Radioimmunoassay of the A-chain of Insulin

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SUMMARY

A radioimmunoassay for isolated A-chain is described. The immunospecificity of A-chain is shown to vary depending on the variation in structure about the cysteine residues. The presence of one or two intrachain disulfide bonds alters the immunoreactivity greatly in a system in which A-chain with all four cysteine residues carboxymethylated is the homologous antigen. Species specificity is also suggested by differences in the immunoreactivity of the cleaved products of bovine and porcine insulin. Further attempts to assay A-chain in human plasma by immunological methods must take these two factors into consideration. DIABETES *16*:681-86, October, 1967.

Mammalian insulin is composed of two polypeptide chains. The A-chain, which has a glycine residue at its N-terminus, consists of twenty-one residues with an intrachain disulfide bridge between the two cysteine residues at positions 6 and 11 from the N-terminus. The B-chain with a phenylalanine N-terminus is composed of thirty residues and is joined to the A-chain by two disulfide bridges. In recent years, the metabolic activity of these polypeptide chains has been investigated,[1-4] and the hypothesis has been advanced that albumin-bound chains[5] or free chains[6,7] in the circulation may inhibit the action of insulin. Acute experiments in which reduced B-chain was infused into dogs have failed to show insulin antagonism as determined by blood glucose changes.[8] Demonstration of biologically important antagonism may depend, however, on more prolonged exposure to the chains or a more sensitive index of antagonism such as an elevation of plasma insulin. Attempts to demonstrate insulin antagonism by A-chain in vivo have not been made.

In order to study the biological significance of one of the component polypeptide chains of insulin, we investigated the possibility of measuring circulating A-chain by a radioimmunological method. Classical experiments by Wilson, Dixon and Wardlaw with hybrid insulins have indicated that the antigenic determinants of insulin reside on the A-chain. Cod A-bovine B hybrid insulin reacts strongly with antisera to cod insulin and weakly with antisera to bovine insulin, whereas bovine A-cod B hybrid insulin reacts strongly only with bovine insulin antisera.[9] Yalow and Berson studied the immunospecificity of various mammalian insulins with identical B-chains and A-chains of different amino acid sequences and also concluded that the antigenic determinants for insulin were on the A-chain.[10] Nevertheless, the antigenic determinants of isolated A-chain differ from those of intact insulin as indicated by the observation that antibodies to A-chain do not react with insulin[11] and antibodies to intact insulin do not react with A-chain.[10] A specific immunological assay for free A-chain seemed possible.

The present study describes our experience with a radioimmunoassay for the A-chain of insulin. Reduced *bovine* A-chain with all four cysteine residues carboxymethylated was used for immunization and standards. Free A-chain present in human biological fluids would differ from this antigen by a species difference in amino acid sequence and by variation in structure about the cysteine residues. The importance of the species difference was assessed by comparing the immunoreactivity of the products of cleavage of bovine and porcine insulin since porcine and human A-chain have identical amino acid sequences.[12] The effect of variation in structure of the cysteine residues on immunoreactivity was ascertained by testing A-sulfo derivatives of insulin with two, one, or no disulfide bridges generously supplied by Drs. H. Zahn and E. Drechsel.

MATERIALS AND METHODS

Bovine insulin-carboxymethylated chain A-reduced (Lot R 2208) was produced in the Mann Research Laboratories by the method of Crestfield et al.[13]

The A-sulfo derivatives of bovine insulin with two, one, or no disulfide bridges were kindly supplied by Drs. Zahn and E. Drechsel from Deutsches Wollforschunginstitut, Aachen, Germany.

Crystalline pork insulin (Lot 81894) was kindly supplied by Dr. W. R. Kirtley of Eli Lilly Company, Indianapolis, Indiana.

Crystalline bovine insulin (Lot R 2772) was purchased from Mann Research Laboratories.

I-125 was purchased from IsoServe Inc. as NaI.

From the Division of Endocrinology and Metabolism, Louisiana State University School of Medicine, New Orleans.

Bovine Fraction V albumin was obtained from Nutritional Biochemicals Corporation.

Dithiothreitol (Cleland's reagent) was obtained from Calbiochem Company.

Sephadex G-25 and G-75 were obtained from Pharmacia, Uppsala, Sweden.

Dialysis tubing, size 8 was purchased from Visking Company, Division of the Union Carbide Corporation.

