Insulin Complexes and the Effects of Various Agents upon Their Conformation and Assays

Lawrence J. Crolla
Loyola University Chicago

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INSULIN COMPLEXES
AND THE EFFECTS OF VARIOUS AGENTS
UPON THEIR CONFORMATION AND ASSAYS

BY

LAWRENCE J. CROLLA

A Dissertation Submitted to the Faculty of the Graduate School
of Loyola University of Chicago in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy
February
1973
BIOGRAPHICAL SKETCH

Lawrence J. Crolla was born December 13, 1944, in Chicago, Illinois. In June, 1962, he graduated from Saint Patrick High School, Chicago, and then attended Saint Mary's College, Winona, Minnesota, from which he received the degree of Bachelor of Arts in June, 1966, with a major in biology and minors in chemistry and mathematics.

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He is co-author of the following publications:


"A Role for Conformation in the Insulin-Protein Complex," Clin. Chem. 18, 719 (1972)
Many people have helped the author with the preparation of this dissertation. To name them all individually would take another volume. Therefore, to all of my friends I give my heartfelt thanks.

I thank, especially, Dr. Hugh J. McDonald, my adviser, my parents, and my wife, without whose patience and gentle prodding this dissertation would not have been written.
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Insulin Production

The beta cells of the islands of Langerhans of the pancreas are the site of insulin synthesis. Islet cells lie close together with only an occasional space between them. Each cell is apparently encircled by its own plasma membrane but at times it is difficult to determine the limits of individual membranes especially along those portions of a cell which adhere to adjacent cells.

The typical beta cell is many-sided and irregular in outline. It contains a varying number of rather coarse granules. Some cells in a given islet are literally filled with granules while others are almost empty. The granules themselves are not evenly distributed throughout a cell but are often densely packed in a portion of the cell which lies along capillaries. These granules, which are virtually electron-opaque, contain insulin and proinsulin that has been formed within the cell. In man, the granules may be rectangular in outline and crystalline in appearance. Variation in the shape of the granules may reflect a difference in insulin structure or in the type of materials with which the insulin is associated in the granules. Since granules are too large to represent single molecules of insulin, it is possible that the actual amino acid sequence in the insulin might influence the way in which the molecules can be arranged within the granule. Similarly, the struc-
ture of the protein which binds insulin may impart certain characteristics of form to the granule.

In order to function as a hormone, insulin must first break out of its capsule and leave the beta cell. The events which bring insulin to its "target tissue" begin with the migration of fully formed insulin sacs from their formation sites. The mechanism by which insulin granules are released when they reach the cell surface is not fully known. However, it is thought that the cell's plasma membrane and the capsule membrane fuse upon contact and that these membranes rupture at the point of contact (73). As a result, a passageway is thereby provided for the insulin granule across two membranes and thus out of the beta cell. Although it is presumed that granules of insulin shed their enveloping capsules when they leave the beta cell, as described, free granules have not been observed. It is likely, therefore, that each granule undergoes a transformation at the moment of release. The insulin that continues on toward the lumen of the nearest capillary maybe in a non-granular form, or it may even be in the soluble state, although perhaps bound to protein.

The Insulin Molecule

Insulin is a protein consisting of fifty-one amino acids and has a minimum molecular weight of 6,000. The molecule is composed of two polypeptide chains designated "A" and "B" which are connected by two interchain disulfide bridges of cysteine (See Figure I).
THE INSULIN MOLECULE

Figure 1
The "A" chain of insulin contains an intrachain disulfide bridge within which a species difference in amino acid composition occurs primarily at positions 8, 9, and 10. A species difference also occurs at the carboxy terminus, position 30, of the "B" chain (See Chart I).

**Circulating Insulin**

The insulin secreted by the pancreas has a rapid turnover in man, the half-life of labeled insulin being of the order of forty minutes. Rapid release from the pancreas along with this rapid turnover implies that the body is able to call upon a relatively large quantity of insulin to satisfy metabolic needs on short notice and is at the same time protected against the dangers of hypoglycemia.

The insulin secreted by the pancreas enters the portal vein and passes through the liver before it is delivered to the systemic circulation. A substantial amount of this insulin is trapped by the liver. In fact, after portal injection of labeled insulin, approximately fifty percent of the administered dose was found to be bound to the liver in a single transhepatic circulation (55). Thus the amount of available insulin is determined in large part by the liver, which not only traps insulin but degrades it as well. It has also been shown that insulin which is not trapped or degraded may be modified so that it leaves the liver in a physiologically
CHART I

SOME SPECIES DIFFERENCES IN AMINO ACID SEQUENCE OF MAMMALIAN INSULINS

<table>
<thead>
<tr>
<th>Positions</th>
<th>&quot;A&quot; Chain</th>
<th>&quot;B&quot; Chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Threonine</td>
<td>Serine</td>
</tr>
<tr>
<td>9</td>
<td>Alanine</td>
<td>Serine</td>
</tr>
<tr>
<td>10</td>
<td>Threonine</td>
<td>Serine</td>
</tr>
</tbody>
</table>

OTHER SPECIES

<table>
<thead>
<tr>
<th>Species</th>
<th>&quot;A&quot; Chain</th>
<th>&quot;B&quot; Chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>Threonine</td>
<td>Serine</td>
</tr>
<tr>
<td>Dog</td>
<td>Threonine</td>
<td>Serine</td>
</tr>
<tr>
<td>Sheep</td>
<td>Alanine</td>
<td>Glycine</td>
</tr>
<tr>
<td>Horse</td>
<td>Threonine</td>
<td>Glycine</td>
</tr>
</tbody>
</table>
inactive protein-complex form (37). The nature of this complexed form of insulin has been the center of much controversy in the field of diabetes research. A large part of the conflict stems from the way in which insulin is assayed. It is, therefore, appropriate to consider, briefly, the various procedures which have been used for the assay of insulin.
CHAPTER II

ASSAYS OF INSULIN

The most commonly employed procedures for the assay of insulin are: the rat diaphragm bioassay, the epididymal fat-pad bioassay, the radioimmunoassay and in vivo bioassays. The results of most assays are reported in terms of units of insulin. One unit of insulin is defined as 1/24 mg of insulin (81).

The first assay to be employed by researchers was the in vivo bioassay. Since the accepted criterion of insulin action is a fall in the concentration of blood sugar, the first insulin assays made use of this fact. Beigelman, in 1958, measured the change in the level of blood sugar in mice (18). The sensitivity of his method was 1000 uU. This method was modified by Bornstein and Lawrence (22). He employed hypophysectomized, adrenalectomized, alloxan diabetic rats. With these animals, the range of assayable insulin is between fifty and five hundred microunits per milliliter. The preparation of test animals for the in vivo assays is difficult, time consuming and expensive and the mortality rate is very high. It is therefore not surprising that these methods were soon abandoned. Better methods, with improved sensitivity and less inherent problems were soon employed.

Vallence-Owen and Hurlock investigated the addition of insulin to a glucose-containing medium incubated with rat diaphragm
muscle (78). The insulin, they found, stimulated glucose uptake and glycogen deposition in the muscle. Their assay was sensitive to relatively low amounts of insulin, making it possible to measure as little as 25 uU of insulin per ml of test solutions. The assay, however, did present some problems. Yalow and Berson showed that in the range up to 100 uU/ml, the insulin concentration must be increased by about 225% to produce a 1% change in glucose concentration (84). The best form of the assay is to measure glycogen deposition rather than glucose uptake. With $^{14}C$ glycogen incorporation, the test can be used to measure in the range of 10 to 500 uU/ml of insulin. However, when using even this form of the assay another problem still persists, namely, the fact that as the serum to be assayed is diluted, the activity measured by the assay does not fall in proportion with the dilution. A greater amount of insulin is measured as activated, upon dilution. That is, insulin in serum found to contain 74 uU/ml will assay as 200 uU/ml after a four fold dilution. This phenomenon has been explained by saying that insulin inhibitors are diluted and thereby more insulin is freed to metabolize glucose (63). Evidence for such insulin inhibitors in the albumin fraction of serum has been presented by Vallence-Owen et.al. (79).

