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METHYLATION OF THE TYROSINE AND HISTIDINE RESIDUES OF INSULIN WITH METHYL IODIDE

By

Vicki Lynn Knowles

Thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of Master's of Science in Biochemistry.

University of Ottawa,
Ottawa, Ontario

November, 1984

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

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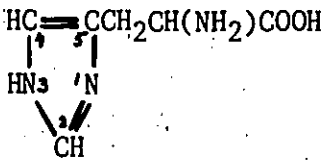
List of abbreviations

DM Hfs	1,3-dimethylhistidine
Dnp-F	1-fluoro-2,4-dinitrobenzene
Dnp-	2,4-dinitrophenyl-
im-	imidazole-
1-M His	1-methylhistidine
3-M His	3-methylhistidine
N-Ac-Tyr	N-acetyltyrosine
N-Ac-O-MT	N-acetyl-O-methyltyrosine
NMR	nuclear magnetic resonance
O-MT	O-methyltyrosine
RP-HPLC	reversed-phase high performance liquid chromatography

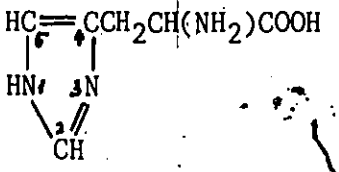
Nomenclature used for histidine

There are at least three nomenclatures in usage for numbering the atoms of the histidine imidazole ring (see "Nomenclature of α amino acids (recommended 1974)" Biochem. J. (1975) 149, 1-16):

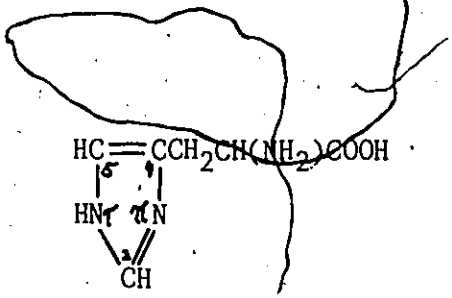
(1) Biochemical



(2) Chemical



(3) I.U.P.A.C.



In this paper, the biochemical nomenclature (#1) is used because it was the most common system found in the literature cited. In N-acetylhistidine, the acetyl group is attached to the amino terminal nitrogen atom.

Abstract

Selective methylation of the tyrosine and histidine residues of insulin was accomplished by citraconylation of the amino groups, incubation with methyl iodide at pH 10.5 and removal of the citraconyl groups by acidification in the presence of 8M urea. Maximum methylation yields an insulin with 3.3 tyrosine residues O-methylated and 2.0 histidine residues dimethylated. The extent of histidine dimethylation was analyzed from acid hydrolysates of methylated insulins after determining the ninhydrin color yield of 1,3-dimethylhistidine to be 0.97 compared to histidine. Since the rates of conversion of O-methyltyrosine to tyrosine during acid hydrolysis of the protein and free amino acid are different, the extent of tyrosine modification in insulin was determined by reacting methylated insulin with 1-fluoro-2,4-dinitrobenzene followed by a 48h acid hydrolysis. Insulin with 1.0 tyrosine and 0.5 histidine residues modified is fully active in producing a decrease in blood glucose levels in fasted rats at 5 x 10^{-8}mol/150g but more extensively methylated insulins are not active at this dose. The alterations in the ultraviolet spectra and the decreased solubility of the extensively modified insulins suggest that a change in conformation may account for the loss of biological activity.

I. Introduction

1. General introduction

Insulin influences a complex array of metabolic and functional responses in various tissues. The importance of insulin in both health and disease has spurred considerable interest in defining the structural and chemical features of the insulin molecule required for biological and immunological activity. A commonly used approach in defining this relationship between insulin structure and function is the use of derivatives which have minor alterations in structure but which result in an abnormal response. The determination of the complete amino acid sequence of insulin by Sanger and coworkers (Ryle et al., 1955) was a major breakthrough in this regard. It allowed the design of chemical experiments to modify certain amino acid residues in the molecule and led to the production of a variety of synthetic insulins and insulin analogues. Hodgkin's group in Oxford was then able to determine the three-dimensional structure of insulin by x-ray crystallography (Blundell et al., 1972). Knowledge of the arrangement of the atoms in space in the insulin molecule permits a more meaningful correlation between structure and function. However, despite intensive investigation, the precise details of insulin structure-function relationships still remain unsolved.

Chemical manipulation of native insulin has been employed for a variety of reasons including the introduction of fluorescent reporter groups or radioactive labels, as well as alteration of structural segments for functional studies (Saunders et al., 1982). Methylation is a particularly attractive modification since the added group is small, introduced under relatively mild conditions, preserves charge

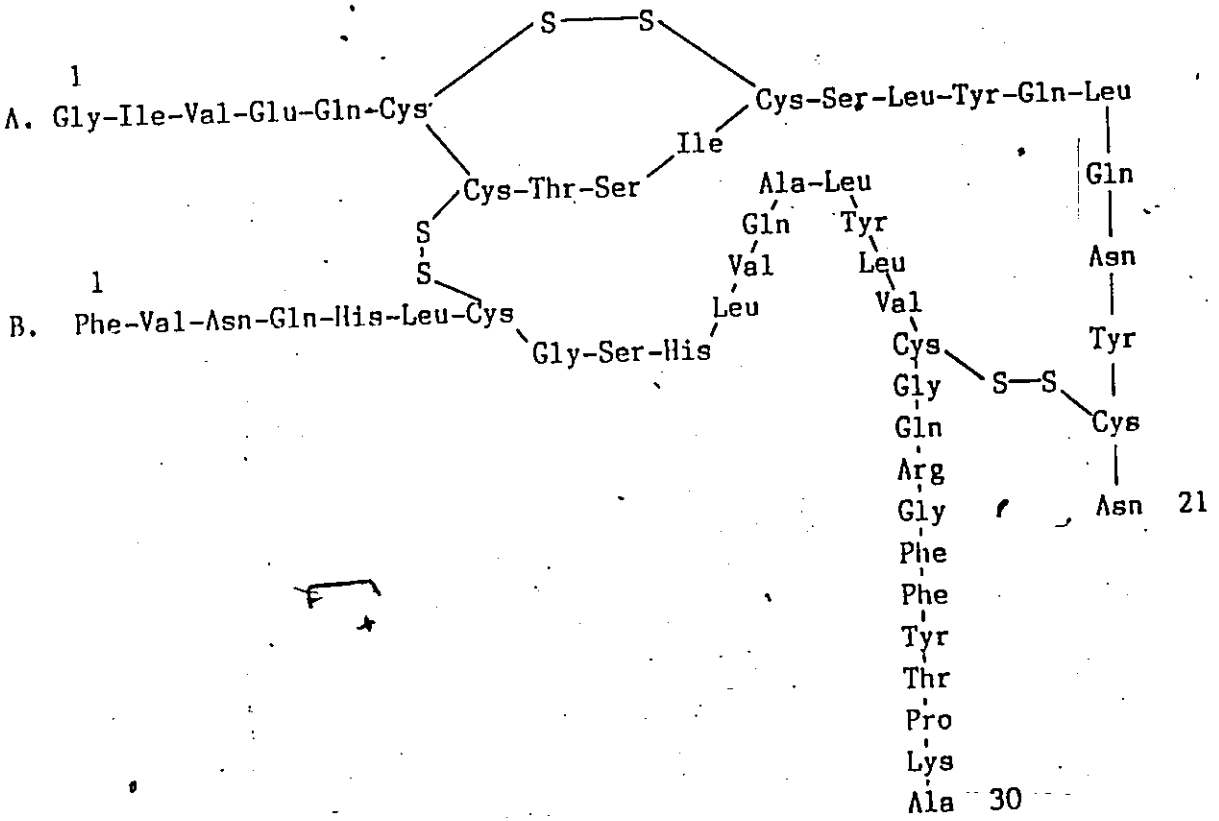
relationships and permits radiolabelling when [^{14}C] and [^{3}H] reagents are used. A variety of proteins, including insulin have been methylated (Means and Feeney, 1971). In early studies, insulin was nonspecifically methylated using methyl iodide (Charles and Scott, 1932) or diazomethane (Jensen et al., 1936) with carboxyl, amino, tyrosine phenol and cystine groups being modified to an unknown extent. More recently, the amino groups of insulin have been reductively methylated (Means and Feeney, 1968; Bradbury and Brown, 1977; Uschkoreit et al., 1980; Marsh et al., 1983). The fully methylated species was separated from native insulin and was found to have 50% biological activity (Marsh et al., 1983).

In this thesis, a method is presented for specifically methylating tyrosine and histidine side chains of insulin using methyl iodide and of quantifying the extent of modification. Preliminary biological data of the methylated insulins are also given. The following review will therefore concentrate on structure and function of insulin with particular emphasis on the involvement of tyrosine and histidine residues.

2. Insulin Structure

The primary structure of porcine insulin (Figure 1) shows two polypeptide chains, A and B, containing 21 and 30 residues respectively with interchain disulphide bridges at residues A7-B7 and A20-B19 and a third disulphide bridge within the A-chain at A6-A11. There are two A-chain (A14 and A19) and two B-chain (B16 and B26) tyrosine residues and two B-chain (B5 and B10) histidine residues. Insulin contains three amino functions (A1 Gly, B1 Phe and B29 Lys) and lacks both methionine

Figure 1: Primary structure of porcine insulin.


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

and tryptophan residues. Determination of the sequences from a number of species has revealed a large degree of homology (Blundell et al., 1972). Human insulin differs from porcine insulin in that its B-chain C-terminal amino acid is a threonine residue and not an alanine residue. Bovine insulin has alanine and valine residues at positions B8 and B10 respectively.

Under physiological conditions, insulin exists predominately in the monomeric form (Goldman and Carpenter, 1974; Pekar and Frank, 1972). Two insulin monomers associate to form an asymmetrical dimer which is held by hydrogen bonding and Van der Waals contacts (Blundell et al., 1972). In the presence of Zn^{2+}, the dimers aggregate to form rhombohedral hexamer crystals. The degree of aggregation depends on a number of factors including pH, ionic strength, metal ions, temperature and insulin concentration (Blundell et al., 1972; Paselke and Levy, 1974). Initial x-ray analysis of the insulin hexamer determined the structure at 1.9Å resolution (Blundell et al., 1972) with later refinements to 1.5Å (Blundell and Wood, 1982). The higher resolution reduces the uncertainty in the position of nearly all the main chain atoms and the side chain atoms to less than 0.2Å (Chothia et al., 1983).

Aggregation to dimer and hexamer, which may be similar in many respects to receptor binding, involves close contacts of tyrosines at the two interunit interfaces (Blundell et al., 1972). The tyrosine residues involved in the formation of the dimer are the B16 Tyr from one monomer subunit which interacts with B'8 Gly, B'9 Ser and B'12 Val from the other subunit, and B26 Tyr which interacts with B'16 Tyr, B'23 Gly, and B'24 Phe (the prime denoting residues of the second monomer).

Similarly, in the formation of hexamers from dimers, A14 Tyr interacts with A'14 Tyr and B'Phe from the adjacent dimer unit. The A14 Tyr is similarly accessible in all association states, whereas B16 Tyr and B26 Tyr are probably only accessible in the monomer (Muszkat et al., 1984). The A19 Tyr is not only on the surface of the monomer, but is also exposed on the surface of the dimer and hexamer (Blundell et al., 1972).

X-ray analysis suggests that the B10 His is important for the formation of the Zn hexamers. Thus, guinea pig (Smith, 1966), coypu (Smith, 1972) and hagfish (Peterson et al., 1975) insulins which do not contain a histidine at B10 do not form stable zinc insulin hexamers. In the dimer, this histidine should be exposed. The B5 His lies on the surface of the monomer, and its accessibility is unchanged on formation of dimers or hexamers (Blundell et al., 1972). However, both histidine residues were found to be inaccessible in the hexamer, but accessible in the monomer when assessed by the photochemically induced dynamic nuclear polarization method (Muszkat et al., 1984).

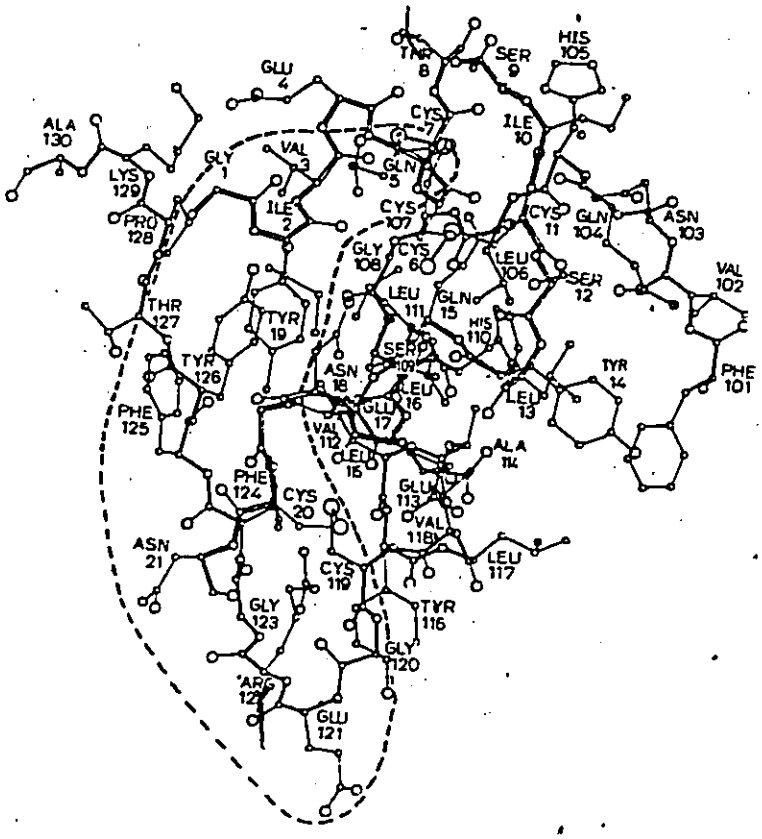
It has generally been assumed that the hexamer found in the crystals has the same structural conformation as that observed in zinc insulin solutions, but this has not been conclusively proven. Further uncertainty is introduced by extrapolating from the monomeric structure in the crystal hexamer to its unaggregated form in dilute solution. The conformation of insulin in solution has been studied by circular dichroism in an attempt to resolve this issue. Blundell's group (Wood et al., 1975) have concluded that there is no difference in the secondary and tertiary structure between the monomer in its free form and in its associated states, but Pocker and Biswas (1980, 1981) found

that there are substantial changes. Because of these difficulties, the exact conformation of physiologically active form of insulin is still tentative.

The conformation of the insulin monomer (molecule 2, see Figure 2) reveals a hydrophobic core and two predominantly hydrophobic surfaces (Blundell et al., 1972). The B-chain contains 3 loops of α-helix while the A-chain contains 2 regions of α-helix and lies within the cleft of the B-chain. The main chain structures for the two independent molecules of the dimer are similar except at the N-terminus of the A-chain and the C-terminus of the B-chain. This ability of the insulin molecule to adopt different conformations was suggested to be an important factor in the expression of its biological activity (Dodson et al., 1979).

Studies of the biological activities and receptor affinities of chemically modified insulins and sequences of insulin from a variety of species have strongly suggested that a largely invariant region on the surface of the insulin monomer is involved in receptor binding (Wood et al., 1975; Pullen et al., 1976; De Meyts et al., 1978; Blundell et al., 1983). The three-dimensional structure shows that this putative functional surface includes both A-chain residues A1 Gly, A5 Gln, A19 Tyr and A21 Asn plus adjacent B-chain residues B24 Phe, B25 Phe, B26 Tyr, B12 Val and B16 Tyr. Chemical studies at the B-chain N-terminus suggest that B5 His is also involved in the expression of bioactivity (Schwartz and Katsoyannis, 1978), although distant from the other implicated residues (Figure 2).

Figure 2: Three dimensional structure of the insulin monomer outlining the surface residues which constitute the putative receptor binding region. The A-chain residues are numbered 1-21 and the B-chain residues 101-130 (from Wood et al., 1975).


