Comparative Immunology of Bovine, Porcine and Human Proinsulins and C-peptides

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SUMMARY

The immunological cross reactions of human, porcine and bovine proinsulins and C-peptides are described. In order to iodinate the C-peptides to high specific activity, tyrosine was coupled to the molecule using N-carboxy-tyrosyl anhydride. The tyrosylated bovine I-131-C-peptide was bound by bovine proinsulin antisera, but did not react with porcine proinsulin or insulin antisera, while the tyrosylated human-I-131-C-peptide did not react with high concentrations of these three antisera.

The cross reaction of human and porcine proinsulin with bovine proinsulin antisera was weak and resembled that of insulins of all three species. Similar results were observed with a porcine proinsulin antiserum. Removal of the insulin-binding antibodies from a proinsulin antiserum eliminated its reactivity with heterologous proinsulins. Using an assay system (bovine proinsulin antiserum: tyrosylated-I-131-C-peptide) which did not respond to insulin, human and porcine C-peptides reacted approximately 250 and 750 times less efficiently than the bovine molecule. These results indicate that the relatively large degree of variability in the amino acid sequence of the proinsulin-connecting segments gives rise to unique immunological determinants in the three species of proinsulin studied. DIABETES *19*:546-53, August, 1970.

The compositions of human, porcine and bovine insulins differ by only three amino acids in a total of fifty-one residues.[1] Thus antisera raised against each of these proteins usually cross react efficiently with the other two molecules. Provided that appropriate standards are available, homologous or heterologous antibodies can be used to measure these three species of insulin by immunological methods.

Soon after the discovery of proinsulin in human islet cell tumors,[2,3] small quantities of this protein were separated from crystals of bovine[4] and porcine[5] insulin. Sufficient material was available for structural studies and immunization.[5-8] On the other hand, extraction of proinsulin from the limited supplies of human insulin available did not yield sufficient amounts of proinsulin for immunization.[9,10] The hope that the cross reactivity among porcine, bovine and human proinsulin would be sufficient to permit the measurement of endogenous proinsulin levels in human plasma was not fulfilled, because antisera to porcine and bovine proinsulins proved to be highly specific in their reaction with the connecting region of the molecule, and thus unsuitable for measuring heterologous proinsulins.[6,9]

This paper describes the immunological interrelationships of proinsulin of these three species. Availability of the isolated connecting peptides (C-peptides), which consist of all the amino acids present in the proinsulin-connecting segment except for the pairs of basic residues at each end through which it is linked to the insulin chains, has greatly facilitated the study.[11] A preliminary report of these findings has been presented.[12]

MATERIAL AND METHODS

Preparation of standards

The preparation of bovine and human proinsulin has been described.[4,9,10] The bovine[11] and human C-peptides[13] were extracted from pancreatic tissue with acid-ethanol and purified by gel filtration on Sephadex G-50, paper electrophoresis in two systems (30 per cent formic acid and pyridine: acetic acid, pH 6.5) and ion exchange chromatography. Porcine proinsulin and a connecting peptide (residues 33 through 63 prepared by tryptic digestion of porcine proinsulin) were a gift of Dr. R. E. Chance of the Lilly Laboratories. In order to test for insulin contamination, the three peptides were assayed against their respective insulins using I-131-insulin and insulin antiserum. One microgram human C-peptide and porcine tryptic peptide were contaminated with the equivalent of 2.0 and 35.0

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mμg. insulin respectively, while insulin was not detected in the bovine C-peptide preparation.

Radioiodinated proinsulin, insulin and C-peptide

Human and bovine C-peptides and the porcine tryptic peptide do not contain tyrosine or histidine. In order to achieve iodinations to high specific activity, we have coupled tyrosine to amino groups present in human and bovine C-peptides.[14,15] The C-peptides (0.5 mg.) were dissolved in 0.15 ml. 0.0025 N. hydrochloric acid and 0.15 ml. 0.1 M. sodium phosphate buffer (pH 7.6) was added. Two milligrams N-carboxytyrosyl anhydride (Cyclo Chemical Corp., Los Angeles) were dissolved in 0.1 ml. anhydrous dioxane and added to the C-peptides in an ice bath at 2° C. The mixture was allowed to react for sixteen hours at 4° C. with continual stirring. The small precipitate which formed was removed by centrifugation and the supernatant diluted in 0.8 ml. 3 M. acetic acid and applied to a 1 × 50 cm. Bio-Gel (P-30, 100-200 mesh) column. The fractions containing the tyrosylated C-peptide were identified by the ninhydrin reaction and subsequently combined and concentrated in vacuo.