An assay somewhat similar to the diaphragm assay but using epididymal fat pads was developed in 1958 by Martin and coworkers (56). Use is made of the fact that adipose tissue responds to insulin with an increase in glucose uptake and with a preferential
oxidation of the sugar via the pentose cycle. In performing this assay, the insulin sensitive parameter which is measured may be glucose uptake, formation of $^{14}$C CO$_2$ from $^{14}$C-glucose or total gas exchange. In its most common form, that of measuring $^{14}$CO$_2$ formation, this assay can measure as little as 10 uU/ml of insulin. However, the levels of insulin obtained with this fat pad assay are higher than those obtained with the diaphragm assay. For this reason the fat pad assay is believed to measure not only insulin itself but also insulin-like activity (ILA) which reflects the presence of other factors which mimic the effects of insulin in the assay procedure.

The final procedure to be discussed is the radioimmunoassay technique. Because of its sensitivity and specificity, this assay, which was developed by Yalow and Berson, has been heralded as the most satisfactory of the in vitro assays.(85)

The radioimmunoassay is based on the competition of insulin, in the sample to be assayed, and of radioactive insulin, for reaction with an antibody which is specific to insulin. The amount of radioactive insulin bound to antibody, therefore, varies inversely with the concentration of insulin in the assay sample (See Figure II).

Several procedures based on this principle have been worked out; they differ in minor details and in the method used for separating insulin bound to antibody from insulin remaining free. However, the most commonly used method is that of Hales and Randle
Insulin (free) + Antibody to insulin (guinea pig) ⇄ Complex of antibody + insulin (bound)

Control

*Only* radioactive Insulin is used. Out of 5 molecules, 4 are bound to the antibody and 1 is free.

\[
\text{Ratio of bound insulin} : \text{free insulin} = \frac{4}{1} = 4
\]

(80% of the radioactive Insulin is antibody-bound)

Measurement

Nonradioactive Insulin is added to the same quantity of radioactive Insulin. Out of 5 radioactive molecules, 2 are now bound and 3 are free.

\[
\text{Ratio of bound insulin} : \text{free insulin} = \frac{2}{3} = 0.66
\]

(40% of the radioactive Insulin is antibody-bound)

**ILLUSTRATION OF THE THEORY OF THE RADIOIMMUNOCASSAY**

**Figure II**
This method is also known as the Double Antibody Method. In this version of the assay procedure, the complex of insulin and anti-insulin serum is rendered insoluble by a second antibody. This insoluble complex precipitates and is separated from free insulin by filtration. The radioactivity of the precipitate is measured and the concentration of insulin in a given sample is found by referring to a calibration curve prepared from measurements on pure insulin. The radioimmunoassay can measure insulin levels as low as 1 uU/ml of insulin, thus this is the most sensitive of the assay procedures described.

A comparison of value obtained using the different assay procedures is shown in Chart II, while the sensitivity of each is shown in Chart III. It is important to remember that all the assay procedures, except the radioimmunoassay, measure biologically active insulin-like activity (ILA), while the radioimmunoassay measures immunologically active free insulin. One cannot make the assumption, therefore, that material assayed by the immunoassay, which shows little or no insulin, complexed to a protein, will not be detected by radioimmunoassay but will be shown to be biologically active in the fat pad assay. This important fact is one which has led to much controversy in that one investigator may have tried to repeat studies which were performed by another investigator who employed a different assay procedure, thereby obtaining conflicting results.
## CHART II

**INSULIN ASSAY VALUES COMPARED**

<table>
<thead>
<tr>
<th>Method of Assay</th>
<th>Fasting</th>
<th>After Glucose or Feeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat Diaphragm Bioassay (Serum or plasma)</td>
<td>64 (40-80)</td>
<td>(130-800)</td>
</tr>
<tr>
<td>Rat Epididymal Fat Pad Bioassay (Serum or plasma)</td>
<td>250 (31-900)</td>
<td>Three to six-fold increase</td>
</tr>
<tr>
<td>Radioimmunoassay (Competitive inhibition of binding of labeled insulin)</td>
<td>21 (0-66)</td>
<td>140 (30-300)</td>
</tr>
</tbody>
</table>
# Chart III

**Sensitivity of Insulin Assays**

<table>
<thead>
<tr>
<th>Test</th>
<th>Sensitivity</th>
</tr>
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<tbody>
<tr>
<td>Radioimmunoassay</td>
<td>1 uU to 1000 uU</td>
</tr>
<tr>
<td>Fat Pad</td>
<td>10 uU to 10 U</td>
</tr>
<tr>
<td>Diaphragm</td>
<td>10 uU to 10 U</td>
</tr>
<tr>
<td>Mouse Hypoglycemia</td>
<td>1 uU to &gt; 100 U</td>
</tr>
<tr>
<td>Rabbit Hypoglycemia</td>
<td>&gt; 100 uU to &gt; 100 U</td>
</tr>
</tbody>
</table>
CHAPTER III

INSULIN-LIKE ACTIVITY

Moloney and Coval in 1955 were able to isolate a specific anti-insulin antibody serum which negated the physiological effect of crystalline insulin when assayed using adipose tissue or muscle (61). However, when the antibody was added to human serum, only part of the insulin-like activity in the fat pad assay was suppressed. In 1961, Slater and coworkers confirmed these findings and used the terms "typical" and "atypical" insulin to describe the materials assayed (72). "Typical" insulin is described as that form of insulin which is immunologically reactive and "atypical" insulin that form which is not.

By using ultracentrifugation techniques, other investigators were able to separate these forms of insulin-like activity (67). They reported that the "atypical" form had a molecular weight in excess of 30,000. They also found prolonged acid-ethanol extraction of the "atypical" form could convert more than 2/3 of it to typical insulin. Analysis of the relative amounts of these two forms showed that typical insulin, measured in the pancreatic venous blood, accounted for 90 to 95% of the total ILA, while that in hepatic venous blood, accounted for only 50% of the ILA. From these studies it was concluded that insulin produced in the beta cells was being altered in some way, in the liver, rendering it
immunologically unreactive.

In 1963, Froesch and coworkers used gel chromatography, fractional precipitation and electrophoresis to isolate and partially purify "atypical" insulin (35). The material they isolated was immunologically unreactive when assayed by either the diaphragm or adipose tissue assay. It showed an activity of 2 uU/mg compared to about 25 uU/mg for pure crystalline insulin. Its properties, however, were very similar to crystalline insulin. It stimulated glucose uptake, and carbon dioxide formation from glucose. It induced hypoglycemia when injected into normal animals and it also inhibited lipolysis in the absence of glucose. When passed through Sephadex G-75 in 5 molar acetic acid, material was found with a molecular weight of approximately 6,000, thus resembling insulin under the same conditions. However, when this atypical insulin was oxidized with performic acid, no insulin "A" chain was found, using electrophoretic mobility as the identification method. Froesch postulated from this that some change in the "A" chain occurred in the liver which alters its physical properties so that large aggregates of individual molecules are formed. Under the proper conditions these aggregates may disassociate into units of the same molecular weight and biological activity as native insulin.

Keen isolated a fraction of serum protein composed largely of albumin which had many insulin-like properties (51). It increased glucose uptake and glycogen deposition as measured by the diaphragm assay. When incubated with the fat pad, the extract
accelerated glucose uptake and incorporation of labeled glucose into lipid. However a large portion of the activity was not suppressible by insulin antibodies.

