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STRUCTURE-FUNCTION RELATIONSHIPS IN INSULIN AND GLUCAGON :

By

Mary Alice Hefford

A thesis submitted to the School of Graduate Studies and Research of the University of Ottawa in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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July, 1984.

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

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DEDICATION

This thesis is dedicated to my father who has contributed more to my education than words could ever tell.

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SYNOPSIS

The pancreatic polypeptide hormones, glucagon and insulin, exhibit a complex, concentration dependent, self-association pattern in solution. The x-ray crystal structures of hexameric insulin and trimeric glucagon have been determined. Current evidence, however, indicates that, in each case, it is the free monomeric form of the polypeptide which binds to the plasma membrane receptor. Because this form predominates only in very dilute solutions (concentrations of 10^{-6} or less), few attempts to directly study the structure of the insulin or glucagon free monomers have been reported in the literature. New methodology has been developed by which the chemical and, by inference, structural properties of proteins or polypeptides in dilute solution can be determined. Application of this methodology to the study of insulin at various concentrations shows that, at physiological pH in 0.1M-salt solution, insulin adsorbs to the surface of pyrex glass, tefzel and polystyrene reaction vessels. The addition of relatively large amounts of a second protein, i.e., calf thymus histone proteins, 10^{-3}M, to the buffering medium does not prevent this adsorption of insulin to pyrex glass. Under conditions where adsorption effects are negligible (1.0M-KCl, pyrex glass reaction vessels), concentration-dependence studies at physiological pH demonstrate that there are no gross conformational differences in the monomeric unit of insulin in its free and associated forms. In solution, insulin self-association is accompanied by locallized changes in the microenvironments of some of the functional groups. The B1-phenylalanine, the B29-lysine and the histidine residues have abnormal chemical properties in associated forms of insulin. Competitive labelling of insulin in dilute solution demonstrates that these groups have normal properties in the free insulin monomer. These results are discussed in terms of the structure of the monomeric unit determined by x-ray crystallography.

Most proteins or polypeptides have only one functional group at the N-terminal residue. Polypeptides of the glucagon family have an N-terminal histidine residue and thus have two functional groups at the N-terminal position, i.e., an α-amino and an imidazole group. New procedures, based on the competitive labelling approach of Duggleby and

Kaplan (Biochemistry, 14, 5168-5174, 1975), have been developed by which the chemical reactivity of each functional group in such a residue can be determined as a function of pH. Using this approach, the reactivity of the α-amino group of the glucagon free monomer towards 2,4-fluorodinitrobenzene gave an apparent pK_a value of 7.60 ± 0.04 which is in good agreement with the value of 7.32 assigned to this by Rothgeb et al. (Biochemistry 17, 4564-4571, 1978) using proton titration and ^{13}C-NMR. The reactivity of the imidazole group, however, gave an apparent pK_a value of 7.43 ± 0.09 as compared to the value of 5.32 assigned to this group on the basis of proton titration. The chemical properties of the N-terminal histidine residue of the model compound histidylglycine were determined. The apparent pK_a of this α-amino group was the same whether determined by the competitive labelling method or by acid-base titration. The reactivity-pH profile of the imidazole group does not indicate the pK_a value of 5.85 expected on the basis of conventional titration but, instead, parallels that of the α-amino group. Similar results were obtained for the N-terminal histidine polypeptides, secretin and vasoactive intestinal peptide. In both cases, the imidazole groups have a reactivity-pH profile which resembles that of the corresponding α-amino group. It has been concluded that the large difference in apparent pK_a values of the imidazole moiety obtained by proton titration and chemical reactivity is an inherent property of an N-terminal histidine residue which results from a large decrease in the inductive effect on the imidazole moiety by the α-amino when the latter loses its proton. While the apparent ionization behaviour of the N-terminal histidine in the glucagon free monomer does not differ from that in model compounds, the reactivity of the imidazole function is substantially greater than that in histidyl-glycine and the reactivity of the α-amino group is a factor of two lower. In addition, the pK_a and reactivity of the solitary lysine in the glucagon free monomer are very different than those of a normal ε-amino group in solution. These results indicate that glucagon has sufficient organized structure in dilute solution (10^{-6}M) to alter the chemical properties of these groups.

LIST OF ABBREVIATIONS

Å	angstrom(s)
BOC	t-butoxycarbonyl
BSA	bovine serum albumin
CD	circular dichroism
Ci	Curie(s)
DNP-	2,4-dinitrophenyl-
DNP-OH	2,4-dinitrophenol
dpm	disintegrations per minute
ESR	electron spin resonance
FDNB	1-fluoro-2,4-dinitrobenzene
k	rate constant
K_a	acid dissociation constant
NMR	nuclear magnetic resonance
ORD	optical rotatory dispersion
pI	isoelectric point
TFA	trifluoroacetyl-
TLC	thin layer chromatography
TNP	trinitrophenyl-
V	volt(s)
VIP	vasoactive intestinal peptide

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INTRODUCTORY COMMENTS

In all research, one endeavors to contribute useful information to the body of scientific knowledge. The merit of any scientific study lies in the problems it attempts to solve and the validity and originality of the experimental approach used. Within the field of protein chemistry, great progress has been made in the elucidation of structure-function relationships as a result of the development of sequencing techniques and physical methods, particularily x-ray crystallography. Despite these advancements, several questions remain unanswered. In some areas, such as the studies of receptor-ligand interactions and of structure-function relationships in membrane-bound proteins or in proteins in dilute solution, the questions which remain are technically difficult to answer using existing methodology. In these cases, it appears that new experimental techniques or approaches will be required.

While the pancreatic polypeptide hormones represent only a minute fraction of known proteins, considerable effort has been expended in the hope of elucidating their mechanism of action. Insulin, in particular, has been the subject of much research in several fields including physiology, protein chemistry and molecular biology. Until recently, glucagon was less extensively studied. Evidence indicating a primary glucagon disorder in some diabetics, however, has resulted in a renewed interest in glucagon research. These polypeptides play complementary roles in the maintenance of normal plasma glucose levels through the regulation of metabolic pathways within target cells. The first event in this cascade of biochemical repercussions is the binding of the hormone to its plasma membrane receptor. In spite of extensive investigation, the details of this event remain unclear. More research designed to augment our present understanding of the mechanism(s) of action of glucagon and insulin is clearly required. In addition to their importance of their study to the understanding of both normal and abnormal metabolism, insulin and glucagon, as peptidyl hormones, may represent simple systems in which one may examine general principles of the chemistry of proteins in solution and multi-protein complexes. The elucidation of any such principles is, in itself, a worthwhile undertaking.

While several important and interesting questions concerning protein structure-function relationships remain to be asked, the answering of these question depends on the availability of suitable methodology. Both insulin and glucagon exhibit a complex, concentration-dependent, self-association pattern. In concentrated solution, oligomeric forms predominate, in dilute solutions, the monomeric form. In the human blood stream, both insulin and glucagon are found at nanomolar concentrations. It thus appears that it is the free monomer which binds the receptor. Study of the relationship between structure and the function of receptor binding, therefore, requires the study of the free monomeric form. Such studies have been limited because of the low concentrations (approximately 10^{-6}M) required to obtain monomeric insulin and glucagon. At present, few techniques are available to determine structure-function relationships of protein in dilute solution (see below). Although no one method can hope to give a complete picture of the mechanism of action of a given protein, new approaches to dilute solution study are clearly needed to complement those currently available.

The x-ray crystal structure of insulin and glucagon are known. In the crystal both insulin and glucagon are oligomeric: insulin crystallizes as a hexamer, glucagon as a trimer. Many investigators have used x-ray crystal data as a basis for postulating the structure of the free monomers in dilute solution. The process of receptor binding in polypeptide hormones has also been extensively probed using chemically modified analogues of insulin and glucagon. The role played by specific regions or individual residues of the hormone in the binding process remains unclear.

Interaction of any one molecule with any other molecule is, fundamentally, a chemical interaction. The chemical properties of a protein, then, determine the types of and the extent of interactions it may have with other molecules. Chemical properties of the functional groups are determined by their microenvironments which, in turn, are determined by the structure (primary, secondary, tertiary and quaternary) of the protein in question. Chemical properties, therefore, are an intergal part of protein structure-function relationships. Elucidation of these properties is necessary to a complete understanding of protein

action. The competitive labelling approach (Duggleby and Kaplan, 1975; Kaplan et al., 1972) provides one method of determining the chemical properties of functional groups in protein in concentrated solution. Application of this approach to the determination of the chemical properties in the insulin and glucagon free monomers requires modification of the method to facilitate use in dilute protein solutions, and to accommodate the fact that glucagon has two nucleophilic groups at its N-terminal residue. Development of this new methodology, then, affords a means of obtaining novel experimental data regarding structure-function relationships in the polypeptide hormones, glucagon and insulin.

GENERAL OBJECTIVES

It is the overall objective of this research to utilize physical-chemical properties to determine structure-function relationships in insulin and glucagon. Of major interest is the elucidation of these relationships in order to relate them to the primary event of hormone action: the binding of the hormone to the specific plasma membrane receptor. Also of interest is the examination of structure-function relationships in insulin and glucagon as they might be relevant to hormone storage, secretion or inactivation and in as much as they may reflect structure-function relationships of proteins in general.

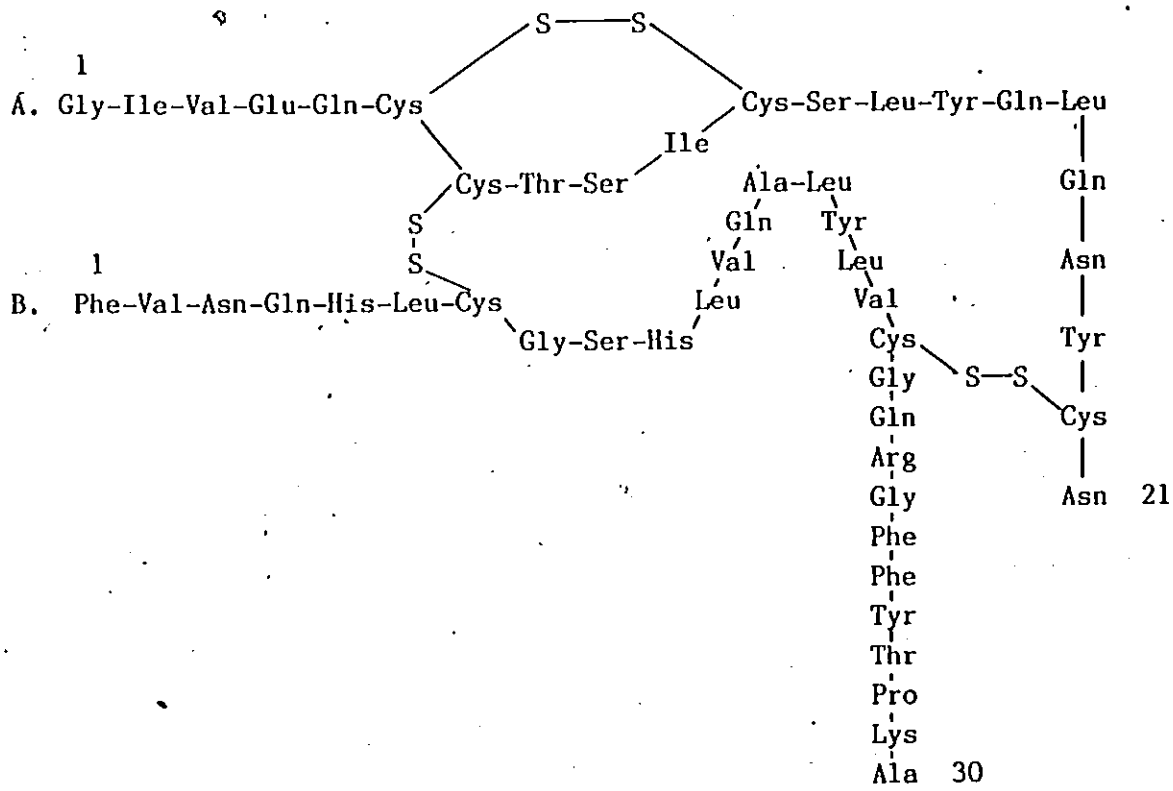
REVIEW OF PERTINENT LITERATURE

Insulin

Insulin was discovered in 1921. Its essential role in maintenance of blood sugar levels was immediately recognized. Since that time much research has been devoted to the study of insulin from both a physiological and a chemical standpoint. Despite the quantity of insulin research, its mechanism of action has remained elusive. The lack of definitive information in this regard, may, in part, arise from the complex nature of insulin structure-function relationships. Insulin is a relatively small protein molecule comprised of fifty-one amino acid residues in two, disulphide-linked chains. (The primary structure of insulin is shown in Figure 1.) This ostensibly simple arrangement of amino acids must afford insulin the versatility to be stored in a condensed form, to be secreted and transported, to recognize and bind the appropriate membrane receptors, to exert its biological effect and to be eliminated from the system at the appropriate time. Part of this versatility may be accounted for by the fact that insulin exhibits a concentration-dependent, self-association pattern (Milthorpe et al., 1977; Jeffery and Coates, 1976; Goldman and Carpenter, 1974; Pekar and Frank, 1972).

Secretion of insulin results in a variety of metabolic effects. Such short term effects as the regulation of glucose transport and of the activities of enzymes involved in glucose utilization pathways are thought to result directly from the binding of insulin to plasma membrane receptors. Insulin-induced modifications to protein synthesis and other long term effects presumably require entry of insulin and/or its receptor into the target cell (Goldfine, 1978). In either case, the first step of insulin action appears to be the binding of the hormone to a specific plasma membrane receptor. Transduction and biological effects ensue. In the blood, insulin exists at low concentrations (10^{-9}M to 10^{-10}M) (Blundell, 1979; Pekar and Frank, 1972) where, on the basis of sedimentation velocity data (Milthorpe et al., 1977; Goldman and Carpenter, 1974; Pekar and Frank, 1972), the free monomeric form of insulin predominates. It is this free monomer, then, that is the biological species relevant to receptor binding.

Figure 1: The primary structure of porcine insulin. Human insulin has the same amino acid sequence except that the C-terminal residue of the B-chain is threonine. Bovine insulin differs from porcine in that it has a B8-alanine and a B10-valine residue.

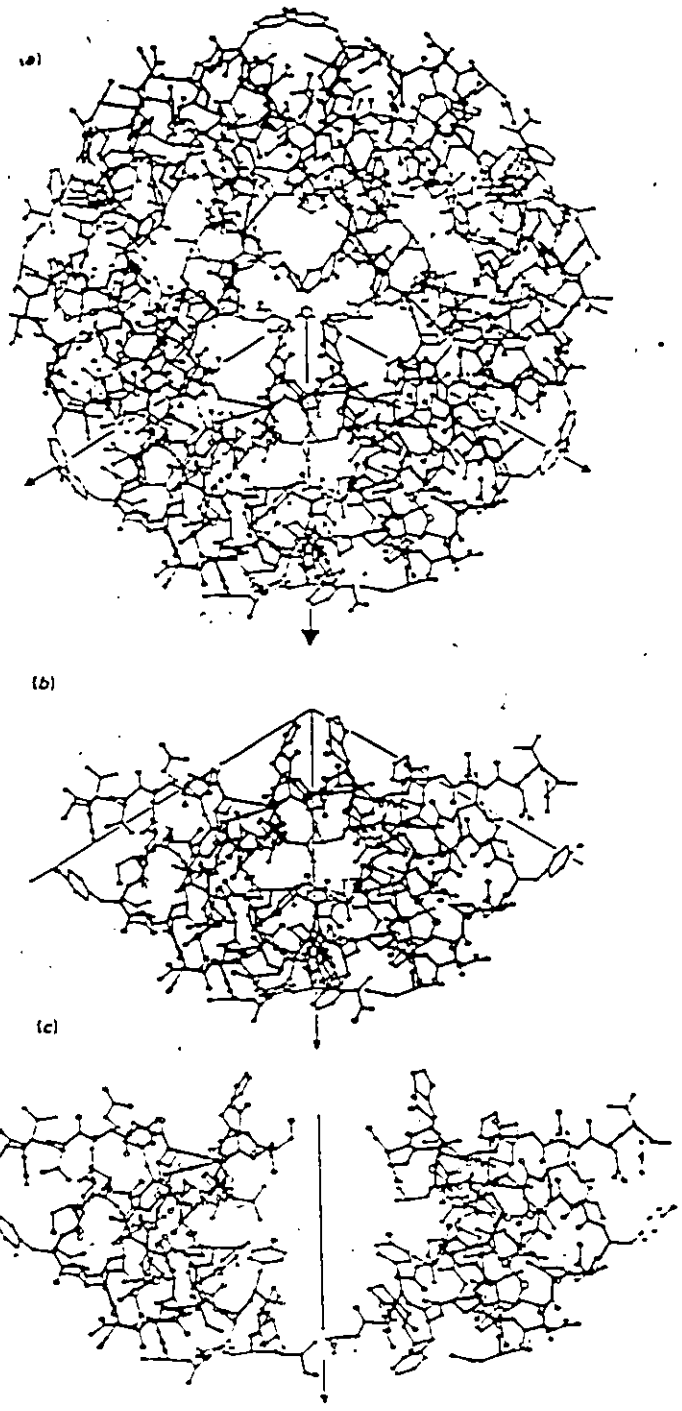

S—S
1
A. Gly-Ile-Val-Glu-Gln-Cys
Cys-Ser-Leu-Tyr-Gln-Leu
Ile
Cys-Thr-Ser
Ala-Leu
Gln
S
Gln
Tyr
1
S
Val
Leu
Asn
B. Phe-Val-Asn-Gln-His-Leu-Cys
Leu
Val
Gly-Ser-His
Cys
Tyr
Gly
S—S
Gln
Cys
Arg
Gly
Asn 21
Phe
Phe
Tyr
Thr
Pro
Lys
Ala 30

Most mammalian insulins form rhombohedral crystals of insulin hexamers in concentrated insulin solutions in the presence of zinc ions and exhibit similar biological potencies. Guinea pig and coypu insulins are notable exceptions to both rules (Zimmerman and Yip, 1974). Porcine and bovine insulins are very similar in primary structure (differing in only two residues), crystal structure, binding affinity and biological potency. Their similarity to human insulin afford their use as models of human insulin and their therapeutic uses in treatment of human disorders. Both 2Zn- and 4Zn-insulin hexamers of porcine insulin are known (Chothia et al., 1983; Blundell, 1979; Bentley et al., 1976; Blundell et al., 1971). These two crystal forms have slight differences in structure (Chothia et al., 1983; Hodgkin et al., 1983 and references cited therein); interconversion of the two is quite facile (Bentley et al., 1978).

Both types of zinc hexamer contain three identical dimers related by a three-fold rotational axis of symmetry. Each dimer is, in turn, comprised of two similar but not identical monomers (Molecule 1 and Molecule 2) which are approximate mirror images (Figure 2). Both monomeric units within the crystal have a compact structure with two essentially hydrophobic surfaces. The first of these is involved in monomer-monomer interactions along the local, two-fold axis and the second in interactions which relate the ensuing dimers within the hexamer. Differences between 2Zn- and 4Zn insulin occur mainly in the structure of Molecule 1. The structure of Molecule 2 of 4Zn-insulin is very similar to that of Molecule 2 in 2Zn-insulin as well as to that in the monomeric unit of hagfish insulin. (This type of insulin forms dimers of two identical monomers but does not hexamerize.) Molecule 2, therefore is thought to represent the "preferred" conformation of the monomer and is postulated to persist in dilute solution (Chothia et al., 1983; Blundell et al., 1971).

In each monomeric unit of porcine 2Zn-insulin, the A-chain is compact. An α-helix starts (A2-A9) and terminates (A12-A19) the chain while the central portion forms an internal ring via a disulphide bridge between A6 and A11 cysteines. The B-chain is divided into three roughly equal segments by glycine residues at B8 and B20. The central portion is helical, the two ends, extended β-sheet. The B9-B19

Figure 2: The 2Zn-insulin hexamer. The hexamer (a) is comprised of three identical dimers (b) which are related by a three-fold rotational axis of symmetry. This crystal dimer is composed of two similar, but not identical monomers (c), Molecule 1 and Molecule 2 (Blundell et al., 1972).


(a)
(b)
(c)

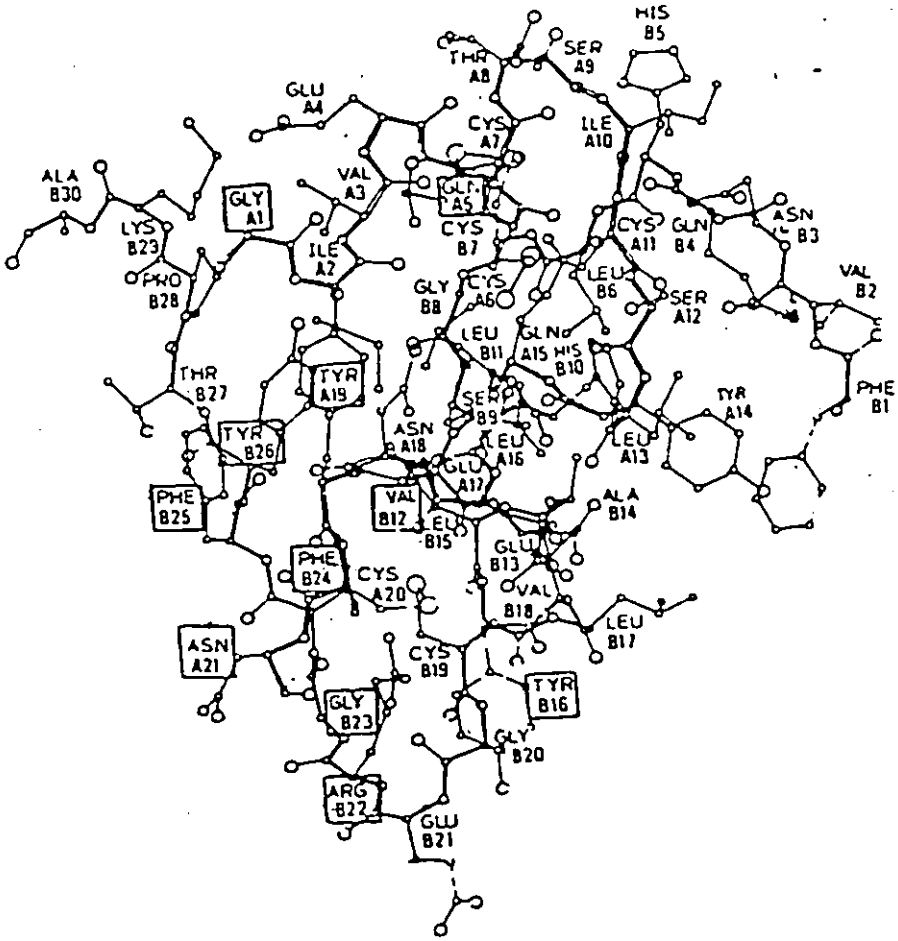
residues form a tight, classical α-helix with well-defined hydrophobic (residues B16-B19) and hydrophilic (residues B9, B10 and B13) sides. The residues B20 to B23 turn the C-terminal portion of the B-chain back against the hydrophobic side of this helix in an antiparallel fashion. In 2Zn-insulin, the B10 histidine residue of each monomeric unit is involved in zinc coordination (Blundell et al., 1972).

In Molecule 1 of 4Zn-insulin, the B1 to B6 residues are not involved in interactions between dimeric units of the hexamer but are extended towards the surface. This results in a wider separation of the B1-Phe and the A14-Tyr in this molecule than in Molecule 2 of 4Zn-insulin of in either monomeric unit of 2Zn-insulin. The α-helical structures in regions A2-A8, A13-A19 and B9-B19 are not well conserved in Molecule 1 of 4Zn-insulin. Both the B5- and the B10-histidine residues bind zinc in 4Zn-insulin crystals.

From the crystalline structure and the invariance of certain residues in insulins from various sources (Blundell and Wood, 1975), Pullen et al. (1976) tentatively mapped out a receptor binding region on the insulin monomer (Figure 3). This region is partly analogous to that involved in monomer-monomer interactions within the crystal (residues B24-Phe, B25-Phe, B26-Tyr, B12-Val and B16-Tyr) but includes at least some of the peripheral polar groups (A1-Gly, A4-Glu, A5-Gln, A21-Asn, B9-Ser, B10-His, B13-Glu, B21-Glu, B22-Arg and B27-Thr). The inclusion of the latter group of residues accounts for the fact that insulin -receptor binding ($K = 10^{9}M$ in rat liver) (Cuatrecasas, 1971) is more favourable than insulin self-association ($K = 10^{5}M$) (Milthorpe et al., 1977; Goldman and Carpenter, 1974; Pekar and Frank, 1972). The Pullen model has two features which render it amenable to experimental validation. (1)Because the model postulates the involvement of several residues in receptor binding, affinity is expected to be additive over a number of hydrophobic interactions and specific hydrogen bonds. The modification or elimination of one residue, therefore, should alter but not completely eliminate receptor affinity. (2)Residues important to dimerization are purported to be involved in the binding process. Consequently, modification of certain residues should affect both processes in a parallel fashion.

Several insulin analogues (resulting from either semisynthesis or

Figure 3: The putative receptor binding region of insulin as postulated by Pullen et al. (1976).


HIS B5
THR A8
SER A9
GLU A4
CYS A7
ILE A10
ALA B30
GLY A1
VAL A3
GLU A5
ASN B3
LYS B23
CYS B7
CYS A11
GLN B4
ILE A2
PHO B28
GLY B8
CYS A6
LEU B6
VAL B2
SER A12
LEU B11
GLN A15
HIS B10
THR B27
TYR A19
TYR A14
PHE B1
SER B9
TYR B26
ASN A18
LEU A16
LEU A13
GLU A17
PHE B25
VAL B12
ALA B14
LEU B15
GLU B13
PHE B24
CYS A20
VAL B18
LEU B17
ASN A21
CYS B19
TYR B16
GLY B23
GLY B20
ARG B22
GLU B21

chemical modification) have been tested for their ability to bind insulin receptors in various tissues and/or to elicit certain biological effects. Some of these have also been tested for their ability to form regular, crystalline structures (see below). The alteration of structure in one portion of a protein molecule, however, may induce a conformational change in a region of the protein molecule quite remote from the site of modification. It is, therefore, difficult to provide an unequivocal interpretation of the data obtained from studies which utilized insulin analogues. Nonetheless, information obtained in this way may provide some insight into the possible roles of specific residues in insulin action.

The N-terminal residue of the B-chain, according to the Pullen model, is not involved in receptor binding. This is consistent with the observation that its removal does not lead to any changes in biological activity at the receptor level (Gliemann and Gammeltoft, 1974; Brandenburg, 1969). The CD spectrum of Des-B1-Phe is essentially the same as that of native insulin (Wollmer et al., 1977). The residue, however, is invariable in vertebrates except in fish where it is replaced by an alanine. In addition, many fish have an additional residue at the N-terminal of the B-chain. Cod and toad fish insulins (B0-Met) and Atlantic hagfish insulin (B1-Arg) show a decrease in biological potency from that of most mammalian insulins despite the fact that the folding of the backbone has been shown by x-ray crystallography to be very similar to that of porcine insulin (Cutfield et al., 1979). X-ray crystal data shows that B1-Phe, A13-Leu, A14-Tyr and A17-Glu residues are close together on the monomer and cluster with equivalent residues in the hexamer (Blundell et al.,1972). Evolutionary changes in one residue are usually accompanied by complementary changes in another to allow zinc hexamer formation. Changes in these residues also affect crystal packing. Cod insulin (B0-Met, B1-Ala, A13-Ile, A14-Phe, A17-Gln), for example, forms orthorhombic rather than rhombohedral crystals (Baker and Dodson, 1970).

Modification studies involving alterations of the N-terminal phenylalanine residue are summarized in Table 1. Perturbation of the N-terminal region of the B-chain by the addition of hydrophobic or hydrophilic side chains at the B1-residue result in derivatives which

TABLE 1: Chemical Modifications of the B1-Phenylalanine

Modification	Change in CD	% Potency					Reference
		RB	MC	BSD	FC	RIA	
Des Phe^{B1}	small			90			Brandenburg (1969) Zahn et al. (1972)
Des Phe^{B1} Des Val^{B2}	large				0.2	2	Brandenburg et al. (1971)
Desamino Phe^{B1}					82		Brandenburg et al. (1977)
L-Tyr^{B1}					102		Brandenburg et al. (1977)
Desamino Tyr^{B1}					73		Brandenburg et al. (1977)
L-Met^{B1}					59		Brandenburg et al. (1977)
D-Phe^{B1}					61		Brandenburg et al. (1977)
I-HPP					69		Brandenburg et al. (1977)
p-I Phe^{B1}					65	85	Krail et al. (1971)
PTC-Phe^{B1}				48	57	94	Brandenburg et al. (1971)
FTC-Phe^{B1}			38				Brandenburg et al. (1971)
(PFTC)-Phe^{B1}			100			31	Lindsay and Shall (1971)
carbamyl-Phe^{B1}			100			23	Lindsay et al. (1972)
methylthiocarbamyl-Phe^{B1}			100			28	Lindsay et al. (1972)
acetoacetyl-Phe^{B1}	small		96	100		10	Lindsay and Shall (1971)
hemisuccinyl-Phe^{B1}	small		65		80	80	Blundell et al. (1977)
biotinyl-Phe^{B1}					94		Hofmann et al. (1977)
citraconyl-Phe^{B1}			78		100	75	Naithani and Gattner (1982)
L-Met-Phe^{B1}		54			63	65	Yeung et al. (1979)
L-Lys-Phe^{B1}		69			78	58	Yeung et al. (1979)
L-Arg-Phe^{B1}		43			38	40	Yeung et al. (1979)
L-Gly-Phe^{B1}					53		Brandenburg et al. (1977)
L-Glu-Phe^{B1}					64		Brandenburg et al. (1977)
L-Leu-Phe^{B1}					75		Brandenburg et al. (1977)

RB, receptor binding assay; MC, mouse convulsion test; BSD, blood sugar depression; FC, lipogenesis in fat cells; RIA, radioimmunoassay.

have decreased binding affinity, potency in the mouse convulsion assay, and immune response. Examination of the CD spectra of these analogues indicates a decrease in β-sheet content at concentrations where dimeric insulin is expected to predominate. Since β-sheet structure is stabilized only in insulin oligomers, these results may reflect the inability of the altered insulin to self-associate (Yeung et al., 1979). Brandenburg et al., (1977), using analogues with modified B1-residues, have concluded that a nucleophilic group at the amino terminus is important but not essential to lipogenic activity in insulin.

Although the B1-phenylalanine residue is not implicated in either dimerization or receptor binding, some modifications at this position result in impairment of one or both functions (Table 1). In addition, the use of N-α-B1-derivatives of insulin as photoaffinity receptor labels (Yeung et al., 1980; Yip et al., 1980) suggests that this residue is either part of or very near to the receptor binding region of insulin.

Work involving insulin analogues resulting from modification of the A-chain amino terminal glycine has recently been reviewed by Rosen et al. (1980) and is summarized in Table 2. Impaired binding ability results in a parallel loss of biological potency in all cases. Modifications which retain the potential for positive charge at this position result in the retention of more activity in both assays than do those which give a neutral or negative group. Amidinated or guanylated A1-Gly derivatives of insulin have CD spectra that are analogous to that of native insulin and form well-developed rhombohedral crystals (Brandenburg et al., 1972). Acetylation at this position, however, results in a substantial shift in the positions of the A2-A9 helix and the A19-tyrosine residue relative to native insulin. The addition of basic residues to the N-terminal glycine resulted in derivatives with greater binding affinity than lipogenic potency (Rosen et al., 1980).

The valine at position B12 is part of the rigid α-helical segment, B9-B19, which forms part of the hydrophobic core of insulin molecules. It is invariant in all insulins characterized thus far and postulated to take part in both insulin dimerization and receptor binding (Blundell et al., 1972). Replacement of this residue with an aspa-

TABLE 2: Chemical Modifications of the A1-Glycine

Modification	% Activity RB	% Activity FC	Reference
acetyl-Gly^{A1}		38	Brandenburg et al. (1972), Gliemann and Gammeltoft (1974).
	23	37	Rosen et al. (1980)
amidino-Gly^{A1}		88	Freisen et al. (1977)
	84		Rosen et al. (1980)
amidino-Gly^{A1}, amidino-Lys^{B29}		75	Freisen et al. (1977)
BOC-Gly^{A1}		17	Pullen et al. (1976)
	15	17	Rosen et al. (1980)
thiazolidine-Gly^{A1}		13	Pullen et al. (1976)
	6		Rosen et al. (1980)
carbamoyl-Gly^{A1}		31	Freisen et al. (1977)
	26	43	Rosen et al. (1980)
L-Lys-Arg-Gly^{A1}		26	Freisen et al. (1977)
	75	36	Rosen et al. (1980)
L-Arg-Gly^{A1}		45	Freisen et al. (1977)
	107	39	Rosen et al. (1980)
L-Arg-Arg-Gly^{A1}		42	Freisen et al. (1977)
	90	35	Rosen et al. (1980)
L-Arg-Arg-Arg-Gly^{A1}		40	Freisen et al. (1977)
	125		Rosen et al. (1980)

RB: receptor binding assay; FC: lipogenesis as assayed in fat cells.

ragine residue results in a substantial decrease in binding affinity and in ability to stimulate lipogenesis at physiological concentrations. The maximal effect of native insulin can, however, be achieved at higher concentrations of the analogue. This indicates that, while B12-Val is important to stabilizing the hormone-receptor complex, it has no apparent role in transduction (Schwartz et al., 1981).

On the basis of x-ray data, monomer-monomer interactions in insulin are postulated to be partially stabilized by a salt bridge between the carboxylate function of the A21-asparagine residue and the guanidine function of B22-arginine residue. Loss of the negative charge on the C-terminal A21-asparagine residue through imidylation results in decrease in the ability of insulin to elict biological response but has no apparent effect on its ability to form crystals (Brandenburg et al., 1977). Replacement of the A21-Asn with an arginine residue yields a derivative with an additional positive charge in the C-terminal region of the A-chain and a decreased affinity for the insulin receptor (Frederigos et al., 1979).

The B9-serine residue begins the tight helical coil of the B-chain and has been conserved in all mammals with the exception of the rat where it is replaced with a proline. Rat insulin has comparable biological potency with other mammalian insulins despite the fact that a proline residue at position B9 is not expected to participate in the α-helical structure (Wood et al., 1978). It is surprising, therefore, that replacement of the B9-serine with a leucine residue results in a loss of approximately 48% of the biological function of insulin as tested by mouse convulsion tests and radioimmunoassay (Schwartz and Katsoyannis, 1977). The loss in biological activity in this derivative was tentatively attributed to a disruption of the cluster of hydrophobic residues of the B9-B19 helix. This residue is not part of the receptor binding region according to the Pullen model.

The B10-histidine residue is involved in zinc binding in both 2Zn- and 4Zn-insulins. Dilution of insulin to form the free monomer is accompanied by release of the zinc ion (Milthorpe et al., 1977; Pekar and Frank, 1972). The B10- but not the B5-histidine is part of the putative receptor binding region. Replacement of this residue with a leucine results in the formation of an analogue with reduced biological

activity and immune response. These results are attributed to a change in polarity at the B10-position and subsequent disruption of the B-chain α-helix (Schwartz and Katsoyannis, 1977).

According to the Pullen model the phenylalanine residues at positions 24 and 25 of the B-chain and three of the four tyrosine residues(A19-Tyr, B16-Tyr and B26-Tyr) are part of the receptor binding region of the free insulin monomer. Kikuchi and co-workers (1981a and 1981b) found that the pentapeptide, Arg^{B22}-Gly^{B23}-Phe^{B24}-Phe^{B25}-Tyr^{B26}, potentiated the action of submaximal concentrations of insulin and insulin Des-octapeptide. The pentapeptide is postulated to increase the binding of insulin by shifting the equilibrium of insulin receptors of various affinities and/or aggregation states.

Because of the utility of insulin derivatives iodinated at one or more of the tyrosine residues to receptor binding studies, the potency of several iodoinsulins has been tested. In general, di-iodinated insulin derivatives show less potency in receptor binding than do mono-iodinated insulins or insulin itself (Maceda et al., 1982). Under some iodination conditions, preferential reaction of the A14-tyrosine residue is possible. This derivative, when separated from reaction side-products by DEAE-sephadex (Hamlin and Arquilla, 1974) or by HPLC (Welinder et al., 1983) has been shown to have an affinity for rat adipocyte plasma membrane receptors equal to that of the native hormone. Iodination of insulin at the A19-tyrosine residue decreases the receptor affinity relative to that of iodo-Tyr^{A14}-insulin in rat adipocytes and hepatocytes. The B16-mono-iodinated analogue has 100% binding potency when isolated adipocyte receptors are used in the assay system but greater potency than the A14-tyrosine derivative in cultured hepatocyte systems. The corresponding B26-derivative has enhanced affinity relative to iodo-Tyr^{A14}-insulin with both types of receptors. In all the derivatives, the lipogenic activity parallels the adipocyte receptor binding affinity (Sonne et al., 1983; Linde et al., 1981). The enhanced binding of the B26-mono-iodinated insulin derivative is consistent with the postulation of a hydrophobic interaction of insulin with its receptor. Removal of the C-terminal tetrapeptide segment (B27-B30) of the insulin B-chain results in an analogue in which the terminal carboxyl group is on the B26-tyrosine residue and in approx-

imately 50% loss in the biological potency of the hormone in the mouse convulsion test (Katsoyannis et al., 1973). The Des-tripeptide (B28-B30) insulin analogue, however, has a potency equal to that of the native hormone in this assay (Katsoyannis et al., 1971).

The accessibility of the tyrosine residues has been probed in insulin hexamers, dimers and monomers using photochemical induction of nuclear polarization. The dimeric and monomeric forms of insulin in this study were generated in concentrated insulin solutions by decreasing the pH to 2.3 and/or increasing the solution temperature to 57°C. The A14-tyrosine is equally accessible in all forms of insulin while the B16- and B26-residues are accessible only in the monomer. A19-tyrosine is inaccessible in all insulin association states (Muszkat et al., 1984).

The B24- and B25-phenylalanine residues are invarient in normal mammalian insulins (Pullen et al., 1976). In 1979 the abnormally low receptor affinity of the insulin of one human patient was attributed to a substitution of leucine for the phenylalanine residue at either position B24 or B25 (Tager et al., 1979). The semi-synthetic insulin analogue with replacement of the B24-Phe with leucine shows 20-30% potency in binding studies while the B25-Leu-derivative has only 2% of the affinity of native insulin (Inouye et al., 1981; Tager et al., 1980). CD analysis, however, indicates that the conformational change resulting from leucine substitution for phenylalanine at the B25-position is small. The CD spectrum for the more active analogue is very different from that of native insulin (Wollmer et al., 1981). Substitution of the D-isomer of phenylalanine for the L-isomer at position B25 gives a derivative which has only 4% lipogenic potency. D-Phe^{B24}-insulin, however, has enhanced binding affinity (180%) and biological activity (140%) relative to the native hormone (Kobayashi et al., 1982).

The carboxyl terminus of the B-chain is not directly implicated in insulin receptor binding. A gradual decrease in biological activity, however, accompanies the elimination of residues from the C-terminal (Weitzel et al., 1976; Gattner, 1975; Katsoyannis et al., 1973). Because the C-terminal residues of the B-chain fold back against the hydrophobic residues of the B-chain helix in the crystal, it has been postulated that the observed decrease in activity results from a

disruption of this helix by the introduction of a negative charge. Amidation of the carboxylate function of insulins with truncated B-chains, however, does not result in a total restoration of biological potency (Cosmatos et al., 1976).

The binding of insulin to its receptor is non-classical both in that the data yields a curvilinear Scatchard plot (Cuatrecasas, 1971) and in that the temperature dependence of binding to the higher affinity sites gives a non-linear van't Hoff plot (Waelbroeck et al., 1979). Various explanations of the former have been advanced (DeMeyts et al., 1976; Jacobs and Cuatrcasas, 1976; Kahn et al., 1974), each involving intrinsic properties of the receptor. The thermodynamics of hormone-receptor complex formation, however, are expected to be influenced by properties of both the receptor and insulin itself.

Examination of the changes in thermodynamic parameters of complex formation as the reaction temperature increases reveals that the reaction is endothermic and entropically driven at low temperatures and exothermic and enthalpically driven at higher temperatures (including physiological temperatures). These observations are consistent with either a hydrophobic interaction or a two-step reaction mechanism (Edelhoch and Osborne, 1976). The process of removing the hydrophobic residues of the putative receptor binding region of insulin (A19-Tyr, B24-Phe, B25-Phe, B12-Val and B16-Tyr) from an aqueous milieu and placing them in a hydrophobic one is calculated to result in a change of heat capacity of -460cal/mol-degree at 25°C. The remainder of the experimentally-determined change in heat capacity is attributed to a similar dehydration of a complementary hydrophobic surface on the receptor. (Hydrophobic interactions are also postulated to be the major contributors to the negative $\Delta G°$ of association of insulin monomers (Pocker and Biswas, 1981).) Hydrophobic interactions alone are incapable of producing the negative $\Delta H°$ and $\Delta S°$ values observed in most protein-protein interactions. Ross and Subramanian (1981) contend that protein-protein interactions are two-step processes in which electrostatic and van der Waals interactions follow the initial hydrophobic association. The net $\Delta G°$ for the reaction is then determined by the positive entropy of the first step and the negative enthalpy of the second. One might thus envision a situation in which the hydrophobic

residues on the surface of insulin are involved in an initial binding step which is followed by specific interactions of other residues on the insulin monomer with complementary residues on the receptor surface.

In general, the studies of the interaction of insulin or of insulin analogues with the plasma membrane receptor provide results which are consistent with the putative binding region proposed by Pullen et al. (1976). The role of any specific residue in this first step of insulin action, the receptor binding process, remains unclear.

Because concentration of insulin in the blood stream is small, the free insulin monomer is predicted to be the insulin species involved in receptor recognition and binding. Various cross-linked insulin dimers have been tested for receptor binding ability (Saunders and Freude, 1982; Tatnell and Jones 1981; Brandenburg and Wollmer, 1973; Brandenburg et al., 1973). In all cases, the dimer binds to plasma membrane receptors less efficiently than the monomer. Nevertheless, because of the high dilutions required to obtain solutions of monomeric insulin, most studies of insulin structure-function relationships are performed at concentrations where the dimer and higher oligomers predominate. At concentrations where associated forms of insulin predominate, the amino groups have unusual chemical properties (Chan et al., 1981; Sheffer and Kaplan, 1979). Preliminary experiments using acetic acid as a chemical probe (Table 3, Kaplan et al., 1984) suggest that these chemical properties may be substantially different in the free monomer. Under appropriate conditions, interconversion of 2Zn- and 4Zn-insulin crystals can be easily affected (Bentley et al., 1976). This conformational plasticity within the constraints of the crystal lends credence to the view that the monomeric unit of insulin is inherently flexible and assumes a different conformation in the free and associated forms of insulin. Two attempts to study the dependence of insulin conformation on its concentration using CD have been reported in the literature. Blundell and Wood (1975) claim that all differences in the CD spectra resulting from the dilution of insulin can be accounted for by changes in the environment of the chromophores as the result of dissociation of insulin oligomers and removal of the zinc ions. On this basis, the structure of the free monomer in solution is postulated

TABLE 3: Competitive Labelling of Porcine Zinc Insulin with Acetic Anhydride at 37°C in Pyrex Glass *

Amino Group	Conc. (μM)	pH						
		6.55	6.75	7.00	7.25	7.50	7.75	8.00
Gly A1	200	2.96	11.5	6.79	215	293	307	315
($\alpha_x r \times 10^3$)	10	0.397	2.97	6.72	12.0	21.0	36.7	58.3
	5	0.512	2.44	5.54	7.03	18.9	31.1	43.6
Phe B1	200	9.31	23.3	155	335	311	175	162
($\alpha_x r \times 10^3$)	10	1.61	4.40	6.93	8.98	10.7	13.5	14.7
	5	0.493	3.29	4.55	5.32	9.76	9.65	13.6
Lys B29	200	0.422	0.926	7.00	21.6	25.4	22.4	31.6
($\alpha_x r \times 10^3$)	10	1.13	1.83	2.11	3.64	6.24	10.7	15.7
	5	0.366	0.957	1.19	2.52	6.20	6.76	16.1

*Kaplan et al. (1984).

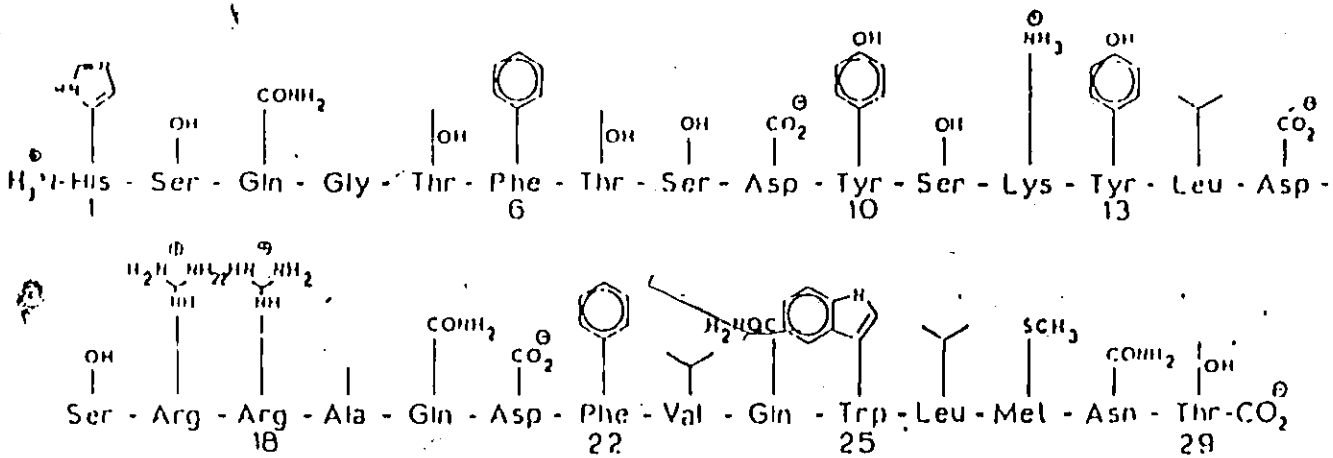
to be the same as that seen in the monomeric unit in the insulin crystal. More recent evidence, again using circular dichroism, is interpreted as showing substantial differences in α-helical and β-sheet content in the monomeric unit of insulin in its free and associated forms (Pocker and Biswas, 1980, 1981). More research designed to yield novel information as to the structure of the free insulin monomer is clearly required.

Glucagon

The maintenance of plasma glucose levels in normal, healthy individuals depends on the interplay of the two pancreatic polypeptides, insulin and glucagon. Since the discovery of glucagon in 1923 (Clough et al., 1923) and its subsequent sequencing (Figure 4, Bromer et al., 1957, 1971), parts of the biochemical chain of events linking glucagon secretion to its metabolic repercussions have been elucidated. Secretion of glucagon by pancreatic α-cells in response to the hypoglycemic state results in the stimulation of glycogenolysis and gluconeogenesis in the liver and of lipolysis in white adipose tissue and in the modulation of insulin and somatostatin secretion by the pancreas. It is well known that some of these metabolic changes within target cells are achieved by the phosphorylation of a variety of regulatory proteins as a result of increased levels of intracellular cAMP following activation of adenylate cyclase within the plasma membrane. Long term effects of glucagon secretion such as increased production of enzymes like phenylalanine pyruvate transaminase (Shih and Chan, 1979) and PEP carboxykinase (Oliver et al., 1978), increased respiration rate (Siess et al., 1979) and increased calcium retention (Prpic et al., 1978) are less understood. The primary event in glucagon action is the binding of the hormone to a specific plasma membrane receptor (Johnson et al., 1981; Rodbell et al., 1971; Birnbaumer et al., 1969). The details of this event and the consequent stimulation of adenylate cyclase remain unclear.

Under certain conditions, glucagon forms crystals of rhombidodecahedral symmetry (Sasaki et al., 1975). The unit cell of these crystals is a trimer of glucagon momomers. The monomer in each trimer is

Figure 4: The primary structure of porcine glucagon.


H3N-His - Ser - Gln - Gly - Thr - Phe - Thr - Ser - Asp - Tyr - Ser - Lys - Tyr - Leu - Asp -
1
6
10
13
Ser - Arg - Arg - Ala - Gln - Asp - Phe - Val - Gln - Trp - Leu - Met - Asn - Thr - CO2
18
22
25
29

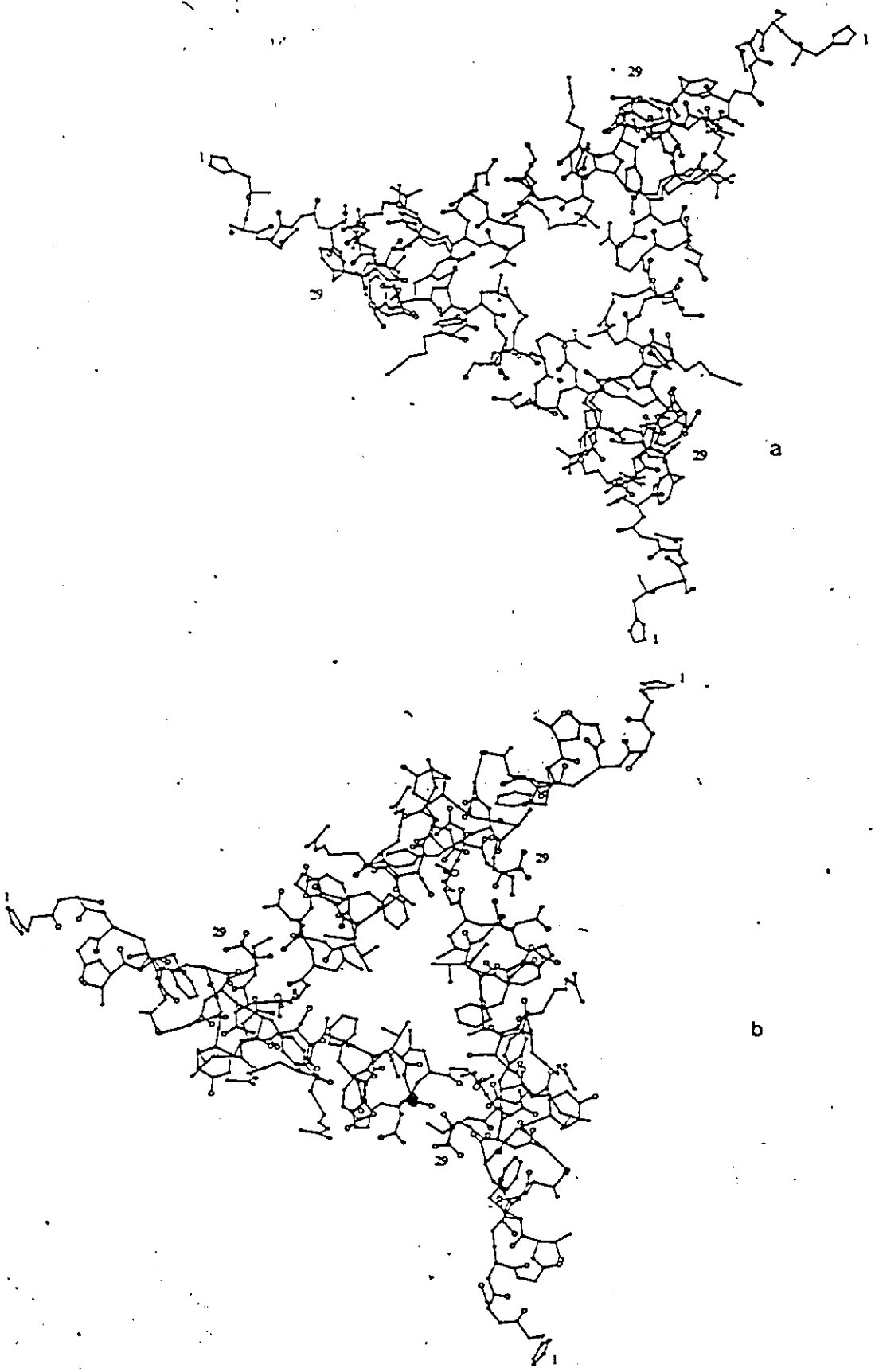
cylindrical in shape and comprised of two α- helical segments interrupted by the arginine-arginine sequence at residues 17 and 18. Two types of trimers are possible. The first, a triangular structure, results from the packing of hydrophobic clusters (residues 6-14 and residues 22-27) against equivalent residues in other monomers. Arginine and aspartic acid residues are exposed to an interior triangle of solvent. In the second type of trimer, monomers are closely joined through the contact of residues 22-27. The N-terminal region of each momomer is free (Figure 5).