The tyrosylated C-peptides, proinsulins and insulins were iodinated with I-131 (Union Carbide Corp., New York) by the method of Hunter and Greenwood[16] as modified by Yalow and Berson.[17] Details of the procedure have been previously described.[6] Unlike I-131-insulin and proinsulin, the labeled C-peptides did not bind firmly to powdered cellulose, and were thus purified by gel filtration on Bio-Gel P-30 in 3 M. acetic acid. The 50 × 1 cm. column and collecting tubes were first coated with bovine albumin (Pentex Corp., Illinois) in 50 per cent acetic acid (10 mg. per ml.) to minimize adsorption onto the glass. The peak of radioactivity was identified by counting an aliquot of each fraction and the center fractions were pooled and evaporated to dryness. The labeled C-peptides were then redissolved in the buffer used for immunoassay and stored at 4° C. until used.

Antiserum

Antisera to insulin (11-0) and bovine proinsulin (A_6) were prepared in guinea pigs. Porcine proinsulin antiserum (Lilly) was a gift from Dr. M. A. Root of the Lilly Laboratories. Purified bovine proinsulin antiserum (Y) was a gift from Dr. C. Yip of the Banting and Best Institute. The insulin-reactive antibodies had been removed from this antiserum by passage of the serum over an immunoadsorbent of insulin coupled to Sephadex.[18] The reactions of antiserum 11-0, A_6, Y and Lilly with the insulin, proinsulin and C-peptide standards of the same species are shown in figure 1. Constant amounts of labeled antigen were mixed with increasing concentrations of unlabeled protein and then incubated with a dilution of antiserum known to bind 40 to 70 per cent of the tracer in the control tubes.

Immunoassay

A modification of the method of Morgan and Lazarow[19] was used. The buffer was 0.1 M. Tris-HCl, pH 7.7, containing 0.05 M. sodium chloride and 0.25 per cent bovine albumin. The volume of the first reaction was 1.0 ml. and its incubation time three days. The antibody-bound complex was precipitated by adding 0.1 ml. rabbit anti-guinea pig globulin (Sylvana Co., New Jersey) and 0.1 ml. carrier guinea pig serum (1:300). After centrifugation at 4° C. for twenty minutes at 2,500 r.p.m., the supernatant was decanted and both the precipitate and supernatant were subsequently counted in an automatic gamma-spectrometer. The result of each sample was expressed as the percentage of total radioactivity found in the precipitate.

RESULTS

Reaction of insulin, proinsulin and C-peptide with proinsulin and insulin antisera

In the insulin assay (figure 1, top left), insulin reacted more strongly than proinsulin, while the C-peptide did not compete with the label for antibody binding sites. On the other hand, standards of proinsulin (top right, bottom left) gave the steepest slope when incubated with I-131-proinsulin and proinsulin antisera. Removal of the insulin-binding antibodies from a proinsulin antiserum (bottom right) resulted in a system which reacted equimolarly with proinsulin and the C-peptide, but did not respond to the addition of high concentrations of insulin.

Characterization of radioiodinated C-peptides

The tyrosylated human and bovine C-peptides were iodinated to specific activities of 50 to 200 mc. per mg. using 2 to 3 mc. I-131 (specific activity greater than 150 mc. per ml.) and 5 μg. peptide. The peak obtained by gel filtration of the labeled C-peptides on Bio-Gel P-30 was asymmetrical on the descending limb and therefore only fractions from the center of the peak were combined for the immunological studies (figure 2). The tyrosylated I-131-C-peptides emerged later from the column than did the unmodified protein. Similar retardation of polytyrosyl trypsin on gel filtration has been reported.[14]