Antoniades and coworkers found that serum, when passed through a cationic exchange resin, lost almost all of its non-immunologically reactive insulin-like activity (6). The activity could, however, be regained from the column by elution with dilute ammonium hydroxide. The eluted substance had no effect on the diaphragm assay but was found to possess considerable insulin-like activity when assayed by the fat pad bioassay. This activity could also be observed using the diaphragm assay if the extract was first incubated with adipose tissue extract.

Shaw and Shuey found this regained activity to be inhibited by anti-insulin serum indicating that there was true insulin in the eluate (7). These results, along with other experiments from his own laboratory, led Antoniades to postulate that the insulin-like activity retained by the resin was in the form of a native insulin complex with a basic protein (4,7). He postulated that the binding of the insulin to the resin could only be explained by means of the basic protein complex, since insulin itself, at physiological pH, is negatively charged and migrates electrophoretically as an anion. The fact that it is bound to a cation exchange resin indicated that the insulin in the blood may be transported along with a substance having a higher isoelectric point, most likely a protein. Antoniades further postulated that the fat pad extract was able to dissociate the insulin protein complex and re-
store normal activity to the insulin. He renamed these two forms of insulin in serum as "free" and "bound". He reported that an equilibrium existed between the two forms and that the equilibrium was shifted toward the free form upon ingestion of a glucose load (4). It was further stated that, in some diabetics, their problem might well be based on an inability to convert complexed or "bound" insulin into the free form rapidly enough or to a great enough extent to handle the glucose load. Further characterization studies were undertaken by Antoniades (1,4). Chart IV shows the current and available information on the insulin complex.

Studies on the insulin binding protein were conducted by subjecting the resin eluate to centrifugation at pH 9.8. Collected precipitates were pooled, dissolved in acetate buffer and subjected to paper electrophoresis (5). Only one substance was evident and it appeared to have a substantial positive charge suggesting that it was indeed basic in nature. Attempts to form the complex in vitro, by incubating serum samples with radioactively tagged insulin, however, failed. This implied that either there is not a large amount of free insulin binding protein in the blood or that the formation of the complex is more intricate in nature than the simple combination of insulin and protein. Further studies by Gershoff, in 1970, however, have shown that perfusion of liver with media containing 74 uU/ml of insulin resulted in a rapid loss of immunoassayable insulin, accompanied by the appearance of non-immunologically reactive insulin-like activity when using the
<table>
<thead>
<tr>
<th></th>
<th>bound insulin</th>
<th>free insulin</th>
<th>crystalline insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dowex-50x8 (Na⁺)</td>
<td>Cation</td>
<td>Anion</td>
<td>Anion</td>
</tr>
<tr>
<td>Electrophoretic mobility</td>
<td>γ, β-globulins</td>
<td>α₁-globulins/albumin</td>
<td>α₁-globulins/albumin</td>
</tr>
<tr>
<td>Molecular weight (pH 8.0)</td>
<td>40,000-60,000</td>
<td>12,000</td>
<td>12,000</td>
</tr>
<tr>
<td>Reactivity with anti-insulin antisera</td>
<td>unreactive</td>
<td>reactive</td>
<td>reactive</td>
</tr>
<tr>
<td>Treatment with reduced glutathione</td>
<td>inactivation</td>
<td>inactivation</td>
<td>inactivation</td>
</tr>
<tr>
<td>Solubility in acid-ethanol</td>
<td>soluble</td>
<td>soluble</td>
<td>soluble</td>
</tr>
<tr>
<td>Origin</td>
<td>Free insulin</td>
<td>Pancreas</td>
<td>Pancreas</td>
</tr>
<tr>
<td></td>
<td>(metabolism catalyzed in vivo by liver and possibly other extra-pancreatic tissue)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Controversy Over Insulin-Like Activity

Ever since the naming of "free" and "bound" insulin for serum insulin-like activity by Antoniades, a controversy has continued as to whether there really is such an entity as "bound" insulin. Through the years various names have been given to the insulin-like activity found by the various investigators, such as, "big" and "little" insulin and "suppressible" and "nonsuppressible" insulin-like activity. However no one has proven completely whether "bound" or "free" insulin exists or doesn't exist. What appears to have been recognized however is that there is some compound that may be retained by a cation exchange resin which will elicit effects similar to insulin when assayed by in vitro bioassay techniques. The big problem in understanding the issue is the fact that most investigators use different assay techniques in trying to compare findings. That is, an interchange of the radioimmunoassay with the rat diaphragm and rat adipose tissue assays is made. As an example of the confusion, in 1968 papers by Jakob (49) and one by Roth (64) came out in the affirmative of the problem, while a paper by Meade (58), using his own isolated materials and those given to him by Antoniades, came up with negative findings, that is to say, he was unable to reproduce previous results.
The problem may be clarified by first realizing that the isolation procedure for bound insulin is very difficult, in fact, in our own laboratory, because of a change in the resin lot, we were unable for a time to isolate the material in a repeatable fashion. Also, the problem may be more readily understood if in using the terms "bound" and "free" insulin one can assume insulin-like activity to occur in the bound form without a separation from the binding protein. If this idea is accepted, then it can be seen how the radioimmunoassay would show no presence of "free" insulin while that of an in vitro bioassay would show physiological activity. Thus the key to the problem may indeed be based on the afore-mentioned hypothesis.

It is hoped that the following chapters will help to demonstrate this possibility of physiological action without a separation of the attached insulin from the binding protein.
CHAPTER IV

ANTIBODIES AND INSULIN

Introduction

Insulin forms non-covalent bonds when put in contact with antibodies to it. This insulin complex has a number of properties which are common to it and to insulin complexes which are formed in vivo. It is possible, in fact, that the in vivo complex is part of an antibody molecule. The reasons for this postulation are as follows: bound insulin which is formed in vivo is known to migrate with the gamma globulin fraction during serum electrophoresis (4). This protein fraction is the one which contains the antibodies of the serum. Secondly, in vivo bound insulin is immuno-nonreactive and shows no glucose uptake as measured by bioassay (4). Thus, if already antibody bound, the insulin could not combine further. Thirdly, tolbutamide has been shown to exert an effect on both in vivo and in vitro complexes as measured by the rat diaphragm assay (46). That is to say, both complexes regain biological activity when tolbutamide is added to the preparation. Fourthly, the Fab fragment of antibody molecules, that is the active antibody site, can be separated from an intact antibody molecule by enzymatic digestion. This fragment still retains the ability to bind antigen and has a molecular weight of approximately 50,000. This is approximately the weight attributed to
the \textit{in vivo} protein which binds insulin. Because of these similarities, investigational experiments have also been conducted on insulin antibody complexes to see if more information can be obtained as to the nature of the insulin protein complexes. A brief review of antibody structure is therefore useful in order to aid in understanding the nature of these types of insulin complexes.

\textbf{Antibody Structure}

Antibodies of the gamma G class have a molecular weight of about 150,000 and when their rate of sedimentation is measured, under the force produced by high-speed centrifugation, they are found to have a sedimentation coefficient of about 7 Svedberg units. When treated in strong urea solutions with agents such as mercaptoethanol, which reduce disulfide bonds, they regularly fall apart into small components. These components consist of two groups of polypeptide chains which differ in size. The smaller is termed the light (L) and the larger the heavy (H) polypeptide chain. The molecular weight of light chains from rabbit gamma globulin is about 25,000, while that of the heavy chain is 50,000. Figure III shows how these chains are attached in an intact molecule. It will be seen that each L chain is attached at the carboxy terminus by one disulfide bond to a heavy chain and that two heavy chains are also linked together by disulfide bonds.