While the storage granules of certain teleosts contain the rhombi-dodecahedral crystals visualized by x-ray crystallography (Lange and Klein, 1974), those in most mammals contain amorphorous glucagon (Blundell, 1979). Solutions of glucagon will form gels or fibrils with β-sheet structure on standing (Beaven et al., 1969; Gratzer et al., 1968). Theoretical considerations indicate that either a β-sheet conformation or an α-helical conformation of glucagon will be stabilzed in concentrated solution (Chou and Fasman, 1975). While it is generally agreed that glucagon is monomeric in dilute solution (less than 10^{-5}M) and oligomeric in concentrated solution, sedimentation velocity data have been interpreted as consistent with a monomer-dimer-hexamer equilibrium (Swann and Hammes, 1969) or with a monomer-trimer equilibrium (Formasano et al., 1977; Gratzer et al., 1972; Blanchard and King, 1966).

The secondary structure observed in the monomeric unit of glucagon within the crystal trimer is stabliized by quaternary rather than tertiary structure. It is therefore unlikely that the α-helical conformation of the crystalline glucagon monomeric unit will persist on dissociation of oligomers in dilute solution (Blundell and Wood, 1982; Blundell, 1979). The results from solution studies designed to determine the conformation of the free monomer appear contradictory. The decreased Cotton effect observed on dilution of glucagon solutions is interpreted as showing the monomer as having less than 15% helical content (Eddelhoch and Lippoldt, 1969; Gratzer et al., 1968; Blanchard and King, 1966). Proton NMR (Boesch et al., 1978) detects only one structured region in the momomer. The lack of tryptophan fluorescence quenching is taken to preclude a globular structure for the free monomer

Figure 5: The x-ray crystallographic structure of glucagon trimers:
(a) trimer formed by heterologous contacts between hydrophobic clusters of residues 6-14 and residues 22-27;
(b) trimer formed by homologous contacts of residues 22-27 in each monomeric unit.

(After Sasaki et al., 1975)


a
b

(Eddelhoch and Lippoldt, 1969). On the basis of these results, the glucagon free monomer has been described as an "equilibrium mixture of flexible conformers" (Blundell, 1979). Several conformations of glucagon are possible in dilute solution; some of these will become relatively more stable on interaction of the glucagon monomer with other molecules. The fact that association with lipids (Robinson et al., 1982), detergents (Brandenburg, 1979), or organic solvents (Gratzer et al., 1972) increases the α-helical content of glucagon monomers (as measured by CD) lends some credence to this viewpoint.

There is, on the other hand, some data based on x-ray diffraction (Korn and Ottensmeyer, 1983) and viscosity studies (Epand, 1971) which indicate that the free glucagon momomer has a compact structure. Tritium exchange studies show slowly exchanging hydrogen ions even in dilute glucagon solutions (McBride and Epand, 1972). These results imply that regions of the free glucagon monomer have local structure. Glucagon has limited solubility in aqueous solutions at physiological temperature and pH. To overcome this problem, most studies of glucagon in solution were performed at either pH 2.0 or pH 10.0. Interpretation of results of solution studies of glucagon is, therefore, complicated.

The primary structure of glucagon, like that of β-endorphin and some lipoproteins, lends itself to the formation of an amphipathic α-helix as every third or fourth residue is hydrophobic. This helix would be stabilized by the appropriate environment. Thus, while the x-ray crystal structure of glucagon appears to bear little resemblance to the structure of glucagon in storage or that in dilute solution, several investigators postulate that this primarily α-helical structure is again stabilzed on receptor binding (Kaiser and Kézdey, 1983; Epand, 1983; Blundell and Wood, 1982; Blundell, 1979; Sasaki et al., 1975). The two α-helical segments of each monomeric unit in crystalline glucagon trimers are separated by arginine residues at positions 17 and 18. Replacement of these two residues with amino acids which are more conducive to helical formation results in an analogue with increased affinity for the receptor (Epand and Liepnieks, 1983). The ability to form an amphipathic α-helix would thus seem important to receptor binding.

The C-terminal region of glucagon contains several hydrophobic

residues which are involved in the intermolecular contacts within the crystal trimer. If the α-helical structure of glucagon seen in the crystal is stabilized on receptor binding, these residues may be essential to the binding event. Glucagon-receptor binding appears to be a primarily hydrophobic process (Birnbaumer et al., 1968). Removal of residues from the C-terminal of glucagon to give glucagon$_{1-21}$ or glucagon$_{1-26}$ (the cyanogen bromide fragment) produces analogues which have CD spectra similar to that of glucagon at high dilution but do not self-associate or form helical structures at increased concentrations (Wright et al., 1980; Epand, 1972). Coupling of amides to the cyanogen bromide fragment of glucagon results in analogues which, despite their decreased affinity for the receptor, are full agonists at high concentration (Wright et al., 1980). The methionine side chain (residue 27) appears important to receptor binding but not to transduction. Methylation at this position results in a 100- to 200-fold decrease in biological potency (England et al., 1982; Rothgeb et al., 1977). S-methyl-glucagon does not aggregate in concentrated solution but apparently has a backbone conformation similar to that of native glucagon at high dilutions (Rothgeb et al., 1978). These data and the fact that the introduction of taurine at the C-terminal decreases the potency 10 fold (Wheeler et al., 1974) indicate that this region is of particular importance to the binding process but not to the subsequent activation of adenylate cyclase. Since several of these analogues do not aggregate at higher glucagon concentrations, it would appear that the ability to form an α-helical structure in this region is important but not essential to receptor binding. Some modifications to the C-terminal portion of the glucagon molecule, however, do not result in a decrease in biological potency. Glucagon analogues selectively oxidized at either tryptophan-25 or methionine-27 (Wright and Rodbell, 1980) have the same ability as native glucagon to bind the receptor and to activate adenylate cyclase. In the absence of GTP, glucagon-NAPS, a photoaffinity label for glucagon receptors, has a higher affinity for the receptor than does the native hormone (Desmoliou-Mason and Epand, 1983).

Antibodies raised against the C-terminal fragment of glucagon

have been used to probe the structural integrity of this region in several glucagon analogues. As expected, any modification or deletion to the C-terminal resulted in a decreased immune response relative to that of native glucagon. Synthetic glucagon analogues, Des(10-15)- and Des(16-21)-glucagon, show no reduction in reaction with this antibody despite their reduced potency in adenylate cyclase bioassays (Frandsen et al., 1984). These results indicate that portions of the glucagon molecule other than the C-terminal portion are important to glucagon action. The deleted sequence in Des(10-15)-glucagon contains two tyrosine residues at positions 10 and 13 and the solitary lysine of glucagon, residue 12. Iodination of either of the tyrosine residues results in an increase in binding affinity indicating that these residues are responsible for important hydrophobic interactions with the receptor (Lin et al., 1976). The tyrosines are also involved in concentration-dependent aggregation of glucagon (Sasaki et al., 1975); spectrophotometric titration of glucagon shows the pK_a of one of the two tyrosine residues is dependent on the state of association (Gratzer and Beaven, 1969). Because introduction of a nitro-group at tyrosine-13 does not remove this concentration-dependence, it would appear that the tyrosine-10 residue is directly involved in the aggregation process (Frank and Pekar, 1974). The biological potency of this derivative was not tested.

The ϵ-amino group of lysine-12 can be selectively modified under appropriate conditions. Several glucagon analogues with modifications to this residue have been prepared and tested for biological activity and α-helical content. N-ϵ-BOC-glucagon (Lande et al., 1972), N-ϵ-pyridoxyl-glucagon (Faloona, 1970) and N- -guanidyl-glucagon (Bregman et al., 1980; Epand et al., 1973) all have approximately 10% potency in adenylate cyclase activation. When CD was used as a probe of secondary structure (Bregman et al., 1980; Faloona, 1970), no change in α-helical content was apparent. N-ϵ-4-hydroxyphenylamido-glucagon displays approximately 50% potency in both binding and adenylate cyclase assays and is a full agonist at increased concentrations despite the fact that it shows a decreased helical content in solution (Wright and Rodbell, 1980).

In acylated N-ϵ-derivatives, the α-helical content as measured by CD in helix-inducing solvents decreases as chain length increases. N-ϵ-

hexanoyl- and N-ε-decanoyl-glucagon have slightly less ability to bind glucagon receptors than does the native hormone while N-ε-acetyl-glucagon has substantially reduced binding affinity. These results are taken to suggest that hydrophobic interactions at the lysine-12 position are important to receptor binding (Carrey and Epand, 1982). In the adenylate cyclase assay, N-ε-acyl-glucagons show potencies which reflect their binding capabilities. The maximal level of stimulation by native hormone cannot be achieved even at increased concentration of N-ε-acyl-analogues. In contrast, the introduction of aromatic groups such as 4-azido-2-nitrophenyl- (Bregman et al., 1972) and phenylthiocarbamoyl- (Wright and Rodbell, 1980) at the N-ε-position results in increased helical content in the monomer and in more ability to bind the receptor than to activate adenylate cyclase. N-ε-acetoamidine-glucagon has the same potency as native glucagon in both receptor binding and adenylate cyclase assays (Wright and Rodbell, 1980). Homoarginine-12-glucagon gives 100% activation of adenylate cyclase but has only 20% of the potency of native glucagon. Since the CD spectrum is essentially the same as that of the native hormone, this loss in potency does not appear to be the result of a conformational change (Bregman et al., 1980). In general, analogues where the positive charge has been retained at the position-12 give the same maximal stimulation of adenylate cyclase as does native glucagon (albeit at higher concentrations) whereas those in which this charge has been lost give some fraction of the maximum at any concentration despite full occupancy of the receptor. A positive charge at position-12 in glucagon thus appears necessary to transduction (Wright and Rodbell, 1980). In the x-ray crystal structure, the α-carbon of lysine-12 is located on the polar side of the N-terminal α-helix while the side chain is either extended into the solvent or in contact with residues 22-26 of an adjacent glucagon molecule in the trimer.

Most of the glucagon molecule is part of a fairly rigid α-helical cylinder in the crystalline state. The N-terminal extends out from this cylinder as a small flexible arm which terminates with the amino terminal histidine residue. The functional role of this region of the molecule has been greatly debated. Early studies in which the N-terminal histidine was removed by a single cycle of Edman degradation

produced a derivative that is a glucagon antagonist, the analogue binds the receptor but does not produce a biological response (Rodbell et al., 1971). In order to explore the possibilty that the antagonistic effect was due to the reaction of the Edman reagent with the ϵ-amino group of lysine-12, the Edman product of N-ϵ-BOC-glucagon (Lande et al., 1972) and a Des-His-glucagon analogue produced using an insoluble Edman reagent (Lin et al., 1975) were assayed. While neither of these derivatives inhibit the activation of adenylate cyclase by native hormone, both can elicit less than 70% of the maximal stimulation achieved by native glucagon. The N-terminal histidine residue appears important to receptor binding and important but not essential to transduction.

This conclusion has been supported by several studies in which the α-amino function of the N-terminal histidine has been modified. All glucagon analogues modified solely at the N-α-position show a decreased ability to activate adenylate cyclase in rat liver membranes. N-α-TFA-glucagon retains 30 to 40% of the potency of the native hormone (Lande et al., 1972). N-α-acetyl-glucagon (Desbuquois, 1975) and N-α-carbanoyl-glucagon (Bregman et al., 1980; Epand et al., 1973) exhibit approximately 10% potency in adenylate cyclase assays. Although the latter derivative was originally thought to be a full agonist (Epand et al., 1973) a more recent study (Bregman et al., 1980) indicates that it is capable of only 27% of maximal stimulation of adenylate cyclase. N-α-TNP-glucagon retains only 0.07% potency in adenylate cyclase assays and competes with glucagon in a dose dependent manner (Wheeler et al., 1974; Epand et al., 1973). Each of these N-terminal derivatives demonstrates a greater ability to bind the receptor than to activate adenylate cyclase.

The N-ϵ-acetoamido-derivative is as potent as native glucagon in both receptor binding and in adenylate cyclase activation. When a second acetoamido-group is incorporated at the N-terminal, the ensuing analogue loses 90 to 95% potency and gives only 80% maximal adenylate cyclase activation even at higher concentrations. Homoarginine-12-glucagon is capable of 100% activation of adenylate cyclase but has 20% of the potnecy of the native hormone. Removal of the N-terminal residue from this derivative (to give Des-His-homoarginine-12-glucagon) results

in 22% of maximal activation. As the CD spectrum is virtually the same as that of the native hormone, the loss of activity does not appear to result from a major conformational change (Bregman et al., 1980).

While the N-terminal region appears to take part in the generation of a biological signal by receptor-bound glucagon, there is some debate as to the specific group involved in this process. Most researchers suggest a functional role for the α-amino group. Since N-α-TNP-glucagon shows much lower potency than analogues with smaller modifying groups at the α-amino position, Epand et al.(1981) propose that it is the imidazole function rather than the α-amino group which is required for biological activity. They propose that modification at the α-amino sterically hinders the interaction of the imidazole moiety with the receptor. This idea is supported by the fact that, while N-α-acyl-glucagons have some ability to activate adenylate cyclase, reaction of N-α-acetyl-glucagon with ethoxy formic acid results in modifications of the imdazole function and loss of all adenylate cyclase activation. Recent studies using synthetic glucagon analogues indicate that other residues in the N-terminal region are required for transduction. Des-(1-4)-glucagon has 5.7% potency in receptor binding assays but does not activate adenylate cyclase. Des(5-9)-glucagon also shows less ability to activate adenylate cyclase than to bind the receptor. It is not apparent whether residues 5 to 9 are directly involved in transduction or merely necessary for the proper orientation at the N-terminal (Frandsen et al., 1981).

On the basis of chemical modification studies, it would appear that the entire glucagon molecule is required for full receptor binding. The N-terminal region, particularily the N-terminal histidine residue, and the lysine residue at position-12 have been implicated in the transduction process. It is not readily apparent what conformation of glucagon is required for binding. The ability of glucagon analogues to form α-helical structures appears to have some relationship to their affinity for the glucagon receptor.

Methods for the Study of Proteins in Dilute Solution.

There are a variety of tools at the disposal of the protein chemist for the study of structure-function relationships in a given protein. These include physical methods (such as x-ray crystallography, ORD, CD, ESR, and NMR) as well as chemical methods (proton titration, chemical modification and kinetic studies, for example). Despite the limitations inherent in each technique, application of any of these can yield some information about the protein in question. It is only through the consolidation of data obtained by various approaches that a comprehensive picture of a protein and its mechanism of action can be obtained.

In 1965, the crystal structure of lysozyme was determined (Blake et al., 1965). X-ray crystal data for a variety of proteins is now available. This single technique has completely altered the traditional view of protein structure-function relationships; much of our current understanding of the mechanism of action of hemoglobin and of the serine proteases, for example, is based on their x-ray crystal structure. Dynamic properties such as the effects of temperature, pH or environment are not immediately apparent from the x-ray structure of a molecule and must be determined by solution studies. In addition, some proteins, including insulin and glucagon, self-associate in concentrated solution. Thus, while the structure of the associated form of the protein can be determined through the x-ray crystallography, there is no a priori reason to assume that this structure provides an accurate picture of the free monomer in solution. In such cases, structural features of the protein and their relationship to function must be examined by solution studies.

The application of physical methods to the study of protein structure in dilute solution has, in general, met with limited sucess. CD and ORD have been extensively used to give estimations of the amount of secondary structure, viz. α-helix or β-sheet, in proteins in concentrated solution. New instrumentation may make these techniques applicable to dilute solution studies. Recently, Pocker and Biswas (1980) have obtained CD spectra for insulin at concentrations as low as 60nM. The validity of the method used, however, has been questioned (Blundell and Wood, 1982).

Some proteins contain natural fluorescent chromophores (the

pyridoxal phosphate in tryptophan synthetase, for example) which can be used as a sensitive probe of structure-function relationships in dilute solution. In cases where natural chromophores are absent, the effect of dilution on structure can be followed by the introduction of a fluorescent probe. Similarly, information regarding conformation can be obtained from ESR using a suitable probe. While these methods are sensitive, they involve the introduction of a bulky reporter group into the molecule which may be accompanied by unwanted steric effects. NMR, on the other hand, can be used to give specific information concerning the structure and the dynamic properties of proteins without the use of an extrinsic reporter group. Despite refined instrumentation, this technique lacks the sensitivity required for dilute solution studies.

Techniques which measure the extent of some protein function, ie., receptor binding, enzyme activity, immune response, are generally more applicable to dilute solution studies than those which attempt to define structural features. Sensitivity is achieved by the use of a radioactive or fluorescent label in the substance to be measured. These methods are commonly used for the detection rather than for characterization of the protein in dilute solution. The functional role of specific residues in polypeptide hormones is, however, often probed by testing the ability of hormone analogues to displace radioactive tracers from membrane receptors. While the extent of reaction can be measured for nanomolar concentrations of analogue, the interpretation of results obtained is complicated by the fact that modification of protein molecules at any one site may induce conformational, and therefore functional, changes in a portion of the molecule which is quite remote from the site of modification.

The relationship between quaternary structure and enzyme activity in fructose-1,6-diphosphate aldolase (Chan et al., 1973; Tiepel and Koshland, 1971) and in aspartate transcarbamylase (Chan and Mort, 1973) has been investigated by the renaturation of these oligomeric enzymes in dilute solution. These studies and that of Pocker and Biswas (1980) mentioned above represent the few attempts reported in the literature to determine structure-function relationships in proteins in dilute solutions. Because the pancreatic hormones insulin and glucagon self-associate, the elucidation of structural properties related to the

binding of the free monomer to its receptor necessitates study of these proteins at low concentrations. Despite extensive study of these polypeptides, their mechanisms of action remain unclear. New methodology designed to yield novel information as to structure-function relationships in proteins at high dilution is needed.

RATIONALE:

It is generally accepted that the structure and function of a protein molecule are intimately connected; the function of a molecule necessitates certain structural properties while the presence of a defined structure dictates the mode and the extent of the interactions of the molecule. This tenet is the basis of a plethora of research into the structure of proteins to shed light on their function and into the function of proteins in order to define their structure. The use of physical-chemical properties viz. pK_a and reactivity, of the functional groups in a protein as probes of structure-function relationships is an approach which is less often taken. The interdependence of the three i.e., structure, function and physical-chemical properties, is, however, readily apparent. The pK_a and reactivity of a particular functional group within a protein are dependent not only on the type of group but also on the microenvironment of that group and, hence, on protein structure. The degree of ionization and/or reactivity of any given group will prescribe its involvement in various types of interactions with other groups and, thus, the mode in which that particular part of the protein can function.

The observation, then, of "unusual" chemical properties of a given functional group indicates the presence of a structured region of the protein in the vicinity of the group in question and provides a basis for the elucidation of the utility of these unusual features in terms of protein function. Postulated structure-function relationships consistent with the observed physical-chemical properties must then be subjected to experimental verification.

Competitive labelling approaches afford a means of determining the physical-chemical properties of distinguishable functional groups in protein molecules by quantifiying the ability of each group to compete for reaction with a limited amount of reagent. The approach has three essential features which render it an effective tool for the study of proteins. Firstly, the reactive groups of the protein themselves are used as probes of structure and only trace amounts of labelling reagent are employed. As a result, the properties determined by this method are for the native protein. Secondly, the protein can be studied in a variety of environments. Thirdly, parameters can be unambiguously

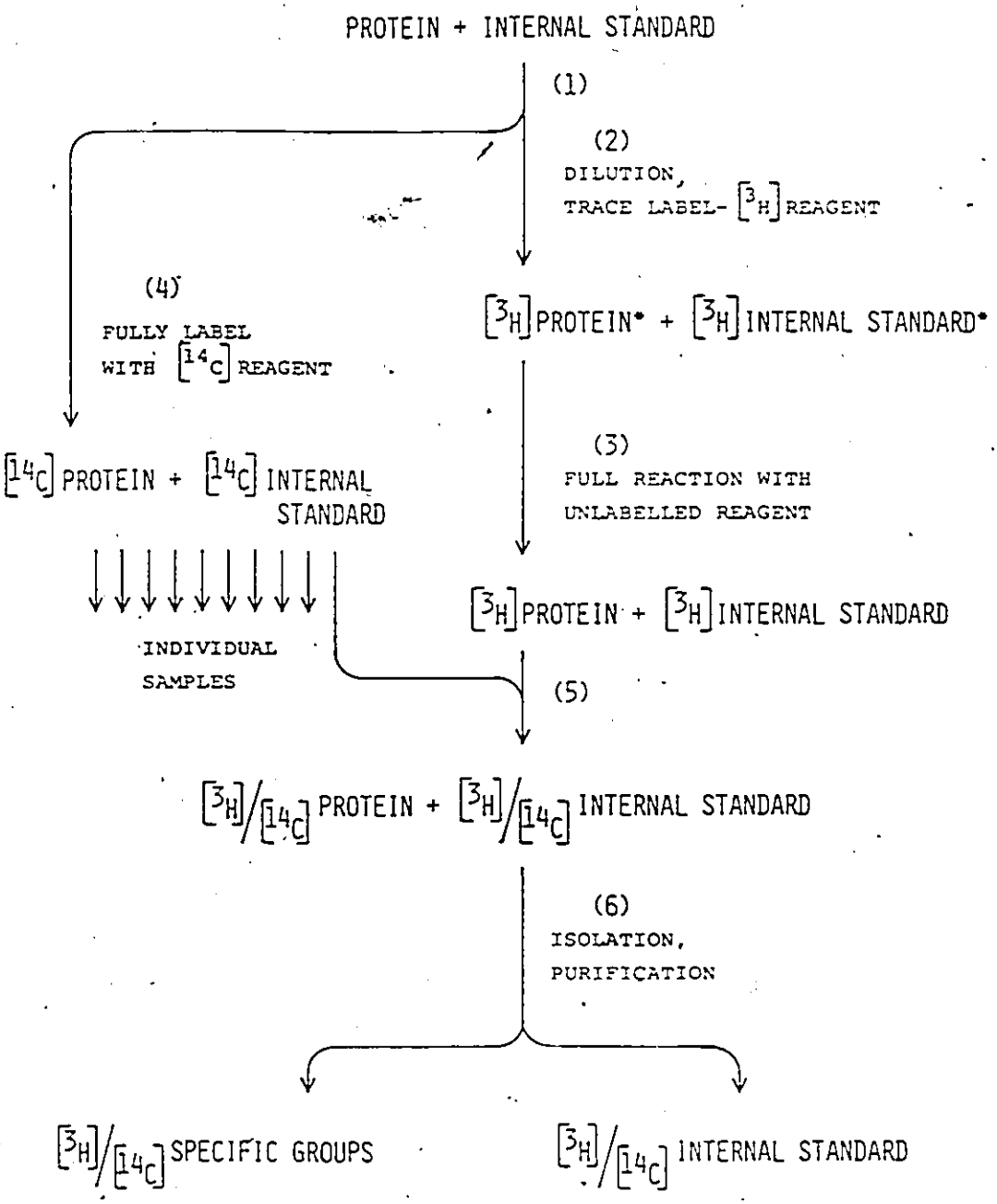
assigned to specific amino acid residues within the protein.

The competitive labelling method (Kaplan et al., 1971) as modified by Duggleby and Kaplan (1975) consists of four main steps: (i) the treatment of a mixture of the protein under study and a suitable standard nucleophile with a trace amount of [^{3}H]labelled electrophilic reagent; (ii) treatment of the mixture with [^{14}C]labelled reagent under denaturing conditions to render the protein and internal standard mixture chemically homogeneous; (iii) separation and purification of the internal standard followed by quantification of its [^{3}H]/[^{14}C]-ratio; and (iv) partial or total hydrolysis of the protein, followed by purification of a peptide or amino acid containing the functional group under study and quantification of its [^{3}H]/[^{14}C] ratio.

Dilute Solution Studies: Insulin Application of the competitive-labelling approach to the study of structure-function relationships in proteins has been limited to studies of proteins at concentrations greater than approximately 5×10^{-5}M. As the amount of protein is decreased, the limiting factor in obtaining accurate quantification of the [^{3}H]/[^{14}C]ratio is the amount of [^{14}C]labelled reagent incorporated in step (ii). To overcome this difficulty, a stock solution of protein and internal standard is prepared. This aliquot is diluted to the appropriate concentration and step (i) carried out as before (Figure 6, step 3). The other aliquot is fully reacted with [^{14}C]labelled reagent (Figure 6, step 4) and equal aliquots of this are added to each [^{3}H]trace-labelled sample (Figure 6, step 5). Steps (iii) and (iv) remain unchanged.

In this modified procedure, sufficient [^{14}C]radioactivity is assured by labelling an appropriate amount of the protein and internal standard separately from the [^{3}H]trace-labelled sample. Because the [^{14}C]labelled sample and experimental sample (which has been diluted to the appropriate concentration before trace-labelling) are derived from the same stock solution, they must have the same protein-to-internal-standard ratio. It is important to note that the rates being measured for the reaction of groups in the protein with the [^{3}H]electrophile are relative rates, i.e., relative to that for the internal standard. Thus, combining the two samples achieves the same result as direct [^{14}C]-

Figure 6: Competitive labelling procedure for proteins in dilute solution.


PROTEIN + INTERNAL STANDARD
(1)
(2)
DILUTION,
TRACE LABEL- [3H] REAGENT
[3H] PROTEIN* + [3H] INTERNAL STANDARD*
(4)
FULLY LABEL
WITH [14C] REAGENT
(3)
FULL REACTION WITH
UNLABELLED REAGENT
[14C] PROTEIN + [14C] INTERNAL STANDARD
[3H] PROTEIN + [3H] INTERNAL STANDARD
INDIVIDUAL
SAMPLES
(5)
[3H]/[14C] PROTEIN + [3H]/[14C] INTERNAL STANDARD
(6)
ISOLATION,
PURIFICATION
[3H]/[14C] SPECIFIC GROUPS
[3H]/[14C] INTERNAL STANDARD

labelling of the [^{3}H]trace-labelled samples.

Polypeptides with an N-Terminal Histidine Residue Most proteins and polypeptides have only one functional group at the amino terminal residue. In these cases, the chemical reactivity of this group can be unequivocally determined by isolation of the α-amino derivative after competitive labelling. A polypeptide with an amino terminal histidine residue, however, presents an unusual problem in that it has two functional groups at the amino terminus, an α-amino and an imidazole function, the reactivities of which must be determined separately. If the procedure of Duggleby and Kaplan (1975) outlined above were followed, the trace-labelling of a protein or polypeptide with an amino-terminal histidine using FDNB as a chemical probe would give the di-DNP-histidine derivative at the N-terminal. The reactivity obtained would therefore be an average of the reactivities of the α-amino and the imidazole group. The unequivocal assignment of reactivities to the α-amino and imidazole functions of an amino terminal histidine residue requires that the rate of reaction of each functional group be independently quantified. Three methods by which this can be accomplished are described.

(a) Histidylglycine. As noted above, the competitive labelling procedure using FDNB, when applied to a protein or polypeptide with an N-terminal histidine residue, yields the di-DNP-derivative. Determination of the reactivity of the α-amino group of the histidine residue is possible, however, since the DNP-group can be selectively removed from the imidazole function by thiolysis under appropriate conditions (Shaltiel, 1967). Quantification of the [^{3}H]/[^{14}C]ratio of the ensuing N-α-mono-DNP-histidine derivative affords a means of determining the chemical reactivity of the α-amino function towards FDNB. Alternatively, the chemical properties of this group could be determined by the use of a reagent like acetic anhydride, which forms stable derivatives of primary amines but not imidazole nitrogens. As the purpose of this study is to determine the chemical properties of both functions on an amino terminal histidine and to determine how the properties of one group influence the properties of the other, the use of such a selective reagent is, therefore, not well suited to this purpose. Similarly, the chemical properties of the imidazole function could be unequivocally

determined from the mono-imidazolyl-DNP-histidine derivative. This derivative was isolated by exploiting one of the essential features of the competitive labelling approach, namely that the number of functional groups competing for reaction greatly exceeds the number of reagent molecules available for reaction. It is therefore statistically very unlikely that any one polypeptide molecule will react with more than one reagent molecule. Thus, the trace-labelling of a polypeptide with an amino terminal histidine residue yields a heterogeneous mixture containing polypeptide molecules in which only the α-amino function or only the imidazole function has reacted with the [^{3}H]reagent as well as unmodified polypeptide molecules. If, after the [^{3}H]trace-labelling step, the α-amino function is selectively blocked using, for example, acetic anhydride, the mono-imidazolyl-DNP-histidine can be isolated and its reactivity quantified after full reaction of the polypeptide and standard nucleophile with [^{14}C]reagent.

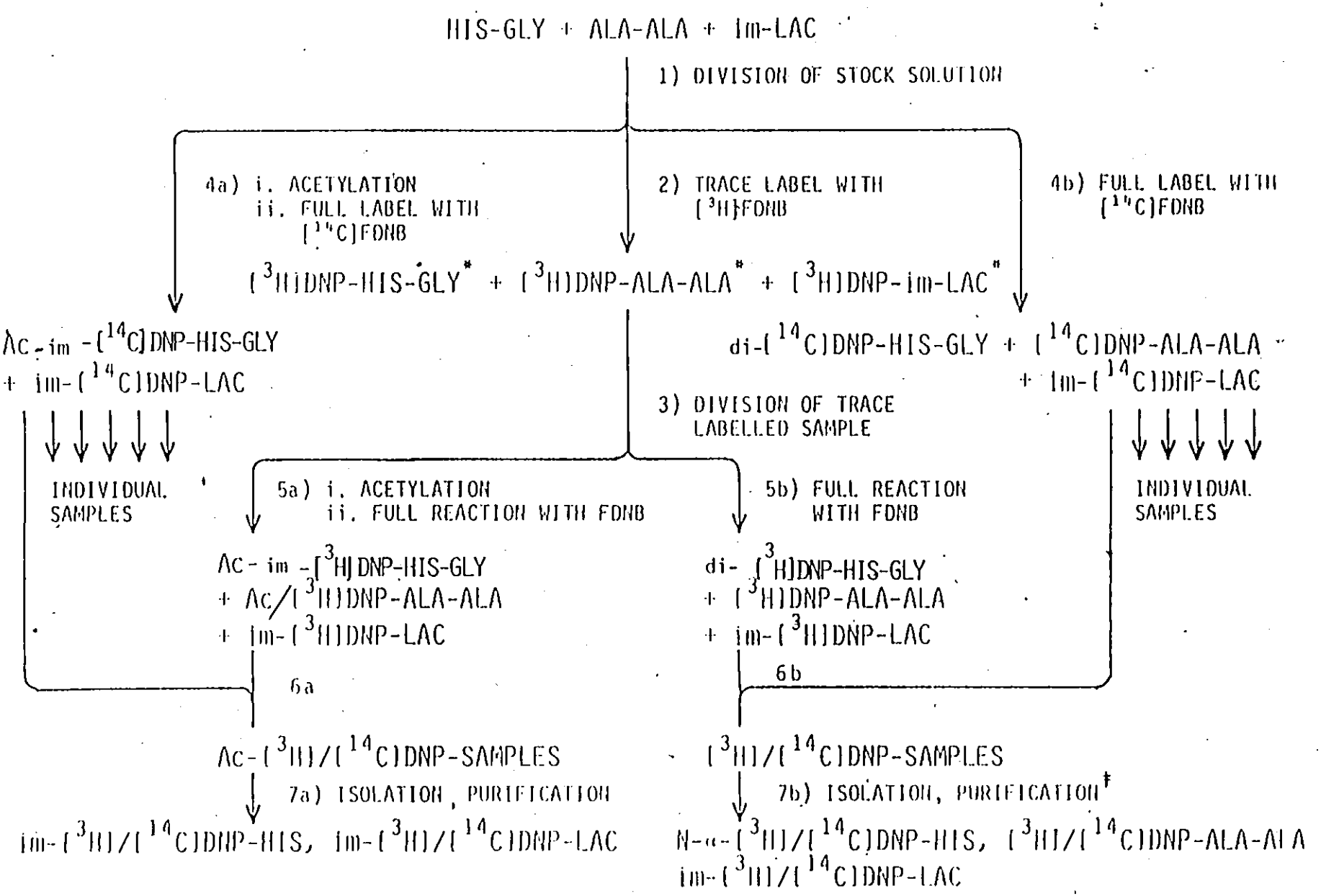
This is essentially the approach used for the determination of the chemical properties of the N-terminal histidine residue of histidylglycine (Figure 7) except that aliquots of the stock solution containing histidylglycine and internal standard were removed for use in the [^{14}C]labelling steps (Figure 7, step 4a and step 4b) before dilution for the [^{3}H]trace-labelling (Figure 7, step 2) as this modification of the procedure leads to more efficient use of the [^{14}C]-reagent. After [^{3}H]trace-labelling, a homogeneous mixture was achieved by reaction with non-radioactive reagents (Figure 7, steps 5a and 5b) prior to the addition of the appropriate [^{14}C]labelled material (Figure 7, steps 6a and 6b).

(b)Glucagon: Preliminary Studies. Studies of the glucagon free monomer are complicated by two factors: firstly, low concentrations (approximately 10^{-6}M) are required to assure that the free monomeric form of glucagon predominates in solution; secondly, glucagon has an N-terminal histidine residue. The first difficulty can be overcome by using the modification of the competitive labelling method described for dilute insulin solution studies in which an aliquot of the stock solution containing protein and internal standard is removed prior to dilution and used for the [^{14}C]labelling. As noted above, trace-labelling of glucagon yields a heterogeneous mixture of unreacted glucagon molecules

Figure 7: Competitive labelling procedure used to determine the chemical properties of the N-terminal histidine residue of histidylglycine.

* Trace-labelled

† Di-[^{3}H]/[^{14}C]DNP-Histidine was isolated, a portion was quantified and the remaining portion thiolyzed to give N-α-[^{3}H]/[^{14}C]DNP-histidine (see Methods).


HIS-GLY + ALA-ALA + im-LAC
1) DIVISION OF STOCK SOLUTION
4a) i. ACETYLATION
ii. FULL LABEL WITH [14C]FDNB
2) TRACE LABEL WITH [3H]FDNB
4b) FULL LABEL WITH [14C]FDNB
[3H]DNP-HIS-GLY* + [3H]DNP-ALA-ALA* + [3H]DNP-im-LAC*
Ac-im-[14C]DNP-HIS-GLY
+ im-[14C]DNP-LAC
di-[14C]DNP-HIS-GLY + [14C]DNP-ALA-ALA
+ im-[14C]DNP-LAC
3) DIVISION OF TRACE LABELLED SAMPLE
INDIVIDUAL SAMPLES
5a) i. ACETYLATION
ii. FULL REACTION WITH FDNB
5b) FULL REACTION WITH FDNB
INDIVIDUAL SAMPLES
Ac-im-[3H]DNP-HIS-GLY
+ Ac/[3H]DNP-ALA-ALA
+ im-[3H]DNP-LAC
di-[3H]DNP-HIS-GLY
+ [3H]DNP-ALA-ALA
+ im-[3H]DNP-LAC
6a
6b
Ac-[3H]/[14C]DNP-SAMPLES
[3H]/[14C]DNP-SAMPLES
7a) ISOLATION, PURIFICATION
7b) ISOLATION, PURIFICATION†
im-[3H]/[14C]DNP-HIS, im-[3H]/[14C]DNP-LAC
N-α-[3H]/[14C]DNP-HIS, [3H]/[14C]DNP-ALA-ALA
im-[3H]/[14C]DNP-LAC

and molecules which have reacted at only the α-amino or the imidazole function. The relative amounts of each derivative depends on the chemical properties of the functional groups. These chemical properties would be determined if these derivatives could be isolated and quantified without further derivation.

In the procedure of Duggleby and Kaplan (1975), quantification of the extent of reaction of each functional group in proteins was facilitated by complete reaction with [^{14}C]reagent under denaturing conditions. In the present study, complete reaction would yield the di-DNP-histidine derivative and was therefore avoided. If the amount of [^{14}C]reagent is limited, however, the amount of di-DNP-histidine-glucagon formed can be minimized. Accordingly, a portion of the stock solution containing glucagon and internal standard is removed, diluted and reacted with trace amounts of [^{3}H]FDNB as per step (i) of the procedure of Duggleby and Kaplan. In place of step (ii), a second portion of the stock solution is trace-labelled with [^{14}C]FDNB and equal aliquots of this reaction mixture are added back to each of the [^{3}H]trace-labelled samples. Separation and purification of the internal standard and functional groups of glucagon (steps (iii) and (iv) of the procedure of Duggleby and Kaplan (1975)) is achieved by hydrolysis of the total protein-internal standard mixture and HPLC separation of the hydrolysate. [^{3}H]/[^{14}C]Ratios are calculated as before except that they must be corrected for the relative amounts of [^{14}C]DNP-derivatives formed in the [^{14}C]trace-labelling step.

(c)Glucagon, Secretin and VIP. In principle, either of the two modifications of the procedure of Duggleby and Kaplan could be used to determine the chemical properties of the N-terminal histidine residue in glucagon, secretin and VIP. The first methodology (that employed in histidylglycine studies) requires the use of relatively large amounts of protein. In cases where the protein under study is costly or available in only minute amounts, this method is not satisfactory. The second method (that employed in the preliminary glucagon studies), while requiring less protein, suffers from the disadvantage that the relative proportions of [^{14}C]DNP-derivatives formed in the [^{14}C]trace-labelling reaction must be determined. A new method (Figure 8) based on the competitive labelling approach was therefore developed. Step (i) (the

- Figure 8: Competitive labelling procedure used to determine the chemical properties of the N-terminal histidine residue in the polypeptide hormones: glucagon, VIP and secretin.

H_2N-HIS-POLYPEPTIDE + STANDARD NUCLEOPHILES

↓ 1) TRACE LABEL WITH [^{3}H] FDNB

N-α-[^{3}H] DNP-HIS-POLYPEPTIDE

[^{3}H] DNP -STANDARD NUCLEOPHILES

im-[^{3}H] DNP-HIS-POLYPEPTIDE +

STANDARD NUCLEOPHILES

H_2N-HIS-POLYPEPTIDE

↓ 2) ADDITION OF
a) N-α-[^{14}C] DNP-HISTIDINE
b) im-[^{14}C] DNP-HISTIDINE
c) [^{14}C]DNP -STANDARD NUCLEOPHILES

↓ 3) HYDROLYSIS

↓ 4) ISOLATION AND PURIFICATION

N-α-[^{3}H]/[^{14}C] DNP-HISTIDINE

im-[^{3}H]/[^{14}C] DNP-HISTIDINE

[^{3}H]/[^{14}C] DNP- STANDARD NUCLEOPHILES

dilution and [^{3}H]trace-labelling of a solution of protein and internal standard) of the procedure of Duggleby and Kaplan (1975) is not changed. In place of full reaction with [^{14}C]reagent in step (ii), however, a mixture containing an equal number of dpm in each of N-α-[^{14}C]DNP-histidine, imidazolyl-[^{14}C]DNP-histidine and [^{14}C]DNP-internal standard is added. The entire mixture is then subjected to acid hydrolysis. Isolation of the [^{3}H]/[^{14}C]DNP-derivatives and quantification of their [^{3}H]/[^{14}C] ratios can then be carried out. Addition of equal [^{14}C]dpm of each mono-DNP-derivative achieves here what full reaction with [^{14}C]FDNB achieved for each solitary functional group in the procedure described by Duggleby and Kaplan (1975).

In all cases, the pH-dependent second order velocity constant ($\alpha_x r$) for the reaction can be calculated from the same expression, viz.

$$\alpha_x r = \alpha_s \frac{(^3H_x/^{14}C_x)}{(^3H_s/^{14}C_s)}$$

where α_s is the degree of ionization of the internal standard, α_x the degree of ionization of the functional group under study, and $(^3H_x/^{14}C_x)$ and $(^3H_s/^{14}C_s)$ the corresponding radioactivity ratios for the groups and internal standard respectively.

EXPERIMENTAL PROCEDURES

Materials

Porcine zinc insulin (0.35% zinc) was obtained as a gift from Connaught Laboratories (Toronto, Ontario). Porcine VIP and glucagon were supplied by Sigma Chemical Company (St. Louis, MO, U.S.A.) as were FDNB, L-β-imidazole lactic acid, histidine, N-α-acetylhistidine, N-α-acetyllysine, N-α-acetyltyrosine, calf thymus histone, alanylalanine, and histidylglycine. Secretin was from Bachem Fine Biochemical Inc. (Torrence, CA, U.S.A.). Amersham Corporation (Oakville, Ontario) supplied the radioactive reagents and NEN Canada (Lachine, P.Q.) the Aquasol-2 for scintillation counting. Porapak Q was supplied by Waters Associates (Toronto, Ontario); the thin layer silica plates for chromatography were from Eastman Kodak (Rochester, NY.). Spectra/Por S dialysis tubing came from Spectrum Medical Industries (Los Angeles, CA, U.S.A.). Pyrex (Corning, 16 x 125mm), polystyrene (Kimble, 16 x 125mm), and Tefzel ETFE (Nalgene, 13 x 100mm) tubes were obtained from Canlab (Ottawa, Ontario). All other reagents were of high purity and supplied by Fisher Scientific Limited (Ottawa, Ontario).

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; 1:4:45 (by volume).

Preparation of Imidazolyl-DNP-Histidine and Imidazolyl-DNP-Lactic Acid

Unlabelled imidazolyl-DNP-histidine used as carrier was routinely prepared from L-histidine dissolved in a minimum volume of water. The α-amino group was blocked by reaction with a fifty-fold molar excess of citraconic anhydride. During the citraconylation, the pH was maintained

at 9.0 by the addition of 5M-KOH using a pH-stat. Sodium bicarbonate was the added to give a saturated solution. After the addition of FDNB (in a twenty fold molar excess over imidazole group), the reaction was allowed to proceed 18h at room temperature in the dark. The pH was then brought to 2.0 with concentrated HCl; unreacted FDNB and DNP-OH, the reaction by-product, were removed immediately by extraction with three volumes of diethyl ether. The acidified aqueous phase was allowed to stand 8h to ensure unblocking of the α-amino group. The sample was diluted approximately six-fold with water and introduced to the top of a Porapak Q column (7mm x 30mm) which had been pre-equilibrated with 95% ethanol then washed with 0.01M-HCl. After washing with 15ml of 0.01M-HCl, the imidazolyl-DNP-histidine was eluted from the column using 80% acetone (v/v in water) and purified by two-dimensional high voltage paper electrophoresis (first dimension: pH 3.5, 3500V, 75min; second dimension: pH 2.1, 3500V, 30min). Unlabelled imidazolyl-DNP-lactic acid was prepared from L-β- imidazole lactic acid by reaction with excess FDNB in a manner similar to the preparation of imidazolyl-DNP-histidine except that the citraconylation reaction was omitted. In some preparations, a Dowex column (Dowex 50WX8, cation exchange resin, 100-200 mesh, 7mm x 30mm) was used in place of a Porapak Q column to effect the removal of salts. In these cases, the sample was introduced to a Dowex column which had been pre-equilibrated with 0.01M-HCl, the column washed with 15 to 30ml of water and the sample eluted with a 2% ammonia solution. The eluted sample was then purified by paper electrophoresis as described above.

Preparation of Modified Histone Proteins

Two 150mg portions of a mixture of histone proteins were dissolved in 5ml of an acidic solution of 8M-urea. The pH of each solution was then adjusted to and maintained at 9.0 by the addition of 5M-KOH using a pH-stat. To each sample, 0.75g of succinic anhydride was added in 0.05g portions. The reaction was allowed to proceed to completion after each addition. In order that reaction volumes be more easily managed, the samples were split in half. Sodium bicarbonate (0.5g) was added to each followed by the addition of 500 l of 50% FDNB (v/v in acetonitrile). The reaction was allowed to proceed in the dark at room

temperature for 18h with shaking on a wrist-action shaker, The pH was then brought to 2.0 by the addition of concentrated HCl and DNP-OH and residual FDNB removed by extraction with three volumes of diethyl ether. This resulted in the precipitation of the protein from the aqueous phase. Upon dialysis against a 4M-urea solution which had been adjusted to approximately pH 9.0 using N-methylmorphorline (5 drops N-methylmorphorline in 4l of 4M-urea) the modified histone protein mixture was again soluble. The proteins remained in solution upon subsequent dialysis against solutions of decreasing urea concentration (2M, 1M, 0.5M, 0.025M) provided that four to five drops of N-methyl-morphorline were added per 4l of dialysate. After dialysis against two 4l portions of distilled water containing N-methylmorphorline, the samples of modified histone proteins were combined and lyophilyzed to dryness. A 1mg sample was removed, redissolved in 6M-HCl and hydrolyzed 24h at 110°C in a sealed, evacuated tube as was a 1mg sample of unmodified histone proteins. One tenth of each hydrolysate was used for amino acid analysis using a Technicon TSM analyzer. In order to confirm that the modified protein was unreactive toward FDNB, another 5mg portion of the modified histone protein mixture was redissolved in 1.5ml of water containing 0.1g of sodium bicarbonate. An aliquot of FDNB (50μl) was added and the reaction allowed to proceed 18h in the dark. After acidification and ether extraction, this sample was dialyzed against 500ml solutions of decreasing urea content as described above. Following hydrolysis, amino acid analysis of a sample containing approximately 0.2mg of protein was carried out.

Preparation of [^{14}C]DNP-Derivatives

N-ϵ- [^{14}C]DNP-lysine, O-[^{14}C]DNP-tyrosine and imidazolyl-[^{14}C]DNP-histidine were prepared from N-α-acetyllysine, N-α-acetyltyrosine and N-α-acetylhistidine, respectively. A sample (2mg) of each N-α-acetylated-amino acid was dissolved in 1ml of distilled waster. After addition of 0.25g of sodium bicarbonate, 125μl of acetonitrile containing [^{14}C]FDNB (2.38 mol, specific radioactivity 21mCi/mmol) was added to each and the reaction allowed to proceed 18h in the dark. Complete dinitrophenylation was assured by reaction with unlabelled FDNB (25μl, 50% v/v in acetonitrile) for a further 18h. The pH was then brought to

2 with concentrated HCl. Following hydrolysis in 6M-HCl (3h, 110°C), each sample was extracted with three 5ml portions of diethyl ether, diluted approximately six-fold with water, and introduced to the top of a Porapak Q column (7mm x 30mm) which had been pre-equilibrated with 95% ethanol then washed with 0.01M-HCl. The [^{14}C]DNP-amino acid was eluted with 80% acetone (v/v in water) and then purified by high voltage electrophoresis on Whatman 3MM paper at pH 2.1 (3500V, 45min).

N-α-[^{14}C]DNP-histidine and L-β-imidazolyl-[^{14}C]DNP-lactic acid were prepared from histidine and L-β- imidazole lactic acid in a manner similar to that described above except that the samples were not hydrolyzed prior to ether extraction. Following elution from the Porapak Q column, L -β-imidazolyl-[^{14}C]DNP-lactic acid was purified by two-dimensional high voltage electrophoresis at pH 3.5 (3500V, 75min) and pH 2.1 (3500V, 30min). The N-α-,N-imidazolyl-di-[^{14}C]DNP-histidine derivative was dissolved in 10ml of 20% acetone, 2% N-methylmorphorline (v/v in water, pH 8.0); 100μl of β-mercaptoethanol was added and thiolysis allowed to proceed for 8h in the dark. (This thiolysis procedure is a modification of that used by Goren and Fidkin (1978).) After evaporation of the acetone, the sample was acidified and extracted with three 10ml portions of diethyl ether. The N-α-[^{14}C]DNP-histidine was isolated from the aqueous phase by high voltage paper electrophoresis at pH 2.1 (3500V, 45min). In all electrophoretic procedures, authentic, unlabelled derivatives (obtained either from Sigma Chemical Company or prepared as described above) were spotted as markers at the side of the sample. All derivatives were detected by their ultraviolet absorption and eluted from the paper with 10% acetic acid.

[^{14}C]DNP-glycine, [^{14}C]DNP-phenylalanine, and [^{14}C]DNP-alanyl-alanine were prepared from L-glycine, L-phenylalanine and L-alanyl-alanine, respectively by the reaction of a 2mg sample with 2.38 mol [^{14}C]FDNB (specific radioactivity 21mCi/mmol) under conditions described for the preparation of other [^{14}C]DNP-derivatives. Again, complete dinitrophenylation was assured by reaction with unlabelled FDNB (25μl, 50% v/v in acetonitrile) for a further 18h. The pH was the brought to 2 with concentrated HCl. The aqueous reaction mixture was extracted 5 times with 5ml portions of diethyl ether. Since DNP-OH extracts into the ether phase along with the desired [^{14}C]DNP-

derivative, the ether was evaporated and the sample redissolved in chloroform saturated with dibasic phosphate solution (1g Na_2HPO_4 in 100ml water). Dinitrophenol was removed by passage of this sample through a silica gel G column (7mm x 30mm) as suggested by Steven (1962). In this method, the sample is applied to the column, dinitrophenol removed by washing with 20 to 30ml of basic choloroform solution and the desired [^{14}C]DNP-derivative eluted with a solution of 80% acetone, 1% acetic acid (v/v in water). [^{14}C]DNP-glycine and [^{14}C]DNP-phenylalanine were purified using two-dimensional TLC on silica plates in Gelman descending chromatography chambers (first dimension: toluene: pyridine: 2-chloroethanaol: 0.8M-ammonia; 10:3:6:6, second dimension: benzene: pyridine: acetic acid; 40:10:1). [^{14}C]DNP-alanylalanine was purified by TLC on silica plates using three different solvent systems: 1) toluene: pyridine: 2-chloroethanol: 0.8M-ammonia; 10:3:6:6, 2) benzene: pyridine: acetic acid; 40:10:1, and 3) chloroform: benzyl alcohol: acetic acid; 70:30:3. In all cases, the overflow technique of Brenner and Niederwiesser (1961), in which the solvent is allowed to run off the bottom of the TLC plate, was used. Authentic derivatives obtained from Sigma Chemical Company were run as markers in each dimension. The yellow samples were scraped from the plates and eluted from the silica with 80% acetone.

Procedures for Insulin Concentration-Dependence Studies

Sample Preparation. Stock solutions containing equimolar (3mM) insulin with L-β-imidazole lactic acid were prepared in 5mM-sodium monohydrogen phosphate with the desired KCl concentration (0.1, 0.5 or 1M).

[^{3}H]Trace-Labelling. Aliquots of the stock solution containing insulin and L-β-imidazole lactic acid were diluted to concentrations of $1x10^{-3}$M, $5x10^{-4}$M, $1x10^{-4}$M, $5x10^{-5}$M, $1x10^{-5}$M, $5x10^{-6}$M, $1x10^{-6}$M, $5x10^{-7}$M and $1x10^{-7}$M in appropriate test-tubes, adjusted to pH 7.5 with 1M-KOH and maintained at 37°C in a water bath. To each 2.5ml aliquot of the above, 25μl of acetonitrile containing [^{3}H]FDNB (2nmol, specific radioactivity 12.5 Ci/mmol) was added and the reaction allowed to proceed 18h in the dark. Concentrated HCl was added to bring the pH to 2 (Figure 6, step 2). After addition of 2.5g of urea and 0.25g of

$NaHCO_3$, each sample was completely dinitrophenylated with unlabelled FDNB (50µl, 50% v/v in acetonitrile) for a further 18h at 22°C in the dark (Figure 6, step 3).

[^{14}C]Labelling. A portion of the stock solution containing 2mol of insulin and L-ρ-imidazole lactic acid was lyophilized and then dissolved in 2 ml of a solution of 8M-urea containing 2.5g of $NaHCO_3$. The sample was fully dinitrophenylated with 31.3µmol of [^{14}C]FDNB (specific radioactivity 200mCi/mol) by reaction for 18h at 22°C. The final volume was adjusted to 25ml and 2.0ml was added to each [^{3}H]-labelled sample (Figure 6, step 5). After addition of 5mg of DNP-insulin and 0.5mg of imidazolyl-DNP-lactic acid to each sample (as carrier), the pH was adjusted to 2.

