T CELL EPITOPE ANALYSIS:
USING INSULIN AS A MODEL ANTIGEN

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Abbreviations

TNP - Trinitrophenyl

PI - Pork insulin

BI - Beef insulin

HI - Human insulin

SI - Sheep insulin

TPI - TNP-pork insulin

TBI - TNP-beef insulin

Abstract.

T cells recognise antigen only on the surface of another cell, the antigen presenting cell, in association with a cell surface molecule encoded by the major histocompatability complex. The nature of these interactions is still not fully understood. For instance, it is not clear how many antigen binding sites are present on MHC molecules, or whether the T cell receptor recognises both antigen and MHC, or just the antigen. Those regions of an antigen which T cells recognise are known as epitopes and attempts to define them has been the focus of many studies.

The objective of this thesis was to investigate T cell epitopes on the protein insulin. A hapten group TNP was used to modify the protein and the initial aim was to generate T cell clones against the hapten, while altering the insulin molecule in specific ways to determine the effect of those changes on response to the hapten. However it was noted at an early stage that in $H\text{-}2^b$ mice the addition of TNP to pork insulin, to which $H\text{-}2^b$ mice are non-responders, stimulated a response. This finding, and the investigation of the mechanisms involved, form the basis of this thesis.

It will show that the TNP group was allowing the recognition of an epitope which in the unhaptenated form of the protein is functionally silent. The TNP was not directly involved in T cell receptor recognition, but may be able to increase the affinity of binding between

pork insulin and H-2^b MHC molecules.

A study on the derivatisation of insulin is discussed. The site of haptenation on the insulin molecule was determined by high performance liquid chromatography. This showed clearly, contrary to previous reports, the the TNP group was binding to the glycine residue at the carboxy terminal of the A chain.

In addition, in an attempt to produce hybrid insulin proteins, site-directed mutagenesis was performed on the human proinsulin gene. The use of molecular biology techniques for the production of these hybrids is discussed.

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Chapter 1:1

General introduction.

The T lymphocyte plays a central role in the immune response to most antigens. The diversity of the lymphocyte repertoire is so enormous that it would seem likely that there are few, if any, antigenic structures that could not be recognised by one or another lymphocyte. It is equally clear however that there is great variety in the capability of different antigens to elicit an immune response. A central question therefore is what properties of an antigen make it more or less stimulatory to the cells of the immune system in general, and individual parts of the system in particular. The study of this question is the province of epitope analysis (reviewed Berzovsky et al, 1987).

A fundamental difference exists between recognition of epitopes by B cells and T cells. The B cell, through antibody molecules on its surface, can interact directly with antigen. Antigen-antibody reactions can therefore be reduced to a two molecule system, and can be studied in solution. In contrast, the T cell antigen receptor interacts with antigen and products of the genes of the major histocompatability complex (MHC) on the surface of another cell. Epitope analysis in the T cell compartment therefore, involves at least a trimolecular interaction, and so far this can only be studied in a cellular system. A molecular

model of the tripartite interaction between the T cell receptor, antigen and MHC molecules has been proposed by Heber-Katz and collegues (Heber-Katz et al, 1983). In this model an epitope is defined as that part of an antigen which interacts with the T cell receptor. An agretope is that part of an antigen that interacts with the major histocompatability complex (MHC) molecules on the surface of an antigen presenting cell (APC). Functional evidence for the existence of agretopes came initially from experiments in which the relative potency of two closely related antigens for a single, homogeneous T cell population depended on the MHC haplotype of the presenting cell. A further complication in T cell epitope analysis is that most antigens are recognised only after they have been proteolytically digested or processed by the presenting cell (reviewed by Chain et al, 1988). This processing unfolds the protein and exposes sites that are necessary for the interaction with MHC or the T cell receptor. The nature and extent of processing is therefore another factor in determining the immunogenicity of a particular segment of a complex protein antigen (Hansburg et al, 1985).

The structure of an antigen is not the sole deciding factor in determining whether it will elicit a response. This is also dependent on the bias of the immune system in a specific host. Mammalian proteins

injected into mammalian hosts will be regulated by the level of self tolerance to the homologous host protein. Even with non-mammalian proteins for which there is no host counterpart, (e.g. viral or bacterial proteins), self-tolerance, immune suppression and idiotypic-anti-idiotypic networks will play a part in determining the response.

The aim of this thesis is to extend our understanding of what determines the immune response of $CD4^+$ T cells to a simple model antigen trinitrophenyl (TNP)-insulin.

Chapter 1:2

The molecular basis of T cell recognition.

T cells recognise antigen via a receptor on their surface (TcR). In the mouse the receptor consists of a heterodimer, a 45kD α chain and a 40kD β chain, which are disulphide linked and glycosylated (Allison et al, 1985). The α and β chains show extensive structural homology with the heavy and light chains of immunoglobulin, and the gene organisation and and mechanisms for generating diversity are similar (Hedrick et al, 1984, Chein et al, 1984, Saito et al, 1984, and Gascoigne et al, 1984).