Before the chain structure of immunoglobulin G (IgG) was
CLEAVAGE SITES OF PAPAIN AND PEPSIN

Figure III
known, it had already been discovered that the enzymes papain and pepsin, which hydrolyze peptide bonds specific for each enzyme, could split IgG antibodies into large fragments which retain many of the biological properties. Figure III shows where the site of cleavage occurs. Papain splits the molecule into three pieces, two of which, termed Fab fragments, are identical and consist of the L chain, linked to the amino terminus of the heavy chain by a single disulfide bond. The third fragment, termed Fc, consists of the carboxyl portions of the heavy chain joined together by one or two disulfide bonds. The Fab fragments carry one of the two antigen binding sites of the original molecule. Since Fab fragments are univalent they cannot agglutinate particles nor form a lattice structure with antigens or lead to specific precipitations. Disruption of the disulfide bond separates the light chain from the N terminal-end of the heavy chain, termed the Fd fragment.

The Fc fragment has no antibody activity but contains part of the heavy chain which determines various other biological properties.

Pepsin treatment yields an F(ab')₂ fragment. The site at which pepsin cleaves the heavy chain is on the side of the disulfide bonds opposite from the site of action of papain. Therefore the two light chains and the Fd fragments of the heavy chains remain linked by disulfide bonds into a single molecule which carries both antibody combining sites. Thus it is bivalent, and
apart from being smaller, combines with antigen in much the same way as the original molecules would.

**Antibody Binding Sites**

The available data suggest that the antibody combining site is composed of certain hypervariable regions within variable sections of both polypeptide chains (25). The size of the active site has been estimated by measuring the affinity of a specific antibody for a series of homologous ligands of different sizes. In one study, antibodies to dextran were employed. Glucose oligosaccharides of increasing size were then tested for their capacity to inhibit the specific antigen-antibody reaction. Maximum inhibition was found with six residues but 90% of the activity could be inhibited by a tetramer (25). It was also found, by reacting antibodies with chemicals which modify certain amino acids, that the amino acid most frequently implicated by such experiments is arginine. It is estimated that of approximately 650 amino acid residues of a light heavy chain pair, between 15 and 150 are involved in the antibody combining site.

The combination between antigen and antibody is reversible and does not involve stable chemical bonds, although formation of some reversible hydrogen bonds does occur. It is thought that the three dimensional arrangement of amino acids at the combining site presents a surface pattern complimentary to the hapten or to the
particular determinant group on the antigen. Figures IV and V show how this might occur. The forces binding the two together are mainly due to the presence of oppositely charged groups in close contact, ionic binding and partly van der Waal's forces which act between molecules independently of their charge and whose strength falls off very sharply with the distance between them. The strength, therefore, depends to a large extent on the preciseness of fit and the nearness to which the components can approach one another. The forces are usually maximal at physiological pH and ionic strength, and at pH values between 3 and 4 or above 10.5 they are often so weak that antigen antibody complexes dissociate.
Figures IV and V are diagrams to illustrate the shape of the combining site of a molecule of antibody against the hapten group and the shape of the hapten group itself, of a related hapten which is known to cross-react strongly, and of another hapten which cross-reacts very weakly. (From Pauling L. (1948) Endeavour 7, 43.)
ILLUSTRATION SHOWING HOW ANTIBODY BINDING MIGHT OCCUR

1 - Hapten
2 - Related Strong Reacting Hapten
3 - Weakly Reacting Hapten

Figure V
CHAPTER V

MATERIALS AND METHODS

Materials

1. Solvents

   Heavy water, warranted to contain a minimum of 99.75% D₂O
   - Isotopes Inc. Westwood, New Jersey

2. Oral Hypoglycemic Agents and Investigative Materials

   Chlorpropamide
   - Pfizer Inc. Brooklyn, New York
     Lot # 07332-17000
   Phenformin
   - USV Pharmaceutical Corp. Yonkers, New York
     Lot # CUN-A-148
   Acetohexamide
   - Eli Lilly Co. Indianapolis, Indiana
     Lot # 48E72
   Sodium Tolbutamide
   - Upjohn Co. Kalamazoo, Michigan
     Lot # SP964G9
   d-Biotin
   - Sigma Chemical Co. St. Louis, Missouri
     Lot # 31C-2300
   Heparin
   - Nutritional Biochemicals Corp. Cleveland, Ohio
3. Immunological Materials

Anti Insulin Serum
- Wellcome Research Lab. Beckenham, Kent, England
  Lot # K4273

Insulin Binding Reagent
- Wellcome Research Lab. Beckenham, Kent, England
  Lot # K1918

Human Insulin Standard
- Wellcome Research Lab. Beckenham, Kent, England
  Lot # K3139

4. Buffer Solutions

Buffer A - 40mM phosphate buffer, pH 7.4, for dilution of antisera and iodinated insulin.

  7.0 gms sodium phosphate monobasic-monohydrate
  0.25 gms domiphen bromide (Mann Laboratories, No. 6405)
  1.0 gm polyvinylpyrrolidinone (Matheson Coleman and Bell Norwood, Ohio)

  Sodium Hydroxide, 2N, to pH 7.4

  Distilled, de-ionized water to 1 liter pH readjusted to 7.4

  Stored at 4°C

Buffer B - Buffer for dilution of standard insulin and plasma samples.

  9.0 gms sodium chloride

  Buffer A, to 1 liter

  Stored at 4°C
Buffer C - Washing buffer for washing antibody precipitates.

39 gms polyvinylpyrrolidinone (Matheson Coleman and Bell, Norwood, Ohio)

Buffer A, to 1 liter

Stored at 4°C
Methods

1. Extraction of Serum Fraction with "Bound Insulin" Activity
Method of Antoniades (1)

A 100 x 2.5 cm column was packed with Dowex Analytical Grade resin, AG50-x8, (Bio-Rad Laboratories, lot #5369). The resin was first equilibrated with 0.15 N NaCl for three hours before being packed in a glass column to a final wet volume of 175 ml. After packing, two liters of 0.15N Na₂HPO₄ were passed through the column, making the final pH 6.6. A 100 to 300 ml sample of pooled human serum was passed through the column at a rate of 8 ml/minute. After passage of the serum, the column was washed with two resin volumes of 0.15 N NaCl, followed by elution of the "bound" insulin fraction with one resin volume of 0.1 N NH₄OH. The pH of the eluate was monitored with a Radiometer pH meter, and kept at 7.0 ± 0.5 with 2N H₂SO₄. After elution of the "bound" insulin fraction, the eluate was lyophilized and stored as the dry powder at -10°C.

2. Modified Isolation of the in vivo Insulin Protein Complex

Because of the fact that the previous procedure caused problems of reproducibility in isolating an assayable "bound" insulin fraction, and the fact that it required a high pH of approximately 10, which might cause some denaturation of the protein, a modified procedure, reported by Guenther and McDonald,
was employed for the isolation of the "bound" insulin (41).

A volume of 500-1000 ml of pooled human serum was stirred for 45 minutes at 4°C with approximately 10 gms of Sephadex C-50 resin, which had been allowed to hydrate overnight prior to use. The mixture was then filtered on a Buchner funnel. The recovered resin was subjected to repeated washings, using 2-3 liters of cold (4°C, approx.) de-ionized water. A pre-cooled, jacketed column, measuring 100 x 2.5 cm, was packed with the pre-washed resin. To remove further anionic and neutral proteins, a 0.005 N aqueous solution of (NH₄)₂CO₃, pH 7.8, was run through the column. Once a steady base line was reached on a UV monitor (LKB) a gradient of ammonium carbonate, from 0.005 N to 1.0 N, at a pH of 7.8, at 25°C, was applied. The flow rate was adjusted to 0.5 ml per minute.

