese, T., Lazarus, N. R., Devrin, S., and Recant, L., Synthesis and release of proinsulin and insulin by isolated rat islets of Langerhans, *J. Clin. Invest.*, 49, 1394, 1970.
100. Burr, I. M., Stauffacher, W., Balant, L., Renold, A. E., and Grodsky, G., Dynamic aspects of proinsulin release from perifused rat pancreas, *Lancet*, 2, 882, 1969.
101. Poffenbarger, P. L., Chick, W. L., Levine, R. L., Soeldner, J. S., and Flewelling, J. H., Insulin biosynthesis in experimental hereditary diabetes, *Diabetes*, 20, 677, 1971.
102. Sorenson, R. L., Steffes, M. W., and Lindall, A. W., Subcellular localization of proinsulin to insulin conversion in isolated rat islets, *Endocrinology*, 86, 88, 1970.
103. Kemmler, W. and Steiner, D. F., Conversion of proinsulin to insulin in a subcellular fraction from rat islets, *Biochem. Biophys. Res. Commun.*, 41, 1223, 1970.
104. Yip, C. C., A bovine pancreatic enzyme catalyzing the conversion of proinsulin to insulin, *Proc. Natl. Acad. Sci. USA*, 68, 1312, 1971.
105. Kemmler, W., Peterson, J. D., and Steiner, D. F., Studies on the conversion of proinsulin to insulin. I. Conversion *in vitro* with trypsin and carboxy peptidase. *J. Biol. Chem.*, in press.
106. Wang, S. S. and Carpenter, F. H., A compositional assay for insulin applied to a search for "proinsulin," *J. Biol. Chem.*, 240, 1619, 1965.
107. Stoll, R. W., Touber, J. L., Ensinck, J. W., and Williams, R. H., Substances immunologically related to proinsulin or connecting peptide in swine plasma, *Hormone Metab. Res.*, 2, 153, 1970.
108. Melani, F., Rubenstein, A. H., and Steiner, D. F., Human serum proinsulin, *J. Clin. Invest.*, 49, 497, 1970.
109. Sherman, B. M., Gorden, P., Roth, J., and Freychet, P., Circulating insulin: The proinsulin-like properties of "big" insulin in patients without islet cell tumors, *J. Clin. Invest.*, 50, 849, 1971.
110. Gorden, P. and Roth, J., Plasma insulin: Fluctuation in the "big" insulin component in man after glucose and other stimuli, *J. Clin. Invest.*, 48, 2225, 1969.
111. Gorden, P., Griggs, R. C., Nissley, S. P., Roth, J., and Engle, W. K., Studies of plasma insulin in myotonic dystrophy, *J. Clin. Endocrinol. Metab.*, 29, 684, 1969.
112. Gutman, R. A., Lazarus, N. R., Penhos, J. C., Fajans, S., and Recant, L., Circulating proinsulin-like material in patients with functioning insulinomas, *N. Engl. J. Med.*, 284, 1003, 1971.

113. Goldsmith, S. J., Yalow, R. S., and Berson, S. A., Significance of human plasma Sephadex fraction, *Diabetes*, 18, 834, 1969.
114. Gorden, P., Sherman, B., and Roth, J., Proinsulin-like component of circulating insulin in the basal state and in patients and hamsters with islet cell tumors, *J. Clin. Invest.*, 50, 2113, 1971.
115. Melani, F., Ryan, W. G., Rubenstein, A. H., and Steiner, D. F., Proinsulin secretion by a pancreatic beta cell adenoma, *N. Engl. J. Med.*, 283, 713, 1970.
116. Blackard, W. G., Garcia, A. R., and Brown, C. L., Jr., Effect of streptozotocin on qualitative aspects of plasma insulin in a patient with a malignant islet cell tumor, *J. Clin. Endocrinol. Metab.*, 31, 215, 1970.
117. Duckworth, W. C. and Kitabchi, A. E., Hyperproinsulinemia in hypoglycemic subjects, *Hormone Metab. Res.*, in press.
118. Brush, J. S., Purification and characterization of a protease with specificity for insulin from rat muscle, *Diabetes*, 20, 140, 1971.
119. Elliott, R. B., O'Brien, D., and Roy, C. C., An abnormal insulin in juvenile diabetes mellitus, *Diabetes*, 14, 780, 1965.
120. Elliott, R. B., Abnormal insulin, proinsulin, and big insulin in diabetes, *Lancet*, 2, 1076, 1969.
121. Duckworth, W. C., Heinemann, M., and Kitabchi, A. E., Proinsulin response in normal and diabetic subjects, *Clin. Res.*, 19, 677, 1971.
122. Duckworth, W. C. and Kitabchi, A. E., Direct measurement of plasma proinsulin in normal and diabetic subjects, *Am. J. Med.*, in press.
123. Mashiter, K. and King, T. M., Changes of immunoreactivity of proinsulin induced by rat tissue extracts, *Nature*, 224, 696, 1969.
124. Mirsky, I. A., Insulinase, insulinase inhibitors, and diabetes mellitus, *Recent Prog. Horm. Res.*, 13, 429, 1957.
125. Williams, R. H., Degradation of insulin and glucagon, in *Textbook of Endocrinology*, Williams, R. H., Ed., W. B. Saunders, Philadelphia, 1968.
126. Katzen, H. M. and Stetten, D., Hepatic glutathione-insulin transhydrogenase, *Diabetes*, 11, 271, 1962.
127. Ansorge, S., Bohley, P., Kirschke, H., Langner, J., and Hanson, H., Metabolism of insulin and glucagon, breakdown of radioiodinated insulin and glucagon in rat liver cell fractions, *Eur. J. Biochem.*, 19, 283, 1971.