As discussed further below, T cells recognise antigen only in the context of MHC molecules. This phenomenon is known as restriction (Zinkernagel et al, 1979). A fundamental question in T cell biology is therefore the molecular basis for the dual recognition of antigen and MHC. The question of whether one receptor recognised antigen and another recognised MHC, or whether one receptor recognised both has been resolved by experiments in which α and β receptor genes were transfected into a cytotoxic T cell hybridoma. It was demonstrated that when receptor genes were expressed, both antigen specificity and MHC restiction were transferred to the recipient (Dembic et al, 1986). The gene products which form a single receptor could therefore recognise both the antigen and MHC.

In order to accomodate a "one receptor" model of dual

recognition, it has been proposed that processed antigen binds directly to the MHC restricting elements. In addition, this model attributed differences in immune responses to antigens, in animals expressing different MHC haplotypes (Immune Response (Ir) gene effects), to differences in binding affinity between antigen and MHC. Direct support for this hypothesis, known as the determinant selection theory has come from experiments using detergent solubilised, affinity purified class II MHC antigens (I-a) of two different haplotypes. A peptide from hen egg lysozyme, known to be immunogenic in mice expressing one MHC haplotype but not in mice expressing the other, was added to the purified MHC molecules, and the association measured by equilibrium dialysis (Babbitt et al, 1985). The peptide was shown to bind to the appropriate MHC molecules, derived from mice able to respond to the peptide, with a 10-fold greater affinity than to MHC molecules from non-responding mice. The reaction was also shown to be saturable and specific, and that the binding is dependent on amino acid sequence and not merely configuration, hydrophobicity or peptide length.

Other experiments used phospholipid vesicles containing I-A^d MHC antigens which when coated onto a glass slide fuse together to form a continuous planar membrane. A T cell hybridoma, specific for ovalbumin

and resticted by I-A^d was added to the planar membrane, and in the presence of an ovalbumin peptide known to be immunogenic for the hybridoma, could be stimulated to release IL-2 (Watts et al 1986). In these experiments the use of a fluorescent labelled peptide showed that MHC and antigen interact directly, but in this case only in the presence of the T cell receptor.

A molecular basis for understanding MHC/antigen interactions has come from the recent crystallographic X-ray analysis of the structure of one of the human Class I MHC (HLA) molecules. The human class I antigen, HLA-A2, has recently been crystallised. The α1 and α2 extracellular domains are helices which are orientated to lie at right angles atop strands of β-pleated sheet to form a cleft into which antigen is putatively bound (Bjorkman et al 1987).

Class II molecules are heterodimeric glycoproteins with an α and a β chain 33 kD and 28 kD respectively. Both chains have two extracellular domains and the membrane proximal domains (α2 and β2) are thought to form immunoglobulin-like structures with intrachain disulphide bonds. The detail of the NH_2-terminal domains is unknown, but they are assumed to have a similar site to that defined on class I, into which protein or peptide could possibly be bound. (Travers et al, 1984).

Unlike T cell receptors, MHC molecules are not

variable within an individual and must therefore bind "permissively" to many antigens. However, as mentioned above, this interaction shows some specificity since different MHC haplotypes have different binding characteristics. A number of studies have compared the structure of different antigens which are known to be restricted by the same MHC element. This has given rise to algorithms which have been used to predict epitopes on different antigens. One such model proposes that T cell epitopes will preferentially adopt a stable amphipathic, helical configuration, with hydrophobic residues on one side of the helix and hydrophilic residues on the other (De Lisi et al 1985).

An alternative algorithm based on the analysis of the primary structure of known epitopes proposes that T cell epitopes contain a central motif of a charged amino acid residue, or a glycine, followed by two hydrophobic amino acids. Residues on either side of this motif will form characteristic sub patterns which give rise to the MHC restiction pattern observed (Rothbard et al 1988a).

Assuming the T cell receptor is highly variable any structural pattern of immunogenic epitopes would arise from the interaction with the MHC. In certain cases it may be that the sequence motif proposed by Rothbard et al selects for a conformational structure similar to

that proposed by De Lisi et al, in that a helical configuration may be adopted. The hydrophobic moieties would form one surface of the helix while the charged residues would form the other. Therefore this may present a possible correlation between the two algorithms (Rothbard et al, 1988b).

Chapter 1:3.

T cell epitope analysis.