Binding of radioiodinated proteins to antisera

When 0.2 to 1.0 mμg. human, porcine and bovine

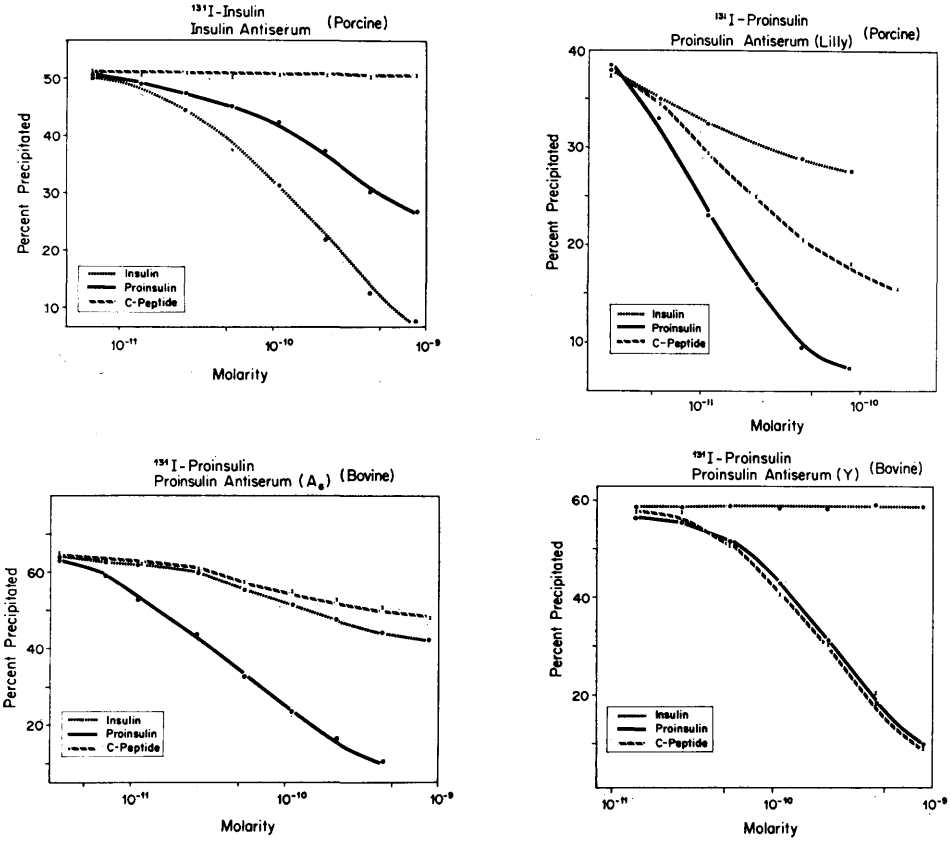

FIG. 1.
Reaction of homologous insulin, proinsulin and C-peptide standards with porcine insulin (11-0) (top left), porcine proinsulin (Lilly) (top right), bovine proinsulin (A_6) (bottom left) and purified bovine proinsulin (Y) (bottom right) antisera. The ordinate indicates the percentage counts in the precipitate (antibody bound) after addition of the rabbit antiguinea pig globulin serum.

I-131-proinsulin were incubated in 1.0 ml. buffer containing 0.1 ml. 1:100 (final dilution 1:1,000) bovine (A_6) and porcine (Lilly) proinsulin and porcine insulin antisera, more than 93 per cent of the tracer was bound to the antisera. On the other hand, bovine I-131-C-peptide (0.5 mμg.) reacted with only bovine proinsulin antisera (A_6 and Y) and the labeled human C-peptide (0.5 to 1.0 mμg.) did not bind significantly (less than 3 per cent) to 1:1,000 dilutions of the porcine or bovine antisera (figure 3). A progressively smaller percentage of bovine I-131-C-peptide (0.5 mμg.) was bound to antiserum A_6 as its final dilution was increased from 1:1,000 (92 per cent bound) to 1,000,000 (12 per cent bound). Addition of 100 mμg.