THR 8
SER 9
HIS 105
GLU 4
CYS -7
ILE 10
ALA 130
GLY 1
VAL 3
GLN 5
LYS 129
ILE 2
CYS 107
CYS 11
GLN 104
ASN 103
PRO 128
GLY 108
CYS 6
LEU 106
SER 12
VAL 102
THR 127
LEU 111
GLN 15
TYR 19
HIS 110
SER 109
TYR 126
ASN 18
LEU 16
TYR 14
PHE 101
PHE 125
GLU 17
LEU 13
VAL 112
ALA 114
LEU 15
PHE 124
CYS 20
GLU 113
VAL 118
ASN 21
LEU 117
CYS 119
GLY 123
TYR 116
GLY 120
ARG 122
GLU 121

3. Insulin Function

Insulin is an anabolic hormone which regulates the metabolism of target cells at a variety of subcellular sites. Thus, insulin influences plasma membrane transport, cytoplasmic enzyme activity, ribosomal protein synthesis and nuclear DNA and RNA synthesis (Goldfine 1978; Goldfine et al., 1982). It is generally accepted that insulin triggers its various metabolic responses in target tissues by specifically binding to a cell surface protein, the insulin receptor (Roth et al., 1975; Cuatrecasas and Hollenburg, 1976; Czeck 1977; Reed et al., 1981; Ronnett et al., 1983). However, the exact mechanism by which the insulin-receptor complex initiates the biochemical effects is unknown.

Much recent work has centred on the insulin receptor. Insulin receptors from several sources have been characterized as membrane glycoproteins with molecular weights of 300,000-350,000 (Cuatrecasas, 1972; Jacobs et al., 1979; Pollet et al., 1982). The carbohydrate moiety of the insulin receptor contains N-acetyl glucosamine, mannose and galactose (Hedo et al., 1981). Two major insulin binding proteins have been identified: a smaller protein (β subunit) which demonstrates linear Scatchard binding and a larger protein (α subunit) which gives a curvilinear plot (Maturo and Hollenberg, 1978; Krupp and Livingston, 1978; Roth et al., 1982). In addition a minor subunit has been identified which is speculated to be a proreceptor or some closely associated effector protein (Kasuga et al., 1982). The insulin receptor possesses two classes of disulphide bonds which differ in their susceptibilty to reducing agents (Massague et al., 1980; Jacobs et al., 1979). Two α subunits and two β subunits are disulphide linked within

the receptor complex (Czech et al., 1981).

The precise chemical structures of the insulin receptor which are required for the recognition of insulin are unknown. Chemical and enzymatic modification of cells has suggested that protein functional groups, sialic acid, carboxyl, tyrosyl and histidyl groups but not phospholipids are important for the interaction of insulin with its receptor (Verspohl et al., 1982; Pilch, 1982). Upon binding, insulin stimulates phosphorylation of serine, tyrosine and possibly threonine residues of the β subunit (Kasuga et al., 1982a & b). Clark and Harrison (1982) have concluded that a fraction of bound insulin becomes covalently linked to its receptor via disulfide-sulfhydryl exchange on the cell surface. However, the significance of these modifications in initiating insulin action are not understood.

When insulin binding to receptors is studied over a wide range of insulin concentrations, Scatchard analysis of the resulting data yields curvilinear plots (DeMeyts et al., 1976; Ginsberg, 1977). It has also been demonstrated that when receptor occupancy is increased with unlabelled insulin, the dissociation rate of previously bound [^{125}I]insulin is greatly increased (DeMeyts et al., 1973). DeMeyts has suggested that these studies provide evidence for negatively cooperative receptor interactions. However, some workers have failed to detect this phenomenon (Gliemann et al., 1975) and others have questioned its interpretation (Pollet et al., 1977). Donner (1980) has found that fragments of [^{125}I]insulin remain associated with the cell and when binding data are corrected to assay only for [^{125}I]insulin, curvilinear Scatchard plots were linearized. This suggests that insulin binding is not a negatively cooperative process. Further

evidence is needed to resolve this controversy.

Binding of insulin to its receptors induces clustering of insulin receptors (Schlessinger et al., 1978; Jarrat and Smith, 1975) which subsequently leads to internalization of the ligand-receptor complex (Bretscher et al., 1980). Chronic exposure of cells to insulin causes a decrease, or down regulation in the level of cell surface insulin receptors (Krupp and Lane, 1981; Blackand et al., 1978). It has been proposed that down regulation of insulin receptors involves internalization (Krupp and Lane, 1981; Peterson et al., 1983) or increased degradation of receptors (Van Obberghan et al., 1981; Kosmokos and Roth, 1980). Insulin induced receptor down regulation has also been demonstrated in vivo (Goldfine et al., 1973; Harrison et al., 1976). A reduction in the number of insulin receptors can account for the lowered sensitivity to insulin in certain pathological states (Harrison et al., 1976).

Several of the diverse metabolic processes resulting from the combination of insulin with its receptor have been exploited to measure the biological function of insulin and insulin analogues. The relative ability of analogues and insulin to stimulate these metabolic effects can be expressed as a biological potency. In vivo methods depend on the ability of insulin to produce blood sugar depression or hypoglycemic spasms in mice (Burn et al., 1952). In vitro methods rely on the augmentation of glucose uptake in the isolated rat diaphragm (Lambert et al., 1972) or the oxidation of [^{14}C]glucose to [^{14}]carbon dioxide in rat adipose tissue (Moody et al., 1974). Other indices of the effects of insulin on adipose tissue include glucose uptake or incorporation of [^{14}C] into fatty acids. Measurements of the ability

of modified insulins to compete with radioactively labelled insulin in a radioimmunoassay have also been used to give an estimate of their relative potency (Lindsay and Shall, 1970; Busse and Carpenter, 1976).

The bioactivities of insulin derivatives vary with the bioassay systems used. In vitro bioassay systems are generally more sensitive to conformational changes as demonstrated by circular dichroism than are in vivo systems (Brandenberg et al., 1971; Freychet et al., 1974). The reason for this discrepency is not clear but may be due to prolonged in vivo circulation times of the modified insulins. Geiger and coworkers (1980) have shown that sulfopropionyl-B1-phenylalanine insulin possesses full biological activity in vivo and in vitro, and receptor binding affinity was unchanged, but the immunological activity of this derivative is greatly reduced compared to native insulin. This suggests that the residues responsible for insulin's receptor binding and immunological activity are not identical and that immunological activity is not necessarily a measure of biological activity induced by insulin receptor binding.

Modification of insulin may alter the biological activity by either reducing the affinity of the hormone for the receptor or by decreasing the ability of the complex, when formed, to elicit a biological response. Either or both of these effects may be brought about by direct interference of the modifying group, by changes of charge distribution or by induced conformational changes in the hormone. Therefore, the position of the modifying group and the conformational changes induced by it must be precisely defined. Most insulin analogues have shown parallel alterations in both binding affinity and biological potency although they may vary widely in both

of these parameters (DeMeyts et al., 1978). The similarity of the potency figures in the receptor binding and bioactivity assays shows that the altered potency in the latter assay can be ascribed to the change in binding affinity toward insulin receptors. The receptor-derivative complexes, once formed, are fully capable of triggering the next event in the chain leading to the biological response. Interestingly, one group of insulin derivatives containing one or more basic amino acids attached to the A1 glycine shows no correlation between binding activity and biopotency (Rosen et al., 1980). Therefore, the translation of binding into a biological effect can be altered by chemical modification of insulin.

4. Analogues and derivatives of insulin:

The role of various residues in the expression of the biological activity of insulin has been studied by chemically modified derivatives and synthetic insulin analogues. Chemical modification with reagents that are more or less selective for specific residues is also useful for determining their disposition in insulin. However, the difficulty in preparing "single site " chemically modified derivatives of insulin is a disadvantage with this technique. Preparation of synthetic insulin analogues allows for a specific alteration in which one amino acid residue may be substituted or deleted. While such analogues can shed valuable information on insulin structure-function relationships, their synthesis is time consuming and costly (Saunders et al., 1982). Thus, most information has been obtained from chemically modified insulins.

Chemical modification of the tyrosyl residues of insulin has taken

two routes: (1) electrophilic ring substitution; and (2) nucleophilic attack of the unprotonated oxygen atom of tyrosine. Iodination (Hamlin and Arquilla, 1974), nitration (Morris et al., 1970) and diazotization (Suzuki et al., 1969) of insulin are examples of the former type of reaction. Reactions with cyanuric fluoride (Aoyama et al., 1965), 2-iodo-N-methylpyridinium iodide (Drewes et al., 1980), 1-fluoro-2,4-dinitrobenzene (Chan et al., 1981) and 1-fluoro-2-nitro-4-trimethylammoniobenzene iodide (Sutton et al., 1972) are examples of the latter reaction mechanism.

Nitration of the tyrosyl residues of insulin at pH 7.4 with tetranitromethane gives rise to mono(A14)-3-nitrotyrosine and di(A14;A19)-nitrotyrosine insulins (Morris et al., 1970). In a similar study, Gattner (1971) found that the reactivities to tetranitromethane over the range from pH 7 to pH 3 are A14 Tyr > A19 Tyr > B16 Tyr > B26 Tyr. When the degree of nitration is increased, the reactivities of A14 Tyr, A19 Tyr and B16 Tyr are nearly equal whereas B26 Tyr retains a low reactivity. These findings agree well with the x-ray model which predicts that all tyrosine residues of insulin are accessible but B26 Tyr should be accessible only with difficulty in the dimer and hexamer (Blundell et al., 1972). The biological activities of mono- and dinitrated insulins as measured by blood glucose depression in chronically diabetic mice are the same as that of native insulin (Morris et al., 1970). However, immunological activities of these derivatives are greatly reduced (Morris et al., 1970; Gattner, 1971).

Iodination of the tyrosine residues of insulin has been extensively investigated not only for structure-function studies, but also for preparation of labelled tracers for the study of action and

metabolism of insulin (Hamlin and Arquilla, 1974; Sodoyez et al., 1975; Linde et al., 1981; Sonne et al., 1983) and the estimation of insulin present in body fluids by immunoassay (Berson and Yalow, 1966). Upon iodination, a heterogeneous mixture of insulin and iodinated insulins with one or more iodine atoms distributed among the four tyrosine residues is produced. The A-chain tyrosine residues are much more reactive than the B-chain residues (Linde et al., 1981). At low levels of iodination and at pH 9.2, A19 Tyr is more readily derivatized than A14 Tyr. With higher levels of iodination, the relative incorporation of iodine is reversed, which agrees with the nitration results. Since the A14 Tyr is readily diiodinated and dinitrated (Morris et al., 1970) but A19 Tyr is only mono-substituted, it has been suggested that one of the ortho positions of the A19 Tyr is buried and therefore inaccessible to chemical reagents. In the presence of 8M urea or organic solvents, iodine is also incorporated into the B-chain (Massaglia et al., 1969).

Early studies with unfractionated mixtures of iodinated insulin revealed that increasing the degree of iodine substitution of insulin results in a loss of biological activity which is of a greater magnitude than the loss of immunological activity (Izzo et al., 1964; Glover et al., 1967; Garratt et al., 1972). To prevent the loss of biological activity, iodination conditions have since been designed to minimize the degree of iodine substitution. The resulting monoiodinated insulins have been fractionated by DEAE-Sephadex A-25 (Hamlin and Arquilla, 1974), DEAE-cellulose (Bihler and Morris, 1972) and anion exchange (Sodoyez et al., 1975) chromatographies.

Biological activity of monoiodinated insulin with 70% of the iodine on the A14 Tyr and 30% on the A19 Tyr was 100% in the fat cell

assay (Freychet et al., 1971), the blood sugar depression test (Hamlin and Arquilla, 1974) and the rat adipocyte (Gliemann et al., 1979). These results suggest that iodination of the A14 Tyr does not affect the bioactivity of insulin. However, in the mouse convulsion assay, activity was 73-74% (Hamlin and Arquilla, 1974; Bihler and Morris, 1972) and no activity was observed in the rat diaphragm assay (Lambert et al., 1972). Immunological activity was 6% compared to zinc insulin (Bihler and Morris, 1972) but almost 100% compared to zinc free insulin (Hamlin and Arquilla, 1974) as measured by the passive immune hemolysis assay.

All four isomers of monoiodoinsulin were prepared to a purity of 97% by Linde and her coworkers (1981) using a combination of polyacrylamide gel electrophoresis and ion exchange chromatography. The heirarchy of the apparent binding affinities of the monoiodinated isomers to rat adipocytes was B26 > A14 = B16 > A19. The biological potencies of these monoiodoinsulins as determined from the rat adipocyte assay corresponded within ±8% to the observed change in binding affinities (Sonne et al., 1983). While the A14 isomer has the same biological activity as native insulin, the potency of the A19 isomer was decreased and that of the B26 isomer was enhanced. Thus, iodination of the A19 Tyr reduces both the binding affinity and biological potency of insulin supporting the hypothesis that this residue is part of the receptor binding region.

The B26 tyrosine is also part of the putative receptor binding region of insulin (Pullen et al., 1976) and the increase in affinity (Linde et al., 1981) and biological potency (Sonne et al., 1983) after iodination (as compared to the A14 isomer) may be due to the presence

of iodine itself or to secondary conformational changes in this region. Very few insulin derivatives have been reported to be superpotent to native insulin. Koboyashi and colleagues (1982) have prepared an insulin with B24 D-Phe and this analogue was more potent than native insulin.

Markussen and Larsen (1980) have used reversed-phase high performance liquid chromatography (RP-HPLC) to separate the A14 and A19 monoiodinated insulin isomers. However, there was some insulin contamination of the A19 isomer. The four monoiodinated insulin isomers and native insulin have recently been completely separated using RP-HPLC (Welinder et al., 1983). This technique should prove extremely useful in the preparation of "single site" iodinated insulins for future receptor binding and biological studies.

There have been few studies in which the tyrosine phenolic groups of insulin have been modified. Reaction of insulin with cyanuric fluoride results in the modification of the A19 and B16 residues, whereas A14 and B26 were unreactive (Aoyama et al., 1965). This is in contrast to the reactivities towards tyrosine ring substitution in which the A-chain but not the B-chain tyrosine residues are reactive. Similarly, two of the four tyrosine residues react with 1-fluoro-2-nitro-4-trimethylammoniobenzene iodide (Sutton et al., 1972). The average reactivities of the tyrosine residues of insulin towards 1-fluoro-2,4-dinitrobenzene are substantially less than for a free phenolic group (Chan et al., 1981). These data support the observation (Menendez et al., 1969; Blundell et al., 1972) that one or two phenolic groups of insulin are "buried" and are therefore inaccessible to chemical reagents.

Purified insulin derivatives substituted with methylpyridinium or trimethylammonionitrate at A14 Tyr have been prepared (Drewes et al., 1980). Biological activity of both derivatives was drastically reduced as measured by the mouse convulsion assay and the fat cell assay. Both A14-modified derivatives were obtained in crystalline form suggesting that the conformation in these derivatives is not different from that of native insulin. Further studies of the conformation of these derivatives are clearly needed. Modification of the A14 Tyr may cause a disturbance in the tertiary structure of the hormone resulting in the decreased bioactivity.

Studies of the receptor binding and biological activities with synthetic analogues of insulin in which a tyrosine residue has been substituted have been performed by two laboratories. Danhoe's group have prepared analogues of porcine insulin in which the A14 Tyr (Danhoe et al., 1980a) or the A19 Tyr (Danhoe et al., 1980b) are replaced with phenylalanine. The purified A14 Phe insulin analogue had the same biological activity as native insulin when measured by lipogenesis in rat adipocytes, supporting the concept that A14 Tyr is not essential for biological activity of the hormone. The biological activity of A19 Phe insulin was only 22.6%. Ferderigos and coworkers (1983) have also synthesized an insulin analogue with A19 Phe. In the stimulation of glucose oxidation by rat adipocytes, this analogue had only about 8% the potency of native insulin and receptor binding affinity was also reduced. These data strengthen the postulation that a hydrogen bond between the hydroxyl function of A19 Tyr and the carbonyl function of the A1 or A5 residues is critical in the stabilization of the insulin monomer and in the establishment of a conformation commensurate with

high biological activity (Blundell et al., 1972). A Van der Waals contact between A19 Tyr and A2 Ile sidechains also appears to be necessary to permit the molecule to assume a biologically active conformation (Kitagawa et al., 1984).

Investigations aimed at elucidating the role of histidine residues in the biological expression of insulin have not been conclusive. Comparative studies of guinea pig, coypu and hagfish insulins which lack a histidine at position B10 have shown that these insulins are considerably less active (<9%) than mammmalian insulins (Smith, 1972; Peterson et al., 1975; Zimmerman et al., 1972). However, the decreased biological activity cannot be attributed solely to the lack of B10 His because the sequences of these three insulins differ substantially from mammalian insulins. The B5 histidine is invariant in all insulin species suggesting that it is essential for biological activity of the hormone.

Few investigations of chemical modification histidine residues in insulin have been performed. Reaction of either zinc or zinc free insulin with diazonium-1-H-tetrazole results in diazotization of one histidine residue while the other residue remains in the monoazotized stage (Suzuki et al., 1969). However, other workers have found that this reagent reacts uniformly with both histidine residues in insulin (Hornishi et al., 1963). Carboxymethylation of zinc free insulin modifies both histidine residues (Covelli and Wolf, 1967).

