Circular Dichroic and Immunologic Studies of Structure Relationships of Insulin and Derivatives†

Thomas M. Brugman*,‡ and Edward R. Arquilla

ABSTRACT: Desoctapeptide- and desalanine-desasparagine insulins have similarly altered circular dichroic spectra compared to insulin. In contrast, the results of immunological studies clearly demonstrate significant conformational differences between these two types of structurally modified insulin molecules. Removal of alanine B-30 alone from insulin yields a derivative indistinguishable from insulin. It is concluded, therefore, that changes in structure of desalanine-desasparagine-insulin are due to the removal of Asn A-21 and not Ala B-30. The presence of a one-way cross-reaction between insulin and antibodies against desalanine-desasparagine-insulin indicates the presence of an antigenic site on desalanine-desasparagine-insulin which is not expressed when insulin is used as the antigen. This site, possibly involving the dimer region, has a similar conformation in both molecules. The B-chain tyrosines of desalanine-desasparagine-insulin were shown to be more accessible for iodination than the same tyrosines of insulin. It is concluded that loss of Asn A-21 results in conformational alterations that weaken the relationship of the COOH terminal of the B chain with the core of the molecule, causing decreased aggregation and exposure of the dimer region as an antigenically active site.

The structural and functional integrity of the insulin molecule is dependent on the interaction of the COOH-terminal B chain with the core of the molecule (Carpenter, 1966; Arquilla and Bromer, 1967; Arquilla *et al.*, 1972; Hodgkin and Mercola, 1972). This interaction results in a hydrophobic center rich in aromatic amino acids (Arquilla *et al.*, 1969; Hodgkin and Mercola, 1972). Specific molecular alterations which affect the interaction of the COOH-terminal B chain with the core of the molecule are, therefore, of interest. The two derivatives most widely studied in this regard have been desalanine (B-30)-desasparagine-(A-21)-insulin and desoctapeptide (B-23 to B-30)-insulin, both of which have attenuated biologic activity (Carpenter, 1966; Arquilla *et al.*, 1969), as well as similarly altered circular dichroic (CD) spectra (Morris *et al.*, 1970), immunologic properties (Arquilla *et al.*, 1969), and ability to aggregrate (Goldman, 1967).

We previously proposed that the structural role of Asn A-21 may involve stabilization of the COOH-terminal B chain *via* electrostatic interaction with the guanidinium residue of Arg B-22, suggesting that deletion of Asn A-21, or the octapeptide of the COOH-terminal B chain may induce similar conformational changes.

In order to investigate possible structural similarities between insulin, desoctapeptide-insulin, and des-Ala-des-Asp-insulin, antisera prepared against each of them were tested for cross-reactivity. These experiments have shown that desoctapeptide- and des-Ala-des-Asp-insulins differ immunologically and hence, structurally in spite of their similar circular dichroic spectra. In addition, we demonstrate an antigenic determinant which is expressed in des-Ala-des-Asp-insulin but not in native insulin.

Materials and Methods

Insulin. Zinc-free insulin was prepared by the method of Shuytman (1955) using Lilly zinc-crystallized bovine insulin (lot T2842). Purity was confirmed by amino acid analysis and electrophoresis in polyacrylamide gel (Figure 1). Bovine zinc-free insulin was used as the reference in all comparisons with desoctapeptide- and des-Ala-des-Asp-insulins which include circular dichroism, immunological activities, and acrylamide gel electrophoresis. It is referred to throughout the manuscript as insulin.[1]

Desalanine-insulin. Desalanine-insulin (lot 516-6213-290) was a gift of the Eli Lilly Co.

Desoctapeptide-insulin. The two samples of desoctapeptide-insulin used in these experiments were prepared as described by Bromer and Chance (1967). No discernable differences or impurities of these two preparations were noted by amino acid analysis, immunological activity, acrylamide gel electrophoresis (Figure 1), or circular dichroism.

Desalanine-desasparagine-insulin. Two samples of des-Ala-des-Asp-insulin were prepared by the exhaustive digestion of insulin with carboxypeptidase A as described by Slobin and Carpenter (1963). Purifications of des-Ala-des-Asp-insulin from the lyophilized reaction mixture was accomplished by column chromatography on Sephadex G-75 equilibrated with 50% acetic acid (v/v). The lyophilized reaction mixture was dissolved in 50% acetic acid with stirring overnight at 4° at an approximate concentration of 5 mg/ml. Approximately 30 ml (±5 ml) of this solution was applied to a 3.5 × 75 cm column with a flow rate of 20 ml/hr. The enzyme peak emerged in the void volume with des-Ala-des-Asp-insulin following immediately. The derivative peak was pooled and lyophilized.