Isolation and Purification of [^{3}H]/[^{14}C]DNP-Derivatives. The solution from step 5 (Figure 6) was extracted with several volumes of diethyl ether to remove excess DNP-OH and aid in the precipitation of the protein. Centrifugation at 1000g for 10min separated the supernatant, which contained the imidazolyl-[^{3}H]/[^{14}C]DNP-lactic acid, from the [^{3}H]/[^{14}C]DNP-protein. The precipitate from the aqueous phase was washed twice with 0.5ml of water. After adding the washings to the supernatant phase, the internal standard was purified using the Dowex column and electrophoretic methods described above for the preparation of unlabelled imidazolyl-DNP-lactic acid. The protein was dissolved in 1ml of 6M-HCl and hydrolyzed at 110°C in evacuated, sealed hydrolysis tubes. Because of the known lability of the N-α-DNP-glycine derivative (Leggitt Bailey, 1962), the hydrolysis was done in two stages. After 8h of hydrolysis, the tubes were opened, extracted with three 5ml portions of diethyl ether, and resealed after evaporation of residual ether. Following hydrolysis for a further 10h, the tubes were reopened and the acid phase extracted a second time with three 5ml portions of diethyl ether. The ether extracts were combined, the ether evaporated and the N-α-[^{3}H]/[^{14}C]DNP-glycine and N-α-[^{3}H]/[^{14}C]DNP-phenylalanine dissolved in 50µl of 95% ethanol. One half of this sample (25µl) was spotted onto a silica plate in a 3cm band. The [^{3}H]/[^{14}C]DNP-derivatives were separated by two-dimensional TLC using toluene: pyridine: 2-chloroethanol: 0.8 ammonia; 10:3:6:6 (upper phase) in the first dimension and benzene: pyridine: acetic acid; 80:20:2 in the

second dimension. The yellow separated spots were scraped from the plates and eluted with 80% acetone (v/v in water).

The acid phase was evaporated and the sample redissolved in 1.0ml of electrophoresis buffer, pH 2.1. An aliquot (0.5ml) was spotted in a 5cm band on Whatman 3MM paper. Imidazolyl-[^{3}H]/[^{14}C]DNP-histidine was separated from O-[^{3}H]/[^{14}C]DNP-tyrosine and ϵ-[^{3}H]/[^{14}C]DNP-lysine by high voltage electrophoresis at pH 2.1 (3500V, 45min). Bands containing O-[^{3}H]/[^{14}C]DNP-tyrosine and ϵ-[^{3}H]/[^{14}C]DNP-lysine were cut and repurified using a second longer electrophoresis at pH 2.1 (3500V, 75min). Authentic samples of unlabelled DNP-derivatives were spotted as markers at both sides of sample band before each electrophoresic step. Bands containing DNP-derivatives were detected by their ultraviolet absorption.

Procedures for Insulin Concentration-Dependence Studies in the Presence of Modified Histone Proteins

Sample Preparation. Stock solutions containing equimolar (3mM) insulin, L-β-imidazole lactic acid and alanylalanine were prepared in 5mM-sodium monohydrogen phosphate/0.1M-KCl with the appropriate concentration of modified histone protein (10^{-6}M, 10^{-5}M, 5×10^{-5}M, 10^{-4}M, 5×10^{-4}M and 10^{-3}M). Similar solutions without insulin were prepared for the blank reactions.

[^{3}H]Trace-Labelling. Aliquots of the stock solutions were diluted to insulin and internal standard concentrations of 10^{-4}M, 10^{-6}M, 10^{-7}M and 10^{-8}M using buffer containing the appropriate concentration of modified histone protein, temperature equilibrated at 37°C in a thermostated water jacket and adjusted to pH 7.5. To each 3.0ml sample, 50μl of acetonitrile containing [^{3}H]FDNB (11.08nmol, specific radioactivity 16.6Ci/mmol) was added and the reaction allowed to proceed 18h in the dark with shaking.

Addition of the [^{14}C]DNP-Amino Acids. To each of the above [^{3}H]-trace labelled samples was added 0.7ml of a solution containing 12,500 dpm in each of N-α-[^{14}C]DNP-phenylalanine, N-α-[^{14}C]DNP-glycine, N-α-[^{14}C]DNP-alanylalanine, O-[^{14}C]DNP-tyrosine, ϵ-[^{14}C]DNP-lysine, imidazolyl-[^{14}C]DNP-histidine and imidazolyl-[^{14}C]DNP-lactic acid in addition to 0.1mg of each unlabelled DNP-derivatives. An equal volume

(3.7ml) of 12M-HCl was added and each sample hydrolyzed 18h at 110°C in evacuated sealed tubes.

Isolation and Purification of [^{3}H]/[^{14}C]DNP-Derivatives. Each hydrolyzed sample was extracted with three 5ml portions of diethyl ether. The ether layer was evaporated; the sample redissolved in chloroform saturated with dibasic phosphate solution (1g Na_2HPO_4 in 100ml water) and one half of the sample applied to a silica gel G column (7mm x 30mm). The column was washed with 20ml of basic chloroform to remove the dinitrophenol. The N-α-[^{3}H]/[^{14}C]DNP-derivatives of alanine, glycine and phenylalanine were eluted using 80% acetone, 1% acetic acid (v/v in water) after the method of Steven (1962). Separation of the three derivatives was effected using two-dimensional TLC silica gel plates (first dimension: toluene: pyridine: 2-chloroethanol: 0.8M-ammonia; 10:3:6:6, second dimension: benzene: pyridine: glacial acetic acid; 80:20:2) using the overflow technique. Samples were eluted from silica gel using 80% acetone and evaporated to dryness.

The acid phase was lyophilyzed, redissolved in 2.0ml of 0.01M-HCl; 1.0ml was applied to a Porapak Q column. The column was washed with 15ml distilled water and the [^{3}H]/[^{14}C]DNP-derivatives eluted with 80% acetone as described above and subjected to high voltage electrophoresis at pH 2.1 (3500V, 45min). Imidazolyl-[^{3}H]/[^{14}C]DNP-histidine and imidazolyl-[^{3}H]/[^{14}C]DNP-lactic acid bands were further purified by electrophoresis at pH 3.5 (3500V, 30min). O-[^{3}H]/[^{14}C]DNP-tyrosine and ε-[^{3}H]/[^{14}C]DNP-lysine bands were subjected to a second, longer electrophoresis at pH 2.1 (3500V, 75min). The DNP-derivatives were eluted from the paper using a 10% acetic acid solution and lyophilyzed to dryness.

Calculations. The ratio of the [^{3}H]/[^{14}C]ratio of the functional groups to the [^{3}H]/[^{14}C]ratio of the internal standard $[(^3H_x/^{14}C_x)/(^3H_s/^{14}C_s)]$ for each sample was corrected for background due, in part, to residual unmodified histone protein by the subtraction of the $(^3H_x/^{14}C_x)/(^3H_s/^{14}C_s)$ratio calculated for the appropriate blank reaction in which insulin was absent.

Procedures for the Study of the Insulin Free Monomer

Sample Preparation. An aqueous solution (100ml, pH 4.0) containing 5μmole each of insulin, alanylalanine and L-β-imidazole lactic acid was prepared. An aliquot (0.998ml) was removed and added to a buffer consisting of 5mM-N-methylmorphorline/5mM-acetic acid/5mM-sodium borate /1.0M-KCl to give a final volume of 100ml (final insulin concentration, $5x10^{-7}$M) (Figure 6, step 1). This solution was used for the [^{3}H]trace-labelling. The remainder of the stock solution was used in the [^{14}C]labelling.

[^{3}H]Trace-Labelling. An aliquot (2.5ml) of the the stock solution containing equimolar amounts ($5x10^{-7}$M) insulin, alanylalanine and L-β-imidazole lactic acid were temperature equilibrated at 37°C and adjusted to the desired pH using either 1M-KOH or 1M-HCl. To each sample, 50μl of acetonitrile containing [^{3}H]FDNB (1.20nmol, specific radioactivity 16.6Ci/mmol) was addded and the reaction allowed to proceed 18h in the dark at 37°C (Figure 6, step 2). The pH was then adjusted to 2.0 with concentrated HCl. After the addition of 2.5g urea and 0.5g sodium bicarbonate to each sample, complete dinitrophenylation was assured by reaction for a further 18h in the dark with 100μl of unlabelled FDNB (50%, v/v in acetonitrile) (Figure 6, step 3).

[^{14}C]Labelling. The second portion of the original solution containing insulin, alanylalanine and L-β-imidazole lactic acid was concentrated and redissolved in 6ml of an acidic 8M-urea solution. After addition of 1.0g sodium bicarbonate, [^{14}C]dinitrophenylation was accomplished by reaction with 0.5mmol of [^{14}C]FDNB (specific radioactivity 250Ci/mmol) for 18h in the dark with shaking (Figure 6, step 4). The [^{14}C]DNP-mixture was diluted to 12.5ml with an 8M-urea solution and 0.5ml of this resulting solution was added to each [^{3}H]trace-labelled sample (Figure 6, step 5).

Isolation and Purification of [^{3}H]/[^{14}C]DNP-Derivatives. The solutions from step 5 (Figure 6) were extracted with four 5ml volumes of diethyl ether to remove [^{3}H]/[^{14}C]DNP-alanylalanine and dinitrophenol as well as to aid in the precipitation of the protein. Imidazolyl-[^{3}H]/[^{14}C]DNP-lactic acid and [^{3}H]/[^{14}C]DNP derivatives of the functional groups were isolated from the aqueous protein suspension and purified as described above. [^{3}H]/[^{14}C]DNP-alanylalanine was isolated

by evaporation of the ether phase, resuspension in basic chloroform and removal of DNP-OH on a silica gel G column as previously described. Purification was accomplished by separation of DNP-alanylalanine from trace contaminants by TLC using the three different solvent systems as described for the purification of the [^{14}C]DNP-alanylalanine (see "Preparation of [^{14}C]DNP-Derivatives" above).

Procedures for Histidylglycine

Sample Preparation. A stock solution containing equimolar (10^{-3}M) L-β-imidazole lactic acid, alanylalanine and histidylglycine was prepared in 5mM-N-methylmorphorline/5mM-sodium borate/5mM-acetic acid/0.1M-KCl.

[^{3}H]Trace-Labelling. 5.0ml aliquots of the stock solution were temperature equilibrated and adjusted to the desired pH as described above. To each sample 50μl of acetonitrile containing [^{3}H]FDNB (0.95nmol, specific radioactivity 26Ci/mmol) was added and the reaction allowed to proceed 18h in the dark. Concentrated HCl was added to bring the pH to 2 (Figure 7, step 2). The sample was then divided in half. One half was used to quantify the reactivity of the imidazole function and the other half to quantify the reactivity of the α-amino group (Figure 7, step 3).

Determination of the Reactivity of the Imidazole Function of Histidylglycine

Acetylation. The first half of each [^{3}H]trace-labelled sample was adjusted to pH 9.0 using 5M-NaOH. The sample was fully acetylated with unlabelled acetic anhydride (60μl) at 22°C. After the addition of 0.5g $NaHCO_3$ and 2.5g urea, each acetylated sample was fully dinitrophenylated with unlabelled FDNB (500μl, 50% v/v in acetonitrile) for a further 12h at 22°C in the dark (Figure 7, step 5a).

[^{14}C]Labelling. A portion of the stock solution containing 0.9 mol each of histidylglycine, L-β- imidazole lactic acid and alanylalanine was lyophilyzed and then redissolved in 3ml of an 8M-urea solution containing 2.5g of $NaHCO_3$. The pH was adjusted to 9 and the sample fully acetylated using 40μl of unlabelled acetic anhydride then dinitrophenylated with 2.98μmol of [^{14}C]FDNB (specific radioactivity

21mCi/mmol) by reaction for 18h at 22°C in the dark (Figure 7, step 4a). The final volume was adjusted to 20.0ml with 8M-urea solution. An aliquot (1.0ml) of this solution was added to each of the above [^{3}H]trace-labelled samples. After addition of 0.5mg of N-α-acetyl-imidazolyl-DNP-histidine and 0.5mg of imidazolyl-DNP-lactic acid to each sample (as carrier), the pH was adjusted to 2 with concentrated HCl (Figure 7, step 6a).

Isolation and Purification of [^{3}H]/[^{14}C]DNP-Derivatives

Each sample was extracted three times with a 5ml aliquot of diethyl ether. A portion (approximately one half) of the aqueous phase was passed over a Dowex column (7mm x 30mm) which had been pre-equilibrated at pH 2 using 0.01M-HCl. The column was washed with 15ml of water to remove the urea. The L-β-imidazolyl-[^{3}H]/[^{14}C]DNP-lactic acid and N-α-acetyl-imidazolyl-[^{3}H]/[^{14}C]DNP-histidine were eluted with a 2% ammonia solution. After lyophilyzation, the sample was dissolved in 1.0ml of 6M-HCl and hydrolyzed 18h at 110°C. L-β-imidazolyl-DNP-lactic acid was separated from imidazole-DNP-histidine by paper electrophoresis at pH 2.1 (3500V, 45min). The derivatives were eluted from the paper with 10% acetic acid.

Determination of the Reactivity of the α-Amino Group of Histidylglycine

Dinitrophenylation. To the second (see above) half of the [^{3}H]trace-labelled sample, 0.5g $NaHCO_3$ and 2.5g urea were added and the samples dinitrophenylated using the procedure described above without prior acetylation (Figure 7, step 5b).

[^{14}C]Labelling. A portion of the stock solution containing 0.9 mol in each of histidylglycine, L-β-imidazole lactic acid and alanyl-alanine was lyophilyzed, then redissolved in 2.0ml of 8M-urea containing 2.5g $NaHCO_3$. The sample was dinitrophenylated using 2.98μmol of [^{14}C]FDNB (specific radioactivity 21mCi/mmol) (Figure 7, step 4b). The final volume was adjusted to 20.0ml with 8M-urea solution. An aliquot (1.0ml) of this solution was added to each of the above [^{3}H]trace-labelled samples. In this case, 0.5mg di-DNP-histidine, 0.5mg L-β-imidazolyl-DNP lactic acid and 0.5mg N-α-DNP-alanylalanine was added as carrier and the pH adjusted to 2 (Figure 7, step 6b).

Isolation and Purification of [^{3}H]/[^{14}C]DNP-Derivatives. Each sample was extracted with three 5ml aliquots of diethyl ether. The aqueous phase was passed over a Dowex column and hydrolyzed as described for the first half of the solution. L-β-imidazolyl-[^{3}H]/[^{14}C]DNP lactic acid was separated from di-[^{3}H]/[^{14}C]DNP-histidine by paper electrophoresis at pH 2.1 (3500V, 45min). N-α-[^{3}H]/[^{14}C]DNP-histidine was prepared from di-[^{3}H]/[^{14}C]DNP-histidine by thiolysis at 22°C for 8h in 2% N-methylmorphorline, 20% acetone at pH 8.0 using 30µl of β-mercaptoethanol. After reaction, the solution was brought to pH 2 using concentrated HCl, the acetone evaporated and the aqueous phase extracted several times with diethyl ether to remove S-DNP-β-mercaptoethanol. The N-α-[^{3}H]/[^{14}C]DNP-histidine was purified by two-dimensional thin layer chromatography (first dimension: toluene: pyridine: 2-chloroethanol: 0.8M-ammonia; 10:3:6:6, second dimension: benzene: pyridine: acetic acid; 40:10:1) followed by paper electrophoresis at pH 2.1 (3500V, 40min).

Procedures for Preliminary Glucagon Experiments*

Sample Preparation. An equimolar mixture of glucagon and alanyl-alanine (1mM) was prepared in 0.1M-KCl/5mM-sodium borate.

[^{3}H]Trace-Labelling. A portion of the above solution was diluted 100-fold with a buffer containing 0.1M-KCl/2mM-acetic acid/2mM-sodium monohydrogen phosphate/2mM-sodium borate. Aliquots (5ml) containing approximately 5nmol of glucagon were adjusted to the desired pH using 1M-KOH and reacted with 25µl of acetonitrile containing [^{3}H]FDNB (6.24nmol, specific radioactivity 14Ci/mmol) for 36h at 20°C. The reaction was quenched by the addition of taurine.

[^{14}C]Trace-Labelling. In order to assure high incorporation of [^{14}C]label at all functional groups without excessive production of the di-DNP-derivative at the N-terminal histidine residue, the [^{14}C]labelling was performed on two separate portions of the stock solution containing glucagon and alanylalanine. One portion was adjusted to pH 6.5, the other to pH 10.5 using 1M-KOH. Both portions were reacted with 1mM

*This experiment was performed in collaboration with Drs. S. Cockle and N. M. Young of the National Research Council of Canada, Ottawa.

[^{14}C]FDNB (specific radioactivity 0.209Ci/mol) added in 50μl of acetonitrile at 20°C for 24h. The reactions were quenched by the addition of taurine and the portions combined. The solution was diluted 25ml with water and an aliquot of 1.0ml added to each of the [^{3}H]trace-labelled samples. A separate aliquot of this solution containing approximately 50nmol of glucagon was hydrolyzed and subjected to HPLC separation in order to determine the proportions of [^{14}C]DNP-derivatives.

Isolation and Purification of [^{3}H]/[^{14}C]DNP-Derivatives using HPLC*. One half of each sample was evaporated to dryness then redissolved in 0.5ml of 6M-HCl and hydrolyzed for 18h at 110°C in sealed, evacuated tubes. After evaporation of the HCl, the samples were redissolved in 0.5ml of water and applied to a C18 SepPak Cartridge (Waters Scientific Ltd, Toronto, Ontario). Buffer salts were removed by rinsing with 4ml of water then the DNP-derivatives eluted with 3ml of acetonitrile. The samples were evaporated to dryness and redissolved in 200μl of the HPLC running solvent to which addition unlabelled imidazolyl-DNP-histidine had been added as carrier. HPLC separations were carried out with equipment from Beckman-Altex using a reverse phase C18 column (5μm, 0.46 x 25cm Beckman Ultrasphere-ODS) and a 10μm C18 precolumn (3.2 x40mm). The columns were maintained at 62°C by means of a thermostated water jacket to effect separation. The eluate was monitored at 320nm with a Beckman Model 155 variable-wavelength detector. Fractions (0.4ml) were collected in polypropylene plates on a Gilson FC80 collector. From an initial isocratic HPLC procedure (27:73; acetonitrile: 35mM-ammonium formate, pH3.0, flow rate = 0.5ml/min), fractions containing N-α-DNP-histidine and imidazolyl-DNP-histidine were collected, pooled and rechromatographed using a running solvent of 8:92; acetonitrile: 35mM-ammoniun formate, pH 3.0. Fractions from the initial isocratic separation containing O-DNP-tyrosine , N-ϵ-DNP-lysine and N-α-DNP-alanine were pooled and recycled in 25:75; acetonitrile: 35mM-ammonium formate, pH 3.0.

*The protocol followed for HPLC separation of the DNP-derivatives is the work of Drs. S. Cockle and N. M. Young of the National Research Council of Canada, Ottawa.

Calculations. The calculated [^{3}H]/[^{14}C]ratio for each of the desired DNP-derivatives were corrected according to the relative proportions of the [^{14}C]DNP-derivatives formed in the [^{14}C]trace-labelling.

Procedures for Subsequent Glucagon, Secretin and VIP Experiments.

Sample Preparation. Stock solutions containing equimolar (10^{-6}M) L-β-imidazole lactic acid, alanylalanine and one of glucagon, secretin or VIP were prepared in 5mM-N-methylmorphorline/5mM-sodium borate/0.1M-KCl.

[^{3}H]Trace-Labelling. Aliquots (3.0ml) of the stock solution containing L-β-imidazole lactic acid, alanylalanine and one of the polypeptides were temperature equilibrated at 37°C in a thermostated water jacket and adjusted to the desired pH using either 1M-HCl or 1M-KOH. An aliquot (50μl) of acetonitrile containing [^{3}H]FDNB (2.08nmol, specific radioactivity 16.6Ci/mmol) was added to each sample and the reaction allowed to proceed 18h in the dark. Concentrated HCl was added to bring the pH to 2.0 (Figure 8, step 1).

Addition of [^{14}C]Amino Acids. The following was added to each trace-labelled glucagon sample in 2.0ml of a solution containing 1% N-methylmorphorline, 25% acetone (v/v in water): imidazolyl-[^{14}C]DNP-histidine (2500 dpm), L-β-imidazolyl-[^{14}C]DNP-lactic acid (2500 dpm), N-α-[^{14}C]DNP-histidine (2500 dpm), N-α'-[^{14}C]DNP-alanylalanine (2500 dpm), N-ε-[^{14}C]DNP-lysine (2200 dpm). The solution also contained 0.03mg of each unlabelled DNP-derivative as carrier. For VIP and secretin, [^{14}C]derivatives were added in 175μl of solution which contained 12,500 dpm of each of the above derivatives. An aliquot (500μl) of another solution containing 0.1mg of each unlabelled derivative as carrier was added (Figure 8, step 2). After removal of the acetone by partial evaporation, an equal volume of 12M-HCl was added to each sample (5.00ml in the case of glucagon samples and 3.68ml in the case of VIP and secretin samples). Each sample was hydrolyzed 18h at 110°C (Figure 8, step 3).

Isolation and Purification of [^{3}H]/[^{14}C]Labelled Derivatives. Each hydrolyzed sample was extracted with three 5ml aliquots of diethyl ether. The ether layer was evaporated; the sample was redissolved in 100μl of chloroform saturated with dibasic phosphate solution (1g

Na_2HPO_4 in 100ml water) and 50µl was applied to a silica gel G column (7mm x30mm). The column was washed with 20ml of basic chloroform to remove the dinitrophenol. The N-α-[^{3}H]/[^{14}C]DNP-alanine was eluted using 80% acetone (v/v in water) and purified by two-dimensional TLC on silica plates (first dimension: chloroform: benzyl alcohol: glacial acetic acid; 70:30:3, second dimension: benzene: pyridine: glacial acetic acid; 40:10:1) using the overflow technique of Brenner and Niederwiesser (1961).

The acid phase was lyophilyzed, redissolved in 1.0ml of 0.01M-HCl and 0.5ml applied to a Porapak Q column. After washing of the column with 15ml of distilled water, the [^{3}H]/[^{14}C]DNP-amino acids were eluted with 80% acetone (v/v in water) and subjected to high voltage paper electrophoresis at pH 2.1 (3500V, 45min). Imidazolyl-[^{3}H]/[^{14}C]DNP-histidine and L-β-imidazolyl-[^{3}H]/[^{14}C]DNP-lactic acid bands were further purified by electrophoresis at pH 3.5 (3500V, 30min). A band containing N-ε-[^{3}H]/[^{14}C]DNP-lysine and N-α-[^{3}H]/[^{14}C]DNP-histidine was cut and subjected to electrophoresis at pH 6.5 (3500V, 120min). The N-α-[^{3}H]/[^{14}C]DNP-histidine isolated from this electrophoresis was the purified by electrophoresis at pH 3.5 (3500V, 30min).

Histidylglycine and Alanylalanine Titrations and pH Measurements

A 10ml solution containing 20 mol of either histidylglycine or alanylalanine in 0.1M-KCl was titrated at 37°C under nitrogen with 0.2M-KOH. The titrant was added from an Agla micrometer syringe apparatus. Blank corrections were made using a 10ml solution of 0.1M-KCl titrated under the same conditions. A Radiometer pH Meter26 fitted with a type GK 232/C glass electrode was used for pH measurements.

Liquid Scintillation Counting

All samples were dissolved in 500µl of 0.01M-HCl and 100-500µl of this solution was added to 10ml of Aquasol-2. Scintillation counting was performed on a programmable LKB 1215 Rack Beta scintillation counter equipped with automatic quench correction and a disintegrations per minute converter.

Analysis of Data.

After correction for background, liquid scintillation counts for the DNP-derivatives of the internal standards and functional groups were analyzed using the following equation (Kaplan et al., 1971):

$$\alpha_x r = \alpha_s({}^3H_x/{}^{14}C_x)/({}^3H_s/{}^{14}C_s)$$

$$\text{where } \alpha_x = \frac{1}{1 + [H^+]/K_x}$$

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

$$r = k_x/k_s$$

K_x and K_s are acid dissociation constants of the group under study and the internal standard, respectively, and k_x and k_s are the second-order rate constants for the dinitrophenylation reactions of the functional group and the internal standards, respectively. Parameters for the theoretical titration curves were obtained by non-linear regression analysis of the resulting data.

RESULTS

Insulin: Concentration-Dependence. A study of the concentration-dependence of reactivity of the functional groups in insulin using FDNB with L-β-imidazole lactic acid as the internal standard (Table 4, Figure 9) at pH 7.5 shows a large decrease in the reactivity of the amino groups which occurs at a concentration between $5x10^{-5}$M and $1x10^{-5}$M. While the reactivity of the amino groups remain constant down to a concentration of $5x10^{-5}$M, the average reactivity of the imidazole groups (histidines B5 and B10) decreases over the entire concentration range. At the lowest concentrations studied ($1x10^{-6}$M to $1x10^{-7}$M), all functional groups are non-reactive.

In order to investigate the possibility that adsorption of insulin to the containing vessel occurs at low insulin concentration, the concentration-dependence was studied under identical reaction conditions but in Tefzel (an analogue of Teflon fluorocarbons) tubes (Table 4, Figure 10) and in polystyrene tubes (Table 4, Figure 11). The results obtained demonstrate that the glycine N-terminus remains reactive down to a much lower concentration than in Pyrex glass reaction vessels (in Tefzel to $1x10^{-5}$M and in polystyrene to $1x10^{-6}$M). The phenylalanine N-terminal residue, however, exhibits a more gradual decrease in reactivity over these concentration ranges while the histidine residues show a very similar concentration dependence in all three types of tubes. Again, at very low concentrations ($1x10^{-7}$M) all the groups are non-reactive.

Figure 12 and Table 4 show the effects of 1.0M-salt on the concentration-dependence of the reactivity in Pyrex glass containers. The reactivity of the glycine N-terminus remains constant at all concentrations down to $1x10^{-7}$M. The phenylalanine N-terminus shows a substantial decrease in reactivity below $5x10^{-6}$M. Again the histidines show a decline in reactivity as the insulin concentration is decreased but, while the absolute reactivities of the N-termini remain approximately the same as in 0.1M-salt, the average histidine reactivity has more than doubled. In contrast with the 0.1M-salt, all groups are reactive at a concentration of $1x10^{-7}$M. The results obtained in 0.5M-salt (Table 4, Figure 13) are intermediate to those obtained in 0.1M-salt and 1.0M-salt.

TABLE 4: Summary of Reactivity Data for Insulin Concentration-Dependence Studies

Insulin (M)	0.1M-KCl Glass Tubes α_x^r					0.1M-KCl Tefzel Tubes α_x^r				
	Gly	Phe	Lys	Tyr	His	Gly	Phe	Lys	Tyr	His
1×10^{-3}	6.80	17.1	3.75	*	27.3	8.20	26.5	1.29	2.17	21.5
5×10^{-4}	7.20	17.7	2.28	*	21.9	7.61	18.7	1.31	1.64	16.9
1×10^{-4}	7.13	20.6	0.963	1.17	9.47	8.51	21.6	1.37	1.86	12.3
5×10^{-5}	7.64	21.2	1.16	1.41	8.52	5.78	14.2	0.827	0.991	7.05
1×10^{-5}	1.66	3.01	0.774	0.566	1.98	7.03	12.4	1.01	1.57	2.27
5×10^{-6}	1.01	1.58	0.195	0.183	0.397	1.84	2.55	0.304	0.467	0.391
1×10^{-6}	0.642	0.529	0.101	0.0441	0.126	0.795	2.40	0.436	0.154	1.20
5×10^{-7}	0.233	0.233	0.258	0.176	0.195	0.441	1.47	0.216	0.178	0.642
1×10^{-7}	0.365	0.365	0.485	0.157	0.535	0.110	0.261	0.608	0.127	0.504

Insulin (M)	M-KCl Poystyrene Tubes α_x^r					0.5M-KCl Glass Tubes α_x^r				
	Gly	Phe	Lys	Tyr	His	Gly	Phe	Lys	Tyr	His
1×10^{-3}	9.57	29.8	1.86	1.86	24.5	9.71	21.5	2.04	3.48	53.3
5×10^{-4}	10.8	24.8	1.13	1.46	21.3	7.67	18.2	1.88	2.52	35.8
1×10^{-4}	9.38	26.0	1.49	1.71	15.8	10.7	21.4	2.39	3.61	47.8
5×10^{-5}	9.92	25.2	1.47	1.88	11.7	10.2	24.4	2.07	3.35	43.1
1×10^{-5}	9.92	18.0	1.56	1.90	4.31	9.40	13.5	1.83	2.75	12.1
5×10^{-6}	9.94	16.3	1.35	3.84	3.15	5.42	8.90	1.46	1.60	2.55
1×10^{-6}	7.62	6.34	0.381	0.378	0.155	7.73	8.45	2.54	2.66	4.90
5×10^{-7}	0.0938	0.0988	0.153	0.0315	0.0850	3.91	4.25	1.63	1.50	2.70
1×10^{-7}	0.238	0.208	0.608	0.497	2.12	5.82	3.28	2.33	2.13	4.18

Insulin	1.0M-KCl Glass Tubes α_x^r				
	Gly	Phe	Lys	Tyr	His
1×10^{-3}	11.8	29.0	2.67	4.94	86.9
5×10^{-4}	11.1	28.4	2.59	4.93	89.4
1×10^{-4}	11.7	25.9	2.34	5.30	68.6
5×10^{-5}	12.3	25.7	2.34	5.30	68.6
1×10^{-5}	11.6	25.4	2.35	5.28	58.6
5×10^{-6}	12.1	24.0	2.10	3.87	11.5
1×10^{-6}	13.4	13.5	3.82	5.10	8.75
5×10^{-7}	9.38	6.99	2.64	2.98	5.99
1×10^{-7}	9.82	4.85	4.59	4.10	8.50

* sample lost.

Figure 9: Concentration-reactivity profiles of the functional groups of insulin in Pyrex glass vessels. Reagent, FDNB; solvent, 0.1M-KCl, 37°C, 5mM-phosphate, pH 7.5. ●, glycine; x, phenylalanine; ○, lysine; ▲, tyrosine; △, histidine. ⊡ indicates data points of too close proximity to be differentiated graphically.

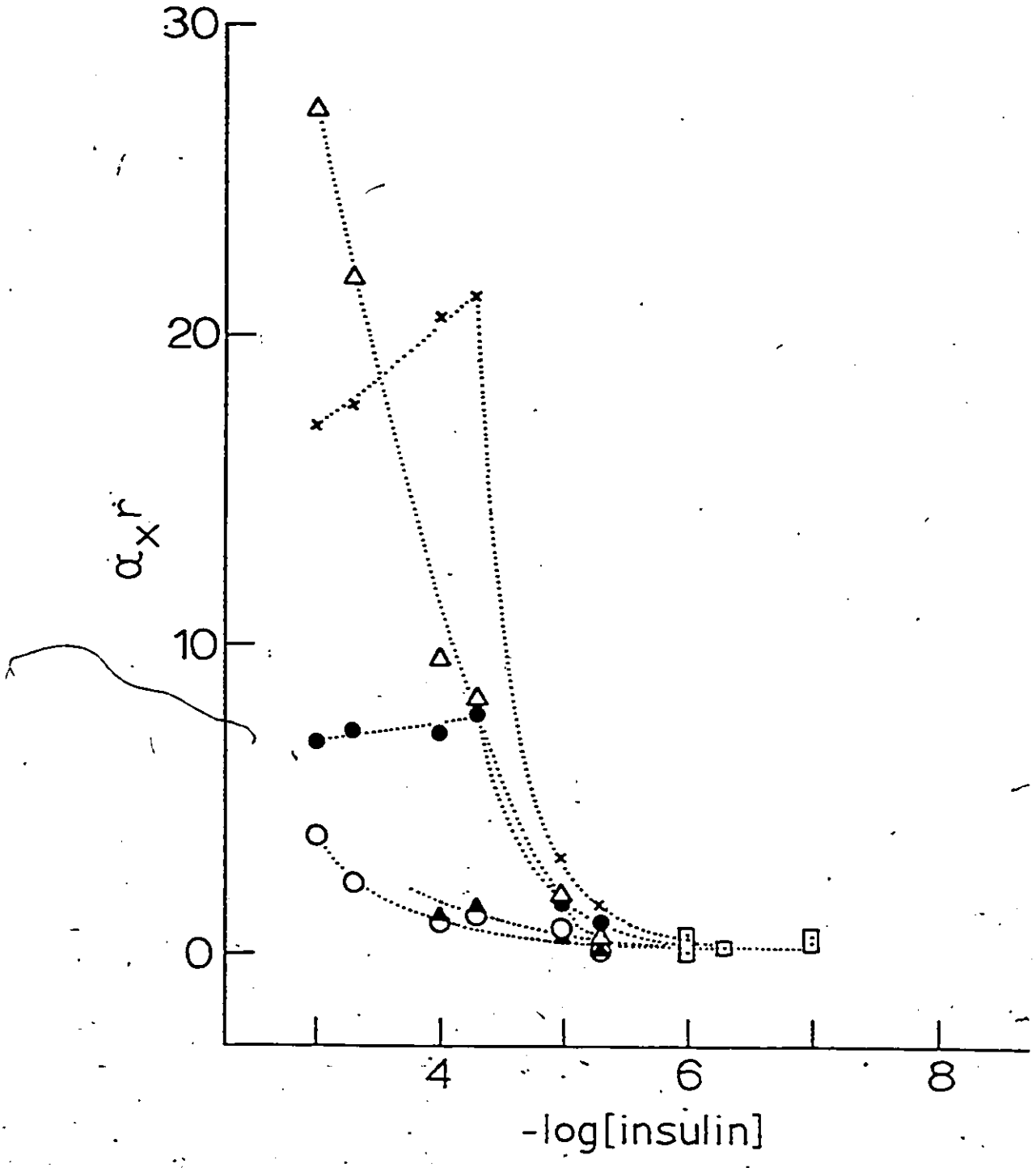

30
20
10
0
$\alpha_x r$
4
6
8
-log[insulin]

Figure 10: Reactivity-concentration profiles of the functional groups of insulin in Tefzel vessels. Reagent, FDNB; solvent, 0.1M-KCl, 37°C, 5mM-phosphate, pH 7.5. ●, glycine; x, phenylalanine; ○, lysine; ▲, tyrosine; △, histidine. ⊡ indicates data points of too close proximity to be differentiated graphically.

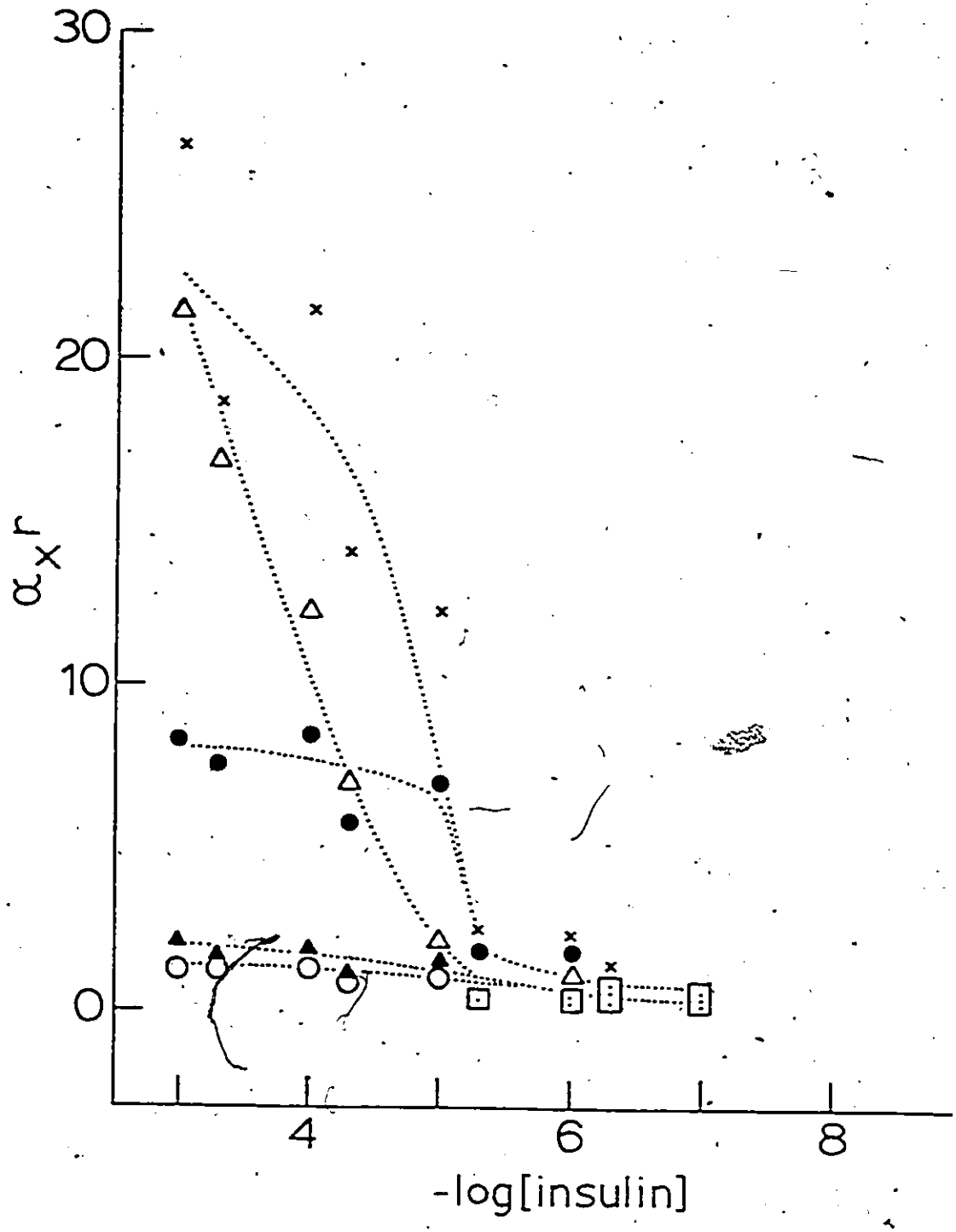

30
20
10
0
α_{xr}
4
6
8
-log[insulin]

Figure 11: Concentration-reactivity profiles of the functional groups of insulin in polystyrene vessels. Reagent, FDNB; solvent, 0.1M-KCl, 37°C, 5mM-phosphate, pH 7.5. ●, glycine; x, phenylalanine; ○, lysine; ▲, tyrosine; △, histidine. ⊡ indicates data points of too close proximity to be differentiated graphically.

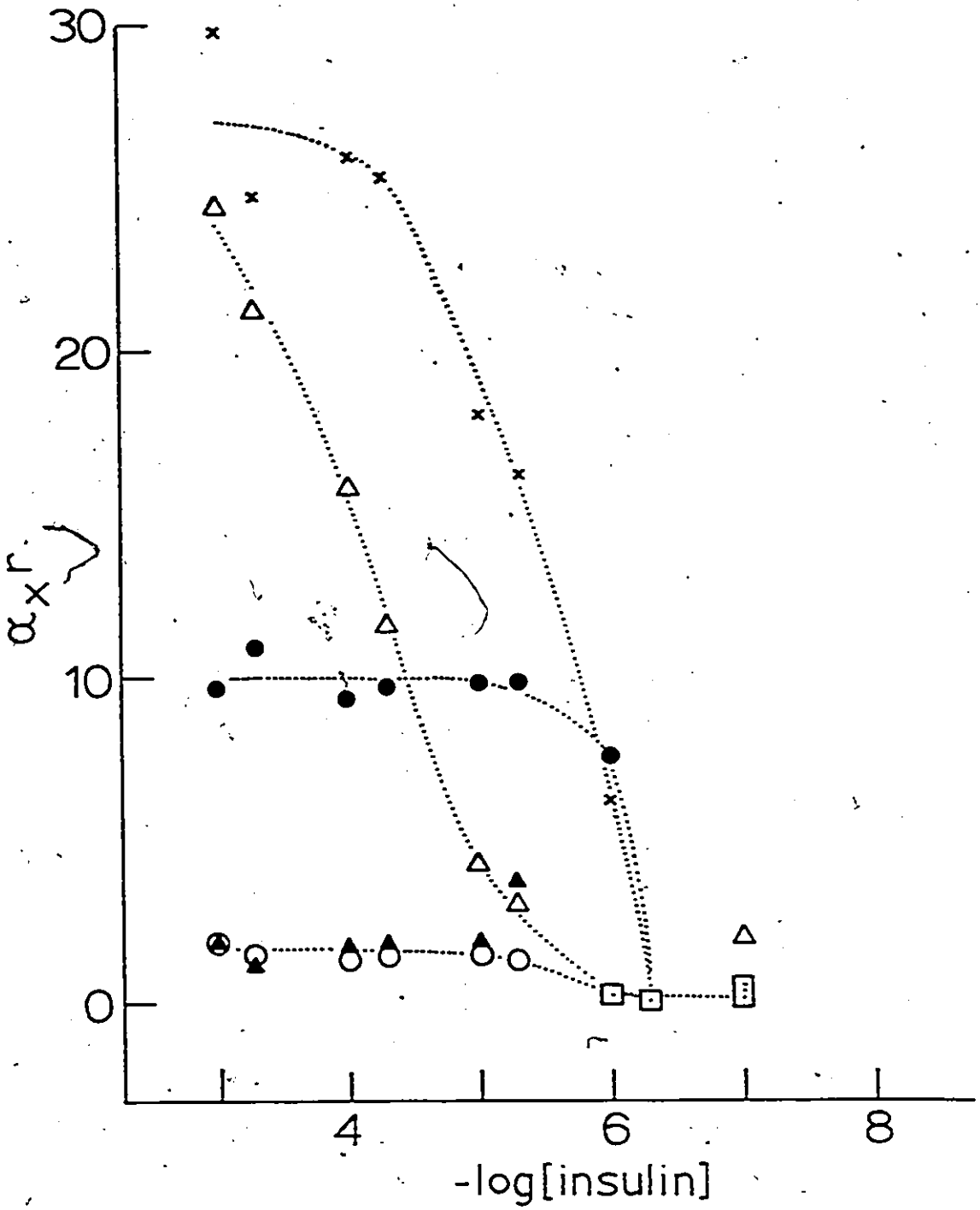

30
20
10
0
$\alpha x r$
4
6
8
-log[insulin]

Figure 12: Reactivity-concentration profiles of the functional groups of insulin in Pyrex glass vessels. Reagent, FDNB; solvent, 1.0M-KCl, 37°C, 5mM-phosphate, pH 7.5. ●, glycine; x, phenylalanine; ○, lysine; ▲, tyrosine; △, histidine. ⊡ indicates data points of too close proximity to be differentiate graphically.

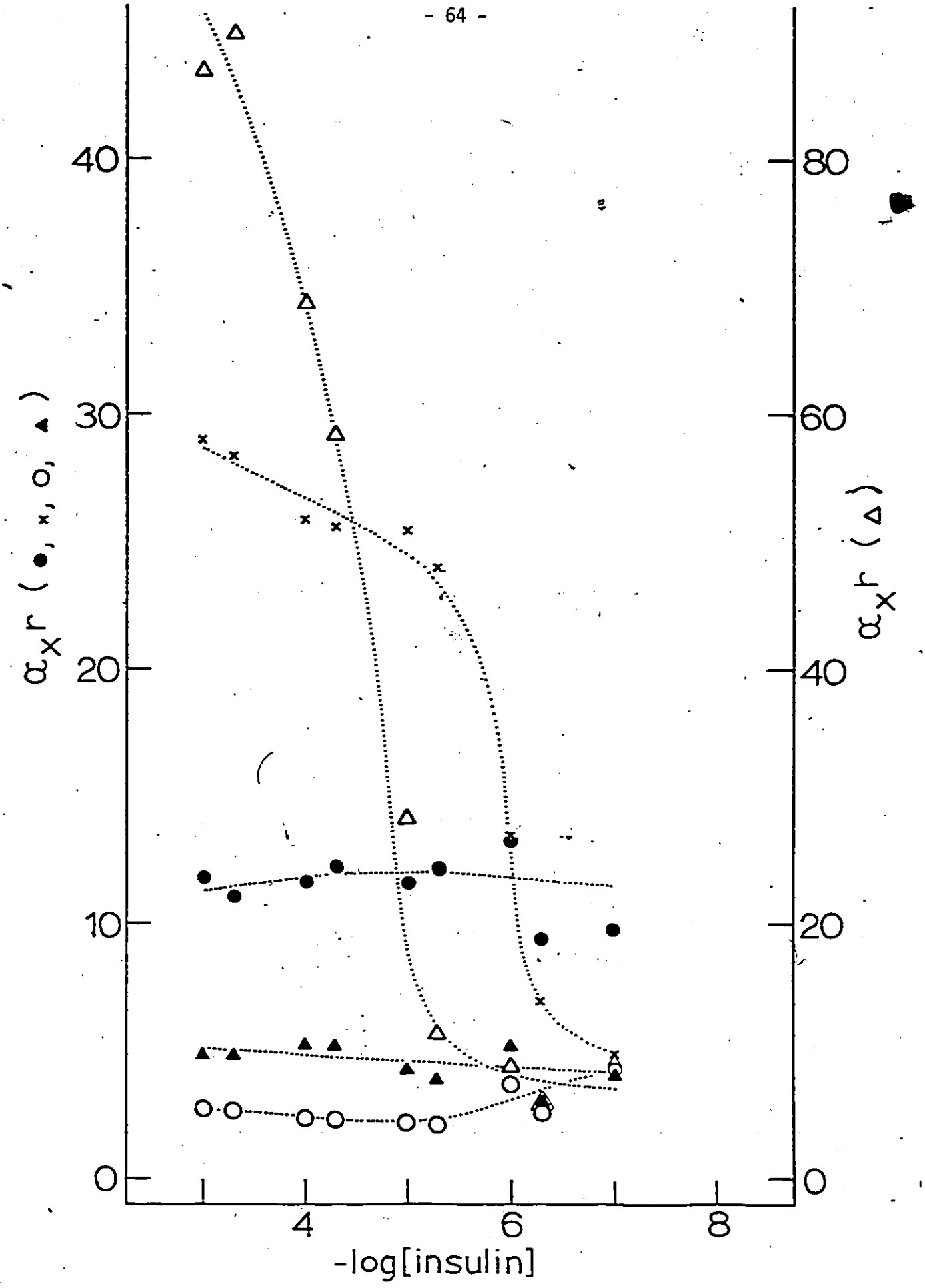
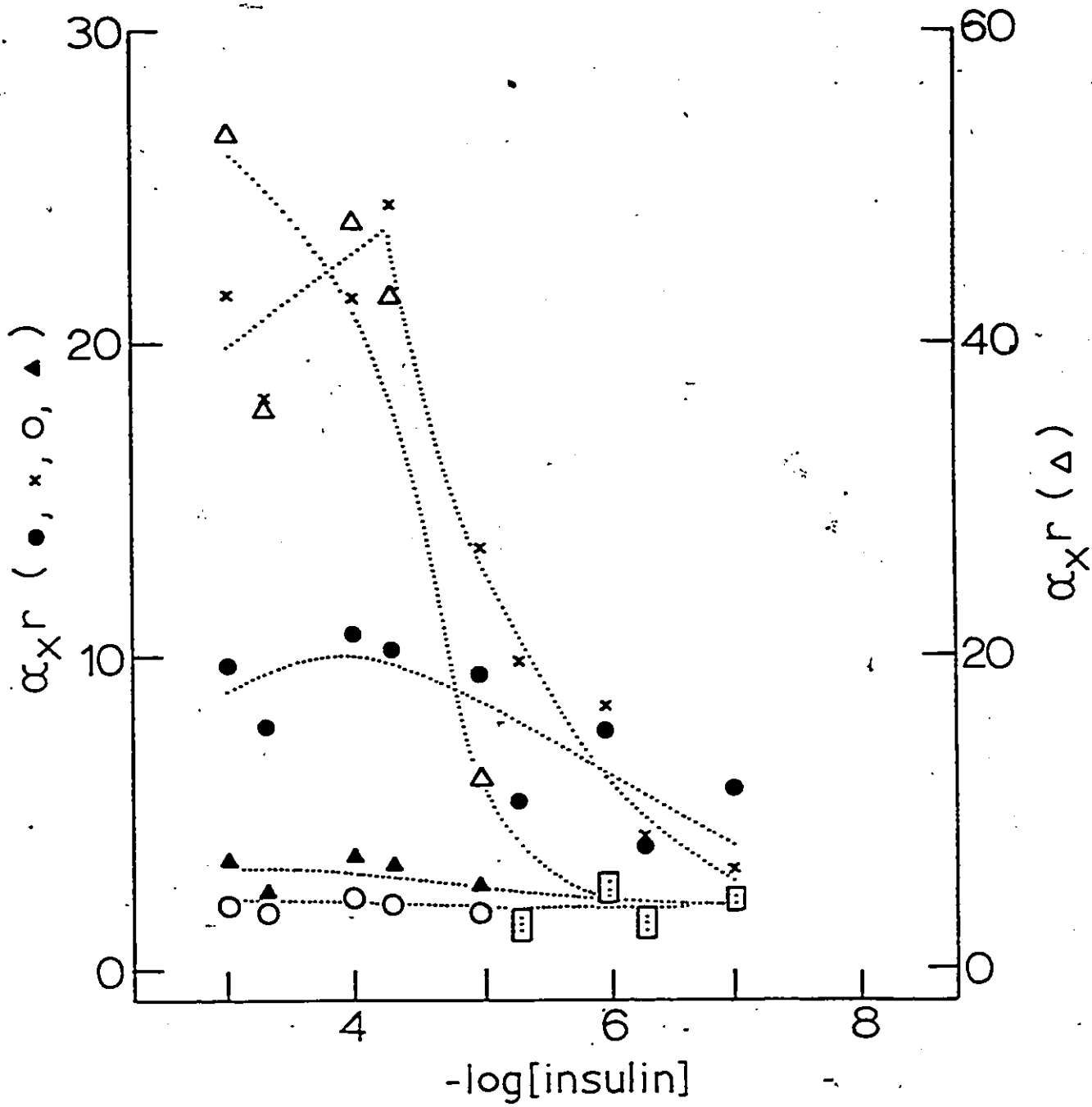

40
30
20
10
0
80
60
40
20
0
4
6
8
-log[insulin]
$\alpha_X r$ (●, ×, o, ▲)
$\alpha_X r$ (△)

Figure 13: Reactivity-concentration profiles of the functional groups of insulin in Pyrex glass vessels. Reagent, FDNB; solvent 0.5M-KCl, 37°C; ●, glycine; x, phenylalanine; ○, lysine; ▲, tyrosine; △, histidine. ⊡ indicates data points too close proximity to be differentiated graphically.


30
20
10
0
60
40
20
0
4
6
8
-log[insulin]
αXr (●, ×, ○, ▲)
αXr (△)

In an attempt to prevent insulin adsorption to Pyrex glass surfaces at low insulin concentrations in 0.1M-salt, various concentrations of a mixture of modified histone proteins were added to the buffer solutions used for the insulin dilutions. The mixture of histone proteins was modified by succinylation followed by reaction with FDNB under denaturing conditions. The amino acid analysis of the modified and unmodified histone mixture is shown in Table 5. Recovery of more than 95% of the lysine residue after treatment with FDNB indicates that the succinylation reaction went at least to 95% completion. The absence of peaks for histidine and tyrosine in the modified histone mixture indicates that these residues have derivatized. Further reaction with FDNB gave no further change in the amino acid analysis of the modified histone preparation.

From the results in Table 6, it is apparent that even a high concentration of a second protein (10^{-3}M histone) does not prevent the adsorption of insulin to the glass surface of the reaction vessel. There is a sharp decrease in the reactivity of both the glycine and phenylalanine amino termini when the insulin concentration is decreased to 10^{-6}M from 10^{-4}M regardless of the concentration of modified histone protein mixture employed. In view of the concentration-dependence of reactivity in polystyrene reaction vessels, one would expect the reactivity of the glycine amino terminus to remain constant in this concentration range in the absence of adsorption effects.

Insulin: Free Monomer. In an effort to determine the chemical properties of the functional groups in the free insulin monomer in the absence of adsorption effects, solutions of 5×10^{-7}M insulin in 1.0M-KCl were trace-labelled at various pH values using FDNB as the chemical probe and alanylalanine as the internal standard. The results obtained are summarized in Table 7. Figure 14 shows a plot of $\alpha_x r$ against pH for the glycine amino terminus in the free monomer. The experimental points closely follow the theoretical titration curve with a pK_a value of 8.21 and a reactivity of 2.22 relative to alanylalanine. Similar plots for the ϵ-amino group of the lysine at B-29 and the four tyrosine residues are shown in Figures 15 and 16. Again the data lie very close to the theoretical titration curves, pK_a values of 9.80 ± 0.05 and 9.18 ± 0.05 and reactivities of 25.4 ± 1.9 and 4.45 ± 0.18 relative to

TABLE 5: Amino Acid Analysis of a Mixture of Calf Thymus Histone Proteins Before and After Chemical Modification.