Synthetic analogues in which the B5 His or the B10 His are substituted with other amino acids have been prepared. Substitution of B5 His with L-alanine resulted in a decrease in activity of approximately 50% suggesting that B5 His is only partly essential for

the biological action of insulin (Weitzel et al., 1970). Synthesis of des (tetrapeptide B1-4) and des(pentapeptide B1-5) insulins and their subsequent biological evaluation revealed activities of 54% and 5% respectively (Schwartz and Katsoyannis, 1978). Since des (B1), des(dipeptide B1-2) and des(tripeptide B1-3)-[pyroglutamic acid B4] insulins possess identical or only slightly reduced bioactivities compared to native insulin (Geiger and Langner, 1973), the drastic decrease in bioactivity of des (pentapeptide B1-5) insulin was attributed to the deletion of the B5 His. This finding indicates that the B5 His is directly involved in the expression of insulin's bioactivity.

While B10-Ala insulin has the same potency as native (B10 His) insulin in the mouse convulsion test (Weitzel et al., 1970), B10-Leu insulin has 45% (mouse convulsion test) or 36% (radioimmunoassay) activity of the native hormone (Schwartz and Katsoyannis, 1977). These results suggest that the B10 His is not directly involved in the biological activity of insulin. Substitution of B10 histidine with lysine produced an insulin analogue with only 14-15% of the potency of native insulin in stimulating lipogenesis and in radioimmunoassays (Schwartz et al., 1982). In insulin receptor binding to rat liver membranes, B10 Lys insulin was 17% as potent as the natural hormone. Schwartz and co-authors have suggested that the relative size of the amino acid residue at B10, rather that its polarity, is the most important factor in maintaining insulin structure conferring high biological activity. However, this is inconsistent with the fact that substitution of the B10 histidine with alanine, a relatively small amino acid, resulted in an analogue with full biological activity.

While the involvement of certain tyrosine residues in the biological expression of insulin activity has been fairly well established, further evidence is needed to determine the importance of histidine residues in the function of insulin. The lack of chemical reagents with specificity for histidine residues has limited our knowledge as to the role of this residue in the structure and function of insulin as well as other proteins. Methylation of the tyrosine and histidine residues of insulin provides a method for studying the structure-function relationship of these residues in insulin. In addition, methylation of insulin with radiolabelled reagents may prove useful in preparing radiolabelled insulin for receptor binding and metabolic studies if it can be shown that methylated insulin retains full biological activity.

II. Materials

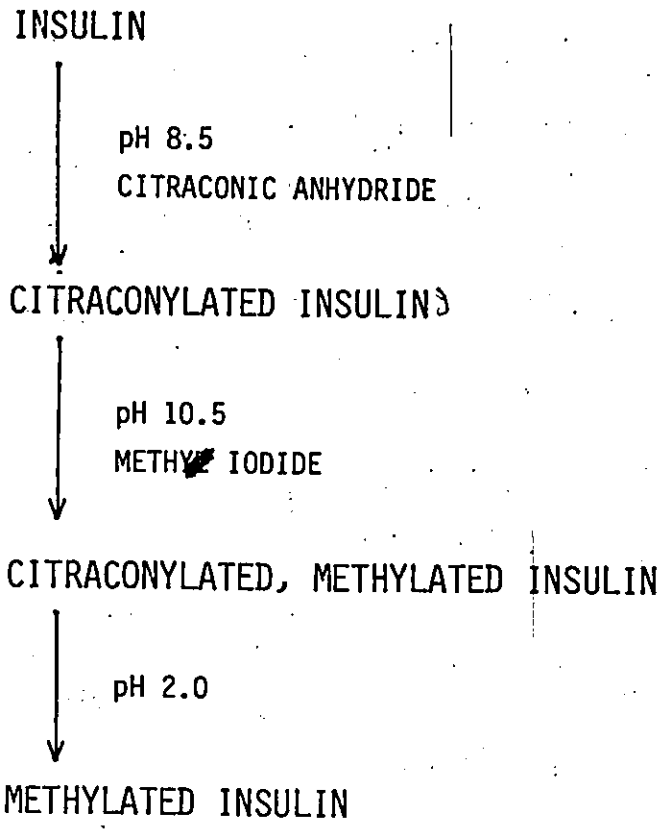
Porcine zinc insulin (0.35% zinc by weight) was obtained as a gift from Connaught Laboratories (Toronto, Ontario). The Glucose kit No. 635, 1-fluoro-2,4-dinitrobenzene (Dnp-F), O-methyltyrosine (O-MT), 1-methylhistidine (1-M His), 3-methylhistidine (3-M His), N-acetylhistidine and N-acetyltyrosine amide (N-Ac-Tyr amide) were purchased from Sigma Chemical Co. (St. Louis, Missouri). [^{14}C]Labelled methyl iodide was obtained from Amersham Corporation (Oakville, Ontario) and NEN Canada (Lachine, Quebec) supplied the Aquasol-2 for scintillation counting. Spectra/Por 3 dialysis tubing was obtained from Spectrum Medical industries (Los Angeles, Calfornia). Male Wistar rats (150-200g) were obtained from Charles River Canada Inc. (Montreal, Quebec).

III. Methods

1. Preparation of methylated insulin

A novel yet simple procedure for specifically methylating tyrosine and histidine residues of proteins using methyl iodide is presented below (see Figure 3). In order to prevent methylation of the amino groups, they are first blocked by reaction with citraconic anhydride (citraconylation). Methyl iodide is added at a sufficiently high pH (pH 10.5) so that most of the tyrosine phenolic and histidine imidazole functions are in their deprotonated forms. The citraconyl groups are removed by acidification yielding a protein with only methylated tyrosine and histidine residues. Specific details are given here for methylation of insulin, but the principles and procedures employed may

Figure 3: Reactions involved in the preparation of methylated insulin. See text for details.


INSULIN
pH 8.5
CITRACONIC ANHYDRIDE
CITRACONYLATED INSULIN
pH 10.5
METHYL IODIDE
CITRACONYLATED, METHYLATED INSULIN
pH 2.0
METHYLATED INSULIN

be applied to other proteins.

(a) Citraconylation of the amino groups of insulin

Insulin (40mg) was dissolved in 5ml of 0.5M borate in the presence or absence of 8M urea. The pH was adjusted to 8.5 by the addition of 5N KOH from an Agla micrometer syringe. A 900 fold molar excess of citraconic anhydride (400 µl) was added and the pH maintained at 8.5 by the addition of 5N KOH controlled by a pH stat apparatus. The reaction was considered complete when uptake of base stopped. Final concentration of insulin was 0.9mM.

(b) Methylation of insulin

The citraconylated insulin solution was adjusted to pH 10.5. A 50% (v/v) solution of methyl iodide in acetonitrile was added to a concentration of 3mM methyl iodide (5000 fold molar excess over insulin). This solution formed a layer beneath the aqueous phase. Solutions were placed in a shaker bath at 40°C and aliquots removed at various time periods. The reactions were terminated by either adjusting the pH to 2 or immediate dialysis against water if retention of the citraconyl groups was desired.

(c) Unblocking of amino groups

The citraconylated, methylated insulin solutions were made 8M in urea, adjusted to pH 2 with 6N HCl and left at room temperature for 18h. The addition of urea was found to be necessary to keep the citraconylated, methylated insulins in solution at low pH.

(d) Renaturation of insulin

Prior to biological and spectroscopic studies, the methylated insulins were renatured by gradually removing the urea. Samples were dialyzed in presoaked 3500 molecular weight exclusion dialysis tubing. The dialysis sacs were placed in one litre of a solution containing 8M urea, 0.5% acetic acid, adjusted to pH 4 with NaOH. Eight litres of sodium acetate buffer (0.5% acetic acid, pH 4) without urea was pumped through the urea sodium acetate buffer at the rate of 200ml/h with a peristaltic pump. The samples were then dialyzed three times against 4l of distilled water brought to pH 4 with acetic acid, lyophilized and stored at -20°C.

2. Quantification of methylation

(a) Conversion of O-methyltyrosine to tyrosine

A solution containing 2 μmol O-methyltyrosine and 2 μmol norleucine in 20ml 6N HCl/1mM phenol was prepared. An aliquot (1ml) of this solution was pipetted into each of 16 pyrex tubes which were sealed under vacuum. Duplicate samples were hydrolyzed at 110°C for 0.5, 1, 2, 4, 8, 16, 24 and 48 hours. The hydrolysates were dried under vacuum and quantified by amino acid analysis with a TSM Technicon auto analyzer using a single column system and eluting with buffers of pH 3.25, 4.25, 7.50, and 9.50. Percent O-methyl tyrosine remaining was calculated from amino acid analysis as follows:

$$\% \text{ O-MT remaining} = \frac{\text{O-MT at time t/ Norleu at time t}}{\text{O-MT at time 0/ Norleu at time 0}} \times 100$$

The % tyrosine recovered was calculated using the ratio of O-methyltyrosine to norleucine at time zero, and correcting for the

difference in color yields between O-methyltyrosine and tyrosine by the formula

$$\% \text{ Tyr} = \frac{\text{Tyr at time t/ norleu at time t}}{\text{O-MT at time 0 x 0.932/ norleu at time 0}} \times 100$$

(b) Quantification of 1,3-dimethylhistidine

To quantify 1,3-dimethylhistidine (DM His) from amino acid analysis, the color yield of this derivative was determined. N-acetylhistidine (40mg) was dissolved in 4ml 0.5M borate and the pH adjusted to 10.5 with 5N KOH. An aliquot containing 20mg N-acetylhistidine was removed and reacted with a 100 fold molar excess (1.2ml) of methyl iodide in 50% (v/v) acetonitrile. The reaction was allowed to proceed for 72h in a shaker bath at 40°C. The product of this reaction and 20mg of unreacted N-acetylhistidine were hydrolyzed separately in 6N HCl for 16h at 110°C in vacuo. The hydrolysates were dried under vacuum, and redissolved in cartridge buffer. Equal volumes of each were combined and subjected to amino acid analysis.

To verify that the structure of this compound is 1,3-dimethylhistidine, 100mg of N-acetylhistidine was methylated and hydrolyzed as described above. The hydrolysate was dried and redissolved in 0.01N HCl. This was applied to a Dowex 50W-X8 column which had been washed with 0.2N NaOH, 6N HCl, and then 0.01N HCl. After washing with 15ml of water, the 1,3-dimethylhistidine was eluted from the column with 5% ammonia. After drying in the presence of sulphuric acid, the sample was redissolved in 0.01N HCl and dried. The purified 1,3-dimethylhistidine was examined by nuclear magnetic resonance (NMR) spectroscopy (Varian 360-60MHz) by Dr. L. Benoiton and

F. Chen.

(c) Hydrolysis of methylated insulin

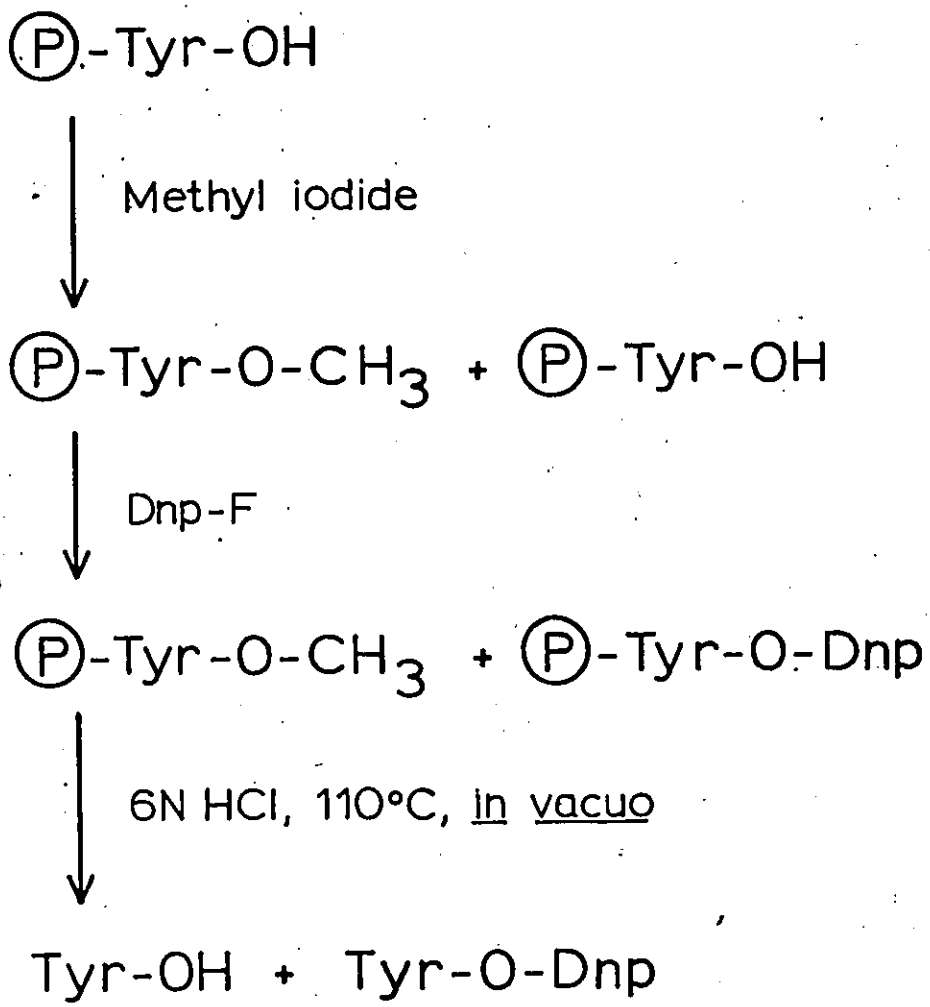
The citraconylated, methylated insulin samples were hydrolyzed in 1ml 6N HCl/1mM phenol for 12h at 110°C in vacuo. Norleucine served as an internal standard. These are the usual conditions for the hydrolysis of proteins and this results in hydrolysis of the peptide bonds of insulin and acidolysis of O-methyltyrosine. For simplicity, the cleavage reactions of methylated insulin under these conditions are referred to as hydrolysis reactions.

(d) Dinitrophenylation of methylated insulins

A second means of quantifying the degree of O-methyltyrosine formation in the methylated protein was devised. The method involves the use of Dnp-F, a nucleophilic reagent which reacts with hydroxyl, amino and imidazole functions of amino acids. If these reactive groups have been citraconylated or methylated, they are unavailable for reaction with Dnp-F. After complete acid hydrolysis of the dinitrophenylated, methylated protein, any tyrosine which appears on amino acid analysis will have resulted from the hydrolysis of O-methyltyrosine (see Figure 4). Tyrosine residues which did not react with methyl iodide will be derivatized by Dnp-F. The resulting O-Dnp-tyrosine is stable to acid hydrolysis and is not detected by the amino acid analysis procedure employed.

Dinitrophenylation of methylated insulin was performed in the presence of 8M urea to maintain the solubility of the reaction mixture. An excess of $NaHCO_3$ (approximately 0.2g/ml of solution) was added followed by the addition of Dnp-F which was in acetonitrile (50% v/v)

Figure 4: Schematic diagram of reactions involving tyrosine residues of proteins during methylation, dinitrophenylation and hydrolysis of insulin.


Ⓟ-Tyr-OH
Methyl iodide
Ⓟ-Tyr-O-CH3 + Ⓟ-Tyr-OH
Dnp-F
Ⓟ-Tyr-O-CH3 + Ⓟ-Tyr-O-Dnp
6N HCl, 110°C, in vacuo
Tyr-OH + Tyr-O-Dnp

(150µl/mg protein). After 18h at 25°C in the dark on a wrist action shaker, the reaction was terminated by adjusting the pH to 2 with concentrated HCl. Unreacted Dnp-F and other side products were extracted with diethyl ether. This procedure results in precipitation of Dnp-protein which was then centrifuged at 10,000g for 10min. The supernatant was discarded and Dnp-protein was washed three times with 1ml water and then hydrolyzed in 6N HCl in vacuo for 48h at 110°C.

(e) Dinitrophenylation of methylated N-acetyltyrosine amide

The rate of methylation of N-acetyltyrosine amide was determined to compare the reaction to that of tyrosine groups in insulin. N-acetyltyrosine amide (45µmol) was dissolved in 10ml of 0.5M borate and the pH adjusted to 10.5 with 5N KOH. A 1000 fold molar excess (5.0ml) of 50% methyl iodide in acetonitrile (v/v) was added. The solution was placed in a shaker bath at 40°C and aliquots containing 4.5µmol N-acetyltyrosine amide were removed at various times. An excess of $NaHCO_3$ (approximately 0.2g/ml of solution) was added followed by the addition of Dnp-F (50% v/v acetonitrile) (400µl/mg protein). The reaction was allowed to proceed 18h at 25°C in the dark and then terminated by adjusting the pH to 2 with 6N HCl. After three extractions with diethyl ether, the product was lyophilized and then hydrolyzed 48h in 6N HCl with norleucine as an internal standard.