Des-Ala-des-Asp-insulin migrated slower than insulin in acrylamide gel electrophoresis in spite of having a charge equal to insulin (Figure 1). This slower rate of migration can be

† From the Department of Pathology, University of California, Irvine, California. *Received August 28, 1972.* Supported by a Public Health grant (AM13441-04) and the Diabetes Association of Southern California. A preliminary report of part of these data was presented in Atlantic City, April 1970 (*Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29* (2), 313).

‡ Research fellow of Training Grant GM02049-03; Department of Pathology, University of California, Irvine, Calif.

[1] Abbreviations used are: des-Ala-des-Asp-insulin, desalanine-(B-30)-desasparagine-(A-21)-insulin; desoctapeptide-insulin, desoctapeptide-(B-23 to B-30)-insulin; desalanine-insulin, desalanine-(B-30)-insulin.

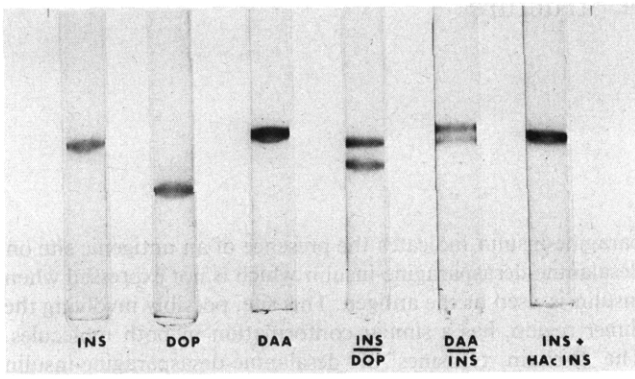

FIGURE 1: Acrylamide gel electrophoresis of insulin (Ins), desoctapeptide-insulin (DOP), des-Ala-des-Asp-insulin (DAA), and an insulin–acetic acid treated insulin (HAc Ins) mixture. Samples of 0.05 mg were applied to each gel and electrophoresed at 4°, pH 8.4 at 3 mA/gel. The increased negative charge of desoctapeptide-insulin due to the loss of Lys B-29 accounts for the more rapid migration toward the anode. The migration of des-Ala-des-Asp-insulin is retarded compared to insulin (see text).

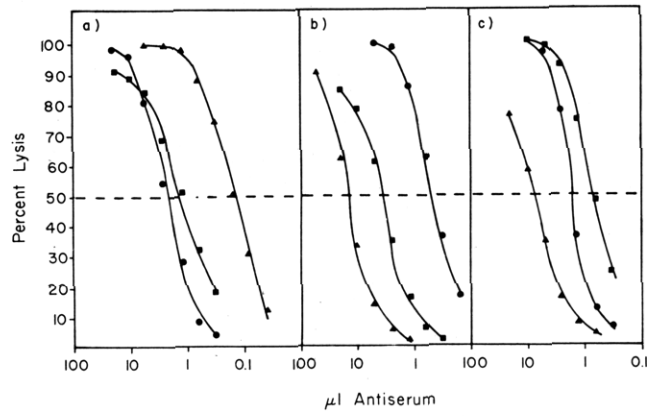

FIGURE 2: Heterologous immune hemolysis of insulin (▲), desoctapeptide-insulin (●), and des-Ala-des-Asp-insulin (■) antisera with insulin (a), desoctapeptide-insulin (b), and des-Ala-des-Asp-insulin (c) coated red cells. The per cent cross-reactivity was calculated as explained in Table II.

attributed to the less compact structure of des-Ala-des-Asp-insulin.

Acrylamide gel electrophoresis performed on a mixture containing insulin filtered through Sephadex G-75 equilibrated with 50% acetic acid to which was added an equal amount of native zinc-free insulin, migrated as a single band with the mobility identical with insulin (Figure 1). The acid-treated insulin was also found to be identical with insulin by circular dichroism and immunologically. It is therefore likely that conformational changes noted in des-Ala-des-Asp-insulin are not the result of the method of preparation and purification.

Acrylamide gel electrophoresis was performed in 7% polyacrylamide gel (pH 8.4) (Zweig and Whitaker, 1967) for 70 min at 3 mA/gel. The gels were stained in 0.5% acid Alizarin Blue BB (Allied Chemical) in 7% acetic acid and destained in 7% acetic acid. It was necessary to apply current to the gels for a minimum of 2 hr prior to applying the samples in order to avoid persulfate oxidation and the appearance of an artifactual component in the samples tested.

Antisera to Insulin, Desoctapeptide-, and Desalanine-desasparagine-insulins. Immunization of adult male mongrel guinea pigs with either zinc crystallized insulin, desoctapeptide-, or des-Ala-des-Asp-insulins was accomplished by injecting 0.125 mg of antigen in 0.2 ml of complete Freund's adjuvant into each paw pad. Fourteen days later, three equally spaced booster injections were given intradermally during a 7-day period. Each guinea pig immunized with insulin was given 0.1 mg/booster. Due to the decreased antigenicity of desoctapeptide- and des-Ala-des-Asp-insulins, animals immunized with these two antigens were given 0.2 mg/booster. Seventeen days later this procedure was repeated. Ten days following the last booster injection, 1-ml blood samples were taken from the hind leg of each animal and titrated for antibody levels.