128. Tomizawa, H. H., Nutley, M. L., Narabara, H. T., and Williams, R. H., The mode of inactivation of insulin by rat liver extracts, *J. Biol. Chem.*, 214, 285, 1955.
129. Katzen, H. M. and Tietze, F., Studies on the specificity and mechanism of action of hepatic glutathione-insulin transhydrogenase, *J. Biol. Chem.*, 241, 3561, 1966.
130. Rudman, D., Garcia, L. A., DiGirolamo, M., and Shank, P. W., Cleavage of bovine insulin by rat adipose tissue, *Endocrinology*, 78, 169, 1966.
131. Rudman, D., Garcia, L. A., and Del Rio, A., Effects on mammalian adipose tissue of fragments of bovine insulin and of certain synthetic peptides, *Biochemistry*, 7, 1875, 1968.
132. Sumner, K. and Doisy, R. J., Degradation of insulin by a particulate fraction from adipose tissue, *Biochem. J.*, 116, 825, 1970.
133. Brush, J. S. and Kitabchi, A. E., Metabolic disposition of [^{131}I] iodoinsulin within the rat diaphragm, *Biochim. Biophys. Acta*, 215, 134, 1970.
134. Burghen, G. A., Brush, J. S., Solomon, S. S., and Kitabchi, A. E., Inhibition of liver insulin specific protease by plasma inhibitors, *Clin. Res.*, 19, 81, 1971.
135. Burghen, G. A., Kitabchi, A. E., and Brush, J. S., Purification and properties of a rat liver protease with specificity for insulin, submitted for publication.
136. Madison, L. L., Combes, B., Unger, R. H., and Kaplan, N., The relationship between the mechanism of action of the sulfonylureas and the secretion of insulin into the portal circulation, *Ann. N. Y. Acad. Sci.*, 74, 548, 1959.
137. Stoll, R. W., Touber, J. L., Menahan, L. H., and Williams, R. H., Cleavage of porcine insulin, proinsulin, and connecting peptide by the isolated rat liver, *Proc. Soc. Exp. Biol. Med.*, 111, 894, 1970.
138. Varandani, P. T. and Nafz, M. A., Enzymatic destruction of immunoreactivity in proinsulin and insulin and activation of their scrambled forms, *Arch. Biochem. Biophys.*, 141, 533, 1970.
139. Challoner, D. R., Degradation of porcine insulin and proinsulin by rat adipose tissue, *Diabetes*, 20, 276, 1971.
140. Cuatrecasas, P., Desbuguois, B., and Krug, F., Insulin-receptor interaction in liver cell membranes, *Biochem. Biophys. Res. Commun.*, 44, 333, 1971.
141. Markussen, J., Sundby, F., Smyth, P. G., and Ko, A., Preparation of human C-peptide, *Hormone Metab. Res.*, 3, 229, 1971.
142. Sundby, F. and Markussen, J., Preparative method for the isolation of C-peptides from ox and pork pancreas, *Hormone Metab. Res.*, 2, 17, 1970.
143. Steiner, D. F., Cho, S., Oyer, P. E., Terris, S., Peterson, J. D., and Rubenstein, A. H., Isolation and characterization of proinsulin C-peptide from bovine pancreas, *J. Biol. Chem.*, 246, 1365, 1971.
144. Yalow, R. S. and Berson, S. A., Immunoassay of endogenous plasma insulin in man, *J. Clin. Invest.*, 39, 1157, 1960.
145. Block, M. B., Mako, M., Steiner, D. F., and Rubenstein, A. H., Serum C-peptide immunoreactivity: An indicator of Beta cell activity in diabetics, *Diabetes*, 20 S-1, 332, 1971.
146. Mirsky, I. A. and Kawamura, K., Heterogeneity of crystalline insulin, *Endocrinology*, 78, 1115, 1966.

147. **Steiner, D.**, personal communication.
148. **Morgan, C. R. and Lazarow, A.**, Immunoassay of insulin: Two antibody system. Plasma insulin levels of normal, subdiabetic and diabetic rats, *Diabetes*, 12, 115, 1963.
149. **Kitabchi, A. E., Stentz, F. B., Yu, S., Duckworth, W. C., and Heinemann, M.**, Biochemical and immunological properties of insulin, proinsulin, and their polypeptides, *Fed. Proc.*, 30, 1318, 1971.
150. **Adams, M. J., Blundell, T. L., Dodson, E. J., Dodson, G. G., Vijayan, M., Baker, E. N., Harding, M. M., Hodgkin, D. C., Rimmer, B., and Sheat, S.**, Structure of rhombohedral 2 zinc insulin crystals, *Nature*, 224, 491, 1969.
151. **Kitabchi, A. E., Stentz, F. B., Duckworth, W. C., and Heinemann, M.**, Studies on the degradation of insulin, proinsulin, and their related polypeptides by insulin specific protease, *Clin. Res.*, 19, 477, 1971.
152. **Curry, D. L., Bennett, L. L., and Grodsky, G. M.**, Dynamics of insulin secretion by the perfused rat pancreas, *Endocrinology*, 83, 572, 1968.
153. **Porte, D., Jr. and Pupo, A. A.**, Insulin response to glucose: Evidence for a two pool system in man, *J. Clin. Invest.*, 48, 2309, 1969.
154. **Bray, G. A.**, Effects of epinephrine, corticotropin and thyrotropin on lipolysis and glucose oxidation in rat adipose tissue, *J. Lipid Res.*, 8, 300, 1967.