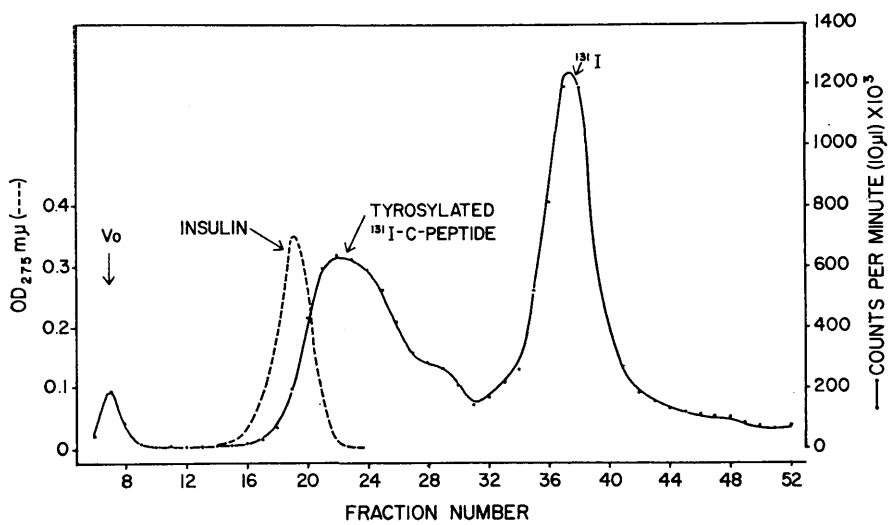

FIG. 2.
Gel filtration of human I-131-tyrosylated C-peptide on Bio-Gel P-30 in 3 M acetic acid. The fraction size was 1.8 ml. Note the retarded elution position of the labeled protein compared to that of insulin.

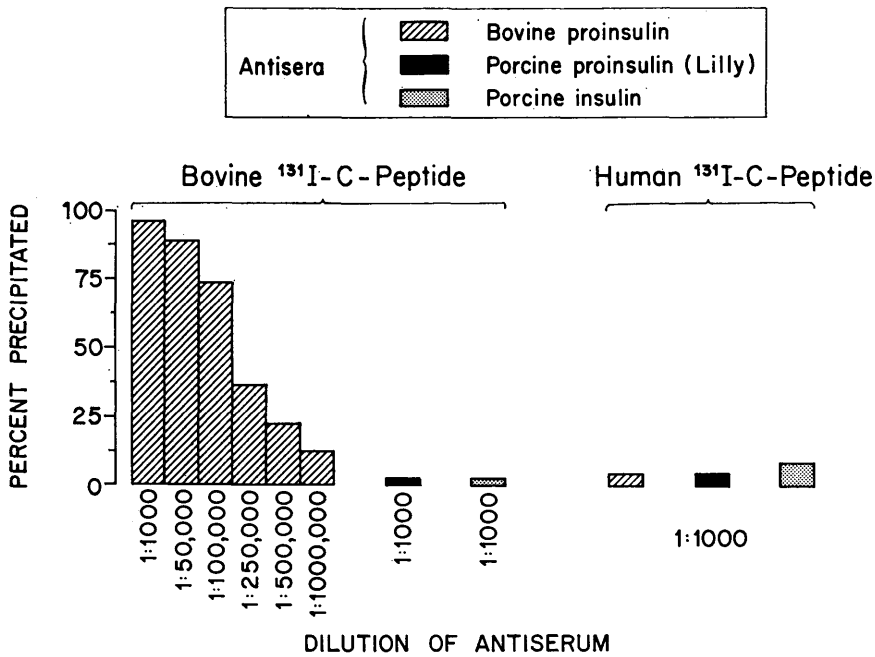

FIG. 3. Incubation of bovine (0.5 mμg.) and human (0.5 mμg.) I-131-tyrosylated C-peptide with bovine proinsulin (A_6), porcine proinsulin (Lilly) and porcine insulin (11-0) antisera. The antisera were added to 1.0 ml. buffer to give final dilutions of 1:1,000 to 1:1,000,000 (bovine proinsulin antiserum), and 1:1,000 (porcine proinsulin and porcine insulin antisera). After seventy-two hours' incubation, the free and bound tracer was separated by the addition of rabbit anti-guinea pig globulin serum and the percentage bound (on the ordinate) was calculated by counting both the precipitate and supernatant after centrifugation.

bovine proinsulin or C-peptide completely prevented the binding of this label to the antiserum.

Cross reaction of bovine, porcine and human proinsulins

The reactions of the three species of proinsulin with bovine (A_6) and porcine proinsulin (Lilly) antisera and I-131-bovine and porcine proinsulin are shown in figure 4. The heterologous proinsulins reacted sim-