T cell epitopes have been investigated in many systems, and by several different approaches. One of the most studied is the mitochondrial respiratory protein cytochrome c. Initial analysis of the antigenic regions within this peptide was possible because of the existence of numerous species variants of cytochrome c, which differ from each other only at a few defined positions. Thus for example, peritoneal exudate T-lymphocytes from B10.A mice ($H\text{-}2^a$) immunised with pigeon cytochrome c were tested against a panel of species variants of the protein. The T cells did not respond to autologous mouse cytochrome c or to the human variant. They did respond in a heteroclitic manner to moth cytochrome c, and to a lesser extent hippopotamus cytochrome c. On analysis of the differences between the variant forms, pigeon differed from mouse by seven amino acids. The important residues, based on the comparison of the sequence of immunogenic and non-immunogenic forms, were determined to be at positions 100 and 104. If a particular variant did not have the same residues as the priming antigen at these positions it would not elicit a response (Solinger et al 1980).

Another well studied protein, in which species variants have been used to identify antigenic sites is

myoglobin. A series of T cell clones were prepared from H-2^d cell lines specific for sperm whale myoglobin. By comparing the response to numerous species homologues of known sequence, it was demonstrated that there were two epitopes recognised by the clones. One group recognised an epitope around position 109 and were restricted by I-A^d, while a second group recognised an epitope around position 140 and were restricted by I-E^d (Berkower et al 1985).

A second method of identifying T cell epitopes, in systems where no species variants exist has been to synthesize overlapping peptides, covering the whole protein structure and testing them on T cell clones. Most T cells which respond to a native proein, also react with a peptide fragment containing the appropriate epitope suggesting that T cell epitopes are determined primarily by the amino acid sequence and not three dimensional conformation. Using this approach, B6A F1 clones (H-2^b x H-2^a) specific for Staphylococcus nuclease could be separated into two populations. H-2^b restricted clones were highly responsive to the peptide 91-110, whereas H-2^a restricted clones responded to the peptide 81-100, and neither responded to the other peptide (Finnegan et al 1986).

Having established which region of a protein is antigenic, synthetic peptides can be used to further define the structural features involved in T cell and

MHC recognition. For example, a series of sequentially truncated peptides, in the region of residues 81-103, of pigeon cytochrome c were tested on specific T clones. The results showed that the smallest peptide capable of stimulating a full response was seven amino acids long. Only conservative changes made within the seven amino acid peptide were able to maintain immunogenicity (Schwartz et al, 1985). Additional residues were shown not to be directly involved in binding with the T cell receptor, but enhanced immunogenicity, perhaps by contributing to the stabilisation of the secondary helical structure of the peptide.

Chapter 1:4.

Immune response gene effects on epitope analysis.

The major generalisation arising from the studies documented above, and many others of a similar nature, is that within any protein molecule only very restricted regions can act as effective stimulators of a T cell response. For example the recognition by a T helper cell, through it's antigen specific receptor, of a peptide bound to a class II molecule leads to T cell activation and in turn an immune response to the antigen from which the peptide was derived (Sette et al, 1987, Guillet et al, 1987). Furthermore, immunogenic regions are determined both by the structure of the antigen, and by the MHC haplotype of the host (Germain & Malissen, 1986).

Differences in both overall immunogenicity and fine specificity of the responding T cell populations to a particular antigen, which can be attributed to the genetic background of a particular host, have been termed immune response gene (Ir) effects. Some of the earliest evidence for genetic control of immune responses came from studies in guinea pigs. A soluble insulin-cellulose conjugate was saturated with antibodies from rabbit sera. The ability of anti-insulin antibodies from strain 2 and strain 13 guinea pigs to bind to the insulin-cellulose conjugate was then tested. It was shown that antibodies from strain

2 could bind to the conjugate while strain 13 animals could not (Arquilla & Finn, 1963). Further evidence was provided from experiments using the branched multichain polypeptide poly(L-Tyr, L-Glu) poly DL-Ala - poly L-Lys (TGAL). When CBA and C57\BL mice were immunised with this antigen CBA mice responded poorly while C57\BL mice responded well. Substitution of the tyrosine moiety with histidine resulted in a reversal of response CBA mice responded well, while C57\BL did not. Both antigens elicited a good response in CBA x C57 hybrid mice (McDevitt & Sela, 1967, 1968). Control experiments ruled out differences due to dose, adjuvants, age or sex, and the results indicated that the murine response to TGAL was a genetically controlled, qualitative, dominant trait. The discovery of immune response genes in the I regions of the MHC (Benacerraf & McDevitt 1972), led to the hypothesis that Ir gene products form together with antigen to give an immune complex which stimulates T cells. In addition the Ir gene product was, via it's ability to bind a particular antigen, able to dictate the T cell response (Kapp et al, 1974, Benacerraf et al 1974, Benacerraf, 1978).

The phenomenon of Ir gene control of immune responses has been studied in great detail in many protein antigen systems such as lysozyme (Allen et al, 1984), cytochrome c (Hannum et al, 1985a,b, Ogasawara et al, 1987) and myoglobin (Streicher et al,1984). These

studies showed that for many protein antigen systems the genetic background of the recipient could determine the level of response. The major controlling elements of immune responsiveness to simple protein antigens have been mapped to the class II regions of the MHC complex.