The radioimmunological method for the determination of insulin A-chain employed in this paper is similar to the Yalow and Berson procedure for insulin[14] as modified by Genuth, Frohman, and Lebovitz.[15]

Immunization of guinea pigs. Five guinea pigs were injected subcutaneously at monthly intervals with 0.75 mg. carboxymethylated A-Chain of bovine insulin emulsified with complete Freund's adjuvant. Ten days after the fourth booster injection, the guinea pigs were bled and the sera were tested for the presence of antibodies. Only two of the five animals developed sufficient antibody concentrations to bind 50 per cent or more tracer A-chain at a serum dilution of 1:50. The antiserum which gave the assay the greatest sensitivity was used at a 1:200 dilution.

Preparation of tracer A-chain I-125. Tracer bovine carboxymethylated A-chain was prepared by the method of Greenwood, Hunter and Glover[16] using 1 mc. I-125 and 2.5 μg A-chain. Separation of labeled A-chain from inorganic I-125 was accomplished by placing 0.2 ml. of the reaction mixture on a G-25 Sephadex column (12 × 1 cm.). Prior to gel filtration, 0.1 ml. diluting fluid containing 1 per cent bromphenol blue stained albumin had been added to the reaction mixture to serve as a marker. When one-milliliter aliquots were collected, two peaks of radioactivity were observed. The first peak occurred just prior to and coincident with the appearance of the stained albumin and the second peak followed several milliliters after the void volume. The first peak contained 75 per cent of the radioactivity to give a specific activity of approximately 300 μc./mg.

Purification to remove damaged components was achieved by incubating small amounts of labeled polypeptide (1-2 μc.) with normal guinea pig serum in a total volume of 0.25 ml. for twenty-four hours at 4° C. The incubation mixture was then placed on a 12 × 1 cm. G-75 Sephadex column and eluted. The radioactive fraction after the void volume was used for tracer. The following solution was employed for elution of all columns: 0.025 M phosphate buffer *p*H 7.5, 0.9 per cent NaCl, 0.1 per cent methiolate, and 0.2 per cent albumin. For dilution of tracer, antisera, standards, and test samples, the albumin concentration was raised to 1 per cent and normal guinea pig serum was added to a concentration of 2 per cent.

Immunoassay procedure. The antiserum, which was used at a 1:200 dilution, was made up with EDTA so that the final incubation mixture would contain .01 M EDTA. The empirical use of EDTA increased the slope of the standard curve. Greater consistency was observed when the tracer was purified daily by gel filtration and the standards were prepared fresh daily.

Tracer (.05 ml.), standard or specimen (0.5 ml.), and antibody (0.1 ml.) were mixed in 15 × 85 mm. test tubes and diluted to a final volume of 0.25 ml. with diluting fluid containing 1 per cent bromphenol blue stained albumin. These tubes, along with others serving as damage controls (without antiserum), were incubated at 4° C. for twenty-four hours. After incubation, free and antibody-bound A-chain were separated on Sephadex G-75 columns (12 × 1 cm.). Three fractions were collected from each column. The first fraction to the end of the blue dye (4-4.5 ml.) contained the antibody-bound A-chain and the next two fractions of 4 ml. each contained free A-chain. Fractions in tubes in which they had been collected, were counted in a Nuclear-Chicago autogamma counter for ten minutes under conditions giving a background of 15 cpm. Sufficient tracer was added to incubation mixtures to give a total of approximately 1,000 cpm.

The number of counts in the first fraction (antibody-bound tracer) was adjusted for the slight variation in total counts from each column. These adjusted figures were employed in calculations suggested by Hales and Randle[17] to give R values, a linear measure of isotope dilution:

$$R = C_0/C_a = \frac{C\text{-}DB}{(U\text{-}DB) \text{ or } (S\text{-}DB)}$$

where C_0 = radioactivity of tracer-antibody complexes in incubations without competing cold A-chain;

C_a = radioactivity of tracer-antibody complexes in incubations with standards or unknowns;

or where C = control (tracer + antiserum)

DB = damage blank (tracer + specimen or dilution fluid)

S = standard (tracer + A-chain standard + antiserum)

U = unknown (tracer + specimen + antiserum).

RESULTS

Radioimmunoassay of carboxymethylated A-chain of bovine insulin

A representative standard curve using homologous carboxymethylated A-chain of bovine insulin as standard is shown in figure 1. The R values on the vertical axis are the ratios of antibody-bound tracer in the absence of unlabeled A-chain (C_0) to antibody-bound tracer in the presence of A-chain standards or unknowns (C_a). A linear relationship is observed between R values and A-chain concentrations over the range of 10-100 nanograms per milliliter. Cross reactivity between bovine insulin and antibody to carboxymethylated A-chain was practically nil. At .1 mg. per milliliter concentration, bovine insulin gave R value of 1.29; at .01 mg. per milliliter or less, the R value did not exceed unity. At the former concentration bovine insulin may be contaminated with detectable amounts of A-chain.

