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Role of the amino groups in the structure and function of insulin

Thesis presented by

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to the

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Abstract

By means of the competitive labelling technique using acetic anhydride as the labelling reagent, the ionization constants of the three amino groups of insulin at 10 °C were found to be 7.3, 7.9 and 7.8 for the phenylalanyl B1, glycyl A1 and lysyl B29 amino groups, respectively. The Phe B1 and Gly A1 amino termini were found to be super-reactive towards acetic anhydride, whereas the Lys B29 ε-amino group appeared to be buried towards this reagent. Under physiological conditions, the three amino groups of insulin are largely deprotonated; it is suggested that this deprotonation is associated with monomer formation. The unusual chemical properties of the three amino groups allow them to readily react with carbon dioxide to form carbamino derivatives. This reaction was shown to cause both a decrease in the specific binding of ^{125}I-insulin to its membrane receptor, and an increase in the dissociation of the insulin-receptor complex. The physiological significance of these findings is discussed.

Acknowledgements

The author would like to gratefully acknowledge the National Research Council and the Medical Research Council for their financial assistance.

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List of Abbreviations

AMP	adenosine monophosphate
ATP	adenosine triphosphate
BAWP	butanol/acetic acid/water/pyridine
BSA	bovine serum albumin
cal	calorie(s)
CD	circular dichroism
Ci	Curie(s)
cpm	counts per minute
FDNB	2,4-dinitrofluorobenzene
g	gram(s)
GMP	guanosine monophosphate
H	enthalpy
I	insulin
K	acid dissociation constant
l	litre(s)
m	metre(s)
M	molar
mol	mole(s)
NMR	nuclear magnetic resonance
ORD	optical rotatory dispersion
PBS	phosphate buffered saline
rpm	revolutions per minute

UV	ultraviolet
V	volt(s)
w	weight
x g	times gravity

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CHAPTER I: Introduction

1. General introduction

The discovery of insulin by Banting and Best in 1921 was a major scientific milestone of the century. Although much effort over the last fifty-eight years has been devoted to exploring the mechanism of action of insulin, still little is known about how this potent protein hormone influences the metabolism and function of most tissues. In the search for its mechanism of action, insulin has acted as an important stimulus to the development of many scientific fields, including x-ray crystallography, cell biology, molecular genetics, intermediary metabolism, endocrinology and protein chemistry.

At the present time, insulin action at the cellular level is considered to reside at four distinct biochemical levels (27, 53, 87):

(a) The actual binding of insulin to its specific membrane receptor.

(b) The transformation of the insulin-receptor interaction into some form of transmembrane signal. Evidence showing enhancement of biological activity by the crosslinking of bound insulin and the

movement of insulin receptors in the plane of the membrane (82, 87, 138) suggests that receptor clustering or aggregation is essential to the exertion of a transmembrane signal. Disruption of groups of insulin receptors by cytochalasin B does not, however, prevent insulin from exerting its action (82). Evidence of lateral diffusion of the insulin receptor in the plane of the membrane (27, 138) gives support to the mobile receptor hypothesis (80), which postulates a receptor which reversibly associates with effectors, the association resulting in changes in reactivity in membrane proteins, enzymes, and carrier systems.(76). An alternative hypothesis suggests that mobile receptors are unnecessary, as the insulin receptor could be in direct physical contact with the effector (27). More generally, binding could cause a conformational change in the receptor, this change then being propagated throughout the membrane (27). Oxidation of membrane sulfhydryls have been indirectly implicated in at least one of insulin's biological actions (40, 69). The suggested importance of membrane fluidity in the insulin-receptor interaction (27, 138) indicates that besides membrane receptor and effector proteins, phospholipids may act as membrane components mediating the cellular effects of insulin (40). The demonstration that insulin binding is accompanied by an increase in lipid microviscosity which mediates a non-specific increase in the degree of exposure of membrane proteins to both sides of the membrane suggests that this also can modulate the response to insulin (109).

(c) The generation of an intracellular message or messenger

at the cell surface. To date, no single intracellular messenger which carries out all the intracellular effects of insulin has been discovered, although several candidates for the role have been proposed, including calcium (15, 40, 69, 72, 76, 87, 115), ATP (69), cyclic AMP (15, 36, 38, 40, 47, 69, 72, 76, 87, 115, 150), cyclic GMP (15, 38, 40, 69, 72, 76, 87, 115), modulators of protein kinase and/or phosphatase activities (40, 87, 143), insulin itself (71, 72, 78, 87, 143, 154), and one of insulin's degradation fragments (15, 49, 69, 72, 87, 143).

(d) Chemical modifications of various enzymes and transport systems, resulting in the final anabolic effects of insulin on such diverse activities as membrane transport of glucose, amino acids, nucleic acid precursors and certain ions, and synthesis of glycogen, proteins, lipids and nucleic acids (27, 36, 40, 72, 143).

In recent years, much detailed information has been accumulated on the first step of insulin action, that of the interaction between insulin and its membrane receptor. This review will concentrate primarily on this hormone-receptor interaction. The structure of insulin itself will first be discussed using x-ray crystallography and chemical modification studies to elucidate the relationship of insulin structure to insulin function; the roles of the three amino groups of insulin in this structure-function relationship will be discussed. The physicochemical characteristics and biochemical prop-

erties of the receptor will then be described, as obtained from such techniques as gel filtration, density gradient centrifugation, and selective enzymatic degradation. The actual interaction between the hormone and its receptor will next be examined in detail, primarily with respect to its characteristics of binding, dissociation, and degradation in liver and adipose tissues. Finally, the clinical significance of such binding studies will be briefly discussed with particular reference to obesity and diabetes.

2. Insulin structure

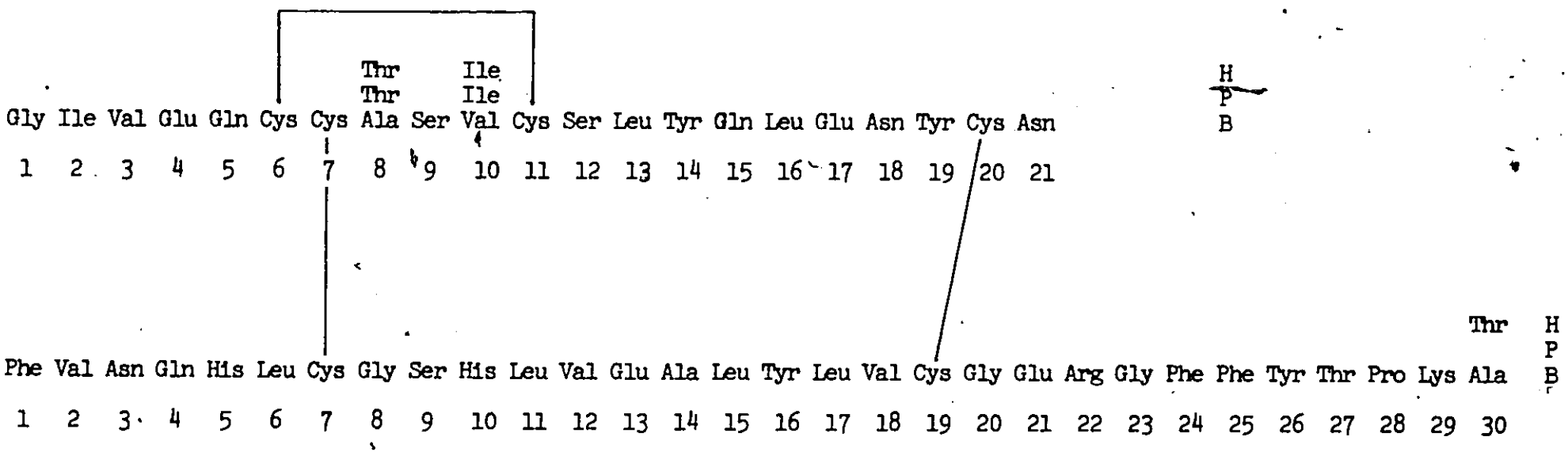
A. X-ray crystallography

Insulin is a low molecular weight (5870 daltons) polypeptide hormone consisting of two disulfide-linked peptide chains (Figure 1), the A chain usually containing 21 amino acid residues, the B chain, 30 residues. The complete determination of the amino acid sequence of insulin by Sanger and co-workers in 1955 provided the framework within which to examine the three-dimensional relationships of the structural moieties present (14). As the solution structure of insulin closely resembles that found in crystals (14, 15), x-ray crystallography is an excellent tool for the determination of these three-dimensional relationships.

Insulin crystallizes as a rhombohedral crystal, containing two zinc ions to every six molecules of insulin (14). In solution,

Figure 1

The amino acid sequences of human (H), porcine (P) and bovine (B) insulins. The upper sequence represents the A chain; the lower sequence represents the B chain. All amino acid substitutions are shown.


Thr Ile
Thr Ile
H
P
B
Gly Ile Val Glu Gln Cys Cys Ala Ser Val Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Thr H
P
Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ala B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

insulin exists primarily as a monomer, but also as a dimer and higher aggregates, depending on such conditions as pH, ionic strength, metal ions, temperature, insulin concentration, and the presence or absence of zinc (14, 56, 126, 135). Most recent evidence suggests that under physiological conditions, insulin exists primarily as a monomer (5, 6, 14, 15, 30, 44). In addition to its self-association properties, the small insulin protein molecule also contains such important structural features as α-helix, antiparallel β-pleated sheet (in the dimer), and a hydrophobic interior and hydrophilic exterior, due to the arrangement of its interchain disulfide bonds (14, 15, 143). Those nonpolar surface residues and polar interior residues which exist in the monomer are progressively shielded or exposed, respectively, in the aggregation of the monomer and dimer (14, 15). Nearly all hydrophobic and hydrogen-bonding interactions between monomers and dimers originate from B chain residues, reflecting the surrounding of the A chain by the B chain (1, 14). The insulin dimer therefore contains an antiparallel β-pleated sheet structure of hydrogen bonding between the two monomers, both of which are almost identical in conformation (14, 79).

Detailed analysis of the x-ray crystallographic structure of insulin has implicated each of the three amino groups of insulin in a stabilizing reaction important for the tertiary structure of insulin, either hydrogen bond or salt bridge formation. For example, crystallographic data indicate that in the hexamer, Phe B1 is nicely

accommodated in a hydrophobic surface pocket between Leu A13 and Tyr A14 of the adjacent molecule (14, 126), as part of an intricately organized region of contacts between dimers (1, 14). This arrangement brings its positively charged α-amino group next to the negatively charged carboxylate group of Glu A17 of the adjacent dimer, enabling a salt bridge to form upon hexamer formation (14, 16). In addition, Phe B1 may be associated with Val B18 and Phe B1 of the adjacent dimer, and may form a hydrogen bond with Glu A17, also of the adjacent dimer (14). Large substituents bound to Phe B1 amino groups impair hexamer formation, since the additional bulky groups prevent formation of the three pairs of Phe B1 residues (1, 14, 105).

Gly A1 resides in a predominantly hydrophobic region of the insulin molecule near to the β-pleated sheet that joins the two terminal parts of the B chains in the insulin dimer (106). The Gly A1 α-amino group is located on the surface of the hexamer (14, 68, 85, 126), occupying a compact surface pocket; its environment is slightly different in the two molecules of the dimer in the crystal structure (14). As this α-amino group is close to ordered water crystals, in solution it may form either a salt bridge with the Glu A4 carboxylate group of the same monomer (14, 16), or hydrogen bond with the hydroxyl function of Tyr A19 (14). Gly A1 is probably located on the periphery of the receptor-binding region (130) described in a later section.

Gly A1 and Lys B29 are in close proximity both in the insulin crystal and in solution, as confirmed by x-ray crystallography and cross-linkage experiments (14, 16). The ε-amino group of Lys B29 lies at the end of the extended B chain on the surface of the hexamer (14, 16, 85), in a position where it could form a salt,bridge with the Glu A4 carboxylate group in solution (1, 14). In the monomer, Lys B29 is in the general region of those residues directly involved in dimerization (14, 16, 130);although it makes no direct contacts on dimerization (14), a small change in its environment on dimerization has been observed (16). Lys B29 is also close to those residues believed to be involved in receptor binding (130).

Detailed x-ray crystallographic data is available not only on the three amino groups of insulin, but also on many additional groups and residues in the insulin molecule (14). Knowledge of the x-ray crystallographic structure of insulin has been of tremendous importance in interpreting the results of the chemical modification studies described in the next section.

B. Chemical modification studies

(1) General introduction

The relationship of structure to the binding and biological activity of insulin has been extensively studied using a variety of

chemical and enzymatic modification techniques. Although the activity of insulin depends to a large extent on its integrity or shape (14, 15, 21, 54, 68, 130), certain regions of the insulin molecule cannot be modified without loss of activity. Therefore, the selective modification of specific residues and measurement of the effect of these modifications on the binding and biological activity of insulin provide an indirect study of the role of different functional groups in the action of insulin (14). Many of these modification studies have been performed on the Gly A1, Phe B1, and Lys B29 amino acid residues, either by chemically modifying their amino groups, or by enzymatically removing the entire residues. In all cases, it is believed that the mechanism by which these and other modifications of insulin lead to a decrease in activity is by decreasing the affinity of the insulin molecule for its receptor, whereas the modified insulin-receptor complex exhibits full activity (61, 68).

(2) Chemical reactivities of the three amino groups

The relative reactivities of the three amino groups toward chemical modifying reagents depend on a variety of reaction conditions, including solvent, pH, temperature, and the reactivity and steric hindrance of the reagent's functional group (14, 106). In almost all cases, reaction of such reagents as phenylisothiocyanate (2, 19),

phenylisocyanate (2, 14, 19), trinitrotoluene (14, 113), and hydroxysuccinimide esters (14, 106) with the three amino groups of insulin indicate that the ε-amino group of Lys B29 is by far the least reactive of the three groups, whereas the relative reactivities of the α-amino groups of Phe B1 and Gly A1 depend on the conditions of the reaction.

(3) Ionization constants of the three amino groups

Until recently, determination of the ionization constants of the three amino groups of insulin has been largely a matter of estimation. For example, in 1954, by means of a hydrogen ion titration curve, Tanford and Epstein (144, 145) determined the pK values of the α-amino groups and the ε-amino group to be 7.4 and 9.6 respectively, using the rather tenuous assumption that all ionizable groups of a given type were intrinsically identical. Using these values, Africa and Carpenter (2) attempted to explain the order of reactivity of the three amino groups with phenylisothiocyanate by assigning them pK values of 7.45, 8.2 and 9.6 for the amino groups of Phe B1, Gly A1 and Lys B29 respectively. Not until 1977, with the work of Bradbury and Brown (16), has there been any real progress in the accurate determination of the ionization constants of the three amino groups of insulin. By means of ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy at 20 °C, these workers obtained pK values of 6.7, 8.0 and

11.2 for the Phe B1, Gly A1 and Lys B29 amino groups of methylated bovine insulin, respectively. More recently, Chan, Oda and Kaplan (24) used the technique of competitive labelling with 2,4-dinitrofluorobenzene as the labelling reagent to determine the pK values of the three amino groups of porcine zinc-free insulin at 20 °C to be 6.9, 7.7 and 7.0 for the Phe B1, Gly A1 and Lys B29 amino groups, respectively.

(4) Effects of modification on the biological activity of insulin *

The biological activities of insulins modified at the three amino groups have been measured directly in vitro by stimulation of glucose oxidation by epididymal rat fat pads, stimulation of glucose uptake by rat diaphragm, and incorporation of ^{14}C-glucose into lipids in rat fat cells, directly in vivo by blood sugar depression in rats, and mouse convulsion tests, or indirectly in vitro by the binding affinity for rat liver or adipocyte plasma membranes.

* For more complete details on the effects of various chemical modifications of the three amino groups of insulin on its biological activity, refer to References 14, 51, 68, 106 and 158.

Measurement of the biological activities of various Lys B29-modified insulin analogues confirm the apparently minor importance of Lys B29 for the activity of insulin (14, 51, 105). For example, modification of the Lys B29 ε-amino group with acetyl, acetoacetyl, thiazolidine carbonyl, phenylthiocarbamoyl and butyloxy carbonyl groups (14, 68, 106) did not affect the biological activity of the insulin molecule to any great extent. Complete removal of the tripeptide Pro B28 Lys B29 Ala B30 (14, 68, 95) also caused very little decrease in biological activity. However, removal of between five to eight amino acid residues from the carboxy terminal end of the B chain (14, 18, 68) caused a tremendous decrease in the biological activity of insulin (to less than 17%), indicating that the Lys B29 residue is quite proximal to a region of the insulin molecule that is crucial to its biological action. The lack of importance of Lys B29 is emphasized by its variability in mammalian insulins (14); in one of the two rat insulins, Lys B29 is replaced by Met, and one of the two toadfish insulins completely lacks Lys B29 (14).

It is clear that although modification or deletion at the B chain amino terminus gives quite a large decrease in immunoreactivity (14, 106), biological activity is affected to a much lesser extent (51). Modification of the Phe B1 α-amino group with acetyl, acetoacetyl, thiazolidine carbonyl, fluorescein isothiocyanate, phenylisothiocyanate, phenylthiocarbamoyl, and butyloxy carbonyl groups (14)

19, 51, 68, 106) caused relatively small changes in biological activity, the largest decreases occurring with the largest reagents (14). Complete removal of Phe B1 or its α-amino group had little or no effect on the biological activity of insulin (17, 68, 105, 106, 158). In addition, in chicken and turkey insulins, Phe B1 is replaced by Ala (14, 141); as well, the amino terminus of the fish B chain often has the sequence Met B0 Ala B1 (14).

Of the three residues containing amino groups in insulin, only Gly A1 and its α-amino group appear to be of any great importance to the biological activity of insulin. Substitution of increasingly bulky groups at Gly A1 progressively lowers the activity of insulin (14, 51, 106, 130); although acetyl-Gly A1 insulin appears to retain most or all of its activity (106, 130), modifications by acetoacetyl, thiazolidine, hemisuccinyl, arginyl, arginyl-lysyl, thiazolidine carbonyl, phenylthiocarbamoyl, butyloxy carbonyl, and 2-dimethyl-3-formyl-L-thiazolidine-4-carbonyl groups all caused decreases in biological activity (14, 68, 106, 130). Replacement of the α-amino group of Gly A1 by a hydrogen caused a loss of 65% of its biological activity (29). Substitution of one hydrogen of the α-amino group by a methyl group decreased biological activity by 17% (29). Replacement of Gly A1 with Leu, Val or Pro decreased biological activity to between 2 and 16% (68). Cross-linkage of Gly A1 to Phe B1 or Lys B29 considerably lowered the biological activity of insulin (51, 60, 68,

85). Complete removal of Gly A1 almost totally eliminated the biological activity of insulin (2, 14, 68, 106). In addition, Gly A1 is one of the few invariant insulin residues, indicating that its geometry or chemical properties are critical to insulin's structure and function (14, 106).

All of the information provided by chemical modification studies of the three amino groups of insulin indicates that the Phe B1 and Lys B29 residues are relatively unimportant when considering the biological activity of the insulin molecule. On the other hand, while Gly A1 may not be directly involved in the biological activity of insulin, an area extremely close to it may form the active region. It must be emphasized that this conclusion is based on many chemical modification studies, some of them involving the formation of neutral derivatives at the Gly A1 α-amino group; therefore, not only could the modifying reagents induce conformational changes in the insulin molecule, or sterically hinder its receptor-binding region, but also they could weaken stabilizing interactions involving the positively charged amino group. However, because the ionization constants of all three amino groups have not been determined conclusively, this is merely speculative, as it was not known whether these amino groups are uncharged or positively charged under physiological conditions.

It must also be emphasized that studies combining x-ray analysis, CD, ORD, UV spectroscopy and the binding and activity of

chemically modified insulins (14, 130, 151) all indicate that it is the conformation or tertiary structure of insulin that plays the most critical role in the interaction of insulin with its receptor, by maintaining the proper spatial relationships among structural components (14, 15, 130). Full receptor binding is probably attained by interaction of several insulin residues with the receptor, and loss or modification of any one residue without large accompanying conformational changes would not completely abolish binding, but simply decrease it (130).