At this point, guinea pigs immunized with des-Ala-des-Asp-insulin showed little antibody activity and it was necessary to administer another set of three boosters. Not until 72 days following the initial immunization were sera with significant, but low, des-Ala-des-Asp-insulin antibody titers found. Continued boosting was found to have little effect on antibody levels.

When satisfactory antibody levels were obtained, guinea pigs were exsanguinated immediately following the titration. Blood samples were allowed to clot at room temperature for 1 hr and then overnight at 4°. The following day the serum was separated by centrifugation and the complement was inactivated by heating to 56° for 30 min. Samples were then divided into aliquots and stored at −80°.

The insulin antiserum used was a pool from seven guinea pigs. Each of the desoctapeptide and des-Ala-des-Asp-insulin antisera used were pools from three of ten guinea pigs immunized.

Antibody Titration. The immunological studies were based on the passive immune hemolysis reaction. The amount of hemoglobin released following the reaction of antibodies with antigen-coated red cells in the presence of excess complement has proven to be an accurate measure of relative antibody concentration (Arquilla and Stavitsky, 1956; Arquilla and Finn, 1963; Arquilla *et al.*, 1966). Levels of insulin, desoctapeptide-insulin, and des-Ala-des-Asp-insulin antibodies in the respective antisera were measured in this manner.

The immunological indicator used in these experiments consisted of sheep red blood cells to which insulin, desoctapeptide-insulin, or des-Ala-des-Asp-insulin was covalently conjugated with bisdiazobenzidine (Arquilla and Finn, 1965). Titrations of the antisera were performed by serially diluting each antiserum. A 0.1-ml volume of each dilution of antiserum was added to 0.1 ml of Veronal-buffered saline (pH 7.4), containing 5×10^{-4} M $MgCl_2$, 1.5×10^{-3} M $CaCl_2$, and 1.5 g of bovine albumin per l. (Kabat and Mayer, 1961). The appropriate homologous red cell conjugate in 0.1 ml (8.7×10^6 red cells) was then added to each of the various antiserum dilutions and the suspension was incubated at 4° for 1 hr in a New Brunswick Scientific gyrotory water-bath shaker. Excess complement (3 units) in 0.1 ml was then added and the incubation was resumed for another 30 min in a second gyrotory shaker at 37°, at which time the reaction was immediately stopped by adding 1.0 ml of cold (4°) 0.01 M phosphate-buffered saline at pH 7.4. The nonhemolyzed red cells were pelleted by centrifugation at 1000*g* for 10 min at 4°. The supernatants were decanted and the hemoglobin was

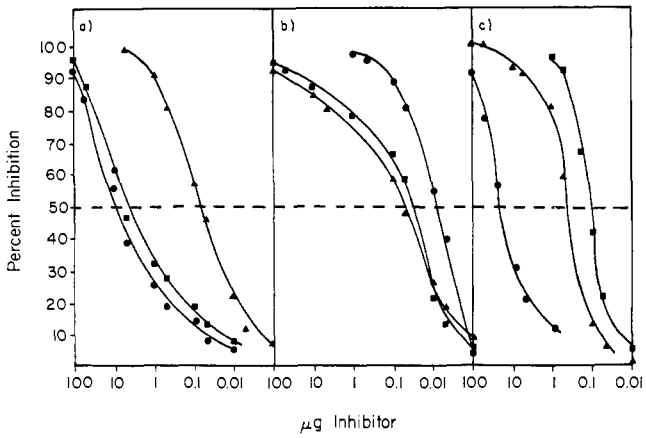

FIGURE 3: Inhibition of immune hemolysis with insulin (▲), desoctapeptide-insulin (●), and des-Ala-des-Asp-insulin (■). (a) Homologous insulin system: pooled insulin antiserum and insulin-coated red cells. Insulin inhibition was from 5.0 to 0.001 μg. Desoctapeptide-insulin and des-Ala-des-Asp-insulin inhibition was from 100 to 0.01 μg. (b) Homologous desoctapeptide insulin system: pooled desoctapeptide-insulin antiserum and desoctapeptide-insulin coated red cells. Desoctapeptide-insulin inhibition levels varied from 1.0 to 0.001 μg. Insulin and des-Ala-des-Asp-insulin varied from 100 to 0.001 μg. (c) Homologous des-Ala-des-Asp-insulin system: pooled des-Ala-des-Asp-insulin antiserum and des-Ala-des-Asp-insulin coated red cells. Inhibition with des-Ala-des-Asp-insulin varied from 1.0 to 0.01 μg. Insulin and desoctapeptide insulin levels varied from 100 to 0.01 μg and 100 to 1 μg, respectively. For each experiment the per cent cross-reaction of an antiserum with a heterologous inhibitor was calculated as explained in Table I. The activity of the homologous inhibitor was taken as 100%.