The different elution peaks were pooled and the pH was adjusted to 7.3 ± 0.1, at 25°C, using acetic acid. Following pH adjustment, the pooled solutions were de-salted and concentrated with an Amicon Ultrafiltration apparatus, using a UM 10 membrane which permits passage of substances below a MW of 10,000. Since "bound" insulin has a MW much greater than 10,000, it would be retained. This filtration procedure also served to remove the last traces of free insulin which might have been trapped by the Sephadex resin. The concentrated eluats were freeze-dried.
3. Optical Rotation Studies

When investigating a molecule by optical rotation studies, one can use plane polarized light. Plane polarized light may be described as the vector sum of two circularly polarized rays, one moving clockwise ("right-handed") or counterclockwise ("left-handed") traveling in phase. Alternately, the circularly polarized beams may be used directly in a difference absorbance measurement. The measurement of the angle of rotation of plane polarized light when it is plotted as a function of wavelength results in an optical rotatory dispersion curve (ORD) while the plot of the difference absorbance of circularly polarized beams results in a circular dichroism curve (CD).

Light being electromagnetic in origin interacts with a molecule by causing a momentary change in the relative spatial positions of positive nuclei and negative electrons. A molecule containing a plane or center of symmetry will interact equally with left and right circularly polarized beams. However, a di-symmetric molecule will cause a preferential interaction with one of the circularly polarized beams. In an absorption region this interaction will be seen as a differential absorption (a dichroism). Circular dichroism bands are closely related to absorption bands and often the circular dichroism maximum coincides with an absorption maximum. The absorbance difference between the left and right components, $\Delta A$ equals $A_L - A_R$. $\Delta A$ is dependent on concentra-
tion and optical path length and leads to the term "molar circular dichroism," $\Delta \varepsilon$, which is equal to $\Delta A/cd$, when:

$$c = \text{concentration in moles per liter}$$
$$d = \text{optical path length in centimeters}$$

For use in protein solution, the molar circular dichroism term can be reduced to a concentration of 1g/100ml and an optical path of 1 cm. This reduced circular dichroism is given by the symbol $\Delta \varepsilon_{1\%}^{1\text{cm}}$ or $\Delta D_{1\%}^{1\text{cm}}$. 
A. Circular Dichroism Measurements

Circular dichroism spectra were run on a Jasco Model ORD/UV-5 recording spectropolarimeter. Samples were run in 10 mm or 1 mm cells especially designed and checked for optical rotation studies. The instrument was calibrated before each set of experiments using camphor sulfonic acid. A sensitivity setting of 0.02 was used, at a gain setting of 7, and a chart speed of 4 mu/cm. All samples were run in phosphate buffer, at pH 7.4, at a room temperature approximating 27°C.

4. Radioimmunoassay of Insulin

The radioimmunoassay carried out was a form of the procedure of Hales and Randle (43). The method uses iodine-125 antiserum prepared to crystalline pig insulin, in guinea pigs and anti guinea pig serum prepared in rabbits. The insulin in the test sample competes with added radioactive insulin for binding sites on the antibody (See Figure II ). The insoluble insulin-antibody-antibody complex is filtered out and measured for radioactivity. The level of activity is related in an inverse manner to the amount of insulin present in the test sample.

A. Reagents

Insulin binding reagent - Wellcome Brand # MR48, obtained
from Wellcome Research Laboratories, Beckenham, Kent England.
The commercial preparation is prepared as stated previously by
forming antisera to crystalline pig insulin in guinea pigs. After
adjusting the titre of the anti-insulin serum, it is diluted in
buffer containing ethylenediamine tetraacetic acid and mixed with
a predetermined amount of the anti guinea pig precipitating serum
prepared in rabbits. The mixture is allowed to react at 4°C for
18 hours and is then freeze dried. When reconstituted to a volume
of 8 ml, each vial contains guinea pig serum precipitate diluted
to 1 in 16,000 and 0.03 M EDTA in buffer A. This reagent binds
approximately 40% of the standard dose of 250 picograms iodinated
insulin (6uU).

B. Working Solution of Iodinated Insulin

Iodinated insulin 125, #IN 38, was obtained from Amersham/
Searle Corporation, Des Plaines, Illinois. The material used
was supplied in 0.1 ug quantities and solubilized in phosphate
buffer, 40 mM, pH 7.4 containing bovine plasma albumin 0.5%
The minimum specific activity was 50 uci/ug. The commercial
material is prepared from specially purified crystalline ox insulin
by iodination with iodine monochloride. Unbound iodine is removed
by gel filtration.

C. Buffer Solutions
Buffer solutions A, B, and C were used as listed on pages 30 and 31.

D. Assay Procedure

Insulin binding reagent was reconstituted with 8 ml of distilled water and 0.1 ml aliquots were dispensed into culture tubes. A volume of 0.1 ml of the unlabeled insulin solution, either sample or standards, was added to the culture tube containing the binding reagent. Along with each series of experiments, blanks consisting of buffer B, were also set up. All tubes were mixed with a Vortex mixer and then placed in a refrigerator for six hours at 4°C. All of the above procedures were carried out in a cold room at 4°C.

After six hours the tubes were removed from the refrigerator and returned to the cold room. One-tenth ml aliquots of insulin 125 working solution were added to the tubes. The tubes were again mixed and returned to the refrigerator for eighteen hours.

At the end of eighteen hours, the tubes were again placed in the cold room. A Millipore filtration apparatus was prepared by first placing the filters onto the 18 wells of the apparatus and applying vacuum. The membrane filters were presoaked overnight in Buffer C and applied to the filter holders by means of a pair of
forceps. The contents of each tube was carefully applied to the filter and the tube washed twice with approximately 1 ml of cold Buffer C, the washing buffer solution being drawn up into the same pipette and applied to the filter. After samples were applied to the filter, the apparatus was disassembled and each filter was immediately inserted into a counting vial using a pair of forceps. The filters, now contained in the counting vials, were dried for a period of 10 minutes at 110°C. When the vials had cooled to room temperature after the drying stage, 10 ml of scintillation fluid was added to each vial. The vials were then placed in a Beckman LS-250 Scintillation Counter, allowed to dark adapt for two hours and then counted. The samples were allowed to count to a 3% error. The gain control was set at 280, optimum operating efficiency for the instrument at this point in time. A full window isoset was employed in the counting. The external standard ratio system of the instrument was used to monitor the relative quenching of the samples and to determine whether or not corrections for quenching would have to be applied to the results. The external standard ratio reading reflects the efficiency of the count for each individual sample. If there is a considerable variation in the external standard ratios of the individual samples, a correction factor must be applied to the results of the count to compensate for a varying efficiency in the counting of different samples.

If the external standard ratio readings are uniform, how-
ever, this reflects the fact that the efficiency of the counts is uniform enough to make any correction factor unnecessary. It was found that the samples were very uniform with respect to the external standard, which meant that no quench correction was necessary.

5. Deuterium Exchange

The technique of deuterium exchange represents a generalized method for the determination of active hydrogen. Chemically reactive hydrogen atoms such as those bonded to oxygen and nitrogen equilibrate rapidly with the deuterium of D₂O to establish a statistical distribution of active hydrogen and deuterium between the exchange compound and D₂O. Hydrogen bonded to carbon will exchange with deuterium but this exchange is extremely slow, being of the order of days and months. Under such conditions, it is impossible to define unambiguously exactly what is meant by active hydrogen, but in this discussion it will be understood to be that hydrogen, which exchanges rapidly (in the order of minutes and seconds) with deuterium.

Rapid hydrogen-deuterium exchange can be explained simply, by a hydrogen-bonding mechanism (44). An alcohol and D₂O may become associated, using the example of Harp (44), in a randomly constructed polymer of the form shown below.
The hydrogen and deuterium rapidly lose their identity with respect to a specific oxygen atom because of a shifting and re-establishment of bonds in the following manner.

Thus, at equilibrium, one finds a statistical distribution of active hydrogen and deuterium between the exchanging compound and D$_2$O. The equilibrium mixture is composed of ROH, ROD, D$_2$O, MOD and H$_2$O. If a sample D$_2$O mixture contains 3 atoms of active hydrogen to 97 atoms of deuterium, then at equilibrium the ratio of ROH to ROD will be 3 to 97.