(f) Radioactive labelling of insulin with [^{14}C]methyl iodide

Insulin (12mg) was citraconylated and the pH adjusted to 10.5. A 50% (v/v) solution of [^{14}C] labelled methyl iodide (59mCi/mmol) and unlabelled methyl iodide (0.5ml)in acetonitrile was added to give a

final specific activity of 6.2 μCi/mmol. Aliquots containing 1.2mg insulin were removed at 0.5, 1, 2, 4, 7, 10 and 24 hours. Samples were dialyzed four times against 4l of 0.01% acetic acid. A portion of each sample containing 0.3mg insulin was dissolved in 10ml of Aquasol 2 and radioactivity determined on a LKB 1215 Rack Beta scintillation counter equipped with automatic quench correction. An aliquot was hydrolyzed 18h with norleucine as an internal standard. Another aliquot of each sample (0.6mg) was lyophilized and redissolved in 3ml of 8M urea. Following reaction with Dnp-F, the Dnp-insulins were hydrolyzed as described above.

3. Preliminary determination of bioactivity of methylated insulins

The biological activity of methylated insulins was assayed by blood glucose depression in vivo. The primary objective here was to carry out a preliminary study to determine whether a detailed investigation of the activity of these derivatives was worthwhile. Wistar rats weighing 150-200g were injected subcutaneously (0.2ml/150g) with control insulin, methylated insulin, or vehicle (0.9% saline, 1mM phosphate, pH 7.35) after a fast of 18h. The range of dosages employed was 10^{-8}M to 10^{-11}M. Blood samples were collected in heparinized capillary tubes from the tail at 0, 15, 30 and 60 min intervals.

Blood glucose was assayed by the o-toluidine method (Glucose No. 635) or by the copper/molybdate method (Folin, 1929). Both methods rely on the reducing ability of glucose. The o-toluidine reagent reacts with glucose to form a colored complex, the absorbance of which is read at 635nm. The copper molybdate method involves reduction of the cupric ion followed by reoxidation with molybdate prior to

absorbance measurements at 670nm. Blood proteins were removed by precipitation with tungstic acid or by trichloroacetic acid prior to the glucose assays.

4. Absorption spectra of renatured methylated insulins.

Renatured, methylated insulins were dissolved in 0.1M acetic acid (pH 3.0) at a concentration of 3.3 x 10^{-5}M. The absorption spectrum of each solution was measured from 200 to 290 nm with a Unicam SP 1800 ultraviolet spectrophotometer equipped with a Unicam AR 25 linear recorder. N-acetyl-O-methyltyrosine amide (N-Ac-O-MT amide) was synthesized by dissolving 10mg N-acetyltyrosine amide in 0.5M borate, adjusting the pH to 10.5 with 5N KOH and reacting with 5ml 50% (v/v) methyl iodide in acetonitrile for 72h at 40° on a shaker bath. A mixed bed ion exchange column was prepared by combining equal volumes of Amberlite and Dowex 50. The Amberlite was pre-equilibrated by washing with 2N HCl, water, 2N NaOH and water. The Dowex 50 was pre-equilibrated by washing with 2N NaOH, water, 2N HCl and water. After washing the mixed bed resin with 10ml 0.1M acetate, the N-acetyl-O-methyltyrosine amide solution was applied to the top of the column and the eluate was freeze dried. The absorption spectra of N-acetyltyrosine amide and its O-methylated derivative were determined in 0.1M acetic acid at concentrations of 3.7 x 10^{-4}M and 2.4 x 10^{-4}M, respectively.

IV. Results

1. Methylation of insulin

Methylation of citraconylated insulin at pH 10.5, 40°C in 0.2M borate for 24h proceeded with liberation of acid as indicated by a small decrease in pH. When the buffering capacity of the medium was increased, no change in pH occurred during the reaction. Subsequent methylations were therefore performed in 0.5M borate. Small increases in the volume of the aquaeous layer and concomitant decreases in the volume of the methyl iodide/acetonitrile layer with prolonged reaction times are attributed to the formation of methanol. After 24h of reaction, insulin was insoluble at pH 10.5.

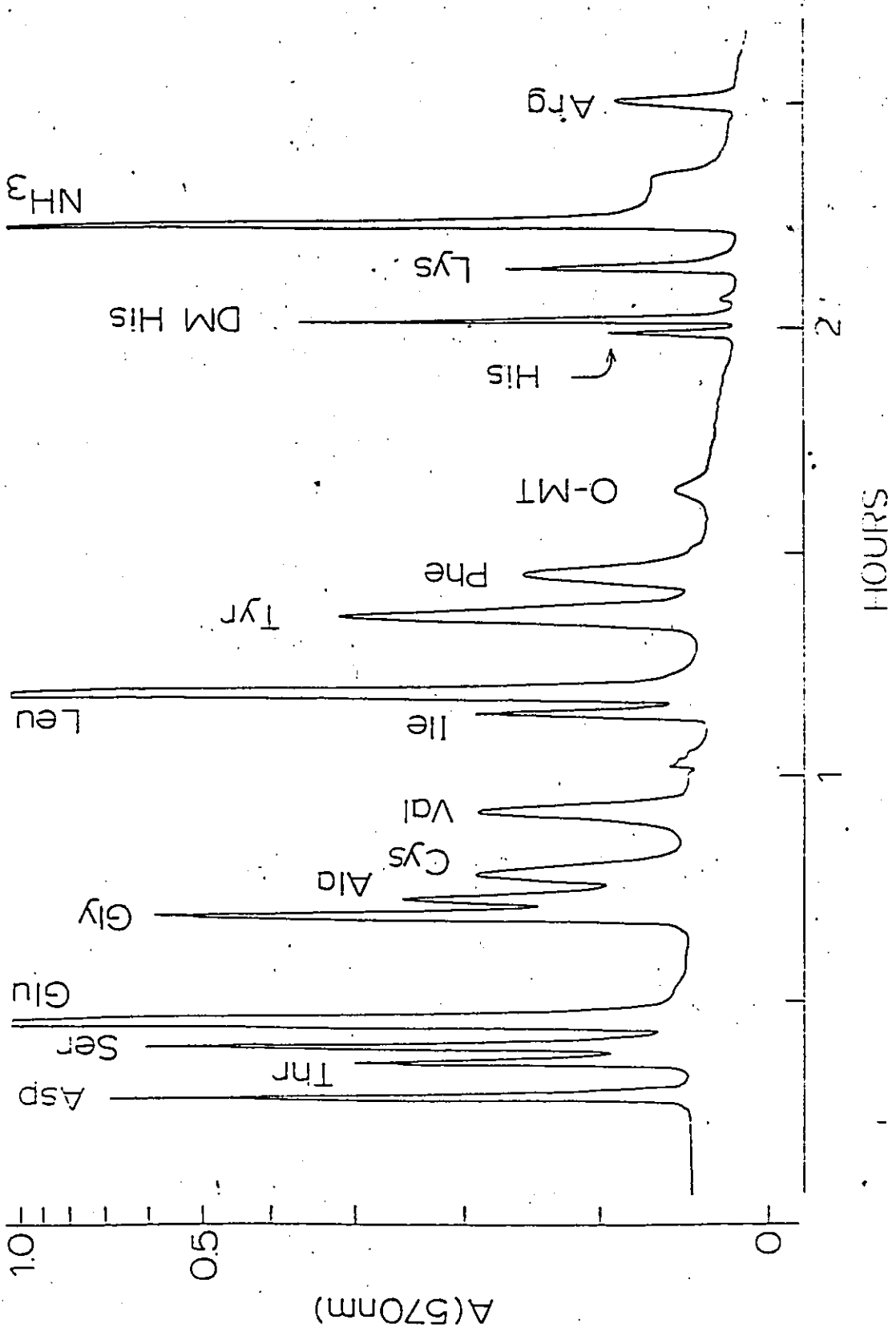
The amino acid composition of methylated insulin and a citraconylated control sample after a 12h hydrolysis is given in Table 1. The composition of the control is in good agreement with the theoretical composition of porcine insulin. Low yields of isoleucine, leucine and valine are probably due to the stability of the peptide bonds involving these amino acids to acid hydrolysis. By contrast, methylated insulin showed a small decrease in tyrosine content and an almost complete disappearance of histidine. The yields of other amino acids from methylated insulin are close to their yield in the control sample. In conjunction with the decreased tyrosine and histidine yields was the appearance of at least 2 new peaks on amino acid analysis (Figure 5). The compound emerging just after phenylalanine was identified as O-methyltyrosine (or p-methoxyphenylalanine) by comparison to a synthetic standard of O-methyltyrosine.

The disappearance of histidine and simultaneous appearance of a

Table 1: Amino acid composition of methylated insulin showing number of residues

Amino Acid	Theory	Control	Methylated
Asp	3	3.00	3.00
Thr	2	1.94	1.97
Ser	3	2.84	2.58
Glu	7	6.01	6.97
Gly	4	4.27	4.26
Ala	2	2.75	2.46
Cys	3	3.12	2.93
Val	4	1.09	1.14
Ile	2	0.37	0.36
Leu	6	5.39	6.05
Tyr	4	3.93	2.37
Phe	3	3.13	3.08
His	2	1.99	0.06
Lys	1	1.06	0.95
Arg	1	1.07	1.07

Figure 5: Typical chromatogram from amino acid analysis of methylated insulin showing the positions of O-methyltyrosine (O-MT) and 1,3-dimethylhistidine (DM His).


A(570nm)
1.0
0.5
0
Asp
Thr
Ser
Glu
Gly
Ala
Cys
Val
Ile
Leu
Tyr
Phe
O-MT
His
DM His
Lys
NH3
Arg
1
2
HOURS

new peak eluting after histidine suggests the formation of a histidine derivative upon methylation. Standard amino acid analyses with 1-methylhistidine or 3-methylhistidine indicate that while good separation of histidine and 1-methylhistidine is obtained when the elution time with the pH 7.5 buffer is elongated, the 3-methylhistidine emerges as a shoulder on the leading edge of histidine. A chromatogram obtained from amino acid analysis with an elongated elution profile illustrates the positions of these histidine derivatives (Figure 6). Manipulation of buffer pH and/or water bath temperature failed to separate these latter compounds. Neither of these monomethyl histidines is compatable with the elution time of the derivative formed on methylation of insulin. The only remaining possibility is therefore the formation of 1,3-dimethylhistidine. This conclusion was confirmed by preparing 1,3-dimethylhistidine and comparing its elution time with that of the derivative formed on methylation of insulin.

2. Quantification of methylation

(a) Conversion of O-methyltyrosine to tyrosine

The conversion of O-methyltyrosine to tyrosine in 6N HCl at 110°C for various times is given in Figure 7. As time of reaction increases the amount of O-methyltyrosine remaining decreases and, within the limits of the accuracy of the analytical procedure employed, is completely converted to tyrosine after 48h. The linear semilog plot (Figure 8) demonstrates that the reaction follows first order kinetics. The rate constant, as calculated by least-squares linear regression, is 0.035 $hours^{-1}$ with a correlation coefficient of 0.986.

The correlation between loss of O-methyltyrosine and appearance of

Figure 6: Chromatogram obtained from amino acid analysis of insulin methylated 4h in the presence of urea and hydrolyzed 12h. Elongation of the elution time with the pH 7.5 buffer enables separation of 1-methylhistidine and histidine.

A (570nm)
0
.05
.1
1
2
3
HOURS
Tyr
Phe
O-MT
3-M His
His
1-M His
DM His
Lys
NH3
Arg

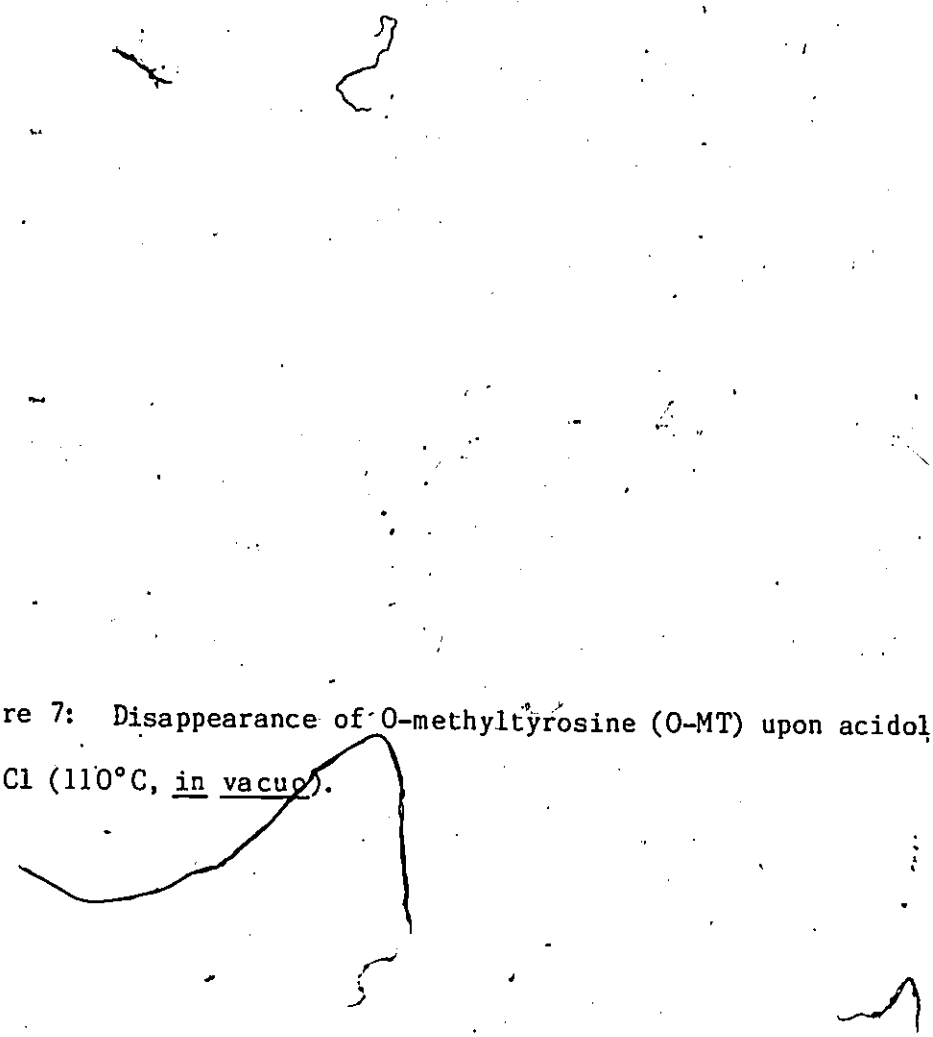


Figure 7: Disappearance of O-methyltyrosine (O-MT) upon acidolysis in 6N HCl (110°C, *in vacuo*).