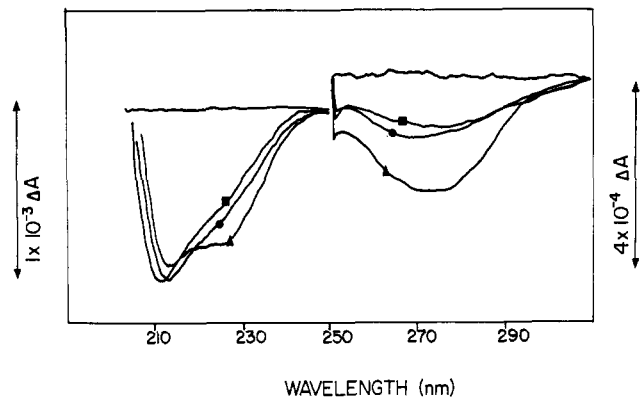

FIGURE 4: The near- and far-uv CD of insulin, desoctapeptide-insulin and des-Ala-des-Asp-insulin. Below 250 nm, the concentration was 1.7×10^{-4} M with an optical path length of 2.0 mm. Above 250 nm, the concentration was 3.0×10^{-4} M with a 5.0-mm optical path length. The circular dichroic bands at 222 and 211 nm, indicative of α-helix content in insulin (▲), are attenuated in desoctapeptide-insulin (●), and des-Ala-des-Asp insulin (■). The cotton effect noted in the near uv CD at 276 and 283 nm, due primarily to tyrosine, is reduced and shifted to the blue region for both insulin derivatives. Spectra were recorded with respect to the base-line signal obtained with buffer alone.

released at each antiserum dilution was measured spectrophotometrically at 414 nm. The relative amount of hemoglobin released (per cent lysis) was plotted against microliters of the antiserum at each of the respective dilutions (see Figure 2, homologous antiserum).

Antibody activity of each of the three antisera is expressed as the microliters of antiserum necessary to hemolyze 50% of the homologous red cell conjugate and was found to be 0.14, 0.45, and 0.65 μl for insulin, desoctapeptide-insulin, and des-Ala-des-Asp-insulin antisera, respectively.

Immune Hemolysis Inhibition. The passive immune hemolysis described above has been shown to be specifically inhibited by free homologous antigen. In the presence of free homologous antigen or other cross-reacting materials, a portion of antibodies in a given antiserum dilution will complex with these materials, resulting in a decreased number of antibodies free to react with the red cell conjugates. The degree of immune hemolysis inhibition can be quantitated by measuring the decrease in the amount of hemoglobin released.

The immune hemolysis inhibition used in these studies employs 8.7×10^6 red cells coated with the homologous antigen, sufficient antiserum to lyse 80% of the red cells, three units of complement, and variable amounts of free homologous or heterologous inhibitor. The reaction is performed in a manner identical with the antibody titration. The degree of cross-reaction of insulin, desoctapeptide-insulin, and des-Ala-des-Asp-insulin is expressed as a molar ratio of homologous antigen (for example, des-Ala-des-Asp-insulin in the des-Ala-des-Asp-insulin immune system) to heterologous antigen (insulin or desoctapeptide-insulin) required for 50% inhibition (Figure 3).

Heterologous Immune Hemolysis. Differences in antigenic determinants on insulin, desoctapeptide-insulin, and des-Ala-des-Asp-insulin were also demonstrated by heterologous immune hemolysis. In these experiments, the ability of the antisera to lyse red cells coated with a heterologous derivative was measured. These reactions were performed in a manner identical with the antibody titration. The per cent cross-reactivity between an antiserum and heterologous red cell conjugate was calculated from the ratio of the microliters of an antiserum required for 50% lysis of homologous red cell conjugate to the microliters of that same antiserum needed for 50% lysis of heterologous red cell conjugates.

Ultraviolet circular dichroism spectroscopy (uv CD) was performed with a Beckman CD spectrophotometer. All spectra used for analysis were averaged by a Hewlett-Packard signal analyzer from at least 200 scans.

Results

Near- and Far-Uv CD of Insulin, Desoctapeptide-, and Desalanine-desasparagine-insulins. The far-uv CD spectrum of desoctapeptide-insulin is similar to the previous CD spectrum we reported (Mercola *et al.*, 1967; Arquilla *et al.*, 1969). The far-uv CD spectrum of des-Ala-des-Asp-insulin is seen to closely resemble the desoctapeptide-insulin spectrum (Figure 4). The $n-\pi^*$ transition at 222 nm present in insulin is markedly attenuated in both desoctapeptide- and des-Ala-des-Asp-insulins.