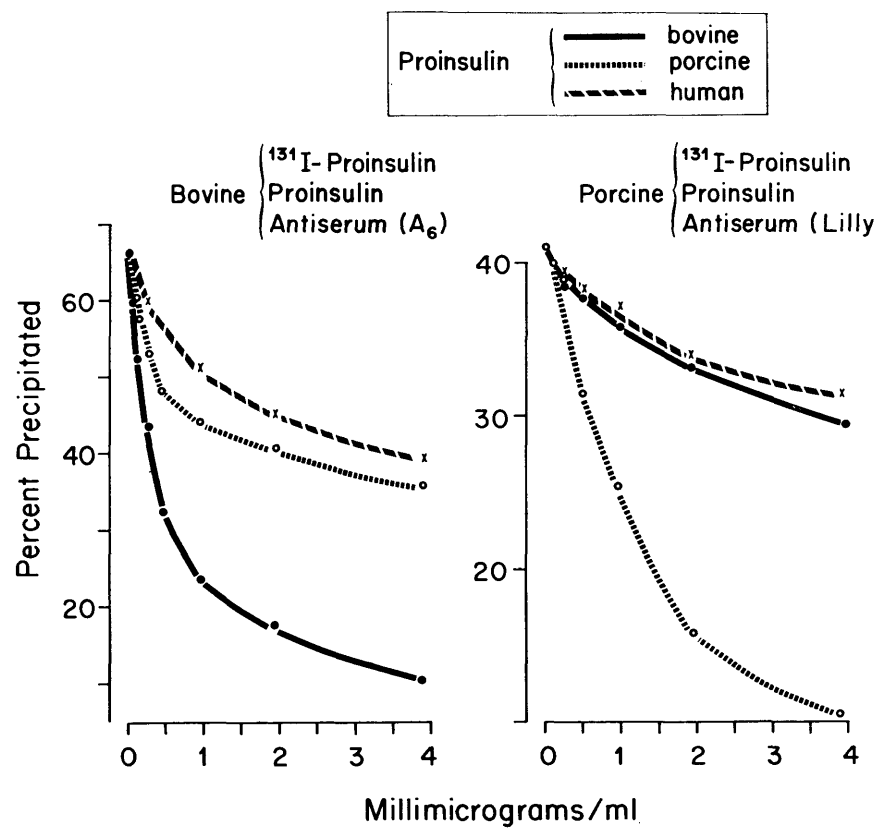

FIG. 4. Competition between increasing concentration of unlabeled proinsulin (bovine, porcine and human) and bovine I-131-proinsulin for binding to antibodies in bovine proinsulin antiserum (left side). The result of a similar experiment using porcine proinsulin antiserum and porcine I-131-proinsulin is shown on the right. In both instances, only the homologous proinsulin standard gave a steep curve.

ilarly to equimolar concentrations of insulins of all three species, while increasing concentrations of the homologous proinsulin standard competed effectively with the tracer to give a steep curve. Using the purified bovine proinsulin antiserum (Y), which did not react with insulin, human and porcine proinsulins (or C-peptides) did not compete with bovine I-131-proinsulin for binding sites on the antibody (figure 5). In the insulin immunoassay, the three proinsulins reacted less efficiently than insulin. The curves of human and bovine proinsulin resembled that of porcine proinsulin in figure 1 (upper left-hand panel).

Cross reaction of bovine, porcine and human C-peptides

Because of the presence of small quantities of insulin in the human and porcine C-peptide preparations, we determined the absolute degree of cross reactivity of the three C-peptides in an assay which did not respond to insulin at all. Large quantities (10, 100 and 1,000 mμg.) of insulin and the three C-peptides were incubated with bovine proinsulin antiserum (A_6) and I-131-C-peptide. Insulin did not compete with the radioactive label for sites on the antibody, while 1,000 mμg. human and porcine C-peptide were equivalent to 4.0 and 1.3 mμg. bovine C-peptide respectively (figure 6). Using antiserum Y, similar results were obtained (1,000 mμg. human and porcine C-peptide measuring 2.5 and 1.0 mμg. respectively against the bovine C-peptide).

DISCUSSION

Proinsulin is a single polypeptide chain of molecular weight about 9,000, which begins at the amino terminus with the B chain of insulin, proceeds through a connecting segment of approximately thirty amino acids and terminates with the A chain.[5,7] The characteristic disulfide bonds of insulin are present in proinsulin,[20] and studies comparing the physical properties of proinsulin and insulin have shown close similarity between the two proteins in regard to their self association characteristics, ultraviolet absorption spectra and optical rotatory dispersion spectra.[21] Exposure of bovine or porcine proinsulin to small amounts of trypsin liberates desalanyl insulin, which is indistinguishable from authentic insulin in its immunologic and biologic properties.[8,22] All these data indicate that the spatial arrangement of the insulin portion of proinsulin is