(5) Effects of modification on the conformation of insulin

As has been previously mentioned, it is believed that the conformation of the insulin molecule is critical to the formation of the insulin-receptor complex. For this reason, it is important to bear in mind the concept that demonstration of inactivity of an insulin analogue is insufficient evidence for the modified residue belonging to an active site; one must also establish that modification of the chemical structure has not induced conformational changes at residues other than the one modified (14, 68). To this end, a few conformational studies have been performed. Phe B1 is apparently not involved in any stabilizing interactions crucial to tertiary structure, as fluorescein thiocarbamyl Phe B1 insulin appears to retain

the tertiary structure of native insulin (14). In addition, the CD spectrum of insulin lacking the Phe B1 residue is very similar to that of native insulin (14, 68). As well, little change in structure is observed upon removal of the tripeptide containing Lys B29 (14), although removal of five or more amino acids from the carboxy terminal end of the B chain causes a marked change in the CD spectrum (14, 68).

On the other hand, CD spectra of insulin with the Gly A1 residue modified with acetyl and arginyl groups show differences from crystalline insulin, possibly indicating some local conformational changes in solution (14). As well, electron density maps of insulin modified at Gly A1 by t-butoxycarbonyl and 2-dimethyl-3-formyl-L-thiazolidine-4-carbonyl groups show that these groups distort the A chain helix and move the side chain of Tyr A19 which in turn gives rise to distortions in the arrangement of the B chain residues involved in dimerization (130). This suggests that chemical modifications at Gly A1 may affect the CD spectrum not by greatly affecting the tertiary structure of the modified insulin but by changing the population of aggregated insulin molecules (130). CD spectra also show that addition of a cross-link between Gly A1 and Lys B29 causes small conformational changes in molecular structure in solution (85). Finally, insulin from which the Gly A1 residue has been completely removed shows a large change in CD (14, 68), suggesting its removal not only removes stabilizing interactions formed by its α-amino and

α-carbonyl groups, but also exposes the hydrophobic interior of insulin to solvent (14).

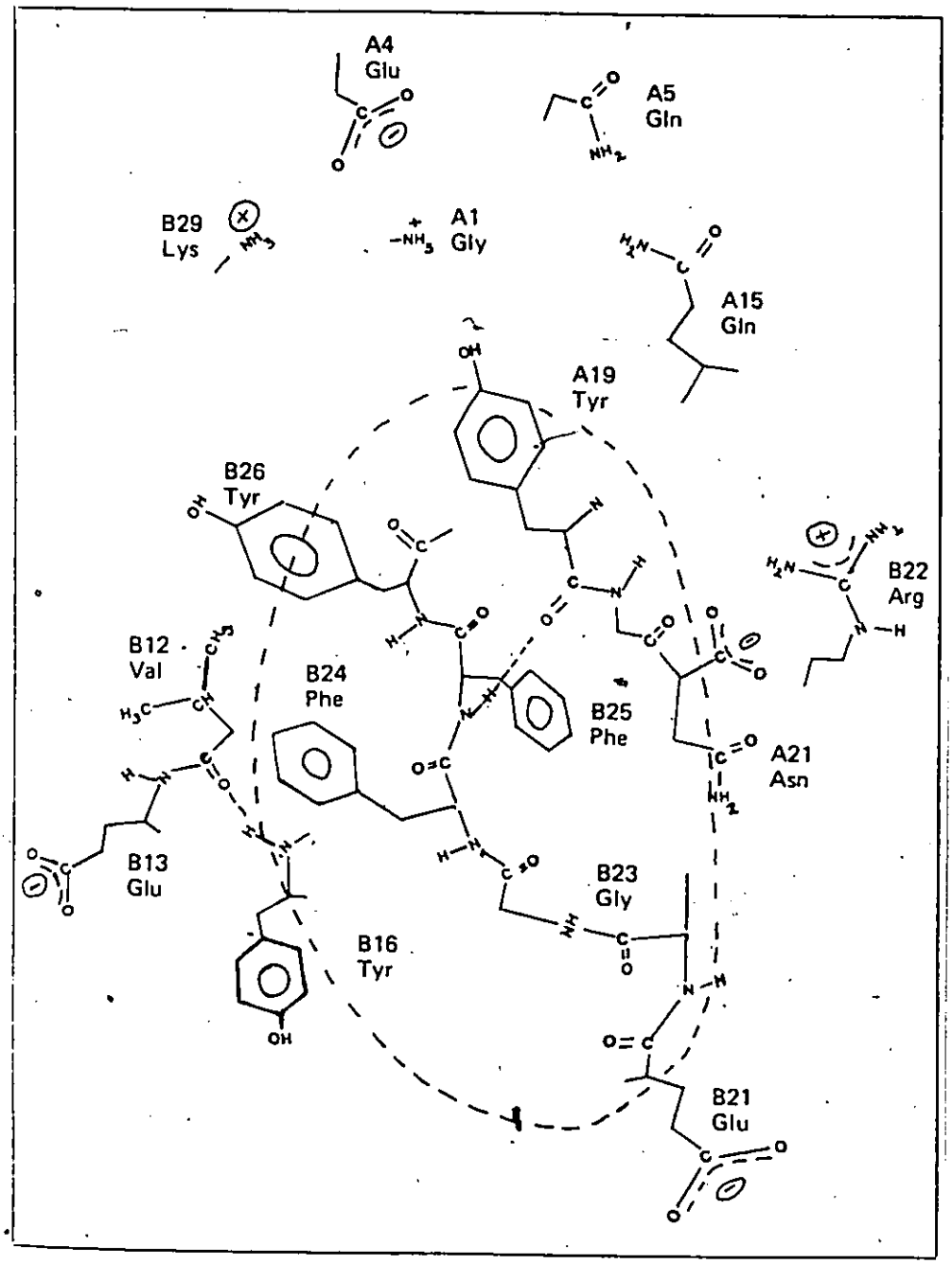
C. The receptor-binding region of insulin

Results of studies involving x-ray analysis, CD, and the binding and activity of chemically modified insulins are consistent with the insulin receptor-binding region containing many hydrophobic residues important to dimerization, in addition to more polar residues (130). A good candidate for this receptor-binding region is a largely invariant region including the residues Gly A1, Gln A5, Tyr A19, Asn A21, Phe B24, Phe B25, Tyr B26, Val B12 and Tyr B16 (14, 15, 46, 61, 85, 130, 155), brought together on the surface of the monomer by molecular folding in the three-dimensional structure (Figure 2). Of these residues, Gly A1, Tyr A19 and Asn A21 play no direct part in dimerization, and are probably on the periphery of the receptor-binding region (15, 61, 85, 130). This postulated region has support in the finding that the structure of the most active peptide with insulin activity is Arg B22 Gly B23 Phe B24 Phe B25 Tyr B26, a nearly invariant region in the insulin monomer (143, 155).

The existence of hydrophobic residues in the receptor-binding region of insulin has led to the suggestion that the deter-

Figure 2

A diagrammatic representation of the arrangement of amino acids on the insulin molecular surface which are probably involved in receptor binding (taken from Reference 15).


A4
Glu
A5
Gln
B29
Lys
A1
Gly
A15
Gln
A19
Tyr
B26
Tyr
B22
Arg
B12
Val
B24
Phe
B25
Phe
A21
Asn
B13
Glu
B23
Gly
B16
Tyr
B21
Glu

minant driving force in the insulin-receptor interaction is the "hydrophobic effect", the change in the organization of water due to the removal of nonpolar residues on the insulin and receptor surfaces from the medium into the hormone-receptor complex. Detailed examination of the thermodynamics of the reaction supports this concept(45, 46, 151).

It has also been suggested by several groups that receptor binding may be analogous to dimerization, involving the formation of an antiparallel sheet structure between insulin and the receptor molecule, with stabilization by hydrogen bonding between main chain functions as well as hydrophobic interactions of the side chains (14, 106, 130). However, since the affinity constant for binding is much greater than that for dimerization, additional interactions must exist (130).

It should be emphasized that certain areas within the postulated receptor-binding region may have different roles, for example stabilizing the hormone-receptor complex, or initiating the biological response (46, 130); to date, however, no such discrete regions have been disclosed (46, 60).

3. The insulin receptor

A hormone receptor, by definition, has a high degree of

specificity and affinity for its hormone, occupies a finite number of sites on the cell plasma membrane, exhibits rapid and reversible binding, and when complexed with hormone, possesses the ability to convey the existence of the recognition of its hormone to other structures responsible for emitting a biologically significant event (36, 61). The insulin receptor is no exception. It can be purified nearly to homogeneity by solubilization from cell membranes with the non-ionic detergent Triton X-100 (35, 37, 81), purification on DEAE-cellulose, and affinity chromatography on insulin-agarose and wheat germ agglutinin derivatives (37, 38, 81, 115). Compared with insulin, surprisingly little is known about the structure of the insulin receptor. This can be understood when one realizes that isolation of pure insulin receptors using rat liver membranes requires a 500,000-fold purification (38), and that the receptors represent only 10^{-4}% of the total protein of rat liver homogenate (37).

Gel filtration and density gradient centrifugation experiments using the soluble insulin binding protein indicate that it has a Stokes radius of 70 Å (37, 81), a sucrose sedimentation coefficient of 11 S, and asymmetric molecular dimensions (35, 36, 37), with a frictional ratio of 1.5 and an axial ratio of 9. In most instances, the molecular weight of the insulin receptor appears to be 300,000 daltons (14, 35, 37, 38, 81, 143). More recently, proteins likely to be the insulin receptor or its subunit have been found with mole-

cular weights of 130,000 (81, 157) and 75,000 (143) daltons. It is therefore apparent that the insulin receptor consists of probably four identical subunits (69, 72, 81, 143) containing two or more binding sites (72); binding of insulin to the receptor appears to enhance its reversible dissociation into subunits with smaller Stokes radii (69, 72, 81, 157).

In the membrane itself, the insulin-receptor has been visualized, using ferritin-insulin, either as a single receptor or in a group of two to six (82). These groups of receptors have been shown to occur before, and independent of, the binding of insulin to its receptor (82). The microfilament system is not believed to be involved in holding these groups of insulin receptors together (82).

Like insulin, much information has been obtained about the structure of the receptor by structural modifications; various enzymatic treatments directed at the receptor moiety have confirmed that the receptor is a protein, due to destruction of binding activity by digestion with such proteases as trypsin (22, 32, 35, 36, 38, 65, 72, 115). It is also known that the insulin receptor is a glycoprotein, although conflicting results have been obtained regarding which carbohydrate residues are involved. Digestion with β-galactosidase appears to destroy certain receptor sites, and plant lectins with galactose specificity largely inhibit the insulin binding process (22); this appears to indicate that the receptor site for insulin

involves galactosyl residues (22, 32, 37). Sequential digestion with neuraminidase and β-galactosidase suggests neuraminidase does not uncover new galactose residues involved in the recognition function of the receptor (22), although other studies (36, 38, 115) found this sequential digestion or digestion with neuraminidase alone decreased the receptor's affinity for insulin. Wheat germ agglutinin does not appear to affect insulin binding (22), although agarose affinity columns containing wheat germ agglutinin effectively adsorb receptor molecules (37). Concanavalin A inhibits insulin binding, implicating mannose residues in the hormonal receptor site; in addition, concanavalin A agarose affinity columns also effectively adsorb the insulin receptor (37, 115).

The specific insulin receptor has been demonstrated in a large number of species, including man, monkey, rat, mouse, guinea pig, rabbit, calf, sheep, pigeon, turkey, chicken, frog, hagfish, and Chinese hamster (96). However, most experiments using membrane receptors of several species exclude the existence of a species-specific receptor; regardless of the affinity of the homologous insulin, most receptors show similar basic characteristics and affinities to different insulin molecules (63, 96). Although the insulin binding site on the insulin receptor has been remarkably conserved throughout evolution (61, 67, 74), even receptors within one species appear to differ immunologically (74).

By examining the insulin receptor-binding region, one may make several speculations concerning the receptor insulin-binding region. The insulin receptor in all likelihood has a concave surface complementary to that of the insulin hormone (15). This receptor probably contains hydrophobic regions complementary to that formed by such residues in the insulin receptor-binding region as Phe B24, Phe B25, Val B12, and possibly part of Tyr A19 (15). The receptor may also have a polypeptide chain capable of forming an antiparallel β-sheet interaction, thus satisfying the hydrogen bond donors of the main chain of residues Phe B24 Phe B25 Tyr B26 (15). The periphery of the insulin-binding region of the receptor probably contains a series of charged groups which are capable of forming ionic interactions with the Gly A1 α-amino group, the Glu A4 carboxylate group, the Asn A21 α-carboxylate group, the Arg B22 guanidinium group and the Glu B13 carboxylate group (15). It is therefore likely that more extensive interactions are involved in the insulin-receptor interaction than in the insulin-insulin interaction, accounting for the higher association constant for receptor binding than for dimerization (15, 130).

As the solubilized insulin receptor has the same binding properties as when in the intact membrane (35), these properties will be discussed at length in the next section only.

4. The interaction between insulin and its receptor

A. General introduction

The general approach to examining the interaction between insulin and its receptor has been the measurement of the binding of radioactively labelled hormone with intact target cells or with isolated membrane preparations. This was not entirely feasible prior to 1971, when Freychet, Roth and Neville (57) clearly demonstrated firstly that ^{125}I-insulin was fully biologically active, and secondly, that highly purified rat liver plasma membranes contained specific binding sites for insulin, as opposed to non-specific adsorption by insulin to the membranes (72, 76, 134). Since that time, the binding of insulin to its membrane receptor has been examined extensively in a variety of tissues (including intact hepatocytes and purified liver plasma membranes (3, 31, 35, 36, 57, 58, 59, 63, 79, 81, 96, 108, 109, 111, 119, 132, 133, 139, 140, 141, 156), intact adipocytes and purified fat cell membranes (4, 33, 34, 35, 36, 47, 57, 70, 88, 101, 102, 109, 123, 124, 132, 133, 139, 140, 157), erythrocytes (48, 62, 64, 66), monocytes (11, 127, 128), lymphocytes (11, 23, 64, 65, 73, 114, 127, 129, 141, 151), fibroblasts (64), kidney cell membranes (13, 48), endothelial cells (8), placenta membranes (112), retinal cells (149), brain tissue (75), and pancreatic cells (100)), from a large number of species (including man (4, 8, 11, 23, 62, 64, 65, 73,

109, 112, 114, 127, 128, 129, 141, 151), monkey (75, 108), rat (3, 31, 47, 57, 58, 59, 63, 70, 75, 79, 81, 102, 109, 111, 119, 132, 133, 139, 141, 156, 157), mouse (100, 142, 149), chicken (96, 140), turkey (66, 67), dog (13), pigeon (75), rabbit (159), guinea pig (96) and calf (96).

B. General binding characteristics

The binding of ^{125}I-insulin to its receptor is specific; unlabelled insulin and insulin derivatives such as desalanine insulin, desoctapeptide insulin, reduced insulin, and proinsulin compete for binding in direct proportion to their ability to stimulate glucose oxidation in isolated fat cells; unrelated peptide hormones such as glucagon, growth hormone and arginine vasopressin have no effect (8, 13, 14, 33, 35, 58, 61, 64, 65, 66, 67, 72, 75, 142, 149). Binding of insulin to both intact membranes (8, 65) and soluble receptor (35, 36) is reversible. Binding is also saturable, with respect to both insulin concentration and cell or membrane protein concentration (8, 13, 14, 31, 34, 35, 36, 58, 65, 66, 67, 72, 119, 127, 133, 134).

The binding of ^{125}I-insulin to its receptor is temperature-dependent (8, 33, 34, 36, 48, 58, 61, 62, 64, 75, 127, 134). In almost all cases, the initial rate of association is proportional to temperature; however, the steady state level of binding is usually

higher the lower the temperature, due to accelerated dissociation and degradation of hormone and receptor at higher temperatures (4, 33, 36, 37, 48, 61, 66, 67, 69, 75, 134, 142, 149). The binding of ^{125}I-insulin is also time-dependent (14, 31, 33, 37, 48, 64, 72, 127, 134). The time course of insulin binding depends on several factors, most particularly temperature (48, 58, 65, 67, 79, 119, 127, 142, 156). In most cases, the time course follows a simple binding curve (48, 58, 65, 67, 119, 127, 142, 156), although in some instances, complex time courses of binding have been observed (62, 79). The steady state level of binding can be reached anywhere from 2 to 120 minutes (58, 61, 79, 96, 119, 141, 149), depending on both the temperature (48, 66, 67, 75, 134) and the insulin concentration (63). Binding for all insulin receptors is extremely dependent on pH, showing a sharp pH optimum between 7.8 and 8.0, depending on the tissue (8, 62, 65, 66, 67, 72, 75, 96, 119, 127, 149).

High ionic strength (2M NaCl) increases binding in almost all membrane preparations, presumably by unmasking new binding sites (31, 33, 36, 37, 38, 72), as the soluble receptor complex is unaffected (36). Similar results are obtained by digesting membranes with phospholipases A and C (31, 32, 33, 35, 36, 37, 38, 115, 133). Depending on the tissue under study, many conflicting results are obtained concerning the ionic requirements of insulin binding to its receptor. For example, Ca^{2+} and Mg^{2+} ions have been found to in-

crease binding (13, 44, 62, 66, 67, 107), decrease binding (67, 133), or have no effect whatsoever on binding (47, 67). Similarly conflicting results are obtained with Na^+, K^+ and Mn^{2+} ions (62, 66, 67, 133). Some groups maintain that binding is completely unaffected by ionic species (33, 36, 37, 65).

C. Dissociation of insulin from its receptor

Dissociation of ^{125}I-insulin from its receptor can be effected by addition of excess unlabelled insulin to the medium (58, 61, 134), by addition of acid to the medium (31, 33, 37, 58, 59, 61, 119, 134), by dilution of the binding medium (61, 134), or by addition of urea to the medium (44). The rate of dissociation is related directly to temperature; the higher the temperature, the faster the dissociation (44, 61, 123). The dissociation process usually does not follow simple first order kinetics (61, 142), and in most cases can be resolved into fast and slow dissociating species which probably represent low and high affinity binding sites, respectively (65). In addition, a dependence of the dissociation rate on the concentration of insulin in the medium has often been observed (43, 69). The implications of these last two facets of the dissociation process will be discussed in detail in the following section on negative cooperativity.

D. Negative cooperativity

A Scatchard plot of insulin binding data gives a curvilinear upward curve (3, 22, 41, 44, 61, 76, 96, 114, 123). This can be interpreted in one of two ways; either two or more discrete populations of receptors with different affinities co-exist, or else, as originally suggested by de Meyts and co-workers in 1973 (43), negative cooperativity between binding sites of a single receptor population is in effect (3, 22, 41, 43, 44, 61, 76, 96, 114, 123, 141, 142). The negative cooperativity model involves insulin binding to a homogeneous class of empty, high affinity sites, which then, with increased fractional saturation of receptors, undergo conformational changes through site-site interactions resulting in their transformation to the low affinity (fast dissociating) state (44, 46, 69, 101, 129, 142). The presence of negatively cooperative site-site interactions can be demonstrated by studying the dissociation of ^{125}I-insulin from its receptor in the absence and presence of unlabelled insulin (43, 44); negative cooperativity exists if the presence of native unlabelled insulin enhances the dissociation of bound ^{125}I-insulin during dilution-induced dissociation (43, 129). Negative cooperativity, as indicated by both curvilinear Scatchard plots and dissociation studies, has been observed in almost all cell fractions studied to date (8, 11, 66, 67, 99, 114). It is dependent on insulin concentration; a decreased effect at higher insulin concentrations is thought to be

due to dimerization of insulin which masks the cooperative site suggested by de Meyts (43, 44, 46, 67, 69, 123, 142).