The mechanism by which Ir gene products influence T cell responsiveness is still an outstanding question. Three major hypotheses have been studied in detail. One is determinant selection, which has been discussed briefly above, whereby antigen recognition depends on its ability to form a complex with some MHC antigens, but not with others. The hypothesis states that there is an intrinsic association between antigen and an MHC product and that it is this association that determines what portion of the antigen will be available to activate T cells (Rosenthal et al, 1978). A second hypothesis is that the pattern of response to any particular antigen is determined by the repertoire of the adult T cell population. Non-responsiveness, in this model, results from the fact that T cells specific for the particular epitope are absent in the adult, because they have been deleted during ontogeny in the thymus. This clonal deletion is a consequence of "self-tolerance", and low responsiveness to a foreign antigen is due to a fortuitous cross-reaction with self. Since MHC antigens determine the self-

repertoire which is selected, they also determine the adult T cell repertoire, and hence the immune response patterns.

The third theory is that certain antigens when bound to MHC molecules preferentially stimulate a population of T suppressor cells which inhibit inducer T cell responses.

The question of differential binding by MHC has been discussed briefly above. The key question in differentiating between theories 1 and 2 above, has been that of experimentally distinguishing between the effects of changes in antigen structure on the interaction with MHC molecules or the T cell receptors. A number of functional studies have been devised to attempt to solve this problem. For example, it was shown that the peptide 86-103 of moth cytochrome c could be presented to the same T cells in the context of both I-A^b and I-A^k with different potency, a phenomenon described as MHC degeneracy (Hansburg et al, 1985). Changes in the peptide structure which affected the relative effectiveness of the I-A molecule were therefore mapped to the "agretope region, while changes which affected T cell recognition equally on both haplotypes were mapped to the epitope. However the agretope and epitope were shown to overlap in this system, and comparisons using different responder T cells showed that it was difficult to clearly distinguish between agretope and

epitope effects.

A more direct way of addressing this question is to use isolated MHC molecules and determine the binding potential of peptides. Babbitt et al used an H-2^k restricted immunogenic peptide and examined the direct binding of this and other peptide analogues to I-A^k antigens in planar membranes,and in functional studies. The majority of the analogues did not stimulate the T cell hybridomas on which they were tested, but they were able to inhibit binding of the immunogenic peptide. This was true both on planar membrane and in functional studies. Perhaps most surprising was the fact that an autologous mouse lysozyme peptide inhibited binding by up to 85% (Babbitt et al, 1986).

The data implies that although some Ir gene effects do correlate with MHC binding differences, other non-immunogenic peptides can associate with Ia molecules but do not stimulate presumably because of an absence of appropriate T cells

Evidence for clonal deletion has come from studies involving tolerising adult mice with trinitrobenzoylsulphonic acid (TNBS), by intravenous injection (Good et al, 1983). Cytotoxic T cell precursors were recovered from these mice and grown in limit dilution cultures, in the presence of Con A supernatants. The cells from the cultures were tested

by cytotoxicity against normal allogeneic spleen cells. The results showed that hapten-specific Tc cells were much depleted in hapten primed mice.

More direct evidence of thymic clonal deletion comes from studies using monoclonal antibodies which recognise specific V_β regions of the T cell receptor. The basis of these experiments was the finding that T cells which recognise certain antigens, in particular allo-MHC antigen I-E, and the minor histocompatability antigen Mls, use receptors which almost always contain a particular V_β regions (V_β8.1 and V_β6 in the case of Mls, and V_β17a in the case of $I\text{-}E^s$ or $I\text{-}E^k$.

In $H\text{-}2^k$ and $H\text{-}2^d$ animals that contain functional germline genes for both Vβ 8.1 and the surface antigen Mls, peripheral T cells expressing the relevant Vβ were severely depleted (Kappler et al, 1988. Similar results were also obtained with V_β6 germline genes and Mls (Macdonald et al, 1988). In addition, T cell hybridomas were prepared by cross linking of TcR by a monoclonal antibody against the relevant V_β to activate the cells and then expanding in IL-2, before being fused with BW5147 T cell thymoma cells. When tested T cell hybridomas expressing the relevant Vβ were shown to recognise the Mls antigen.

Similar results were obtained with T cell hybridomas derived from $I\text{-}E^-$, $H\text{-}2^s$, SJL mice expressing Vβ 17a. SJL mice can only express the $I\text{-}E_\alpha$ chain and it was shown that in these animals $V_\beta17a^+$ T cells form

approximately 10% of the peripheral T cell pool. However, when SJL mice were crossed with BALB\c mice the resultant F1 offspring, which can express either I-E^{d} or I-$E^{d\backslash s}$ had virtually no $V_{\beta}17a^{+}$ T cells (Kappler, 1987).