Detection of A-chain after cleavage of bovine and porcine insulin with dithiothreitol. Bovine and porcine insulin were incubated with dithiothreitol (Cleland's reagent) to cleave insulin into its component polypeptides. After a reaction time of one hour, the mixture was dialyzed for five hours against running distilled water to remove dithiothreitol as this reagent in high concentrations interferes with the immunoassay. No attempt was made to prevent reoxidation of the reduced chains during dialysis. Radioimmunoassay of dilutions of the dialyzed solutions were performed and the results are shown in figure 2. Bovine and porcine insulin solutions not exposed to dithiothreitol gave R values of unity at a concentration of .01 mg. per milliliter or slightly above unity at a concentration of .1 mg. per milliliter (Bovine insulin, $R = 1.29$; porcine insulin, $R = 1.31$). Although the yield of immunoreactive material was small, the increase in R values after reaction with dithiothreitol confirms that the assay detects a component of insulin, presumably A-chain. R values of dilutions of dithiothreitol treated bovine insulin solutions were higher than those of corresponding dilutions of similarly treated porcine insulin.

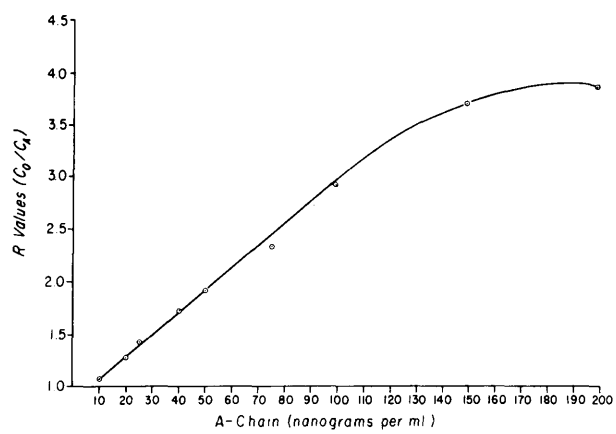

FIG. 1. Representative standard curve for radioimmunoassay of the A-chain of insulin. Standard is reduced carboxymethylated A-chain of bovine insulin.

Loss by dialysis did not account for the poor yield of immunoreactive material after cleavage of insulin as this material diffuses poorly through dialysis membrane. In one experiment in which 1 ml. of a mixture of bovine insulin and dithiothreitol were dialyzed in 5 ml. distilled water for five hours, the R value of the dialysate was 1.39 compared to 3.81 of the dialyzed solution. Monomer A-chain has a molecular weight of approximately 2,750 and should pass easily through a dialysis membrane. However, all of the detectable immunoreactive material in the cleaved insulin solutions and in the carboxymethylated A-chain standards is eluted from a G-25 Sephadex column within the void volume and from a G-75 column after the void volume. This indicates a molecular weight between 5,000 and 50,000 which may account for the observation that little immunoreactive material escapes into the dialysate.

Chemical structure of A-chain and immunospecificity.

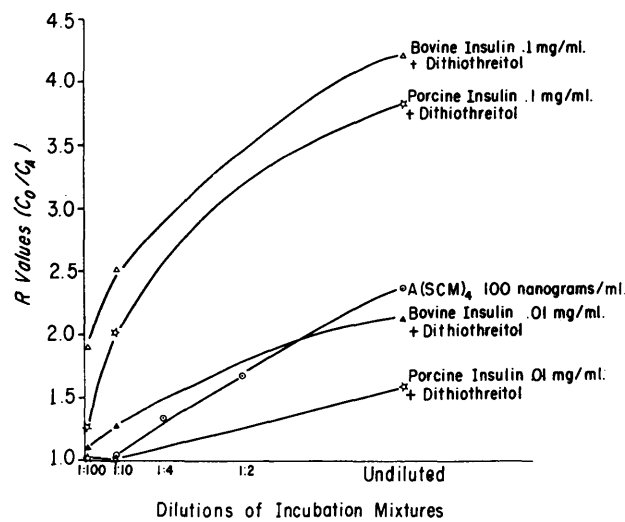

FIG. 2. Immunoreactivity of the products of cleavage of bovine and porcine insulin. Dithiothreitol 0.1 mg. was added to 1 ml. insulin solutions of the concentrations shown. After a reaction time of one hour at room temperature, the solutions were dialyzed against running distilled water for five hours. The dialyzed solutions were diluted with standard diluting fluid and assayed. $A(SCM)_4$ standards are included for comparison.