In some studies, negative cooperativity does not appear to account for all observed kinetic interactions, and functionally distinct high affinity, low capacity, and low affinity, high capacity binding sites must also exist (122, 123, 124). In other studies, receptor heterogeneity alone appears to account for observed kinetic data (22, 47, 65). Recently evidence has accumulated questioning the existence of negative cooperativity under physiological conditions, and suggesting the presence instead of a homogeneous group of non-interacting receptors (4, 33, 34, 47, 69). For example, several studies suggest that negatively cooperative interactions are temperature-dependent, and virtually not apparent above 30 °C (63, 69, 129). It has also been demonstrated by Pollet and co-workers (129) that the dissociation rate of bound insulin is largely independent of binding site occupancy, indicating that enhanced dissociation of bound hormone does not provide evidence of negative cooperativity. Further studies with several membrane preparations (101, 102, 111, 112) have suggested that apparent negative cooperativity between receptors can be explained by the interaction of the insulin receptor with a non-receptor membrane glycoprotein, the interconversion between these two insulin-binding species being mediated by insulin. Finally, the mobile receptor hypothesis, which describes a process in which receptors

diffuse independently in the plane of the membrane, reversibly associating with effectors to regulate activity, could in some instances explain the complicated kinetics of the insulin binding reaction (80, 101, 129).

In conclusion, it is apparent that in most cases, at physiological temperatures and insulin concentrations, insulin receptors behave as a homogeneous class of non-interacting, high affinity binding sites (129), although in some instances data can be explained only if heterogeneous receptor sites exist or negatively cooperative interactions are in effect, or both.

E. Non-specific binding

In ^{125}I-insulin binding experiments, it is necessary to correct for a term commonly called "non-specific binding", that is, radioactivity non-specifically adsorbed to non-receptor regions of the plasma membrane, as well as to a variety of inert materials, including glass and plastic surfaces (37, 39, 58). Experimentally, non-specific binding is determined as the amount of ^{125}I-radioactivity bound to membranes in the presence of a large excess of unlabelled insulin (123). Although the proportion of non-specific binding varies widely, depending on such factors as the concentrations of labelled and unlabelled hormone, the temperature, the age and quality of the ^{125}I-insulin preparation, and the membrane protein concentration (34,

37, 58, 61, 156), it is usually found that a very low fraction of the total radioactivity added, about 5-20% of the total binding, is non-specifically bound (13, 75, 79, 123, 140, 141). Specific binding, that is, binding which is displaceable by native insulin, is calculated by subtracting the percentage of ^{125}I-radioactivity bound to the membrane in the presence of excess unlabelled insulin (when specific binding sites are primarily occupied by unlabelled insulin) from that bound in the absence of unlabelled insulin (59).

Non-specific binding of ^{125}I-insulin has not been studied to any large extent. It appears to be virtually temperature-independent (156). It also appears to increase slowly with time (66, 100, 153), sometimes reaching a plateau, depending on the duration of the experiment (153). Non-specific binding decreases tremendously in the presence of albumin (33, 63). Dissociation of non-specifically bound ^{125}I-insulin appears to be a different process from that of specifically bound insulin, being complete in much less time (119, 123). Intact cell studies have revealed the irreversible association of some non-specifically bound insulin to cells (119).

F. Degradation of the insulin receptor

Insulin receptors are constantly being synthesized and degraded (53). Degradation of the insulin receptor is dependent on time, temperature and membrane concentration (142). It is believed

that the loss of receptors from the cell surface represents the entry of the receptors into the cell where they are degraded (72).

G. Degradation of insulin

The liver is the major organ that removes insulin from the circulation (38, 48, 59, 148), although insulin degrading activity has been observed in all peripheral tissues, including kidney, muscle, adipose tissue, isolated fat cells, and the particulate fractions of fat cells (38, 59). Degradation of insulin is a function of time (3, 48, 59, 142). It is also extremely dependent on temperature, being drastically reduced at lower temperatures (48, 99, 119, 142). Degradation of insulin is a function of cell or membrane protein concentration; increased degradation is observed with increasing protein concentrations (48, 59, 63, 142). Degradation is also dependent on the criteria used in its evaluation, these criteria being the ability of the insulin to specifically bind to a second aliquot of membranes, to bind to anti-insulin antibody, to precipitate in the presence of trichloroacetic acid, and to adsorb to talc (59).

The relationship between the binding of ^{125}I-insulin to its receptor and its degradation is an extremely controversial one. Their independence (11, 13, 34, 36, 38, 59, 61, 65, 67, 88, 134, 142) is strongly suggested by such evidence as different biochemical characteristics (including affinities, analogue specificities, pH and ionic

strength optima, temperature dependence) (13, 40, 59, 67, 88), the recovery of undegraded insulin upon dissociation of the hormone-receptor complex (31, 59), the lack of effect on degradation when insulin binding is blocked with antibodies to the insulin receptor (52, 88), and the decrease in degrading activity upon purification of membranes (35, 59). However, recent work by several groups has indicated that a relationship between insulin binding and insulin degradation does exist. For example, in 1975, Terris and Steiner (146) found that the rate of degradation of insulin was proportional to the amount of insulin bound (40, 69, 70, 143). Another study (63) showed a release of 50% immunoreactive ^{125}I-insulin and non-immunoreactive ^{125}I-activity from rat hepatocytes, suggesting both inactivation and degradation of receptor-bound insulin. Other workers (5, 70, 147) have found both bound degraded insulin and free degraded insulin; some of these studies suggest the presence of two types of insulin receptors in plasma membranes, only one of which is associated with insulin degradation (5, 147). It is therefore apparent that receptor-linked degradation does exist, although depending on the tissue under study and the reaction conditions, it usually accounts for only a small fraction of total insulin degradation (4, 69, 70, 88).

The exact enzymatic mechanism by which insulin is normally degraded in cells is not clear. Most studies appear to indicate that

degradation is primarily a membrane phenomenon, or at least the rate-determining step occurs at the plasma membrane (61, 70, 119). Two major enzymes have been implicated, the microsomal glutathione-insulin transhydrogenase, and the cytosolic insulin-specific protease (63). Glutathione-insulin transhydrogenase is a reductase which cleaves the disulfide bonds of insulin, producing A and B chains, which are then susceptible to further degradation by non-specific cellular proteases (49, 125, 143, 148). Studies with insulin protease have led to the hypothesis that an early step in the degradation of insulin is the cleavage between residues Tyr B16 and Leu B17 that renders the molecule susceptible to further degradation by non-specific proteases (49). Examination of the degradation products of ^{125}I-insulin suggest they consist of small peptide fragments (59, 88) and iodotyrosine (70). It has been speculated by several groups that the degradation of insulin requires its compartmentalization or transport into cells (15, 70, 88, 143, 159).

H. Intracellular binding of insulin

The early finding by Cuatrecasas that insulin covalently bound to large insoluble agarose beads was still biologically active led to the conclusion that insulin elicits all its effects by binding to the external surface of the plasma membrane of the target cell (14, 30, 36, 37, 72). Although this effect was later shown to be

entirely due to the liberation of free insulin from the insulin-agarose complex (72, 76), it was still believed that insulin bound exclusively to the plasma membrane to exert its biological action.

In recent years, however, an abundance of evidence indicating the entry of insulin into intact cells has suggested the existence of intracellular binding sites (72). For example, several studies have shown a non-dissociable specific binding of insulin to intact cells (70, 86, 88, 119), probably representing intracellular radioactivity. In addition, much evidence for the intracellular migration of ^{125}I-insulin and ferritin-insulin has been found using the techniques of electron microscopy, electron microscopic autoradiography, and light microscopic autoradiography (23, 72, 73, 88).

It has been suggested that insulin enters the cell after its initial binding to the cell surface, possibly being interiorized by pinocytosis (with or without its receptor), followed by a transfer through the cytoplasm to one or more intracellular organelles (72, 78, 153, 154). To date, specific high affinity intracellular binding sites for insulin have been discovered on purified intact nuclei and nuclear membranes, smooth and rough endoplasmic reticula, and the Golgi apparatus (3, 71, 72, 78, 88). Not only are these intracellular binding sites immunologically distinct from those on the plasma membrane (71, 72, 153, 154), but they are also biochemically distinct (72); the intracellular nuclear membrane sites have

a lower affinity for insulin (71, 153, 154), different pH optima (between 6.5 and 7.25) (153, 154) and salt optima (no enhancement of binding with 2M NaCl)(153, 154), a single class of high-affinity receptors (153), and a lack of any insulin degrading activity (78, 154).

The biological significance of these intracellular binding sites is unknown. It has been suggested that the Golgi membrane and rough endoplasmic reticulum binding sites may be precursors of those on the plasma membrane (71, 72, 154). As it has been shown conclusively that insulin enters intact cells, it may also be speculated that these intracellular binding sites are involved in the regulation of certain long-term effects of insulin, including stimulation of the synthesis of DNA and RNA (71, 72, 73, 78, 154).

5. The clinical significance of insulin binding studies

In 1889, Minkowski and von Mehring observed that a pancreatectomy leads to the development of diabetes, a discovery that paved the way for Banting and Best over thirty years later (76). Since that time when insulin was first linked to diabetes, a considerable amount of effort has been spent determining the exact nature of the relationship between insulin and both diabetes and obesity, another insulin-resistant state.

Mechanisms of insulin resistance can operate before, at, or beyond the level of the insulin-receptor interaction. Before binding, for example, anti-receptor antibodies can impede access of the active hormone molecule to its site of action (53, 71, 74, 83). At the level of binding, defects in receptor concentration or affinity may be apparent (41, 53, 97, 98, 108, 120, 142). Beyond the receptor, defects in any pathways for insulin action could produce insulin resistance (7, 41, 94, 98, 108, 120). Although all three of these mechanisms are known to exist, most studies have concentrated on insulin resistance at the level of insulin binding. In almost all known cases, it has been found that an inverse relationship exists between circulating levels of insulin and the number of insulin receptor sites (3, 10, 12, 41, 61, 72, 74, 76, 97, 108, 117, 118, 120, 121, 154); in other words, insulin directly regulates its binding capacity on both the plasma membrane and on intracellular membranes (152, 154), a phenomenon known as "down regulation". In cases of diabetes and obesity with concomitant hyperinsulinemia, a characteristic feature of these insulin-resistant states (97, 108), it is found that insulin receptors are decreased in number, but are indistinguishable from normal receptors by such criteria as binding affinity, kinetics of association and dissociation, and temperature dependence of binding (9, 12, 22, 26, 42, 53, 61, 72, 74, 76, 94, 97, 98, 99, 108, 120, 121, 142). It has been suggested that this decrease

in insulin receptors may be simply a reflection of a more generalized alteration in membrane glycoproteins (26). Many exceptions to this decrease in insulin receptors have been observed (3, 7, 41, 53, 61, 93, 107, 108, 117, 118), indicating that more than a single variable is involved. Whether hyperinsulinemia is the cause or the effect of insulin resistance via decreased insulin receptors is unknown (84, 97); regardless of the sequence of events, the cause of the initiating abnormality has not yet been discovered (84, 120).

The relationship between insulin levels and insulin receptor sites is also found in many insulin-sensitive states, such as following an adrenalectomy or hypophysectomy, or in diabetic animals with hypoinsulinemia; in these cases, decreased plasma insulin concentrations lead to increased numbers of insulin receptors (72, 76, 94, 98, 154).

In summary, the mechanisms for insulin resistance are as complex as insulin action itself; decreased insulin effectiveness can be due to single or multiple abnormalities located at any step of the pathway of insulin action, including changes in insulin receptors, alterations in insulin effector systems, or impairment of the coupling between insulin receptor complexes and insulin effector systems (26, 94, 120).

6. The aim of this thesis

Elucidation of the chemical properties of individual functional groups in proteins and the relationship of these properties to the protein macrostructure is fundamental to the understanding of that protein's structure and function. As these chemical properties are determined both by the inherent properties of the functional group under consideration and by its interactions with its microenvironment, determination of the ionization constant and chemical reactivity of the group should allow the deduction of its local environment in the insulin molecule. Where insulin is concerned, determination of the chemical properties of its functional groups is of particular importance in examining structure-function relationships, as the striking pH dependence of insulin binding to its membrane receptor would imply that the ionization states of groups on the insulin molecule and/or its receptor are crucial structural features. The initial aim of this work was, therefore, to determine the chemical properties (pK values and reactivities) of the three amino groups of insulin, using the competitive labelling technique developed by Kaplan, Stevenson and Hartley (89). As the three amino groups of insulin were found to be unusually reactive under physiological conditions, it was speculated that they might react with electrophilic metabolites including carbon dioxide. Due to the observation of an interesting effect of carbon dioxide on the reactivities of these three amino groups, the purpose of this thesis was extended

to include an examination of the effect, if any, of carbon dioxide on the interaction between the insulin molecule and its receptor.

CHAPTER II: Materials and Methods

1. Materials

Porcine zinc insulin was obtained as a gift from Connaught Laboratories, Toronto, Ontario. Bovine insulin (0.3% zinc) and bovine serum albumin (RIA grade) were obtained from the Sigma Chemical Company, Saint Louis, Missouri, U.S.A. Elastase was obtained from Whatman Biochemicals Limited, Maidstone, Kent, England.

Ultra-pure sucrose (density-gradient grade) was obtained from Schwarz/Mann, Orangeburg, New York, U.S.A.

[Acetic-1-^{14}C]anhydride (specific activity 122.8 mCi/mmol) and tritiated acetic anhydride (specific activity 3 Ci/mmol) were obtained from Amersham/Searle Corporation, Arlington Heights, Illinois, U.S.A. ^{125}I-insulin (specific activity 80-100 µCi/µg) was obtained from New England Nuclear, Boston, Massachusetts, U.S.A.

Solutions used are shown in Table 1.

All other reagents and chemicals were high-purity preparations obtained from commercial sources.

Table 1

Preparation of solutions

Solutions	Preparation
1. Performic acid solution	99% Formic acid/30% H_2O_2, 19:1 by volume, prepared at room temperature 2 hours before use
2. Electrophoresis buffers	
(a) pH 6.5	Acetic acid/pyridine/water, 3:100:900 by volume
(b) pH 3.5	Pyridine/acetic acid/water, 1:10:190 by volume
(c) pH 2.1	Formic acid/acetic acid/water, 144:45 by volume
3. Solvent BAWP	Butanol/acetic acid/water/pyridine, 15:3:12:10 by volume
4. Homogenizing medium	0.5mM $CaCl_2$, 1mM $NaHCO_3$, pH 7.5
5. Phosphate buffer	118mM NaCl, 5mM KCl, 1.2mM $MgSO_4$, 24mM KH_2PO_4, 200mM Na_2HPO_4, 3% BSA, pH 7.5
6. Bicarbonate buffer	As in 5, except made 0.4M $NaHCO_3$
7. Salt buffer	As in 5, except made 0.52M NaCl (same ionic strength as 6)
8. Phosphate buffered saline (PBS)	2.7mM KCl, 1.5mM KH_2PO_4, 137mM NaCl, 8.0mM Na_2HPO_4, 1% BSA, pH 7.5

2. Methods for the determination of the chemical properties of the three amino groups of insulin

A. Preparation of porcine zinc-free insulin

Porcine zinc insulin (250 mg) was dissolved in 10 ml 0.1M potassium phosphate buffer pH 7.4 containing 0.01M disodium EDTA and 10 drops 85% phosphoric acid to solubilize the insulin. The solution was dialyzed in pre-soaked 3500 molecular weight exclusion dialysis tubing (Spectrapor) against 0.017M potassium phosphate buffer pH 7.4, then against distilled water, and freeze-dried.

B. Labelling procedure

A sample of insulin (6 mg, 1 µmol) and 1 µmol of L-phenylalanine (internal standard) were dissolved in 5 ml of a buffer consisting of N-methylmorpholine (5mM), boric acid (5mM) and KCl (100mM), pH 5.45. HCl (1M) was added to pH 4.2 to solubilize the insulin. This mixture was allowed 5 minutes to achieve temperature equilibrium in a water-jacketed reaction vessel thermostatically controlled at 10.0 °C or at 37.0 °C. With certain samples, the reaction mixture was saturated with 99.9% CO_2. The pH was adjusted to the desired value with 1M KOH, and 50 µl of acetonitrile containing 0.208 µmol tritiated acetic anhydride (specific activity 3 Ci/mmol) were added. The reac-

tion mixture was maintained at a constant temperature and pH for at least 5 minutes and then the pH was lowered to pH 3 by the addition of concentrated HCl. Urea (5 g) was added to 8M and the reaction mixture was left to stand for 5 minutes to ensure complete denaturation. The reaction mixture was adjusted to pH 9 and the insulin and internal standard were completely acetylated by the addition of 50 µl acetic anhydride containing 0.102 µmol [acetic-1-^{14}C]anhydride (specific activity 122.8 mCi/mmol) in 10 µl aliquots. During acetylation, the solution was maintained at pH 9 using a pH stat with 5M KOH as titrant.

C. Purification of acetylphenylalanine

After complete acetylation, the pH of the reaction mixture was lowered to pH 2 with concentrated HCl and the internal standard was extracted with 4 x 5 ml ethyl acetate. To remove traces of urea, the extracts were evaporated to dryness in a rotatory evaporator, dissolved in 5 ml 0.02M HCl, then re-extracted with 2 x 5 ml ethyl acetate. The extracts were again evaporated to dryness, dissolved in 1 ml 20mM NH_3, and spotted along a 7 cm band of Whatman 3MM paper 20 cm from the anode end. The ^{14}C-acetylphenylalanine was spotted as a marker alongside the internal standard. High-voltage electrophoresis at pH 6.5 was carried out for 40 minutes at 3000 V. The dried electrophoretograms were autoradiographed and the acetylphenylalanine

was eluted with 20mM NH_3.

D. Preparation of ^{14}C-acetylated marker insulin

A sample (5 mg) of insulin (bovine or porcine) was dissolved in 3 ml 8M urea brought to pH 4 with 8.4M HCl. A small amount, approximately 1 mg, of sodium tetraborate was added as buffer. The pH was adjusted to and maintained at pH 9.0 with 5M NaOH. 2 x 125 µl acetonitrile containing 83.3 µCi ^{14}C-acetic anhydride (specific activity 122.8 mCi/mmol) were added to the insulin solution. After 5 minutes, 60 µl unlabelled acetic anhydride were added to the reaction mixture.

E. Preparation of unlabelled acetylated insulin

A sample (15 mg) of insulin (bovine or porcine) was dissolved in 5 ml 8M urea and brought to pH 1 with concentrated HCl. After 5 minutes, the pH was adjusted to and maintained at pH 9.0 with 5M KOH. 5 x 10 µl aliquots of acetic anhydride were added to the reaction mixture and the insulin was allowed to completely react.

F. Digestion of acetylated insulin

All acetylated insulin samples were treated in the following manner. After thorough dialysis against distilled water, the acetylated insulin was freeze-dried. This freeze-dried material was dissolved

in 6 ml 1% ammonium bicarbonate and digested for 3.25 hours at 37 °C with 0.3 mg elastase, freeze-dried again, then performic acid oxidized (77) for 2.5 hours at 4 °C and freeze-dried.

G. Identification of acetylated peptides

(1) High-voltage electrophoresis

The final digest of unlabelled acetylated insulin was dissolved in ammonia solution and pH 6.5 buffer, then spotted along 20 cm of Whatman 3MM paper 20 cm from the anode end. To enable detection of the positions of the acetylated peptides by autoradiography, the digested ^{14}C-acetylated marker insulin was spotted as a 1 cm band alongside each end of the 20 cm strip. Electrophoresis was performed at pH 6.5 for 45 minutes at 3000 V.