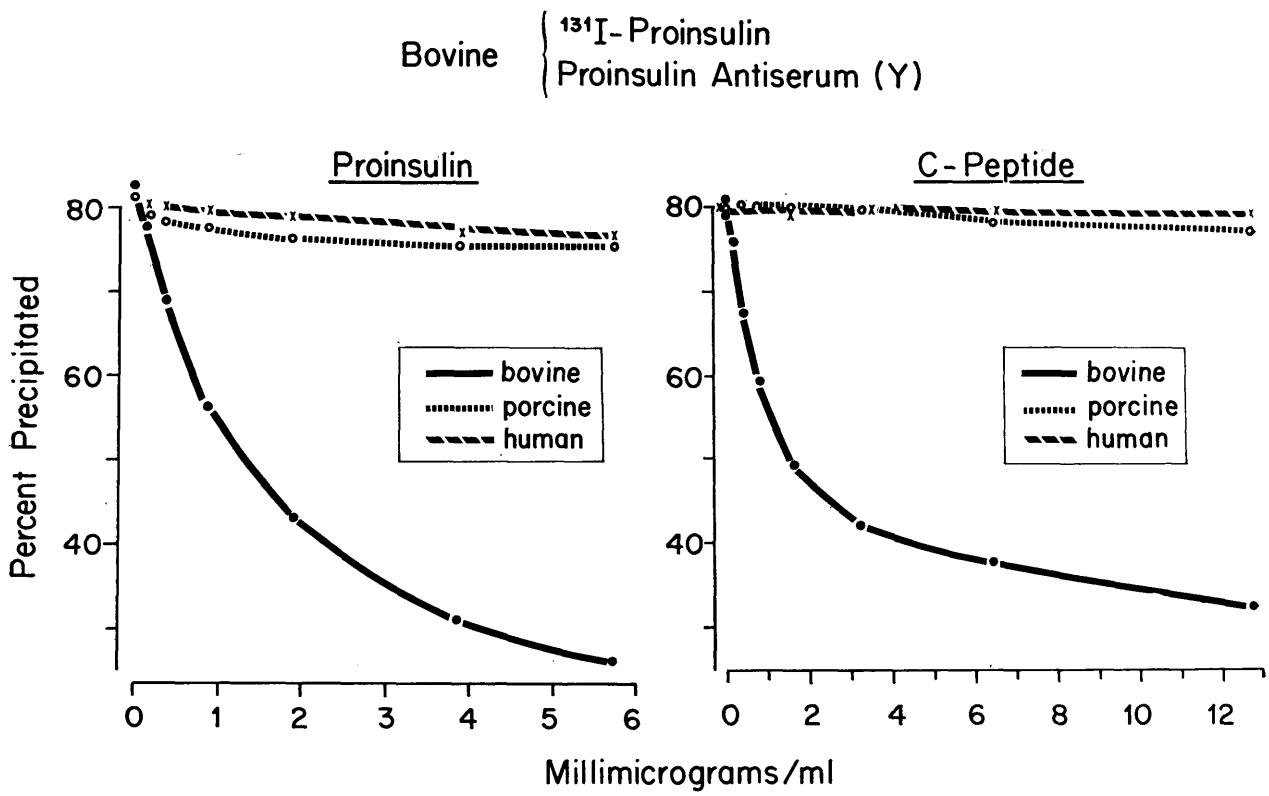

FIG. 5. Competition among bovine, porcine and human proinsulins, the respective C-peptides and bovine I-131-proinsulin for binding to antibodies in purified bovine proinsulin antiserum (Y).