Residue	Histones	Modified Histones	Ratio Modified/Histones
Asp	0.546	0.517	0.95
Thr	0.574	0.581	1.0
Ser	0.301	0.550	1.7
Glu	1.00	1.00	1.00
Gly	1.07	0.802	0.75
Ala	1.05	1.14	1.1
Cys+Val	0.858	1.36	1.6
Met	0.0917	0.0448	0.49
Ile	0.524	0.447	0.85
Leu	1.08	0.837	0.78
Tyr	0.260	0.0	0.0
Phe	0.161	0.143	0.90
His	0.227	0.0	0.0
Lys	1.06	1.01	0.95
Arg	1.10	1.06	0.96

All values are corrected according to colour yields. Glutamic acid has been taken as 1.00.

TABLE 6: Concentration-Dependence of Reactivity of Functional Groups in Insulin in the Presence of Modified Histone Proteins.

modified histone (M)	insulin (M)	$\alpha_x r$ Phe	$\alpha_x r$ Gly
10^{-3}	10^{-4}	2:30	1.18
	10^{-6}	0.289	0.0623
	10^{-7}	*	0.0734
	10^{-8}	0.0170	0.292
10^{-5}	10^{-4}	2.70	1.75
	10^{-6}	1.88	0.588
	10^{-7}	0.0144	*
10^{-6}	10^{-4}	2.82	1.01
	10^{-6}	0.400	0.589
	10^{-7}	0.016	0.131

All $\alpha_x r$ values are relative to alanylalanine. In each case, $[^3H]/[^{14}C]$ values were corrected by subtraction of the corresponding $[^3H]/[^{14}C]$ ratio determined for a blank reaction where insulin had been omitted (see text). Cases where this correction resulted in negative $\alpha_x r$ values are indicated with an asterisk (*).

TABLE 7: Reactivity Data for the Functional Groups in the Insulin Free Monomer

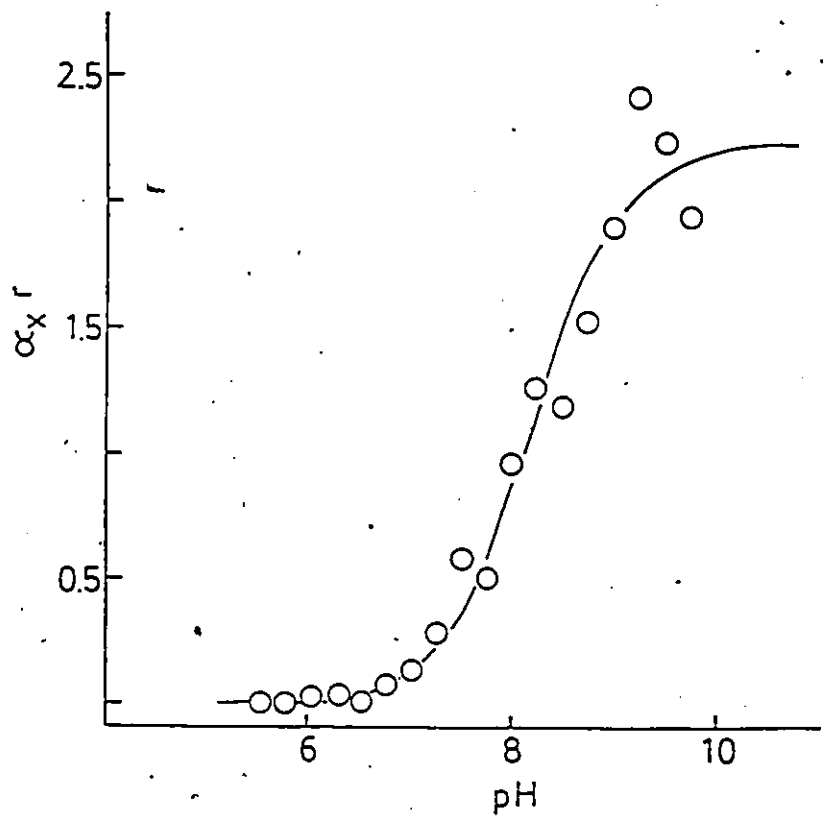
pH	$^{3}H/^{14}C$						α_s	$\alpha_x r$				
	Ala_2†	Phe	Gly	im-His	Lys	Try	Ala_2†	Phe	Gly	im-His	Lys	Tyr
5.50	0.0484	0.681	0.117	1.11	0.0701	0.117	0.00287	0.0404	0.00690	0.0067	0.00420	0.00710
5.75	0.0402	1.01	0.0946	1.05	0.392	0.154	0.00509	0.127	0.0120	0.136	0.0509	0.0199
6.00	0.263	1.28	0.208	0.769	0.161	0.136	0.0159	0.0440	0.0270	0.0264	0.00552	0.00466
6.25	0.106	0.919	0.286	-	-	-	0.0208	0.138	0.0430	-	-	-
6.50	0.486	1.57	0.515	0.700	0.553	0.187	0.0487	0.0670	0.0220	0.0300	0.0237	0.00800
6.75	0.333	1.28	0.587	0.734	0.413	0.225	0.834	0.187	0.0859	0.195	0.0618	0.0336
7.00	0.692	1.50	1.09	0.624	1.06	0.365	0.139	0.181	0.132	0.0752	0.128	0.0440
7.25	0.560	3.05	1.16	0.736	0.644	0.371	0.223	0.758	0.287	0.187	0.164	0.0942
7.50	0.683	1.85	1.77	0.779	0.527	0.696	0.338	0.604	0.580	0.259	0.176	0.232
7.75	1.13	1.54	1.67	0.586	0.532	0.519	0.476	0.462	0.500	0.175	0.159	0.155
8.00	1.03	1.53	2.07	0.612	0.937	0.721	0.618	0.707	0.958	0.287	0.439	0.338
8.25	1.28	1.37	2.62	0.460	1.09	1.03	0.742	0.660	1.26	0.224	0.531	0.502
8.50	1.24	0.682	2.00	0.443	1.59	1.05	0.837	0.408	1.19	0.236	0.951	0.628
8.75	0.868	0.547	1.57	0.337	1.85	1.08	0.901	0.528	1.52	0.326	1.79	1.04
9.00	0.615	0.495	1.29	0.935*	1.89	9.89*	0.942	0.726	1.89	1.37*	2.77	14.5*
9.25	0.603	0.473	1.54	0.337	3.66	1.66	0.966	0.739	2.41	0.527	5.72	2.60
9.50	0.495	0.307	1.14	0.308	4.22	1.53	0.981	0.600	2.23	0.601	8.24	2.99
9.75	0.429	0.269	0.845	0.380	5.00	1.50	0.989	0.616	1.93	0.869	11.4	3.43
10.00	0.512	0.329	0.732	0.270	6.74	1.61	0.994	0.635	1.41	0.522	13.0	3.11
10.25	0.438	0.267	0.773	0.258	5.86	1.41	0.996	0.606	1.75	0.586	13.3	3.20

* value obviously aberant and not used in calculation

† Ala_2: alanylalanine

Figure 14: Reactivity-pH for the α-amino function of the glycine, A-chain, N-terminal residue in the insulin free monomer. The solid line is the theoretical titration curve with a pK_a value of 8.21 and a reactivity of 2.22 relative to alanylalanine.

Figure 15: Reactivity-pH profile for the ε-amino function of the B29-lysine residue in the insulin free monomer. The solid line is the theoretical titration curve with a pK_a value of 9.80 and a reactivity of 25.4 relative to alanylalanine.


2.5
1.5
0.5
$\alpha_x r$
6
8
10
pH

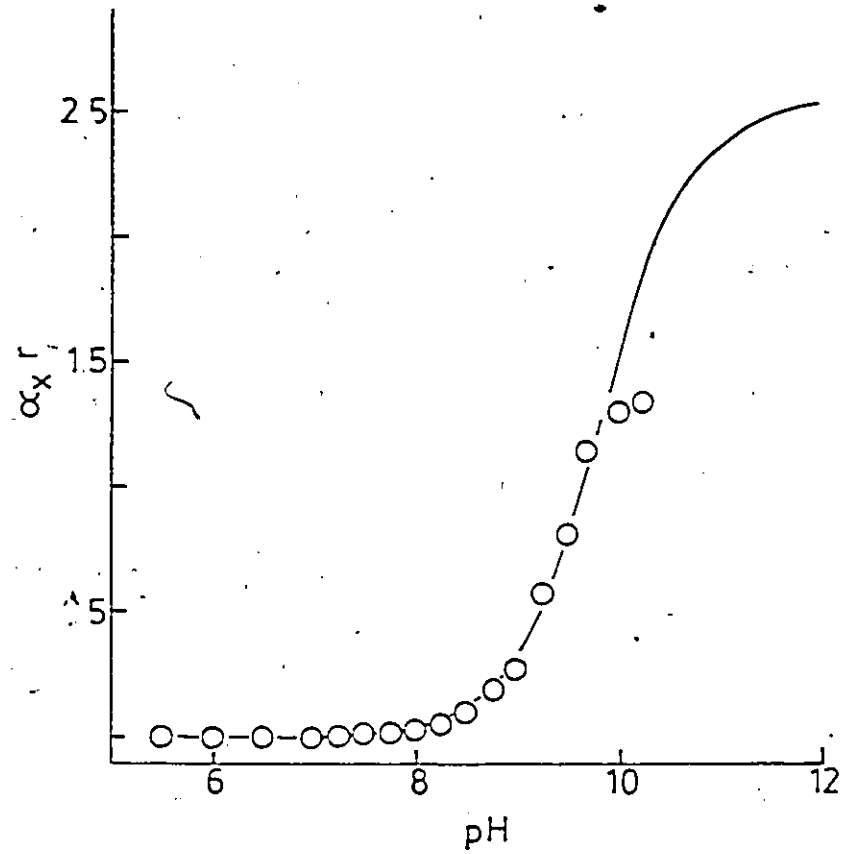
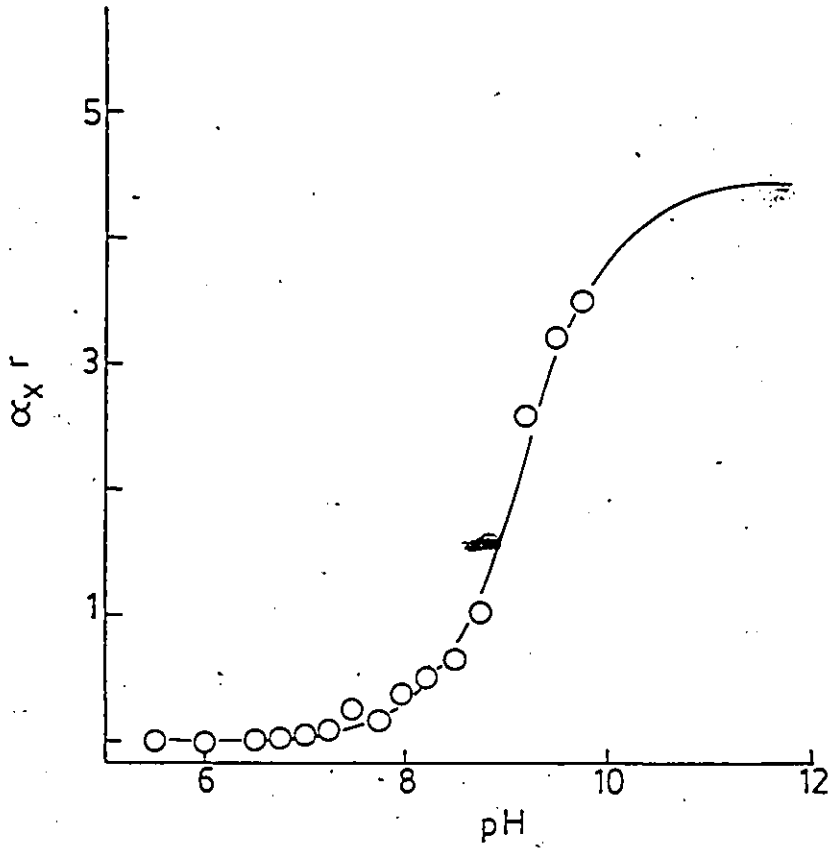
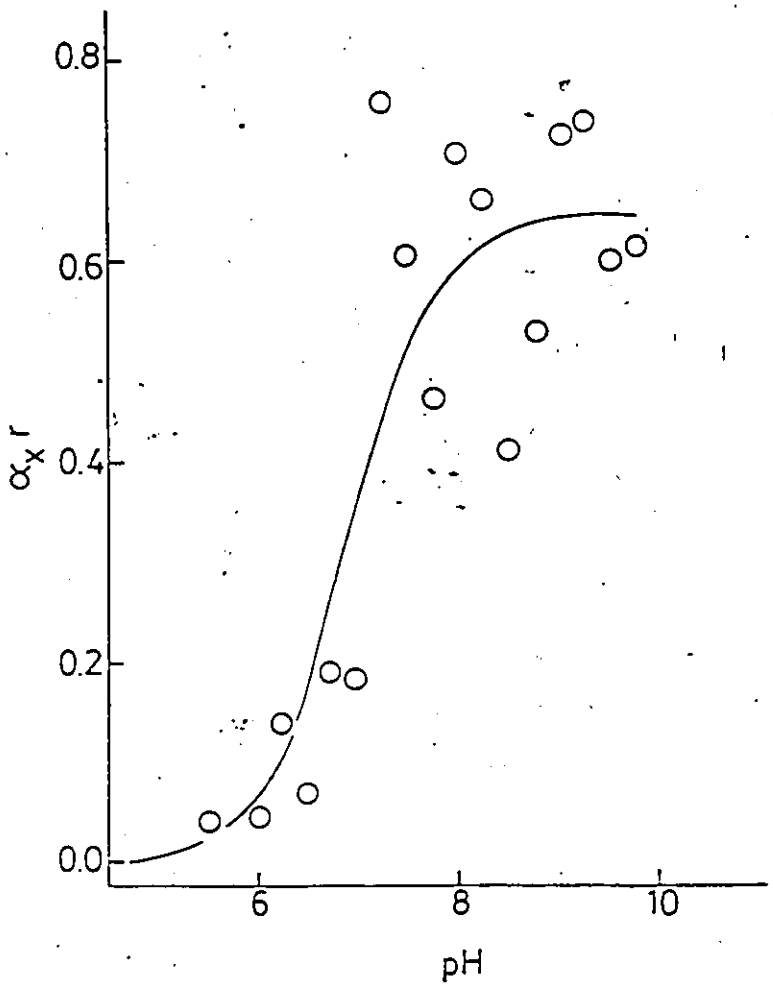

2.5
1.5
.5
$\alpha_x r$
6
8
10
12
pH

Figure 16: Dependence of the average reactivity of the four tyrosine residues in the insulin free monomer upon pH. The solid line is the theoretical titration curve with a pK_a of 9.18 and a reactivity of 4.45 relative to alanylalanine.

Figure 17: Reactivity-pH profile for the α-amino group of the B-chain phenylalanine amino terminus in the insulin free monomer. The solid line is the theoretical titration curve with a pK_a of 6.92 and a reactivity of 0.644 relative to alanylalanine.


5
3
1
α_X r
6
8
10
12
pH


0.8
0.6
0.4
0.2
0.0
α_X r
6
8
10
pH

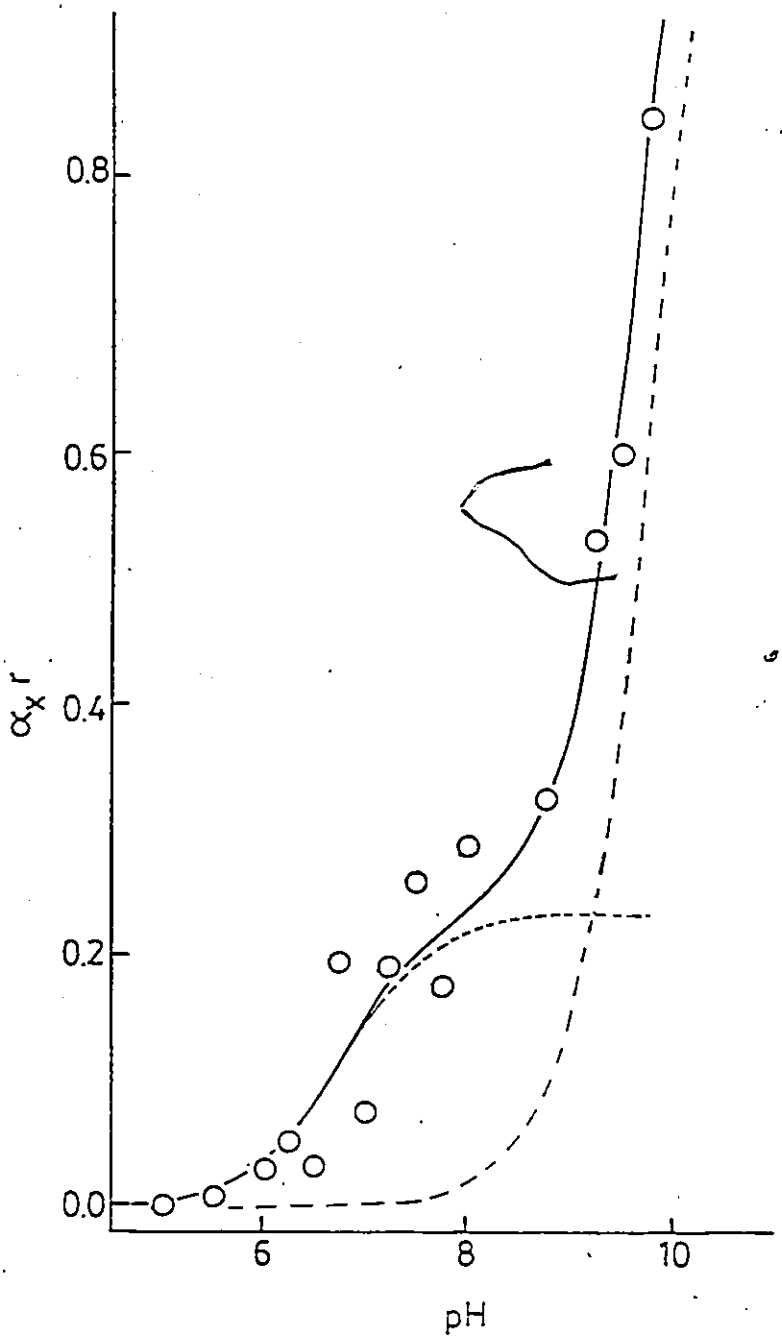
alanylalanine were determined for the lysine and tyrosine residues respectively.

The reactivity-pH profile for the two histidine residues is shown in Figure 18. These groups exhibit normal titration behaviour below pH 8.25. When the pH is further increased, there is a substantial increase in the average reactivity, relative to the internal standard, of the two histidines towards FDNB indicating that the second ionization of the imidazole moiety results in a very reactive species. Non-linear least squares analysis of the data assuming two ionizations gives a pK_a value of 6.80 ± 0.26 and a reactivity relative to alanylalanine of 0.234 ± 0.033 for the first ionization and a pK_a value of 10.12 ± 0.50 and a reactivity of 2.1 ± 1.7 for the second. Analysis of the data below pH 8.25 gives a very similar pK_a value (6.78 ± 0.23) for the first ionization.

The change in reactivity of the phenylalanine N-terminus with pH was similarily analyzed. Figure 17 shows the fit of the experimental data to a theoretical titration curve with a pK_a of 6.92 and a reactivity of 0.644 relative to alanylalanine. It is clear that this functional group has an unusual reactivity-pH profile. While low reactivity is observed below pH 7.0 and substantially higher reactivity above pH 7.0, the intermediate $\alpha_x r$ values expected at pH's near the pK_a value are absent.

If the internal standard, alanylalanine, is assumed to lie on a Brønsted plot for the reaction of a series of standard amines with FDNB (Chan et al., 1981), a second order, pH-dependent rate constant of $0.615 M^{-1}.min^{-1}$ at 20°C is calculated. Using this value and the further assumption that the increase in temperature from 20°C (used in the determination of the Brønsted plot) to 37°C (in the present experiments) affects all reaction rates equally, the second order rate constants for the reactions of the functional groups in monomeric insulin with FDNB were estimated. Figure 19 shows that all three amino groups in the insulin free monomer lie slightly above the Brønsted curve. The B-1 phenylalanine and A-1 glycine lie very close to the curve and thus appear to have "normal" properties while the B-29 lysine shows "slightly enhanced" reactivity on this basis. Deviations from the Brønsted plot can be represented as relative reactivities (RR), ie., the

Figure 18: Dependence of the average reactivity of the B5- and B10-histidine residues of the insulin free monomer upon pH. The solid line is the theoretical titration curve for a residue with the two ionizable groups: (a) a group with a pK_a of 6.80 and and a reactivity of 0.234 relative to alanylalanine; and (b) a group with a pK_a of 10.12 and a reactivity of 2.1 relative to alanylalanine. The theoretical titration curves for the individual titrations are shown in the dashed lines.


0.8
0.6
0.4
0.2
0.0
$\alpha_X r$
6
8
10
pH

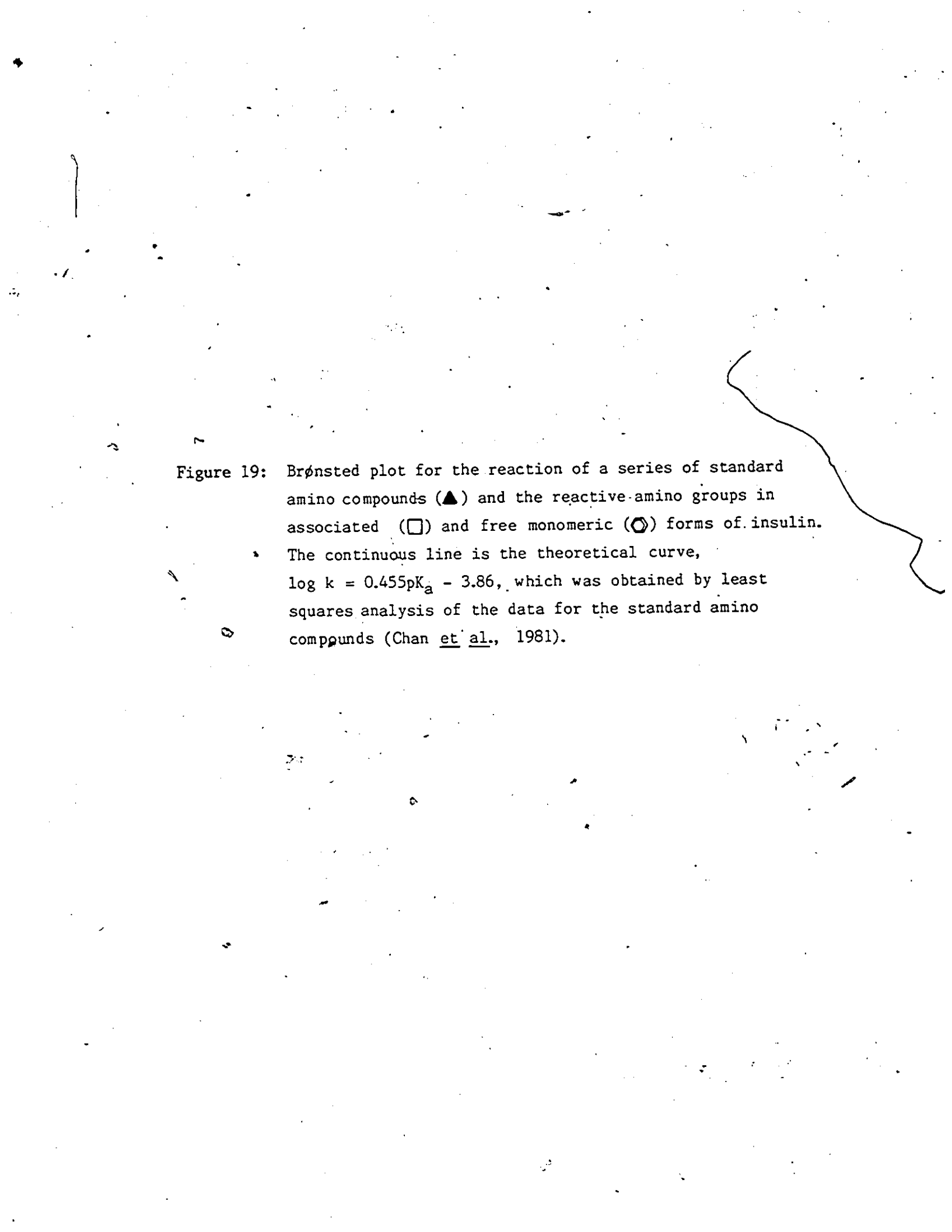
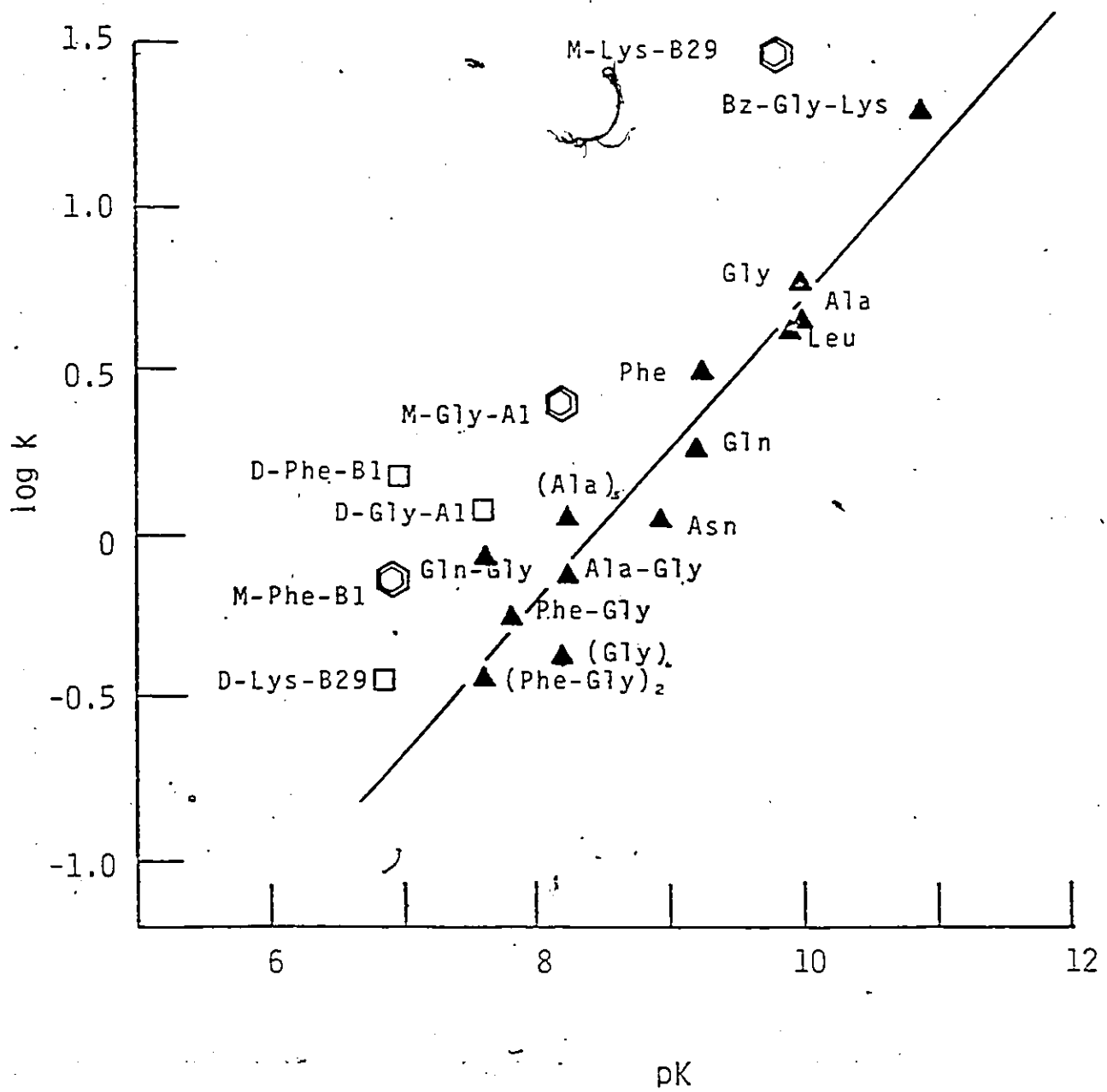


Figure 19: Brønsted plot for the reaction of a series of standard amino compounds (▲) and the reactive amino groups in associated (□) and free monomeric (○) forms of insulin. The continuous line is the theoretical curve, $\log k = 0.455pK_a - 3.86$, which was obtained by least squares analysis of the data for the standard amino compounds (Chan et al., 1981).


1.5
1.0
0.5
0
-0.5
-1.0
log k
6
8
10
12
pK
M-Lys-B29
Bz-Gly-Lys
Gly
Ala
Leu
Phe
M-Gly-A1
Gln
D-Phe-B1
(Ala)3
D-Gly-A1
Asn
Gln-Gly
Ala-Gly
M-Phe-B1
Phe-Gly
(Gly)4
D-Lys-B29
(Phe-Gly)2

reactivity relative to that of a standard amine with the same pK_a that lies on the plot. Table 8 summarizes the pK_a values and relative reactivities obtained for the amino groups in insulin in its free monomeric (this study) and associated forms (Chan et al.,1981; Sheffer and Kaplan, 1979). It is clear that these parameters are similar for the glycine amino terminus in all three studies but different for the lysine and phenylalanine in monomeric and associated forms of insulin. Second order, pH-dependent rate constants of $0.144 M^{-1}.min^{-1}$ and $2.64 M^{-1}.min^{-1}$ were calculated for the reaction of the two histidine and four tyrosine residues, respectively, with FDNB. Comparison of this parameter for the histidine imidazole function with those of standard imidazole groups (Table 9, Cruickshank and Kaplan, 1975) show that at least one of the two histidine residues in the insulin free monomer has more than "normal" reactivity. Comparison of the average rate constant for the four tyrosine residues with that of the phenolic hydroxyl group of N-acetyltyrosine amide ($k = 29.M^{-1}.min^{-1}$ Chan et al., 1981) indicates that some of these residues are buried in the free insulin monomer.

Histidylglycine. Tables 10 and 11 show the $\alpha_x r$ values obtained for the reaction of the α-amino and imidazole functions of histidylglycine with FDNB using L-β-imidazole lactic acid or alanylalanine as the internal standard as well as those for the α-amino group of alanylalanine relative to imidazole lactic acid. When di-DNP-histidine was isolated, the reactivity-pH profile is indicative of a single ionization with a pK_a value of approximately 7.5 whether imidazole lactic acid (Figure 20) or alanylalanine (Figure 21) is used as the internal standard. Acid-base titration of histidylglycine, however, indicates that the N-terminal histidine residue has the two ionizations (pK_{a1} = 5.85, pK_{a2} = 7.69) expected for the imidazole and α-amino functions (Table 12, Figure 22). The pK_a value for the α-amino function of alanylalanine is the same whether determined by competitive labelling (Figure 23) or conventional acid-base titration (Table 11, Figure 24).

When the reactivities of the imidazole and α-amino groups of histidylglycine are separated, the reactivity-pH profile for the imidazole function (Figure 25) does not indicate the low pK_a value anticipated from acid-base titration but, instead, closely resembles

TABLE 8: Chemical Properties of Associated and Free Insulin Monomers

Functional Group	Chemical Property	Insulin Concentration, Labelling Reagent, Internal Standard		
		2×10^{-4}M[a] Acetic Anhydride Phenylalanine 10°C	7×10^{-5}M[b] FDNB imidazole lactate 20°C	5×10^{-7}M FDNB alanyl alanine 37°C
Glycine	pK_a	7.9	7.7	8.21 ± .08
	RR	2.6	2.5	3.3
Phenylalanine	pK_a	7.2	6.9	6.92 ± .19
	RR	6.6	7.5	3.7
Lysine	pK_a	7.8	7.0	9.80 ± .05
	RR	.023	1.7	3.9
Histidine	pK_a	--	6.5	6.80 ± .05
	RR	--	20	5.9
Tyrosine	pK_a	--	8.7	9.18 ± .05
	RR	--	.08	.09

[a]Sheffer and Kaplan, 1979
[b]Chan et al.,1981
RR relative reactivity

TABLE 9: Reactivity of Imidazole Compounds Towards FDNB at 20°C*.

Imidazole compound	pK_a	$k \times 10^2$ ($M^{-1}.min^{-1}$)
4-hydroxymethylimidazole	6.30	2.57 ± 0.11
N-α-acetyl-L-histidine	7.20	4.35 ± 0.22
L-β-imidazole lactic acid	7.27	4.43 ± 0.08
imidazolylacetic acid	7.31	4.93 ± 0.21
imidazole	6.93	5.20 ± 0.28

* From Cruickshank and Kaplan (1975).

TABLE 10: Summary of Reactivity Data for Histidylglycine: Reactivity of the α-Amino Group

pH	$^3H/^{14}C$				α_s(im-lac)*	α_s(ala_2)*	α_{xr}(im-lac)*			$\alpha_{x\eta}$(ala_2)*	
	im-lac	ala_2	N-His	Bis-His			ala_2	N-His	Bis-His	N-His	Bis-His
5.00	0.404	1.22	13.9	1.34	0.00530	0.000909	0.0160	0.182	0.0147	0.0104	0.00100
5.50	0.529	1.22	5.22	2.42	0.0167	0.00287	0.0385	0.164	0.0763	0.0123	0.00569
6.00	0.592	2.14	5.61	3.04	0.0597	0.00902	0.216	0.566	0.306	0.0239	0.0129
6.50	1.02	4.09	9.39	5.02	0.145	0.0280	0.582	1.34	0.715	0.0643	0.0344
7.00	1.51	9.12	6.39	7.75	0.349	0.0834	2.11	1.47	1.75	0.0581	0.0709
7.25	1.60	12.8	13.7	7.71	0.488	0.139	3.91	4.18	2.35	0.149	0.0839
7.50	1.20	11.9	10.7	6.11	0.629	0.224	6.24	5.61	3.20	0.201	0.115
7.75	1.13	13.9	11.1	5.85	0.751	0.338	9.24	7.38	3.89	0.207	0.142
8.00	0.922	13.7	9.54	5.13	0.843	0.476	12.5	8.72	4.69	0.332	0.178
8.25	0.109	18.9	10.2	6.02	0.905	0.618	15.7	8.47	4.91	0.334	0.197
8.50	0.920	17.4	9.19	5.28	0.944	0.742	17.9	9.43	5.61	0.392	0.225
8.75	0.808	18.1	9.34	4.87	0.968	0.836	21.5	11.2	5.83	0.434	0.225
9.00	0.784	18.9	8.64	4.69	0.982	0.901	23.7	10.8	5.87	0.412	0.224

* internal standard nucleophile used: (im-lac), L-β-imidazole lactic acid; (ala_2), alanylalanine.

TABLE 11: Summary of Reactivity Data for Histidylglycine
Reactivity of the Imidazole Function

pH	$^{3}H/^{14}C$		α_s	$\alpha_x r$
	im-lac	im-His		im-His'
5.00	0.317	0.569	0.00530	0.0951
5.50	0.474	1.21	0.167	0.0426
6.00	0.532	1.10	0.597	0.123
6.50	0.733	1.38	0.145	0.273
7.00	1.03	1.23	0.349	0.417
7.25	1.25	1.33	0.488	0.520
7.50	1.41	1.67	0.629	0.745
7.75	1.09	1.17	0.751	0.806
8.00	1.15	2.29	0.843	0.946
8.25	1.05	1.18	0.905	1.02
8.50	0.993	1.02	0.944	0.970
8.75	0.931	1.11	0.968	1.15
9.00	0.802	0.769	0.982	0.941

Figure 20: Reactivity-pH profile of the N-terminal histidine residue of histidylglycine as determined by the isolation of the di-DNP-histidine derivative using L-β-imidazole lactic acid as the internal standard. The solid line is the theoretical titration curve with a pK_a of 7.44 and a reactivity of 5.98 relative to L-β-imidazole lactic acid.

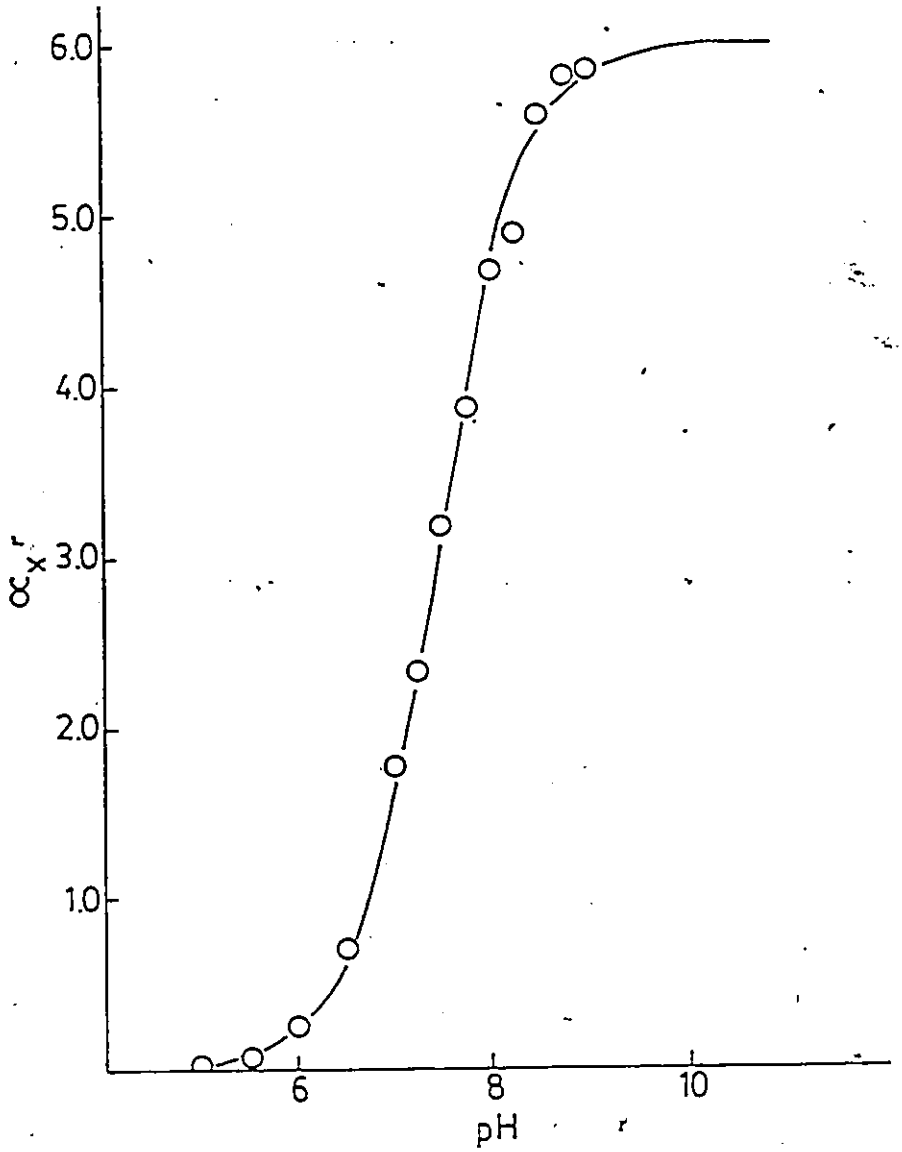

6.0
5.0
4.0
3.0
2.0
1.0
$\alpha_{X^{r-}}$
6
8
10
pH

Figure 21: Reactivity-pH profile of the N-terminal histidine residue of histidylglycine as determined by the isolation of the di-DNP-histidine derivative using alanylalanine as the internal standard. The solid line is the theoretical titration curve with a pK_a of 7.49 and a reactivity of 0.235 relative to alanylalanine.

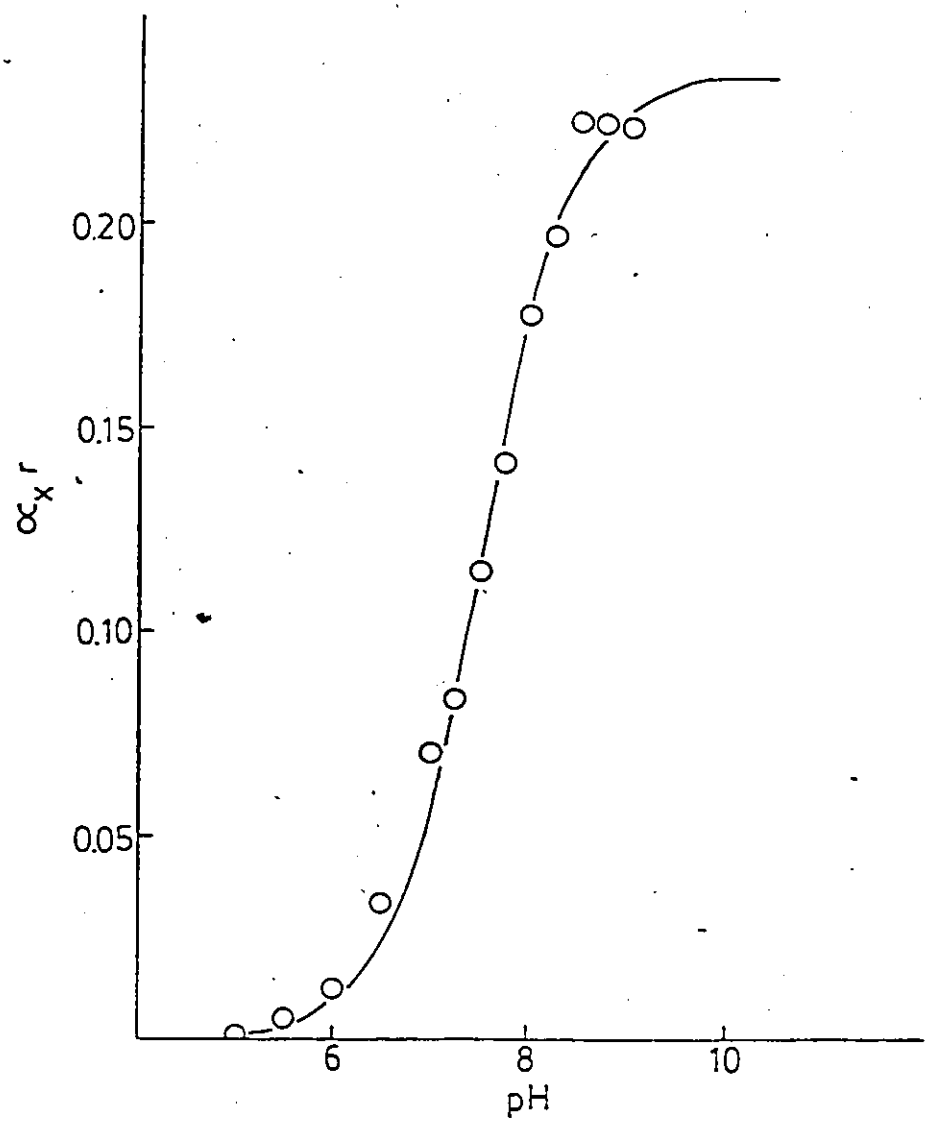

0.20
0.15
0.10
0.05
$\alpha_{x,r}$
6
8
10
pH

Table 12: Summary of Data for the Acid-Base Titration of Histidylglycine and Alanylalanine

pH	volume of titrant * (μl)		pH	volume of titrant * (μl)		pH	volume of titrant * (μl)	
	his-gly	ala-ala		his-gly	ala-ala		his-gly	ala-ala
2.90	-	-	5.90	112	44	8.80	265	138
3.00	21	-	6.00	118	44	8.90	267	142
3.10	26	-	6.10	124	44	9.0	270	145
3.20	30	-	6.20	136	45	9.10	273	147
3.30	34	3	6.30	136	45	9.20	275	149
3.40	38	7	6.40	142	46	9.30	278	151
3.50	41	10	6.50	147	47	9.40	279	153
3.60	44	14	6.60	153	47	9.50	280	154
3.70	48	19	6.70	159	48	9.60	281	157
3.80	50	22	6.80	163	48	9.70	283	158
3.90	51	26	6.90	173	49	9.80	285	159
4.00	53	28	7.00	179	51	9.90	286	161
4.10	54	30	7.10	185	54	10.0	289	162
4.20	55	32	7.20	190	56	10.10	290	163
4.30	56	34	7.30	196	59	10.20	292	164
4.40	57	36	7.40	201	63	10.30	294	165
4.50	59	38	7.50	208	66	10.40	295	165
4.60	61	39	7.60	215	71	10.50	297	165
4.70	63	39	7.70	218	77	10.60	298	165
4.80	65	41	7.80	224	83	10.70	299	165
4.90	67	41	7.90	230	96	10.80	300	165
5.00	71	41	8.00	236	101	10.90	300	
5.10	74	42	8.10	240	106	11.00	300	
5.20	79	43	8.20	245	112	11.10	300	
5.30	82	43	8.30	249	116	11.20	300	
5.40	88	43	8.40	253	122	11.30	300	
5.50	92	43	8.50	255	126	11.40	300	
5.60	98	44	8.60	258	131	11.50	300	
5.70	105	44	8.70	261	135			

* All volumes are corrected by subtraction of the corresponding value obtained by titrating an equal volume of 0.1M-KCl under the same conditions.
his-gly, histidyglycine; ala-ala, alanylalanine

Figure 22: Titration of Histidylglycine. 200μmol of histidylglycine in 10ml of 0.1M-KCl was titrated with 0.2M-KOH under nitrogen. Blank corrections were made using a 10ml solution of 0.1M-KCl titratred under the same conditions. The data between pH 4.50 and pH 8.50 was analyzed using a non-linear regression procedure assuming two ionizations. pK_a values of 7.69 ± 0.02 and 5.85 ± 0.01 were obtained.

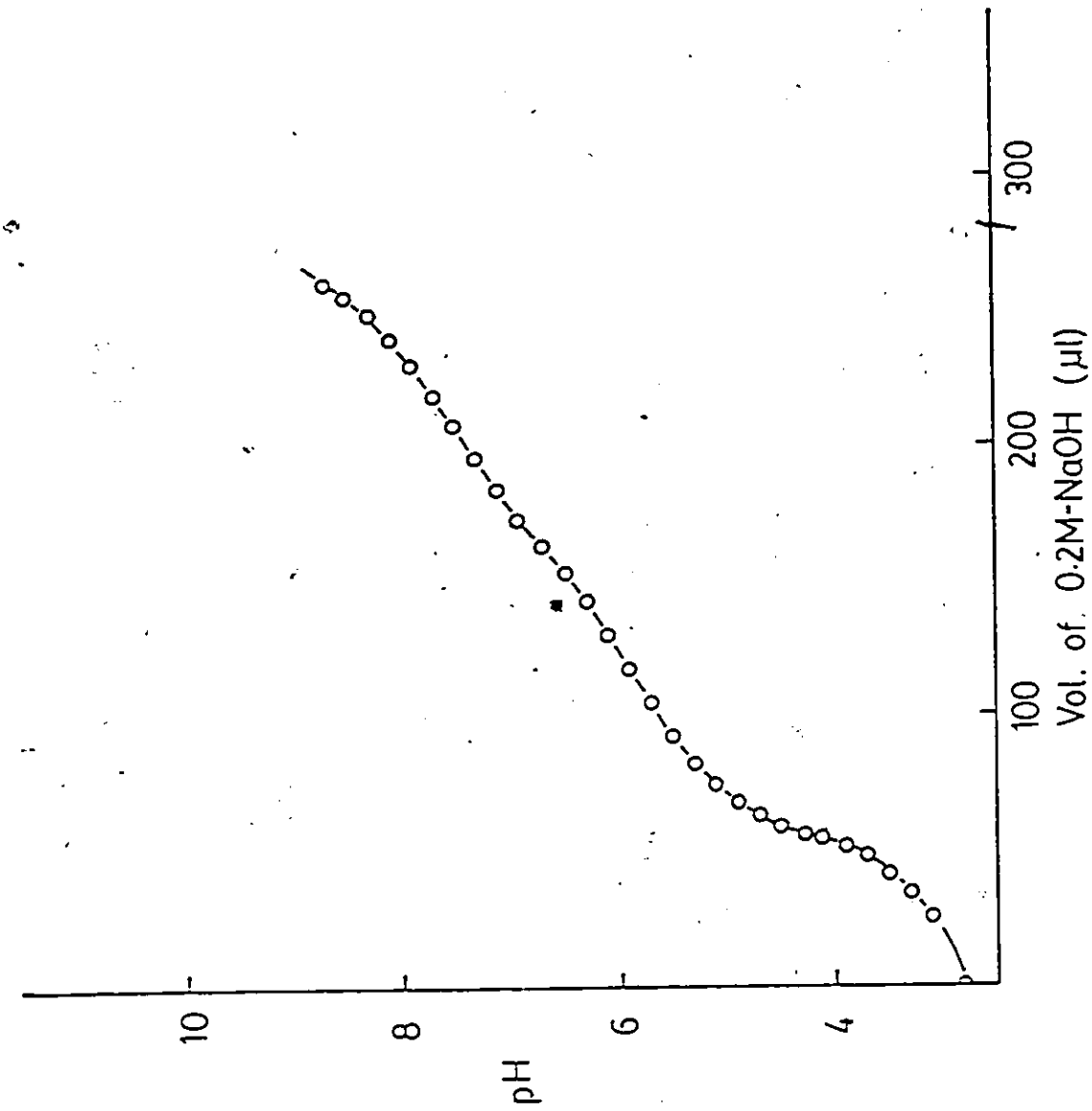

pH
10
8
6
4
100
200
300
Vol. of 0.2M-NaOH (µl)

Figure 23: Reactivity-pH profile of the N-terminal α-amino group of alanylalanine. The solid line is the theoretical titration curve with a pK_a of 8.02 and a reactivity of 25.4 relative to L-β-imidazole lactic acid.

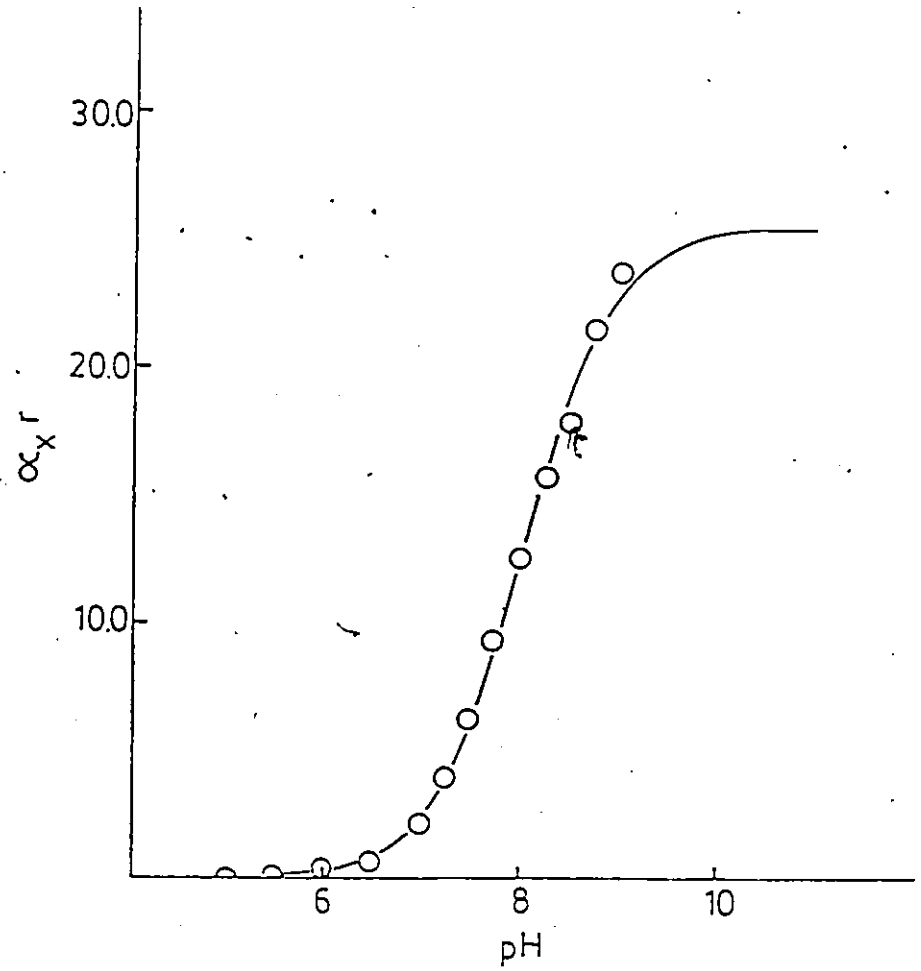

30.0
20.0
10.0
α_{χ} r
6
8
10
pH

Figure 24: Titration of Alanylalanine. 200μmol of alanylalanine in 10ml of 0.1M-KCl was titrated with 0.2M-KOH at 37°C under nitrogen. Blank corrections were made using a 10ml solution of 0.1M-KCl titrated under the same conditions. The data between pH 4.50 and pH 8.70 were analyzed using a non-linear regression procedure. A pK_a value of 8.03 ± 0.008 was obtained.