(2) Autoradiography

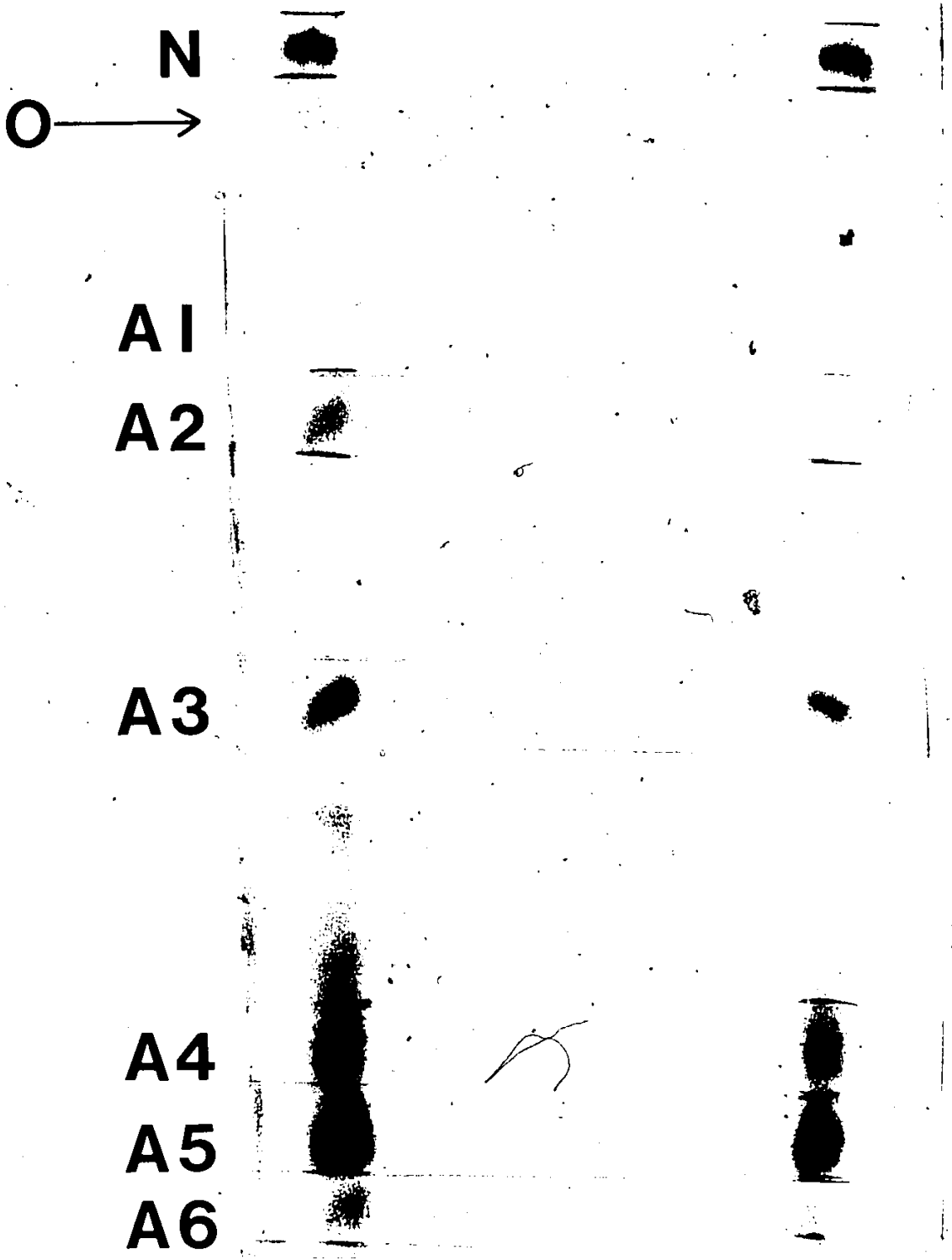
Figure 3 shows an autoradiogram obtained by this procedure. Several dots were made with radioactive ink on the electrophoretogram, which was attached to an x-ray film in a cardboard folder and stored in the dark under even pressure. After development, the dots helped to align the film with the original electrophoretogram.

(3) Purification of acetylated peptides

The various radioactive peptides in Figure 3 were purified

Figure 3

pH 6.5 electrophoretogram of performic-acid oxidized and elastase digested acetylated insulin. The dark spots are ^{14}C-acetylated peptides derived from ^{14}C-acetylated marker insulin used to locate the bands containing the ^{3}H/^{14}C-acetylated peptides derived from the competitive labellings.


N
O
A I
A2
A3
A4
A5
A6

as follows. The neutral band, N, was cut out with its ^{14}C-marker spots, stitched onto a sheet of Whatman 3MM paper 20 cm from the cathode end and subjected to high-voltage electrophoresis for 60 minutes at pH 2.1. The fastest migrating band was cut out with its marker spots, stitched onto a second sheet of Whatman 3MM paper 10 cm from the top and chromatographed overnight in a descending manner with the solvent BAWP. The band was then cut out with its marker spots, stitched onto a third sheet of Whatman 3MM paper 20 cm from the cathode end and subjected to high-voltage electrophoresis at pH 3.5 for 45 minutes at 3000 V. After autoradiography, the ^{14}C-marker at either end of the strip was removed and the remainder of the band was eluted with 20mM NH_3.

Acidic band A2 was cut out with its ^{14}C-marker spots, stitched onto a sheet of Whatman 3MM paper 10 cm from the top and chromatographed overnight in a descending manner with the solvent BAWP. The band was then cut out with its marker spots, stitched onto a second sheet of Whatman 3MM paper 30 cm from the anode end and subjected to high-voltage electrophoresis at pH 3.5 for 1 hour at 3000 V. After autoradiography, the ^{14}C-marker spots were removed from the fastest migrating band and the remainder of the band was eluted with 20mM NH_3.

Acidic bands A5 (bovine insulin) and A(4&5) (porcine insulin)

were cut out with their ^{14}C-marker spots, stitched onto a sheet of Whatman 3MM paper 10 cm from the top and chromatographed overnight in a descending manner with the solvent BAWP. The slowest migrating bands were cut out with their marker spots, stitched onto a sheet of Whatman 3MM paper 20 cm from the anode end and subjected to high-voltage electrophoresis at pH 3.5 for 30 minutes at 3000 V. The ^{14}C-markers were removed from the A5 band and the faster migrating A(4&5) band, and the remainders of the bands were eluted with 20mM NH_3.

(4) Amino acid analysis

The dried acetylated peptides were hydrolyzed with 25 nmol norleucine standard in 6M HCl at 110 °C for 24 hours in sealed, evacuated tubes. After evaporation, the samples were analyzed using a Technicon model amino acid analyzer. Table 2 gives the analyses for each of the three acetylated peptides quantitated in this study.

H. Purification of acetylated peptides from competitive labelling procedure

The neutral band, N, and the acidic band, A2, were both purified as described above with the elimination only of the BAWP chromatography steps. The acidic bands A5 and A(4&5) were purified

Table 2

Composition of acetylated peptides of insulin

Peptide	Amino acid composition
B24-B30 (Bovine N)	Phe - Phe - Tyr - Thr - Pro - Lys(Ac) - Ala 0.94 0.94 0.92 1.04 1.15 0.88 1.16
B24-B30 (Porcine N)	Phe - Phe - Tyr - Thr - Pro - Lys(Ac) - Ala 0.93 0.93 0.90 1.04 1.27 0.93 0.97
B1-B8 (Bovine A2)	Ac - Phe - Val - Asn - Gln - His - Leu - Cys - Gly - 0.99 1.12 0.81 0.95 1.03 1.09 1.00 0.97
B1-B9 (Porcine A2)	Ac - Phe - Val - Asn - Gln - His - Leu - Cys - Gly - Ser - 0.83 0.89 1.06 1.00 0.92 0.90 1.34 1.04 0.43
A1-A13 (Bovine A5)	Ac - Gly - Ile - Val - Glu - Gln - Cys - Cys - Ala - Ser - 1.09 0.58 0.98 1.13 1.13 0.83 0.83 1.15 0.97 Val - Cys - Ser - Leu - 0.98 0.83 0.97 1.07
A1-A13 (Porcine A5)	Ac - Gly - Ile - Val - Glu - Gln - Cys - Cys - Thr - Ser - 1.05 0.69 0.73 1.26 1.26 0.90 0.90 1.14 0.88 Ile - Cys - Ser - Leu - 0.69 0.90 0.88 0.80

The sequences are taken from the established sequences of bovine and porcine insulins (20, 136, 137).

exactly as described above.

I. Liquid scintillation counting

A Nuclear Chicago Isocap/300 liquid scintillation counter was used to measure radioactivity. Dried acetylphenylalanine samples were counted in 10 ml Aquasol. Dried acetylated peptides were dissolved in 100 µl formic acid, then counted in 10 ml Aquasol.

J. pH measurements and titrations

A Radiometer pH meter 26 fitted with a type GK 2321C glass electrode was used for pH measurements. Titrations were performed by adding titrant from an Agla micrometer syringe apparatus.

3. Methods for the determination of the effect of carbon dioxide on the insulin-receptor interaction

A. Preparation of rat liver plasma membranes (131)

All preparation was performed at 4 °C. A portion of liver (10 g) from one male Sprague-Dawley rat (180-220 g) was cut into small pieces in 100 ml homogenizing medium, then homogenized in a Dounce homogenizer (loose pestle) with 25 gentle strokes. The homogenate

was diluted to 1 l with homogenizing medium, then allowed to stand for 5 minutes with occasional shaking. The diluted homogenate was poured through one layer of Miracloth, then centrifuged in 250 ml glass cups at 1450 x g (2750 rpm) at 4 °C for 30 minutes in a swinging bucket rotor HS-4 in a Sorvall Superspeed RC2-B centrifuge. The supernatant was discarded and the pellet resuspended in a small amount of medium and homogenized gently (4-5 strokes). This suspension was diluted to 500 ml with homogenizing medium and centrifuged again for 15 minutes at 1230 x g (2500 rpm). The supernatant was discarded and the pellet was resuspended by gentle homogenization (4-5 strokes) in a small amount of homogenizing medium, diluted to 250 ml with medium, and re-centrifuged at 1230 x g (2500 rpm) for 15 minutes. The supernatant was discarded, and the final pellet was suspended in a small volume of homogenizing medium (final volume 4 ml). Sixteen ml 60% (w/w) ice-cold sucrose solution were added with thorough mixing. The suspension was divided equally between 6 Spinco SW-25.I plastic tubes. Eight ml 45% (w/w) ice-cold sucrose solution were layered over each suspension, followed by 10 ml 41% (w/w) ice-cold sucrose solution. The tubes were balanced with approximately 10 ml 37% (w/w) ice-cold sucrose solution. The tubes were centrifuged in a Spinco SW27 swinging bucket rotor in a Beckman L2-65B ultracentrifuge at 4 °C for 2 hours (brake off) at 90,000 x g (25,000 rpm). The thin, compact membrane layer at the interface between 37% (w/w) and 41% (w/w) sucrose was removed using a Pasteur pipette. The

membranes were diluted with 1mM $NaHCO_3$, then centrifuged in a JA-20 rotor in a Beckman J-21 centrifuge for 1 hour at 8,000 x g (10,000 rpm). The supernatant was discarded, and the membrane pellet re-suspended in a small volume of 1mM $NaHCO_3$ and re-centrifuged for 30 minutes at 8,000 x g (10,000 rpm). The membrane pellet was resuspended in 1 ml 1mM $NaHCO_3$ by vigorous aspiration through a 1 ml syringe.

B. Protein assay

Membrane protein concentrations were assayed using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, California, U.S.A.). Bovine serum albumin in 1mM $NaHCO_3$ was used as the standard. The total yield of protein was approximately 1 mg per g wet liver.

C. Storage of membranes

Membranes were aliquoted in volumes suitable for experiments into 1.5 ml microfuge tubes and stored at -80 °C.

D. Preparation of unlabelled insulin for binding assays

A sample of porcine zinc insulin (12 mg) was dissolved in 24 ml 1% NH_4HCO_3, then, in some cases, brought to approximately pH 7 with acetic acid. The insulin solution was aliquoted in 500 µl portions and freeze-dried. Aliquots were reconstituted in 500 µl buffer

prior to each experiment.

E. Acetylation of ^{125}I-insulin

Four aliquots containing a total of 70 µl acetic anhydride were added to a solution containing ^{125}I-insulin (~1-2 µCi/ml) in 6 ml PBS maintained at pH 7.5 with 5M KOH by means of a pH stat. After 15 minutes, the solution was dialyzed overnight at 4 °C in pre-soaked 3500 molecular weight exclusion dialysis tubing against PBS pH 7.5. The solution was then freeze-dried.

F. Binding assays

The following is a representative example of a binding experiment. Exact details for each experiment are found in figure and table legends. Membranes diluted in 150 µl buffer (final concentration ~250 µg membrane protein/ml) and ^{125}I-insulin in 150 µl buffer (final concentration ~10^{-10}M) were added to 100 µl buffer (for total binding) or 100 µl 1 mg/ml unlabelled insulin in buffer (final concentration ~40µM) (for non-specific binding) in a 400 µl microfuge tube, which was then vortexed vigorously. It was found useful to centrifuge briefly (~ 1 second) after each addition of ^{125}I-insulin and buffer or unlabelled insulin in buffer, to reduce foaming upon addition of membrane. In certain experiments, 99.9% CO_2 was bubbled into the solution for 20 seconds. After a specific time interval at room temperature,

the microfuge tube was centrifuged at 4 °C for 5 minutes in an Eppendorf microcentrifuge (∿15,000 rpm). The supernatant was then removed with a syringe. The pH of the supernatant was immediately measured using an Ingold surface electrode 6020. The surface of the membrane pellet was rinsed by the addition of 300 µl 10% sucrose in phosphate buffer, followed by centrifugation for 5 minutes at 4 °C. The supernatant was discarded. The tip of the microfuge tube was excised and counted in a Nuclear Chicago gamma counter.

CHAPTER III: Results and Discussion

1. Chemical properties of the three amino groups of insulin

A. Introduction

In order to determine the precise structural requirements for the binding of the insulin molecule to its specific membrane receptor, the elucidation of the chemical properties (ionization constants and reactivities) of its individual functional groups is essential. As the striking pH dependence of insulin binding to its membrane receptor would imply that the ionization states of groups on the insulin molecule and/or its receptor are crucial structural features (24, 33, 67), it is of particular importance to determine the chemical properties of those functional groups believed to be involved in the binding interaction between insulin and its receptor. To this end, the first part of this thesis involved the determination of the ionization constants and reactivities of the three amino groups of insulin, by means of the competitive labelling technique (24, 25, 50, 89, 90, 91). The principle of this technique is as follows: When an ionizable standard nucleophile S of known ionization constant and reactivity is made to compete with another ionizable nucleophile A of unknown properties for a trace amount of ^{3}H-labelled reagent RX

at a series of pH values according to the scheme

$$A + RX \xrightarrow{k_a} RA + X$$

$$S + RX \xrightarrow{k_s} RS + X,$$

the rate constant and ionization constant for nucleophile A can be determined after complete reaction with ^{14}C-labelled reagent RX from the relation

$$\alpha_a r = \alpha_s \times \frac{^3H/^{14}C \text{ of RA}}{^3H/^{14}C \text{ of RS}} \qquad (1)$$

where $\alpha_a = 1/1 + [H^+]/K_a$

$\alpha_s = 1/1 + [H^+]/K_s$

$r = k_a/k_s$

and K_s and K_a are acid dissociation constants. (For complete derivation of equation (1), refer to Reference 89). In this study, the ionizable standard nucleophile S is L-phenylalanine, the ionizable nucleophile A is one of three peptides containing the amino groups of insulin, and the reagent RX is acetic anhydride.

B. Ionization constants and reactivities of the three amino groups at 10 °C

(1) pH-reactivity profiles

In order to make an unequivocal assignment of parameters (pK and reactivity), it was first necessary to isolate peptides containing only the functional groups of interest. To this end, both bovine and porcine zinc insulins were digested with elastase, followed by performic acid oxidation, to give the radioactive bands shown in Figure 3. After complete purification, each peptide was analyzed as to its amino acid composition (Table 2), and the three smallest peptides containing the acetylated phenylalanyl B1 (band A2) and glycyl A1 (band A5) amino termini and the ε-amino group of lysine B29 (band N) were chosen for quantitation of the parameters associated with their respective amino groups. An identical purification was carried out for each of the acetylated peptides obtained from competitive labelling experiments. After correction for background and spillover, the $^{3}H/^{14}C$ ratio of each, as determined by scintillation counting, was substituted in equation (1) to calculate the relative rate constants $\alpha_a r$, listed in Tables 3, 4 and 5. The value of α_s was calculated by taking pK_s equal to 9.50 at 10 °C, determined from the titration curve obtained by titrating 23.5 μmol phenylalanine in 0.10M KCl at 10.0 °C with 0.150M NaOH.

Table 3

Competitive labelling of porcine insulin

with acetic anhydride at 10.0 °C

pH	N-acetyl-phenylalanine		Ac-Gly A1 (peptide A1-A13)		Ac-Phe B1 (peptide B1-B9)		Ac-Lys B29 (peptide B24-B30)	
	$(^3H/^{14}C)$	$(\alpha_s x10^3)$	$(^3H/^{14}C)$	$(\alpha_a rx10^2)$	$(^3H/^{14}C)$	$(\alpha_a rx10^2)$	$(^3H/^{14}C)$	$(\alpha_a rx10^3)$
6.24	13.8	0.549	156	0.622	1230	4.91	15.7	0.625
6.49	14.9	0.976	149	0.976	1350	8.88	26.7	1.75
6.77	18.2	1.84	183	1.84	1370	13.8	31.8	3.21
7.02	16.2	3.26	264	5.32	1270	25.7	59.1	11.9
7.15	23.2	4.44	285	5.45	1120	21.5	23.4	4.48
7.29	25.5	6.12	366	8.80	1110	26.6	20.9	5.03
7.49	25.5	9.67	361	13.7	985	37.4	26.4	10.0
7.77	32.1	18.3	460	26.2	861	49.1	31.5	17.9
8.01	42.3	31.3	657	48.6	525	38.8	45.7	33.8
8.51	59.6	92.8	416	64.8	276	42.9	55.4	86.2

Table 4

Competitive labelling of porcine zinc insulin

with acetic anhydride at 10.0 °C

pH	N-acetyl-phenylalanine		Ac-Gly A1 (peptide A1-A13)		Ac-Phe B1 (peptide B1-B9)		Ac-Lys B29 (peptide B24-B30)	
	$(^3H/^{14}C)$	$(\alpha_s x10^2)$	$(^3H/^{14}C)$	$(\alpha_a rx10^2)$	$(^3H/^{14}C)$	$(\alpha_a rx10^2)$	$(^3H/^{14}C)$	$(\alpha_a rx10^3)$
6.23	20.4	0.054	292	0.759	817	2.13	47.2	1.25
6.51	23.4	0.102	274	1.21	1070	4.71	41.2	1.77
6.76	28.6	0.179	391	2.47	1060	6.67	-	-
7.02	29.8	0.326	337	3.65	906	9.81	-	-
7.15	31.2	0.444	532	7.68	914	13.2	41.2	5.78
7.31	46.4	0.641	452	6.22	924	12.7	33.9	4.70
7.51	51.7	1.01	533	10.5	723	14.3	61.1	11.8
7.76	58.1	1.78	559	17.3	664	20.6	49.3	14.9
8.06	75.7	3.46	893	41.1	453	20.9	50.6	23.0
8.55	88.5	10.1	904	105	233	27.0	56.9	64.1

Table 5

Competitive labelling of bovine zinc insulin

with acetic anhydride at 10.0 °C

pH	N-acetyl phenylalanine		Ac-Gly A1 (peptide A1-A13)		Ac-Phe B1 (peptide B1-B8)		Ac-Lys B29 (peptide B24-B30)	
	$(^3H/^{14}C)$	$(\alpha_s \times 10^3)$	$(^3H/^{14}C)$	$(\alpha_a r \times 10^2)$	$(^3H/^{14}C)$	$(\alpha_a r \times 10^2)$	$(^3H/^{14}C)$	$(\alpha_a r \times 10^3)$
6.48	20.7	0.954	249	1.15	773	3.57	30.1	1.39
6.73	24.1	1.70	206	1.45	847	5.97	30.0	2.12
7.03	26.9	3.38	282	3.55	793	9.97	39.8	5.00
7.15	27.1	4.44	309	5.06	819	13.4	54.2	8.87
7.25	30.8	5.59	293	5.32	773	14.0	44.0	7.99
7.50	36.5	9.90	375	10.2	677	18.4	40.9	11.1
7.76	37.9	17.8	296	13.9	379	17.8	26.7	12.5
8.02	37.4	31.7	592	50.3	464	39.4	43.9	37.2
8.51	70.4	92.8	666	87.8	262	34.5	49.6	65.3
9.02	74.4	249	545	182	133	44.4	101	338

A plot of $\alpha_a r[H^+]$ against $\alpha_a r$ gives a straight line with slope -K (results not shown). Using this estimated pK value and least squares analysis to determine the maximal value of r, titration curves for each of the three amino groups can be derived. Figure 4 is a plot of $\alpha_a r$ values obtained for the glycyl A1 amino terminus from porcine and bovine zinc insulins and porcine zinc-free insulin at 10 °C. It can be seen that the pH-reactivity profiles for the glycyl amino terminus in all three insulins are very similar. Below pH 8, the data can be approximated to a titration curve with a pK of 7.9 and an r value of 0.37. Above pH 8, the reactivity data do not fit a titration curve, and the reactivity of the Gly A1 amino group increases markedly in all three cases.