The near-uv CD spectra of these two derivatives are also similar; however, they both differ from that of insulin (Figure 4). They are reduced in intensity and shifted to the blue region, suggesting that in both derivatives the tyrosyl environment is altered.

Immunological Cross-Reactivity of Insulin, Desoctapeptide-, and Desalanine-desasparagine-insulins. A limited cross-reaction of desoctapeptide-insulin antiserum with des-Ala-des-Asp-insulin and des-Ala-des-Asp-insulin antiserum with desoctapeptide-insulin was observed in both immunological systems (Tables I and II). The antigenic determinants on desoctapeptide-insulin are therefore significantly different than the antigenic determinants on des-Ala-des-Asp-insulin.

TABLE I: Per Cent Immunologic Cross-Reaction of Insulin, Desoctapeptide-insulin, and Desalanine-desasparagine-insulin by Immune Hemolysis Inhibition.[a]

	Insulin Antibody	Desocta-peptide-insulin Antibody	Des-Ala-Des-Asp-insulin Antibody
Insulin	100	5.0	33.7
Desoctapeptide-insulin	3.2	100	0.5
Des-Ala-des-Asp-insulin	3.4	15.2	100

[a] Free inhibitor was added to the corresponding antibody–homologous antigen conjugated red cell system. The per cent cross-reaction is calculated from the ratio of the amount of homologous inhibitor to the amount of heterologous inhibitor required to inhibit 50% of the immune hemolytic activity induced by a given dilution of antiserum.

TABLE II: Per Cent Immunologic Cross-Reaction of Insulin, Desoctapeptide-insulin, and Desalanine-desasparagine-insulin by Heterologous Immune Hemolysis.[a]

	Insulin Antibody	Desocta-peptide-insulin Antibody	Des-Ala-des-Asp-insulin Antibody
Insulin cells	100	22.6	64.4
Desoctapeptide-insulin cells	0.9	100	19.7
Des-Ala-des-Asp-insulin cells	2.3	29.2	100

[a] Antibodies were allowed to cross-react with the corresponding antigen conjugated red blood cells in the absence of any inhibitor. The percent cross reactivity between an antiserum and heterologous red cells conjugate was calculated from the ratio of the microliters of an antiserum required for 50% lysis of homologous red cell conjugate to the microliters of that same antiserum needed for 50% lysis of heterologous red cell conjugates.

The degree of cross-reaction of des-Ala-des-Asp- and desoctapeptide-insulins with insulin antisera was very slight (Tables I and II). This is presented as evidence that there are few determinants on desoctapeptide- and des-Ala-des-Asp-insulins that react well with antibodies obtained when insulin is used as the antigen. Similar results have also been noted with other pools of insulin antisera (Arquilla *et al.*, 1969).

When excessive amounts of a heterologous derivative (100–1000 times the homologous inhibitor) were used to inhibit immune hemolysis it was possible to achieve 100% inhibition (Figure 3). It is therefore likely that most of the antigenic determinants on insulin, desoctapeptide-, and des-Ala-des-Asp-insulins can cross-react, although poorly, with the antibodies induced by each of them.

The cross-reaction of insulin with desoctapeptide-insulin antibodies in the immune hemolysis inhibition was 5.0% compared to 22.6% in the heterologous immune hemolysis system. The immune hemolysis inhibition system in this case measures the effectiveness of insulin binding to desoctapeptide-insulin antibodies in competition with the binding of these antibodies to desoctapeptide-insulin-coated cells. The lower degree of cross-reaction of insulin in this case could be an expression of decreased avidity of insulin compared to desoctapeptide-insulin for binding to desoctapeptide-insulin antibodies. The greater cross-reaction of insulin in the heterologous immune hemolysis system could be an expression of the lack of competition since the ability of desoctapeptide-insulin antibodies to bind with insulin coated cells is measured directly.

Similar results were also noted in the case of desoctapeptide-insulin binding to des-Ala-des-Asp-insulin antibodies (Tables I and II).

Insulin was found to cross-react with des-Ala-des-Asp-insulin antibodies to a significantly greater degree than des-Ala-des-Asp-insulin cross-reacted with insulin antibodies (Tables I and II). Consequently, there appears to be a substantial number of antibodies induced when des-Ala-des-Asp-insulin is used as an antigen which combine well with a restricted portion of insulin.

Since des-Ala-des-Asp-insulin reacts poorly with insulin antibodies, it is not reasonable to attribute the cross-reaction between insulin and des-Ala-des-Asp-insulin antibodies to contamination of this derivative by insulin. Furthermore, the determinants on insulin which cross-react well with des-Ala-des-Asp-insulin antibodies induce relatively few antibodies when insulin is used as the antigen.