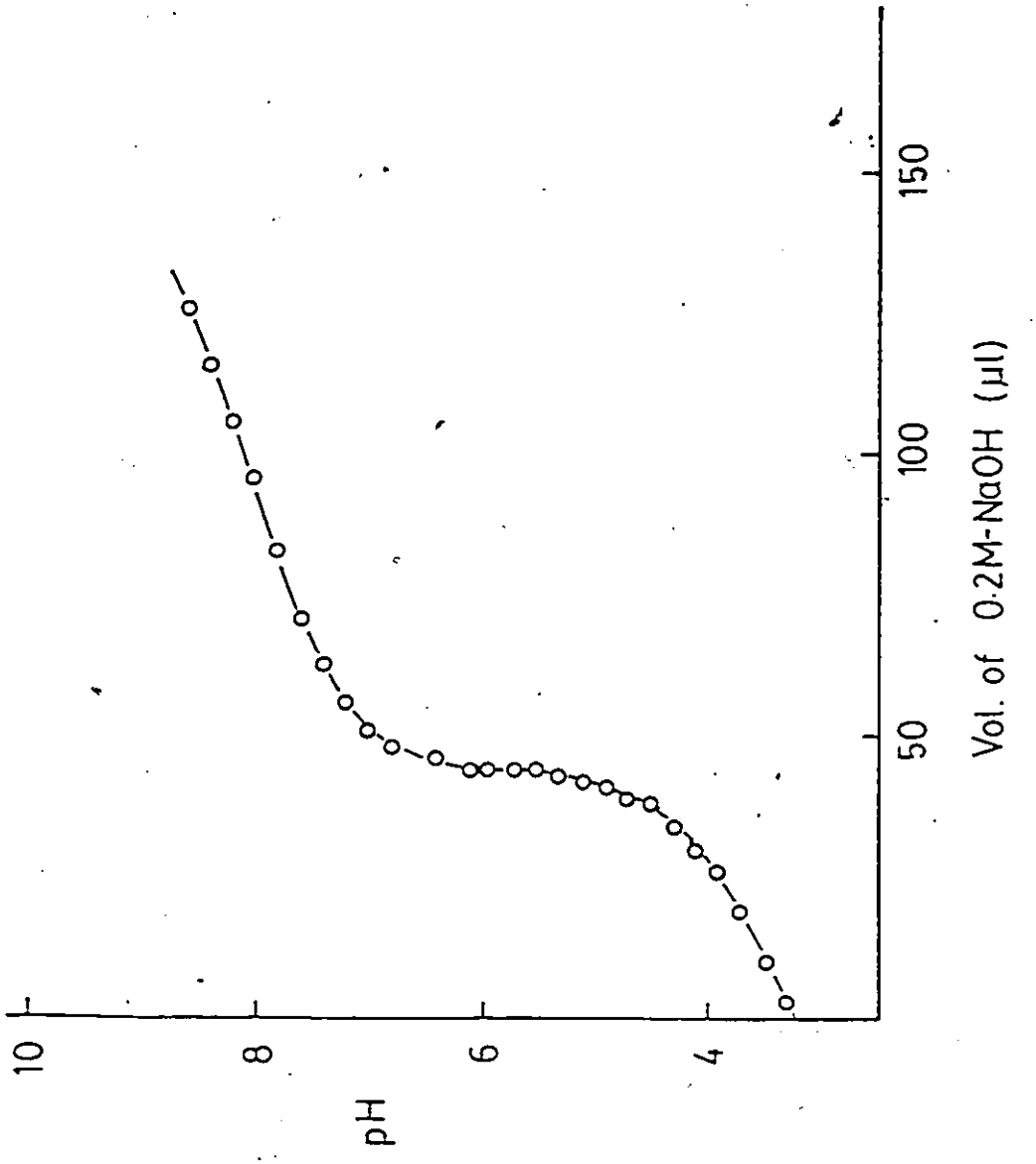
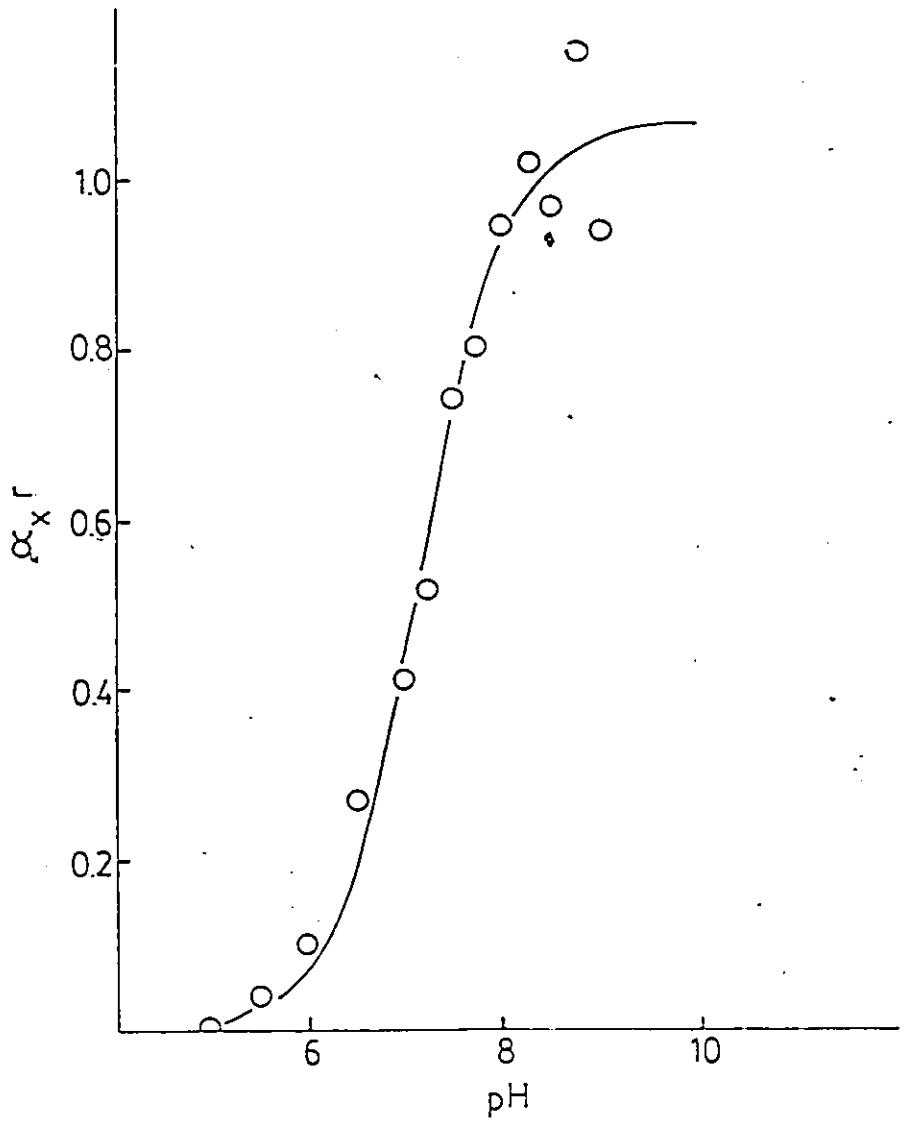

pH
10
8
6
4
50
100
150
Vol. of 0.2M-NaOH (µl)

Figure 25: Reactivity-pH profile for the imidazole group of the N-terminal histidine residue of histidylglycine. The solid line is the theoretical titration curve with a pK_a of 7.16 and a reactivity of 1.06 relative to L-β-imidazole lactic acid.


1.0
0.8
0.6
0.4
0.2
$\alpha_{x\,r}$
6
8
10
pH

the reactivity-pH profile for the α-amino function (Figures 26 and 27). A pK_a value of 7.16 ± 0.07 and a reactivity of 1.06 ± 0.03 relative to that of imidazole lactic acid were calculated for the imidazole function of histidylglycine using non-linear regression analysis of the data. A similar analysis of the experimental data for the α-amino function gave a pK_a value of 7.64 ± 0.07 and a reactivity of 0.413 ± 0.017 when alanylalanine was used as the internal standard and a pK_a of 7.49 ± 0.06 and a reactivity of 10.75 ± 0.03 when imidazole lactic acid was the internal standard. In all cases the experimental data show good fit to the theoretical titration curves.

Glucagon, Secretin and VIP. Preliminary studies with glucagon utilized HPLC to separate the DNP-derivatives. Figure 28A shows the isocratic separation (27:73; acetonitrile: 35mM-ammonium formate, pH 3.0) on a 5 m, reverse phase C18 column at 62°C of standard DNP-derivatives. The typical separation obtained for [^{3}H]/[^{14}C]labelled samples obtained by this procedure is shown in Figure 28B as monitored by the absorbance a 320nm and in Figure 28C as monitored by [^{3}H]counts in the eluant fractions. The various DNP-derivatives exhibit very different absorbance maxima; the wavelength 320nm chosen to monitor their elution from the column is therefore a compromise. In order to obtain sufficient purity to accurately determine [^{3}H]/[^{14}C] ratios, the fractions containing imidazolyl-DNP-histidine and N-α-DNP-histidine were pooled and subjected to a second isocratic separation using a 8:92; acetonitrile: 35mM-ammonium formate buffer at pH 3.0. Figure 29A shows the separation obtained for standard compounds and Figure 29B, that for the pooled fractions. In addition to the imidazolyl- and α-amino-DNP-derivatives of histidine, this pooled fraction contained DNP-taurine and a breakdown product (Pp) presumably from the photolysis of imidazolyl-DNP-histidine and an unidentified by-product resulting from the hydrolysis of DNP-alanylalanine. The recovery of radioactivity in this step was 82%. ε-DNP-lysine, O-DNP-tyrosine and DNP-alanine containing fractions from the first chromatography step were also pooled and rechromatographed using 25:75; acetonitrile: 35mM-ammoniun formate, pH 3.0 as the eluting buffer. In this step, 92% of the radioactivity was recovered from the column.

Figures 30 through 33 show the reactivity-pH profiles for the

Figure 26: Reactivity-pH profiles of the α-amino function of the N-terminal histidine residue of histidylglycine obtained when L-β-imidazole lactic acid is used as the internal standard. The solid line is the theoretic titration curve with a pK_a of 7.49 and a reactivity of 10.75 relative to L-β-imidazole lactic acid.

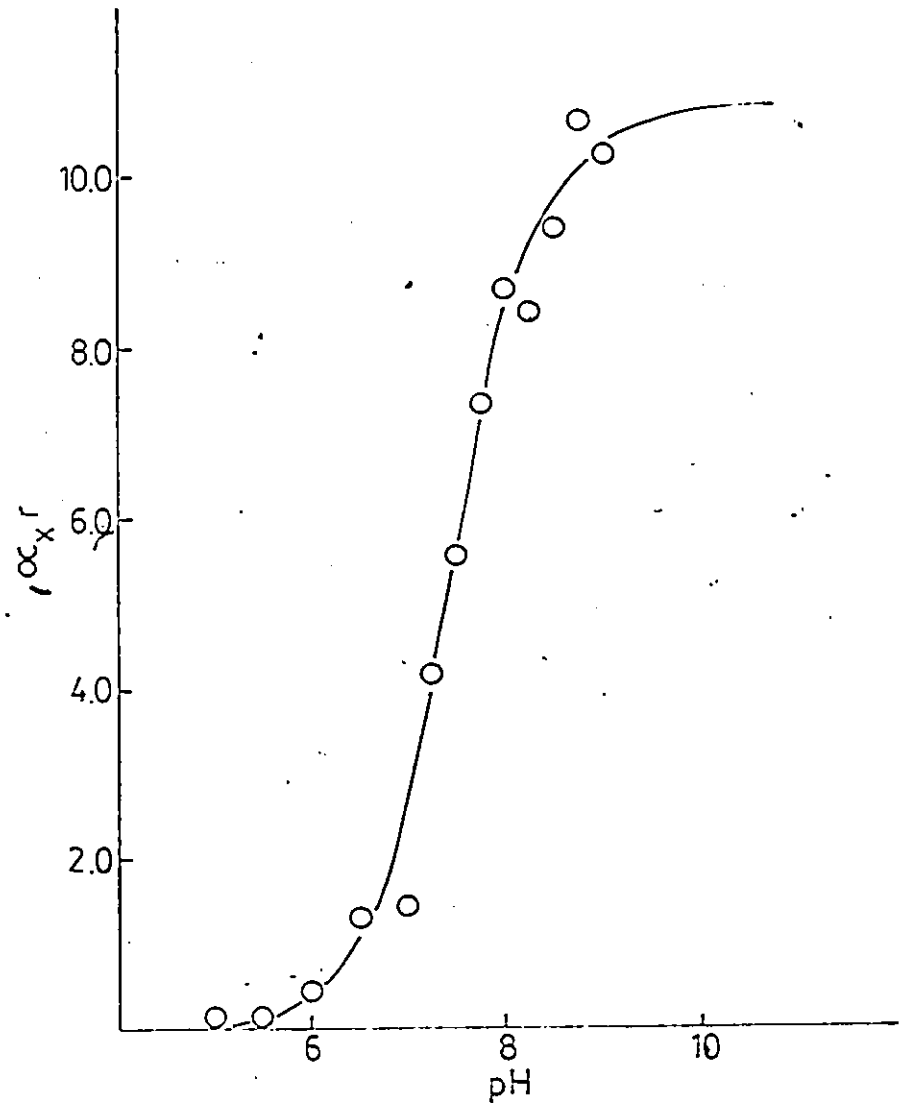

10.0
8.0
6.0
4.0
2.0
α_{x^r}
6
8
10
pH

Figure 27: Reactivity-pH profile of the α-amino group of the N-terminal histidine residue of histidylglycine when alanylalanine is used as the internal standard. The solid line is the theoretical titration curve with a pK_a of 7.64 and reactivity of 0.413 relative to alanylalanine.

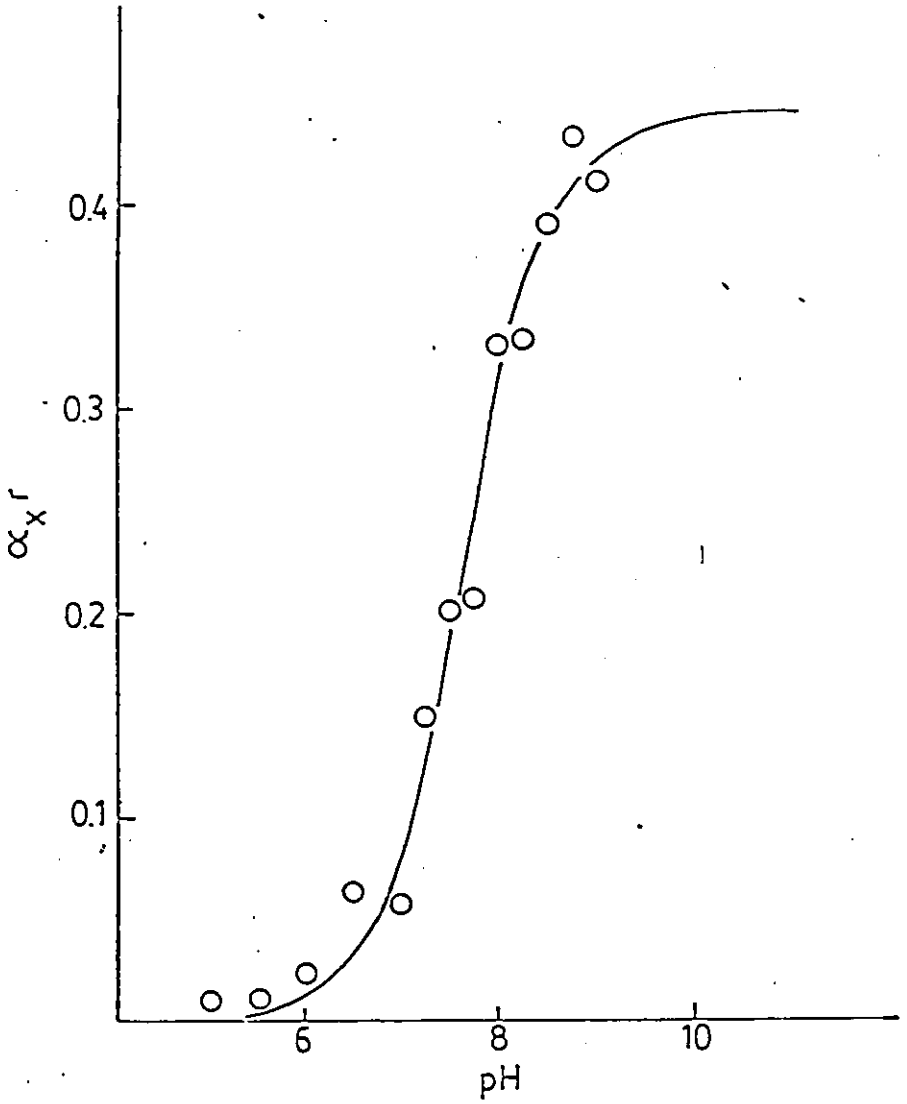

0.4
0.3
0.2
0.1
α_{x^r}
6
8
10
pH

Figure 28: HPLC isocratic separation (27: 3, acetonitrile: 35mM ammonium formate, pH 3.0) on a 5μm, reverse phase C 18 column at 62°C of a group of standard DNP-derivatives (A). The typical separation obtained of [^{3}H]/[^{14}C] derivatives obtained by this procedure is shown in (B) as monitored by the absorbance at 320nm and in (C) as monitored by [^{3}H] counts in the eluant fractions.

H, imidazolyl-DNP-histidine ; α, N-α-DNP-histidine;
K, N-ϵ-DNP-lysine; Y, O-DNP-tyrosine; DNP, dinitrophenol;
A, N-α-DNP-alanine Ta; DNP-taurine.

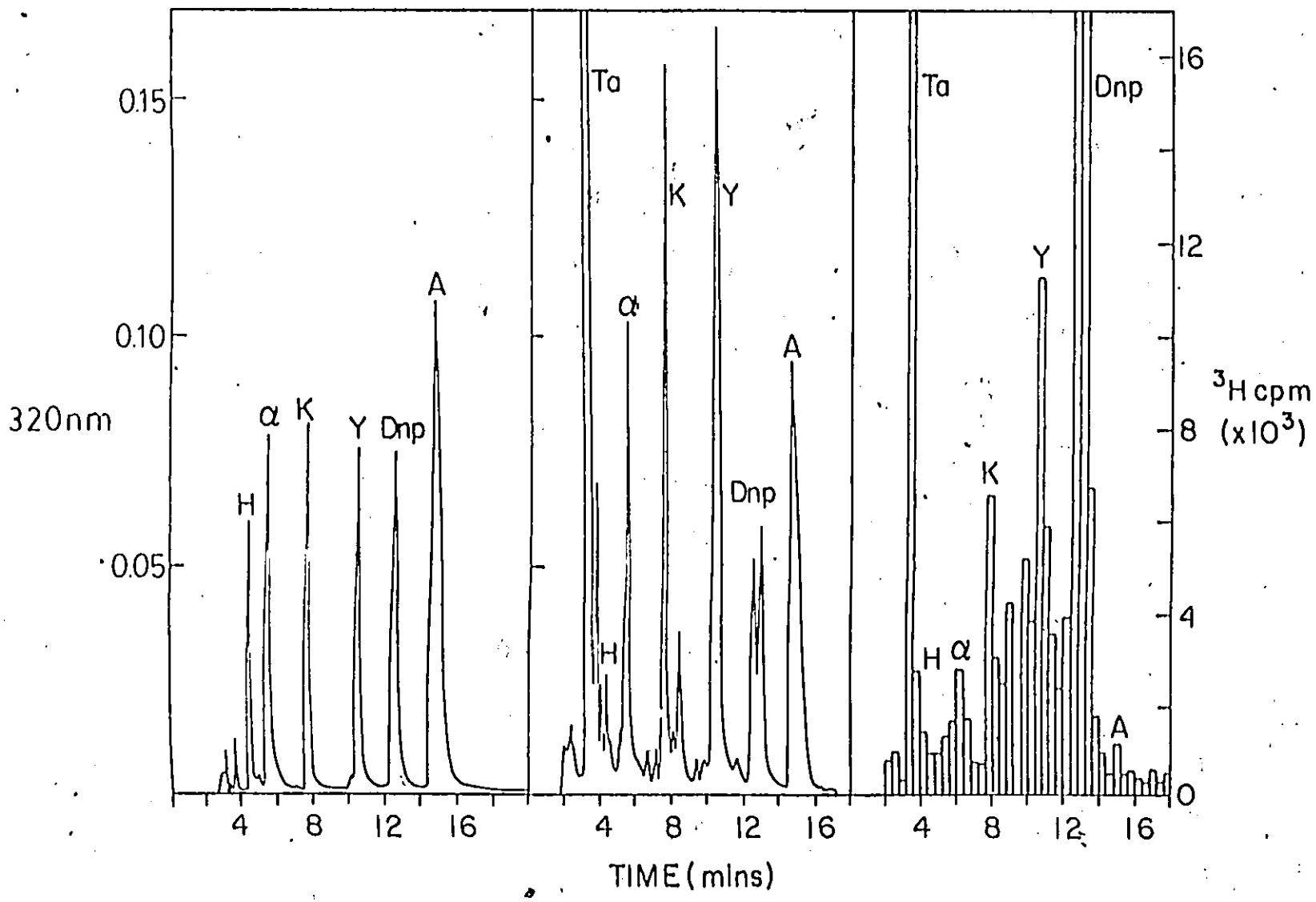

320nm
0.15
0.10
0.05
H
α
K
Y
Dnp
A
Ta
^{3}H cpm (x10^3)
16
12
8
4
0
4
8
12
16
TIME (mins)

Figure 29: HPLC isocratic separation (8:92, acetonitrile: 35mM-ammonium formate, pH 3.0) on a 5 μm, reverse phase C 18 column at 62°C of a group of standard DNP-derivatives (A) and of the pooled fractions containing imidazolyl-DNP-histidine and N-α-DNP-histidine (B) obtained from the isocratic separation shown in Figure 28.
H, imidazolyl-DNP-histidine, C, S-DNP-cysteine.
Pp, photochemical degradation product of imidazolyl-DNP-histidine; α, N-α-DNP-histidine; D, N-α-DNP-aspartic acid; E, N-α-DNP-glutamic acid.

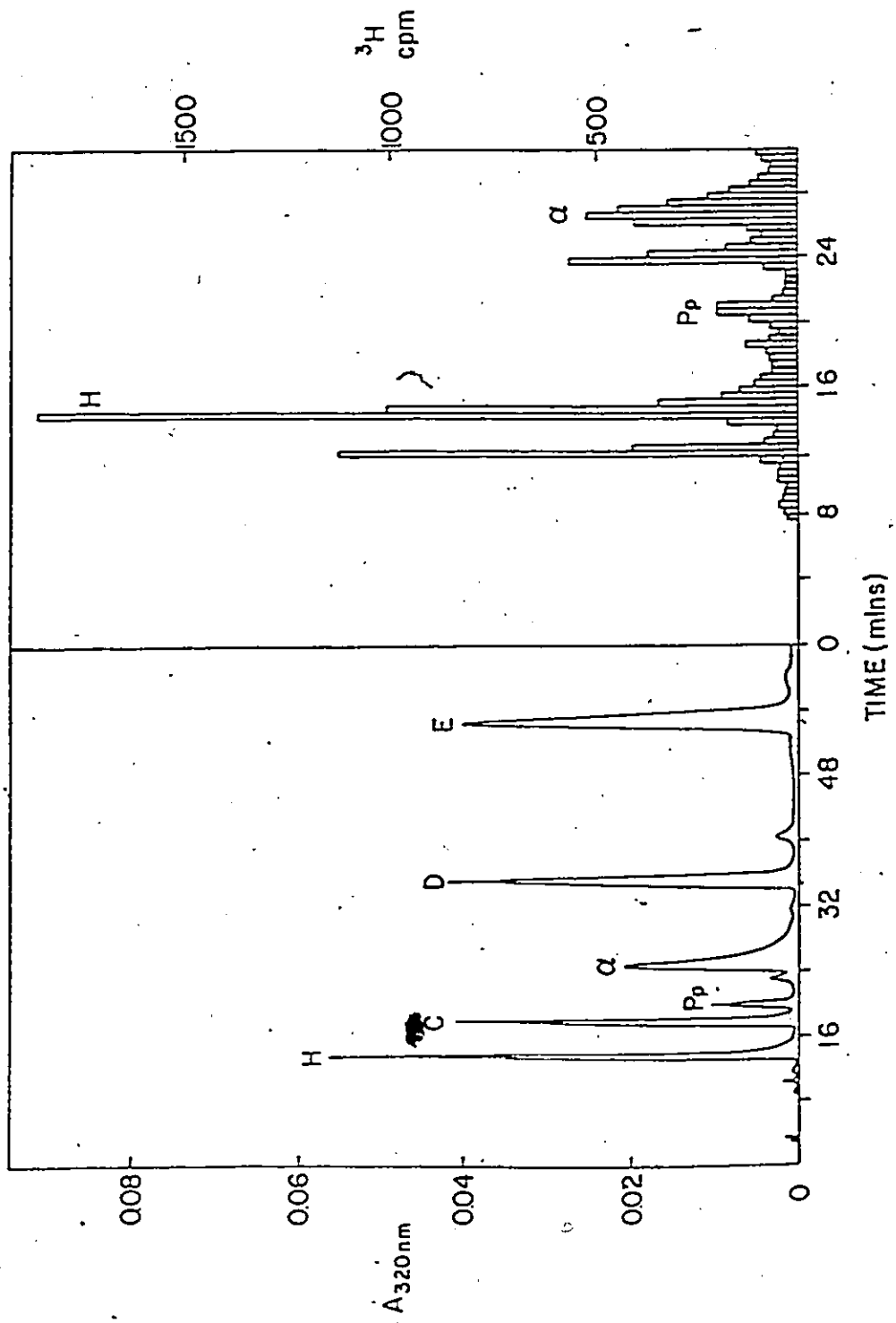

^{3}H cpm
1500
1000
500
α
Pp
H
24
16
8
0
TIME (mins)
E
D
α
Pp
C
H
48
32
16
0
0.08
0.06
0.04
0.02
0
A_{320nm}

Figure 30: Reactivity-pH profile for the imidazole function of the N-terminal histidine residue of glucagon obtained in the preliminary glucagon experiments using HPLC separation. The solid line is the theoretical titration curve obtained by non-regression analysis of the experimental data and has a pK_a value of 7.84.

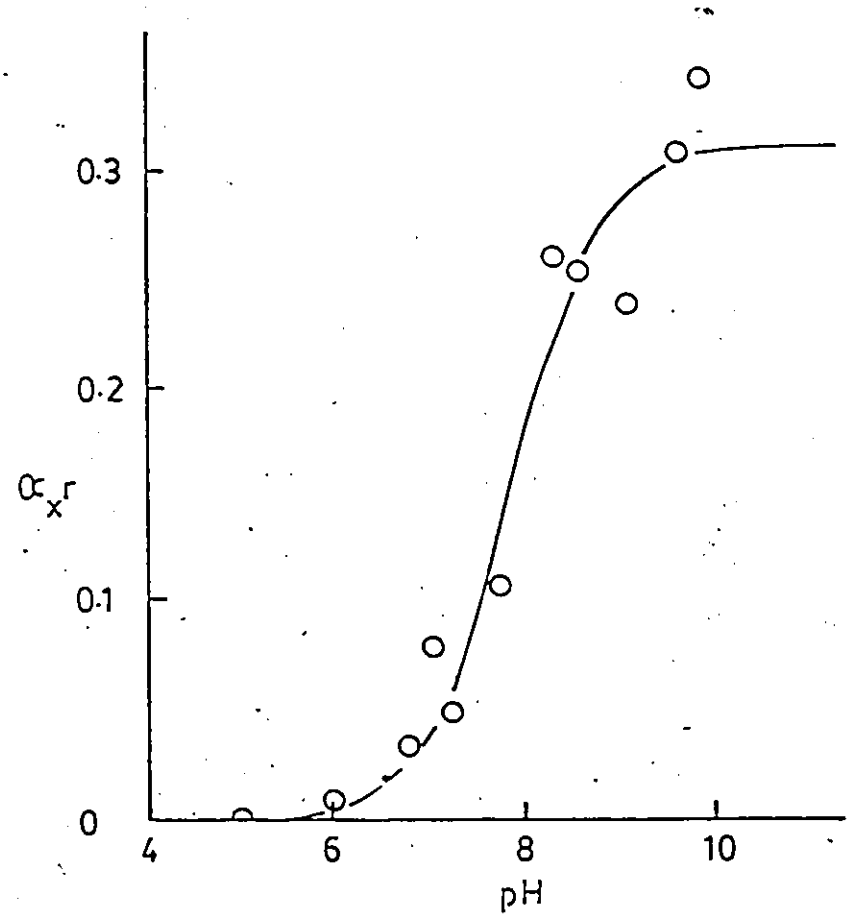

0.3
0.2
0.1
0
$\alpha_x r$
4
6
8
10
pH

Figure 31: Reactivity-pH profile for the α-amino function of the N-terminal histidine residue of glucagon obtained in the preliminary glucagon experiments using HPLC separation. The solid line is the theoretical titration curve obtained by non-linear regression analysis of the experimental data and has a pK_a value of 7.98.

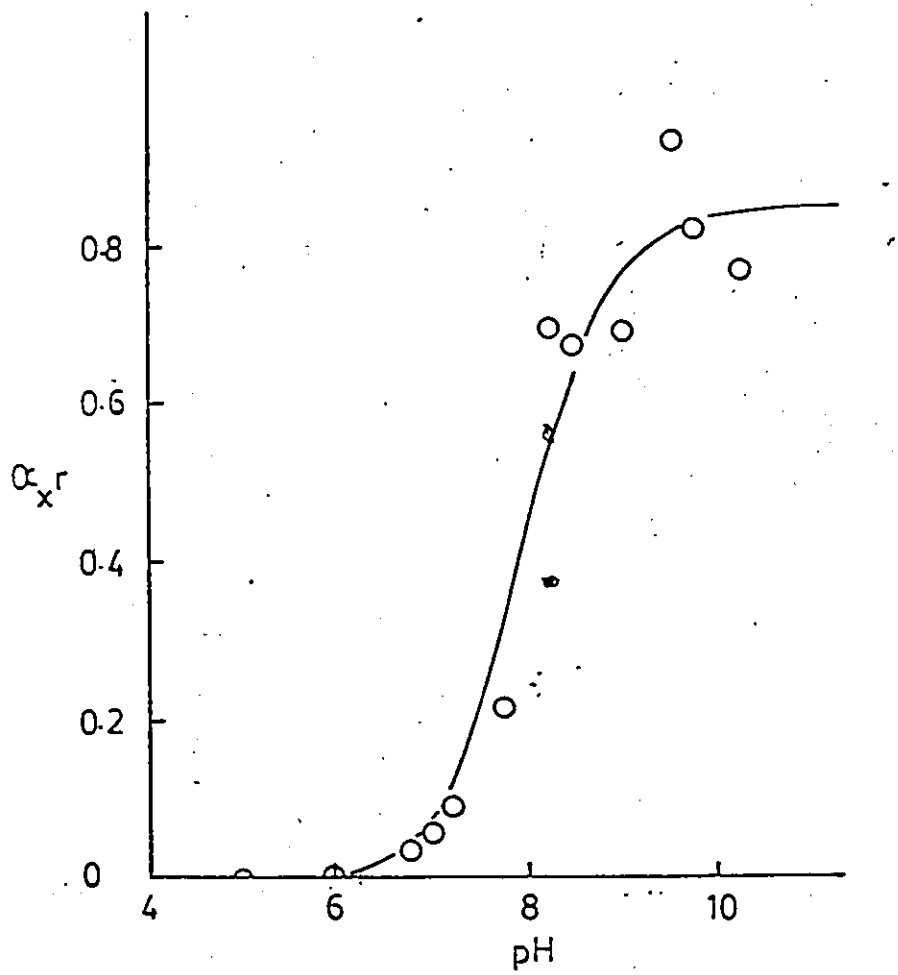

0·8
0·6
α_{xr}
0·4
0·2
0
4
6
8
10
pH

Figure 32: Reactivity-pH profile for the ϵ-amino group of lysine-12 in glucagon obtained in the preliminary glucagon experiments using HPLC separation. The solid line is the theoretical titration curve obtained by non-linear regression analysis of the experimental data and has a pK_a value of 8.46.

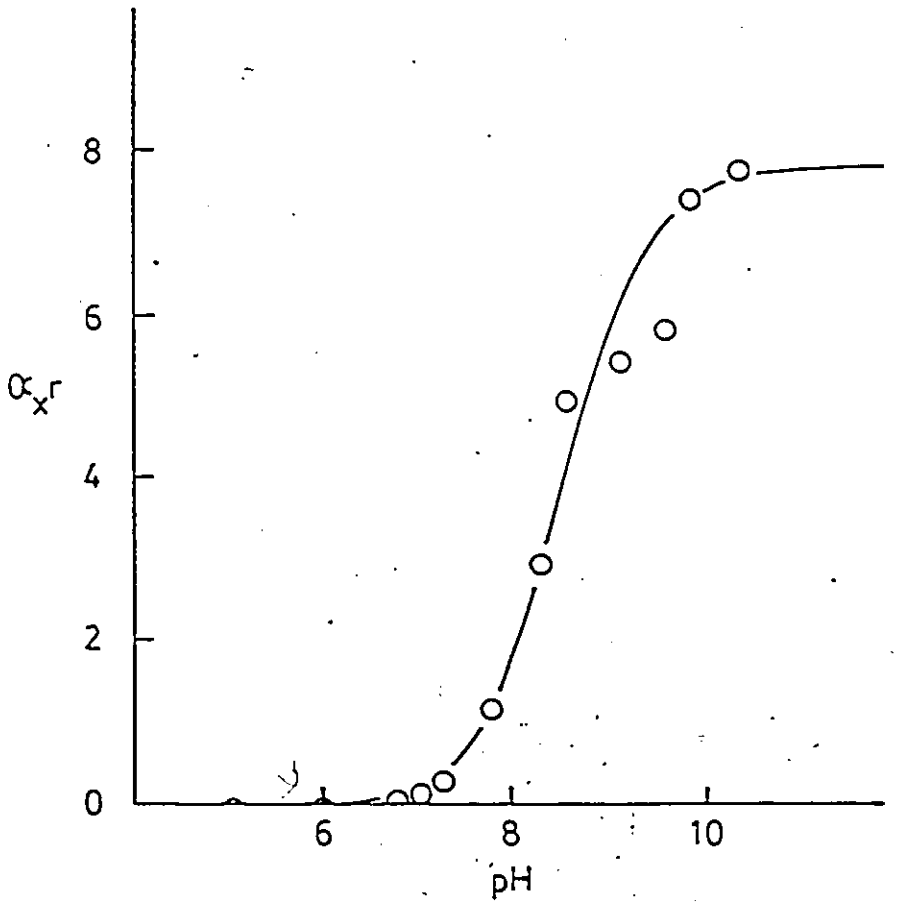
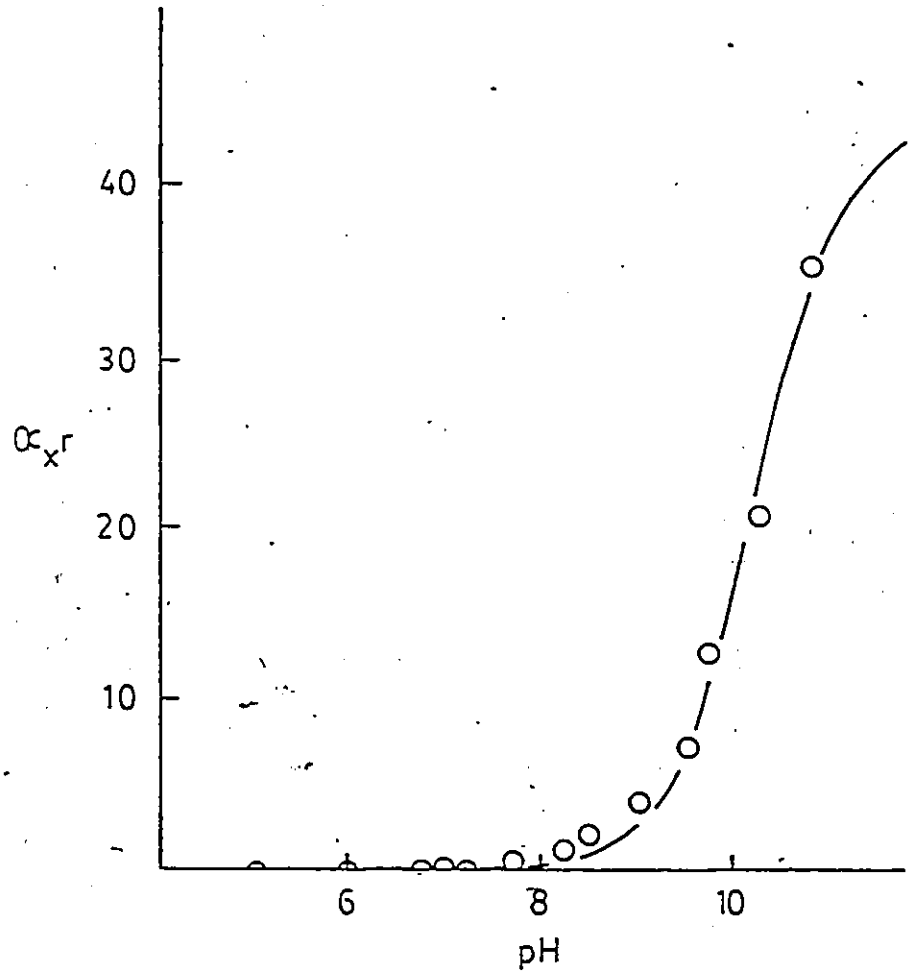

8
6
4
2
0
α_{xr}
6
8
10
pH

Figure 33: Dependence of the average reactivity of tyrosine-10 and tyrosine-13 upon pH obtained in the preliminary glucagon experiments using HPLC separation. The solid line is the theoretical titration curve obtained by non-linear regression analysis of the experimental data and has a pK_a value of 10.22.


40
30
20
10
α_{xr}
6
8
10
pH

imidazole, α-amino, ε-amino and phenolic hydroxyl functions in glucagon obtained in this preliminalry study. As in the case of histidyl-glycine, the imidazole moiety of the N-terminal histidine residue of glucagon gives a reactivity-pH profile which is similar to that of the α-amino function and is indicative of an ionization with a much higher pK_a than that expected from conventional acid-base titration (Rothgeb et al., 1978; Epand et al., 1973). More data points in the lower pH range, however are necessary to state definitely that the first ionization at a lower pH value seen by titration is not also reflected in the reactivity-pH profile.

Figure 33 shows that the average $\alpha_x r$ values obtained for the two tyrosine residues in glucagon lie very close to a theoretical titration curve with a pK_a of 10.22 and a reactivity of 45. The data for the lysine shown in Figure 33 give a pK_a value of 8.46 — an unusually low pK_a value for an ε-amino function in a protein. A low pK_a value has also been observed for the lysine in associated forms of insulin (Chan et al., 1981; Sheffer and Kaplan, 1979). The slight scatter of data points about the top of the theoretical titation curve may be indicative of a second ionization closer to the pK_a expected for this type of group. Again, more data points are necessary to examine this possibility.

More complete studies of the chemical properties of the functional groups in glucagon and those in the N-terminal histidine polypeptides VIP and secretin were undertaken using the modified competitive labelling method shown in Figure 8. The results of these studies are shown in Tables 13, 14, and 15. The parameters calculated by non-linear regression analysis of these data as well as those for histidylglycine are summarized in Table 16. In all polypeptides, the pK_a of the imidazole moiety of the N-terminal histidine residue as determined by competitive labelling was higher than that expected for such a group on the basis of titration and was very similar to the pK_a values for the α-amino function of the same residue.

Reactivity-pH profiles for the imidazole (Figure 34) and the α-amino (Figure 35) functions of the N-terminal histidine residue of glucagon are very similar to those obtained in the preliminary study of glucagon. There is no indication of an ionization at the lower pK_a

TABLE 13: Summary of the Reactivity Data for the Glucagon Free Monomer

pH	$^{3}H/^{14}C$				$\propto_s$	$\propto_x r$		
	im-Iac	im-His	N-His	ϵ-Lys		im-His	N-His	ϵ-Lys
5.00	14.7	25.0	155	21.3	0.00530	0.00901	0.0575	0.00766
5.25	16.2	35.9	137	42.0	0.00946	0.0210	0.0813	0.0245
5.50	21.2	66.9	88.9	20.9	0.0167	0.0527	0.0739	0.0165
5.75	16.9	48.5	92.1	50.9	0.0293	0.0841	0.160	0.0883
6.00	13.6	32.7	39.8	11.2	0.0597	0.144	0.171	0.0492
6.25	7.67	9.53	24.9	16.2	0.0872	0.108	0.284	0.185
6.50	14.9	23.0	56.9	23.6	0.145	0.224	0.547	0.230
6.75	15.6	22.8	58.7	26.6	0.232	0.339	0.873	0.396
7.00	15.2	21.8	56.2	29.1	0.349	0.501	1.31	0.670
7.25	13.0	14.5	54.8	25.1	0.488	0.542	2.02	0.943
7.50	13.7	25.9	48.8	36.4	0.629	1.19	2.21	1.67
7.75	17.9	39.5	54.7	45.9	0.751	1.66	2.37	1.92
8.00	14.3	29.1	59.9	47.5	0.843	1.72	3.58	2.80
8.25	12.2	19.6	56.2	37.0	0.905	1.42	4.38	2.75
8.50	13.2	23.3	60.2	54.3	0.944	1.66	4.48	3.88
8.75	10.9	24.5	51.8	13.3	0.968	2.15	4.60	0.490*
9.00	8.74	17.7	44.5	60.2	0.982	1.84	5.00	6.20
9.25	12.8	23.5	65.3	88.6	0.990	1.81	5.09	6.84
9.50	17.1	43.3	86.0	124	0.994	2.41	5.00	6.90
9.75	14.5	28.1	77.6	141	0.997	1.93	5.41	9.72
10.00	50.6	150	110	196	0.998	2.96	2.31	3.86
10.25	26.6	70.2	57.3	231	0.999	2.64	2.27	8.67
10.50	19.8	36.8	44.5	202	0.999	1.86	2.18	10.2
10.75	25.0	40.7	80.7	165	1.00	1.63	3.14	5.58
11.00	19.9	28.4	96.3	143	1.00	1.42	5.17	7.18

TABLE 14: Summary of the Reactivity Data for Vasoactive Intestinal Peptide

pH	$^{3}H/^{14}C$		α_s(lac)*	α_{xr}(lac)*	$^{3}H/^{14}C$			$\alpha_s(ala_2)$*	$\alpha_{xr}(ala_2)$*	
	im-lac	im-His		im-His	Ala_2	N-His	ϵ-Lysx4		N-His	ϵ-Lysx4
5.00	–	–	–	–	5.13	1.01	5.20	0.000909	0.000179	0.000921
5.50	0.325	1.08	0.0167	0.0550	2.84	0.899	2.82	0.00287	0.000910	0.00286
6.00	0.440	1.41	0.0597	0.192	1.97	1.77	3.59	0.00902	0.00808	0.0164
6.50	0.559	1.71	0.145	0.446	2.26	1.83	4.73	0.0280	0.0169	0.0587
7.00	0.614	2.69	0.349	1.53	3.78	2.50	6.21	0.0834	0.0553	0.137
7.25	1.58	3.41	0.488	1.06	4.02	3.01	5.98	0.139	0.104	0.207
7.50	1.47	3.64	0.629	1.56	5.50	3.30	10.1	0.223	0.134	0.411
7.75	1.39	1.93	0.751	1.04	6.19	3.50	12.2	0.338	0.191	0.666
8.00	0.897	2.20	0.843	2.06	5.66	2.57	16.5	0.476	0.216	1.39
8.25	1.76	3.12	0.905	1.61	5.62	1.95	19.1	0.618	0.214	2.10
8.50	5.26	18.2	0.944	3.28	4.77	2.53	25.2	0.742	0.394	3.92
8.75	4.22	9.18	0.968	2.10	4.48	2.10	33.3	0.837	0.392	6.23
9.00	1.22	3.11	0.982	2.51	4.47	1.96	24.9	0.901	0.395	7.45
9.25	1.47	5.16	0.990	3.47	4.47	1.52	37.2	0.942	0.321	7.85
9.50	1.61	4.20	0.994	2.59	3.64	2.24	32.7	0.966	0.595	8.70
9.75	1.27	4.01	0.997	3.41	4.37	1.27	36.2	0.981	0.286	8.12
10.00	2.52	5.10	0.998	2.03	3.14	1.04	34.5	0.989	0.327	10.8
10.5	1.64	4.25	0.999	2.59	2.67	1.22	23.3	0.996	0.453	8.70
11.0	0.876	1.79	1.00	2.04	1.18	0.710	8.47	0.999	0.601	7.11

* internal standard nucleophile used: (lac), L-β-imidazole lactic acid; (ala_2), alanylalanine

TABLE 15: Summary of the Reactivity Data for the N-Terminal Histidine Residue of Secretin

pH	$^{3}H/^{14}C$			α_s	$\alpha_x r$	
	Ala_2	N-His	im-His		N-His	im-His
5.00	1.42	1.12	0.345	0.000909	0.000717	0.000221
5.50	0.754	1.79	1.42	0.00287	0.00681	0.00539
6.00	0.684	2.22	1.85	0.00902	0.0293	0.0244
6.50	1.50	3.55	2.47	0.0280	0.0663	0.0460
7.00	3.15	2.03	2.15	0.0834	0.0537	0.0570
7.25	2.43	2.82	2.08	0.139	0.161	0.119
7.50	4.62	1.26	1.44	0.223	0.0608	0.0696
7.75	2.52	2.07	5.26	0.338	0.278	0.705
8.00	3.56	10.4	1.97	0.476	1.39	0.263
8.25	6.44	4.87	8.34	0.618	0.467	0.801
8.50	4.36	4.56	2.28	0.742	0.776	0.388
8.75	3.43	2.68	1.98	0.837	0.654	0.484
9.00	2.82	3.69	3.81	0.901	1.18	1.22
9.25	3.43	6.76	2.80	0.942	1.86	0.768
9.50	2.91	3.51	2.85	0.966	1.94	0.945
9.75	2.27	3.81	3.09	0.981	1.65	1.33
10.00	2.57	4.29	--	0.989	1.65	--
10.50	9.44	2.66	--	0.996	0.281	--
11.00	1.26	1.94	--	0.999	1.54	--

TABLE 16: Summary of the Chemical Properties of Amino and Imidazole Groups*

FUNCTIONAL GROUP	pK_a-Acid-Base Titration	pK_a-Competitive Labeling		Reactivity	
		(ala-ala)[a]	(im-lac)[a]	(ala-ala)[a]	(im-lac)[a]
Alanylalanine					
α-amino	8.03±0.008	-	8.02±0.03	-	25.4±0.5
Histidylglycine					
α-amino	7.69±0.02	7.64±0.07	7.49±0.06	0.413±0.017	10.75±0.03
imidazole	5.85±0.01	-	7.16±0.07	0.041[c]	1.06±0.03
Glucagon					
α-amino (his-1)	7.23[b]	-	7.60±0.04	0.202[c]	5.13±0.11
imidazole (his-1)	5.23[b]	-	7.43±0.09	0.080[c]	2.02±0.08
ε-amino (lys-12)	-	-	8.49±0.09	0.338[c]	9.66±0.52
VIP					
α-amino (his-1)	-	7.88±0.18	-	0.42 ±0.04	10.16[c]
imidazole (his-1)	-	-	7.52±0.18	0.111[c]	2.81±0.22
ε-amino (average of four)	-	8.63±0.06	-	2.4 ±0.1	60.96[c]
Secretin					
α-amino (his-1)	-	8.83±0.12	-	2.13±0.18	54.10[c]
imidazole (his-1)	-	8.24±0.24	-	1.07±0.14	27.18[c]

a. internal standard nucleophile used.

b. value obtained by Rothgeb et al. (1978).

c. value calculated based on a reactivity of 25.4±0.5 for alanylalanine (ala-ala) relative to β-L-imidazole lactic acid (im-lac).

* The values of pK_a and reactivity and their standard errors were calculated by fitting the data to theoretical titration curves using non-linear least-squares regression procedure.

Figure 34: Reactivity-pH profile of the imidazole function of the N-terminal histidine residue of glucagon. The solid line is the theoretical titration curve with a pK_a of 7.43 and a reactivity of 2.02 relative to L-β-imidazole lactic acid.

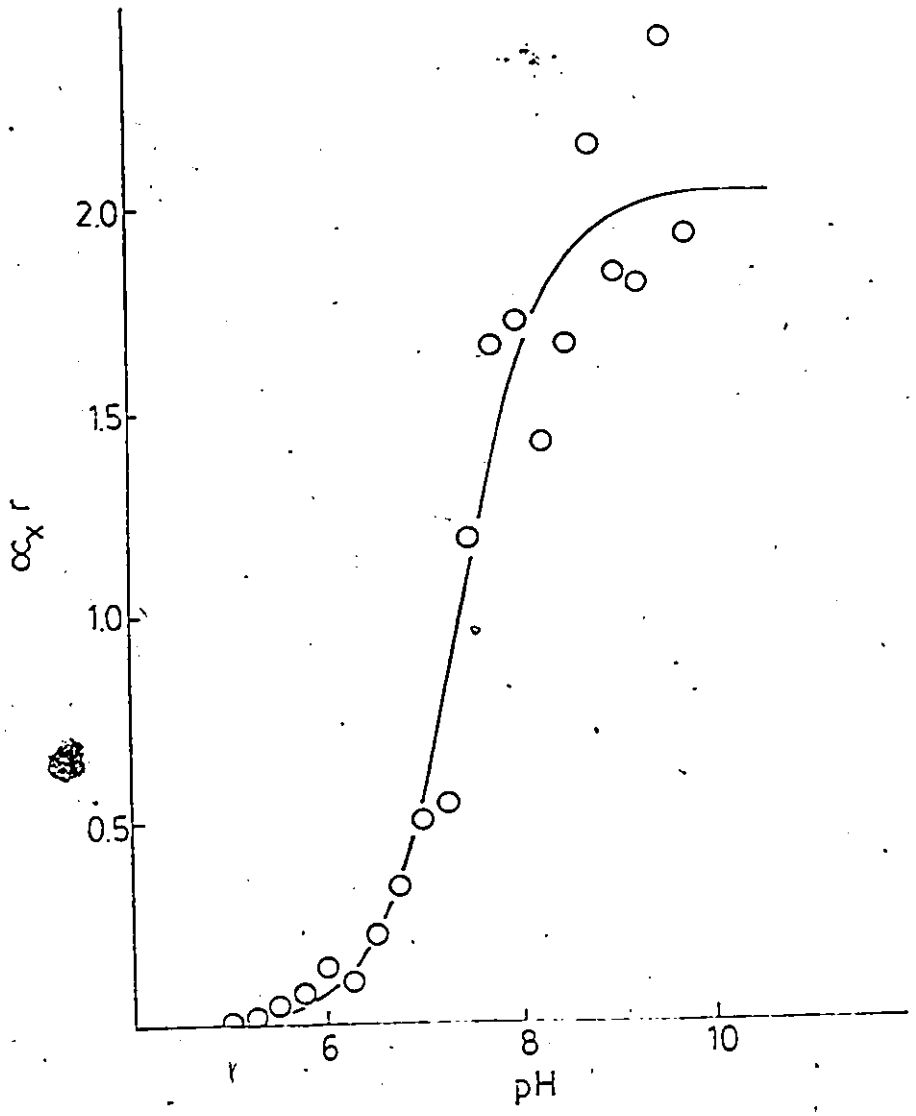
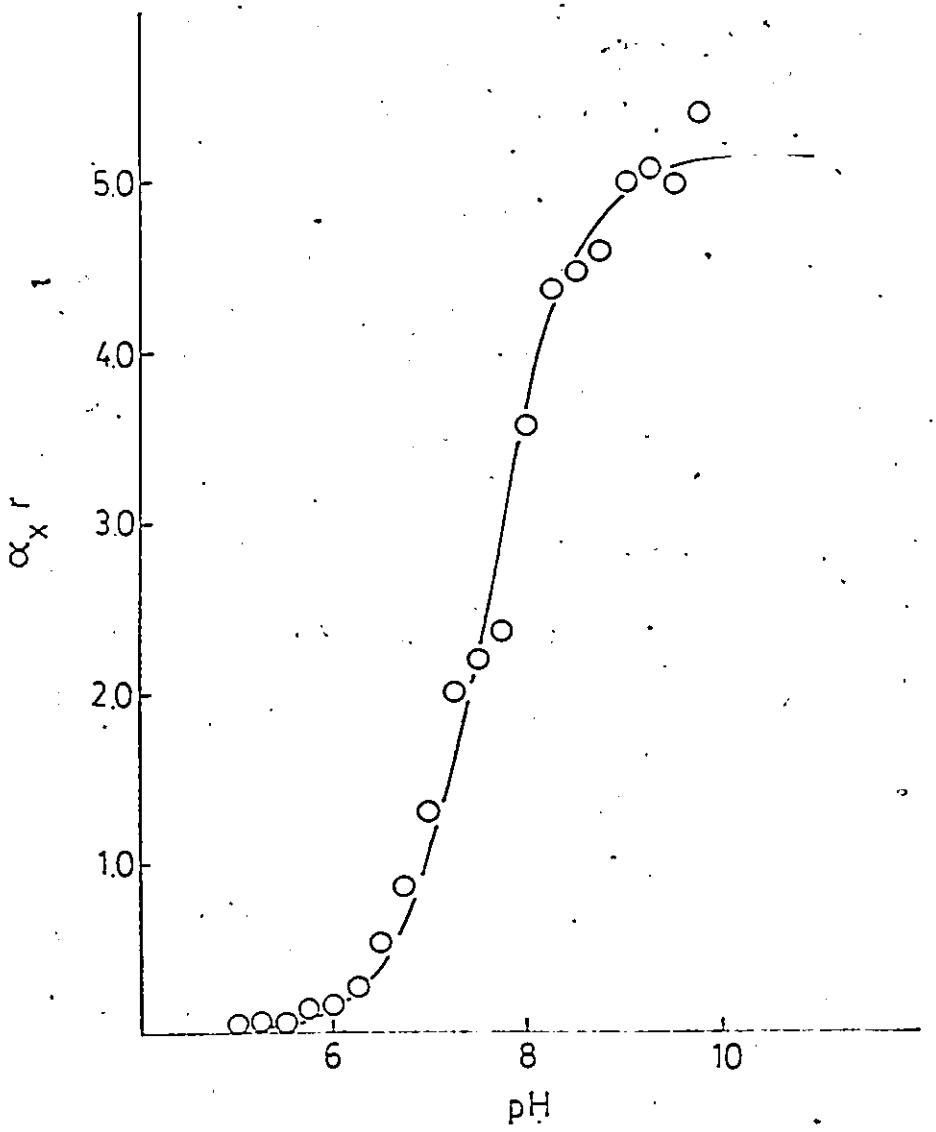

2.0
1.5
1.0
0.5
α_x r
6
8
10
pH

Figure 35: Reactivity-pH profile of the α-amino group of the N-terminal histidine residue of glucagon. The solid line is the theoretical titration curve with pK_a of 7.60 and a reactivity of 5.13 relative to L-β-imidazole lactic acid.