Figure 5 is a similar plot for the phenylalanyl B1 amino terminus. Although the porcine and bovine zinc insulins have very similar pH-reactivity profiles, it is obvious that the Phe B1 amino group has a slightly higher reactivity in zinc-free porcine insulin. Unlike the glycyl A1 amino terminus, there is no marked increase in reactivity above pH 8, and the data fit titration curves with pK and r values of 7.3 and 0.28 for porcine and bovine zinc insulins, and 7.2 and 0.45 for porcine zinc-free insulin.

In Figure 6, the pH-reactivity profile for the ϵ-amino group of Lys B29 for the three insulins is shown. As with the amino group

Figure 4

Plot of $\alpha_a r$ versus pH for the glycyl A1 amino terminus of porcine zinc insulin (●), bovine zinc insulin (o), and porcine zinc-free insulin (Δ). The solid line is a theoretical titration curve with a pK of 7.9 and an r value of 0.37.

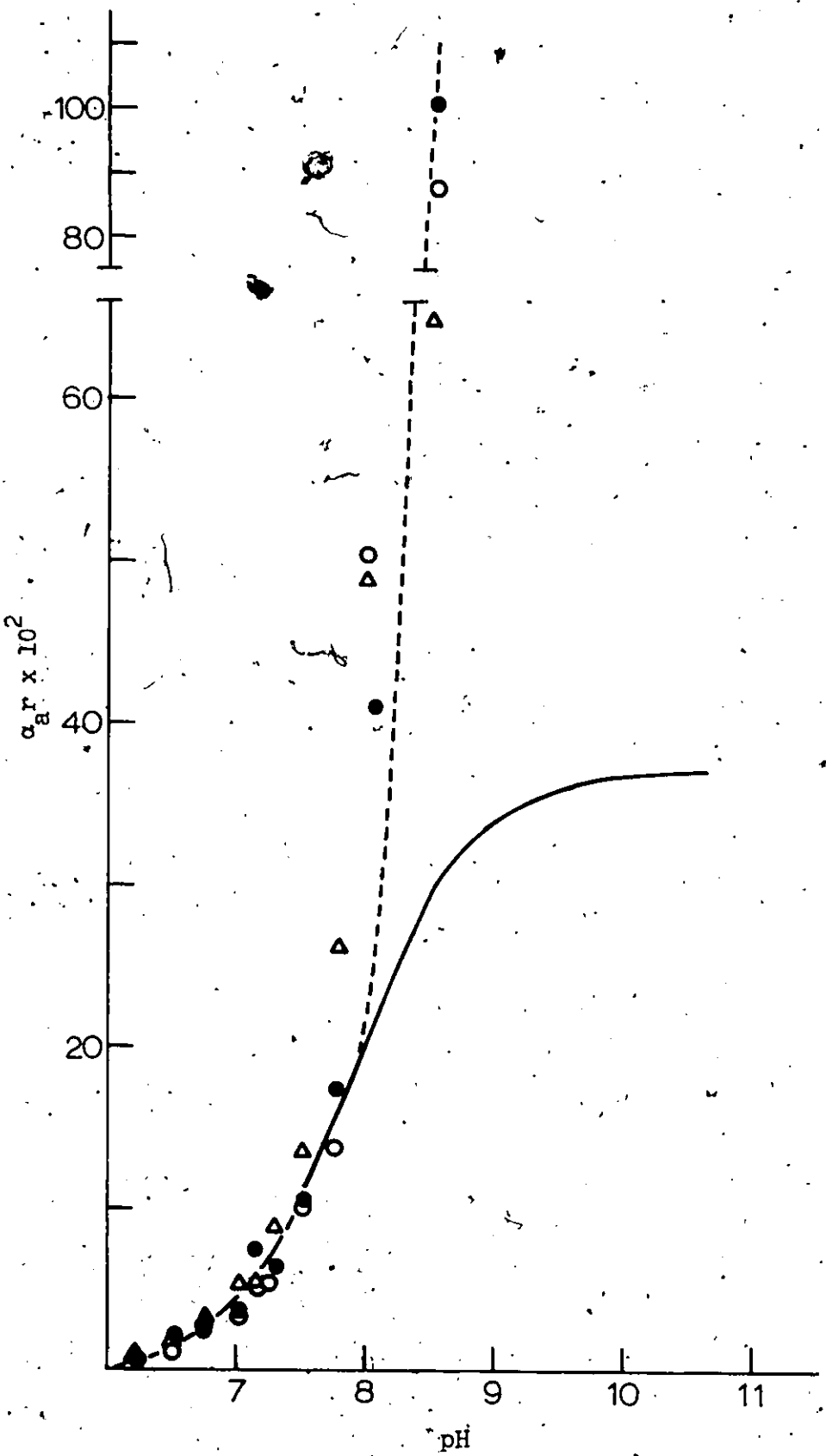

100
80
60
40
20
$\alpha_{ar} \times 10^2$
7
8
9
10
11
pH

Figure 5

Plot of $\alpha_a r$ versus pH for the phenylalanyl B1 amino terminus of porcine zinc insulin (●), bovine zinc insulin (o) and porcine zinc-free insulin (Δ). The solid lines are theoretical titration curves with pK and r values of 7.3 and 0.28, and 7.2 and 0.45.

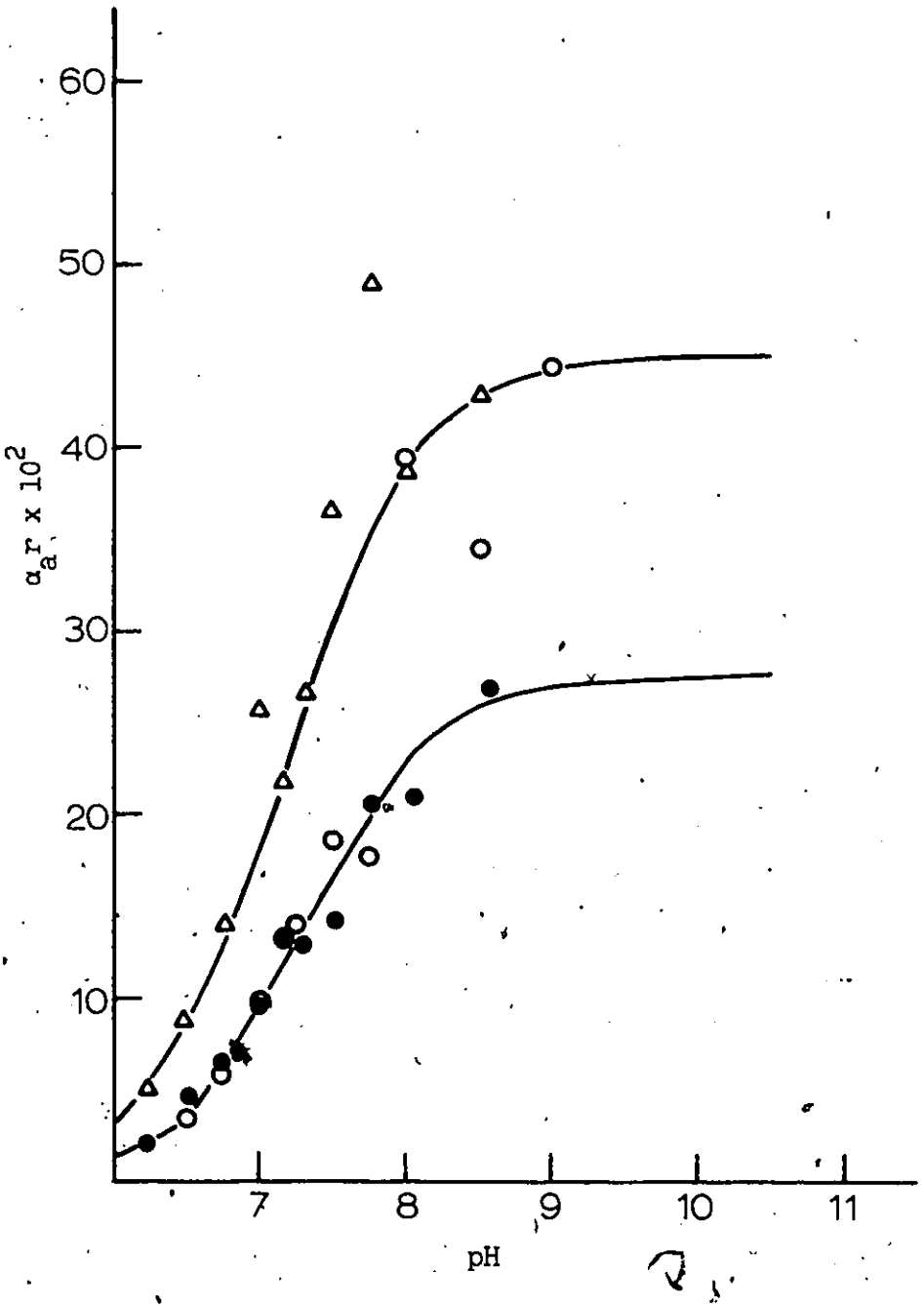
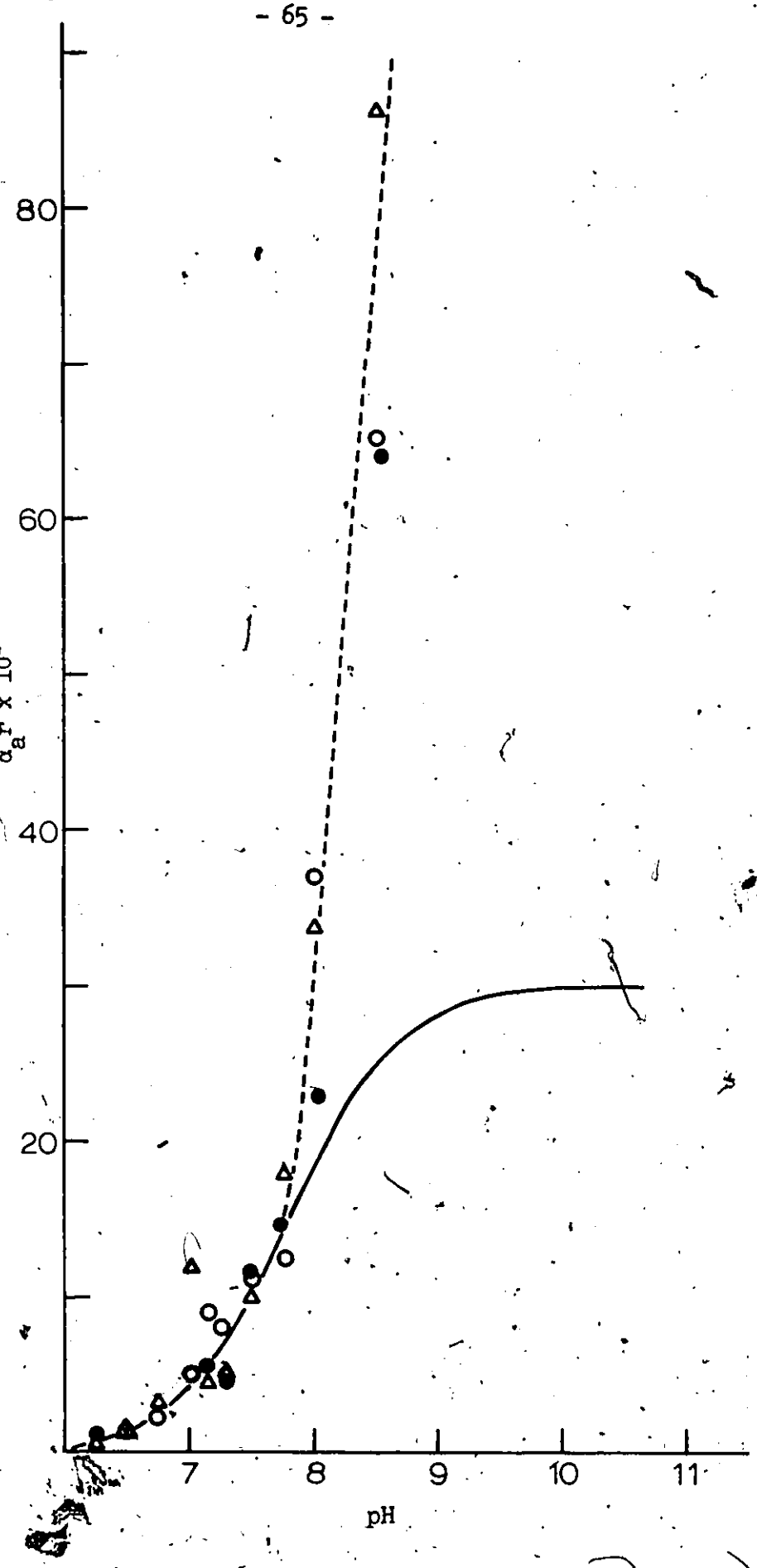

60
50
40
30
20
10
$\alpha_a r \times 10^2$
7
8
9
10
11
pH

Figure 6

Plot of $\alpha_a r$ versus pH for the lysyl B29 ε-amino group of porcine zinc insulin (●), bovine zinc insulin (o) and porcine zinc-free insulin (Δ). The solid line is a theoretical titration curve with a pK of 7.8 and an r value of 0.030.


80
60
40
20
$\alpha_a r \times 10^3$
7
8
9
10
11
pH

of Gly A1, the reactivity data below pH 8 can be approximated to a titration curve with a pK of 7.8 and an r value of 0.030; above pH 8, the reactivity of the Lys B29 ε-amino group shows a marked increase.

(2) Analysis of ionization constants

In a study by Bradbury and Brown (16), pK values of 6.7, 8.0 and 11.2 for the amino groups of Phe B1, Gly A1 and Lys B29 respectively in methylated insulin at 20 °C were obtained using the technique of nuclear magnetic resonance spectroscopy. In a more recent study (24) involving competitive labelling with FDNB as the labelling reagent, the pK values of the three amino groups of porcine zinc-free insulin at 20 °C were determined to be 6.9, 7.7 and 7.0 for Phe B1, Gly A1 and Lys B29 amino groups, respectively. In the present study, using the competitive labelling technique with the smaller, more readily accessible reagent acetic anhydride, the pK values at 10 °C were found to be 7.3, 7.9 and 7.8 for Phe B1, Gly A1 and Lys B29 amino groups, respectively. Assuming a ΔH of approximately 10 kcal/mol (28), the pK values obtained for the phenylalanyl B1 and glycyl A1 amino termini in all three studies are in excellent agreement. However, a discrepancy is noticed in the results for the ε-amino group of Lys B29. In the study involving competitive labelling with FDNB, the relatively large shift in pK values from 7.8 at 10 °C

to 7.0 at 20 °C can probably be explained by an abnormal dependence of the ionization constant of this group on temperature, reflecting the unusual chemical properties of the Lys B29 ε-amino group. However, the pK value of 11.2 obtained for this amino group in the NMR study cannot be explained in this manner; as Bradbury and Brown did not study the parameters for this amino group over the physiological pH range, this possibly indicates that the titration curve for this group was obtained using insulin in an aggregation or conformational state different from that present at physiological pH.

In most proteins, the pK values for α-amino groups normally lie between 8 and 8.5; for ε-amino groups, these pK values usually range between 10.5 and 11. The unusually low pK values obtained in this study for the three amino groups of insulin must somehow reflect their structural relationships within the insulin macrostructure. Since the Phe B1 α-amino group is probably completely deprotonated under physiological conditions, the speculation by Blundell and co-workers (14, 16) that this group may form a salt bridge with the carboxyl group of Glu A17 of the adjacent dimer upon hexamer formation is not supported by these data. It is more likely that the deprotonated form of the Phe B1 α-amino group is involved in hydrogen bonding (24) accounting for its low pK; under conditions of hexamer formation, it could associate with Glu A17 of the adjacent dimer (14).

Depending on its charge under physiological conditions, the

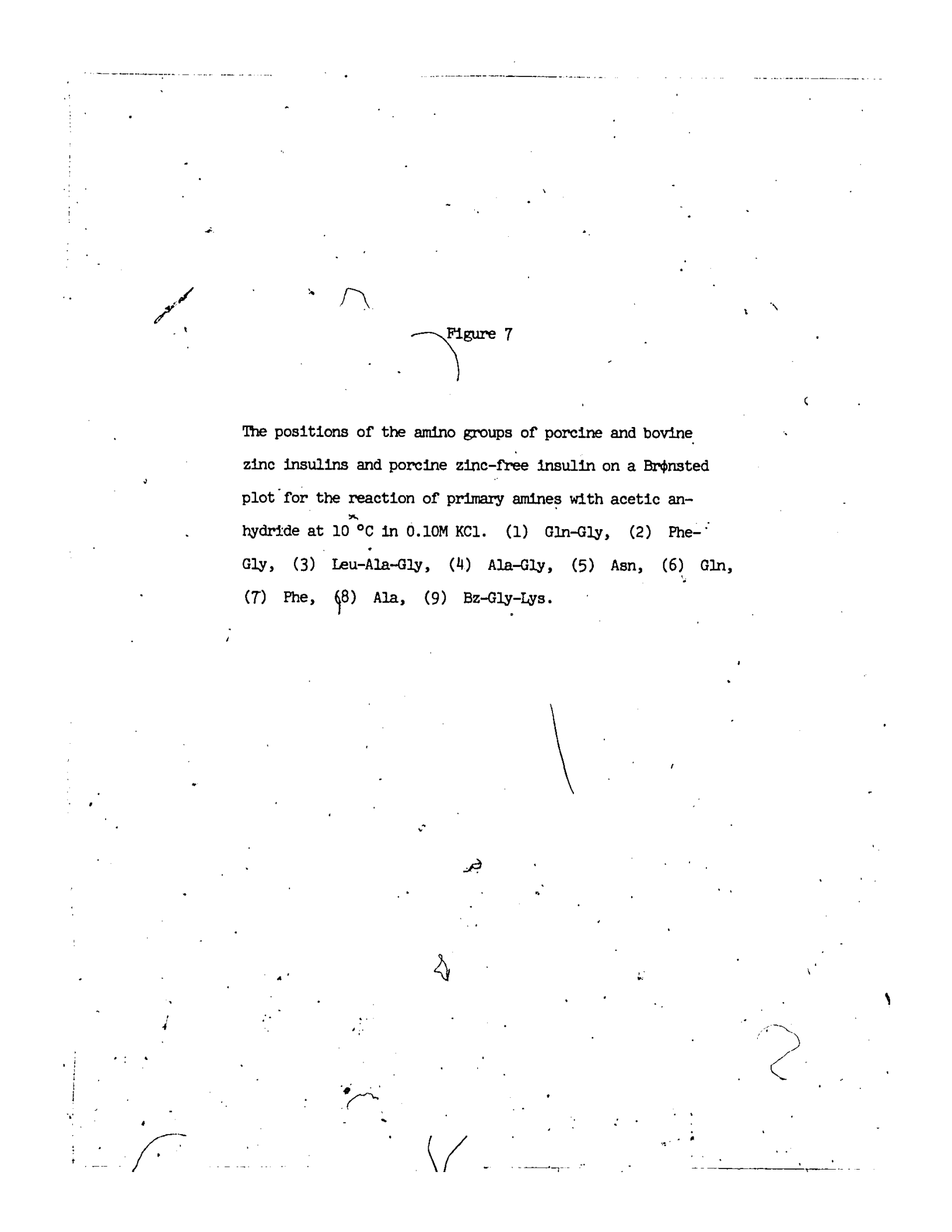
Gly A1 α-amino group may form either a salt bridge with the Glu A4 carboxylate group of the same monomer (14, 16), or hydrogen bond with the hydroxyl function of Tyr A19 (14). The salt bridge would be expected to increase the pK of the amino group (16), whereas the hydrogen bond would tend to lower it (24). As at 10 °C and 20 °C the Gly A1 α-amino terminus has the highest pK of the three amino groups (24), it may under physiological conditions be partially protonated. It is therefore not obvious which of these two possibilities would be of greater significance. In addition, the Gly A1 α-amino group is in close proximity to the Lys B29 ε-amino group. If this latter group is deprotonated under physiological conditions, it will have no effect on the pK of the glycyl A1 α-amino group. On the other hand, if the Lys B29 ε-amino group is proximal to a positive charge on the Gly A1 α-amino group, this would account for its relatively low pK value (16, 24). The speculation that the Lys B29 ε-amino group could form a salt bridge with the Glu A4 carboxylate group in solution at neutral pH values (1, 14, 15) (Figure 2) is not supported by the data in this study.