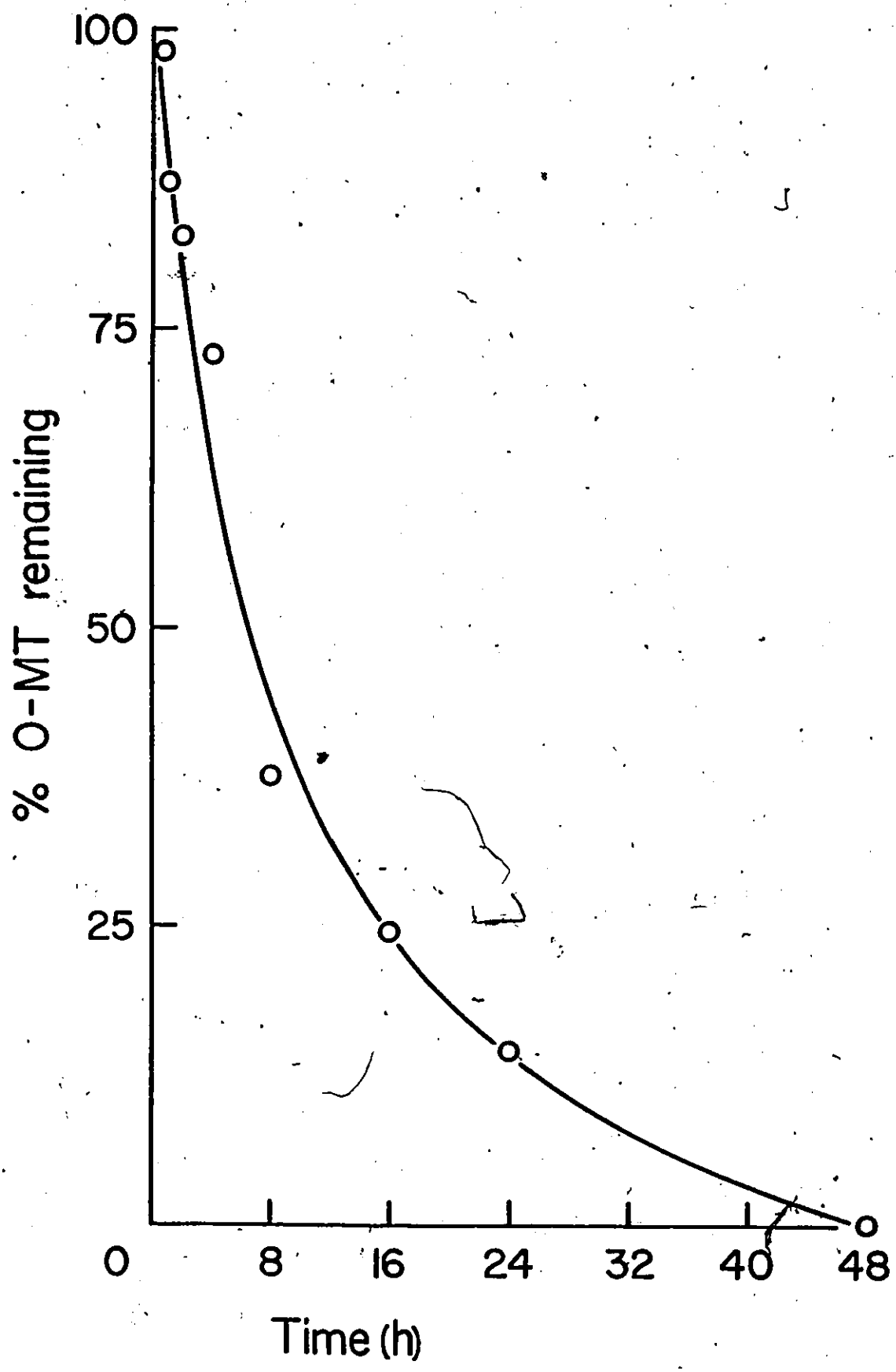
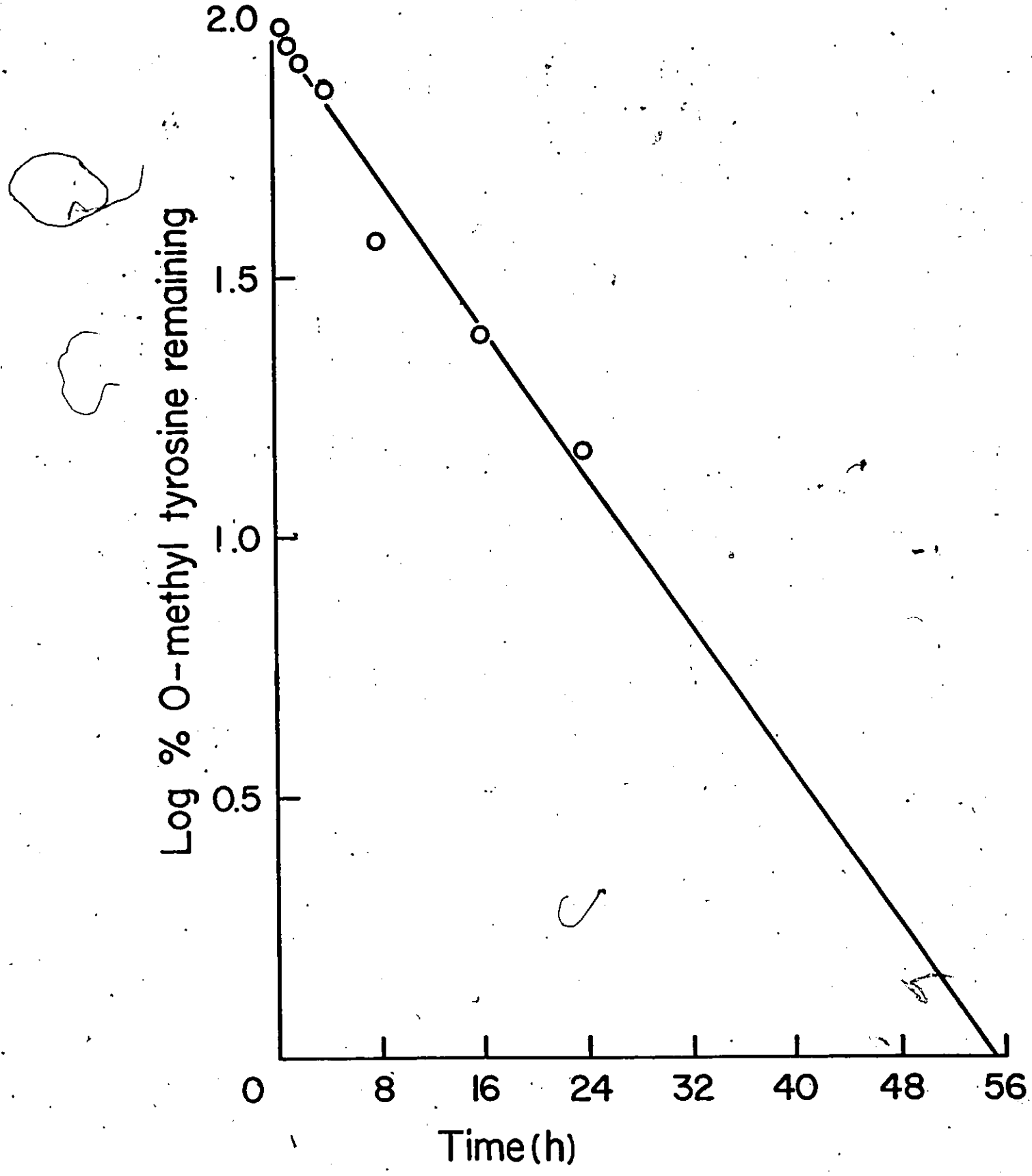

100
75
50
25
0
8
16
24
32
40
48
% O-MT remaining
Time (h)

Figure 8: Semilog plot of the amount of O-methyltyrosine remaining after acidolysis in 6N HCl at 110°C.


2.0
1.5
1.0
0.5
0
8
16
24
32
40
48
56
Log % O-methyl tyrosine remaining
Time(h)

tyrosine is shown by Table 2. By 48h, all O-methyltyrosine has been converted to tyrosine with excellent recovery (98.4%). This indicates that the lysis of O-methyltyrosine proceeds by a single reaction yielding tyrosine and methylchloride (Morrison and Boyd, 1974).

(b) Color yield of 1,3-dimethylhistidine

Amino acid analysis of N-acetylhistidine reacted with methyl iodide for 72h and hydrolyzed in 6N HCl showed that a single reaction product was obtained. Its elution time was identical to that of the histidine derivative formed upon methylation of insulin for 24h. The color yield of 1,3-dimethylhistidine obtained from amino acid analysis is 0.972 compared to histidine (average of three determinations).

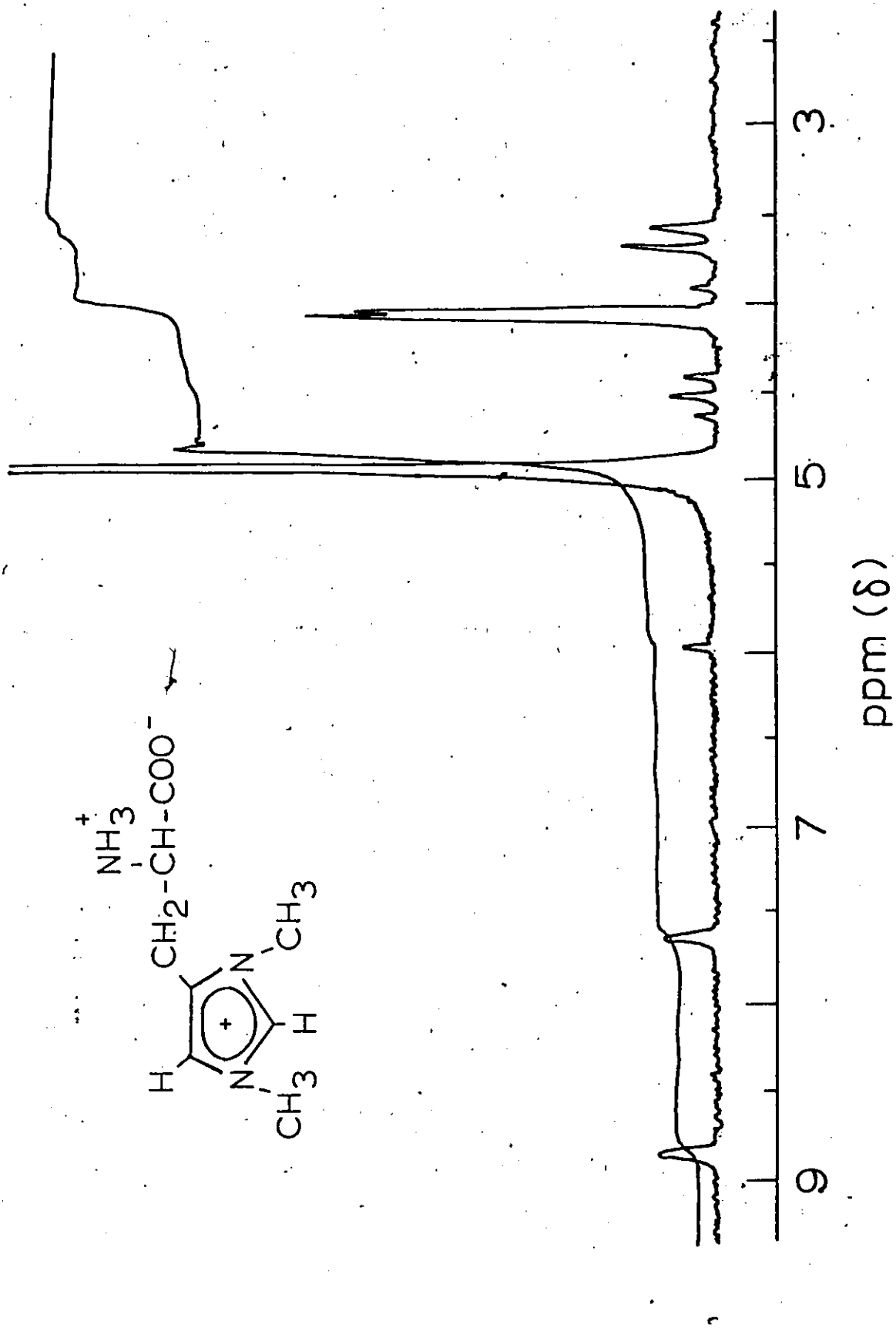
After binding the histidine derivative to a Dowex column, it was eluted with 5% ammonia. The ammonia was removed by drying the product in the presence of sulphuric acid, and the resulting product was an oil. By redissolving this oil in 0.01N HCl, the dried product was obtained in crystal form. This dihydrochloride salt of the methylated histidine derivative was then analyzed by NMR spectroscopy (Figure 9). The β-CH_2 protons appear as a doublet at 3.38ppm and the α-CH proton at 4.28ppm is split into a triplet. At 3.79ppm, the three protons of each of the two methyl groups on the imidazole ring appear as two overlapping singlets. Protons on C-2 and C-4 have been shifted downfield by the imidazole ring system to 8.62 and 7.40ppm. The integration is consistent with the structure of 1,3-dimethylhistidine dihydrochloride and the spectrum therefore verifies that this was the compound formed.

Table 2: Conversion of O-methyltyrosine to tyrosine[1].

Time (h)	% O-MT	% Tyr	Total % O-MT + % Tyr
0.0	100	0.00	100
0.5	98.1	1.81	99.9
1.0	87.0	7.23	94.2
2.0	82.6	13.3	95.9
4.0	72.6	23.4	96.0
8.0	37.7	47.7	85.4
16.0	24.7	69.4	94.1
24.0	14.9	79.4	94.3
48.0	0.00	98.4	98.4

[1]Solutions of O-methyltyrosine (O-MT) in 1ml of 6N HCl were incubated at 110°C for the indicated times. Values given are percentages of O-methyltyrosine at time zero, and are the average of 2 determinations.

Figure 9: [^{1}H]-NMR spectrum of 1,3-dimethylhistidine dihydrochloride in D_2O with tetramethylsilane as internal standard.


NH3+
CH2-CH-COO-
CH3
N
H
H
N
CH3
9
7
5
3
ppm (δ)

(c) Hydrolysis of methylated insulin

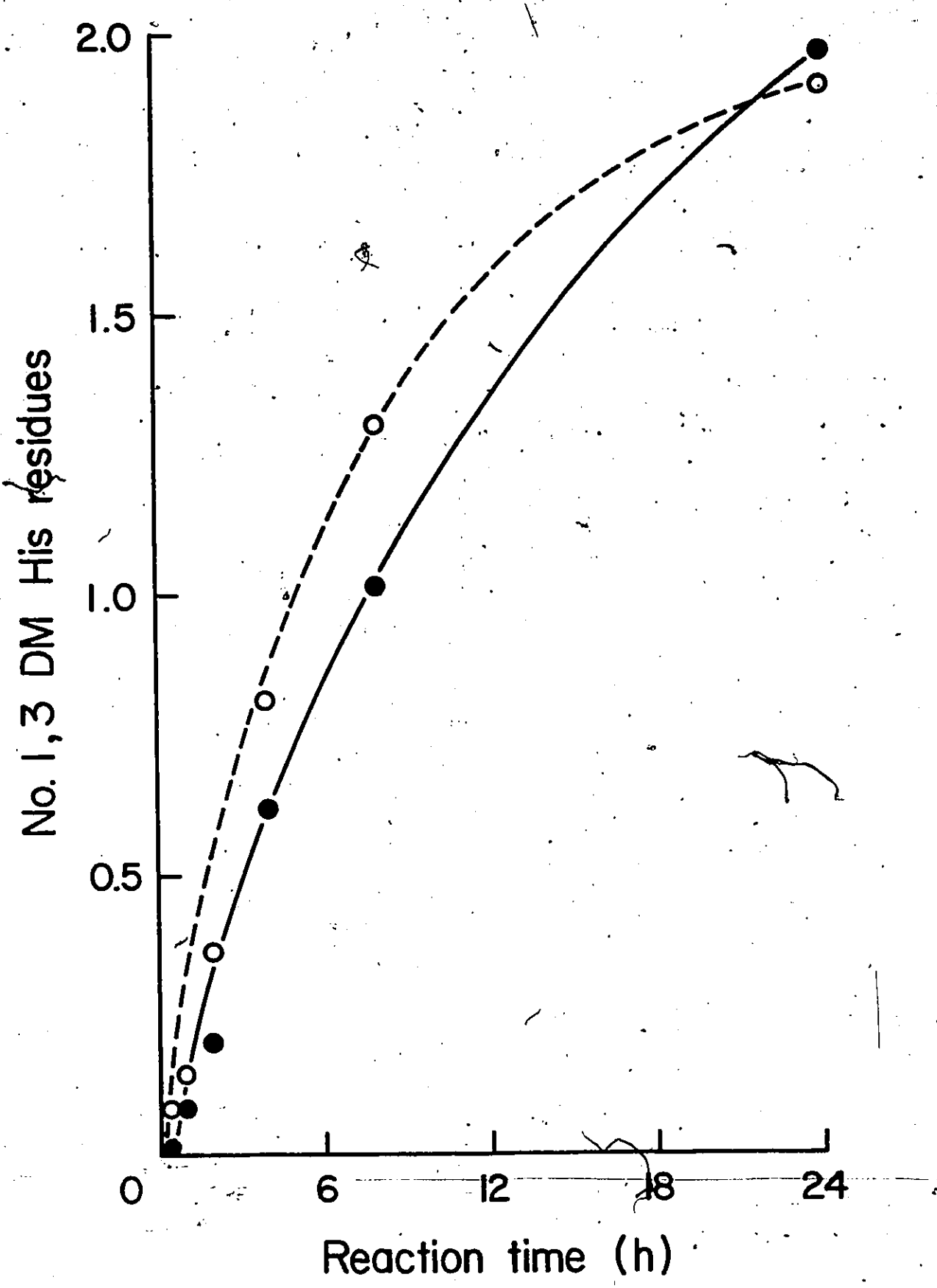
Samples of citraconylated insulin which had been methylated for various periods of time were hydrolyzed 12h and subjected to amino acid analysis. The number of tyrosine residues modified is given in Table 3. Assuming the rate of lysis of free O-methyltyrosine and O-methyltyrosine in insulin are the same, 32% of the O-methyltyrosine (Figures 7 and 8) would remain after a 12h acid hydrolysis. Using this correction factor, 4.59 and 6.88 tyrosine residues are calculated as being modified after 8h and 24h of methylation. Since there are only four tyrosine residues in insulin, these values are clearly in error. Thus, this method is invalid for quantifying the amount of O-methyltyrosine in the methylated insulin samples.