The homologous antigen in the present immunoassay is A-chain in which all four cysteine residues have been reduced and carboxymethylated. Before attempting to measure A-chain in biological systems, an investigation of the effect of structural changes in the cysteine residues on immunospecificity seemed important. A-sulfo derivatives of insulin with two, one, or no disulfide bridges which have the structures shown in figure 3 were kindly supplied to us by Drs. H. Zahn and E. Drechsel.

The inhibition of binding of tracer A-chain with antibody by the various heterologous A-chain derivatives and unlabeled homologous A-chain is shown in figure 4A-C. All compounds are bovine insulin derivatives and all were made in the same laboratory with the exception of $A(SCM)_4$. The A-sulfo derivative with completely ruptured disulfide bridges, $A(SSO_3H)_4$, inhibited the formation of tracer-antibody complexes as well or slightly better than did the homologous A-chain standards, $A(SCM)_4$. Less immunoreactivity was observed with the A-sulfo derivative of insulin with intact 6, 11 disulfide bridge, $A(SS)(SSO_3H)_2$. Compound $A(SS)_2$ with two intact disulfide bridges did not react at all in the concentrations employed in figure 4 but gave a small R value (1.21) at a concentration of 0.1 mg. per milliliter. Rupture of the disulfide bonds of

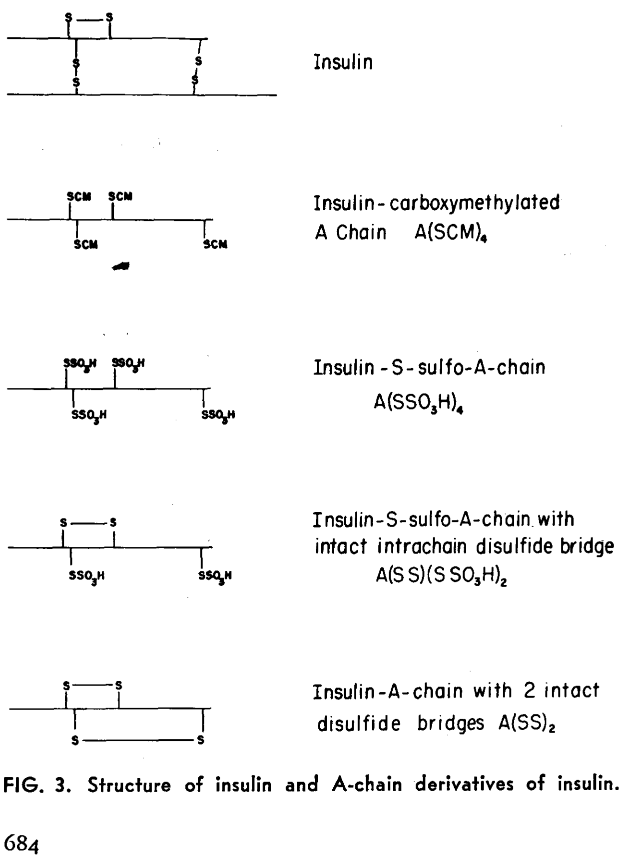

FIG. 3. Structure of insulin and A-chain derivatives of insulin.