(3) Analysis of chemical reactivities

As the striking pH profile for the interaction of insulin with its receptor probably reflects the molecular ionization constants of the functional groups directly involved in the insulin-receptor

interaction (33, 67), the determination of the ionization constants of the three amino groups of insulin is extremely important, particularly since the Gly A1 amino terminus has been implicated in this interaction. However, it is also of considerable importance to deduce the interactions of these amino groups with their microenvironments, a task which is made possible by an examination of their chemical reactivities. Figure 7 shows where the reactivity parameters obtained for the three amino groups at 10 °C below pH 8 (Table 6) lie on a Brønsted plot for primary amines (89). As the phenylalanyl B1 and glycyl A1 amino termini lie above the curve, they are seen to be super-reactive toward acetic anhydride, i.e. more reactive than would be expected for unhindered amines with the same pK values. This is in excellent agreement with results obtained in the competitive labelling study at 20 °C using FDNB (24). At 10 °C, the lysyl B29 ε-amino group lies below the line on the Brønsted plot, indicating it is more buried towards acetic anhydride than would be an unhindered amine with the same pK value. At 20 °C with the bulkier FDNB, the Lys B29 ε-amino group lies closer to the line on the Brønsted plot and appears to be less buried. The finding that the lysyl B29 ε-amino group appears to be buried does not mean that it is never accessible to reagents, but simply that it reacts with reagents at a slower rate, due to the fact that the conformations in which this group is exposed form a relatively minor fraction of the dynamic equilibria existing in the insulin macrostructure.

Figure 7



The positions of the amino groups of porcine and bovine zinc insulins and porcine zinc-free insulin on a Brønsted plot for the reaction of primary amines with acetic anhydride at 10 °C in 0.10M KCl. (1) Gln-Gly, (2) Phe-Gly, (3) Leu-Ala-Gly, (4) Ala-Gly, (5) Asn, (6) Gln, (7) Phe, (8) Ala, (9) Bz-Gly-Lys.

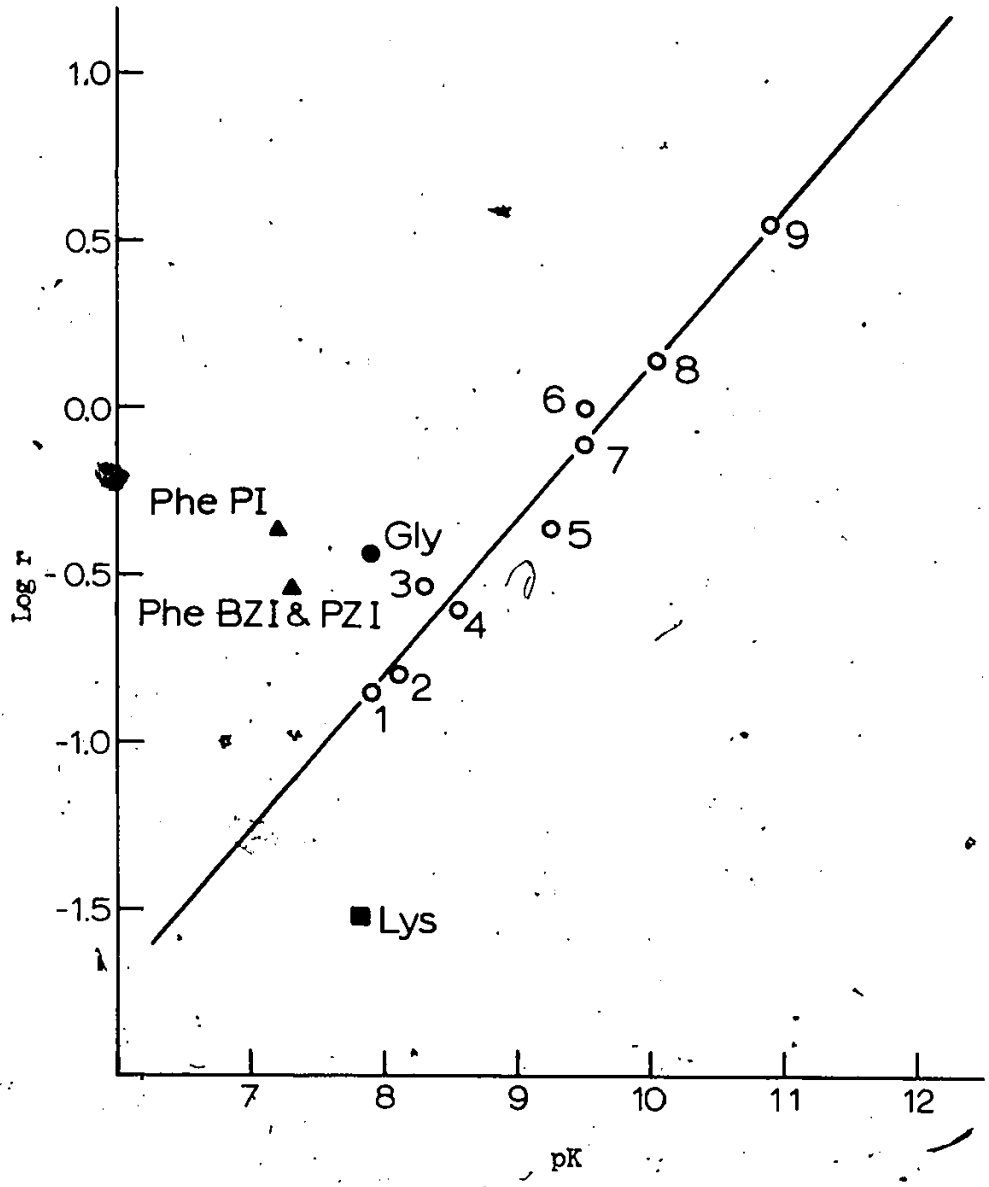

1.0
0.5
0.0
-0.5
-1.0
-1.5
Log r
Phe PI
Gly
Phe BZI & PZI
Lys
1
2
3
4
5
6
7
8
9
7
8
9
10
11
12
pK

Table 6

Summary of the parameters for the three amino groups of insulin in 0.10M KCl at 10 °C

Amino group	pK	r	k^* ($M^{-1}min^{-1}$)
Glycine A1	7.9	0.37	16,300
Phenylalanine B1	7.2 (zinc-free)	0.45	19,800
	7.3 (zinc)	0.28	12,300
Lysine B29	7.8	0.030	1,320

*Calculated by taking k_s = 44,000 $M^{-1}min^{-1}$ (89).

In the case of the lysyl B29 ε-amino group and the glycyl A1 amino terminus, discontinuities in their pH-reactivity profiles occur above pH 8, with marked increases in their reactivities. Similar discontinuities have been observed in several other competitive labelling studies (50, 89, 90), and have been interpreted as arising from changes in conformation. As it has been reported that insulin undergoes a change in its state of association to a lower molecular weight form above pH 8 (14, 126), the observed discontinuities probably reflect this conversion. This hypothesis is substantiated by the fact that the Gly A1 residue is positioned near to the β-pleated sheet joining the two terminal parts of the B chains in the insulin dimer (106), and by the observation that dimerization causes a significant change in the environment of Lys B29 (16, 126). The reason for the marked increase in reactivities concurrent with this conversion is not clear, and may reflect new or stronger hydrogen bonding of the deprotonated forms of these groups upon dissociation. The observation that the phenylalanyl B1 amino terminus does not appear to undergo a change in properties above pH 8 is consistent with this hypothetical conversion to a lower molecular weight form, as the Phe B1 residue is intimately involved only in the interaction between insulin dimers, and is not known to make contacts on dimerization (1, 14). However, a contradictory finding by fluorine NMR studies (126) is that significant alterations in the environment around Phe B1 occur upon disaggregation of insulin above pH 8.

The slightly higher reactivities observed with porcine zinc-free insulin compared with both zinc insulins cannot be adequately explained, and may reflect Zn^{2+}-induced changes in the conformation of the insulin molecule in the region of Phe B1, changes in its state of aggregation, or a combination of both, as suggested by CD spectroscopy studies (6).

It should be emphasized that titration curves are used only as a theoretical framework within which data are to be analyzed. There is no a priori reason why any functional group in a protein should perfectly fit a titration curve. Deviations from ideal behaviour as indicated by discontinuities in reactivity data merely indicate that the insulin molecule is a dynamic structure whose properties, and hence the environments of its functional groups, change with pH.

C. Effect of temperature on the chemical properties of the three amino groups

Ideally, one strives to examine the chemical properties of insulin under physiological conditions, including insulin concentration (10^{-9}-10^{-11}M) (14, 15), temperature (37 °C) and pH (7.4). However, temperatures well below 37 °C are usually used, as they reduce the rates of reaction of the functional groups in the insulin molecule with various reagents, and allow the determination of the chemical properties of these functional groups to proceed with less difficulty.

An attempt was therefore made to determine the chemical properties of the three amino groups of insulin at 37 °C. The relative rate constants, $\alpha_a r$, at 37 °C are listed in Table 7. The value of α_s was calculated by taking pK_s equal to 9.01 at 37 °C, determined from the titration curve obtained by titrating 23.5 μmol phenylalanine in 0.10M KCl at 37.0 °C with 0.150M NaOH. Figures 8, 9 and 10 show the effect of altering the temperature from 10 °C to 37 °C on the pH-reactivity profiles for the three amino groups. At 37 °C, there is a sharp increase in the reactivities of all three amino groups between pH 7 and 7.5. Above pH 7.5, the reactivity profiles become discontinuous. Due to the rapid transitions in the pH-reactivity profiles, these data cannot be fitted to a titration curve; however, the midpoints in the transitions occur between pH 7.0 and 7.3 for all three groups. In view of the results obtained at 10 °C with acetic anhydride (Figures 4, 5 and 6) and at 20 °C with FDNB (24), the most probable explanation of the sharp discontinuities observed in the region of physiological pH is that at 37 °C, insulin undergoes a change in association state, due to the deprotonation of one or more of its three amino groups. As considerable evidence indicates that insulin exists as a monomer under physiological conditions (5, 6, 15, 30), these data probably reflect the conversion of insulin from a dimer to a monomer. The fact that this conversion occurs so rapidly over the narrow physiological pH range suggests that the change in structural properties may be important in the functioning of insulin. As

Table 7

Competitive labelling of porcine zinc insulin

with acetic anhydride at 37.0 °C

pH	N-acetyl-phenylalanine		Ac-Gly A1 (peptide A1-A13)		Ac-Phe B1 (peptide B1-B9)		Ac-Lys B29 (peptide B24-B30)	
	$(^3H/^{14}C)$	$(\alpha_s x10^2)$	$(^3H/^{14}C)$	$(\alpha_a rx10^2)$	$(^3H/^{14}C)$	$(\alpha_a rx10^2)$	$(^3H/^{14}C)$	$(\alpha_a rx10^3)$
6.26	80.5	0.175	91.4	0.198	317	0.687	11.2	0.242
6.49	100	0.296	100	0.296	315	0.931	14.3	0.422
6.75	76.1	0.544	160	1.15	325	2.33	12.9	0.926
6.86	69.3	0.700	106	1.07	437	4.41	17.1	1.73
7.01	22.1	0.986	152	0.679	348	15.5	15.7	7.00
7.15	13.7	1.36	141	13.9	275	27.3	15.8	15.6
7.30	15.3	1.88	174	21.5	272	33.5	17.6	21.6
7.50	22.9	2.99	225	29.3	239	31.1	19.5	25.4
7.63	23.8	3.94	188	31.0	285	47.2	31.6	52.2
7.76	48.6	5.30	282	30.7	161	17.5	20.6	22.4
8.00	78.4	8.87	279	31.5	143	16.2	27.9	31.6
8.54	55.4	25.2	236	108	70.2	32.0	44.0	201

Figure 8

pH-reactivity profiles for the glycyl A1 amino terminus of porcine zinc insulin at 10 °C (●) and 37 °C (■). The open triangles (Δ) are the reactivities in the presence of CO_2 at 10 °C.

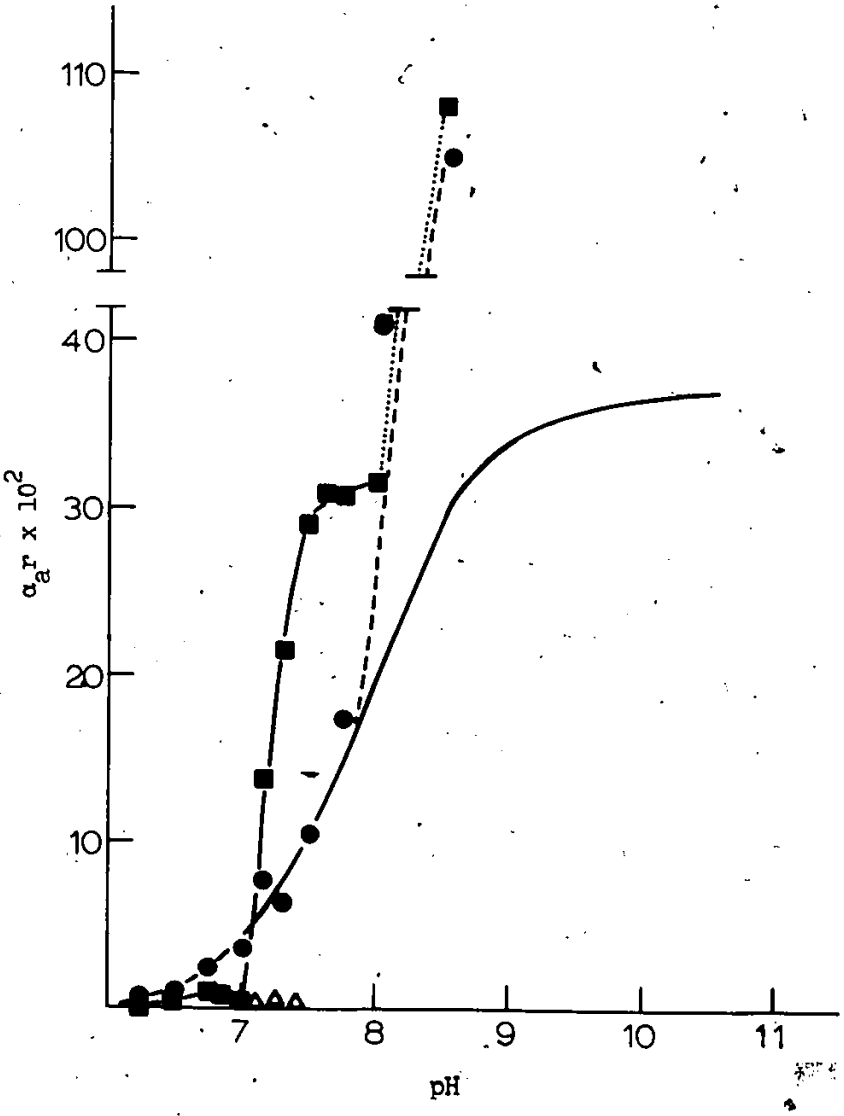

110
100
40
30
20
10
$\alpha_a r \times 10^2$
7
8
9
10
11
pH

Figure 9

pH-reactivity profiles for the phenylalanyl B1 amino terminus of porcine zinc insulin at 10 °C (●) and 37 °C (■). The open triangles (Δ) are the reactivities in the presence of CO_2 at 10 °C.