The same sort of argument applies to a peptide or a protein which is dissolved in D$_2$O. A random distribution of active hydrogen and deuterium exists between the exchanging compound and D$_2$O. However, because of the tertiary properties of protein, the exchange of side chain and peptide hydrogen in the interior of the molecule becomes blocked or extremely slowed down to the order of days. It seems evident, therefore, that by altering the conformation of the molecule, a particular group may become exposed and
an increase in the number of active hydrogens would be expected.

To measure the number of active hydrogens, the sample is dissolved in D$_2$O and the mixture is stirred for a half hour. All of the exchanged hydrogens, regardless of their origin in the equilibrium mixture, become bonded to oxygen atoms and the OH bonds formed show the typical hydrogen-bonded OH stretching which results in an absorption band at 2.97 microns. Thus, after suitable calibration, the active hydrogen content of the unknown can be determined from the OH absorption of the D$_2$O phase. All samples were run in a 0.025 mm CaF$_2$ cell at the slow scan speed on a Perkin Elmer Model 337 Grating Infrared Spectrophotometer. The range from 2.5 to 3.3 microns was scanned. A calibration curve, using H$_2$O as the standard, was prepared. The number of moles of active hydrogen were calculated from the following formula of Harp (44)

\[
\text{Moles of active hydrogen/gram of sample} = \frac{\text{Moles of OH/ liter of solution}}{\text{grams of sample/ liter of solution}}
\]

where moles of OH/ liter of solution is a corrected term, obtained from the calibration curve representing corrected absorbance vs. concentration. "Corrected absorbance" is absorbance corrected for cell blank and residual D$_2$O background.
The calibration curve is prepared by using water (H₂O) in a concentration range of 0 to 6 moles of active hydrogen per liter. Absorbances equal to log To/T are determined for the calibration solutions where To equals the transmittance at 2.50 microns and T equals the transmittance at 2.97 microns, both transmittances being measured relative to air. The transmittance at 2.5 microns is used as a base line reference point since D₂O and the samples analyzed have a maximum transmittance at this wavelength in the range scanned. From these determinants a calibration curve is constructed for use in the analysis of unknown samples.
CHAPTER VI

RESULTS AND DISCUSSION

The first area of research to be discussed will be that of the radioimmunoassay. An ILA extract, isolated according to the procedure published by Antoniades and co-workers was used in the beginning phases of experimentation (1). This extract was added to obtain a concentration of 6 mg/cc to a 0.037 M solution of each of the following substances: three hypoglycemic sulfonylureas, tolbutamide, chlorpropamide, and acetohexamide (See Figure VI) and with one biguanide, phenformin (See Figure VII). The results of this assay are shown in Figure VIII. The ILA extract showed an activity of only 0.7 uU/mg of resin eluate. Chlorpropamide and phenformin were essentially inert in causing a release of free insulin; acetohexamide, however, and tolbutamide caused a 24.0 and a 43.0 uU/mg of resin eluate release, respectively. These latter two compounds, although minimally, are somewhat effective in that they release trace quantities of free insulin-like material. However, the concentration of the various oral hypoglycemic compounds used was 0.037 Molar, a concentration which is quite high when compared to the normal physiological usage concentration of 1.4 mM. The question of whether these drugs may have some effect on the assay itself, that is, the binding equilibrium of the radioimmunoassay, cannot be ignored, especially when one considers the
**STRUCTURE OF SULFONYLUREAS**

*Figure VI*
phenethylbiguanide

STRUCTURE OF PHENETHYLBIGUANIDE
(also referred to as Phenformin and DBI)

Figure VII
BAR GRAPH OF INSULIN-LIKE ACTIVITY IN THE PRESENCE OF ORAL HYPOGLYCEMIC AGENTS

Figure VIII
fact that tolbutamide will cause physiological reactivity of anti-
body bound insulin in the rat diaphragm assay (46). To help an-
swer this question, further radioimmunoassays were carried out.

Radioimmunoassay of ILA Extract and Antibody Bound Insulin Complexes

The radioimmunoassay procedure discussed was carried out using ILA extracts which were obtained using the procedure des-
cribed by Guenther and McDonald, (41) and also antibody bound in-
sulin complexes obtained by combining antibody with a quantity of insulin slightly in excess of the binding capacity of the antibody. The antibody-insulin complex, being soluble, could be assayed with the radioimmunoassay since it would not be retained on the filter. As stated in the introduction, it is hoped that this insulin com-
plex will serve as a model system for the in vivo bound-insulin complex.

The new ILA extract was utilized in this set of radioimmuno-
assays at a concentration of 20 mg/cc and the antibody bound insu-
lin complex was used at a concentration of 3.46 µg/cc. The fol-
lowing compounds were assayed with each complex, at a concentra-
tion of 0.4 mg/cc, which is approximately their physiological concentrations (at 1.4 mM/l); tolbutamide, acetohexamide, phen-
ethylbiguanide and chlorpropamide. Also heparin and biotin were assayed at a concentration of 50 µg/cc, (0.2 mM/l). To help eliminate the possibility of any interference from these agents
with the assay procedure itself, standard curves were prepared in the presence of each of the individual compounds. Figures IX to XV show these curves plotted according to the method of Hales and Randle, using \( \frac{c_0}{c_1} \) versus concentration, to obtain a linear plot (43). The theoretical background for this procedure is described by Hales and Randle as follows:

Suppose:

- \( i \) = the concentration of unlabeled insulin
- \( c_0 \) = the radioactivity (cpm) of the insulin-antibody complex when \( i \) is zero
- \( c_i \) = the radioactivity (cpm) of the insulin-antibody complex when the concentration of unlabeled is \( i \).
- \( I_o \) and \( I \) = the amount of \( ^{125}I \) - labeled insulin bound by antibody under these conditions.
- \( x \) = specific activity of the labeled insulin
- \( i_o \) = concentration of \( ^{125}I \) - labeled insulin

Then:

\[
\frac{I_o}{I} = \frac{i_o + i}{i_o} = \frac{i}{i_o} + 1
\]

\[
c_o = x \cdot I_o
\]

\[
c_i = x \cdot I
\]

\[
c_o/c_i = I_o/I
\]

Then:

\[
c_o/c_i = i \cdot \frac{1}{I_o} + 1
\]
Saline Standard Curve

IMMUNOASSAY STANDARD CURVE

Figure IX
Tolbutamide Standard Curve

\[ \frac{c_0}{c_i} \]

\[ \mu U \]

IMMUNOASSAY STANDARD CURVE

Figure X
Heparin Standard Curve

IMMUNOASSAY STANDARD CURVE
Figure XI
IMMUNOASSAY STANDARD CURVE
Figure XII
Chlorpropamide Standard Curve

IMMUNOASSAY STANDARD CURVE
Figure XIII
Acetohexamide Standard Curve

**IMMUNOASSAY STANDARD CURVE**

*Figure XIV*
IMMUNOASSAY STANDARD CURVE

Figure XV
which is in the form of \( y = mx + b \). Therefore \( \frac{c_0}{c_1} \) will be linearly related to \( i \) and \( \frac{c_0}{c_1} \) will be unity when \( i = 0 \).

This theoretical relationship will only hold in practice if the affinity of antibody for \(^{125}\text{I}\)-labeled insulin and unlabeled insulin is the same and if the amount of insulin bound by antibody is independent of the concentration of the insulin. Departure from this theoretical relationship, in practice, will not interfere with the practical assay of insulin as long as the latter is made to refer to a standard solution of the hormone.

The curves in Figures IX to XV demonstrate that the various chemical agents used affect the assay procedures by changing the slope of the standard curves indicating that the binding of antibody to insulin is altered. It appears from these curves that tolbutamide and heparin enhance the binding capacity of the antibody system while DBI and chlorpropamide inhibit it. On the other hand acetohexamide and biotin have little effect on the binding capacity causing possibly only a slight decrease. If we let the saline curve represent a factor of unity for binding capacity, Table I then shows how much the binding capacity of the system is altered relative to the saline standard curve.