The number of histidine residues modified with increasing times of methylation is also given in Table 3. The area under the 1,3-dimethylhistidine peak is used as a measure of histidine modified. Methylation of histidine is seen to proceed slightly faster in the presence of urea than in the absence of urea at early reaction times as illustrated graphically in Figure 10, and no histidine, 3-methylhistidine or 1-methylhistidine peaks were found. Thus, all histidine residues are converted to 1,3-dimethylhistidine within 24 hours of reacting with methyl iodide. At early times of methylation, small amounts of 1-methylhistidine were seen in addition to 1,3-dimethylhistidine and histidine. Since 3-methylhistidine elutes with histidine, it is not known at what rate nitrogens at positions 1 and 3 are methylated. However, the isolation of the 1-methylhistidine peak shows that at least one monomethyl derivative is formed as intermediate to the dimethyl species.

Figure 10: Methylation of porcine insulin at pH 10.5, 40°C showing the average formation of 1,3-dimethylhistidine (DM His). Samples were methylated in presence (O) or absence (●) of 8M urea and hydrolyzed 12h.

Table 3: Average number of tyrosine residues methylated and histidine residues dimethylated in insulin determined after a 12h hydrolysis. The number of tyrosine residues modified was calculated by correcting for the lysis of O-methyltyrosine to tyrosine. Histidine modification is calculated from the amount of 1,3-dimethylhistidine.

	Number of residues modified		
Reaction time (h)	Tyrosine	Histidine	
	urea	urea	no urea
0.0	0.00	0.00	0.00
0.5	1.72	0.0824	0.00451
1.0	2.16	0.145	0.0853
2.0	3.06	0.360	0.204
4.0	3.69	0.814	0.613
8.0	4.59	1.30	1.08
24.0	6.88	1.91	1.97


2.0
1.5
1.0
0.5
0
No. 1,3 DM His residues
6
12
18
24
Reaction time (h)

(d) Time course of insulin methylation from hydrolyzed Dnp-derivatives

The extent of methylation of tyrosine was determined from the amount of tyrosine present in the citraconylated, methylated insulins after reaction with Dnp-F and 48h acid hydrolysis (Table 4). The hydrolysis period of 48h was chosen because previous results showed that O-methyltyrosine is fully converted to tyrosine under these conditions. The control samples have no tyrosine, as expected if the Dnp-F reaction was complete. This finding also eliminates the possibility that a small amount of O-Dnp-tyrosine is converted to tyrosine by hydrolysis. O-Dnp-Tyrosine is known to decompose slowly in strong acid or strong light (Means and Feeney, 1971) but the decomposition product is not tyrosine.

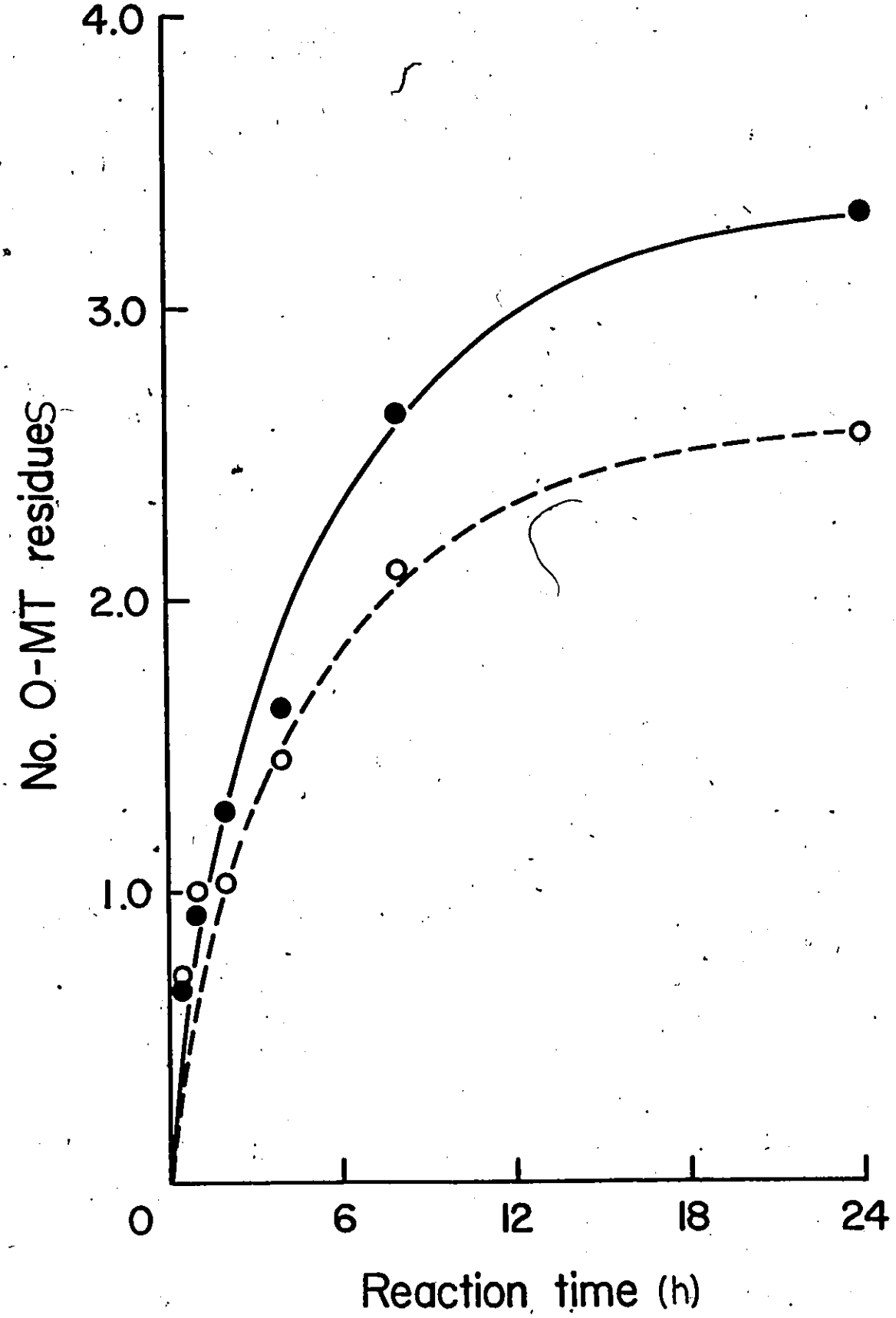
Methylation of tyrosine proceeded slightly faster in the absence of denaturing concentrations of urea than in its presence. No further increase in number of tyrosines modified is seen after 24h, even when more methyl iodide was added to the incubation mixture. Surprisingly, maximum modification is found when no urea is added to the reaction, and is 3.36 out of a possible 4 tyrosines in the native protein. This data is represented graphically in Figure 11. Comparing the methylation of tyrosine with the methylation of histidine (Figure 10) reveals that the two reactions proceed at similar rates at pH 10.5. Dr. Oda in our laboratory has shown that reaction of insulin with methyl iodide (100 fold molar excess over insulin) at pH 7.5 for 24h resulted in the dimethylation of 0.5 histidine residues. Reaction of this insulin with Dnp-F followed by acid hydrolysis revealed that no tyrosine methylation had occurred.

Quantification of histidine from the Dnp, methylated protein

Table 4: Average number of tyrosine residues modified in presence or absence of 8M urea with increasing times of methylation as determined from Dnp-derivatives hydrolyzed 48h.

Reaction time (h)	Number Tyr residues modified	
	urea	no urea
0.5	0.731	0.684
1.0	1.03	0.921
2.0	1.08	1.27
4.0	1.45	1.61
8.0	2.10	2.63
24.0	2.57	3.31
48.0	2.54	3.24
72.0	2.83	3.36

Figure 11 : Porcine insulin methylated for various times at pH 10.5, 40°C in presence (○) or absence (●) of 8M urea, reacted with Dnp-F and hydrolyzed 48h. The average number of tyrosine residues methylated is plotted as a function of the reaction time with methyl iodide.


4.0
3.0
2.0
1.0
No. O-MT residues
0
6
12
18
24
Reaction time (h)

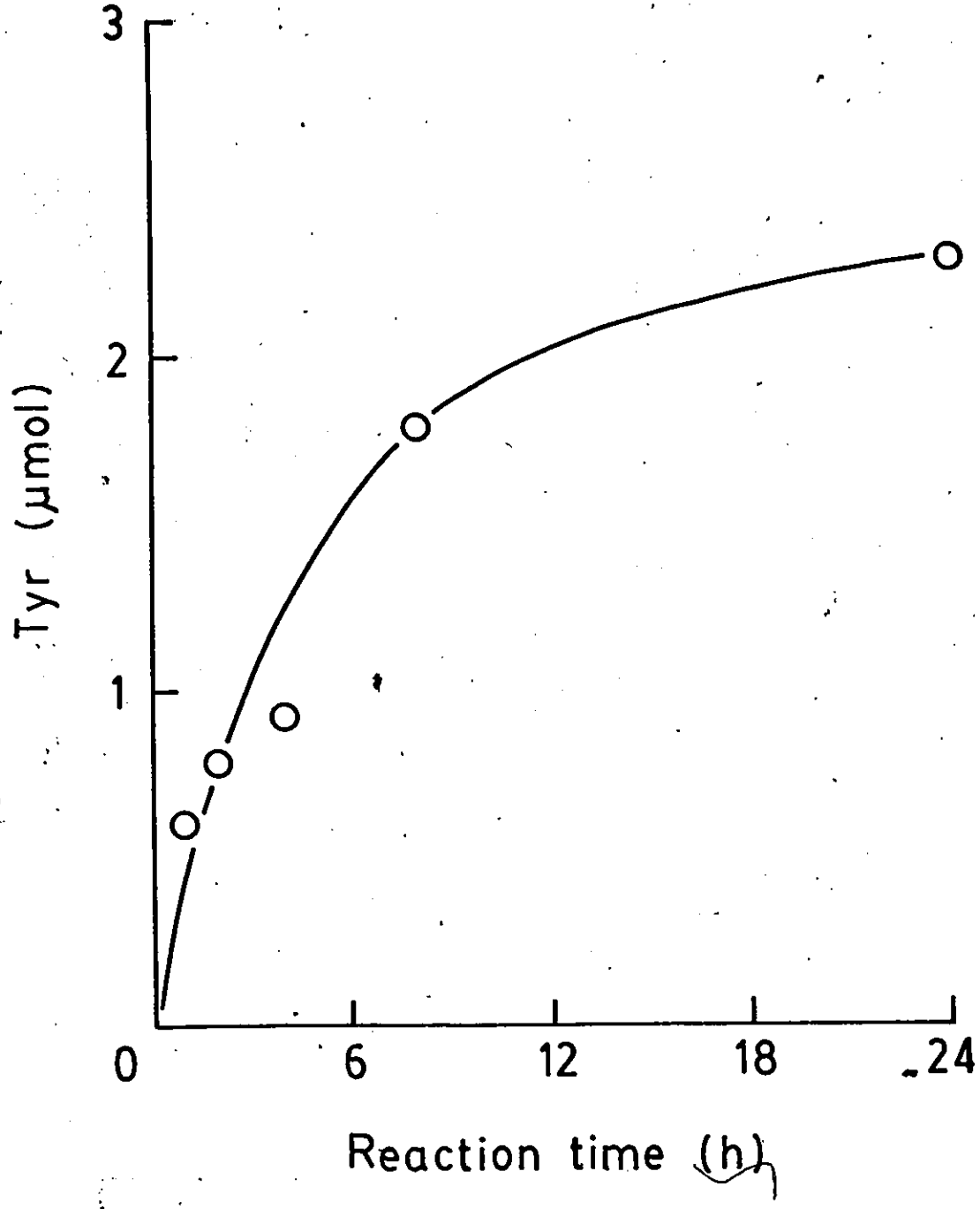
hydrolyzed 48h revealed the time course of 1,3-dimethyl histidine formation to be in excellent agreement with the previous data shown in Table 3 and Figure 11. Since both histidine and tyrosine methylation can be determined using the Dnp-F reaction followed by a 48h hydrolysis, this is the method of choice when one is interested in quantifying both of these amino acids after methylation.

Methylated insulin (reacted with methyl iodide for 24h) which was still citraconylated when reacted with Dnp-F yielded on analysis 0.94 lysine residues, 3.09 phenylalanine residues and 4.31 glycine residues which are close to their theoretical values in porcine insulin. When methylated insulin was decitraconylated by adjusting the pH to 2 for 18h prior to reaction with Dnp-F, the result was 0.00 lysine residues, 2.01 phenylanine residues and 3.13 glycine residues. These results show that the citraconylation reaction had completely blocked the three free amino groups and that acidification for 18h is effective in unblocking these amino groups.

(e) Time course of methylation of N-acetyltyrosine amide from hydrolyzed Dnp-derivatives.

The rate of methylation of N-acetyltyrosine amide was determined from the amount of tyrosine present in the Dnp, methylated derivative (Figure 12). By 24h, 52% of the N-acetyltyrosine amide was converted to N-acetyl-O-methyltyrosine amide. A comparison of the rates of methylation of tyrosine residues in insulin (Figure 11) with that of N-acetyltyrosine amide reveals a similar reaction profile.

Figure 12: Time course for the reaction of N-acetyltyrosine amide (4.5 mol), with methyl iodide at pH 10.5. The amount of N-acetyl-O-methyltyrosine amide was determined as tyrosine after dinitrophenylation and hydrolysis in 6N HCl at 110°C.


3
2
1
0
6
12
18
24
Tyr (μmol)
Reaction time (h)

(f) Radioactive labelling

The total number of methyl groups incorporated upon methylation of insulin with [^{14}C]methyl iodide at pH 10.5 was determined from radioactivity measurements and from amino acid analysis of the Dnp derivatives of these methylated insulins hydrolyzed 48h (Table 5). Calculation of methyl groups incorporated by the dinitrophenylation procedure was not corrected for 1-methyl and 3-methylhistidine formation. The two methods produce similar values, thus substantiating the validity of the dinitrophenylation procedure for the determination of O-methyltyrosine and 1,3-dimethylhistidine formation.

3. Bioactivity of methylated insulins

The blood glucose levels of rats injected with insulin ($5x10^{-8}$ mol/150g) was expressed as a percent of the basal glucose level (Table 6). Native insulin which had been denatured in 8M urea and renatured by gradual dialysis prior to injection produced a fall in blood glucose one hour after injection to 8.6±6.0% of the basal glucose concentration. Similarly, samples A and B in which less than 1.0 tyrosine and 0.5 histidine residues were methylated caused a dramatic decrease in blood glucose. However, when more than 1.5 tyrosine and 1.2 histidine residues are modified (samples C and D), there is little or no blood glucose depression. While native insulin, and samples A and B were soluble, samples C and D were insoluble at pH 7.35 at this concentration and were therefore injected as a suspension. The blood glucose level of animals injected with samples C or D was not decreased from the basal level when assayed 90min after injection.

The blood glucose depression curves following injection with

Table 5: The extent of insulin methylation at pH 10.5 as determined from [^{14}C] incorporation and from dinitrophenylation of the [^{14}C]methyl insulin derivatives hydrolyzed 48h.

Time of reaction with methyl iodide (h)	Number methyl groups incorporated per insulin molecule as determined by	
	Dinitrophenylation[1]	Radioactivity[2]
0.5	0.80	0.92
1.0	1.20	1.06
2.0	2.08	1.99
4.0	3.23	3.09
7.0	5.12	5.21
10.0	6.17	5.61
24.0	7.34	7.05

[1] Calculated using the formula # O-methyltyrosine + 2(# 1,3-dimethylhistidine)

[2] Specific activity of [^{14}C]methyl iodide was 6.2μCi/mmol.