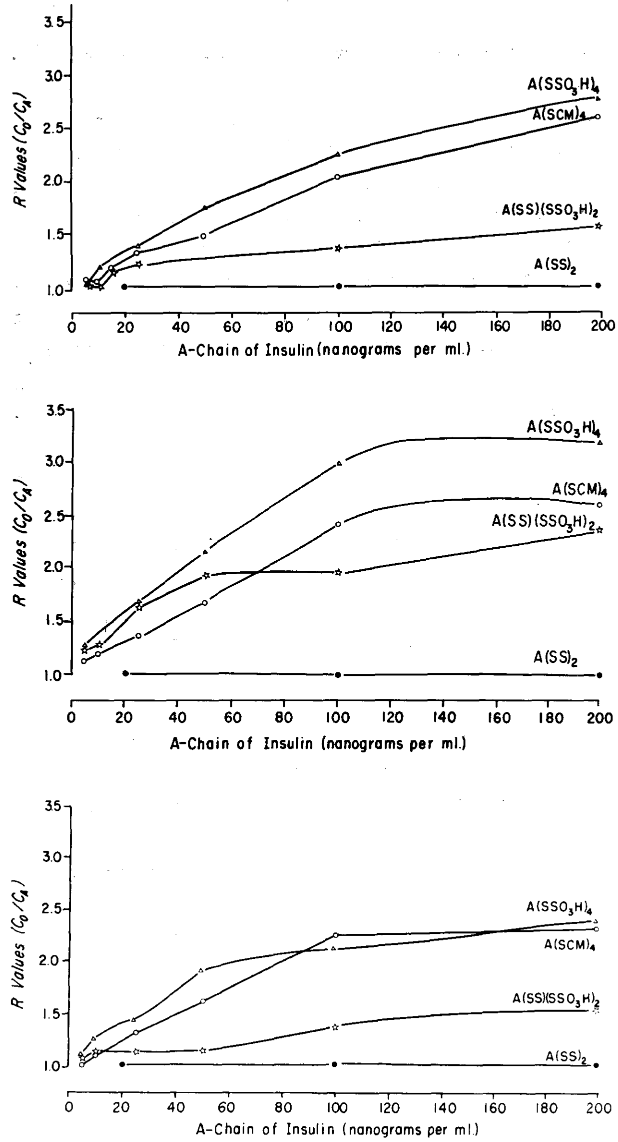

FIG. 4 A-C. Immunoreactivity of A-chain derivatives of bovine insulin. Each figure represents dose-response curves run simultaneously.

$A(SS)_2$ by treatment with dithiothreitol resulted in a compound reactive with $A(SCM)_4$ antiserum (table 1).

In addition to variations in immunoreactivity related to specific structural changes, compounds with supposedly identical structures made in separate laboratories gave slightly different results (figure 5). A-sulfo derivatives of bovine insulin produced in the laboratory of Drs. Zahn and Drechsel and in Mann Research Laboratories had similar immunoreactivity at lower concentrations, but some variation was observed at higher concentrations.

TABLE 1

Effect of dithiothreitol on immunoreactivity of $A(SS)_2$ in $A(SCM)_4$ system

Concentration $A(SS)_2$ (mg./ml.)	Dithiothreitol (mg./ml.)	Dialysis*	Dilution†	*R* value (C_0/C_x)
0.1	None			1.21
0.01	None			1.05
0.1	0.1		1:100	2.46
0.1	0.1		1:1000	1.69
0.01	0.1			2.22
0.01	0.1	+		1.70
0.01	0.1	+	1:10	1.26
0.01	0.1	+	1:100	.99

*Dialysis was performed where indicated by placing 1 ml reaction mixture, $A(SS)_2$ plus dithiothreitol, in Visking dialysis tubing and dialyzing for five hours against running distilled water. Corrections were not made for small increase in volume of dialyzed material.

†Dilutions made after reaction for two hours or after dialysis when performed.

Radioimmunoassay of A-chain in plasma. Results so far have been disappointing. Most blood samples have *R* values greater than unity, but in a region of the curve ($R = 1.0 - 1.24$) which is not believed sensitive enough to assign absolute A-chain values. Two observations have suggested that the *R* values of plasma samples are not a measure of A-chain concentrations. No increase in *R* values has been observed in samples taken after a glucose load. Also the *R* value of plasma from a patient with an islet cell carcinoma having consistently elevated fasting plasma insulin levels (60-80 μU./ml.) was not increased. Attempts are being made to obtain a better antiserum which will make the assay more sensitive. The lowest concentration at which the present assay is sensitive is approximately twenty times the concentration of fasting plasma insulin in normal persons.

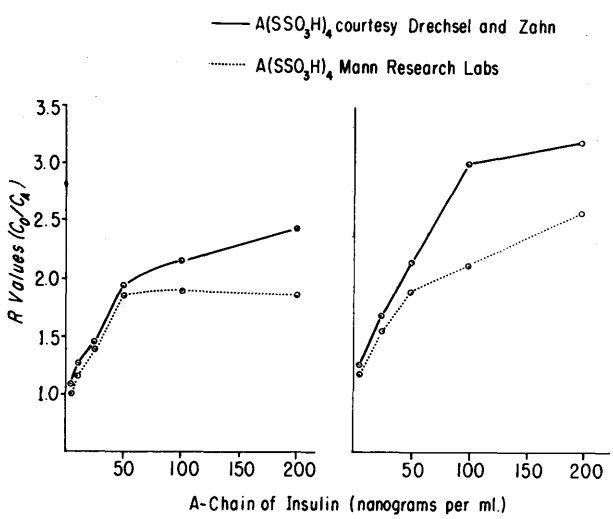

FIG. 5. Immunoreactivity of the A-sulfo derivative of bovine insulin made in two separate laboratories. Dose-response curves on two separate days are shown.