5.0
4.0
3.0
2.0
1.0
α_x r
6
8
10
pH

value expected from acid-base titration. The data for the solitary lysine at residue 12 gives excellent fit to a theoretical titration curve with a pK_a of 8.49 and a reactivity of 9.66 relative to imidazole lactic acid (Figure 36). The low pK_a observed for this residue in the preliminary study is thus confirmed; the possibility of a second ionization can be ruled out. The preliminary finding for the two tyrosine residues could not be confirmed as the O-DNP-tyrosine derivatives could not be separated from a breakdown product of N-α-DNP-histidine in the electrophoretic separations used in this procedure.

Reactivity-pH profiles for the α-amino, imidazole and ε-amino functions of VIP are shown in Figures 37, 38, and 39, respectively. Again the change in the reacivity of the imidazole group with pH parallels that of the α-amino group. The reactivity-pH profile of the lysine residues shows good fit to a theoretical titration curve with a pK_a value of 8.63 and a reactivity of 2.4 relative to alanylalanine. The low pK_a value for lysine is reminiscent of that observed in glucagon but is perhaps even more unusual in VIP as it represents an average value for the four lysine residues in this polypeptide.

The α-amino and imidazole functions of secretin (Figures 40 and 41) give pK_a values which are approximately one pK unit higher than those obtained for the other N-terminal histidine residues studied (Table 16). The results obtained are similar to those obtained for the other polypeptides studied in that the reactivity-pH profile of the imidazole function very closely parallels that of the α-amino function.

While the pK_a values of the imidazole and α-amino functions are close to one another in all peptides studied, the reactivities of these groups are very different. In histidylglycine, the α-amino group is approximately an order of magnitude more reactive than the imidazole; in glucagon and VIP it is approximately four times more reactive than the imidazole group; and in secretin, a factor of 1.3 more reactive. The reactivity of the imidazole function in histidylglycine towards FDNB is the same as that of imidazole lactic acid while the imidazole functions in glucagon and VIP are approximately two- and three-fold higher, respectively. In secretin, the reactivity of this group is about forty times that seen with imidazole lactic acid. Although the imidazole moiety of secretin exhibits a higher pK_a value than the

Figure 36: Reactivity-pH profile for the ϵ-amino group of lysine-12 in the glucagon free monomer. The solid line is the theoretical titration curve with a pK_a of 8.49 and a reactivity of 9.66 relative to L-β-imidazole lactic acid.

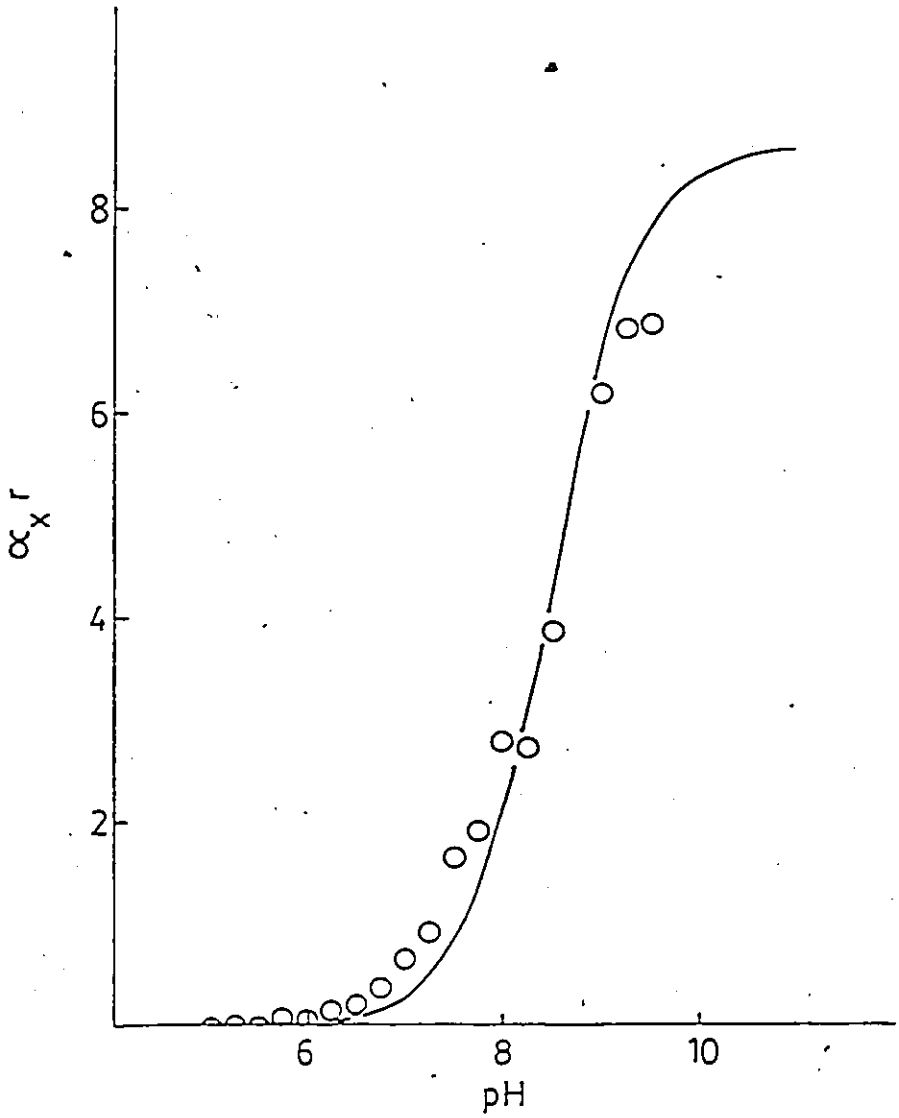

8
6
4
2
$\alpha_x r$
6
8
10
pH

Figure 37: Reactivity-pH profile of the α-amino group of the N-terminal histidine residue of VIP. The solid line is the theoretical titration curve with a pK_a of 7.88 and a reactivity of 0.42 relative to alanylalanine.

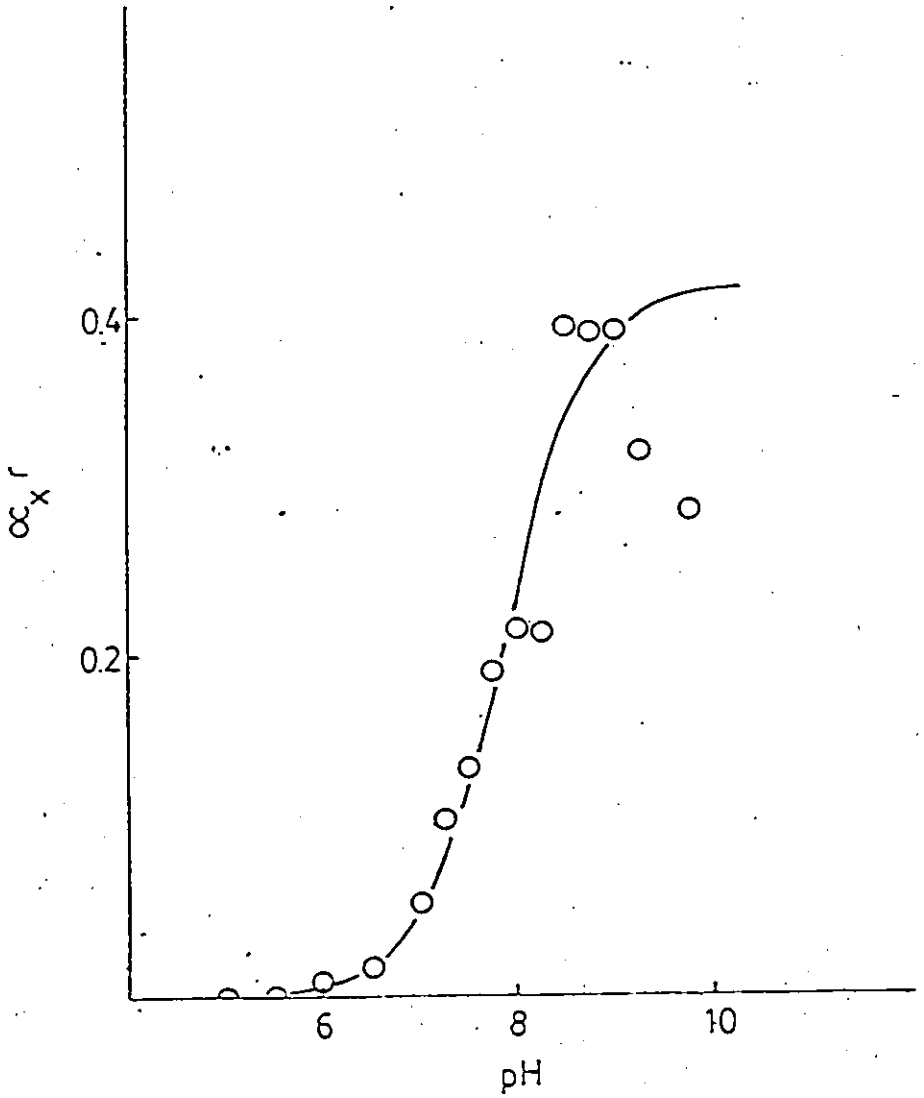

0.4
0.2
α_x r
6
8
10
pH

Figure 38: Reactivity-pH profile of the imidazole function of the N-terminal histidine residue of VIP. The solid line is the theoretical titration curve with a pK_a of 7.52 and a reactivity of 2.81 relative to L-β-imidazole lactic acid.

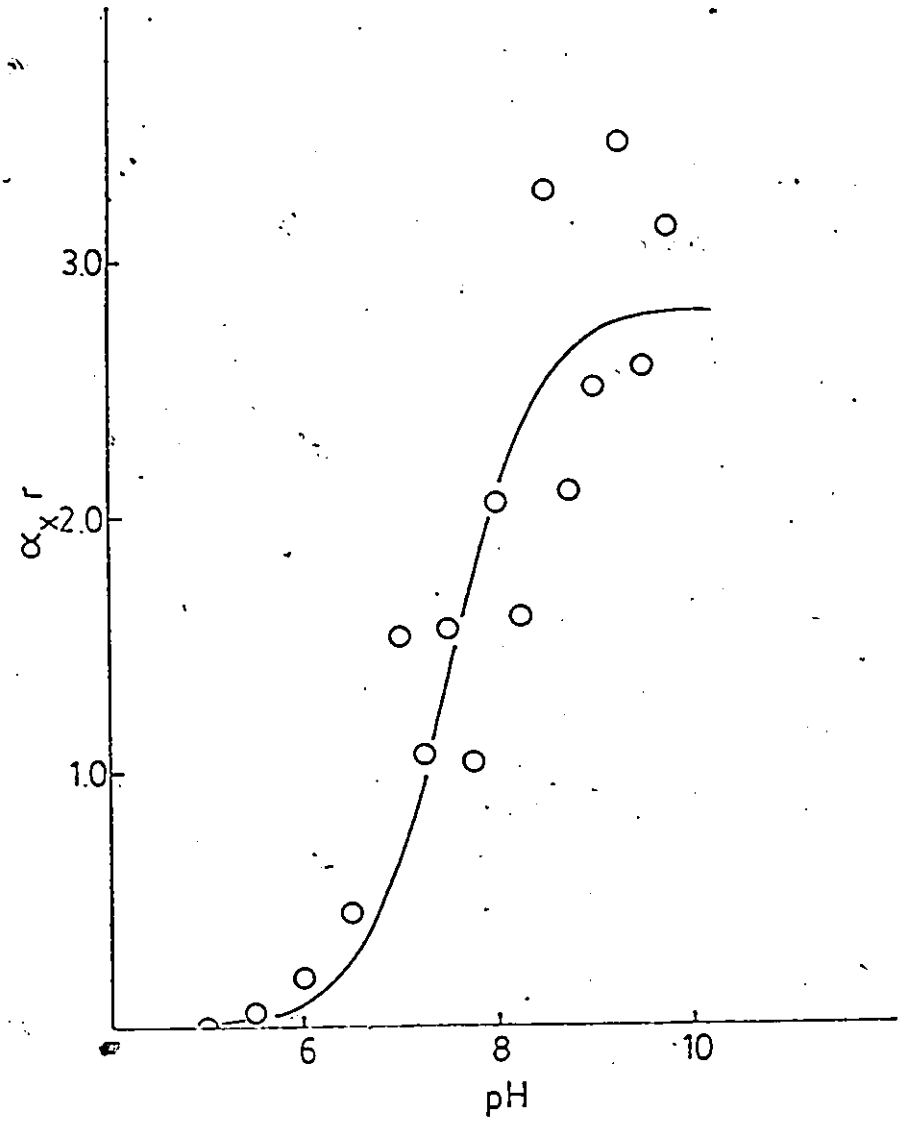

3.0
2.0
1.0
$\alpha_{\times r}$
6
8
10
pH

Figure 39: Dependence of the average reactivity of the four lysine residues of VIP upon pH. The solid line is the theoretical titration curve with a pK_a of 8.63 and a reactivity of 2.4 relative to alanylalanine.

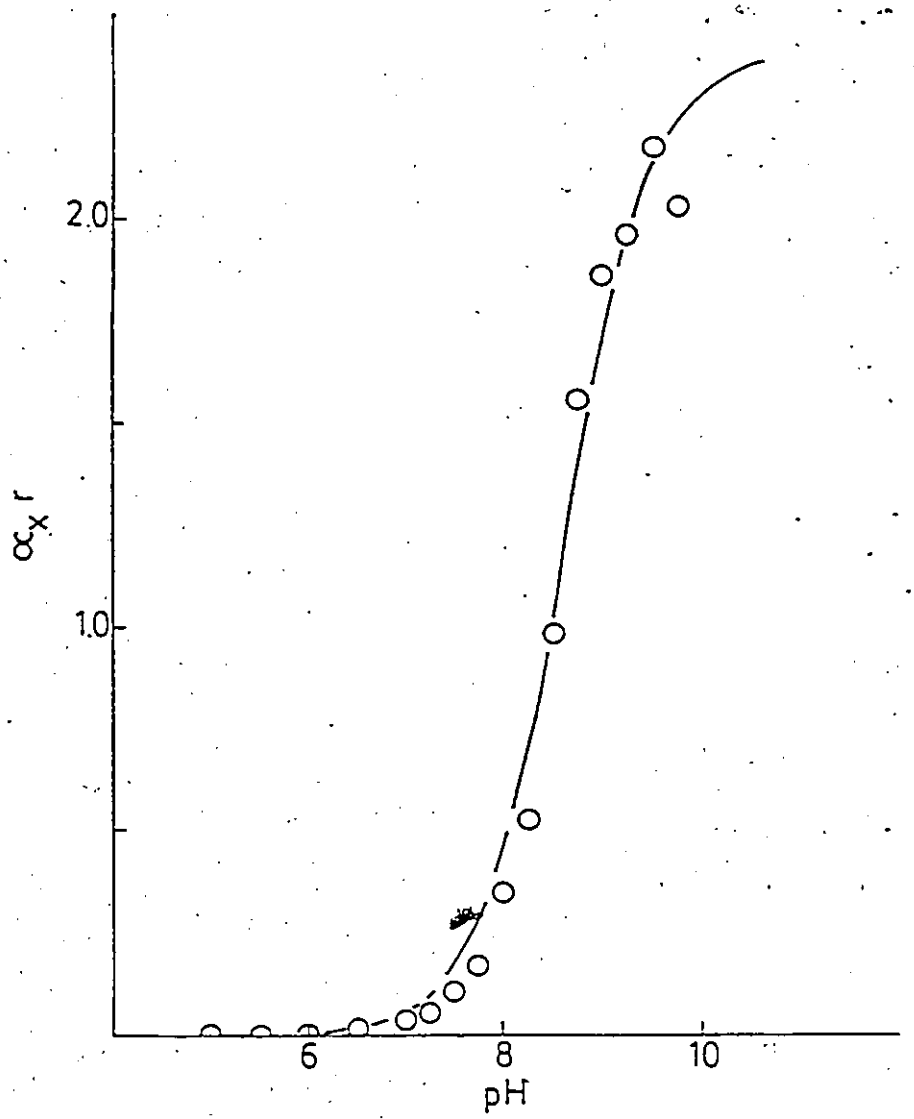

2.0
1.0
α_x r
6
8
10
pH

Figure 40: Reactivity-pH profile for the α-amino function of the histidine residue in secretin. The solid line is the theoretical titration curve with a pK_a of 8.83 and a reactivity of 2.13 relative to alanylalanine.

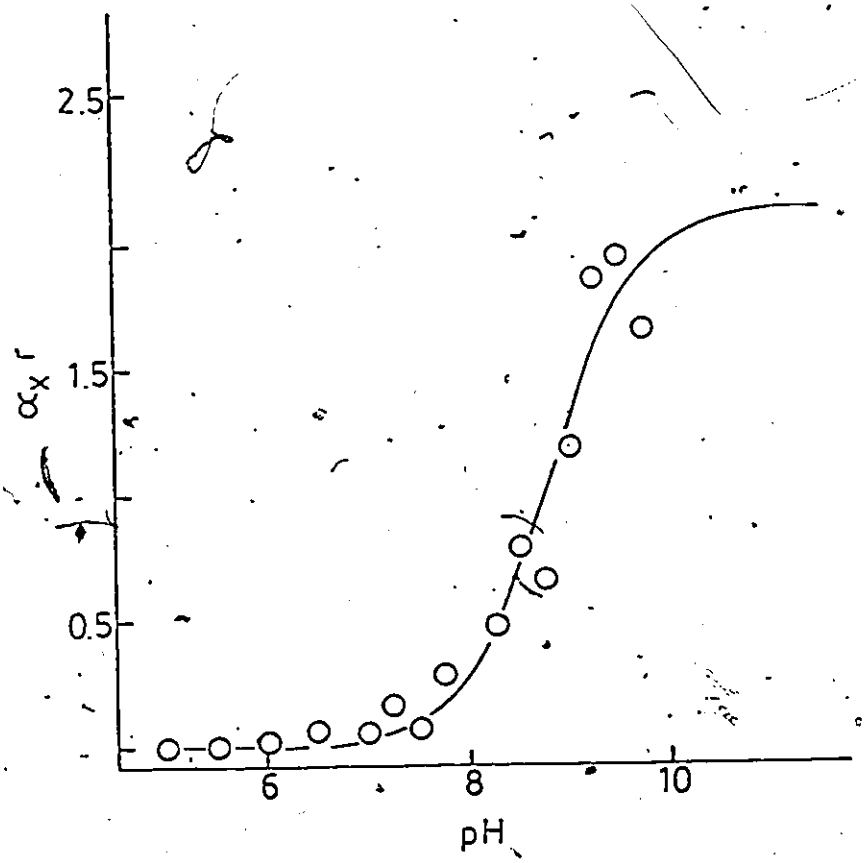
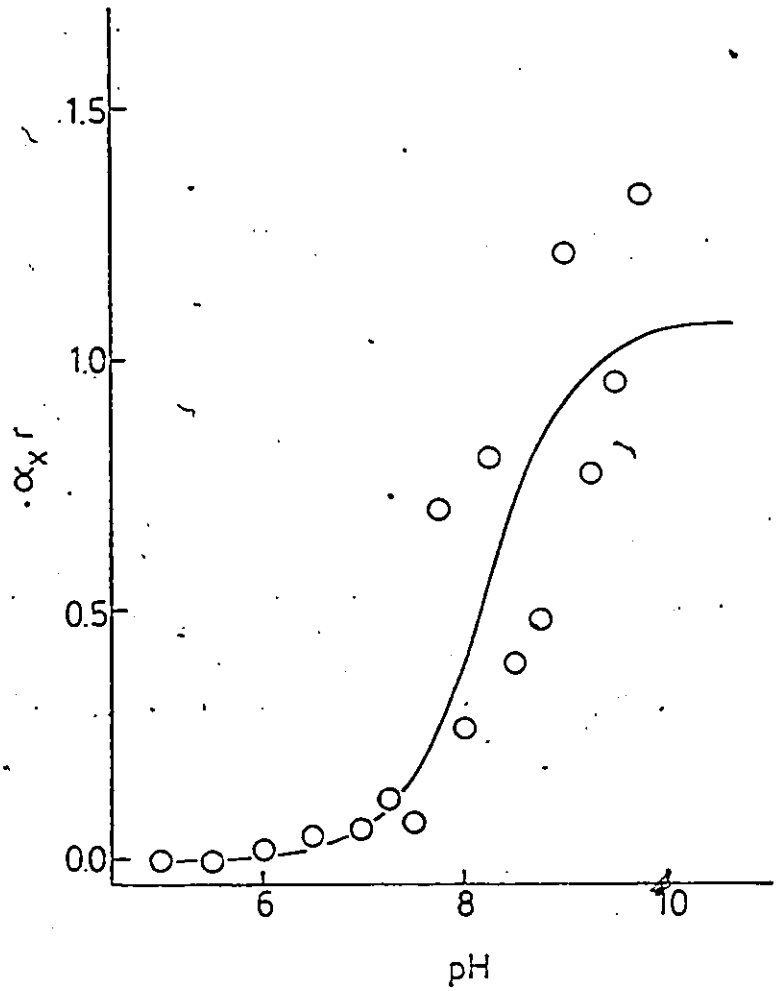

2.5
1.5
0.5
$\alpha\chi^r$
6
8
10
pH

Figure 41: Reactivity-pH profile for the imidazole function for the N-terminal histidine residue in secretin. The solid line is the theoretical titration curve with a pK_a of 8.24 and a reactivity of 1.07 relative to alanylalanine.


1.5
1.0
0.5
0.0
α_{X^r}
6
8
10
pH

corresponding group in the other peptides studied, the increase in reactivity of this group is greater that that which would be expected solely on the basis of its increased pK_a.

The α-amino groups in histidylglycine, VIP and secretin have approximately normal reactivities for their pK_a values on the basis of a Brønsted relationship, whereas, the same group in glucagon shows about half the expected reactivity. Similarily the lysine residues in VIP appear to have "normal" reactivities despite their abnormally low pK_a value; that of glucagon is abnormal in that it has a low pK_a and is less reactive than a standard amine with the same pK_a that lies on the Brønsted curve (Figure 19).

DISCUSSION

Insulin: Concentration-Dependence. In an effort to elucidate structure-function relationships in proteins, a variety of physical and chemical techniques have been developed. Of these, x-ray crystallography has, perhaps, held the most attention and, at present, provides the best basis for postulating the mechanism of action of the protein in question. Because the conditions under which proteins are generally crystallized (extremes of pH, high ionic strength, the presence of heavy metals) bear little resemblance to those in biological systems, one could argue, however, that the structure seen in the crystal is different from that in solution. In cases where the protein under study displays a concentration dependent self-association pattern, the physiological relevance of x-ray crystal data is even more questionable. On the basis of the x-ray crystal structure of insulin, several researchers have proposed functional roles for various regions of the insulin molecule. It is therefore essential to determine whether the monomeric unit seen in the crystal accurately reflects the structure of the free insulin monomer in solution. At present, there is conflicting evidence based on circular dichroism in this regard. Blundell and Wood (1975) conclude that all changes in the CD spectrum seen upon the dilution of insulin can be explained by the removal of zinc and/or residues of adjacent monomers and that the conformation of the monomeric unit remains unchanged on dissociation. Pocker and Biswas (1980, 1981), on the other hand, maintain that there are substantial changes to the secondary structure of insulin as its concentration in solution is decreased.

Previous studies (Kaplan et al., 1982; Kraal and Hartley, 1978; Bresciani, 1977) have demonstrated that, since the chemical properties of functional groups in proteins are dependent on their microenvironment, the competitive labelling approach can be used as a sensitive probe of conformational change. The study of the free monomer of insulin by traditional methods is technically difficult due to the low concentration of protein which must be employed. The results of the present study show that the described modifications to the competitive labelling procedure make it possible to determine the chemical

properties and, by inference, the structural properties of proteins at high dilutions.

Insulin exhibits a complex, concentration-dependent association pattern (Blundell et al., 1972). Various models have been proposed to account for the observed apparent weight-average molecular weight obtained in sedimentation equilibrium studies (Milthorpe et al., 1977; Goldman and Carpenter, 1974; Pekar and Frank, 1972; Jeffrey and Coates, 1966). All models allow for free monomer, dimer and hexamer with the possibility of higher molecular weight forms. In these studies the lowest concentration employed was approximately $1x10^{-5}$M and a dimer dissociation constant of the order of 10^{-5}M was determined. On the basis of these studies it would appear that, at neutral pH values, the free monomeric form of insulin predominates at concentrations below $1x10^{-6}$M. At higher concentrations, determination of the proportions of associated forms, i.e. dimer, tetramer, hexamer and higher oligomers, is dependent on the particular association model chosen for analysis of the sedimentation data.

Competitive labelling of insulin at concentrations where associated forms predominate (Chan et al., 1981; Sheffer and Kaplan, 1979) demonstrate that several of the functional groups in insulin have unusual chemical properties. The amino terminal phenylalanine of the B-chain has an abnormally low pK_a and was found to be "super-reactive" towards both acetic anhydride at 10°C and fluorodinitrobenzene at 20°C when compared to standard amino compounds by means of a Brønsted relationship. The A-chain glycine amino terminus, while exhibiting normal ionization behaviour, showed enhanced reactivity towards both labelling reagents. The B29-lysine residue, on the other hand, has an unexpectedly low pK_a and less reactivity towards acetic anhydride than a corresponding standard amino compound with the same pK_a. When fluorodinitrobenzene (FDNB) was used as the electrophilic labelling reagent, the two histidine residues showed an average reactivity that was an order of magnitude greater than that of the internal standard L-β-imidazole lactic acid while the average reactivity of the four tyrosine residues was substantially less than that of a free phenolic group (Chan et al., 1981). Preliminary studies in Pyrex glass test tubes using acetic anhydride as the electrophilic reagent and phenylalanine as

the internal standard (Table 3) suggest that the reactivities of all the amino groups of insulin are concentration dependent: all groups show high reactivity at a concentration of $2x10^{-4}$M and low reactivity at concentrations of $1x10^{-5}$M and $5x10^{-6}$M. In adddition, none of the groups exhibits the titration behaviour expected for free amines in solution.

The initial experiment in the present series is an attempt to examine more fully the concentration-dependence of the functional groups in insulin using fluorodinitrobenzene as the chemical probe and L-β-imidazole lactic acid as the standard nucleophile. As one can see from the data in Figure 9, all groups show substantial reactivity at the higher insulin concentrations but are non-reactive at concentrations where the free monomeric form of insulin predominates. As expected from the previous studies, the amino and imidazole functions show high reactivity at high concentrations of insulin. The reactivities of the glycine and phenylalanine amino termini drop sharply as the insulin concentration is decreased from $5x10^{-5}$M to $1x10^{-5}$M. Although the effect is less dramatic, the reactivity of the ε-amino group at lysine-B29 also decreases in this concentration range. These results are consistent with previous indications that the reactivity of some of the functional groups of insulin is concentration-dependent under the conditions used in these studies. The fact that at high dilution an extremely low reactivity was observed toward two reagents using different internal standards, i.e. acetic anhydride with phenylalanine and FDNB with L-β-imidazole lactic acid, which have substantially different chemical and physical properties, demonstrates that the observed decreases in reactivity are unlikely to be due to specific interactions of the labelling reagents with insulin in any of its forms.

On the basis of these preliminary studies and the sedimentation equilibirium data, one might be tempted to speculate that the dissociation of insulin oligomers to the free monomer is accompanied by a conformational change in the monomeric unit in which groups which are exposed and "super-reactive" become buried and virtually non-reactive. There is, of course, at least one other explanation which is consistent with the data: adsorption of the protein to the reaction vessels. Each of these initial studies was carried out in Pyrex glass reaction

vessels. Adsorption of insulin to glass containers has previously been reported (Hollenberg and Cuatrecasas, 1976) although the extent of adsorption was not quantified. Indeed, the sharp drop in the reactivities of the amino termini towards FDNB at insulin concentrations of approximately $5x10^{-5}M$ may be indicative of such an adsorption phenomenon. In order to distinguish these two possible scenarios i.e., conformational change and adsorption to the reaction vessel, the concentration-dependence of the reactivity of the functional groups of insulin was studied in Tefzel (Figure 10) and polystyrene (Figure 11) reaction vessels. FDNB was used as the chemical probe because it is possible to obtain data for the tyrosine and histidine groups in addition to the amino groups.

A comparison of the data obtained in polystyrene vessels (Figure 11) with that obtained in Pyrex glass (Figure 9) shows distinct differences in the patterns of the relative reactivities of the amino termini but not the other functional groups. In polystyrene, (Figure 11) the reactivity of the glycine A-chain amino terminus is unaffected by concentration until the insulin concentration is decreased below $10^{-6}M$ where the reactivity of this group becomes negligible relative to that of the standard nucleophile, L-β-imidazole lactic acid. This is in sharp contrast to Pyrex glass, where the reactivity of the glycine α-amino group all but vanishes at $10^{-5}M$ insulin (Figure 9). The data for the B-chain amino terminus, however, parallels that obtained with Pyrex glass in that the reactivity is constant at insulin concentrations above $5x10^{-5}M$ and declines at lower concentrations. This decrease is much more gradual in the polystyrene reaction vessel: at $1x10^{-6}M$ insulin, this group still shows appreciable reactivity. Below $1x10^{-6}M$ there is a sharp decrease in reactivity in all functional groups and, as in the case of Pyrex glass, all the relative reactivities approach zero. In both types of reaction vessel, the average reactivity of the two histidine residues decreases markedly on dilution even at insulin concentrations greater than $5x10^{-5}M$, while the reactivities of the amino termini are unchanged. This indicates that the microenvironment, and hence the chemical properties, of at least one of the two histidine residues in the associated monomeric unit is dependent upon the degree of association. When Tefzel vessels are used (Figure 10),

the change in reactive properties of the amino termini on dilution of insulin is intermediate to that observed in glass and polystyrene. Once again the histidine residues show a decline in reactivity at concentrations where the other groups maintain constant chemical reactivity.

It appears that, in each type of reaction vessel used, insulin is adsorbed to the surface of the container. This conclusion is based on the observations that, on dilution of insulin, all functional groups become non-reactive in all three types of containers but at different concentrations ($5x10^{-5}$M with glass, $1x10^{-5}$M with Tefzel and $1x10^{-6}$M with polystyrene). The concentration-dependence of the reactivity of the histidine residues in all three types of reaction vessels (Figures 9, 10 and 11) and the phenylalanine amino terminal in polystyrene (Figure 11) cannot be explained solely on the basis of an adsorption effect. If adsorption is the only effect, the reactivities of all groups should decrease simultaneously. These results therefore indicate that the microenvironments of at least one of the two histidine residues and of the phenylalanine amino terminus are dependent on the degree of association of insulin. It would be desirable to examine the effect of dissociation of insulin oligomers in the absence of any adsorption effects. Since adsorption was present to some degree in each type of reaction vessel despite the ostensible differences in chemical properties among the materials, the search for an adsorption-free container was abandoned. The alternate strategy, to interfere with the adsorption process, was adopted.

As a working hypothesis, one might envision that each type of reaction vessel has a certain number of sites to which the protein can reversibly bind. Each type of protein would have a characteristic affinity for these sites. At higher insulin concentrations all sites are occupied thereby removing a constant number of insulin molecules from the solution. If the number of sites is relatively small, at high concentrations of insulin, even full occupancy would not result in a significant decrease in the effective concentration of protein in solution. At lower protein concentration, one would expect, on the basis of LeChatelier's principle, that the occupancy would be decreased. This could, however, still represent a significant fraction

of the protein molecules in solution. In the worst case, if the affinity of the protein for sites was very high, almost complete occupancy might be possible even at low protein concentrations. The effective concentration would, therefore, be much less than the nominal concentration. In the case of proteins like insulin, the problem may be further compounded if associated forms of the protein have low affinity for sites on the surface of the reaction vessel while the free monomer has a high affinity. If this were the case, one would expect a simultaneous sharp drop in the reactivity of all groups at low protein concentrations. If a reversible equilibrium between insulin species free in solution and those bound to the surface of the reaction vessel does indeed exist, one would anticipate that molecules of other proteins would be able to compete for these sites. An experiment was therefore designed in which a second protein was added to buffered solutions prior to the addition of a variable concentration of insulin in an effort to prevent the adsorption of insulin to the surface of the reaction vessel.

The competitive labelling approach is based on the fact that all functional groups will compete for reaction with the limited amount of the tritiated labelling reagent. In the present procedure (Figure 6), the reaction mixture is hydrolyzed immediately after the addition of [^{14}C]-derivatized protein and the groups are isolated. In order to determine the reactivity of the functional groups of insulin, it is necessary to ensure that the [^{3}H]counts quantified are due to the reaction of groups on the insulin molecule and not to reaction of those on the added protein. Thus the second protein must be rendered unreactive towards the chemical probe. Since all proteins have nucleophilic functional groups this entails extensive derivatization of the second protein before addition to the solution. Such extensive derivatization would result in the preciptation of most proteins. In order to circumvent this problem, a mixture of histone proteins was selected as the competing protein. Because the high isoelectric point of these proteins is due, at least in part, to the large number of positively charged arginine residues, reaction of this protein mixture with succinic anhydride would give a derivative with a pI which is in the same range as that of most other proteins. The large number of positive charges due to arginine and of negative charges resulting from

succinylation of the primary amino groups should maintain the solubility of the protein in neutral solution even after further derivatization of the imidazole and phenolic functions by reaction with FDNB. The latter reagent would also react with any as yet underivatized amino groups. A derivatized protein mixture with a solublity of up to 10^{-3}M was indeed obtained.

The extent of derivatization was tested by amino acid analysis. Succinylation appears to be at least 95% complete as evidenced by the recovery of 95% of the lysine residues after reaction with FDNB (Table 5). The tyrosine and histidine residues appear to be completely modified as tyrosine and histidine are undetectable by amino acid analysis. Further reaction with FDNB gave no further change in this profile. As FDNB is also used as the chemical probe in the competitive labelling experiment, groups on the histone proteins which did not react with excess FDNB under denaturing conditions were not expected to present significant competition with functional groups of insulin for trace amounts of the labelling reagent. Since radiolabelling methods are more sensitive than amino acid analysis to small amounts of reactive material, all $[^{3}H]/[^{14}C]$ ratios were corrected using controls in which the insulin was omitted in the trace-labelling step.

It is a well known fact that different proteins adsorb to surfaces to different extents. For example, fibrinogen appears to have a higher affinity for sites on glass and polyethylene than does albumin (Vroman et al., 1975). The amount of modified histone mixture required to successfully compete with insulin for sites on the reaction surface can thus not be known a priori. Since losses of insulin due to adsorption seem to be more severe than the corresponding losses of other proteins, one might expect that insulin has a higher affinity for surface sites than most proteins and that relatively large amounts of modified histone would be required to interfere with insulin adsoption to the surface. In this study the histone concentration was varied from 10^{-6}M to 10^{-3}M.

From the results in Table 6, it is evident that the presence of even large amounts (10^{-3}M) of derivatized histone does not inhibit the adsorption of insulin to the reaction vessel. At high concentrations of insulin, the functional groups show substantial reactivity; at 10^{-7}M

they are unreactive. In some cases the [^{3}H]counts in the controls approach those in samples at lower insulin concentrations. This indicates that the modification of the histone mixture to "unreactive protein" was incomplete. While the data are not suited to detailed quantitative analysis, the general trend is readily apparent: the modified histone mixture does not prevent the adsorption of insulin. No attempt was made to obtain a more fully derivatized histone mixture. It is possible, of course, that the sites on the reaction vessel are indeed masked by the addition of a second protein but that new sites for insulin adsorption are created on the protein itself. There is evidence from other studies that, in polystyrene vessels, protein adsorbs to at least two distinct types of sites: primary sites on the vessel surface and secondary sites which appear to represent protein to protein associations (Cantarero et al., 1980). Since the purpose of this study was to find conditions where the effect of concentration on the reactivity of the functional groups of insulin in the absence of adsorption effects and since the addition of relatively large amounts of another protein to dilute insulin solutions does not appear to remove all adsorption effects, the question as to whether insulin (in glass reaction vessels in the presence of a modified histone mixture) is adsorbing to the reaction vessel itself or to the modified histone mixture was not further pursued. It should be noted, however, that the protein, bovine serum albumin, is routinely added to dilute insulin solutions used in insulin receptor binding assays (Hollenberg and Cuatrecasa, 1971) to inhibit interaction of insulin with the walls of the reaction vessel. In view of the results of the present study, the efficacy of a 1%BSA solution in preventing the adsorption of insulin from nanomolar solutions to reaction vessels is questionable.

The physical-chemical basis for the adsorption of insulin to reaction vessels is not known and may indeed be different for each material used in the present study. In the case of Pyrex glass reaction vessels where adsorption is the strongest, this adsorption could well be due to ionic interactions between the glass surface and the insulin molecule. An increase in the salt concentration of the buffer should minimize any such interaction and perhaps provide condition where the effects of insulin concentration on chemical

reactivity could be studied in the absence of adsorption effects. At higher salt concentrations in glass reaction vessels (0.5M-KCl in Figure 13 and 1.0M-KCl in Figure 12), the results obtained at insulin concentrations greater than $1x10^{-6}$M clearly parallel those obtained with 0.1M-salt in polystyrene reaction vessels. Although some adsorption effects are evident in 0.5M-salt, all of the functional groups retain significant reactivity at an insulin concentration of $1x10^{-7}$M. This indicates that the adsorption is not nearly as strong as in 0.1M-KCl (Figure 9). When 1.0M-salt concentration is used, it appears that adsorption is no longer significant since all functional groups retain at least normal reactivity at all concentrations of insulin used.

The reactivity of the glycine amino terminus remains constant over the entire concentration range as does the average reactivity of the four tyrosine residues. The amino groups of the B-chain amino terminus and the lysine at B-29 are constant to an insulin concentration of 10^{-6}M. As the concentration is further decreased, the reactivity of the phenylalanine decreases to a value below that of the glycine but is still in a range which can be considered normal for such a group. The reactivity of the solitary lysine residue increases at concentrations where the free monomer predominates. The average reactivity of the two histidine residues, on the other hand, is again abnormally high at high insulin concentrations and gradually decreases as the insulin concentration is decreased to 10^{-6}M where it is essentially equal to the reactivity of the internal standard, L-β-imidazole lactic acid. This normal reactivity value for the histidine imidazole group is retained when the insulin concentration is further decreased.

From the present study it appears that an increased ionic strength of the solvent inhibited the adsorption of insulin to glass surfaces while addition of an unreactive protein to the solvent did not. Chan et al. (1973) report that loss of aspartate transcarbamylase in dilute solution (Teipel and Koshland, 1971) can be avoided by the addition of BSA. Suelter and DeLuca (1983) have recently found that modification of the solvent by addition of glycerol or Triton X-100 and/or by increased ionic strength reduced losses of the protein luciferase through adsorption to glass or plastic surfaces while

addition of other proteins to the solution did not. Modification of the solvent by the addition of non-physiological substances or by extremes of ionic strength or pH may result in conformational changes in the protein under study. Increased NaCl concentration (up to 2M) has been reported to substantially increase the affinity of insulin for liver and adipocyte plasma membrane receptors (Tatnell and Jones, 1981; Kahn et al., 1974; Cuatrecasas, 1971) indicating that the properties observed in the present study are for a functional insulin monomer. In view of the results shown here, the observed increase in insulin binding at increased salt concentrations may, in fact, be a reflection of an inhibition of insulin adsorption to the surface of the reaction vessel.

The reactivity data obtained in this study clearly demonstrate that the microenvironment of some functional groups changes as insulin dissociates from hexamer to dimer to free monomer. The question thus arises whether these changes reflect a difference in the conformation of the monomeric unit in the free and associated states or merely differences due to disruption of intermonomer interactions in the associated states. Much of the current viewpoint concerning insulin structure-function relationships has been inferred from the x-ray crystallographic data for the monomeric unit of hexameric insulin crystals. At present, there is conflicting evidence concerning the structure of the free insulin monomer in solution. If the conformation of the monomeric unit of insulin is dramatically altered on formation of the free monomer, one would expect simultaneous changes in the reactivities of all functional groups. This is not observed. The reactivity of the A-chain amino terminal glycine and the average of the four tyrosine residues is, in fact, constant in all stages of insulin self-association. The histidine residues decrease in reactivity only as the insulin concentration decreases from $1x10^{-3}$M to $5x10^{-6}$M. On the basis of sedimentation studies, this is the concentration range where hexamers and possibly tetramers are dissociating to form dimers. In the concentration range $5x10^{-6}$M to $1x10^{-7}$M where the dimer is dissociating to form the free monomer, the reactivity of the histidines is constant. The phenylalanine amino terminus and the lysine residues have the opposite reactivity pattern. The reactivity remains unchanged in the

transition from hexamer to dimer, but, on formation of the free monomer, the reactivity of the lysine increases while that of the phenylalanine decreases. These observations indicate that the changes in reactivity of the histidine residues reflects alterations in their microenvironment on dimer-dimer association while those of the phenylalanine and lysine residues reflect alteration on monomer-monomer association. The lack of change in the reactivity of the glycine amino terminus indicates that the environment of this group is essentially the same in all stages of association.

The only approach (other than that reported here) that has been used to investigate the conformation of the insulin monomer is circular dichroism. Wood et al. (1975) have interpreted their results as showing little or no difference between the conformation of the free monomeric unit and that of the monomeric unit in the dimer. More recently Pocker and Biswas (1980), using improved instrumentation and lower insulin concentrations, claim that the transition from dimer to free monomer is accompanied by substantial changes in α-helix, β-sheet and random coil content of the monomeric unit. The reactivity data obtained in this study show that the changes that occur are in the microenvironments of individual functional groups and that there is no gross conformational difference between the monomeric unit in its free and its associated forms. Blundell and Wood (1982) suggest that surface adsorption may have affected the data reported by Pocker and Biswas (1980). In view of the present results which show strong adsorption of insulin to various materials, this appears to be a possible explanation of the C.D. spectra obtained at low insulin concentrations.

On the basis of x-ray crystallographic studies of insulin, several functional groups have been postulated to be involved in either inter- or intra-monomer interactions (Chothia et al., 1983; Blundell et al., 1971). A closer examination of the predictions based on x-ray structure in light of these reactivity data is therefore warranted. Insulin, in the presence of zinc, forms crystals of insulin hexamers. Each hexamer is associated with either two zinc or four zinc molecules depending on the zinc content of the crystallizing medium. There are slight conformational differences in the monomeric units to the 2Zn- and 4Zn-insulins (see above). The zinc content of the insulin used in the

present study corresponds to that of the 2Zn-insulin crystal. Thus, for the purpose of simplicity, the conformations discussed are those of the 2Zn-insulin crystal structure except in the case of the histidine residues where the microenvironment of one of the residues in question is affected by the higher zinc content. In this case the possibility of some 4Zn-insulin formation will be discussed.

A model of the insulin dimer from the 2Zn-insulin hexamer was constructed using the coordinates from the crystal structure refined to 1.5Å (Cutfield et al., 1981) in order to evaluate possible structural alterations in the monomeric unit when the free monomer is formed in solution. The dimer is the symmetry unit in the crystal. It is composed of two similar but not identical monomeric units (denoted Molecule 1 and Molecule 2) which are related by a two fold axis (Figure 2, upper panel of Figures 44 and 45A). The hexamer is formed of three such dimeric units which are related by a local three-fold axis (shown schematically in Figure 42). In 2Zn-insulin the zinc atoms lie on this three fold rotational axis. The B10-histidine residues of each monomeric unit in the crystal are involved in zinc complexation. The positions of the amino and imidazole functions of Molecule 2 of the 2Zn-hexamer are indicated in Figure 43.

The α-amino group of the A1-glycine residue is a surface pocket in the crystal (Figure 44). Its accessibility would not appear, from the crystal structure, to depend on the degree of association, which is in complete accord with the findings of this study.

The corresponding group of the B1-phenylalanine is postulated (Blundell et al., 1972) to be in a position to interact with the A17-carboxyl of an adjacent dimer in the crystal. Such an interaction would be reflected in a change in the reactivity of B1-phenylalanine as insulin hexamers and tetramers dissociate to form dimers. This was not observed: the reactivity of this amino terminus is constant in concentrations where associated forms of insulin, including the dimer, predominate but decrease as the dimer dissociates to form the free monomer. This indicates that this group is involved in a monomer-monomer interaction leading to enhanced reactivity; no dimer-dimer interaction in solution is indicated. The monomeric unit of insulin in the crystal has a hydrophobic surface which is exposed to water and two

Figure 42: Schematic representation of the postulated association patterns of insulin in solution. In dilute solution insulin exists in its free monomeric form (a). In more concentrated solutions, insulin aggregates to form dimers (bi and bii) and hexamers (c). The insulin monomeric unit has two essentially hydropobic surfaces which, in the free monomer, are postulated to be exposed to the solvent. Interaction of insulin monomers at one of these surfaces gives rise to the "symmetric dimer" (bi). Interaction at the other surface results in the formation of the "asymmetric dimer" (bii). (see text.)

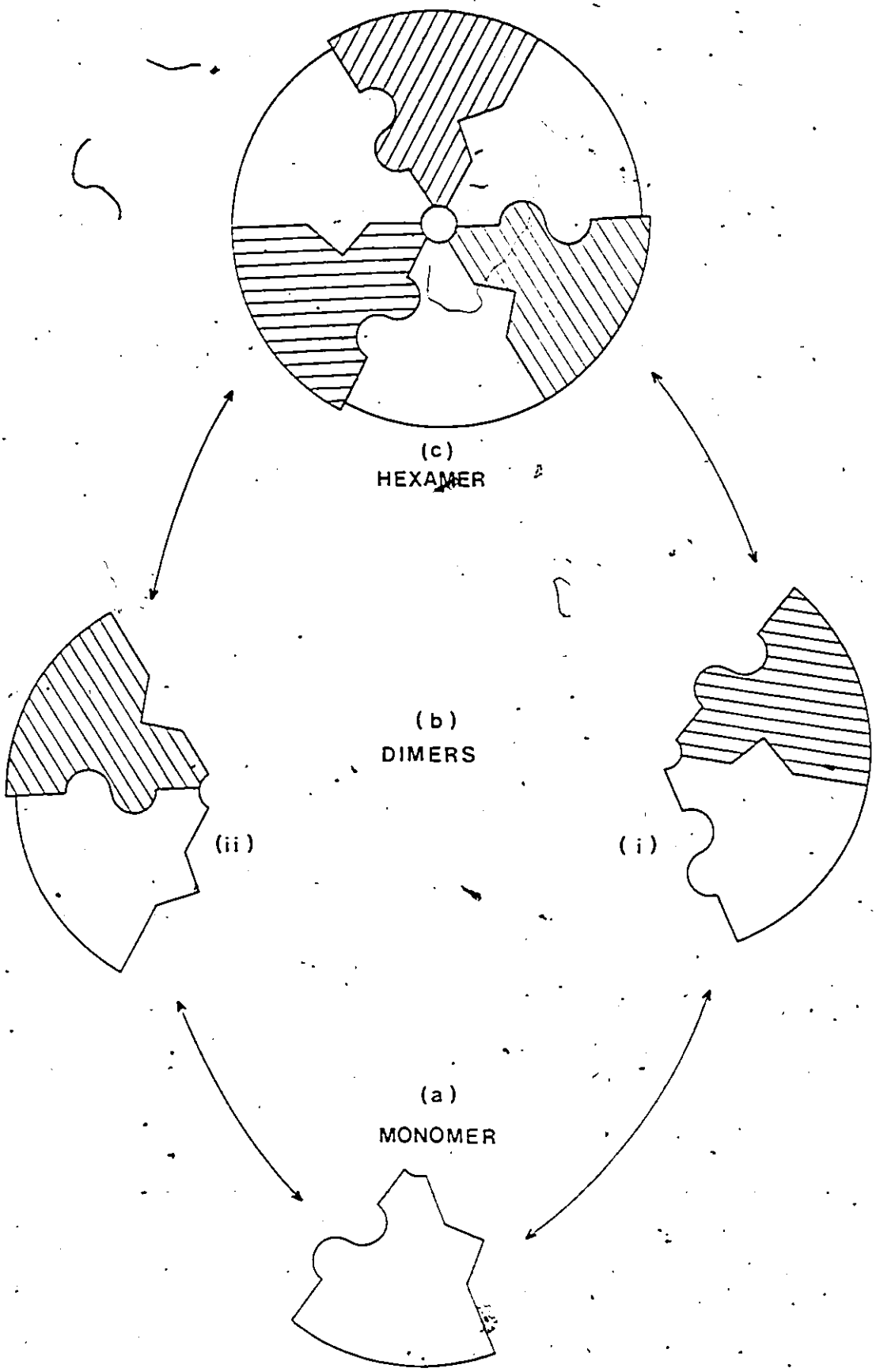
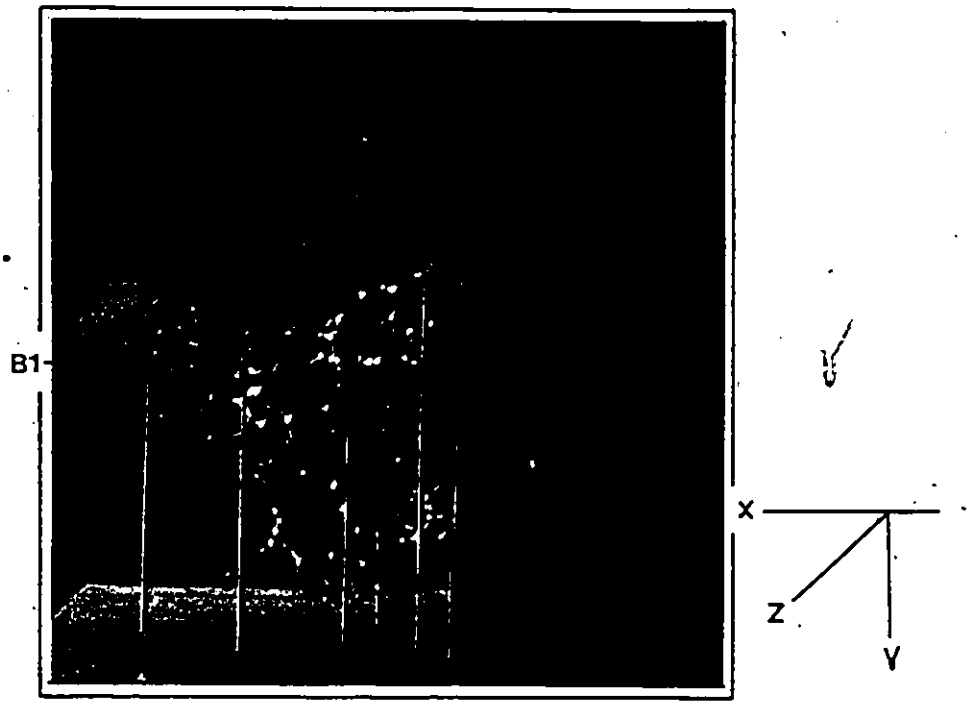

(c)
HEXAMER
(b)
DIMERS
(ii)
(i)
(a)
MONOMER

Figure 43: The positions of the amino and imidazole functions of Molecule 2 from the x-ray crystal structure of 2Zn-insulin.