These results strongly support the theory that tolerance to self MHC, or to modified MHC in the case of Mls, are the result of deletion of reactive T cells before they become part of the peripheral pool.

A third possibility is the role of suppressor mechanisms which render helper T cells functionally silent (reviewed Dorf & Benacerraf, 1984).

An example where MHC linked low responder status, has been attributed to suppression is the response to hen egg lysozyme. Studies on this protein antigen found that the Th and Ts determinants were non-overlapping, with the Ts region situated at the amino terminal and the Th region internal to the protein structure. The Ts determinant was removed by chemical cleavage and the remainder,including the Th epitope was used to prime previously non-responder mice. The mice showed strong proliferative responses to the modified protein (Oki et al, 1985).

A key finding in these and similar experiments is that suppression is a dominant phenomenon. The study of helper epitopes may therefore be masked by the presence of a single "suppressor epitope" on the

antigen.

The role of MHC restriction in suppressor cell activity is still a matter of controversy. While most suppressor cells are reported to be CD8 positive, (and therefore presumably class I restricted), there is now general agreement that these effector cells are activated by a CD4 positive, class II restricted suppressor inducer cell (Oliveira, et al 1988).

The factors which determine whether an epitope gives rise to "helper" or "suppressor" responses are still largely unknown. However, it has been suggested that Ts inducer and Th cells use different MHC antigens as restriction elements. As Ir gene products in complex with antigen will stimulate an immune response, immune suppression,(Is), gene products will in complex with antigen suppress a response. The response to the liver antigen F, which is strictly MHC dependent, shows dominant non-responsiveness in the F1 cross between H-2^k (responder) and H-2^b (non-responder). This non-responsiveness can be mapped to the I-E region of the MHC. In vitro T cell proliferation in responder CBA (H-2^k) mice was blocked by the appropriate α-I-A^k monoclonal antibody, whereas it was enhanced (i.e suppression inhibited) in the F1 strain, in the presence of α-I-E antibodies (Oliveira et al, 1987). Is gene effects linked to a particular restriction element were also demonstrated in experiments on Schistosoma japonicum in humans where suppression was

shown to be DQ restricted (Hirayama et al, 1987).

The conditions which appear to induce Ts cells include excess antigen, in which case suppressor cells prevent the whole immune system being directed to the one antigen, and chronic infection such as parasitic diseases. In the latter case suppressor cells may operate to prevent the possible damage, i.e. immune complex formation, caused by an ongoing immune response, which may have more harmful effects than the infection itself (Mitchison et al, 1986).

On the basis of the above evidence non-responsiveness is regulated at the T cell level as well as by the MHC. For a protein or a peptide to be immunogenic it must be able to bind to class II MHC molecules, but binding does not necessarily result in an immune response. MHC does not discriminate between foreign and autologous peptides, but does so between structurally different peptides. Both Ts cells and clonal deletion may be involved and which takes precedence may depend on the antigen concerned, the antigen load and the route of entry.

For my thesis I have employed a different method for studying epitopes. Insulin was haptenated with trinitrobenzoylsulphonic acid to attach a single trinitrophenyl (TNP) group to the protein. The objective was to produce T cell clones which recognised the hapten as an epitope. The carrier

molecule would then be altered by the use of species variants of insulin, or other insulin analogues. In principle, this method would maintain the epitope as constant (the TNP structure will remain independent of the carrier structure) while the agretope would be altered. In addition the conformational relationship of agretope and epitope could be studied in detail. However as will be shown in practice TNP had complex effects on the immunogenicity of the carrier molecule. For example in H-2^b mice TNP linked to a non-immunogenic carrier protein, pork insulin, altered the molecule to render it immunogenic. The investigation of this result will form the basis of this thesis.

Chapter 1:5.

Insulin as an antigen.

Insulin was chosen as a model carrier antigen for several reasons, (1) it is a small globular protein whose structure is known in great detail from X-ray crystallography (Blundell et al, 1972) Fig 1, (2) naturally occuring species variants are readily available, Table 1, (3) different congenic MHC mouse strains show distinct response patterns to different species variants of insulin, Table 2, (4) most importantly, the fact that TNP will haptenate insulin on a single site (Li, 1956).

In order to analyse the immune responses to insulin, it is essential to understand the detailed structure of the molecule. The monomeric form of insulin is made up of two polypeptide chains (A and B), linked to each other by two disulphide bonds. The A chain consists of 21 amino acids, and the B chain 30.

In the A chain regions A_2 - A_8 and A_{13} - A_{20} are helical, running almost anti-parallel to each other. A_9 - A_{12} is in the form of a an extended polypeptide chain. In the B chain regions B_2 - B_7 and B_{24} - B_{29} are in the form of a β - pleated sheet, while B_9 - B_{19} is a helix.