By computing the equation for the standard curve the amounts of immunologically active compounds were calculated. Table II shows these results for "bound" insulin and the insulin antibody complexes. In the "bound" insulin complexes, it is evident that
<table>
<thead>
<tr>
<th>Substance</th>
<th>Relative Binding Capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>1.0</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>2.5</td>
</tr>
<tr>
<td>Heparin</td>
<td>2.9</td>
</tr>
<tr>
<td>Acetohexamide</td>
<td>0.9</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.8</td>
</tr>
<tr>
<td>Chlorpropamide</td>
<td>0.4</td>
</tr>
<tr>
<td>DBI</td>
<td>0.3</td>
</tr>
</tbody>
</table>

(Numbers over 1 = increase in binding capacity, Numbers under 1 = decrease in binding capacity)
TABLE II

IMMUNOASSAYABLE INSULIN ACTIVITY IN THE PRESENCE OF CHEMICAL AGENTS

<table>
<thead>
<tr>
<th>Sample</th>
<th>Insulin Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank-bound insulin (BI)</td>
<td>1.1 uU/mg extract</td>
</tr>
<tr>
<td>Tolbutamide-BI</td>
<td>2.2 uU/mg extract</td>
</tr>
<tr>
<td>Biotin-BI</td>
<td>1.0 uU/mg extract</td>
</tr>
<tr>
<td>Heparin-BI</td>
<td>0.53 uU/mg extract</td>
</tr>
<tr>
<td>Acetohexamide-BI</td>
<td>0.03 uU/mg extract</td>
</tr>
<tr>
<td>DBI-BI</td>
<td>2.9 uU/mg extract</td>
</tr>
<tr>
<td>Blank-antibody (AB)</td>
<td>26 mU/mg antibody</td>
</tr>
<tr>
<td>Tolbutamide-AB</td>
<td>17 mU/mg antibody</td>
</tr>
<tr>
<td>Biotin-AB</td>
<td>47 mU/mg antibody</td>
</tr>
<tr>
<td>Heparin-AB</td>
<td>11 mU/mg antibody</td>
</tr>
<tr>
<td>Acetohexamide-AB</td>
<td>25 mU/mg antibody</td>
</tr>
<tr>
<td>Chlorpropamide-AB</td>
<td>75 mU/mg antibody</td>
</tr>
<tr>
<td>DBI-AB</td>
<td>135 mU/mg antibody</td>
</tr>
</tbody>
</table>
DBI shows the greatest activity, with tolbutamide also showing activity. On the other hand biotin appears to have little effect while heparin and acetohexamide appear to inhibit activity.

In the experiments conducted on the insulin antibody complexes, DBI again shows the greatest activity, with chlorpropamide, too, showing high activity. Tolbutamide and heparin appear to inhibit activity while acetohexamide and biotin show essentially no change or only a moderate increase, respectively. Also illustrated by the data from the insulin antibody complexes is what was predicted by the standard curves and explained previously, that is, the alteration in binding capacity of the insulin antibody system, by the chemical agents studied. As predicted, tolbutamide and heparin increase binding capacity, thereby inhibiting release and this is what has occurred. Also, binding capacity was predicted to decrease with DBI and chlorpropamide and remain unchanged or increase slightly with acetohexamide and biotin, respectively. This, again, is what has occurred.

**Immunological Insulin**

In both types of complexes, the increase in activity caused by some of the agents appears to be a significant quantity. However, it is not really so if comparison is made to physiological activity. Experiments conducted in the laboratory by coworkers indicate about a ten-fold greater physiological activity
with some of the agents.

A logical interpretation of these experimental results would be that, in addition to the effect which some of the hypoglycemic compounds used might have in causing an actual release of insulin from its bound form, they may act in another completely different manner. This latter effect may possibly be due to an ability of the compounds to change the conformation of the insulin protein complex, thereby rendering it physiologically active without involving an actual release of the insulin from the insulin-protein complex. This concept is credible because of the fact that it has been shown that insulin can cause its physiological effects by disulfide binding to a receptor site on the cell membrane (63). Further discussion of this phenomena will be taken up later. This concept would, then indicate that insulin does not have to pass through the cell membrane to cause its effect but would only have to be available to bind to the cell membrane.

Circular Dichroism: Evidence for Conformational Change

Circular dichroism spectra were run on the same antibody insulin complex and using the same hypoglycemic compounds which were employed in the previous radioimmunoassay studies. The concentrations of all reagents were maintained at the same level as in the previous studies. Figures XVI to XXI show these curves, Figure XXI being a composite of all curves. The curves are plotted
Insulin-antibody Complex Blank

CD CURVE
Figure XVI
Tolbutamide Insulin-antibody Complex

CD CURVE

Figure XVII
DBI Insulin-antibody Complex

CD CURVE

Figure XVIII
Chlorpropamide Insulin-antibody Complex

CD CURVE

Figure XIX
Heparin Insulin–antibody Complex

CD CURVE
Figure XX
1 - Insulin-antibody complex
2 - Insulin-antibody complex with heparin
3 - Insulin-antibody complex with tolbutamide
4 - Insulin-antibody complex with DBI
5 - Insulin-antibody complex with chlorpropamide

CD CURVE COMPOSITE
Figure XXI
using reduced circular dichroisms versus wavelength, the former being $A_L - A_R$ expressed at $1\%$ and at a pathlength of 1 centimeter.

The compounds investigated, as shown by these curves, produced changes in the conformation of the antibody-insulin complex. The region studied, 320 to 240 nm, is indicative of disulfide bond and aromatic group interactions. The circular dichroism effects of the antibody-insulin complex, in the region studied, are weak and complex, however, there is no reliable alternative method with which to check dynamic conformation in solution. The conclusions which were reached, based on this data, must therefore be considered to be somewhat speculative in nature. A few remarks, however, may be pertinent.

The complex seems to show a blue shift (a shift in the spectra to shorter wavelength) over that of the single components as shown in the spectra of other investigators (62 and 68). The addition of heparin causes even more of a shift. This change in spectra may be indicative of tyrosine and phenylaline transitions of insulin possibly with a contribution from disulfide interactions. The remaining compounds investigated caused a decrease in the circular dichroism of the complex at 265 nm and 250 nm, indicative of a change in conformation due again, possibly, to phenylalanine and tyrosine transitions. This is based on the investigations of Ettinger that have shown phenylalanine, tyrosine, and their
derivatives to show circular dichroism transitions between 250 and 274 nm (32).

Deuterium Exchange

Deuterium exchange experiments were conducted on antibody-insulin complexes and on bound insulin complexes, using heparin and tolbutamide. This data is shown in Table III. In every case, the addition of one of these compounds caused a change in the number of active hydrogens present in the complex. However, all of the changes represented a reduction in the number of active hydrogens which is indicative of a conformational change in these complexes due either to the formation of bound water which would thereby reduce the amount of OH stretch measured, or by a dilution effect of the D2O caused by the release of bound water trapped in the insulin protein complex. This latter effect may be more likely what has occurred in light of the interpretation of the circular dichroism studies.
### TABLE III
DEUTERIUM EXCHANGE DATA

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**BI** = "Bound Insulin"

BI used with tolbutamide was isolated according to the procedure of Antoniades. (1)

BI used with heparin was isolated according to the procedure of Guenther. (41)
CHAPTER VII

FURTHER DISCUSSION AND CONCLUSIONS

The previous experimental material has shown that two effects on insulin-protein complexes occur in the presence of oral hypoglycemic agents. These effects involve the liberation of free insulin and a conformational change.