Table 6: Bioactivity of native and methylated insulins (5×10^{-8} mol/150g)

Sample	No. residues modified		No. trials	% Glucose[1] at time (min)		
	Tyr	His		15	30	60
saline	-	-	3	100 ±23	112 ±16	104 ±16
native	-	-	6	54.8± 8.5	21.2± 5.4	9 ± 6
A	0.4	0.0	2	34.2± 3.3	21.9± 1.6	21.6± 2.1
B	1.0	0.5	6	50 ±16	26 ±10	20.1± 8.3
C	1.5	1.2	2	87.5± 5.5	87.4± 5.5	94.7± 5.4
D	2.7	2.0	2	99.4± 0.7	98.4± 1.6	88.7± 4.2

[1] Basal level of each subject taken as 100%

saline, native insulin or methylated insulin ($5x10^{-8}$mol/150g) containing 1.0 tyrosine and 0.5 histidine residues modified (sample B) is shown in Figure 13. The bioactivity of this methylated insulin is not different from that of native insulin when tested by the unpaired student t test ($p > 0.01$).

Animals were then injected with lower doses of native or methylated insulin (Table 7). It is clear from the large amount of variability among animals that a very large sample size is needed to determine activity in vivo at low doses of insulin. Native insulin produced blood sugar depression at $5x10^{-10}$mol/150g but seems less active at $1x10^{-10}$ mol/150g. Methylated insulin (sample B) caused glucose levels to fall at $5x10^{-9}$mol/150g. Below this dose, methylated insulin was active in some animals, but not in others. This may indicate that there is a shift in the dose response relationship upon methylation, such that a 10 fold higher dose is required for activity.

4. Absorption spectra of methylated insulins

The spectra of native and two renatured methylated insulins are shown in Figure 14 and are seen to change upon methylation. All insulins had a major peak at 235nm (not shown) and a minor peak at 274nm. The spectral properties of various insulins are given in Table 8. The molar extinction coefficients at 274nm increase with the degree of insulin methylation. Absorption spectra of N-acetyltyrosine amide and its O-methylated derivative are very similar and the molar extinction coefficients at 274nm are essentially the same (Table 8). Therefore, methylation of the phenol group of tyrosine does not change the spectral properties at 274nm.

Figure 13: Glucose depression curves following injection with saline (□), native insulin (○) or methylated insulin (△). Methylated insulin contains 1.0 tyrosine and 0.5 histidine residues modified (sample B). Glucose concentration is expressed as a percent of basal glucose levels.

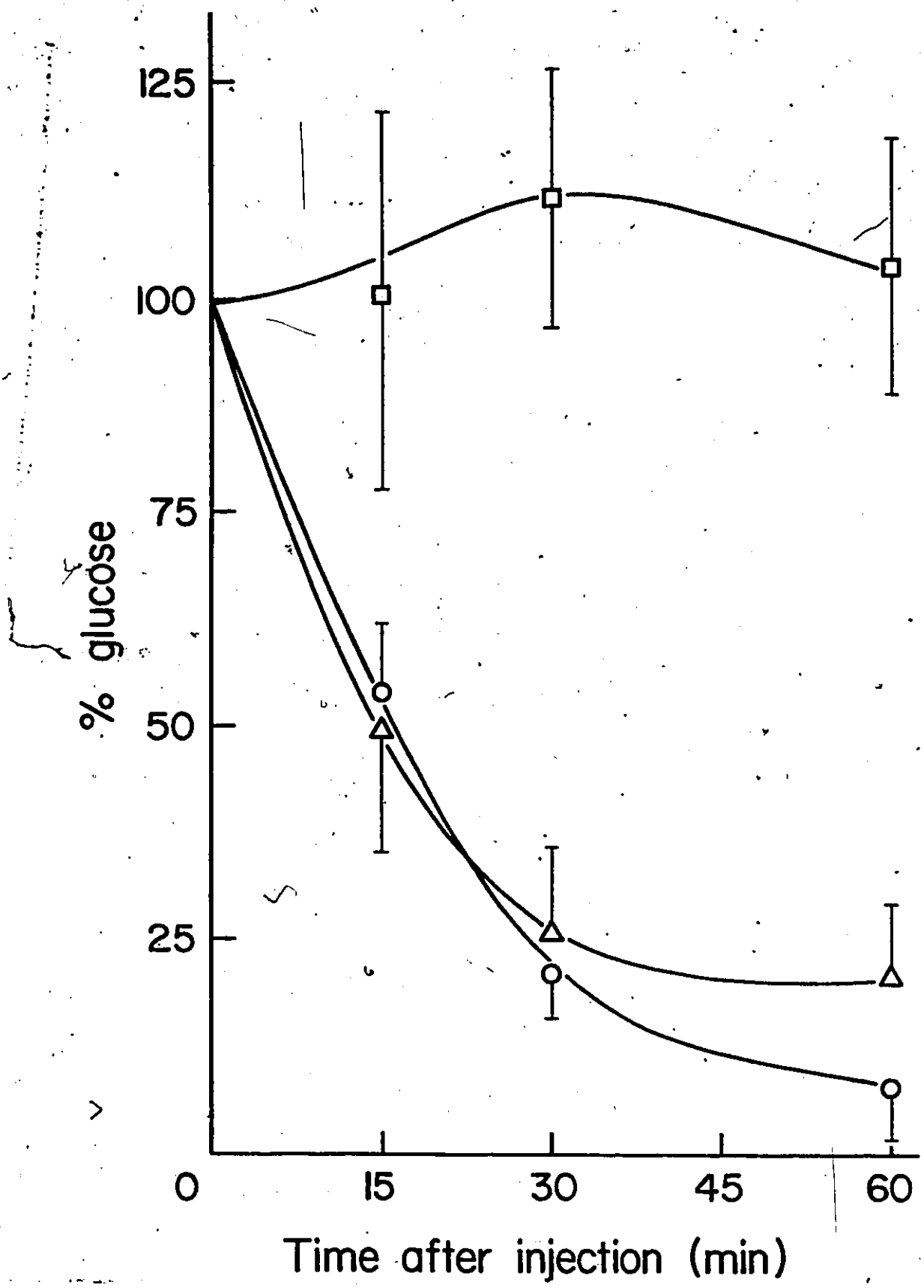

125
100
75
50
25
% glucose
0
15
30
45
60
Time after injection (min)

Table 7: Bioactivity of native and methylated insulin in rats injected with various doses.

Sample[1]	Dose (mol/150g)	% Glucose[2] at time (min) 15	mean	30	mean	60	mean
Native	$5x10^{-9}$	81.0		47.6		47.6	
	$5x10^{-10}$	52.9	66 ±13	63.2	57 ±7	45.9	38 ± 8
		79.2		50.0		29.2	
	$1x10^{-10}$	48.5	76 ±27	55.4	81 ±25	71.8	86 ±14
		102.9		105.9		100.0	
Methyl	$5x10^{-9}$	30.0	71 ±26	10.0	46 ±21	10.0	30 ±14
		68.8		62.7		46.3	
		85.0		60.0		40.0	
		100.0		50.0		22.2	
	$1x10^{-9}$	70.7	88 ±18	61.8	81 ±19	41.2	71 ±29
		106.1		100.0		100.0	
	$5x10^{-10}$	102.1	88 ±14	129.0	87 ±30	81.1	48 ±31
		83.2		100.0		74.9	
		100.0		60.0		10.0	
		66.6		58.3		25.0	

[1]Native insulin was subjected to the renaturation procedure prior to injection. Methylated insulin (sample B) contains 1.0 and 0.5 O-methyltyrosine and 1,3-dimethylhistidine residues, respectively.

[2]Basal level of each subject taken as 100%.

Figure 14: Spectra of native and methylated insulins at pH 3. N, native insulin; B, 1.0 tyrosine and 0.5 histidine residues modified; C, 1.5 tyrosine and 1.2 histidine residues modified; and D, 2.7 tyrosine and 2.0 histidine residues modified.

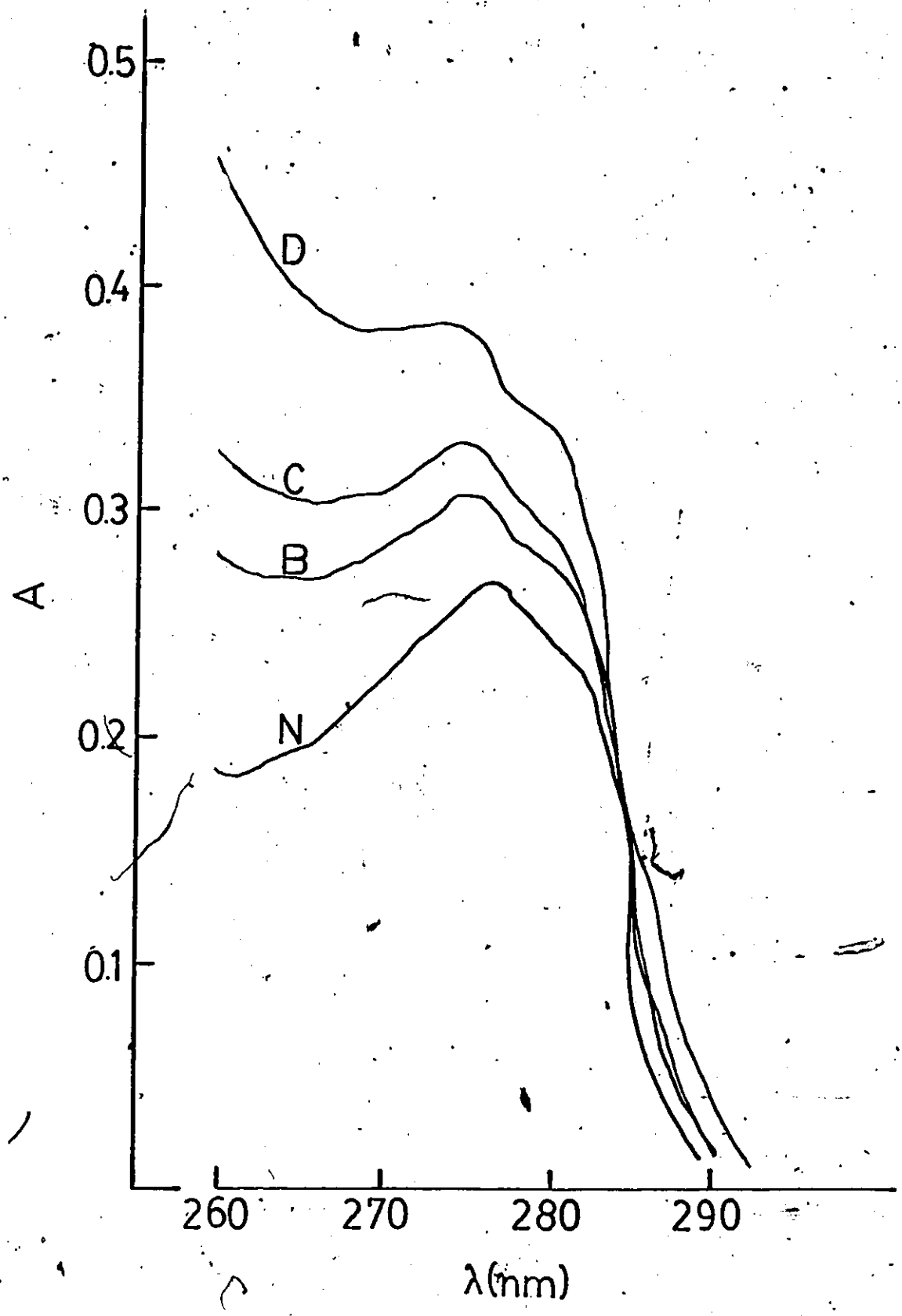

0.5
0.4
0.3
0.2
0.1
A
D
C
B
N
260
270
280
290
λ(nm)

Table 8: Extinction coefficients of various insulins and tyrosine derivatives.

Sample[1]	ϵ[2] at 274nm
Native insulin	8182
A	8788
B	9394
C	10606
D	11515
N-Ac-Tyr amide	1180
N-Ac-OMT amide	1229

[1] See Table 6 for labelling system of methylated insulins.

[2] ϵ, molar extinction coefficient

V. Discussion

For many years, there has been great interest in defining the roles of specific amino acid side chains in the properties of various biologically active proteins. Chemical modification of these functional groups has been widely used towards this end with generally good results. However, there remains a great need for reagents which may be used under mild conditions and are selective for particular types of side chains.

A number of reagents have been employed to specifically methylate various amino acid side chains. Reductive methylation of proteins with sodium borohydride or sodium cyanoborohydride and formaldehyde at pH 9 converts amino groups to mono-methylated and dimethylated products (Means and Feeney, 1968; Uschkoreit et al., 1980; Marsh et al., 1983). Although fairly specific for amino groups, Uschkoreit and colleagues reported that side reactions with tyrosine residues do occur, especially above pH 8. However, initial attempts to methylate the tyrosine residues of insulin at pH 10.5 with sodium borohydride and formaldehyde after citraconylation of the amino groups produced O-methylation of tyrosine in very low yields (less than 15%).

Methylation of the methionine and cysteine residues in proteins has been well studied. Reaction of ribonuclease A (Link and Stark, 1968), myoglobin (Jones et al., 1976), glucagon (Röthgeb et al., 1977) and peptide fragments (Sasgawa et al., 1983) with methyl iodide at pH 2.5 to 5.0, yeilds methyl methionine derivatives. Within this pH range, side reactions were not observed. S-Alkylation of cysteine by the use of alkyl iodides has been described (Banks and Shafir, 1970). Reaction of reduced proteins with methyl iodide (Rochat et al., 1970), methyl-p-

nitrobenzene sulfonate (Heinrikson, 1971) or dimethylsulfate (Eyem et al., 1976) yields S-methyl derivatives. Although specificity for cysteine residues has been claimed, side reactions with methionine were found (Rochat et al., 1970) and no conclusions can be made in these studies as to whether acid labile modifications had occurred. Methylation of histidine-57 of chymotrypsin with methyl p-nitrobenzene sulfonate has been reported (Nakagawa and Bender, 1969) but experimental details were not given. No procedure has yet been reported for the preparation of methylated tyrosine side chains in high yields.

The method described here affords the means of specifically methylating the tyrosine and histidine residues of insulin. An advantage of insulin is that it contains no methionine residues which would presumably react under these conditions. Reversible modification of the amino functions by acylation with citraconic anhydride prevents the methylation of these groups. Subsequent reaction of the citraconylinsulin with methyl iodide at pH 10.5 leads to monomethylation of tyrosine and dimethylation of histidine residues. The reaction of tyrosine and histidine sidechains probably involves nucleophilic attack of the unprotonated phenol and imidazole groups on methyl iodide.

Quantification of the extent of tyrosine methylation is complicated by the acid lability of O-methyltyrosine. Although it has long been recognized that the ether linkage of O-methyltyrosine is cleaved by acid (Rutherford et al., 1940; Herriott, 1947), there have been no reports in which the rate of this reaction was determined. Cleavage of free O-methyltyrosine in 6N HCl is a first order reaction

with a rate constant of 0.035 hours^{-1}. Other amino acid derivatives which are converted to the parent amino acid upon treatment with acid include tyrosine-O-sulfate (Bettelheim, 1954), the O-phosphoamino acids (Jergil and Dixon, 1970; Bylund and Huang, 1976; Hohmann et al., 1975), and S-methyl cysteine (Heinrikson, 1971; Eyem et al., 1976). The rate of O-methyltyrosine lysis is similar to the rate of O-phosphothreonine hydrolysis ($k = 0.043$ hours^{-1}) reported by Bylund and Huang (1976). The rate of phosphoserine hydrolysis (Bylund and Huang, 1976) is much greater and the rate of S-methyl cysteine cleavage (Eyem et al., 1976) and is considerably less than the rate of O-methyltyrosine cleavage.

To determine the extent of tyrosine methylation in insulin, it was assumed that recovery of O-methyltyrosine upon acid hydrolysis was the same for the free amino acid as for the protein. However, calculation of the number of tyrosine residues methylated in insulin gave values which exceeded the number of residues which are present. This suggests that the rate of hydrolysis of O-methyltyrosine from insulin is less than that of the free amino acid. Bylund and Huang (1976) have found that the rate of destruction of O-phosphoamino acids during hydrolysis of peptides is dependent on the identity of the neighbouring amino acid residues which may either accelerate or decelerate the rate of cleavage of the phosphate group. Thus, the hydrolysis of a free amino acid with an acid labile group generally is not a good model for the hydrolysis of that amino acid in a protein. In this regard, it is interesting to note that in analogous attempts to correct for the destruction of certain amino acids to acid hydrolysis, there is poor correlation between destruction of amino acids in the free as compared to in the protein state (Blackburn, 1978).