An intrachain disulphide bond between cysteine moieties at positions 6 and 11 on the A chain push out the intervening amino acids to form a region termed

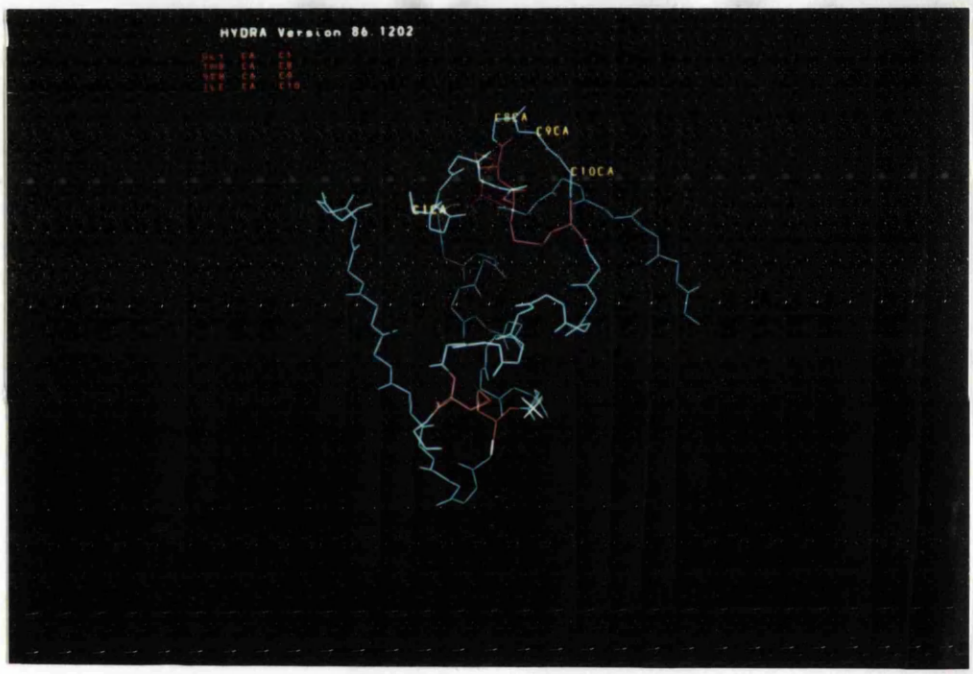

Fig 1. Structure of the insulin molecule.

(a) Monomer structure. The disulphide bonds are coloured red, and the A chain loop residues and A1 glycine are labelled.

(b) Dimer structure. The interacting side chains are coloured orange, and the NH_2 terminal groups are labelled.

My thanks to Alistair McLeod for his help in preparing these figures.

Table 1

Differences in insulin amino acid sequences between species.

Species	Amino Acid Position					
	A chain				B chain	
	4	8	9	10	3	30
Mouse	D	T	S	I	K	S
Cow	E	A	S	V	N	A
Pig	E	T	S	I	N	A
Sheep	E	A	G	V	N	A

Table 2

Response to insulins in different strains of mice.

Haplotype	Insulin		
	Beef	Pork	Sheep
H-2^b	+	-	+
H-2^d	+	+	+
H-2^k	-	-	-

the A chain loop. Two interchain disulphide bonds between A_7 and B_7, and A_{20} and B_{19} maintain the tertiary configuration of the protein.

In vivo, insulin is derived from the single chain precursor proinsulin, in which there is a connecting peptide which joins the C terminus of the B chain and the N terminus of the A chain. The C peptide is the connecting peptide less the two basic amino acid residues at either end. Due to difficulties involved obtaining high yields of insulin from reacting isolated A and B chains together in vitro, insulin is produced synthetically by expressing the whole proinsulin gene, and removing the C peptide by proteolysis. Studies aimed at producing mutant insulins by site-directed mutagenesis of proinsulin are discussed below.

Under physiological conditions insulin is in a dimer-hexamer equilibrium. The hexamer is formed by six monomers with two zinc ions bound into the structure. Insulin will bind zinc more avidly with increasing pH between 4.5 and 8. Below pH 4 and above pH 8 dissociation occurs and the monomeric acid form and the monomeric basic form predominate.

Dimers are formed through non-covalent linkage between the phenylalanine group at position 24 and the tyrosine at position 26 on the two B chains.

Segregation of polar and non-polar residues is more

clearly defined in the hexamer than in any of the other sub-units, with the surface being covered with polar side chains. Any exposed non-polar side chains are utilised in hexamer binding. Conversely, in the monomer the B chain will have many more hydrophobic residues exposed and this may be of importance in binding to MHC or to the T cell receptor.

Species variants of insulin show small differences in sequence, though the overall structure of the molecule is not altered.