It is probable that insulin, when used as an antigen, is in a highly aggregated form. This would result in a paucity of antibodies directed against the residues of the B chain intimately involved in the dimer site (Blundell *et al.*, 1971). These residues may be more accessible to antibody producing cells when des-Ala-des-Asp-insulin is used as the antigen, since it aggregates less well than insulin (Goldman, 1967). Insulin, at the concentration of 6.1×10^{-5} M, was probably a monomer when used as the heterologous inhibitor in the des-Ala-des-Asp-insulin immune hemolysis inhibition system, in which case, the dimer site would be exposed and free to combine with the relatively greater proportion of antibodies in des-Ala-des-Asp-insulin antisera directed against the residues of the B chain which are ordinarily involved in dimer interaction. Conversely, because of a relatively small number of antibodies in insulin antisera directed against the dimer site, a lower level of cross-reactivity between des-Ala-des-Asp-insulin and insulin antibodies would be expected.

This possibility is amenable to testing by determining whether the interactions with functional groups of the residues in the dimer site are enhanced when des-Ala-des-Asp-insulin is compared to insulin. We therefore designed experiments to test whether the tyrosines (B-16 and B-26) in the B chain of des-Ala-des-Asp-insulin were more readily iodinated than the same insulin tyrosines when comparable conditions were employed.

Iodination of Insulin and Desalanine-desasparagine-insulin. Equal concentrations of insulin and des-Ala-des-Asp-insulin were iodinated at levels of 0.75 atom of iodine (with trace ^{125}I)/molecule by the lactoperoxidase method (Marchalonis, 1969; Morrison *et al.*, 1971). Oxidative sulfitolysis of the respective reaction mixtures was performed according to DeZoeten and DeBruin (1961). Separation of the *S*-sulfonate A and B chains was accomplished by oxoid cellulose acetate

electrophoresis in 8 M urea according to Bromer *et al.* (1967). After electrophoresis, the cellulose acetate strips (4.8 × 19.5 cm) were divided into 0.5-cm segments and counted to determine levels of A- and B-chain iodination (Figure 5).

Previous work has shown that iodination at this level results in 90% or more incorporation into the A-chain tyrosines and 10% or less on B-chain tyrosines of insulin (DeZoeten and DeBruin, 1961; Rosa *et al.*, 1967; Arquilla *et al.*, 1968). Figure 5 shows 8.6% iodination to have occurred on the B chain of insulin compared to 41.6% on the B chain of des-Ala-des-Asp-insulin. This is submitted as evidence that the B-chain tyrosines of des-Ala-des-Asp-insulin are more accessible for iodination than the B-chain tyrosines of insulin. It follows that enzymatic cleavage of Asn A-21 causes conformational changes which permit iodination of Tyr B-16 and/or B-26 to proceed more easily than in insulin.

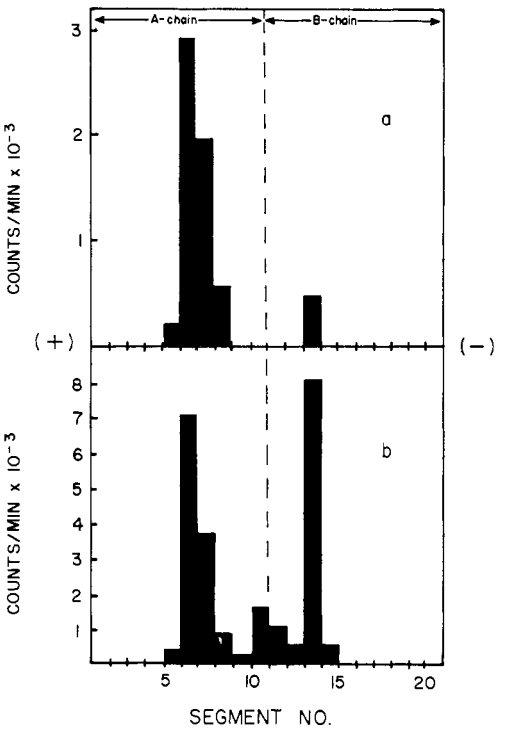

FIGURE 5: Cellulose acetate electrophoresis of *S*-sulfonate A and B chains from [^{125}I]insulin (a) and [^{125}I]des-Ala-des-Asp-insulin (b) was performed on 4.8 × 19.5 cm strips in acetate buffer (pH 3.2) with 8 M urea at 160 V. The *S*-sulfonate B chain migrates toward the cathode. The *S*-sulfonate A chain migrates toward the anode. Following electrophoresis, strips were divided into 0.5-cm segments. Total cpm cathodal from the origin were used to calculate per cent of the total ^{125}I that was attached to the respective B chains. Iodination of the insulin B chain was 8.6% compared to 41.6% for the B chain of des-Ala-des-Asp-insulin.