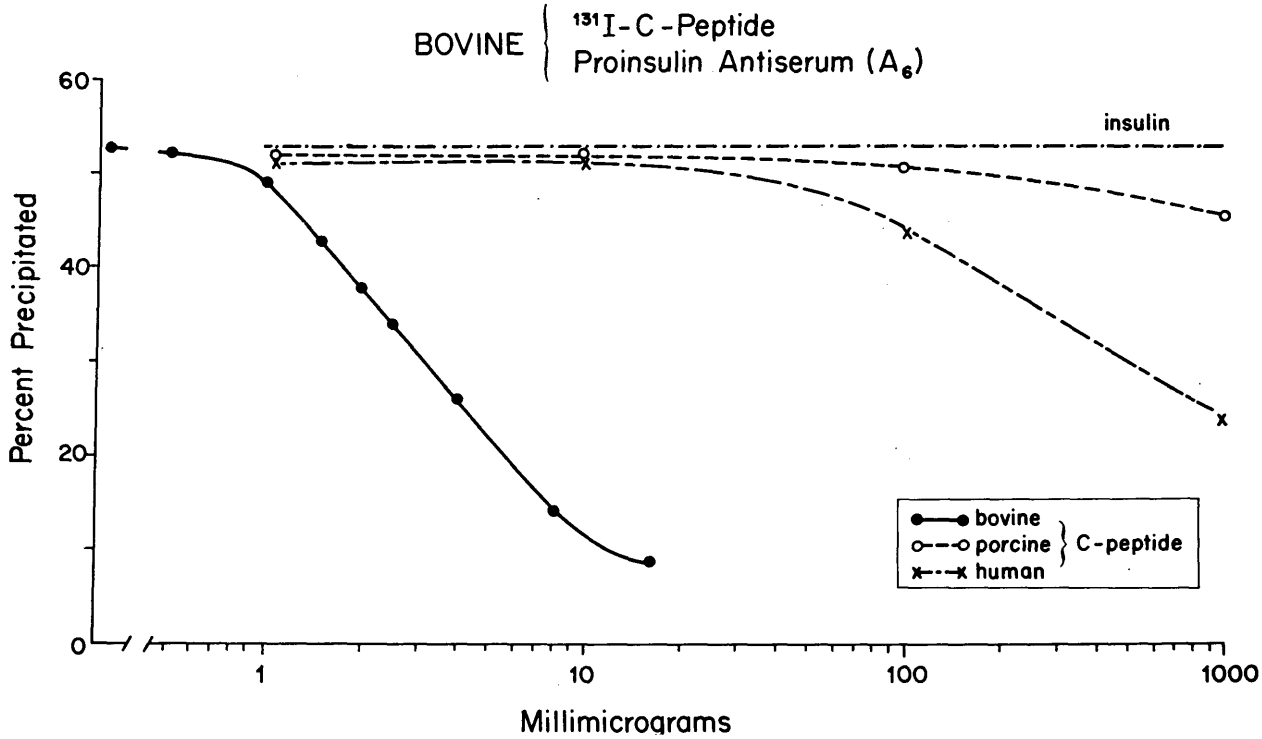

FIG. 6. Competition between increasing concentrations of C-peptides (bovine, porcine and human) and bovine I-131-tyrosylated C-peptide for binding sites in bovine proinsulin antiserum (A_6). Increasing concentrations of bovine C-peptide (1 to 10 mμg.) give a steep slope, while human and porcine C-peptides react weakly. Insulin does not react in this system.

closely similar, if not identical to that of insulin itself. Nevertheless, despite these similarities, proinsulin usually reacts less efficiently with insulin antisera than equimolar concentrations of insulin. It seems probable that the presence of the connecting segment interferes with the access of insulin antibodies to the insulin part of the proinsulin molecule, or that the attachment of the connecting segment to the termini of the A and B chains decreases the affinity of antibodies directed against these two specific areas of the insulin molecule. In addition, the physical properties of proinsulin have not yet been studied in sufficient detail to detect minor configurational differences between its insulin moiety and insulin itself.

The C-peptide is twenty-six to thirty-one amino acids in length, but does not contain tyrosine in the case of the porcine, bovine or human molecules.[5,11] Although successful iodination of secretin,[23] which also does not contain this amino acid, has been described, we elected to couple tyrosine to the C-peptide to facilitate this reaction and ensure high specific activities. The chloramine-T method of labeling was satisfactory, but purification on Bio-Gel P-30 was necessary to remove damaged products and tyrosylation side products which had been iodinated. Other proteins have also been reacted with N-carboxyanhydride derivatives of amino acids with remarkably little alteration in their biological or immunological activities. Fuchs and Sela[24] demonstrated that a poly-DL-alanyl rabbit gamma globulin derivative preserved its capacity to bind antigen, while Canfield[25] showed that human growth hormone, to which twenty-three alanine residues had been added, retained 95 per cent of its biological activity. The immunological reactivity of the bovine C-peptide was similarly unimpaired by tyrosylation and iodination.