At the A chain loop region pork and mouse insulin are identical, whereas beef insulin differs by two amino acids. These differences may have an important role to play in the H-2^b restricted T cell responses.

T cell responses to insulin have been the subject of many studies. In guinea pigs two inbred strains which differ at the MHC were immunised with PI, and their T cell proliferative responses were measured. Strain 2 T cells responded strongly to PI and weakly to BI. Strain 13 responded to PI, BI and isolated B chain. Thus, strain 13 guinea pigs recognised a common epitope on the B chain, while strain 2 guinea pigs were recognising the loop region of the A chain where BI and PI differ (Barcinski et al, 1977). This was confirmed by immunising with isolated B chain. Strain 13 responded to PI, BI and B chain, strain 2 responded to none of the antigens, while F1 primed T cells gave the same pattern as strain 13 cells. In

insulin responses, therefore, as in other antigen systems described above, the fine specificity of responding T cells was controlled by the MHC haplotype.

In mice, experiments using a peritoneal exudate T cell enriched (PETLES) proliferation assay system demonstrated that T cells from H-2^b animals responded to BI, but not to PI or SI,(i.e an A-chain loop epitope). H-2^d mice responded to BI, PI, SI and isolated B chain, (i.e. a B chain epitope). Using recombinant mouse strains the location of the control of the immune responses to both the B chain determinant in H-2^d mice and the A chain loop determinant in H-2^b was mapped to the I-A region of the MHC (Rosenwasser et al 1979).

In H-2^b mice pork insulin failed to stimulate antibody or proliferative responses. However if PI primed H-2^b T cells are transferred to sygeneic recipient mice in adoptive transfer experiments they were shown to provide help to BI primed B cells. Other non-responder forms of insulin, including autologous rat insulin could prime T helper cells in H-2^b and H-2^d mice, but not H-2^k mice (Bucy et al, 1983)

The evidence suggests that different immunodominant epitopes are recognised by different strains of mice. If that epitope involves the A chain loop only those variants that differ from mouse insulin at this region

will stimulate a response. All these experiments have been interpreted in terms of the determinant selection model of MHC gene restriction.

Ir gene effects in these strains of mice are further complicated by experiments in which F1 (b x k) mice responded not only to BI, but also to PI and SI. Both parental strains are low responders to the latter two antigens. It was also shown that the F1 T cells responded to the three insulins on F1 APC, but not on either of the parental strains (Reske-Kunz et al 1982). A likely explanation of these results is that novel MHC restriction elements are formed in F1 mice with an α chain from one parental I-A molecule, and a β chain from the other (Spaeth et al, 1983).

An additional unusual feature of T cell responses to insulin is that there is evidence of "conformation dependent" epitopes. In these experiments, F1 (d x k) high x low responder PI primed T cells were fused with a BW5147, a thymoma line, to give cloned insulin specific T cell hybridomas. The hybridomas could be separated into two groups based on IL-2 production in response to various insulins. Group 1 responded to PI but not to rat insulin which differs only at positions A_4 and B_3. Group 2 could be stimulated by PI,BI,SI,HI and isolated B chain, and were recognising an epitope on the B chain. The fine specificities of group 1 hybridomas suggested that they were recognising an antigenic moiety that included the A chain loop,

asparagine at position A_4 and glutamate at position B_3, which would suggest that the tertiary configuration of the protein is important (Glimcher et al, 1983). This could be possible as positions A_4 and B_3 lie close together in the three-dimensional structure of insulin.

Although data on T cell proliferation and lymphokine secretion has been interpreted largely in terms of determinant selection, independent experiments on antibody responses have provided strong evidence for the existence of suppressor cell pathways in these mice. In H-2^b mice both BI and PI primed T cells are able to stimulate insulin specific PFC responses in the presence of BI, while neither population recognise PI. However if PI primed T cells were selectively irradiated, then added to cultures they gave secondary help in a PI PFC response (Jensen, 1984). In addition when non-irradiated PI primed T cells were added to irradiated PI primed T cells the response could be suppressed. This data suggests that the response to PI in H-2^b mice can be suppressed by a radiosensitive subset of cells. Only PI primed Ts cells were capable of suppressing the response, BI primed T cells did not. Neither BI nor PI primed cells inhibited the response to BI (Jensen et al, 1984).

Extracts from H-2^b $CD8^+$ cells were prepared by sonication, followed by centrifugation and filtration

of the soluble fraction. These extracts were able to suppress in vitro secondary antibody responses in H-2^b mice, but not in other haplotypes (Jensen et al, 1986). As this factor was absorbed by pork but not beef insulin bound on a Sephadex column it suggests that the Ts factor was binding to the A chain loop. This data suggests that non-responsiveness is determined by the repertoire of specific Ts cells rather than through deficiencies in the Th cell repertoire, or defects in antigen presentation.