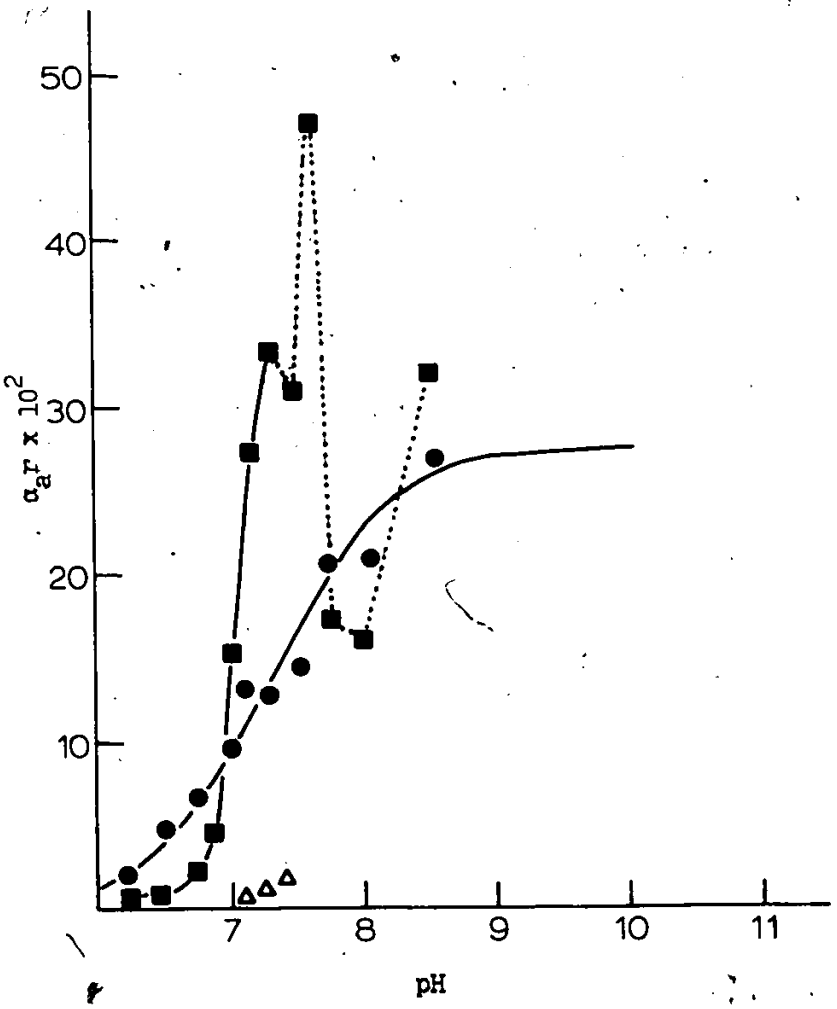
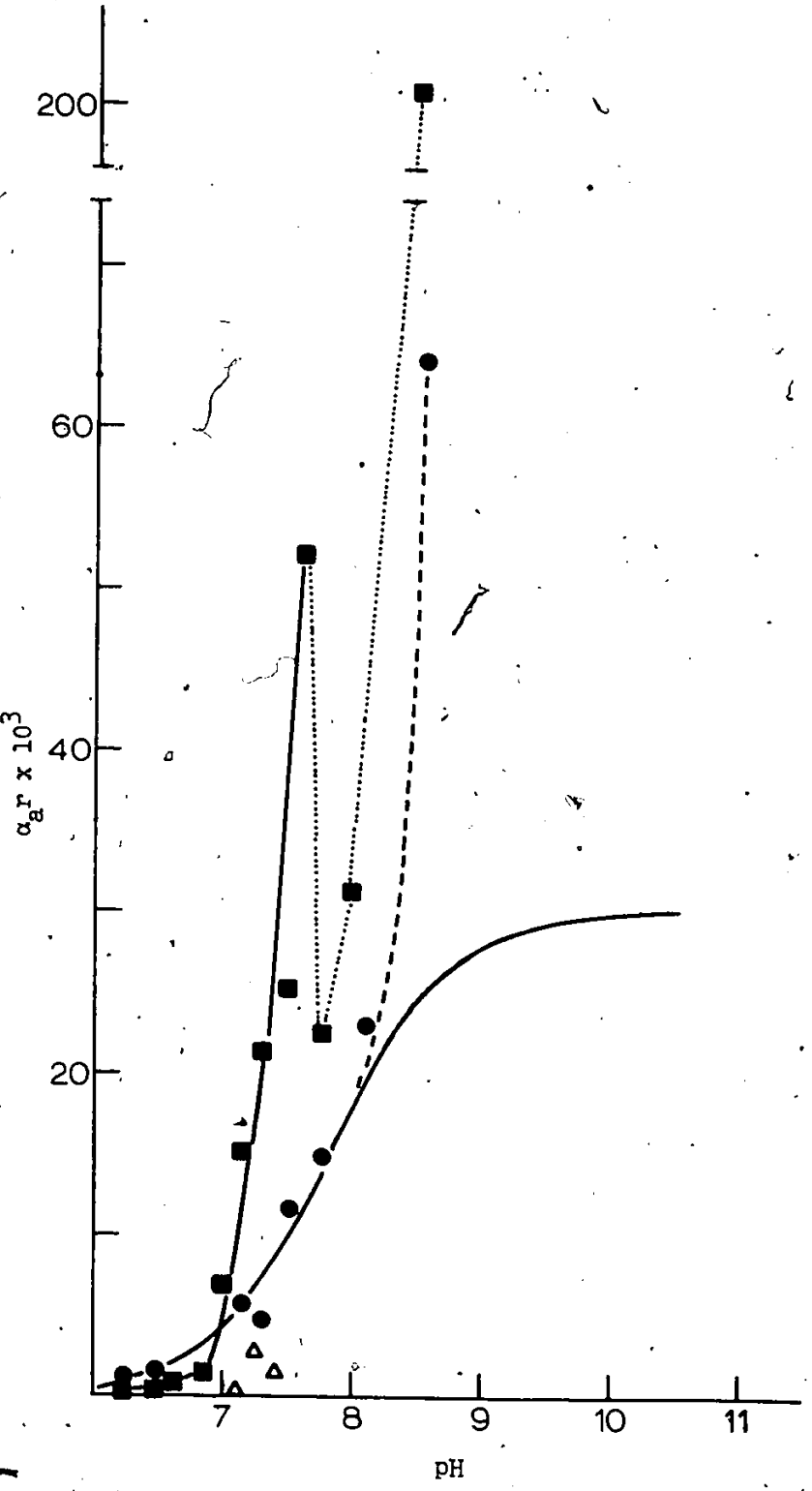

50
40
30
20
10
$\alpha_a r \times 10^2$
7
8
9
10
11
pH

Figure 10

pH-reactivity profiles for the lysyl B29 ε-amino group of porcine zinc insulin at 10 °C (●) and 37 °C (■). The open triangles (Δ) are the reactivities in the presence of CO_2 at 10 °C.


200
60
40
20
$\alpha_g r \times 10^3$
7
8
9
10
11
pH

these data also show that this change in structural properties is strongly dependent on temperature, it should be emphasized that caution must be exercised in interpreting properties of insulin obtained below 37 °C, and stressed that insulin should be studied under physiological conditions wherever possible.

D. Effect of carbon dioxide on the reactivities of the three amino groups

The general nature of the reaction of carbon dioxide with amino groups has been recognized for many years (55, 103, 116). As the present study appears to indicate that at physiological pH the three amino groups of Phe B1, Lys B29 and Gly A1 are largely deprotonated, the amino groups are therefore considerably more nucleophilic than normal amino groups under physiological conditions, and should react with all electrophilic metabolites, including carbon dioxide. The open triangles (Δ) in Figures 8, 9 and 10 show the reactivities (Table 8) at 10 °C of the three amino groups in the presence of saturating amounts of carbon dioxide. In all three cases, the reactivities are markedly reduced, indicating that carbon dioxide has reacted with the amino groups to form carbamino derivatives, and suggesting that a more rapid reaction with carbon dioxide should occur under physiological conditions. The reaction of CO_2 with the three amino groups could not be studied at 37 °C, due to the fact that the

Table 8

Competitive labelling of porcine zinc insulin with acetic anhydride at 10.0 °C in the presence of carbon dioxide

pH	N-acetyl-phenylalanine		Ac-Gly A1 (peptide A1-A13)		Ac-Phe B1 (peptide B1-B9)		Ac-Lys B29 (peptide B24-B30)	
	$(^3H/^{14}C)$	$(\alpha_s x10^2)$	$(^3H/^{14}C)$	$(\alpha_a rx10^2)$	$(^3H/^{14}C)$	$(\alpha_a rx10^2)$	$(^3H/^{14}C)$	$(\alpha_a rx10^3)$
7.10	78.9	0.396	69.5	0.335	160	0.840	7.98	0.418
7.25	76.6	0.559	89.3	0.633	185	1.39	31.2	2.35
7.40	80.0	0.788	33.4	0.322	200	2.02	18.2	1.84

phenylalanine internal standard reacts with carbon dioxide under these conditions.

2. Effect of carbon dioxide on the insulin-receptor interaction

A. Introduction

It has been suggested by Gurd and co-workers (116) that carbamino formation may be a quite general and functionally important phenomenon throughout biology, not limited primarily to hemoglobin. The formation of these derivatives changes the chemical function of the amino terminus of the peptide chain, alters its charge, introduces bulk to the protein, and has an effect on the conformation of the terminal region of the chain (116). An attempt was therefore made to determine whether those changes induced by the formation of carbamino derivatives in the insulin molecule somehow affected the interaction of ^{125}I-insulin with its specific receptor in rat liver plasma membranes.

B. Effect of carbon dioxide on the binding of ^{125}I-insulin to its membrane receptor

Initially, the effect of carbon dioxide on the binding of ^{125}I-insulin was examined using a binding mixture through which 99.9% CO_2 was bubbled to give a final pH of $\sim$ 6.8, and an identical mixture

to which an acetic acid solution had been added to give the same final pH (results not shown). However, it was observed that this low pH decreased the binding of ^{125}I-insulin to such an extent that any effect due to CO_2 was difficult to discern. It was therefore decided to perform all binding studies at higher pH values in the presence of bicarbonate, which produces CO_2 according to the equilibrium

$$HCO_3^- + H^+ \rightleftharpoons CO_2 + H_2O. \qquad (2)$$

Whereas it is difficult to determine the amount of carbon dioxide dissolved in solution, the use of specific amounts of bicarbonate is advantageous in that it allows quantitation of results.

A comparison was made between the effects of 0.2M phosphate buffers containing 0.4M $NaHCO_3$ and NaCl of the same ionic strength on the binding of ^{125}I-insulin to liver plasma membranes. Phosphate buffers of this high ionic strength were used in order to control the pH changes which occur in the presence of bicarbonate. For the same reason, extreme care was taken to prevent atmospheric contact in all experiments, as the diffusion of CO_2 out of solution causes large pH changes. Because the reversible hydration of CO_2 is a rather slow process, it was initially thought advantageous to include a trace amount of the enzyme carbonic anhydrase, which catalyzes the reaction shown in (2), to the reaction mixture, so as to maintain more nearly equilibrium conditions. However, it was observed in several experi-

ments (results not shown) that the presence of carbonic anhydrase had little or no effect on the experimental results, so it was eliminated from the reaction mixtures.

Table 9 shows that the percentage of specific binding varied between 3.6 and 13.7% of the total radioactivity added, depending on such factors as pH, presence or absence of bicarbonate, temperature, and membrane preparation. In all cases it was observed that the specific binding of ^{125}I-insulin to rat liver plasma membranes was lower in the presence of 0.4M $NaHCO_3$. However, due to the difference in pH values between the salt (control) and bicarbonate samples, it was found necessary to determine the pH profile of ^{125}I-insulin binding in the presence of 0.52M NaCl, to allow direct comparison of results. This pH profile of ^{125}I-insulin binding to rat liver plasma membranes is shown in Figure 11. The optimum pH for binding is seen to be pH 7.75, in good agreement with those findings of other workers (72, 96, 119). In the pH range of interest, very little variability is apparent. Large degrees of variability are visible above pH 8, possibly indicating a change in the state of aggregation in the insulin molecule (14, 126). Examination of the pH profile indicates that the results shown in Table 9 are extremely significant. In all cases, the pH of the bicarbonate reaction mixture should be associated with at least equal or higher ^{125}I-insulin binding. Therefore, ignoring small variations in the pH profile, bicarbonate binding should be

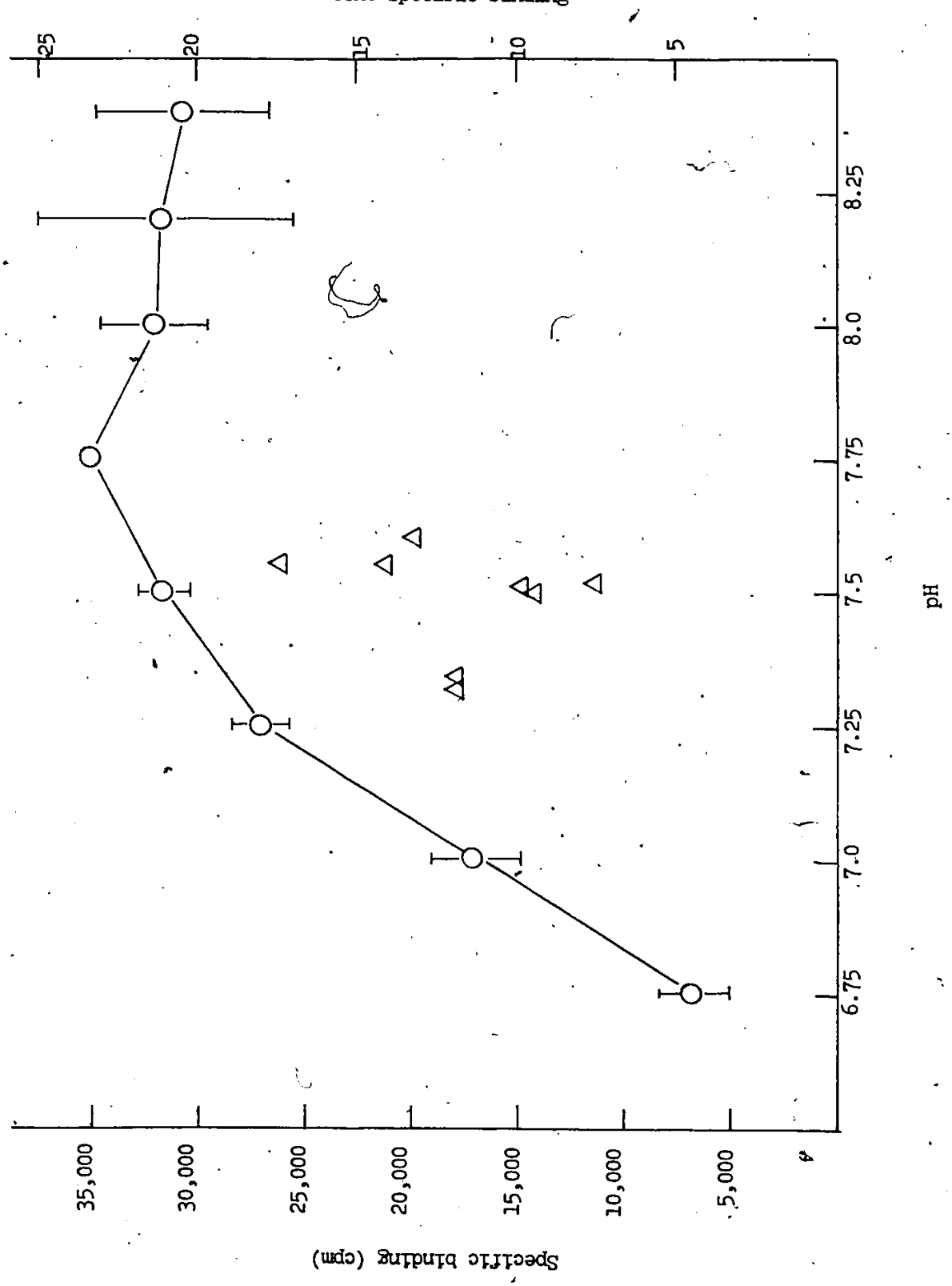
Table 9

Effect of carbon dioxide on the specific binding of ^{125}I-insulin. Membranes diluted in 150 µl salt or bicarbonate buffer (see Table 1) (final concentration ~ 250 µg membrane protein/ml) and ^{125}I-insulin in 150 µl of the same buffers (final concentration ~ 10^{-10}M) were added to 100 µl of the appropriate buffer (for total binding) or 1 mg/ml unlabelled porcine zinc insulin in 100 µl of the appropriate buffer (final concentration ~ 40µM) (for non-specific binding) in a 400 µl microfuge tube. After vigorous vortexing, the closed tubes were allowed to stand for 30 minutes at room temperature. The tubes were then centrifuged at 4 °C for 5 minutes in an Eppendorf microcentrifuge. The supernatants were removed with a syringe, and their pH values immediately measured. The surfaces of the membrane pellets were rinsed by the addition of 300 µl 10% sucrose in phosphate buffer to each, followed by centrifugation for 5 minutes at 4 °C. The supernatants were discarded. The tips of the microfuge tubes were excised and counted in a Nuclear Chicago gamma counter. Experiments were performed in duplicate or triplicate. Specific binding was calculated as the difference between total binding and non-specific binding. Specific binding was corrected for pH variation according to the pH profile in Figure 11.

Experiment	CO_2 Absent		CO_2 Present			Percent decrease in specific binding due to CO_2
	pH	% Specific binding	pH	% Specific binding	Corrected % specific binding	
1	7.34	6.9	8.15	5.1	4.3	38
2	7.51	12.0	7.80	6.6	5.6	53
3	7.55	9.0	7.88	6.9	5.9	34
4	7.55	4.2	7.83	3.6	3.4	19
5	7.60	13.6	7.76	9.1	8.2	40
6	7.50	12.1	7.84	6.3	5.4	55
7	7.52	13.7	7.78	6.0	4.8	65
8	7.32	7.6	8.03	5.7	4.8	37

Figure 11

The pH profile of ^{125}I-insulin binding to rat liver plasma membranes. The experiments were performed essentially as described in the legend to Table 9. Buffers were prepared at specific pH values by the addition of 1M HCl or 1M NaOH. Reaction was allowed to continue for 45 to 55 minutes at room temperature. Results shown are the averages of three experiments performed in duplicate, normalized at pH 7.75. Standard deviations are as shown. Open triangles (Δ) represent the percentage specific binding in the presence of carbon dioxide, corrected for pH variation.


Percent specific binding
25
20
15
10
5
8.25
8.0
7.75
7.5
7.25
7.0
6.75
pH
35,000
30,000
25,000
20,000
15,000
10,000
5,000
Specific binding (cpm)

higher than that in the absence of bicarbonate, but it is in fact considerably lower (Table 9). This leads to the conclusion that the net effect of 0.4M bicarbonate on the specific binding of ^{125}I-insulin to rat liver plasma membranes is to lower it between 19 and 65% of its value at the original pH, depending on the actual pH changes within each experiment. This is shown graphically by the open triangles (Δ) in Figure 11, representing the percentage specific binding of ^{125}I-insulin in the presence of CO_2 after correction for pH differences.

Due to the fact that equilibration of the bicarbonate buffers occurs very rapidly, it was not possible to determine whether the observed effect of bicarbonate buffer on insulin binding was in fact due to carbon dioxide or due to the bicarbonate ion itself. However, as it has been shown in this study that carbon dioxide readily reacts with amino groups to form carbamino derivatives, and since this chemical modification of the amino groups is quite likely to impair binding activity, the most probable explanation is that carbon dioxide is in fact responsible for all observed effects on the insulin-receptor interaction.

In some cases, depending on the preparation of unlabelled insulin used, relatively large amounts of non-specific binding were observed. This could be caused by one of two factors, or a combination of both. Bicarbonate ion, carbon dioxide, or high buffer pH

values could cause the precipitation of high concentrations of unlabelled insulin which, upon dimerization with labelled insulin, could account for higher apparent non-specific binding. In some instances, this was visually substantiated. Another possibility is that additional negative charges due to either the formation of carbamino derivatives, the higher pH, or both, cause a legitimate increase in non-specific binding of ^{125}I-insulin to the membrane; this second explanation could be true only where total binding is higher than non-specific binding. High non-specific binding at high pH values was in fact observed in one of three pH profiles. As a result of these inconsistencies, it is extremely difficult to quantitate results. However, due to the fact that total binding in the absence of carbon dioxide is always higher than total binding in the presence of carbon dioxide, the qualitative effect of carbon dioxide on binding can still be seen to be present, although possibly to a lesser extent.

An attempt was made to quantitate the effect of carbon dioxide on the binding of ^{125}I-insulin by varying the concentration of bicarbonate from 0.0M to 0.4M. Table 10 shows that increasing concentrations of bicarbonate cause decreased ^{125}I-insulin binding. After correcting for the fact that binding in the presence of bicarbonate buffer should actually have in increased due to the effect of pH alone, it is apparent that increasing bicarbonate concentrations cause

Table 10

Effect of increasing bicarbonate concentrations on ^{125}I-insulin binding. Experiments were carried out essentially as outlined in the legend to Table 9. Buffers were made as follows (note that salt concentrations are in addition to those already present in the phosphate buffer):

	[$NaHCO_3$]	[NaCl]
(1)	0.4	0.0
(2)	0.3	0.1
(3)	0.2	0.2
(4)	0.1	0.3
(5)	0.05	0.35
(6)	0.0	0.4

Experiments were performed for 30 (Experiment 1) or 60 (Experiment 2) minutes. Experiments were performed in triplicate. Specific binding was normalized at pH 7.61 and corrected for pH variation according to the pH profile in Figure 11.

Molar bicarbonate concentration	Experiment 1			Experiment 2		
	pH	% Specific binding	Corrected % specific binding	pH	% Total* binding	Corrected % total* binding
0.0	7.50	13.5	15.1	7.52	12.2	12.2
0.05	7.61	9.7	10.2	7.61	10.9	10.4
0.1	7.66	9.4	9.4	7.67	10.6	9.8
0.2	7.74	7.6	6.9	7.78	9.8	8.7
0.3	-	-	-	7.88	9.4	8.8
0.4	7.87	9.4	9.9	7.96	9.4	9.2

* Total binding was used due to the large amounts of non-specific binding in this particular experiment.

decreased ^{125}I-insulin binding, particularly at bicarbonate concentrations <0.2M (Figure 12). At higher bicarbonate concentrations, this effect is less predominant, in all probability due to the fact that the concentration of CO_2 dissolved in solution has decreased due to the higher pH values. Very little difference was observed between experiments conducted for 30 minutes and for 60 minutes (results not shown).