Acid hydrolysis is routinely used to study the amino acid composition of peptides and proteins. Its widespread use for preparing proteins for amino acid analysis is due to the relative ease and efficiency as compared to alkaline or enzymatic hydrolysis. Alkaline hydrolysis of proteins has only limited application as the destruction of arginine, serine, threonine, cystine, histidine and aspartic and glutamic acids precludes its general use (Ray and Koshland, 1962; Harrison and Garratt, 1970). Although ether linkages are stable to base, and hence it can be predicted that O-methyltyrosine is stable to alkali, the decomposition of histidine upon basic hydrolysis suggests that dimethyl histidine would be labile under these conditions. In addition, technical difficulties such as the formation of silicates and salts which must be removed prior to analysis must be overcome (Oelshlegel et al., 1970). The advantage of enzymatic hydrolysis is that acid- or alkali-labile linkages survive the hydrolysis (Royer et al., 1977). However, complex incubation procedures are required and there is no guarantee that enzymes will cleave at amino acid residues which have been modified. For example, chymotrypsin activity is suppressed at tyrosine sites of insulin which have been modified by either N-methyl pyridinium iodide or 4-trimethylammonio-2-nitro fluorobenzene iodide (Drewes et al., 1980) or tetranitromethane (Morris et al., 1970). With these difficulties in mind, it was decided to employ acid hydrolysis of dinitrophenyl derivatives to quantify tyrosine methylation. This procedure is advantageous in that commonly employed laboratory reagents are used and the extent of histidine methylation can be determined simultaneously.

Dnp-F has been employed to identify N-terminal amino acids

(Sanger, 1945), for chemical modification studies (Henkart and Dorner, 1971) and for competitive labelling studies (Chan et al., 1981; Kaplan et al., 1984). Use of Dnp-F to indirectly quantify the extent of tyrosine methylation represents a novel application of this reagent. Determination of tyrosine methylation at pH 10.5 from the acid hydrolyzed Dnp-insulin revealed that the maximum modification occurred within 24h. An average of 3.3 and 2.6 tyrosine residues are methylated in the absence and presence of denaturing concentrations of urea. It is surprising that there is less modification when the protein is denatured. A similar phenomenon has been reported by Marsh and coworkers (1983) who found that the addition of either 8M urea or 5M guanidine HCl diminished the rate of lysine methylation when insulin was reductively methylated. Since at most, an average of 3.3 out of a possible 4 tyrosine residues are methylated, at least one tyrosine residue does not fully react.

Several lines of evidence indicate that at least one tyrosine residue is "buried" and is therefore unreactive (Blundell et al., 1972; Menendez et al., 1969). While this may account for the low reactivity of a tyrosine residue in the absence of denaturing concentrations of urea, the denatured protein has all groups exposed. Even in the absence of 8M urea, citraconylation of insulin would at least partially denature the protein. Thus, the lack of exposure of tyrosine residues to methyl iodide probably does not account for their low reactivity. It has been shown that two tyrosine residues of insulin ionize with an apparent pK of 10.2 and two residues ionize with an apparent pK of about 11.7 (Morris et al., 1970). This suggests that at pH 10.5, ionization of tyrosine residues is incomplete and may account for the

low reactivity of one or two phenolic groups.

At pH 7.5, reaction of insulin with methyl iodide did not methylate tyrosine residues. This is expected since the average pK of the tyrosine hydroxyl groups is above this pH and thus, at pH 7.5, the phenol group is protonated and will not undergo reaction with methyl iodide.

Reaction of N-acetyltyrosine amide with methyl iodide at pH 10.5 followed by reaction with Dnp-F and acidolysis yields tyrosine. This shows that O-methylation of the free amino acid derivative of tyrosine has occurred. The rates of methylation of N-acetyltyrosine amide and tyrosine residues in insulin are similar with the reaction approaching saturation by about 8h.

The methylated, citraconylated insulins were acidified for 18h and this treatment was effective in removing the citraconyl groups as indicated by their yields on reaction of the unblocked protein with Dnp-F. This is in accordance with the data of Dixon and Perham (1968) who found an overnight incubation at pH 3.5 released the blocking groups from citraconyl insulin. However, Naithani and Gattner (1982) found that 70h at pH 3.5 in 4M urea was required to completely remove the citraconyl groups from citraconylinsulin. The higher concentration of urea used in the present study may account for the increase in the rate of unblocking.

During chemical modification studies on proteins it is often desirable to analyze for a modified amino acid rather than for the absence of the unmodified residue since a decrease may represent only a small percentage of the total quantity of that modified amino acid in the protein. Preparation of 1,3-dimethylhistidine allowed the

determination of the ninhydrin color yield of this amino acid and therefore permitted the direct determination of its presence. Reaction of N-acetylhistidine with methyl iodide at pH 10.5 for 72h followed by acid hydrolysis yields 1,3-dimethylhistidine as the sole product as indicated by amino acid analysis. This method of preparation is superior to the previously reported route of synthesis in which methylation of phthaloyl-L-histidine methyl ester with methyl iodide in dimethylformamide and subsequent hydrolysis produced a mixture of monomethyl, dimethyl and unreacted histidine (Cowgill, 1957). The color yield of 1,3-dimethylhistidine was 97.2% of that of histidine on a molar basis. This is considerably higher than the ninhydrin color yield value 80% estimated by Cowgill for the same amino acids.

Since 1,3-dimethylhistidine is stable to acid hydrolysis, it is quantified without the need of correcting for its cleavage. Reaction of insulin with methyl iodide at pH 10.5 for 24h resulted in the complete dimethylation of the two histidine residues both in the presence and absence of 8M urea. Methylation of insulin at pH 7.5 for the same amount of time resulted in the dimethylation of an average of 0.5 histidine residues. Therefore, the formation of 1,3-dimethylhistidine is enhanced at the higher pH. Methylation of N-acetylhistidine with methyl iodide over a pH range of 7.0-11.0 has been performed (Tom Haus, unpublished). The formation of 1,3-dimethylhistidine was progressively enhanced as the pH of the reaction medium was increased. A possible explanation for this phenomenon is that at high pH a proton is removed from the imidazole ring producing a highly reactive anionic species. Since insulin does not contain methionine residues, and since tyrosine is not methylated at pH 7.5 as indicated by analysis of the 48h

hydrolyzed Dnp-derivative of this insulin, methylation of insulin with alkyl halides at this pH offers a method to selectively modify the histidine residues of this protein.

Reaction of methylated insulin with Dnp-F yields imidazole-Dnp-histidine (im-Dnp-histidine) and 1,3-dimethylhistidine among the products but no histidine, 1-methyl or 3-methylhistidine are present. It has been shown that Dnp-F reacts preferentially with the 3-nitrogen of the imidazole ring of N-acetylhistidine and the product, im-Dnp-histidine, does not decompose upon acid hydrolysis (Henkart, 1971; Bell and Jones, 1974). This suggests that 1-methyl but not 3-methylhistidine will react with Dnp-F. Therefore, upon analysis, 3-methylhistidine should be apparent. However, no 3-methylhistidine was found, even at early methylation times. There are two conceivable explanations for the absence of 3-methylhistidine. One possibility is that only 1-methylhistidine is formed as a reaction intermediate which is then converted to 1,3-dimethylhistidine upon further methylation. However, it was shown from the acid hydrolyzed samples of methylated insulin that both 1-methyl and 3-methylhistidine are present at early methylation times. Despite steric hindrance about the 1-nitrogen, this position can be mono-methylated. Alternatively perhaps 3-methylhistidine within the protein in the presence of 8M urea, unlike the free N-acetylhistidine in absence of urea reacts with Dnp-F at the 1-nitrogen, and the lim-Dnp-3-methylhistidine appears on analysis with 3im-Dnp-histidine. This is also unlikely as steric hindrance about the protein would be greater than that of the free amino acid.

The formation of 1,3-dimethylhistidine can be calculated from hydrolyzed methylated insulin samples and from hydrolyzed Dnp,

methylated insulin. Both methods produce comparable results for quantifying 1,3-dimethylhistidine. This supports the validity of the dinitrophenylation procedure for quantifying the extent of methylation.

There are several methods for radio-labelling insulin by chemical modification. [^{125}I]Iodination is the most common means, and purification of monoiodoinsulin from iodination mixtures has resulted in specific activities of 2Ci/μmol (Sodoyez et al., 1975). Most investigation have employed unpurified iodinated insulins with specific activities of 100-200Ci/mmol (Roth, 1975). However, the validity of iodine-labelled insulin as a tracer for determining the mode of action and the metabolism of insulin has been seriously questioned. Evidence against the biological identity of iodinated and native insulin has been obtained from in vivo metabolic studies. Ooms et al. (1968) have shown that irrespective of the degree of iodination, the metabolic clearance rate of iodoinsulin was both reduced and altered in character. In addition, there are health hazards associated with [^{125}I]iodine and its short half life limits its storage time.

There has been a search for other radioactive reporter groups to label insulin. Halban and Offord (1975) have synthesized [^{3}H]B1 phenylalanine insulin with a specific activity of 20μCi/mmol. Tritiation of insulin by exposure to tritium gas activated by microwave radiation has also been reported (Misbin, 1977). Reductive methylation of insulin's amino groups with [^{14}C]formaldehyde and/or sodium boro [^{3}H]hydride has been reported (Uschkoreit et al., 1980; Marsh et al., 1983). However, biological activity of fully methylated insulin was

decreased (Marsh et al., 1983) and the homogeneous dimethylated derivatives possess very low specific activities (Uschkpreit et al., 1980). It is hoped that further studies may show that methylation of the tyrosine and histidine residues with [^{14}C]methyl iodide provides a new and useful method for radiolabelling insulin.

There is a good correlation between the number of methyl groups incorporated into insulin as assayed by [^{14}C]labelling and dinitrophenylation procedures. Thus, the dinitrophenylation procedure gives a reliable measure of tyrosine methylation and histidine dimethylation while the [^{14}C]labelling method enables rapid determination of the total extent of methylation. Since the degree of methylation measured by [^{14}C]labelling does not exceed the values obtained upon dinitrophenylation, methylation of residues other than tyrosine and histidine can be excluded.

In vivo measurements of the blood glucose depression produced upon injection of the modified insulins indicate that partially methylated insulin is active when injected subcutaneously into fasted rats at a dose of 5×10^{-8} mol/150g. At this dose, methylated insulin with an average of 1.0 and 0.5 tyrosine and histidine residues modified (sample B) had the same activity as native insulin. While these results suggest that sample B has full biological activity, it can be argued that since the derivatized insulin is a heterogeneous mixture of unreacted insulin and various monomethylated and/or dimethylated products, the activity observed upon injection of sample B may have been due to blood glucose depression induced by (unreacted) insulin itself. In an attempt to rule out this possibility, native and methylated insulins were injected at lower doses. The rationale here

is that if the threshold dosage of native and methylated insulins required for a given response are the same, then the possibility of activity being due to any unreacted insulin could be ruled out. However, initial experiments to determine the threshold dose required for producing glucose depression showed that animal variability was so great that a very large sample size would be needed. The results suggest that animals may require a higher dose of sample B than native insulin to reach a given level of glucose depression. There are at least two possible outcomes of this experiment should the sampling size be augmented to the point where n is sufficiently large to enable statistical analysis: (1) sample B and native insulin require the same concentration to produce a threshold level of response (i.e. identical dose response curves); or (2) a shift in the dose response curve such that a higher concentration of modified than native insulin is required to reach the threshold level. If result 2 was obtained (as seems likely), then the possibility would still exist that bioactivity of sample B is due to the presence unreacted insulin molecules in the injected sample. At this point, it was decided that a more logical experimental design would be to purify the methylated derivatives to homogeneity before assaying their biological activities.

When more than 1.0 and 0.5 tyrosine and histidine residues are modified (samples C and D), methylated insulins were not biologically active in vivo at a dose of 5×10^{-8} mol/150g. These samples were inactive even though they were injected at a dose which was 100 times greater than the dose at which native insulin was biologically active. It is probable that changes in the three-dimensional structure of these methylated insulins (see below) and their insolubility account

for the failure of the modified insulins to produce blood sugar depression. Insolubility is presumably induced by increased hydrophobicity and conformational changes. Modification of specific tyrosine and/or histidine residues may also have caused the lack of biological activity. Available evidence indicates that A19 Tyr, B16 Tyr, B26 Tyr and B5 His are all involved in insulin receptor binding. Thus, methylation of any or all of these residues could decrease biological activity.

The correlation between changes in protein structure resulting from denaturation or proteolysis with alterations in the ultraviolet absorption spectra of proteins in the range 260 to 320nm was established over two decades ago (Laskowski et al., 1956; Glazer and Smith, 1960; Yanari and Bovey, 1960; Glazer and McKenzie, 1963). In this region, the absorbance spectra contain contributions from phenylalanine, tyrosine and tryptophan residues, whereas the imidazole group of histidine absorbs in the region between 185 and 220nm (Wetlaufer, 1962). Using a variety of denaturation conditions, Glazer and Smith (1960) reported that the absorbancies at 230 to 235nm and 278 to 285nm were enhanced as compared to the native protein. The only amino acids which could make a significant contribution to the absorbance intensity in these regions were determined to be tyrosine and tryptophan. Since insulin does not contain any tryptophan residues, the spectral characteristics of this amino acid are not considered further.

The ultraviolet spectra of methylated insulins show that with increasing alkylation, the molar extinction coefficient at 274nm is progressively enhanced. However, the spectral properties of N-

acetyltyrosine amide and N-acetyl-O-methyltyrosine amide are similar. This is in accordance with the data of Wetlaufer's group (1958) who found that tyrosine and O-methyltyrosine have nearly congruent spectra between 265 and 295nm. Thus, the alterations in absorbance observed upon methylation of insulin resemble the changes in absorbances of a variety of proteins upon denaturation. Since spectral changes due to the formation of O-methyltyrosine can be excluded and since histidine does not absorb in the region tested it can be concluded that methylation of insulin produces a change in conformation. The spectrum of methyl insulin in which 2.7 and 2.0 histidine residues are modified (sample D) was greatly distorted from that of native insulin. Gross structural changes in this insulin probably account for its inactivity in vivo.

VI. Conclusions and future research

Tyrosine and histidine residues of insulin can be selectively methylated by incubation of the protein with methyl iodide at pH 10.5 after blocking the amino groups by citraconylation. Since O-methyltyrosine is acid labile, tyrosine methylation cannot be quantified by analysis of acid hydrolysates. The extent of tyrosine residue O-methylation was determined by reacting the methylated protein with Dnp-F, followed by analysis of acid hydrolysates. Of the 4 tyrosine residues of insulin, a maximum of 3.3 residues were methylated on average, indicating that at least one tyrosine residue does not fully react. It would be useful to verify these data by analyzing hydrolysates of methylated insulin obtained from enzymatic or alkaline hydrolysis. Histidine residues are converted primarily to 1,3-dimethylhistidine residues when insulin is reacted with methyl iodide at pH 10.5. The acid stability of this derivative enables quantification of histidine modification from analysis of acid hydrolysates of methylated insulin. Both histidine residues are dimethylated within 24h of reaction.

Insulin with 1.0 tyrosine and 0.5 histidine residues modified on average is as active as native insulin in producing blood glucose depression at a dose of 5×10^{-8} mol/150g. More extensively methylated insulins are not active *in vivo*, probably due to a change in conformation of these methylated insulins. Purification of the heterogeneous sample which was active *in vivo* could be achieved by HPLC if a suitable solvent system is found to separate the derivatives. Biological activity of the purified methylated insulins could then be

determined in a more sensitive system such as the rat diaphragm assay or the fat cell assay. If the activity of purified methylated insulin can be shown to be identical to native insulin, methylation with [^{14}C]methyl iodide would be a useful method for preparing radiolabelled insulin. Identification of the residues which are modified would be possible by enzymatic digestion of radiolabelled methylated insulin followed by separation of the resulting peptides by high voltage electrophoresis and amino acid analysis of hydrolysates of the peptides. Once the identity of the methylated residues is known and the methylated insulins are purified to homogeneity, assessment of their biological activities will give valuable information towards elucidating the structure-function relationship of this important hormone.

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Claims to original research

1. New methodology

(a) A new method for methylating the tyrosine and histidine residues of proteins is presented.

(b) A method of quantifying tyrosine O-methylation is described.

(c) A procedure for the preparation, identification and quantification of 1,3-dimethylhistidine is described.

2. Novel information

(a) Both histidine residues are dimethylated and an average of 3 tyrosine residues are O-methylated when citraconylated insulin is incubated with methyl iodide at pH 10.5.

(b) The cleavage of O-methyltyrosine to tyrosine in 6N HCl at 110°C is a first order reaction with a rate constant of 0.035 h^{-1}.

(c) Insulin with an average of 1.0 tyrosine and 0.5 histidine residues methylated is as active <u>in vivo</u> as native insulin at a dose of 5×10^{-8} mol/150g. More extensively methylated insulins are not active at this dose.

(d) Methylation of insulin produces alterations in the ultraviolet spectrum indicating that this modification induces conformational changes in insulin.