DISCUSSION

Radioimmunoassay of the A-chain of insulin presents certain problems which are not encountered in the immunoassay of insulin. A-chain is prepared by splitting insulin into its component polypeptide chains by sulfitolysis and depending on reaction conditions, A-chain having one, two, or no disulfide bridges may be formed. The cysteine residues not involved in disulfide bonds are sulfonated by the sulfitolysis procedure after which they may be reduced and carboxymethylated. In the present study, carboxymethylated A-chain with no disulfide bridges was chosen for immunization.

In a system where sulfonated A-chain, $A(SSO_3H)_4$, was the homologous antigen, Yagi, Maier, and Pressman have shown that $A(SCM)_4$ was only 25 per cent as active in inhibiting the formation of tracer-antibody complex.[11] These authors concluded that the nature of the altered cysteine residues affects the antigenic determinant region of the A-chain. In the present study, the A-sulfo derivative, $A(SSO_3H)_4$, had the same or slightly greater activity than homologous $A(SCM)_4$ chain in inhibiting reaction of labeled $A(SCM)_4$ chain with its antiserum. Our results do not support involvement of the S-sulfonate or S-carboxymethyl groups in the determinant region of A-chain. However, differences in immunoreactivity of A-chains with the same chemical formula prepared in different laboratories as shown in this study make one cautious in interpreting changes in immunoreactivity strictly on the basis of presumed primary structure. Subtle changes in conformation or polymerization which might occur during preparation may also affect immunoreactivity.

A more important determinant of immunoreactivity than the different substituents on the reduced cysteine residues is the presence or absence of disulfide bonds between cysteine residues. A single disulfide bridge between cysteine residues at positions 6 and 11 reduced reactivity of the compound in the $A(SCM)_4$ system considerably, whereas the compound with a second disulfide bond between cysteine residues at position 7 and 20 did not react at all. The disulfide bridges alter the tertiary structure greatly which accounts for changes in

immunoreactivity. This apparent immunospecificity of each variant of A-chain presents a problem in the assay of A-chain in biological fluids since its exact structure if present in blood or other biological fluids is unknown.

A second problem in the immunoassay of A-chain is exemplified in experiments in which insulin was cleaved with dithiothreitol. Cleavage of bovine insulin resulted in an apparent yield of A-chain no greater than 3 per cent. The A-chain formed under these circumstances (dithiothreitol was dialyzed away) was free to combine with B-chain or aggregate with other A-chains to form many different types of polymers. Polymerization and possibly low immunoreactivity of the particular A-chain variants formed may explain the low yields. Gel filtration indicated some polymerization even in standards employed for the radioimmunoassay. If the component polypeptides of insulin are released into the circulation, A-chain containing aggregates which are poorly reactive in the immunoassay system may be formed.

The tendency of reduced A-chain to polymerize raises a question concerning the use of Sephadex to separate free from antibody-bound A-chain. If free A-chain formed polymers of molecular weight greater than 50,000, they would be eluted within the void volume of the G-75 column and contribute to the antibody-bound fraction. In radioimmunoassay work, the degree of polymerization of tracer only is pertinent as the determinations depend on the ratio of free to antibody-bound tracer. Since all of the detectable immunoreactive material in solutions of carboxymethylated A-chain used for standards and tracer is eluted from G-75 columns after the void volume, polymers with molecular weight greater than 50,000 either do not form or they are not immunoreactive. Additional evidence against polymerization of tracer to the extent that free tracer might be eluted in the antibody-bound fraction is that less than 5 per cent of the tracer radioactivity was eluted in the void volume fraction from damage blanks which were run with all test solutions.

Dithiothreitol treated bovine insulin exhibited approximately tenfold greater reactivity in the immunoassay system than similarly treated porcine insulin. Although less efficient cleavage or greater polymerization of the end products of the porcine mixture are possible causes, species differences in the amino acid sequence of A-chain is a more attractive explanation for the variance in immunoreactivity. Porcine and bovine A-chain differ in the amino acid residues at position 8 and 10. Since porcine and human A-chain have identical sequences, future attempts to detect A-chain in human plasma by immunoassay should begin with porcine A-chain for immunization and standards.

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