In the event that binding in the presence of carbon dioxide does not follow the same equilibrium as that in the absence of carbon dioxide, it was deemed necessary to follow the time course of binding of ^{125}I-insulin to rat liver plasma membranes in the presence and absence of bicarbonate buffer. Two representative examples are shown in Figure 13 (a) and (b). In the first, a steady state had not yet been reached in the absence of carbon dioxide by 90 minutes, whereas in the presence of carbon dioxide, binding began to decline after 60 minutes. However, at all time points, binding was considerably lower in the presence of carbon dioxide, consistent with previous results. In the second experiment, binding in the absence of CO_2 began to decline after 30 minutes, whereas in the presence of CO_2, binding reached an apparent steady state between 30 and 45 minutes. At all time points between 0 and 60 minutes, binding is higher in the absence of carbon dioxide. Due to the inconsistency of these results, primarily as a result of the use of different membrane preparations and varia-

Figure 12

The dependence of ^{125}I-insulin binding on bicarbonate concentration. Plot of corrected percent specific binding or total binding of ^{125}I-insulin to rat liver plasma membranes as a function of molar bicarbonate concentration. This is a graphic representation of results shown in Table 10 (Δ, Experiment 1; o, Experiment 2).

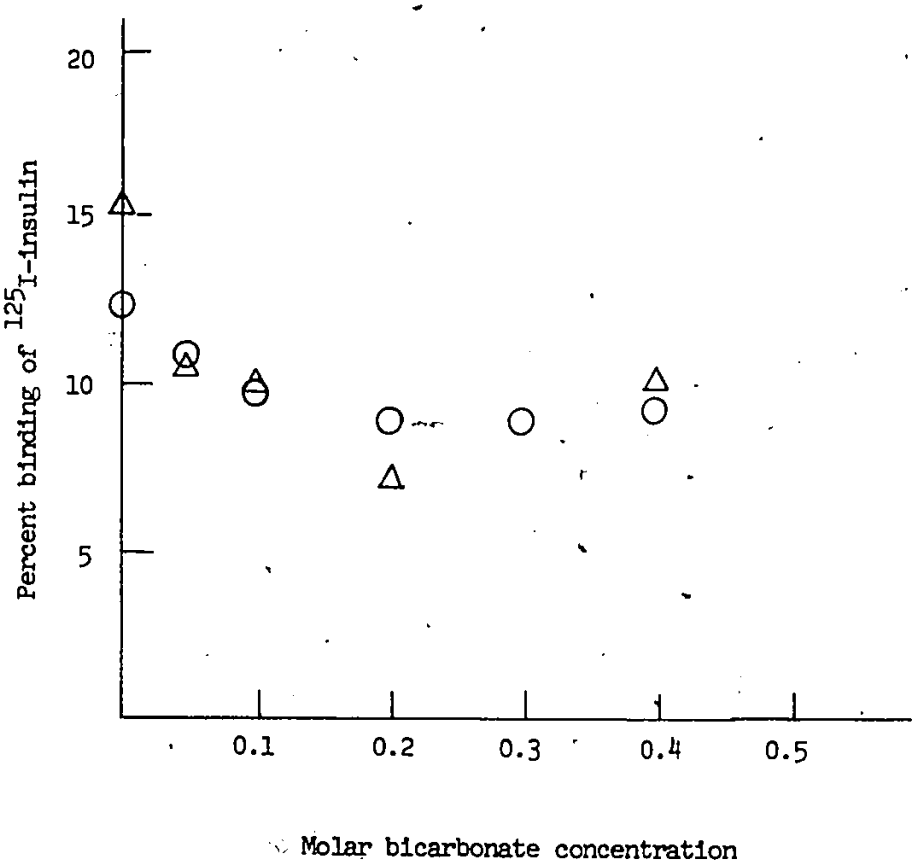
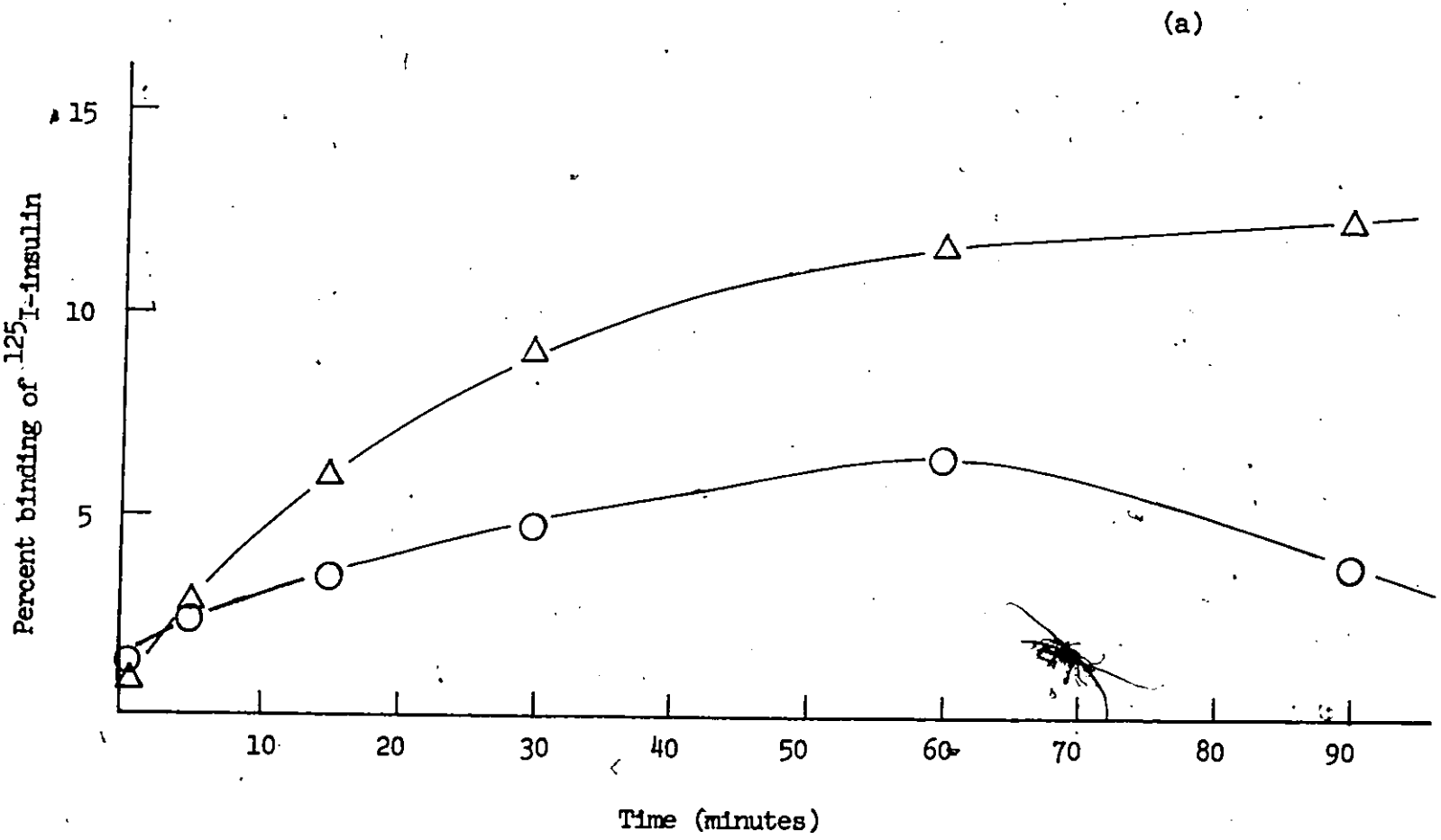
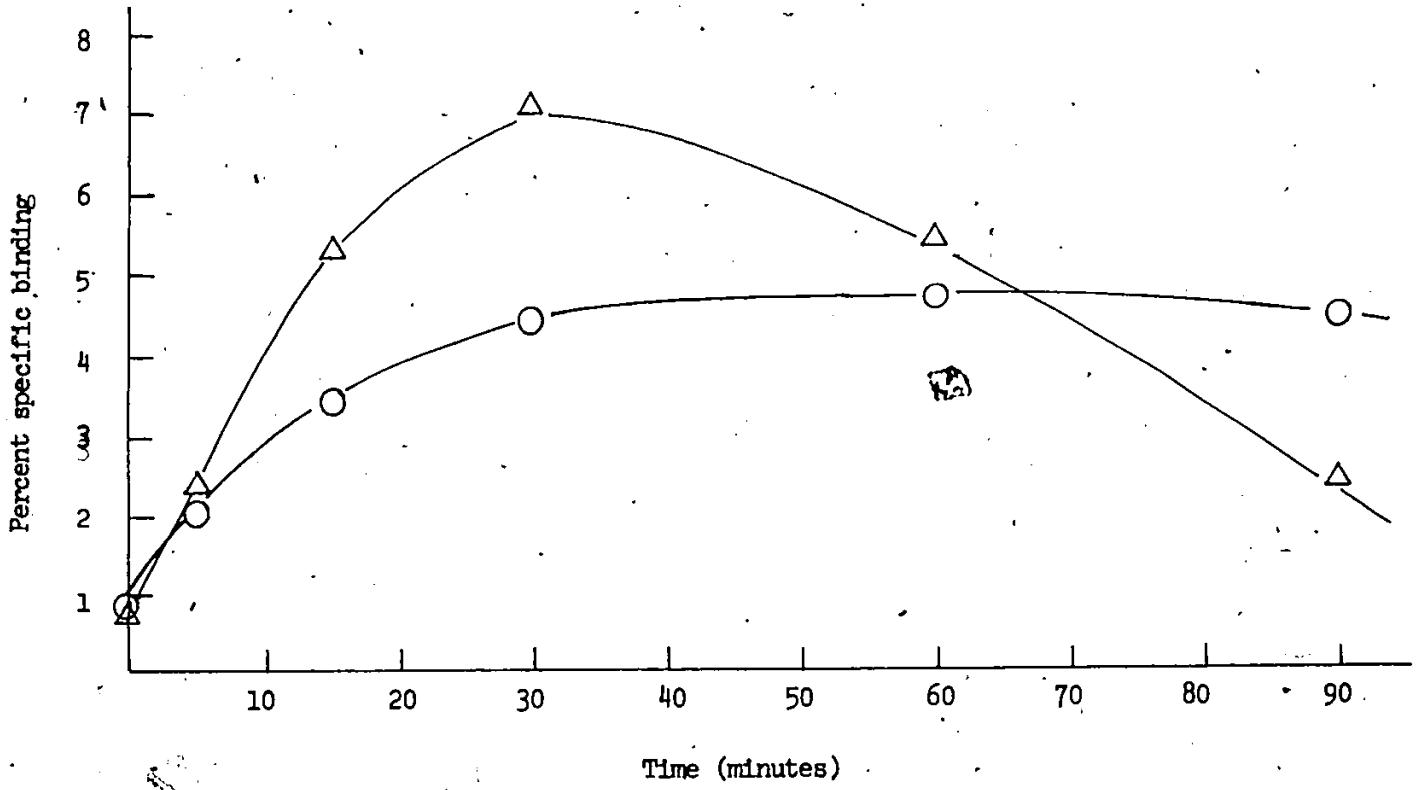

Percent binding of ^{125}I-insulin
20
15
10
5
0.1
0.2
0.3
0.4
0.5
Molar bicarbonate concentration

Figure 13

The time course of ^{125}I-insulin binding in the presence and absence of carbon dioxide. Experiments were carried out as described in the legend to Table 9, except that separate, identical tubes were prepared for each time point. The buffer was lowered to pH 6.9 with concentrated HCl before addition of $NaHCO_3$ so that the final pH values in the presence and absence of carbon dioxide would be approximately the same. Experiments were performed in duplicate. Figure 13 (a) shows the specific binding of ^{125}I-insulin in the absence of carbon dioxide (Δ) at pH 7.50, and the total binding of ^{125}I-insulin in the presence of carbon dioxide (o) at pH 7.47. Figure 13 (b) shows the specific binding of ^{125}I-insulin in the absence of carbon dioxide (Δ) at pH 7.49, and in the presence of carbon dioxide (o) at pH 7.43.


(a)
15
10
5
Percent binding of ^{125}I-insulin
10
20
30
40
50
60
70
80
90
Time (minutes)

(b)


Percent specific binding
8
7
6
5
4
3
2
1
10
20
30
40
50
60
70
80
90
Time (minutes)

tions in room temperature (affecting degradation of hormone and receptor), it is difficult to choose a best time at which to perform "equilibrium" measurements; however, it is apparent that a 30 minute time point is in most cases the most appropriate time at which to perform these measurements. Ideally, a time course determination should be made on each membrane preparation; however, due to the small yield of membrane obtained in each preparation from rat liver, this is not entirely feasible.

Throughout this thesis it has been assumed that carbon dioxide is responsible for the decreased binding ability of ^{125}I-insulin by its reaction with the three amino groups of insulin. If this is in fact the case, then it would be expected that the binding of ^{125}I-insulin fully acetylated at all three amino groups would not be affected by the addition of CO_2. However, the binding ability of acetylated ^{125}I-insulin in the absence of CO_2 was almost totally abolished in three separate experiments (results not shown); as a result, no significant conclusions concerning the effect of CO_2 on the binding of acetylated ^{125}I-insulin to rat liver plasma membranes could be drawn. The binding ability of triacetyl insulin <u>in vitro</u> has been found to vary between 8 and 100% by different experimenters (60, 92, 104); no attempts have been made to determine the binding affinity of acetylated ^{125}I-insulin.

The physiological significance of the finding of decreased

binding of ^{125}I-insulin in the presence of carbon dioxide is not clear. Since high concentrations of carbon dioxide exist near the plasma membranes of rapidly metabolizing cells such as muscle cells, any insulin present should form significant amounts of carbamino derivatives. Since it is well known that insulin which is chemically modified at its amino groups has decreased biological activity (14, 51, 68, 106, 158), it is not surprising that carbamino insulin will not bind as readily as native insulin to its receptor on plasma membranes. This reversible reaction of insulin with carbon dioxide could therefore act as part of a negative feedback mechanism, modulating the activity of insulin and controlling the entry of glucose into rapidly metabolizing tissue. A further possibility is that high levels of carbon dioxide at the membrane surface could affect the interaction of insulin with its receptor by a combination of a local decrease in pH and reaction with the amino groups to form carbamino derivatives; this is supported both by the observation in the present study that aggregation occurs rapidly below pH 7 at 37 °C, as indicated by the sharp decreases in reactivities (Figures 8, 9 and 10), and by the pH profiles of insulin binding to its receptor which show sharp decreases in binding activity at pH values below 7.8 (this study, 72, 96, 119). In addition, the presence of negatively charged carbamino groups on the insulin molecule may increase the non-specific binding of insulin to the plasma membrane, thus preventing its binding to its specific membrane receptor; this hypothesis, however, is not consistent with

all experimental results.

C. Effect of carbon dioxide on the dissociation of the ^{125}I-insulin- receptor complex

The physiological significance of the effect of carbon dioxide on insulin binding may not be as important as previously supposed, for the following reason. As full biological effects of insulin on membrane functions are elicited when less than 10% of the total cell surface insulin receptors are occupied (27, 41, 72, 120), this suggests that the number of receptors on the cell surface occupied by insulin is not the rate-limiting step in insulin action. As a result, unless CO_2 reacted with a large majority of the amino groups of insulin, it would not be expected to have a large effect on the binding and biological activity of insulin. An attempt was therefore made to determine whether carbon dioxide affected another aspect of the interaction between insulin and its receptor, that of dissociation of the insulin-receptor complex.

As it is known that a drop in pH causes dissociation of insulin from its receptor (31, 33, 37, 58, 59, 61, 119, 134), and since the addition of CO_2 will obviously lower the pH of the reaction mixtures, it was necessary to carry out a pH control, so that a comparison could be made between the effects of a drop in pH alone, and a drop in pH concurrent with the addition of CO_2. One problem is

that when using carbon dioxide, one is placed in the pH region where binding is quite low, so that significant errors may arise. However, in all three experiments (Table 11), it is apparent that there is an effect of CO_2 above and beyond that of the drop in pH on the dissociation of insulin from its receptor, not explainable by variations in pH.

Table 11

Effect of carbon dioxide on the dissociation of the insulin-receptor complex. Membranes diluted in 50 µl salt buffer (final concentration ∿ 250 µg membrane protein/ml) and ^{125}I-insulin in 50 µl of the same buffer (final concentration ∿ 10^{-10}M) were added to 50 µl buffer (for total binding) or 50 µl 1 mg/ml unlabelled insulin in buffer (final concentration ∿ 55µM) (for non-specific binding) in a 400 µl microfuge tube. After vigorous vortexing, the closed tubes were allowed to stand for 30 minutes (Experiment 3) or 1 hour (Experiments 1 and 2) at room temperature. After this time, 5 µl buffer were added to the experimental control tubes, 5 µl of an acetic acid solution (glacial acetic acid:buffer, 1:10) were added to the pH control tubes, and 5 µl buffer were added to the experimental CO_2 binding mixtures, over whose surfaces 99.9% CO_2 was then bubbled vigorously for 20 seconds. After vigorous vortexing, the closed tubes were allowed to remain an additional 10 minutes at room temperature. All tubes were then centrifuged for 5 minutes at 4 °C, and subsequently treated as described in the legend to Table 9. All experiments were performed in triplicate. Specific binding was corrected for pH variation according to the pH profile in Figure 11.

Experiment	Experimental Control		pH Control		Experimental CO_2			
	pH	% Specific binding	pH	% Specific binding	pH	% Specific binding	Corrected % specific binding	% Change in specific binding caused by CO_2
1	7.56	10.6	6.87	6.1	6.92	4.1	3.0	51
2	7.53	6.5	6.78	3.0	6.89	2.6	0.9	70
3	7.50	10.3	6.85	4.4	6.83	2.5	2.9	34

CHAPTER IV: Summary

In summary, the present investigation has shown that under physiological conditions, the three amino groups of insulin are deprotonated, and that this deprotonation is very likely associated with monomer formation. Due to the unusual chemical properties of the three amino groups, they readily react with carbon dioxide to form carbamino derivatives. Furthermore, this reaction has been shown to cause both a decrease in the specific binding of ^{125}I-insulin to its membrane receptors and an increase in the dissociation of the insulin-receptor complex.

CHAPTER V: Suggestions for Future Research

The results presented in this thesis have paved the way towards a new field of insulin research. Before pursuing this new field, however, it is first necessary that these results be confirmed in a more quantitative manner, using either nuclear magnetic resonance spectroscopy (110, 116) or a completely closed system in which no interactions between CO_2 and the atmosphere are allowed. It is also necessary to repeat the CO_2 experiments at physiological temperatures, in view of the changes in structural properties of insulin at 37 °C.

A number of additional experiments are suggested by the results of this study. For example, since most chemical modifications of the amino groups of insulin have involved their reaction to form neutral derivatives, it would be interesting to examine the effect of the negative charge of the carbamino group on binding by forming a slightly larger negatively charged amino derivative, using a reagent such as iodoacetate. As the monomeric state of insulin is assumed to be the biologically active form of the hormone (5, 6, 15, 30), an examination of the effect of carbon dioxide on the aggregation state of insulin under physiological conditions might provide significant results. Whether CO_2 affects the binding of insulin

to purified insulin receptors, from both plasma and nuclear membrane preparations, should also be determined. With respect to intracellular insulin receptors, an examination of the effect of CO_2 on the transport of insulin into the interior of the cell might prove interesting. Variations in time courses of insulin binding in the presence and absence of carbon dioxide suggest a possible effect on the degradation of insulin and/or its receptor. It would also be extremely important to reproduce these experiments using other target tissues of insulin, for example, muscle and adipose tissue. In view of the clinical importance of insulin, a comparison of the effects of carbon dioxide on insulin binding to normal livers with binding to liver tissue from diabetic or obese animals may provide clinically significant results.

Note added in proof

Recent gel filtration experiments performed in the laboratory of Dr. H. Kaplan have shown that the fraction of insulin existing in the monomeric state at 37 °C increases markedly above pH 7.0. In addition, a larger proportion of insulin exists as a dimer at pH 7.0 at 20 °C than at 37 °C. These findings support the hypothesis presented in this study that the discontinuities in the pH-reactivity profiles of the three amino groups of insulin at both 10 °C (Figures 4, 5 and 6) and 37 °C (Figures 8, 9 and 10) reflect the conversion of insulin from a dimer to a monomer.

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