The reactions of the four selected antisera with homologous proinsulin, insulin and C-peptide are representative of many other insulin and proinsulin antisera which we have tested.[26] Thus proinsulin cross reacts to a greater or lesser degree with different insulin antisera, while the C-peptide does not. Although antisera to proinsulin react with proinsulin, insulin and C-peptide, the degree of cross reaction with the latter two proteins varies considerably. Removal of the antibodies directed against insulin yields an antiserum which binds both proinsulin and C-peptide efficiently.

A similar result can be achieved with I-131-C-peptide and proinsulin antisera. Insulin does not compete with the label under these conditions, while proinsulin and C-peptide react similarly on a molar basis.

The amino acid compositions of the connecting segments of rat,[27] bovine,[7,28] porcine,[5] human,[11,13] and cod fish[29] proinsulin are known. Although they have some similar features, such as the large number of glycine and glutamic acid residues, the amino acid sequences of the two proinsulins which have been analyzed to date differ considerably. The connecting segments of porcine[5] and bovine proinsulin[7] vary in length (thirty-three and thirty amino acids, respectively) and the two sequences differ by about 30 per cent. The human C-peptide sequence also differs substantially from that of the porcine or bovine molecules[13] (figure 7). The structural variations are reflected in the high degree of immunological specificity shown by these proteins with proinsulin antisera. Thus, when insulin-binding antibodies have been removed from these antisera, their capacity to react with insulin as well as with proinsulins from other species is abolished. Without such treatment heterologous proinsulins react similarly to insulin over a ten to thirty-fold range.

The immunological specificity of the C-peptides isolated from human and bovine pancreatic tissue has confirmed and extended these findings. Bovine I-131-C-peptide alone binds to concentrated bovine proinsulin antisera, while high concentrations of the heterologous C-peptides only compete to a small extent with this label for binding sites in bovine proinsulin antisera. Although these results indicate that specific and sensitive immunoassays for each species of proinsulin and C-peptide will depend upon purification of sufficient protein for immunization, it is possible that an antiserum directed against those regions of the proinsulin-connecting segment, which are similar in the different species, will be produced. Proinsulin is an excellent antigen, but we have had difficulty raising antibodies to the isolated C-peptide. Preliminary polymerization or coupling to albumin will probably be necessary to enhance antibody formation, and are currently under study.

The role of proinsulin in the biosynthesis of insulin within the pancreatic beta cells is well established.[30] The single-chain precursor form appears to facilitate the formation of the correct disulfide bonds of insulin,[22] which is subsequently liberated from the larger proinsulin molecule prior to secretion. In contrast to the virtual absence of insulin-like immunological activity of the free A and B chains, the proinsulin C-peptide continues to react well with proinsulin antisera after being detached from the proinsulin molecule.[6] This apparent retention of ordered structure may be an indication of orienting and stabilizing forces inherent in its primary structure, which initiate and/or maintain correct folding of the proinsulin molecule.

Because of the considerable variability in the amino acid sequence of the connecting segment in different proinsulins, comparisons in a wide variety of species will be required to determine those features which may be essential for the correct folding of the molecule. Nevertheless, it is obvious that those regions of the C-peptide which are similar in the human, porcine and bovine molecules are immunologically insufficient to stimulate a high proportion of cross reacting antibodies.

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	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Human:	Glu	Ala	Glu	Asp	Leu	Gln	Val	Gly	Glu	Val	Gln	Leu	Gly	Gly	Gly	Pro
Porcine:	Glu	Ala	Glu	Asn	Pro	Gln	Ala	Gly	Ala	Val	Glu	Leu	Gly	Gly	Gly	Leu
Bovine:	Glu	Val	Glu	Gly	Pro	Gln	Val	Gly	Ala	Leu	Glu	Leu	Ala	Gly	Gly	Pro

	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Human:	Gly	Ala	Gly	Ser	Leu	Gln	Pro	Leu	Ala	Leu	Glu	Gly	Ser	Leu	Gln
Porcine:	Gly	—	Gly	—	Leu	Gln	Ala	Leu	Ala	Leu	Glu	Gly	Pro	Pro	Gln
Bovine:	Gly	Gly	Gly	—	—	—	—	—	Ala	Leu	Glu	Gly	Pro	Pro	Gln

FIG. 7. Comparison of the amino acid sequences of human,[13] porcine[5] and bovine[7] C-peptides. Arbitrary deletions in the porcine and bovine molecules have been assumed to enable the best comparisons to be made. The numbers refer to the amino acid positions in bovine proinsulin. The amino acids which are identical in all three C-peptides have been enclosed in boxes.

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