Discussion

We previously suggested that desoctapeptide-insulin and des-Ala-des-Asp-insulin may have similar structures (Morris *et al.*, 1970). This speculation was based upon apparent structural similarities of these two derivatives. Earlier studies (Arquilla and Bromer, 1967; Arquilla *et al.*, 1969) demonstrated perturbations in the aromatic core regions of these molecules with which guinea pig strain 13 insulin antibodies bind (Arquilla *et al.*, 1969). Both desoctapeptide- and des-Ala-des-Asp-insulins have markedly reduced biological activities (Carpenter, 1966; Arquilla *et al.*, 1969; Surmaczynska *et al.*, 1969). The near- and far-uv CD spectra are also very similar (Morris *et al.*, 1970).

Deletion of Ala B-30 alone by mild carboxypeptidase A hydrolysis results in a derivative which has a circular dichroic spectrum indistinguishable from insulin in both the near- and far-uv (Arquilla *et al.*, 1972) regions. Other observations have shown that desalanine-insulin has biological (Harris and Li, 1952; Nicol, 1960; Akre *et al.*, 1964) and immunological properties (Grodsky *et al.*, 1959; Berson and Yalow, 1963) indistinguishable from those of insulin. The loss of biological activity, change in structure measured by CD, and alteration of antigenic determinants (Arquilla *et al.*, 1969) noted with des-Ala-des-Asp-insulin can be therefore attributed to removal of Asn A-21 and not to the removal of Ala B-30.

In this report we describe experiments that test for possible conformational similarities between desoctapeptide- and des-Ala-des-Asp-insulins. Antisera were obtained against each of these derivatives and the immunological cross-reactivities were determined. These studies show that desoctapeptide- and des-Ala-des-Asp-insulins have conformational differences easily demonstrable by the immunologic studies based upon passive immune hemolysis techniques. By comparison, circular dichroism is a less sensitive indicator of alterations in conformation than are these immunological methods. In addition to being less sensitive the near- and far-uv CD spectral changes noted in these derivatives are nonspecific in that they can also be induced by exposing insulin to heat or nonaqueous solvents (Ettinger and Timasheff, 1971).

When excessive amounts of insulin, desoctapeptide-insulin, or des-Ala-des-Asp-insulin were used in the immune hemolysis inhibition experiments (Figure 3) it was possible to demonstrate 100% cross-reaction. This suggests that the gross features of the structure of each of these derivatives possess similarities. There exist, however, distortions of the antigenic determinants which markedly affected the immunological cross-reactivities of desoctapeptide-insulin with des-Ala-des-Asp-insulin that are not discernable by CD. The speculation which we previously proposed (Morris *et al.*, 1970), suggesting that the conformation of des-Ala-des-Asp-insulin was similar to the conformation of desoctapeptide-insulin must be regarded as excluding fine structural differences, the presence of which have been measured only by the present immunological results.

Our additional speculation that Asn A-21 may stabilize the chain folding of the COOH terminal of the B chain *via* electrostatic interaction with Arg B-22, which is within van der Waals radii of Asn A-21 (Hodgkin and Mercola, 1972), is still reasonable.

These studies show enhanced cross-reactions between insulin and des-Ala-des-Asp-insulin antibodies compared to the reciprocal cross-reactions of des-Ala-des-Asp-insulin with insulin antibodies. This one-way cross-reaction is probably best explained by the presence of antibodies in des-Ala-des-Asp-insulin antisera which react with a portion of insulin which in turn induces few antibodies when insulin is used as the antigen. In view of the poor cross-reaction between insulin antibodies and des-Ala-des-Asp-insulin (Arquilla *et al.*, 1972), it is reasonable to propose that many of the native determinants on this derivative are distorted and combine poorly with insulin antibodies. It follows that the antibodies induced by des-Ala-des-Asp-insulin which cross-react with insulin are directed to a restricted region of antigenic determinants which has maintained its native conformation. If insulin, when used as an

antigen, is in a highly aggregated state, induction of antibodies against residues buried within the dimer would be minimal. Consequently, insulin antisera would contain a paucity of antibodies directed to the exposed dimer site.

The iodination of insulin compared to des-Ala-des-Asp-insulin suggests that at comparable concentrations and similar reaction conditions, the B-chain tyrosines B-16 and/or B-26 of insulin are less accessible for iodination than the same tyrosines in des-Ala-des-Asp-insulin. Tyrosine B-26 is buried in the dimer site (Hodgkin and Mercola, 1972). Tyrosine B-16 although partially exposed in the insulin dimer is probably hydrogen bonded to water (Hodgkin, 1972). As we proposed earlier, it is probably the removal of Asn A-21 that results in conformational alterations that weaken the COOH-terminal B-chain relationship with the core of the molecule resulting in a less compact molecular structure in the case of des-Ala-des-Asp-insulin. The slower migration in acrylamide gel electrophoresis, the increased accessibility to iodination of the B chain tyrosines, and the attenuated aggregation demonstrable by ultracentrifugation (Goldman, 1967) of des-Ala-des-Asp-insulin compared to insulin are all consistent with such a proposal.