The backbone atoms of the A-chain are shown in yellow while those of the B-chain are in red. Aliphatic carbons in side chains are grey in colour; aromatic carbons are purple. Functional groups containing nitrogen are shown in blue, those containing oxygen in orange.

The molecule in the upper panel has been rotated 180 to give the view in the lower panel. The β-sheet structure in the C-terminal region of the B-chain (B) is involved in monomer-monomer interactions in the crystal dimer. A second β-sheet structure which includes the B1 to B8 residues is stabilized by dimer-dimer interactions in the crystal.


B1
X
Z
Y


B 29-Lys
Y
Z
X

Figure 44: The position of the α-amino group of the A1-glycine residue in the x-ray crystal structure of insulin.
The upper panel shows a view of the symmetry unit of the crystal, the dimer. Molecule 1 (white and green) and Molecule 2 (red and yellow) are related by an approximate two-fold axis; symmetry is more strictly maintained in molecules closer to the local axis. The α-amino groups of the A1-glycine residue of Molecule 1 (A1-Gly I) and Molecule 2 (A1-Gly II) lie in surface pockets exposed to the solvent in the 2 Zn-insulin hexamer. Because Molecule 1 lies above Molecule 2 in the crystal dimer the micro-enviornment of the A1-glycine amino terminus is postulatd to remain essentially unchanged on dissociation of insulin oligomers to form the free monomer in solution. The enviornment of the amino group of the A1-glycine of Molecule 2 is seen more clearly in the lower panel. Only the backbone atoms are shown in this model.


Z
X
Y


Z
-Y
X

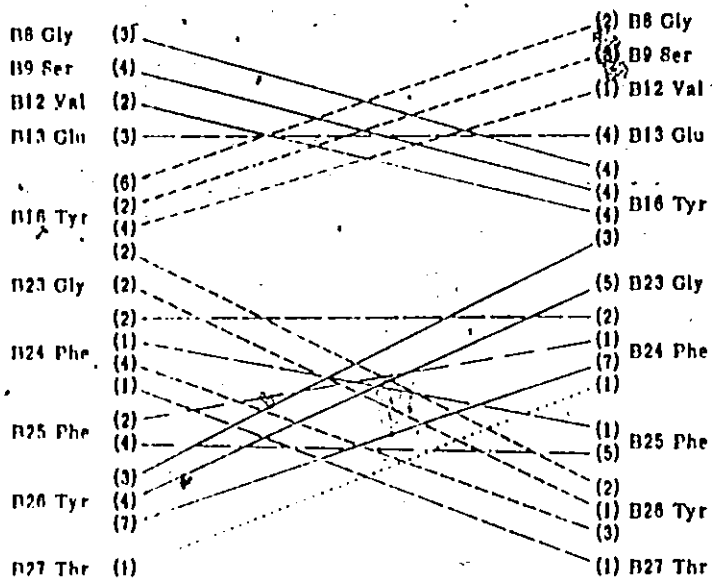
hydrophilic surfaces: one along the local two fold axis which relates Molecule 1 to Molecule 2 in the crystal dimer unit; the other is involved in the interaction of one dimeric unit of the crystal with the next dimeric unit to form the hexamer. Blundell et al. (1972) have examined the number of intermolecular contacts of less than 4 angstroms possible at each of the two hydrophobic surfaces of the monomeric unit (Table 17) and have tentatively predicted that the formation of the "symmetrical dimer" (Figure 42) seen in the crystal would be thermodynamically more favourable than that of the "asymmetric dimer" and is therefore the first dimeric unit formed in solution (Figure 42).

The formation of this "symmetrical dimer" in solution does not involve an obvious change in the environment of the B1-phenylalanine α-amino group (this group is part of the hydrophobic surface which extends away from the surface postulated to be involved in monomer-monomer interactions) and thus does not account for the increase in reactivity seen on dimer formation in the present study. These data could, however, be interpreted on the basis of x-ray crystal structure if one assumed that both the "asymmetric dimer" and "symmetric dimer" of the crystal exist simultaneously in solution . The formation of this dimer would result in a change in the local environment of the B1 amino-terminal phenylalanine bringing the phenyl ring in a position to overlap with that of the tyrosine at A14 of the adjacent monomer and α-amino group close to the A17-carboxyl (Figures 45A, B and C). Formation of a hydrogen bond between these two residues would account for the observed increase in reactivity of the α-amino group in associated forms of insulin.

The thermodynamic favourability of any reaction depends on the relative energy levels of the products and the reactants. If one assumes that the driving force for the self-association of insulin monomers is the removal of hydrophobic residues from an aqueous milieu (the interaction of polar molecules with water in the free monomer would be compensated for by the formation of hydrogen bonds or salt bridges between monomeric units), then the energy of interaction can be, to a first approximation, calculated by the Van der Waals interactions afforded between molecules on dimerization. The free energy of each interaction depends on the type of interaction (i.e., carbon to

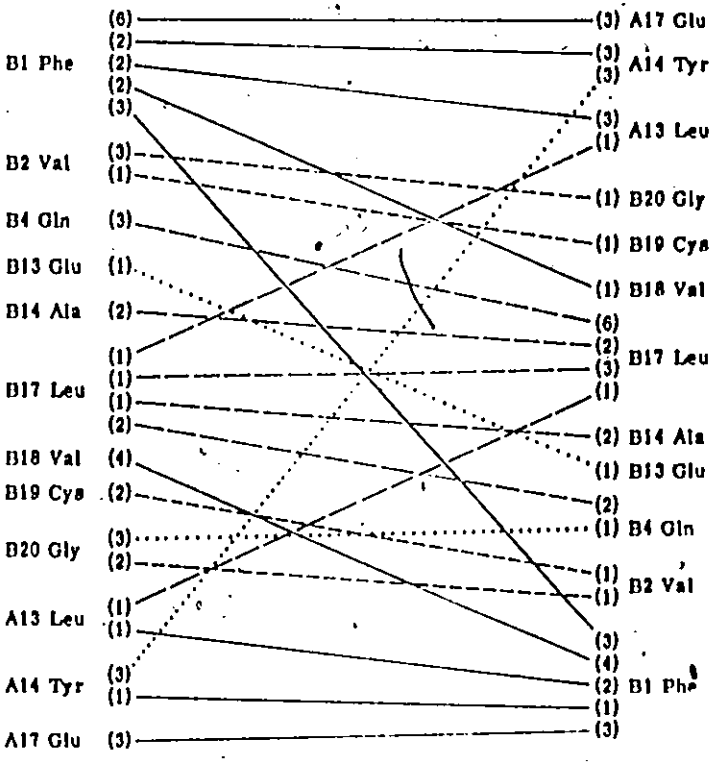
TABLE 17: A Listing of the Number of Atoms Involved in Contacts of Less Than 4Å between Pairs of Residues in the 2Zn-Insulin Crystal
a)Monomer-Monomer Contacts in the Crystal
b)Dimer-Dimer Contacts in the Crystal
(Blundell et al., 1972)

a. Monomer - Monomer contacts



20 residues with 111 atoms in contact

b. Dimer - Dimer contacts

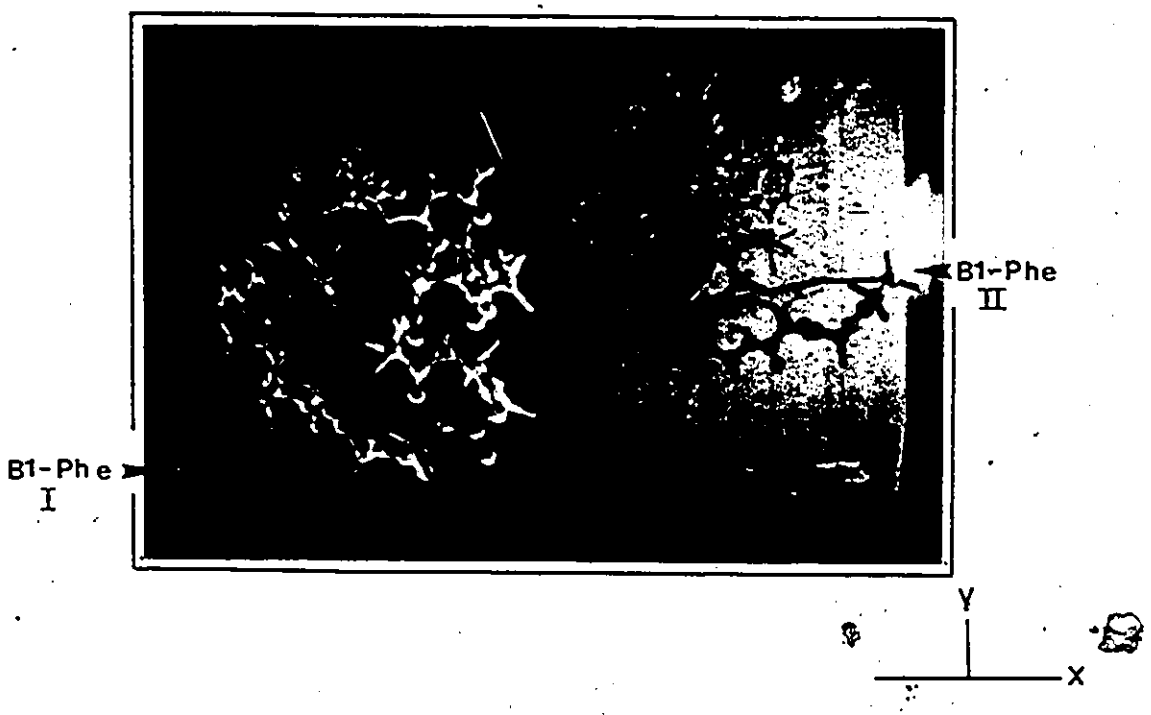


24 residues with 99 atoms in contact

Figure 45A: Position of the α-amino group of the B1-phenylalanine in the x-ray crystal structure of insulin.

Upper Panel: A view looking down the three-fold rotational axis at the "symmetric dimer" of the crystal: The backbone atoms of the A-chain of Molecule 1 are shown in white and the B-chain in green. The backbone atoms of the A- and B-chains of Molecule 2 are in yellow and red respectively. The positions of the α-amino groups (shown in light blue) are indicated. The α-amino group of the A1-glycine residue of Molecule 2 is on the under side of the dimer and cannot be seen in this view. The other two dimeric units in the crystal can be placed by rotation of this dimer through an angle of 120° and 240° about the threefold axis (denoted 0).

Lower Panel: A view of the α-amino group of the B1-phenylalanine in Molecule 1 including side chains. Again, the backbone atoms of the A-chain are shown in white and those of the B-chain in green. The aliphatic carbon atoms in the side chains are grey; the aromatic carbon atoms are purple. Functional groups containing a nitrogen atom are coloured dark blue while those containing an oxygen atom are orange. The α-amino group (B1-Phe I) and the aromatic ring of the B-chain amino terminus can be seen at the bottom of the picture at the end of a β-sheet structure in the crystal. The side chain of the A14-tyrosine and the carboxylate of A17-glutamate of the same monomer are situated to the left of the B1 residue in this view.


B1-Phe
II
B1-Phe
I
Y
X

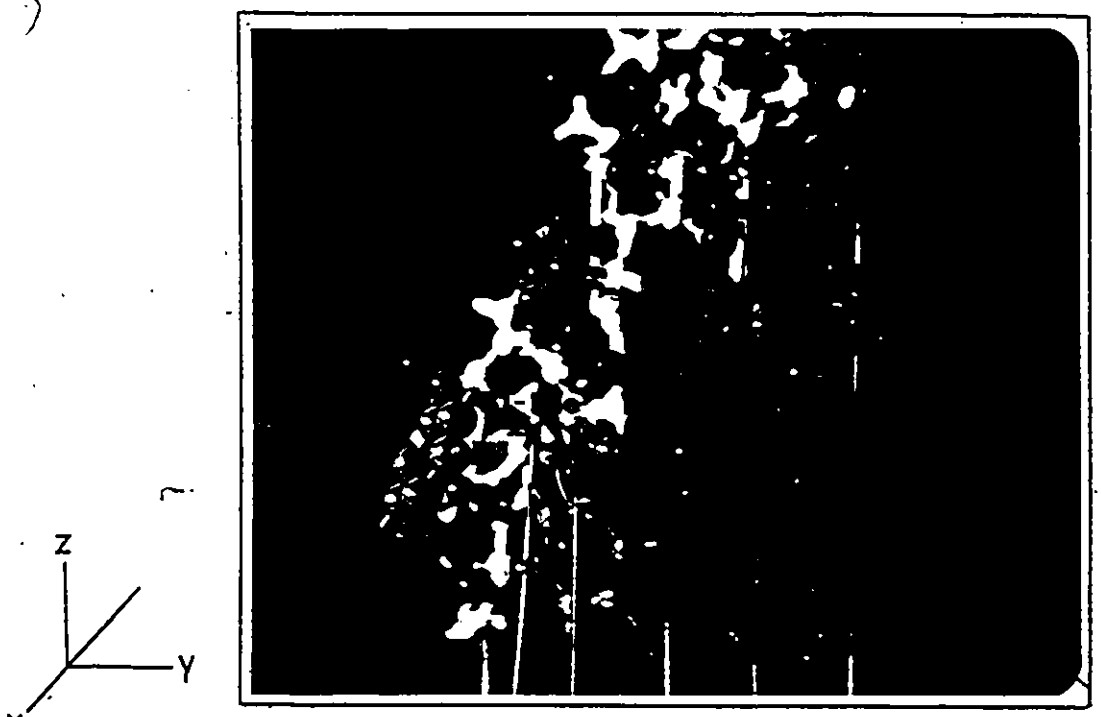
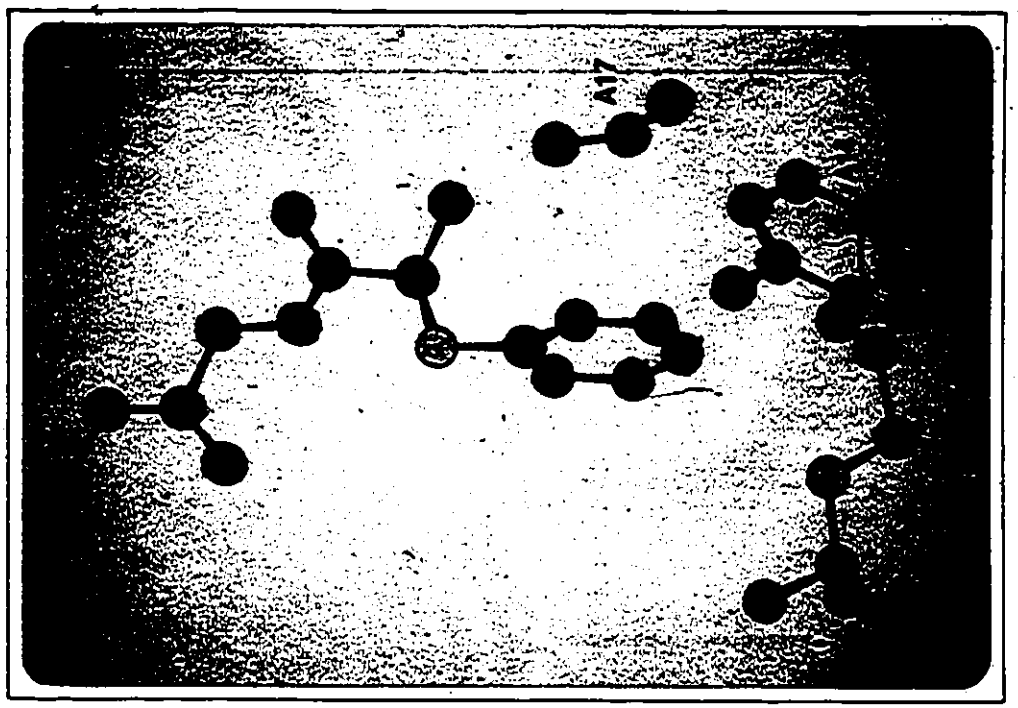
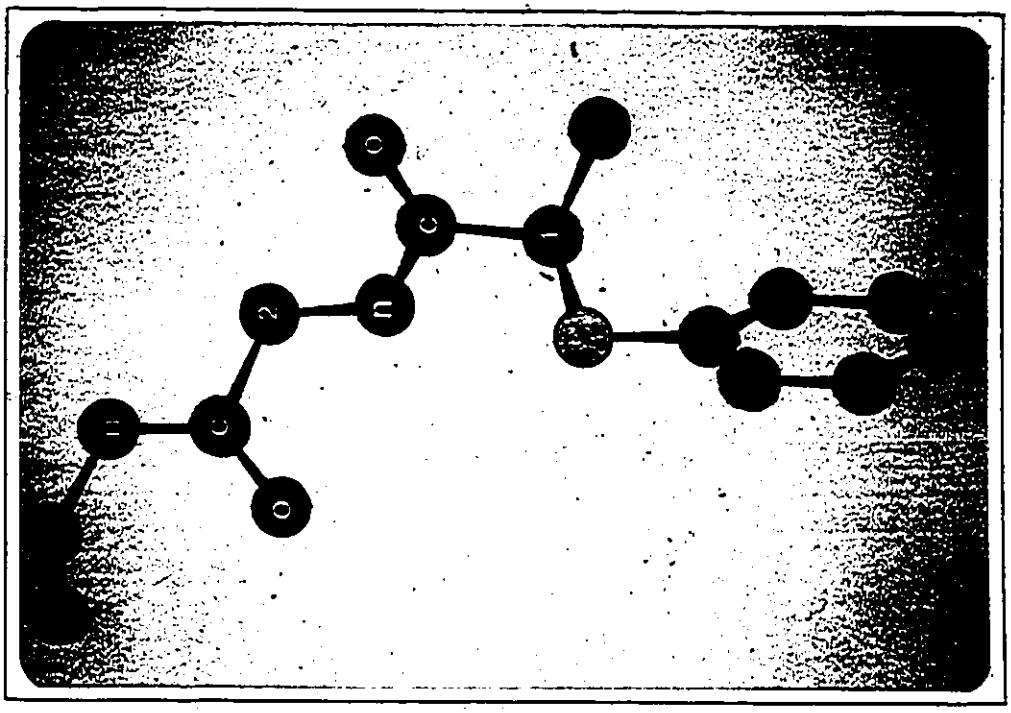

Z
Y
X

Figure 45B: Three-dimensional projections* showing the environment of the B1-phenylalanine of Molecule 1 in the crystal monomer and crystal dimer (left) and in the crystal hexamer (right). Molecule 1 has been rotated 240° from its position in the upper panel of Figure 43. "x = 0" indicates that the drawing is of the z-y plane as viewed from the centre of the hexamer. This view is, therefore, at approximately 180° to that in the lower panel of Figure 44A.
While the microenviornment of the B1-phenylalanine of Molecule 1 is the same in the crystal or "symmetric" dimer as in the monomer (left), dimer-dimer interactions in the crystal bring the phenylalanine of Molecule 1 (backbone in green) close to the carbocylate of A17-glutamate and the phenolic function of the A14-tyrosine of Molecule 2 (backbone carbons in yellow) of the adjacent dimer (right). The interactions of the N-terminal phenylalanine of Molecule 2 are similar.

* The three dimensional projections presented in this thesis were drawn from the x-ray crystal co-ordinates (Cutfield et al., 1979) at the National Research Centre, Ottawa, Canada, using a PLUTO (IBM) plotting program.



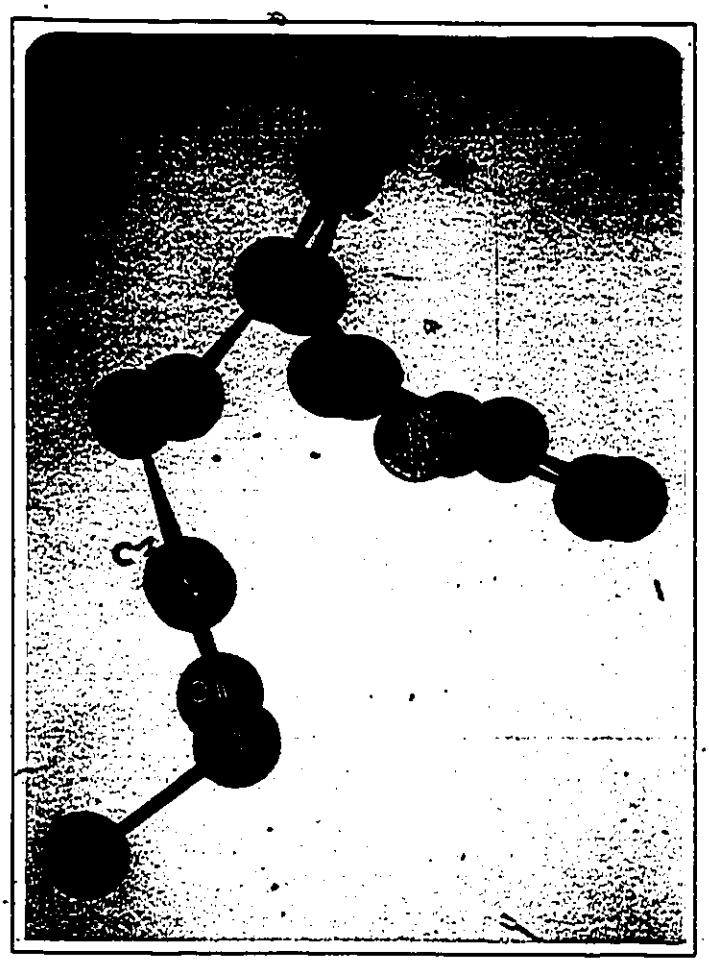
X=O



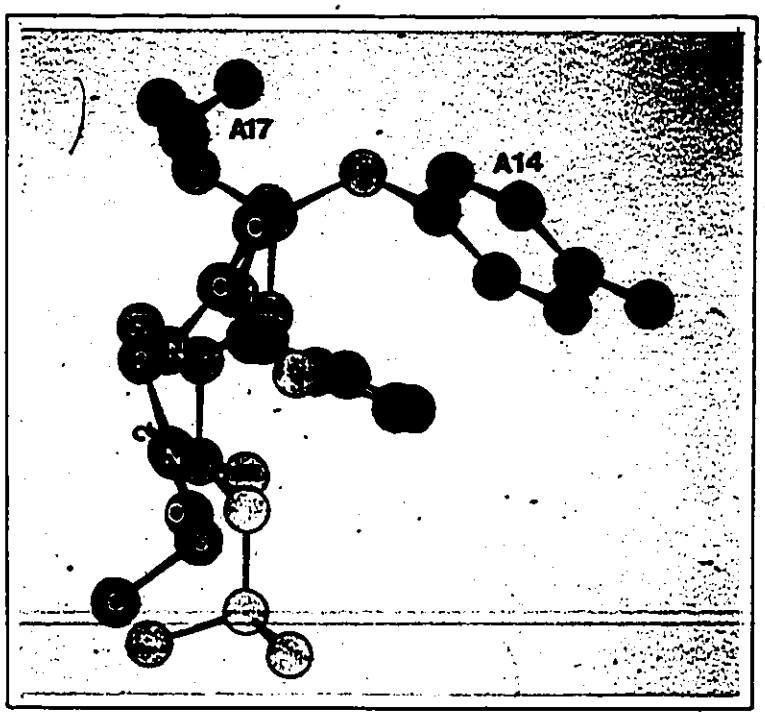
X=O

Figure 45C: Three dimensional projections showing the B1-phenylalanine of Molecule 1. The view is of the x-y plane (z = 0) as seen from the center of the crystal hexamer. Molecule 1 has again been rotated 240° from its position in the upper panel of Figure 43.

The upper panel shows the position of α-amino and the phenyl functional group relative to the backbone atoms of Molecule 1 in the crystal monomer and dimer. This amino and the A17-carboxylate of the adjacent Molecule 2 (lower panel) in the crystal hexamer are close enough to allow for hydrogen bond formation. The reactivity data indicate that the B1-phenylalanine residues are involved in monomer-monomer interactions rather than dimer-dimer interactions in solution. (See text).



Z = 0



Z = 0

carbon, carbon to oxygen, oxygen to oxygen etc.), and the distance between molecules. The actual values assigned for these interactions are dependent on empirical calculations and vary with the means of determining the thermodynamic parameter (Schultz and Schirmer, 1979). Determination of the relative stabilities of the "symmetric" and "asymmetric" insulin dimer would therefore require more detailed calculations which take into account these several parameters. At present there is no reason, on thermodynamic grounds, to rule out the existence of both dimers in solution.

The position of the B29-lysine ϵ-amino group is not well-defined in the crystal structure. Our reactivity data indicated that, like the phenylalanine amino terminus, it is involved in a monomer-monomer interaction. The lysine residue is part of an extended β-sheet region along the local two-fold axis of the insulin crystal. Presumably this β-structure would be unstable in the free monomer but stabilized by the self-association of insulin along this hydrophobic surface i.e., in the formation of the "symmetric dimer". How the formation of the "asymmetric dimer" in solution would affect the local environment of this residue is not immediately apparent. The lack of resolution attainable in this region by crystallographic techniques indicates that, in associated forms of insulin, the position of the ϵ-amino group is not constant.

Sedimentation equilibrium studies of insulin solutions with similar zinc content indicate that the Zn-hexamer predominates in concentrated solution (Milthorpe et al., 1977). In this structure, the B10-residue of each monomeric unit is involved in binding the zinc. Therefore, the abnormally high reactivity of the histidines in associated states of insulin observed here and in a previous study (Chan et al., 1981) cannot be due to this particular residue. (While one cannot rule out the possibility of an increased zinc content due to contamination of the distilled water, the reaction vessel, etc., with trace amounts of zinc, formation of the 4Zn-insulin hexamer is not consistent with the data. In the later structure both the B10- and the B5-histidine residues are involved in zinc binding and should be non-reactive). Based on the x-ray crystal structure of 2Zn-insulin, the B5-histidine is postulated to form an intermonomer hydrogen bond through the N^{τ} to the backbone of the A1-helix of an adjacent hexamer (Chothia et al., 1983). The B5-histidine

residue of the Molecule 2, but not Molecule 1, (Figure 46 and Figures 47A and B), is in a position to be involved in an intramolecular hydrogen bond through the N^{δ} with the A7-carbonyl (Blundell et al. 1972). The structure of Molecule 2 is thought to be the "preferred" conformation of insulin (Chothia, et al., 1983; Hodgkin et al., 1983; Blundell, 1979) and thus to represent the structure of the free monomer in dilute solution. The N^{δ} interaction should lead to the enhanced reactivity at the N^{ε} as postulated previously (James, 1980; Cruickshank and Kaplan, 1975) to explain the enhanced reactivity observed for the active centre histidine of chymotrypsin. Since FDNB can react only at the N^{ε} (Henkart, 1971), the postulated interaction at this position should lead to a decreased reactivity of the imidazole toward FDNB. Neither of these postulated interactions in the crystal explains the observed dependence of reactivity on concentration. These data indicate that, in solution, the N^{δ} is involved in strong hydrogen bonding only on dimer-dimer interactions. In the dimer and free monomer the histidine have a reactivity which is closer to that normally expected for such a group. Thus, the microenvironment of at least one of the two histidine residues appears to change on dissociation of insulin oligomers to form dimers or free monomers in solution. Other studies suggest that only the zinc free monomer exist at high dilutions (Milthorpe et al., 1977; Pekar and Frank, 1972). One would therefore expect the B10-histidine as well as the B5-histidine to be reactive towards FDNB. These data imply that if Molecule 2 does, as predicted, exist in dilute solution, it exists in a conformation in which the hydrogen bond between the N^{δ} of the B5-histidine and the A7-carbonyl is much weaker than in the dimer. As very little in the change in the relative postions of the two atoms is required to weaken a hydrogen bond, the conformational change on dissociation to the dimer need not be large. In 1M-KCl the reactivity of the histidine in the hexameric or tetrameric form of insulin is further increased. This cannot be due to a specific imidazole effect as the internal standard is L-β-imidazole lactic acid but may reflect a strengthening of hydrogen bonding in 1M-salt.

From the x-ray crystal structure of 2Zn-insulin, it would appear that the microenvironments of three of the four tyrosine residues would change on association of insulin. These changes are not apparent from

Figure 46: The B5-histidine of Molecule 2.

The positions of the B10- and B5-histidine residues of Molecule 2 of the 2Zn-insulin crystal are shown in the upper right. In the 2Zn-hexamer, the B10-histidine is involved in coordination of the zinc atoms. On the basis of this structure, the N^{δ} (d) is postulated to be in a position to form a hydrogen bond with the A7-carbonyl function (C-O) of the same monomer. This would account for the high reactivity of the N^{τ} (t) towards FDNB (see text).

The relative positions of the imidazole function and the A7-carbonyl (denoted C7) are more clearly seen in the three-dimensional projections. The left panel shows the z-y plane (x = 0) and the lower right panel (z = 0), the x-y plane. Both views are from the centre of the hexamer.

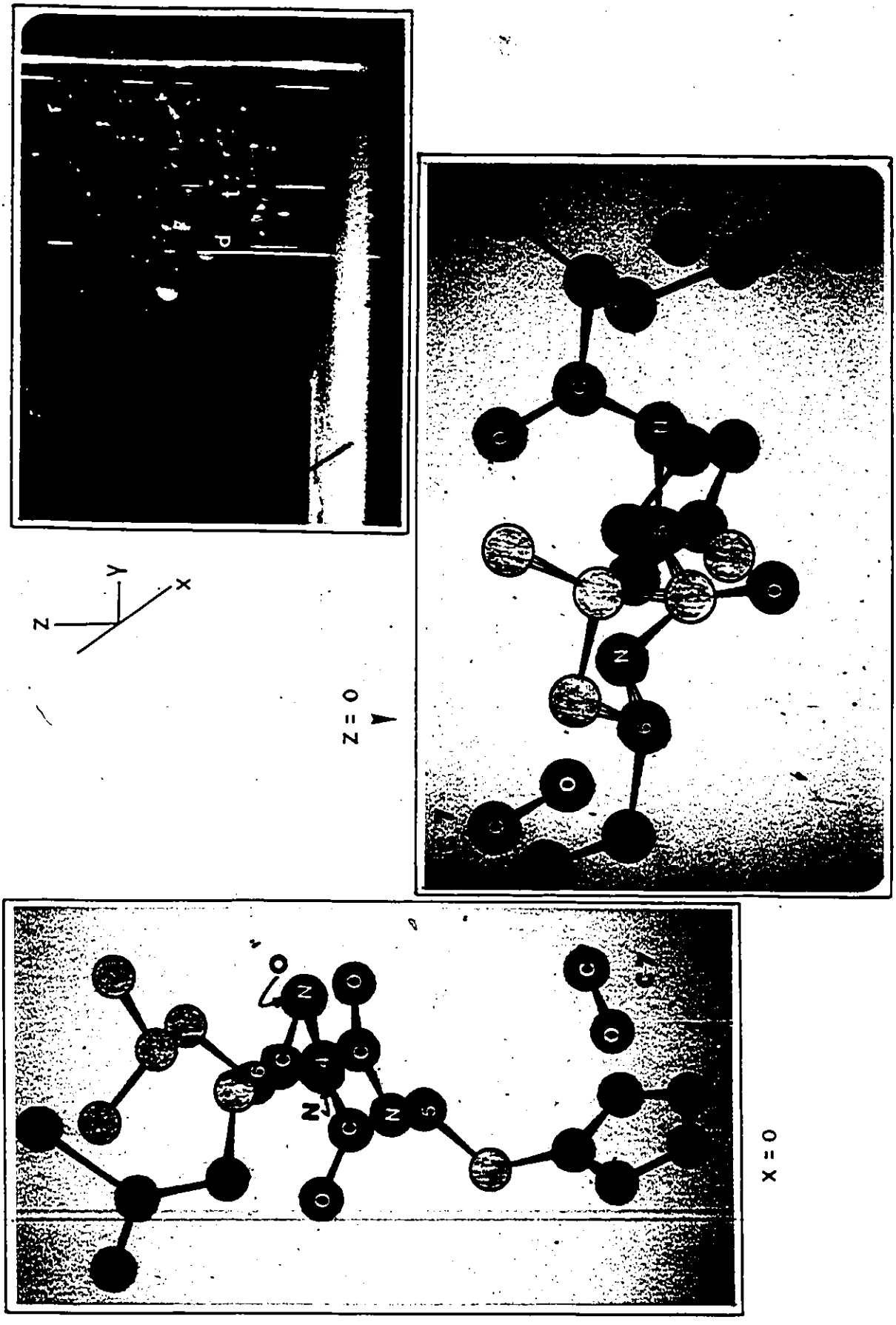
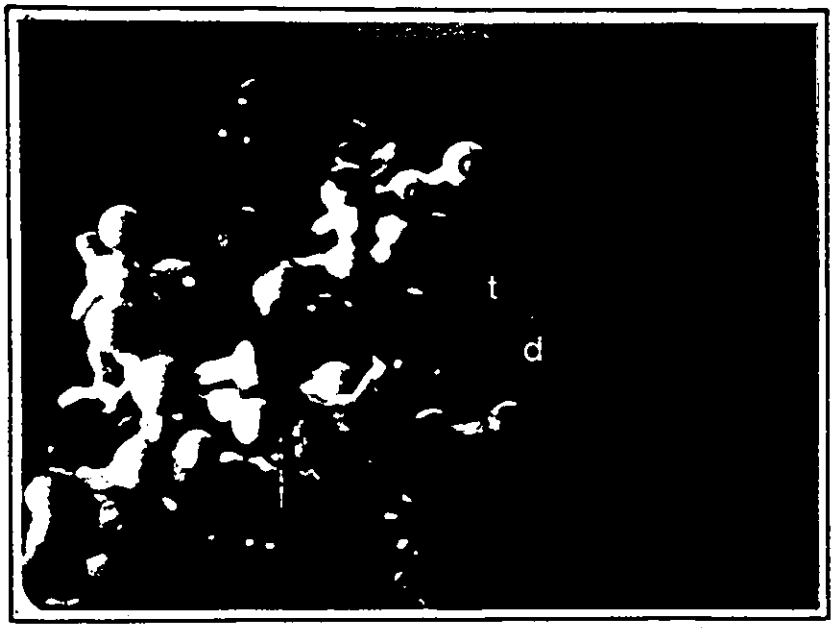
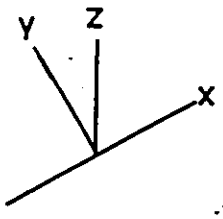

Y
X
Z
Z = 0
X = 0

Figure 47A: The B5-histidine of Molecule 1.

The relative positions of the A7-carbonyl (C-O) and the N^{δ} (d) of the imidazole moiety of the B5 histidine of Molecule 1 shown in a model of the crystal structure of 2Zn-insulin: The upper and lower panel give two different views of the same residue. The viewing angle shown in the lower panel suggests that, in Molecule 1, the N^{ε} (t) rather than the N^{δ} (d) is in a position to form a hydrogen bond with the A7-carbonyl of the same monomeric unit.


t
d


Y
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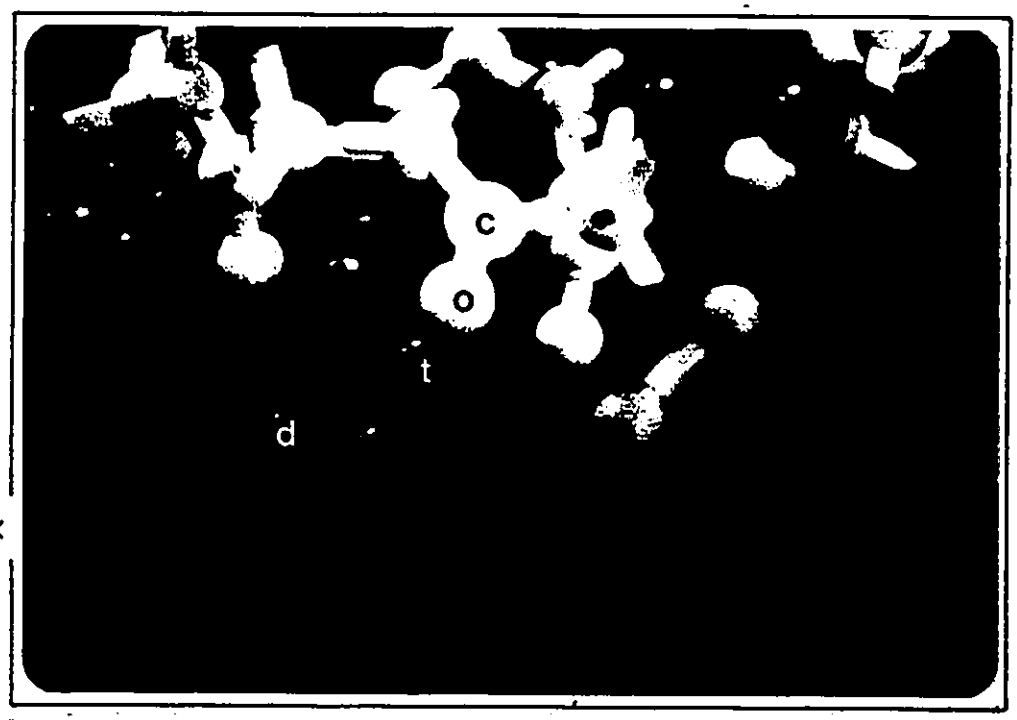

c
o
t
d

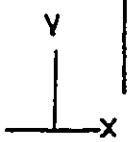
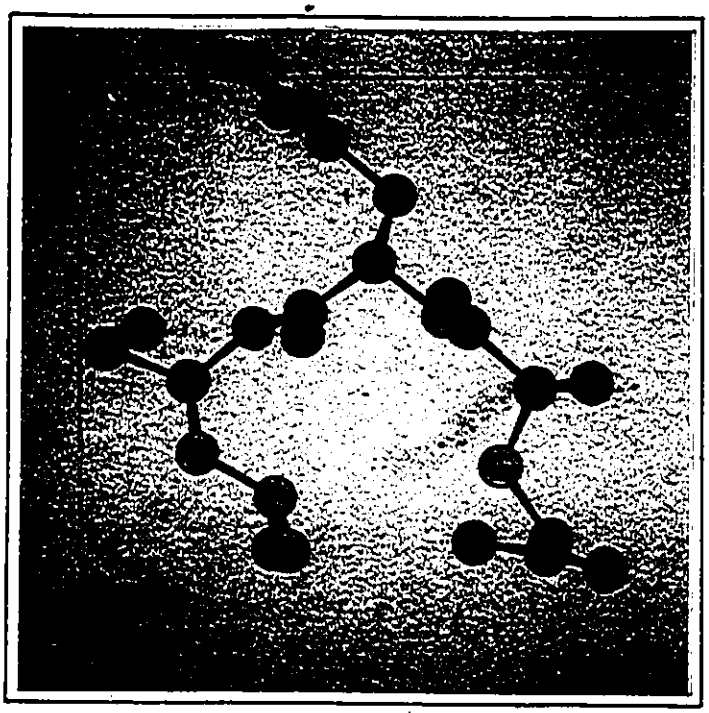

Y
X

Figure 47B: Three-dimensional projections showing the B5-histidine of Molecule 1:

The backbone atoms of the B-chain are in green; carbons of the imidazole function are purple and the nitrogen atoms, blue. All aliphatic carbons in side chains are shown in grey. The three-dimensional projection of x-y plane (z = 0, lower panel) supports the suggestion of an interaction of the N^{ε} of the imidazole function with the neighbouring carbonyl function at residue A7 (shown in brown). A view of the z-y plane (x = 0, upper panel) indicates that any interaction of the A7-carbonyl with the imidazole function at B5 is unlikely. (See text)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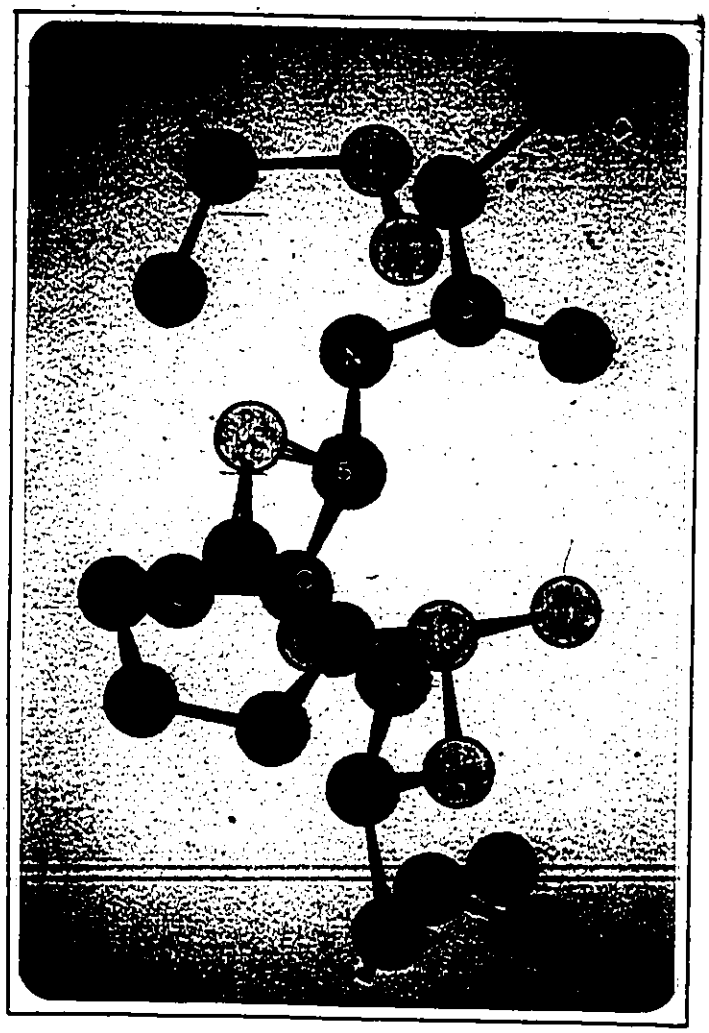
Z = 0

the reactivity data. The tyrosine reactivity in the present study, however, represents the average of the reactivities of the four tyrosine residues in the insulin monomeric unit. The effect of association on the environment of individual tyrosine residues would be more effectively examined by a study in which these four reactivities were separated.

Insulin: Free Monomer. From this study of the concentration dependence of the reactivities of the functional groups in insulin it is apparent that the chemical properties of some of the groups are different in the free monomer of insulin and in its associated forms. Insulin circulates in the human blood stream at concentrations where the free monomeric form predominates (Blundell et al., 1979 and references cited therein). It is the free monomer, then, which is involved in recognition of and binding to the insulin receptor on the plasma membrane of target tissues. It is thus of interest to determine the chemical properties of the free insulin monomer. Competitive labelling of insulin at a concentration of 5×10^{-7}M (where the free monomeric form predominates) in 1.0M-KCl at 37°C was therefore undertaken.

Previous competitive labelling studies of 2×10^{-4}M and 7×10^{-5}M insulin in 0.1M-KCl (Chan et al., 1981; Sheffer and Kaplan, 1979) demonstrate that, in the associated forms of insulin, the glycine amino terminus has a "normal" pK_a and a "normal" reactivity on the basis of a Brønsted plot (Cruickshank and Kaplan, 1975). From the examination of concentration-dependence of reactivity at pH 7.5 reported here, one would predict that properties of this group do not change when insulin is diluted. Isolation of N-α-DNP-glycine after competitive labelling of insulin with FDNB at 5×10^{-7}M bears out this prediction: a pK_a value of 8.21 ± 0.08 and a reactivity of 2.22 ± 0.01 relative to alanyl-alanine is obtained (Figure 14).

In insulin solutions where associated forms predominate pK_a values of 7.9 at 10°C (Sheffer and Kaplan; 1979) and 7.7 at 20°C (Chan et al., 1981) were obtained for this functional group using the competitive labelling procedure while a pK_a value of 8.0 at 25°C was obtained by Bradbury and Brown (1977) from an examination of the dimethylated form of amino termini of bovine insulin. These values are in close agreement

with that obtained here for the free monomeric form of insulin. A sharp discontinuity in reactivity-pH profile above pH 8.0 was observed at an insulin concentration of 2×10^{-4}M using acetic anhydride as the labelling reagent. This may reflect a specific interaction of the reagent acetic anhydride with insulin when the A1-glycine α-amino function is deprotonated. When FDNB was used as a labelling reagent (the present study, Chan et al., 1981), discontinuity in the reactivity-pH profile of the glycine amino terminus was not observed. When acetic anhydride is used as the labelling reagent at 37°C, the glycine amino terminus in associated forms of insulin shows an unusual reactivity-pH profile in that the reactivity observed for this group increases too rapidly between pH values of 7.0 and 7.5 to be fitted to a titration curve. Similar increases in the reactivities of the α-amino group of the B1-phenylalanine and the ε-amino function of the B29-lysine were also observed. It was suggested that these observations reflect a change in association state of insulin due to the ionization of one or more the amino groups at 37°C (Sheffer and Kaplan, 1979). The reactivity-pH profile obtained for the glycine amino terminus of the monomeric form of insulin in the present study does not show this sharp increase in reactivity: the data gives good fit to the theoretical titration curve (Figure 14). If the discontinuity in the data in the former study does indeed reflect a change in conformation due to dissociation of insulin, one would expect it to be absent in the reactivity-pH profile in the free monomer which cannot further dissociate. The concentration dependence study, however, indicates that the microenvironment of the glycine amino terminus is the same regardless of the degree of association of insulin. If this were the case, one would expect the reactivity-pH profile of the glycine amino terminus of 37°C to be the same in the two studies. It clearly is not. The data obtained by Sheffer and Kaplan (1979) indicate that there is some change in the conformation of insulin in concentrated solution as the pH is increased which affects the microenvironment of all three amino functions. It should be noted, however, that this conformational change may be reflective of a change in the nature of association as well as the degree of association especially since the properties seen at higher pH values are not reflected in the reactivity-pH profiles of

the functional groups in the free monomer. If indeed a monomer is formed above pH 7.5 in concentrated insulin solutions at 37°C, this monomer need not have the same conformation as the free monomeric unit formed in dilute insulin solutions. It is well known that reconstitution of ribosomes from constituent proteins is temperature dependent (Nomura, 1973) indicating that the conformation of these constituent proteins and/or the oligomers formed on their association is affected by temperature changes. It is not entirely unexpected, therefore, that the conformation of the insulin monomeric unit is temperature dependent at concentrations where associated forms exist.

Figure 19 is the Brønsted plot for the reaction of a series of standard amines with FDNB at 20°C (Chan et al., 1981). The present competitive labelling study was carried out at 37°C. The relative reactivity (RR) of the A1-glycine amino group in the free insulin monomer (i.e., the reactivity relative to that expected, on the basis of a Bronsted plot, for a primary amine with the same pK_a) can be estimated from this plot if one makes two assumptions: firstly, that the reactivity of the internal standard, alanylalanine, towards FDNB parallels to basicity at 20°C (i.e., fits on a Brønsted plot) and secondly, that the temperature dependence of the second order pH-dependent rate constant is the same for the primary amino group of the A1-glycine as for the primary amino groups of the standard amines used to construct the plot. Since alanine, alanylglycine and the penta-peptide, $(alanine)_5$, all lie very close to the curve, the assumption that the dipeptide, alanylalanine, lies on the Brønsted curve seems reasonable. The effect of temperature on reaction rate can be determined using the Arrhenius equation but, as a rule of thumb, a two-fold increase in reaction rate is normally considered to accompany a temperature increase of 10°C. Because the groups in question are all primary amines, the temperature coefficient is likely to be similar in each case. Using these assumptions, the relative reactivity of the glycine amino terminus in the free insulin monomer was calculated to be 1.8 which is very similar to the value obtained for this residue in associated forms of insulin (Table 8). Given that alanylalanine has a reactivity of 25.4 ± 0.2 relative to L-β-imidazole lactic acid (see below), at pH 7.5 the reactivity of this functional group in the free

monomer is approximately ten times that of L-β-imidazole lactic acid (Figure 14). This is in excellent agreement with the reactivity value at pH 7.5 for this residue at an insulin concentration of $5x10^{-7}M$ expected on the basis of the concentration-dependence study (Figure 12). Both of these results confirm that the chemical properties and, hence the microenvironment, of the A-chain amino terminal glycine are very similar in the monomeric unit of insulin in its free and associated states.

The average reactivity of the four tyrosine residues at pH 7.5 is unchanged as the insulin concentration is decreased from $10^{-3}M$ to $10^{-7}M$ (Figure 12). The reactivity-pH profile of these residues at an insulin concentration ($5x10^{-7}M$) where the free monomer predominates (Figure 16) shows a pK_a of 9.18 ± 0.05 and a reactivity of 4.45 ± 0.18 relative to alanylalanine. Using the competitive labelling procedure, Chan et al. (1981) estimated an average pK_a value of approximately 10 for the four tyrosine residues in insulin at a concentration of $6.86x10^{-5}M$, a value which is slightly higher than that obtained here. The average pK_a and reactivity values assigned to the tyrosine residues in associated forms of insulin by these authors, however, were merely estimated from a plot of the data. When this primary data was subjected to more rigorous mathematical analysis, an average pK_a value of 8.69 ± 0.04 was obtained for the four tyrosine residues in insulin in its associated forms. This latter value is slightly lower than that of 9.18 ± 0.05 determined in the present study.

Given that the pK_a and reactivity parameters for tyrosine were calculated using data obtained at pH values below the estimated pK_a (i.e., in the lower half of the curve), it is possible that a significant error accompanies the estimation of these parameters despite the fact that non-linear regression analysis shows a good fit of the data to the theoretical titration curve. Thus the difference in the average pK_a values calculated for the four tyrosines in insulin in its free and associated states may not be significant. Using the assumptions discussed above, the average second order, pH-dependent rate constant for the reaction of the phenolate ions of the tyrosines in the free insulin monomer with FDNB was calculated to be $2.74M^{-1}.min^{-1}$ which again is slightly higher than that of $2.2M^{-1}.min^{-1}$ (1.13 if non-linear least

squares analysis of data is used to calculate the reactivity) estimated for the tyrosine residues in associated forms of insulin (Chan et al., 1981). In both cases, however, the average rate constant is more than ten-fold lower than that of the reaction of N-acetyl-tyrosine amide with FDNB ($k = 29M^{-1}.min^{-1}$ Chan et al., 1981). Thus, by comparison to the reactivity of N-acetyltyrosine amide, at least some of the tyrosine residues must be buried in both the free monomeric and associated states of insulin.

On the basis of the concentration-dependence study, one would predict that the average reactivity of the four tyrosines would be 4.5 times that of imidazole lactic acid at pH 7.5 in both $7x10^{-5}M$ and $5x10^{-7}M$ insulin solutions. The reactivity-pH profile of the tyrosine residues at these concentrations both indicate that the the average reactivity is approximately twice that of imidazole lactic acid. Both the study of pH-dependence of chemical reactivity at two insulin concentrations and the study of the concentration-dependence of chemical reactivity at pH 7.5, however, indicate that there is little change in the average reactivity of the tyrosine as the association state of insulin changes. There is, however, a slight difference in the reactivity value obtained by the two types of experiments. Since these values are taken in the pH range when the tyrosine phenolic function is almost completely protonated and thus unreactive towards FNDB, the significance of the small difference between values from pH-dependence studies and concentration-dependence studies is questionable.