The fact that physiological insulin activity, as reported by Guenther and McDonald, (41) is greater than accounted for by free insulin release would indicate that the conformational modification is the more important effect in eliciting insulin-like activity. The following question can then be raised: How can insulin remain bound as an integral part of an insulin protein complex and still exhibit physiological activity? This seeming contradiction may be resolved by considering the fact that insulin does not need to penetrate the cell in order to cause its effect but only needs to bind to the cell membrane. The studies of Wohltman and Narahara have shown that insulin which is bound to cell membranes exerts a physiological effect on these tissues even after prolonged washing in insulin free solution (83).

Ultrastructural studies of Barrett and Ball also suggest that the first event of insulin action must be its adsorption to the cell, specifically, to the cell membrane (17). Their studies, using electron microscopy, demonstrated the induction of an invagination
in the membrane of the epididymal adipose tissue cell. The question remains, then, how does the combination of insulin with a specific receptor at the cell surface actually occur?

Schwartz proposed that a sulfhydryl-disulfide (SH-SS) interchange reaction might be the basis for the initial reaction (69). Noting that the disulfide ring in oxytocin is of approximately the same size as the intrachain disulfide loop of the A chain of insulin which is known to be essential for activity, it was discovered by Mirsky that synthetic oxytocin exerted an insulin like action on the utilization of glucose by rat epididymal adipose tissue and reduced the free fatty acid concentration in the plasma of normal and alloxan-diabetic dogs (60). Further investigations on the question of insulin attachment by Cadenas, showed in perfused rat heart that brief exposure to the sulfhydryl blocker, N-ethylmaleimide (NEM), abolishes subsequent stimulation of glucose transport by insulin (23). However, such inhibition by NEM does not occur if the hearts are first treated with insulin. Edelman and Schwartz have shown, by further investigation using NEM and labeled insulin, that this disulfide interaction is not interchain but rather intrachain involvement with the cell receptor (27). Figure XXII shows the proposed binding mechanism.

This proposed binding mechanism of insulin to the cell receptor could be the answer to the question of how protein bound insulin is still able to exert its physiological effect, that
PROPOSED INSULIN-MEMBRANE BINDING

Figure XXII
is, by combining with the cell membrane through sulfhydryl-disulfide reactions instead of actually penetrating the cell membrane. In the proposed mechanisms shown in Figure XXII, part A shows how, as Rieser (63) explains, hormone receptor disulfide sulfhydryl reactions could occur with a subsequent change in the tertiary structure of the membrane proteins thereby altering the membrane barriers that limit diffusion of water and specific solutions. In part B is shown how disulfide-linked membrane fibrils could be separated by disulfide interchange thereby causing pores in the membrane to open up for the passage of solution.

Although a mechanism for physiological action of insulin has been proposed, the question of how the "bound" insulin molecule becomes activated still remains. To help answer this question, it is necessary to turn to the tertiary structure of insulin as elucidated by Blundell from x-ray crystallographic studies (20). Figure XXIII shows the structure proposed by Blundell. The most obvious stabilizing forces result from the disulfide bonds. B7 - A7 is on the outside of the molecule while B19 - A20 is slightly more concealed but still accessible to solvent. The A6 - A11 intrachain disulfide bond is completely buried and forms part of the non-polar core of the insulin molecule. As Blundell has shown, the general disposition of these groups is consistent with the ease with which the interchain bridges are reduced while the intrachain disulfide is highly unreactive to chemical agents.
PROPOSED TERTIARY STRUCTURE OF INSULIN

Figure XXIII
Another important feature of this structure is the existence of a completely non-polar core, as Blundell states, "The regions of non-polar intrachain contacts are brought together in the complete molecule and define a hydrophobic center comprising the residues: B6, B11, B15, and A16 leucines; B18 valine; B24 phenylalanine and the phenyl ring of B26 tyrosine; the A6 - A11 and part of the A20 - B19 cystines." (20) Figure XXIV shows the schematic representation of Edmundson and Schiffer for the non-polar core (28). This non-polar core may be the reason for the change observed in the deuterium exchange data, namely, a reduction of solvent OH. This may be explained by the fact that when insulin is bound as an integral part of an insulin-protein complex, as in the case of insulin hexamer formation, solvent is excluded from the exposed non-polar face of the monomer whereas when a conformational change occurs, solvent may be taken up. Blundell states, "Whenever sequence variations involving substantial modification of the non-polar core or stabilizing residues are made, the insulin is found to be inactive... Indeed, it has been emphasized that any change in conformation results in a decrease in biological activity of insulin." (20)

The deuterium exchange data and the circular dichroism spectra shown in the experimental section indicates a definite change in the conformation of the insulin protein complex in the presence of the hypoglycemic compounds investigated. The change
Hydrophobic residues between monomers

Hydrophobic residues of monomer core

Hydrophilic residues on dimer surface in region of 3-fold axis of hexamer

Hydrophobic residues on surface of dimer but in core of hexamer

THE INSULIN NON-POLAR CORE

Figure XXIV
in conformation observed may be a return of the insulin molecule to a more random or native form of conformation from that formed by the association of insulin with protein. This may be explained by looking at the antibody complex of insulin.

Antibodies against a helical structure tend to stabilize or induce the helical tendency of polypeptides (68). This return to the native form, that is, a combination with the cell membrane through sulfhydryl-disulfide interaction, upon the addition of chemical agents, may, then, be what allows the insulin-protein complex to exert its physiological action.

In conclusion, it has been shown that the addition of oral hypoglycemic compounds to insulin complexes causes a dual effect, namely that of free insulin release and that of conformational change, the latter apparently being more significant in relation to physiological activity of insulin. Both bound insulin isolated from human serum and synthetic antibody-insulin complexes appeared to be affected similarly. This observation that both complexes are effected similarly would seem to justify ruling out the possibility that although the isolated bound insulin had some contaminating protein the effect seen in the deuterium exchange studies was not attributable to these other proteins.

The important role played by a conformational change also helps to clear up a controversy that has continued in the literature for over five years concerning the existence of "bound"
insulin. Various investigators have reported that they have been able to isolate it while others have been unable to do so. An important consideration here is the fact that those investigators who have not been able to isolate "bound" insulin have, for the most part, used the radioimmunoassay procedure which measures antibody-combinable insulin only. On the other hand, investigators who have confirmed the existence of "bound" insulin have used bioassay procedures which measure physiologically active insulin.

The conformational changes induced by the various oral hypoglycemic compounds also aid in understanding their function, for example, phenethylbiguanide is known to produce a hypoglycemic effect in pancreatectomized animals. It has been shown previously that phenethylbiguanide works on the hexokinase system (66) but now it has also been shown that it can release free insulin from its bound form and also contribute to a conformational change in the protein-insulin complex.

Of major importance is the demonstration that biotin, a vitamin, causes a similar change in protein-bound insulin and at concentration levels which are in the physiological range. Thus, this vitamin and the other compounds which are discussed may help the body maintain homeostasis upon glucose load by causing a physiological activation of the bound insulin complex. Now that new mechanisms for the action of the oral hypoglycemic compounds have been elucidated, there is no longer any justification for
explaining their hypoglycemic action by a single property, for example, in the case of tolbutamide, the release of insulin from the pancreas.
# APPENDIX

A REPRESENTATIVE SAMPLE OF DATA DERIVED FROM THE RADIOIMMUNOASSAY PROCEDURE

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### APPENDIX

#### COMBINED EXPERIMENTS

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Standard 1 = 10.4 uU of insulin per ml
Standard 2 = 19.9 uU of insulin per ml
Standard 3 = 36.5 uU of insulin per ml
Standard 4 = 62.5 uU of insulin per ml
BIBLIOGRAPHY


63. Rieser, P., Insulin Membranes and Metabolism, the Williams and Wilkins Co., Baltimore (1967)


The dissertation submitted by Lawrence J. Crolla has been read and approved by five members of the faculty of Loyola University of Chicago.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval with reference to content, form, and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

January 9, 1973
Date

Signature of Adviser