The results presented are submitted as evidence to support the previous speculation (Morris *et al.*, 1970) that the COOH-terminal Asn A-21 interacts with Arg B-22 stabilizing the chain folding of the COOH terminal of the B chain in proximity to the core of the molecule. It is further proposed that this interaction is necessary for the stability of the insulin structure and optimal biological activity.

Acknowledgments

We thank Edward J. Stanford for technical aid in the immunological studies, G. Michael Bradbury for assistance in circular dichroic studies, and Ingrid Kazakevicius for the preparation of iodinated insulin and desalanine-des-asparagine-insulin.

References

Akre, P. R., Kirtley, W. R., and Galloway, J. A. (1964), *Diabetes 13*, 135.

Arquilla, E. R., and Bromer, W. W. (1967), *Excerpta Med. Int. Congr. Ser., Proc. 6th Congr. Int. Diabetes Foundation Stockholm Ser. No. 172S*, 28.

Arquilla, E. R., Bromer, W. W., and Mercola, D. A. (1969), *Diabetes 18*, 193.

Arquilla, E. R., and Finn, J. (1963), *J. Exp. Med. 118*, 55.

Arquilla, E. R., and Finn, J. (1965), *J. Exp. Med. 122*, 771.

Arquilla, E. R., Miles, P. V., and Morris, J. W. S. (1972), Handbook of Physiology, Sect. 7, Vol. I, Baltimore, Md., Waverly, p 159.

Arquilla, E. R., Ooms, H., and Finn, J. (1966), *Diabetologia 2*, 1.

Arquilla, E. R., Ooms, H., and Mercola, K. (1968), *J. Clin. Invest. 47*, 474.

Arquilla, E. R., and Stavitsky, A. B. (1956), *J. Clin. Invest. 35*, 458.

Berson, S. A., and Yalow, R. S. (1963), *Science 139*, 844.

Blundell, T. L., Dodson, G. G., Dodson, E. J., Hodgkin, D. C., and Vijayan, M. (1971), *Progr. Hormone Res. 27*, 1.

Bromer, W. W., and Chance, R. E. (1967), *Biochim. Biophys. Acta 133*, 219.

Bromer, W. W., Sheehan, S., Berns, A., and Arquilla, E. R. (1967), *Biochemistry 6*, 2378.

Carpenter, F. H. (1966), *Amer. J. Med. 40*, 750.

DeZoeten, L. W., and DeBruin, O. A. (1961), *Recl. Trav. Chim. Pays-Bas 80*, 907.

Ettinger, M. J., and Timasheff, S. N. (1971), *Biochemistry 10*, 824.

Goldman, J. (1967), Ph.D. Thesis, University of California, Berkeley, Calif.

Grodsky, G. M., Peng, C. T., and Forsham, P. H. (1959), *Arch. Biochem. Biophys. 81*, 264.

Harris, J. I., and Li, C. H. (1952), *J. Amer. Chem. Soc. 74*, 2945.

Hodgkin, D. C. (1972), *Diabetes 21, Suppl. I*, iii.

Hodgkin, D. C., and Mercola, D. A. (1972), *in* Handbook of Physiology, Sect. 7, Vol. I, Baltimore, Md., Waverly, p 139.

Kabat, E. A., and Mayer, M. M. (1961), *in* Experimental Immunochemistry, Springfield, Ill., Charles C Thomas, p 149.

Marchalonis, J. J. (1969), *Biochem. J. 113*, 299.

Mercola, D. A., Morris, J. W. S., Arquilla, E. R., and Bromer, W. W. (1967), *Biochim. Biophys. Acta 133*, 224.

Morris, J. W. S., Mercola, D. A., Miles, P. V., and Arquilla, E. R. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29* (2), 313.

Morrison, M., Bayse, G. S., and Webster, R. G. (1971), *Immunochemistry 8*, 289.

Nicol, D. S. H. W. (1960), *Biochem. J. 75*, 395.

Rosa, U., Massaglia, A., Pennisi, F., Cozzani, I., and Rossi, C. A. (1967), *Biochem. J. 103*, 407.

Shuytman, L. A. (1955), *Biochim. Biophys. Acta 17*, 169.

Slobin, L., and Carpenter, F. (1963), *Biochemistry 2*, 16.

Surmaczynska, B., Metz, R., Barrett, R., and Lucena, G. (1969), *Endocrinology 85*, 577.

Zweig, G., and Whitaker, J. R. (1967), *in* Paper Chromatography and Electrophoresis, Vol. 1, New York, N. Y., Academic Press, p 159.