In total, these studies indicate that there may be small differences in the average chemical properties of the tyrosine residues in the free monomeric and associated forms of insulin. Accurate determination of these properties is somewhat hampered as the pK_a of these groups is in the higher pH range. Generation of a complete titration curve for "normal" tyrosine residues involves the determination of data points above pH 10.0. At high pH many proteins are denatured. If the micro-environment of the groups in question changes during protein denaturation, the chemical properties of the groups will also change. Consequently, pK_a and reactivity values are generally estimated by extrapolation of data in mainly the lower half of the reactivity-pH profile and are possibly in error. The present study may give a more accurate

prediction of these parameters under the conditions of study than does the previous study by Chan et al. (1981) as twice as many data points in the pH range 7 to 10 were used to fit the theoretical curve. Differences in the chemical properties of individual tyrosine residues on insulin self-association must be determined from studies in which the reactivities of these groups are separated. In the pH range which is normally considered to be physiological, the average reactivity of these groups is relatively small and does not appear to change drastically with changes in the association state of the insulin monomeric unit.

On the basis of the study of the concentration-dependence of reactivity of functional groups of insulin, the microenvironments of the histidine, the B_T-chain amino terminal phenylalanine and the solitary lysine residues change on dissociation of insulin oligomers. In the case of lysine, the dissociation to the free monomer is accompanied by an increase in the reactivity of the ϵ-amino function at pH 7.5 (Figure 12). In associated forms of insulin, the ϵ-amino group of the B29-lysine residue has an abnormally low pK_a at both 10°C and 20°C. There are sharp discontinuities in the reactivity-pH profiles above pH 8.0 in which the lysine residue becomes more reactive and may be the reflection of a second ionization curve with a higher pK_a. In fact, Bradbury and Brown (1977) using data determined by NMR above pH 8.0, assign this group a pK_a value of 11.2 at insulin concentrations where associated forms of insulin are expected. Thus, when the monomeric unit of insulin is self-associated, the ϵ-amino group of the B29-lysine exhibits a low pK_a value at pH values below pH 8.0 and a second, much higher pK_a value above pH 8.0. Such a situation would be expected if the loss of positive charge from insulin molecules in their associated forms resulted in a conformational change in which the microenvironment of the lysine was changed.

The exact position of the ϵ-amino group of the B29-lysine is not well defined in the crystal structure. It lies near the end of the extended C-terminal region of the B-chain on the outside of the hexamer (Figure 48). It is postulated that, in solution, the protonated ϵ-amino group is involved in a salt linkage with the carboxylate ion of the A4-glutamate residue of the same monomer (Blundell et al., 1972).


Z
Y
X

Such an interaction would result in the stabilization of the protonated ϵ-amino group relative to the deprotonated group and an increased pK_a for the lysine residue. In dilute solution, the B29-lysine ϵ-amino function has a pK_a which is close to that expected for such a group in the absence of interactions. In more concentrated insulin solution, the lysine residue has an abnormally low pK_a value in the physiological pH range. The postulated salt linkage involving the ϵ-amino group of the B29-lysine, therefore, is not supported by reactivity data.

When the reactivity of the functional groups of insulin in the insulin free monomer are studied, the reactivity-pH profile for the ϵ-amino group of the B29-lysine gives a single titration curve with a pK_a value of 9.8 ± 0.05 and a reactivity of 25.4 ± 1.7 relative to the internal standard, alanylalanine (Figure 15). There is no indication of the unusually low pK_a observed for this group below pH 8.0 at higher insulin concentrations. The pK_a of this group in the free monomer is approximately 1.5 pK units lower than that determined by Bradbury and Brown (1977) at higher insulin concentrations. The NMR experiments of Bradbury and Brown were performed in D_2O solution at pH meter readings above 10 on insulin solutions varying in concentration from 0.23% (3.8×10^{-4}M) to 1.8% (3×10^{-3}M). Under these conditions they report that insulin solutions of 0.9% (1.5×10^{-3}M), 0.25% (4.2×10^{-4}M) and 0.15% (1.7×10^{-4}M) give $s_{20,w}$ values of 1.54, 1.32 and 1.2 respectively, which are more consistent with a dimer to monomer transition than the hexamer to dimer transition expected in this concentration range from other velocity sedimentation studies (Milthorpe et al., 1977; Pekar and Frank, 1972; Goldman and Carpenter, 1972). It has been suggested elsewhere (Sheffer and Kaplan, 1979; Paselk and Levy, 1974; Blundell et al., 1972) that high pH promotes the dissociation of insulin oligomers. On the basis of ^{3}H-NMR data, Ramish (1982) has suggested that a 1.74mM-insulin solution at 37°C undergoes a hexamer to dimer transition at pH 10.3 and a dimer to monomer transition at pH 10.8. Whether the conformation of the monomeric unit formed by base-promoted dissociation of insulin in concentrated solution is the same as that of the free monmer formed in neutral, dilute insulin solutions is questionable given the known tendency for protein denaturation at basic pH and the large difference in pK_a value for the lysine ϵ-amino function in the present

study (dilute insulin solutions) and in the study of Bradbury and Brown (1977) (high pH, concentrated insulin solutions). Direct comparison of the monomeric unit formed under these differing conditions is made difficult by the fact that the latter study used insulin solutions which, on the basis of their calculations, contained significant amounts of dimeric insulin as well as monomeric insulin. In addition, the NMR studies were performed on solutions of insulin in D_2O. The effect of the heavier isotope on the protonation-deprotonation reaction necessitates the use of several assumptions in the assignment of a pK_a value to the reaction.

Using the pK_a and reactivity obtained below pH 8.0, the lysine residue in insulin dimers and higher oligomers appears buried when acetic anhydride is used as the chemical probe but exhibits approximately normal reactivity towards FDNB (Table 8). On the basis of a Brønsted plot (Figure 19), the lysine ϵ-amino function in the free insulin monomer has "normal" to "slightly enhanced" reactivity for a group with a pK_a of 9.8 (Table 8). Concentration-dependence studies indicate that the chemical properties of this functional group depend on the association state of insulin. A comparison of the reactivity-pH profiles of insulin in its free and associated forms confirms this dependence.

The microenvironment of the B1-amino terminal phenylalanine appears to change as monomers associate to form dimers; no further change is seen as the dimer associates to form higher oligomers (Figure 12). Earlier studies (Chan et al., 1981; Sheffer and Kaplan, 1979; Bradbury and Brown, 1977) were performed at concentrations above $5x10^{-5}M$ where, on the basis of sedimentation equilibrium data, an equilibrium mixture of dimer and higher oligomers is expected. The change in the microenvironment of the B-chain amino terminus on monomer-monomer association is, thus, expected to be apparent in a comparison of the chemical properties of this group in the insulin free monomer with those obtained in the previous studies of concentrated insulin solutions. Figure 18 shows how the reactivity data for the phenylalanine amino terminus determined by competitive labelling of solutions of $5x10^{-7}M$ insulin fits a theoretical titration curve with a pK_a of 6.92 and a reactivity of 0.644 relative to alanylalanine. (The parameters were obtained by non-linear least squares regression analysis of the

experimental data.) The reactivity of this functional group changes very rapidly over the physiological pH range. At pH values below pH 7.5 the group has low reactivity; at values above pH 7.5, high reactivity. The intermediate reactivity values which typically occur around the pK_a of a functional group are not observed. At the lower pH values the reactivity-pH profile approximates the general shape expected for a titration curve while at higher pH values the data points are more scattered about the theoretical curve. These data indicate that the microenvironment of the B-chain phenylalanine amino terminus in the free monomeric form of insulin is changing over the physiological pH range. This change is concomitant with the deprotonation of the α-amino function. At higher concentrations of insulin, this α-amino group give a more typical reactivity-pH profile at both 10°C and 20°C whether acetic anhydride or FDNB is used as the labelling reagent (Chan et al., 1981; Sheffer and Kaplan, 1979). The pK_a value determined in these studies is similar to that obtained in the present study. At concentrations of insulin where the dimer associates to form higher oligomers, the phenylalanine amino terminus was found to be "super-reactive" on the basis of a Brønsted plot. Using the assumptions discussed above, this group was found to have a reactivity in the free monomer which is very close to that expected for a primary amine with the same pK_a value (Figure 18, Table 8). Both the lowered reactivity and unusual reactivity-pH profile observed for the B1-phenylalanine in the present study compared to studies at higher concentrations lend credence to the idea that the microenvironment of the B-chain amino terminus of insulin is different in the free monomer of insulin and in insulin dimers and higher oligomers.

The reactivity of the phenylalanine α-amino group at pH 7.5 from the reactivity-pH profile shown in Figure 18 is 12.5 times that of imidazole lactic acid (reactivity of 0.49 relative to alanylalanine) which is slightly higher than the reactivity of 8 times that of imidazole lactic acid expected for this group at pH 7.5 at an insulin concentration of 5×10^{-7}M from the insulin concentration dependence studies (Figure 12). As the reactivity of this group is very sensitive to pH in the pH range 7.25 to 7.50, this difference in reactivity may merely reflect a very small difference in the pH during the competitive

labelling of insulin in the two studies.

As shown above, the reactivity and, hence, the microenvironment of at least one of the two histidine residues changes upon the interruption of dimer-dimer interactions. No further change is observed upon the interruption of monomer-monomer interactions. The chemical properties of the two histidine residues should therefore be the same in the insulin free monomer as in the dimer and different from those observed for insulin tetramers and hexamers. Since insulin solutions containing insulin dimers also contain significant amounts of higher oligomers and/or free monomers, absolute reactivity and pK_a values for the dimer are not easily obtained. The average reactivity of the two histidine residues of insulin towards FDNB was previously obtained in insulin solutions of a concentration of approximately 7×10^{-5}M at 20°C (Chan et al., 1981). On the basis of sedimentation equilibrium studies, an equilibrium mixture of insulin dimers and higher oligomers is expected at this concentration. The relative amount of free monomer is negligible. The dissociation of tetramers and hexamers to dimers results in a decrease in the average reactivity of the two histidine residues at pH 7.5 (Figure 12). The proportion of insulin hexamers and/or tetramers present in solutions at this concentration is sufficient to give a reactivity value for the histidine residues which is significantly greater than that which was obtained for these residues in the free monomer and that expected for the insulin dimer itself. Using the data obtained below pH 8.5, Chan and co-workers (1981) estimate that the histidine residues in 7×10^{-5}M insulin solution have an average pK_a value of 6.5 and a reactivity of 20 times that of imidazole lactic acid.

In the present study the reactivity-pH profile for the histidine residues in the insulin free monomer is indicative of two ionizations of the histidine residues: one in approximately the physiological pH range and a second at a much higher pH (Figure 17). This second ionization is, indeed, expected for all imidazole functions. In the previous study (Chan et al., 1981), this second ionization was not observed: at pH values above pH 8.0 the reactivity of the imidazole functions of the histidine residues decreased. This reactivity, however, was taken relative to the internal standard imidazole lactic

acid which itself has a second ionization at higher pH. These results can be interpreted as showing that the second ionization of the imidazole function results in a greater increase in the reactivity of imidazole lactic acid than in the average reactivity of the two histidine residues. In the present study, alanylalanine was used as an internal standard. Since this reactive moiety is an α-amino function with one ionization, the second ionization of the histidine imidazole function is more readily observed. If one assumes two ionizations, least squares analysis of the reactivity data for the histidines in monomeric insulin (Table 7) gives a pK_a value of 6.80 ± 0.26 and a reactivity of 0.234 ± 0.033 relative to alanylalanine for the first ionization and a pK_a value of 10.12 ± 0.50 and a reactivity of 2.10 ± 1.7 for the second (theoretical curves shown in Figure 18). When the data below pH 8.5 was taken to represent a single titration, a pK_a of 6.78 ± 0.231 and a reactivity relative to alanylalanine of 0.254 ± 0.030 was determined. Since the reactiviy of alanylalanine is 25.4 times that of imidazole lactic acid (see below), both methods of analysis yield an average reactivity for the histidines in the free monomer (5.9 relative to imidazole lactic acid) which is substantially lower than that obtained for these residues in insulin solutions containing dimers, tetramers and hexamers.

At pH 7.5 in 1.0M-KCl the average reactivity of the histidine residues is six times that of imidazole lactic acid ($\alpha_x r$ relative to alanylalanine = 0.244) which is in good agreement with the value predicted from the dependence of reactivity on insulin concentration in 1.0M-KCl. The study by Chan et al. (1981) was performed using solutions which were 0.1M in KCl. The reactivity of the histidines at pH 7.5 is approximately that obtained at pH 7.5 for a 7×10^{-5}M insulin solution in the initial study of the dependence of reactivity on insulin concentration in 0.1M-KCl (Figure 9). A comparison of Figures 9 and 12 shows that increasing the salt concentration of insulin solutions from 0.1M to 1.0M results in approximately a three-fold increase in the reactivity of the histidine residues only. As noted above, this increase in reactivity is not a specific imidazole effect but reflects some localized structure involving at least one of the histidine residues in associated forms of insulin. On the basis of the x-ray crystal data and

the enhanced reactivity of the histidine residues, this structure is suggested to be a hydrogen bond involving the δ- nitrogen of the imidazole ring (see discussion above). The decrease in the reactivity of the histidine residues with the interruption of dimer-dimer interactions suggests that the formation of this hydrogen bond in the insulin dimer and free monomer is less favourable. In the free monomer, the fact that the average reactivity of the histidine residues is approximately six times that of imidazole lactic acid indicates that some localized structure still exists in the vicinity of at least one of the two histidine residues. The decreased reactivity relative to that seen in higher oligomers may indicate the stabilization of a different conformation of the monomeric unit in which the hydrogen bond involving the δ-nitrogen is less directed. Since proteins are dynamic molecules, their conformation in solution is continually changing. The relative amounts of particular conformers, of course, is dependent upon their thermodynamic stability. The reactivity of a particular residue as determined by competitive labelling, or any other physical method, thus represents the weighted average of the reactivity of that residue in all possible conformers. It is therefore possible that the same conformation of the monomeric unit responsible for the abnormal reactivity of the histidines in concentrated insulin solutions is also present to a lesser extent in solutions of the free monomer. The reactivity of the histidine residues in concentrated insulin solutions was further increased when the salt content of the solutions was increased from 0.1M to 1.0M. A similar enhancement of histidine reactivity by increased salt concentration is possible in dilute insulin solutions. Adsorption effects at lower salt concentration, however, make it difficult to ascertain the extent of any such enhancement.

The reactivity-pH profiles for the functional groups in the free insulin monomer support the conclusion reached in the concentration-dependence study: the conformation of the insulin monomeric unit in its free form does not differ greatly from that in its associated forms. Thus, use of the x-ray crystal structure of insulin hexamers as a model for the structure of the monomeric unit in dilute solution appears justified. Postulations concerning the dynamic properties of any protein in either concentrated or dilute solution based on x-ray

crystal data, however, are always tentative and require confirmation by solution studies. In the case of monomeric insulin, such studies have previously been hampered by the high dilutions necesssary to promote dissociation to the free monomer. At present, the method described in this thesis provides the only approach by which the chemical properties of proteins in dilute solution can be directly determined.

Polypeptides with an N-terminal Histidine Residue. There has been much speculation in recent literature concerning the size of polypeptide required for the maintainance of a well defined structure in dilute solution (Blundell, 1982; Kaiser and Kézdy, 1984). The x-ray crystal data for insulin shows that secondary structure is stabilized by tertiary structure in addition to quaternary structure. The structure of the monomeric unit as depicted by x-ray crystallography was predicted to persist when dissociation to the free monomer is promoted by dilution. Application of the competitive labelling procedure to insulin at various concentrations has shown that there are no gross conformational differences in the monomeric unit in its free and associated forms. Polypeptides such as glucagon and β-endorphin, by virtue of their smaller size present less opportunity for the stabilization of secondary structures by tertiary structure. The x-ray crystal data for glucagon shows that the α-helical structure in the trimer is stabilized by quaternary structure. In dilute solution, the absence of intermolecular interactions is predicted to result in the formation of a flexible random coil (Saski et al., 1975). The presence of a hydrophobic amino acid at every third or fourth position in the primary sequence and the observation that it, like β-endorphin and related peptides, forms an amphipathic helix similar to that seen in the crystal structure in the presence of lipids or detergents has led to the prediction that glucagon molecules circulating in the blood stream are of random configuration but that interaction with the plasma membrane of target cells results in the formation of an α-helical structure. This coil to helix transition is postulated to be important in the biological action of glucagon (Kaiser and Kézdy, 1984; Blundell, 1982; Blundell et al., 1976; Sasaki et al., 1975). At present there is conflicting evidence in the literature concerning the structure of the free glucagon monomer: it is described alternately as a "compact structure"

and as an "extended, flexible coil" (see above). Since the determination of the chemical properties of a protein or polypeptide can yield novel information concerning structure, application of the competitive labelling approach to dilute glucagon solution affords a means by which one can determine whether or not this polypeptide hormone is indeed smaller than some critical size needed for the maintenance of defined structure in dilute aqueous solution.

Rothgeb et al. (1979) have used ^{13}C-NMR to obtain pK_a values for the ionizations of the N-terminal histidine residue in S-methyl-glucagon. They noted, in agreement with an earlier study by Epand et al. (1973), that as far as proton titration behaviour is concerned, these groups do not differ from those in model compounds such as histidine amide. Neither of these studies, however, directly evaluates the chemical properties of these groups. Some functional groups in proteins have apparently normal titration behaviour but abnormal chemical reactivities (Cruickshank and Kaplan, 1975; Garner et al., 1975; Hill and Davies, 1967). The fact that a group shows normal titration behaviour does not indicate, therefore, that its properties are normal in all respects. Determination of other chemical properties is also necessary.

Most proteins have only one ionizable group at the amino terminal residue. Glucagon is part of a family of biologically active peptides which have an amino terminal histidine residue and hence two ionizable groups at the amino terminal residue. It is, therefore, not immediately obvious what chemical properties would be deemed "normal" for such a group. The chemical properties and titration behaviour of the N-terminal histidine of the dipeptide histidylglycine were determined to establish a standard of comparison for the properties of the N-terminal histidine residue of glucagon.

Histidylglycine. The pK_a values for the functional groups of the dipeptides alanylalanine and histidylglycine were determined by both conventional acid-base titration and by competitive labelling. As expected, the pK_a value for the α-amino group of alanylalanine was the same within the limits of experimental error regardless of the method of determination. Titration data for histidylglycine, however, gives two ionizations which are separated by approximately two pK units

(Table 16) while the reactivity data of this N-terminal histidine residue as determined by the isolation of di-DNP-histidine after competitive labelling gives an excellent fit to a single titration with a pK_a value of 7.44 ± 0.02 (Figure 20). This corresponds to the higher pK_a value obtained by acid-base titration. There is no indication of a second pK_a value corresponding to the lower value obtained by acid-base titration. When the reactivities of these two groups are separated (Figures 25 and 26), the imidazole group has approximately the same apparent pK_a value as the α-amino and not the low value expected from acid-base titration. There is, however, a large difference in the reactivities of these two groups (Table 16): the α-amino group is an order of magnitude more reactive than the imidazole. The average reactivity of the two groups relative to L-β-imidazole lactic acid from the data in Figure 20, is 5.98 ± 0.08 which is in excellent agreement with an average of 5.90 calculated from the data for the individual groups in Table 16. When alanylalanine is used as the internal standard, isolation of di-DNP-histidine again gives a single ionization with a pK_a value of 7.49 ± 0.04 (Figure 21). The reactivity obtained is 0.235 ± 0.005 relative to alanylalanine which is also in excellent agreement with the average value of 0.242 calculated from the data in Table 16.

An imidazole group of a histidine residue in the interior of a polypeptide chain has a pK_a value of approximately 6.5 (Cohn and Edsall, 1943). When the imidazole function is on an N-terminal histidine, acid-base titration gives a pK_a value that is lowered by approximately one pK unit (Table 16; Rothgeb et al., 1978; Greenstein, 1933). This observation can be explained by the inductive effect of the protonated α-amino on the imidazole moiety. In previous studies (Cruickshank and Kaplan, 1975), the chemical reactivity for the imidazole groups yielded pK_a values with were close to those expected from titration data. In the present study, however, the pK_a value obtained by competitive labelling is substantially higher than that obtained by titration. It can be seen from the data in Figure 25 that the apparent ionization of the imidazole moiety obtained by reactivity is close to that of the α-amino group.

The reactivity-pH profile of a nucleophile should parallel its

titration curve as the reactivity of the protonated nucleophile is negligible in comparison to that of the deprotonated nucleophile. In this regard, the imidazole moiety of histidylglycine appears to be anomalous since the reactivity-pH profile is indicative of an ionization with a much higher pK_a value than that observed by titration. It can only be concluded that there is another factor influencing the reactivity of this group to such an extent that it completely masks the effects of the protonation-deprotonation reaction. An obvious clue as to what this factor may be is the observation that the reactivity of the imidazole moiety very closely parallels that of the α-amino group (Figures 25 and 26). Since the protonated α-amino group has such a large inductive effect on the imidazole group, it follows that the nucleophilicity of the imidazole moiety, and hence its chemical reactivity, will be substantially reduced. This explains the lack of reactivity observed in the pH range where the imidazole group is ionizing. Deprotonation of the α-amino group will remove this inductive effect and the reactivity of the imidazole moiety should increase and reflect the degree of ionization of the α-amino group. When the α-amino group is completely deprotonated, it is expected that, as observed in Figure 25, the reactivity of the imidazole moiety of histidylglycine is virtually identical to that of L-β-imidazole lactic acid.

The reactivity data obtained in this study demonstrate that if a protein of polypeptide has an N-terminal histidine, the imidazole moiety will have chemical properties which are dependent on the state of ionization of the α-amino group. There are several naturally occurring polypeptides such as glucagon, VIP and secretin that have an N-terminal histidine residue which is essential for their biological activity. It is therefore of interest to determine the chemical properties of these groups in order to determine whether or not the special properties of an N-terminal histidine are related to their biological activities and mechanism(s) of action.

Glucagon, VIP and Secretin. In the case of histidylglycine, the reactivity of the imidazole moiety could be separated from that of the α-amino group by acetylating the α-amino group in one half of the trace-labelled sample, isolating the N-acetyl-imidazolyl-DNP-histidine after full derivatization as described in the methods and quantifying its

[^{3}H]/[^{14}C] ratio. The reactivity of the α-amino group was determined by fully derivatizing the other half of the trace-labelled sample with FDNB (to form the di-DNP-histidine derivative), removing the DNP-group from the imidazole moiety by thiolysis, isolating the N-α-DNP-histidine and quantifying its [^{3}H]/[^{14}C]ratio. In principle, the same strategy could be applied to the N-terminal histidine of glucagon and VIP. There are, however, two considerations which make the same approach impractical. Firstly, in order to assure sufficient [^{14}C]counts for accurate quantification of the [^{3}H]/[^{14}C]ratio, relatively large amounts of material would be required making the experimentation costly. Secondly, the problem of assuring sufficient amounts of the di-DNP-histidine derivative for thiolysis and subsequent isolation of the mono-N-α-DNP-histidine derivative would add significantly to the amounts of material required. Two alternate strategies were employed.

a)Preliminary Glucagon Studies using HPLC

In the first procedure an equimolar mixture of glucagon and a standard nucleophile, alanylalanine, was divided into two parts. The first part was diluted, adjusted to the desired pH, and used for the [^{3}H]trace-labelling with [^{3}H]FDNB. In contrast to the procedure used for dilute insulin solutions, the [^{3}H]trace-labelled samples were not subsequently reacted with unlabelled FDNB as this would have resulted in the formation of the di-DNP-histidine derivative at the N-terminal residue. The second part of the mixture was divided again. Both portions were trace-labelled with [^{14}C]FDNB: the one at pH 6.5 and the other at pH 10.5. Taurine was added after all trace-labellings to remove unreacted FDNB. The two portions of [^{14}C]trace-labelled mixture were then recombined and diluted appropriately. Aliquots of this material were added to the [^{3}H]trace-labelled samples. The [^{3}H]/[^{14}C]-ratios for each DNP-derivative were determined after hydrolysis and HPLC separation. A separate aliquot of the [^{14}C]trace-labelled material was used to estimate the relative proportions of each [^{14}C]DNP-derivative after hydrolysis and HPLC separation. These estimates were used to correct the calculated [^{3}H]/[^{14}C]ratios in the samples.

DNP-derivativies of amino acids are photochemically labile. The

N-α-DNP-histidine and imidazolyl-DNP-histidine moieties are particularily unstable. Several breakdown products were present after a single isocratic HPLC separation. Although these materials were present in small amounts as evidenced by their low absorption at 320nm, they contained significant amounts of [^{3}H]cpm's (Figure 28). Consequently, a second isocratic HPLC procedure was used to obtain the N-α-DNP- and imidazolyl-DNP-histidine derivatives in sufficient purity to ensure accurate quantification of their [^{3}H]/[^{14}C]ratios (Figure 29).

The reactivity-pH profiles in Figures 30 and 31 show that the N-terminal histidine residue in glucagon resembles that in histidylglycine in that the reactivity of the imidazole group does not show the low pK_a value expected from titration data (Rothgeb et al., 1979; Epand et al., 1973) but rather follows that of the α-amino group. The pK_a of the imidazole function as determined from its chemical reactivity is 7.84 ± 0.13 while that of the α-amino function is 7.98 ± 0.11. While the deprotonation of the imidazole group is not detectable in this preliminary study, more data points in the lower pH range are necessary. The data for the tyrosine residues (Figure 32) shows that these two residues have an average pK_a value of 10.22 ± 0.05. Titration of glucagon in dilute solution has been followed spectrophotometrically by Gratzer and Beaven (1969). When the data thus obtained was curve-fitted assuming the titration of two groups with differing ionizations, pK_a values of 9.9 and 10.7 were assigned to the two tyrosine residues. The value obtained in the current study is approximately the average of these two values. When the competitive labelling data for the solitary lysine in glucagon was analyzed by a non-linear least squares procedure, a pK_a of 8.46 ± 0.10 was obtained. This is a lower pK_a than one normally expects for an ε-amino function but similar to the low pK_a value for the B29-lysine of insulin in concentrated solutions (Chan et al., 1981; Sheffer and Kaplan, 1979). The slight scatter of data points about the theoretical titration curve at pH values above 9.0 is also reminiscent of the reactivity-pH profile of the lysine in associated forms of insulin and may suggest a second conformation of glucagon exists above pH 9.0 in which the lysine has a substantially higher pK_a value.

In this preliminary experiment, data points were taken at intervals of 0.25 pH-units between pH 7 and pH 8 but at intervals of

0.50 pH-units in the higher and lower pH regions. The data is therefore not sufficient to completely rule out the possiblility of a second pK_a value closer to that normally expected in the case of either the ϵ-amino or the imidazole function. The data do, however, indicate that the N-terminal histidine residue of glucagon is similar to that of the model compound, histidylglycine, in that the imidazole function appears to act as a 'reporter group' for the deprotonation of the α-amino group and, in this regard, are interesting enough to warrent a more complete study of glucagon and related peptides.

The modified competitive labelling procedure used in this preliminary study was designed to circumvent several of the steps needed in the determination of the chemical properties of the N-terminal histidine of histidylglycine and to decrease the amount of material used. The method was unsatisfactory, however, in some respects, most notably in that it was necessary to correct the [^{3}H]/[^{14}C]ratios calculated according to the relative proportions of [^{14}C]labelled derivatives. In previous competitive labelling procedures, the protein and internal standard mixture was completely reacted with [^{14}C]labelled reagent. In these cases, the amount of [^{14}C]label is the same at each site of reaction. In the present procedure, the protein and internal standard mixture was trace-labelled with [^{14}C]reagent. In this case, the amount of [^{14}C]label at each site of reaction depends on the reactivity of that functional group. Since the same [^{14}C]labelled mixture was added to each [^{3}H]trace-labelled sample, the relative amounts of [^{14}C]-labelled derivatives were the same in all samples. Once this [^{14}C]-labelled material is added to the [^{3}H]trace-labelled sample the [^{3}H]/[^{14}C]ratio for each derivative is fixed; breakdown affects [^{3}H]- and [^{14}C]labelled derivatives equally and quantitative recovery is not necessary. In order to calculate absolute reactivites for the various functional groups, however, the amount of each [^{14}C]labelled derivative added to the [^{3}H]trace-labelled samples must be determined. Accordingly, a portion of the [^{14}C]labelled mixture was hydrolyzed and the amount of [^{14}C] quantified for each derivative after purification by HPLC. This method assumes that the [^{14}C]counts in each derivative after hydrolysis and HPLC separation accurately reflects the [^{14}C]counts for that group in the original [^{14}C]labelled mixture.

DNP-derivatives of amino acids differ from one another in their lability during the acid hydrolysis (Leggitt Bailey, 1962) and purification steps (as noted from the breakdown products observed on the HPLC runs (Figures 28 and 29)) as well as in their solubility in the eluting solvents (O-DNP-tyrosine, for instance, has notably poor solubility in both water and acetonitrile). The counting of eluant fractions (Figure 28C) in a typical sample run showed several peaks of high [^{3}H] content but low absorbance at 320nm (Figure 28B). Similar peaks were less evident in the [^{14}C]counting. These peaks may be due to radiolysis of the highly labelled [^{3}H]FDNB; still, the possiblity of differences in the breakdown of the samples and the singly-labelled [^{14}C]glucagon cannot be ruled out. While the overall recovery of radioactivity from the HPLC column was greater than 80% in all steps, quantitative recovery was never achieved. The relative proportions of the [^{14}C]DNP-derivatives after the isolation procedure was, therefore, not likely to be representative of the relative proportions of these derivatives in the initial [^{14}C]mixture. Thus, while the changes in $\alpha_x r$ values with pH for any one group accurately reflect changes in chemical reactivity, the absolute value of $\alpha_x r$ at any pH depends on the relative proportions of the [^{14}C]labelled derivatives and is likely to be in error. This preliminary study, then, affords a means of determining the pK_a of each functional group but not the absolute reactivity. A second procedure, in which the amount of each [^{14}C]DNP-derivative could be accurately determined, was therefore used in the subsequent studies.

b)Subsequent Studies of N-Terminal Histidine Polypeptides

In the second procedure (Figure 8), the N-α-[^{14}C]DNP-histidine and the imidazolyl-[^{14}C]DNP-histidine derivatives are added to the reaction mixture directly after the trace-labelling and prior to acid hydrolysis so that no further derivatization is necessary. Quantification of the [^{3}H]/[^{14}C]ratio for each derivative permits an unequivocal assignment of parameters to the α-amino and imidazole groups. A further technical advantage is that acid hydrolysis can be carried out directly on the reaction mixture thus eliminating several operations required in the procedure described by Duggleby and Kaplan (1975). Much more efficient use is made of the [^{14}C]reagent which gives an

additional cost savings to the method. The chemical properties of lysine, tyrosine and cysteine residues, if they are present, can also be determined by this procedure by adding the appropriate [^{14}C]DNP-derivative to the reaction mixture. In the present study, N-ϵ-[^{14}C]DNP-lysine was added so that the reactivity of the solitary lysine at position twelve in glucagon could be quantified and the average reactivity of the four lysines in VIP obtained.

Figures 35 and 34 show the fit to theoretical titration curves of the reactivity data obtained for the α-amino group and the imidazole moiety of the histidine in glucagon. It is clear that the reactivities of these functional groups in glucagon resemble those of the corresponding groups in histidylglycine in that the reactivity of the imidazole function parallels that of the α-amino group. The results with VIP (Figures 37 and 38) and with secretin (Figures 40 and 41) are similar to those of glucagon and histidylglycine. The pK_a values for both the α-amino and the imidazole functions in secretin are slightly higher than those in the other peptides.

The difference in the apparent pK_a value of 7.43 obtained in this study for the imidazole moiety of glucagon and that of 5.23 obtained by Rothgeb et al. (1978) for S-methyl-glucagon can be explained by the same inductive effect observed in histidylglycine. The approach of Rothgeb et al. (1978) reflects changes in the state of ionization of this group whereas the present approach reflects changes in the chemical properties. For most groups, these changes occur simultaneously but, as this study shows, an incomplete picture of the chemical properties of the imidazole group of an N-terminal histidine residue is obtained if one ignores subsequent chemical changes due to the ionization of the α-amino group. This may be especially relevant if, as evidence indicates, the histidine residue is essential for biological activity (Epand et al., 1981, and references cited therein).

The present results show that the similarity of the reactivity-pH profiles of the α-amino group and imidazole function in glucagon, secretin and VIP is a special property of an N-terminal histidine residue. In the case of histidylglycine, the reactivity of the imidazole function is not detectable when the α-amino group is protonated. However, when the α-amino is deprotonated the imidazole

group's reactivity is the same as that in L-β-imidazole lactic acid. In the cases of glucagon, secretin and VIP, the reactivity of the imidazole is also undetectable when the α-amino is protonated but when the α-amino is deprotonated the imidazole have a significantly higher than expected reactivity; approximately two times higher in the case of glucagon and three times higher in VIP. The reactivity of the imidazole function in secretin is approximately forty times that of imidazole lactic acid. This group also has a pK_a value which is one pK unit higher than that of imidazole lactic acid. N-α-acetyl-L-histidine, which has a pK_a that is one pK unit lower than that of imidazole lactic acid, is about half as reactive towards FDNB (Cruickshank and Kaplan, 1975; Table 9). If this relationship between pK_a and reactivity can be extrapolated to higher pH values, the enhanced reactivity of the imidazole moiety in secretin cannot be explained solely on the basis of its higher pK_a value. This group could therefore be classified as "super-reactive". The α-amino groups in histidylglycine secretin and VIP have the expected reactivity based on a Brønsted relationship (Chan et al., 1981; Kaplan et al., 1971) whereas the α-amino group in glucagon has approximately one half the expected reactivity.

The most obvious explanation for the enhanced reactivity of the imidazole moiety in glucagon and VIP is that this reactivity is reflecting some organized structure in the N-terminal region of the molecule. The evidence from circular dichroism studies for glucagon in dilute solution shows that there is no substantial amount of repeating structure (Blundell and Wood, 1982 and references cited therein). On the other hand, there is evidence from NMR studies (Jardelzky and Roberts, 1981; Wagman et al., 1980; Boesch et al., 1978) that stable localized structures exist in glucagon in solution. Such a local structure could be the presence of a hydrogen bond involving the deprotonated imidazole group. This would account for the increased reactivity observed in the present study. An increased reactivity due to hydrogen bonding has been observed previously in the case of the active center histidine of chymotrypsin (Cruickshank and Kaplan, 1975). One cannot, at present, rule out the possibility that a localized hydrophobic environment exists in the vicinity of the imidazole group which preferentially attracts the FDNB. This also would lead to an increased reactivity.

Nevertheless, each of these explanations requires that the N-terminal portion of the molecule be part of a structured region.

The solitary ϵ-amino group of glucagon has an unusually low pK_a value of approximately 8.5 and one sixth the reactivity expected on the basis of a Brønsted relationship (Chan et al., 1981). Again this is indicative of a localized structure. Epand and Wheeler (1975) measured the rate of reaction of trinitrobenzenesulfonic acid with glucagon at several pH values. On the basis of the data obtained, they assigned a pK_a of 10.36 to the solitary lysine residue. Unfortunately, the glucagon concentration (and hence the association state) is not specified and the experiments lack sufficient data points in the critical region between pH 8 and 10.5. It is therefore not possible to compare their results with those of the present study.

The lysine residues in VIP, while exhibiting "normal" reactivity, have an abnormally low pK_a value. Since this value represents the average of four lysine residues in VIP, it is particularly surprising. If, for example, only one of the four lysine residues in VIP had a lower than expected pK_a value, one would not anticipate that the average pK_a value would be so far removed from the "normal" value. It is likely, therefore, that more than one and possibly all of the lysine residues in VIP are in regions of localized structure. Since the lysine residues in glucagon and VIP exhibit abnormal chemical properties, it is possible that a structure involving a hydrogen bonded ϵ-amino group is important to the mechanism of action in these related polypeptides. While the two hormones have distinct plasma membrane receptors (Rodbell et al., 1970), both exert their biological effect by the activation of adenylate cyclase subsequent to receptor binding. It has previously been suggested that glucagon and VIP share a common mechanism of action (Rodbell et al., 1971).

Korn and Ottensmeyer (1983) have reviewed and summarized the evidence in the literature regarding the structure of glucagon in solution and have come to the conclusion that a compact, well-defined structure exists in dilute solution. They have proposed a model based, in part, on the available experimental data and, in part, on theoretical considerations of intramolecular interactions. One of the major conclusions is that the imidazole moiety of the N-terminal histidine is

hydrogen bonded to glutamine-20 and is the terminal residue in a hydrogen bonding network involving five residues. Such a structure would give rise to the enhanced reactivity we observe. They have also suggested that this hydrogen bonding network may provide the basis of an allosteric mechanism by which adenylate cyclase is activated. Unfortunately, they have not considered the fact that the chemical properties, and hence the hydrogen bonding properties, of the imidazole group will depend on the ionization state of the α-amino group. Since the α-amino group has a pK_a value of 7.6, less than half of the imidazole groups, on average, could take part in hydrogen bonding at normal, physiological pH values. It is possible that on binding to the receptor the pK_a value of the α-amino group is lowered by this new microenvironment with a concomitant increased nucleophilicity, and hence hydrogen bonding capacity, for the imidazole group. In such an event, our results would lend support to their hypothesis that this structural feature is important for the activity of glucagon.

Korn and Ottensmeyer (1983) also predict that the aliphatic chain of lysine-12 is involved in a hydrophobic interaction with leucine-14. If this is the case, one would predict a lowered pK_a value due to the hydrophobic environment and lowered reactivity due to steric hindrance. This is what is observed and, while such an exact agreement may be fortuitous, it does give additional support to the view that glucagon has a well-defined and ordered structure in dilute solution.

Chemical modification of the ε-amino group of lysine-12 suggests that a positive charge at this position is necessary to the activation of adenylate cyclase by receptor-bound glucagon. Most analogues which have this positive charge are full glucagon agonists at increased concentrations but exhibit decreased affinity for the glucagon receptor (Wright and Rodbell, 1980). In these analogues, the prototropic group at residue-12 would be expected to have a higher pK_a value than that obtained here for the lysine ε-amino function. Because the chemical properties of a functional group are dependent on its microenvironment, the pK_a of the solitary lysine in glucagon may increase on binding of the hormone to its receptor. If this is the case, one might postulate that the low pK_a of the ε-amino group is important to receptor recognition and binding but that a higher pK_a for this group in

receptor-bound glucagon is important to transduction.

The method used in the second glucagon experiment and in secretin and VIP experiments has a distinct advantage over the method used in the preliminary glucagon experiment in that a known number of [^{14}C]-counts in each DNP-derivative was added to each [^{3}H]trace-labelled sample. In this case, the [^{14}C]DNP-derivatives are added as amino acid derivatives and the [^{3}H]DNP-derivatives result from the hydrolysis of [^{3}H]trace-labelled protein. Although differences in the stability of DNP-derivatives in proteins and free amino acids have not been reported in the literature, it is possible that the breakdown of [^{14}C]DNP-groups differs from the of [^{3}H]DNP-groups during the hydrolysis procedure. In the case of glucagon, the one group in question is at an N-terminal position and the bond to be hydrolyzed is His-Ser; the other group, a lysine, is in the interior of the protein in the sequence Ser-Lys-Tyr. Hydrolysis of the bond to release the N-terminal histidine is expected to be quite facile; hydrolysis of bonds to release the lysine should also proceed with relative ease (Leggitt Bailey, 1962). Error due to the addition of [^{14}C]counts as DNP-amino acids rather than DNP-proteins is, in this case, minimal. It is conceivable, however, that the hydrolysis of bonds to release a group of interest proceeds relatively slowly. If there is indeed a differential breakdown of free DNP-amino acids and DNP-derivatives of amino acids in proteins, addition of [^{14}C]counts as the DNP-amino acids could result in some error in determining the absolute reactivity of such a group. In the method used for dilute solutions of insulin, where part of the protein-internal standard mixture was fully reacted with FDNB, both the [^{3}H]- and [^{14}C]counts are present as DNP-derivatives of protein residues. The type of error discussed above is thus avoided. Because of the special nature of peptides of the glucagon family, ie., the presence of two functional groups on one residue, complete reaction of the protein with FDNB was avoided. The modification of the dilute solution method used for the determination of the chemical properties of the N-terminal histidine of histidylglycine requires the use of fairly large amounts of protein. The addition of [^{14}C]counts as amino acid rather than protein derivatives limits the protein required to the small amounts necessary for the trace-labelling steps. Given the cost reduction and the fact that

several steps in the procedure for the isolation of specific groups are eliminated, in many cases, this method may provide a viable alternative to those used for the determination of the chemical properties of functional groups in insulin and histidylglycine.

One of the main features of the competitive labelling approach is that the $[^3H]/[^{14}C]$ratios for each functional group are determined in the $[^3H]$- and $[^{14}C]$labelling steps. Quantitative recovery of the derivatives is, therefore, not required. In most cases, the isolation and purification of the derivatives is a multistep process. If any one sample is accidentally lost at any stage of this process or if sufficient purity of the derivative is not attained, the labelling procedure must be repeated. To guard against such unforeseen mishaps, each $[^3H]/[^{14}C]$sample is split in half after acid hydrolysis and ether extraction. (Division of samples prior to acid hydrolysis is impractical as the DNP-protein is often insoluble in 6M-HCl at room temperature.) DNP-derivatives are purified from the first half of each sample and their $[^3H]/[^{14}C]$ratios quantified. Derivatives are isolated from the second half of the sample only when necessary (i.e., loss of the first half of the sample, insufficient purity or insufficient yield obtained for accurate $[^3H]/[^{14}C]$ quantification) or when one desires to check the results obtained from the first half of the sample. Where possible, only part of the purified DNP-derivative is added to the scintillation cocktail.

Determination of the absolute reactivity of each functional group using the competitive labelling procedure is accomplished by measuring its reactivity relative to that of an internal standard of known reactivity. In the later experiments presented in this thesis, a second internal standard was included as a precautionary measure. The data for the functional groups of histidylglycine demonstrate that the pH-dependent reactivity for a particular group is the same regardless of which internal standard is used. L-β-imidazole lactic acid was included as the sole internal standard in the initial experiments in this series. In experiments in which the reactivity of imidazole groups is to be determined, L-β-imidazole lactic acid is a useful internal standard as one can distinguish between effects which are common to all imidazole functions and those which are specific to

imidazole functions within the protein under study (as evidenced in the insulin concentration-dependence study in 1.0M-KCl, Figure 12). In any experiment, however, the internal standard can be lost during the purification procedure or its reactivity altered by specific interactions with the system under study. In the absence of an internal standard of known reactivity, the absolute reactivities of the functional groups in the protein in question cannot be determined. The use of two internal standards with differing chemical properties is a simple means by which the experiment can be protected against such situations .

The photo-lability of DNP-derivatives, paticularily imidazolyl-DNP-histidine and imidazolyl-DNP-lactic acid is well known (Leggitt Bailey, 1962). Because [^{3}H]- and [^{14}C]labelled compounds are indistinguishable from the unlabelled compound, one would expect the [^{3}H]/[^{14}C]ratio in some breakdown products to reflect the [^{3}H]/[^{14}C]-ratio of the parent compound. This, of course is not the case if breakdown occurs via a loss of the labelled carbon atom as $^{14}CO_2$ or of the labelled hydrogen as tritiated water, for example. The parent compound, if successfully separated from breakdown products, will maintain a constant [^{3}H]/[^{14}C]ratio. All samples and unlabelled DNP-derivatives were stored at -4°C in the dark. In high voltage paper electrophoresis of unlabelled imidazolyl-DNP-histidine or unlabelled imidazolyl-DNP-lactic acid markers, breakdown of the derivatives was evidenced by the appearance of new bands as the markers aged. Similar bands developed in the sample runs over time. This breakdown is inconsequential (as long as sufficient counts remain for quantification of [^{3}H]/[^{14}C]ratios) unless a breakdown product co-migrates with the parent compound in the electrophretic processes.

In studies where proteins and internal standards are in concentrated solution, the amount of material present assures that the percentage of label lost through breakdown will be insignificant. Even if the sample co-migrates with a breakdown product resulting from the preferential loss of [^{3}H] or [^{14}C], relative errors in quantification will be small provided that the derivatives are purified within a reasonable time after the labelling steps and that care is taken to protect the samples from light. When less material is present in the samples, relative errors resulting from breakdown and co-migration

will be larger. In the present experiments involving insulin or histidylglycine, sufficient material was present to minimize any possible errors. Additional peptide and internal standard(s) was added to each [^{3}H]trace-labelled sample in the form of [^{14}C]labelled peptide and [^{14}C]labelled internal standard(s) (approximately 1mg of peptide) and in the form of unlabelled carrier (5mg peptide and 0.5mg internal standard).

In order to determine the chemical properties of the N-terminal histidine residues of glucagon, secretin and VIP, the competitive labelling procedure was modified so that only the small amount of peptide present in the trace-labelling steps was used. [^{14}C]Counts and unlabelled carrier were added as DNP-derivatives of amino acids. DNP-derivatives of the functional groups in glucagon were isolated within a period of a few weeks after the labelling steps. The internal consistency of the data indicates that contamination of samples with breakdown products is not significant in this time frame. VIP and secretin samples were stored longer between acid hydrolysis and purification of the derivatives. The reactivity-pH profile of the imidazole function in both VIP and secretin shows a slight scatter of points about the theoretical titration curve. While this scatter could reflect an inherent property of the imidazole function in these systems, the possibility of preferential loss of one label through breakdown was investigated. Portions of the purified derivatives which had not as yet been added to scintillation cocktail (but which had been stored at -4°C in the dark for an approximately six weeks) were repurified by an addition electrophoretic separation and their [^{3}H]/[^{14}C]ratio quantified. In some cases, the imidazolyl-DNP-histidine and imidazolyl-DNP-lactic acid were purified from the second half of the sample. In each instance, aging of the sample resulted in an increase in the [^{3}H]/[^{14}C]ratio of the derivative. This is indicative of a breakdown process in which [^{14}C]counts were preferentially lost and of the co-migration of this breakdown product with the authentic sample. Breakdown of imidazolyl-DNP-histidine appears to be less severe than that of imidazolyl-DNP-lactic acid. The inclusion of the second, more stable standard nucleophile, alanyl-alanine, in these experiments allowed accurate determination of

reactivity-pH profiles for the α-amino functions and for the ε-amino functions in VIP. While some error is associated with the determinations of pH-dependent reactivity for the imidazole function of VIP and secretin, the data obtained is consistent with that obtained for the imidazole functions in glucagon and histidylglycine. In all cases, the reactivty-pH profile of the imidazole function is not indicative of a group with an ionization below pH 6 but parallels the reactivity-pH profile of the corresponding α-amino function. The error resulting from breakdown of the imidazolyl-DNP-derivatives of histidine and imidazole lactic acid may be avoided in any future experiments by the addition of larger amounts of unlabelled carriers. It is always advisable to minimize the time elapsed between the labelling steps and quantification of the $[^{3}H]/[^{14}C]$ratios of the purified DNP-derivatives. With these minor modifications, the method used in the VIP and secretin experiments provides a viable alternative to the other competitive labelling methods described.

CONCLUDING REMARKS

The modifications to the competitive labelling procedure outlined in this thesis provide a means to determine the chemical properties of proteins in dilute solution, of proteins with two functional groups at the N-terminal residue and of proteins which can be obtained in only minute amounts. The studies of the dependence of reactivity on insulin concentration in various reaction vessels and under various solvent conditions demonstrates that insulin adsorbs to glass, tefzel and polystyrene to differing degrees. Adsorption effects are significant in dilute insulin solutions. This adsorption cannot be prevented by the addition of a mixture of chemically modified histone proteins but can be prevented by addition of 1.0M-KCl to the buffer. Determination of the concentration-dependence of reactivty in the absence of adsorption effects indicates that the conformation of the monomeric unit does not change radically on the dissociation of insulin oligomers to form the insulin free monomer. Localized conformational changes affecting the microenvironments of some of the functional groups does accompany insulin self-association. Comparison of the chemical properties of the functional groups determined for the free insulin monomer with those for the monomeric unit in associated forms of insulin (Chan et al., 1981; Sheffer and Kaplan, 1979) shows that these localized structural changes result in "normal" properties for the histidine, lysine and phenylalanine in the free monomer and in "abnormal" properties for these groups at insulin concentrations where the dimer predominates.

The dependence of the chemical reactivity of an imidazole function at the N-terminal residue of a peptide or polypeptide is not reflective of its titration behaviour. The reactivity-pH profile of such a group parallels that of the corresponding α-amino function. The masking of the imidazole ionization appears to result from a decrease in the nucleophilicity of this group as a result of the inductive effect of the protonated α-amino function. When the α-amino group is fully deprotonated, the imidazole moiety has at least normal reactivity for a group of its type. This special property of the imidazole function is observed for the N-terminal histidine residue of all peptides studied.

Local structured regions in glucagon, VIP and secretin are indicated by the fact that each contains a functional group with unusual chemical properties.

Several researchers have made predictions as to the structure of insulin and glucagon in dilute solution of the basis of x-ray crystallographic data. The glucagon free monomer is postulated to be a flexible molecule with very little secondary structure. At present, there is evidence from physical studies both to support and to oppose this view. The unusual chemical properties observed in this study for the N-terminal histidine and the solitary lysine of glucagon indicate regions of local structure in the glucagon free monomeric form. In the case of insulin, the structure of the "preferred" monomeric unit, Molecule 2 of the 2Zn-hexamer, is predicted to persist in dilute solution. While the present competitive labelling study shows that there is no gross conformational change in the insulin monomeric unit on dissocation of insulin oligomers, the chemical properties of the N-terminal phenylalanine, the B29-lysine and at least one of the two histidine residues are different in the insulin free monomer and in associated forms of insulin. Both insulin and glucagon recognize and bind their respective plasma membrane receptors as free monomers. Determination of the chemical properties of these free monomers is one step towards the elucidation of mechanisms of hormone action. The next step is the determination of any changes in these properties on receptor binding. The experiments presented in this thesis set the stage for those involving the receptor-bound hormone.

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CLAIMS TO ORIGINAL RESEARCH

a) New Methodology:

i) Work in this thesis develops new methodology for the determination of the chemical properties of functional groups in proteins at very low concentrations (10^{-6}M or less) using the competitive labelling approach.

ii) Two methods are described which allow the application of the competitive lablelling approach to proteins or polypeptides with an N-termninal histidine residue. The second method (because it requires only the small amounts of protein needed for the trace-labelling step) affords a means of determining the chemical properties of proteins which can be obtained in only minute amounts.

b) Novel Information:

i) Work in this thesis shows that adsorption effects are significant at low insulin concentrations in Pyrex glass, Tefzel and polystyrene reaction vessels. Although insulin was known to adsorb to glass reaction vessels, the concentration at which adsorption becomes significant was not known. Loss of insulin from dilute solution through adsorption to Tefzel or polystyrene has not been reported previously.

ii) Adsorption of insulin to Pyrex glass can be prevented by increased salt concentration but not by addition of modified histone proteins to dilute solutions of insulin.

iii) These solution studies demonstrate that, while insulin self-association is accompanied by changes in the local environment of some of the functional groups, there are no gross conformational differences in the monomeric unit of insulin in its free and associated forms. This result provides experimental basis for using the x-ray crystal data for insulin to predict the structure of the free monomer in dilute solution.

iv) This thesis presents the first experimental evidence to suggest that the insulin dimer formed in solution may be a mixture of the symmetric and asymmetric dimers seen in the crystal structure.

v) An N-terminal histidine residue in a polypeptide has been shown

to have special chemical properties in that the reactivity-pH profile of the imidazole moiety does not reflect its titration behaviour but instead parallels the reactivity-pH profile of the α-amino group.

vi) The work in this thesis represents the first determination of the chemical properties of functional groups in the insulin and glucagon free monomers formed in dilute solution near physiological pH and temperature.

Some of the work in this thesis has been reported in the following publications and presentations:

Publications:

Cockle, S. A., Kaplan H., Hefford, M. A. and Young, N.M. (1982), Applications of High-Performance Liquid Chromatography to Competitive Labelling Studies: The Chemical Properties of Functional Groups of Glucagon, Analyt. Biochem. 125, 210-216.

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