Epitopes both on the A chain and the B chain can be recognised by human T cells. HLA-DR1^{+ve} cells from a single insulin treated diabetic patient were stimulated in vitro,and T cell lines (TCL) derived. Ten lines were studied for responses to insulin variants as measured by (^{3}H) TdR incorporation. The eight TCL derived from stimulation with BI showed no cross reactivity with PI or HI. The two TCL derived from stimulation with PI showed substantial heteroclitic responses with BI, and one was weakly autoreactive with HI (Miller et al, 1987). Therefore the data on the T cell repetoire from a single donor is similar to that of inbred mouse strains and includes fine specificity for one or two amino acids and recognition of autologous insulin.

Chapter 1:6.

The use of haptens to study protein antigens.

Immunogenicity is the ability to stimulate an immune response, whereas antigenicity refers to the ability to be recognised by a product of a previous immune response either an antibody molecule or a T cell receptor.

A hapten is not immunogenic alone but, when attached to an immunogenic carrier it will elicit an immune response. Therefore free hapten is antigenic but not immunogenic.

The antibody response to haptenated proteins has been studied in great detail, and the recognition that the antibody response directed to a hapten could be regulated by the nature of the carrier led to the discovery of T cell help (Mitchison, 1971).

A number of important generalisations have been established for hapten specific antibody responses. Firstly, the interaction between hapten and antibody molecule is indepedent of the carrier molecule. However, the immunogenicity of a hapten\carrier antigen is largely dictated by that of the carrier protein. Where the response to a carrier protein is under the control of Ir genes, the antibody response is usually determined by carrier specific T cells, and the regulation of the immune response to hapten and carrier occurs in parallel (Wrede et al, 1973).

This regulation has been studied, for example by using insulins haptenated at the B_{29} site with a dinitrophenyl group. It was demonstrated that DNP-PI stimulated a high titre of both anti-DNP and anti-insulin antibodies in H-2^d mice. In H-2^b and H-2^k mice, there was no response to either. H-2^b mice primed with DNP-BI gave good responses, while H-2^k mice did not respond to either insulin (Keck, 1975a). As the strains differed only at the H-2 loci this was strong evidence for Ir gene control, and that in H-2^b mice the A chain loop was implicated in T cell recognition.

Some haptenated proteins are also good antigens for inducing T cell proliferation. In contrast to B cells, the T cell response to haptens are often carrier specific. A very extensive study of hapten reactive T cells was carried out by Janeway et al. Guinea pigs immunised with DNP (dinitrophenyl)-Mycobacteria showed delayed hypersensitivity responses to the priming antigen. T cells were purified, by nylon wool separation, from peritoneal exudates from primed animals, and gave in vitro proliferative responses to DNP-Mycobacteria. In addition, some other DNP modified proteins could also elicit proliferative responses in DNP-Mycobacteria primed T cells. Unconjugated proteins failed to stimulate the T cell populations. Since not all DNP conjugates will trigger DNP-Mycobacteria

primed T cells it was postulated that the T cell receptor was not strictly DNP specific and that they also recognise part of the carrier protein. Of those modified proteins which were non-stimulatory, the lack of tyrosine residues was shown to be the limiting factor (Janeway, 1975).

The mechanism by which DNP-Mycobacteria can prime T cells to respond to DNP-proteins other than the priming antigen is unknown. One explaination is that the lipid coat of Mycobacteria absorbs free DNP groups during modification, which are released in vivo to bind to self antigens. A second possibility is that the lipid coat could fuse with the T cell membrane stabilising weak affinity interactions between DNP-protein and the T cell receptor. In agreement with this theory similar results were obtained with DNP-bovine serum albumin which had been further modified by conjugation with fatty acids. When used to immunise guinea pigs delayed hypersensitivity reactions could be demonstrated to several DNP-proteins (Dailey et al, 1974).

The T cell receptor on DNP-Mycobacteria primed guinea pig T cells was shown to recognise a portion of the protein together with the hapten. This was demonstrated when DNP was attached to the protein via a tripeptide linker. Proteins modified in this way could not stimulate a proliferative response Therefore, while B cells recognise haptens regardless

of the carrier, T cells recognise a more extensive determinant, with essential contributions being made by amino acids to which DNP is not directly attached (Janeway et al, 1976a).

Inducer T cells could be demonstrated by priming with TNP and NP, bound to several carriers. The T cell population could be divided into two broad groups, conjugate specific, by far the largest group, which are activated only by hapten conjugated to the same carrier used for priming. The second group, which was much rarer, were hapten specific and recognised hapten on other proteins including the autologous form. This supports the findings of Janeway that the vast majority of inducer cell clones recognise hapten in association with a defined sequence of amino acids. (Clayberger et al, 1983).

The use of haptens can illustrate the fine precision with which T cells can discriminate between closely related antigenic determinants. A series of proteins were modified using 2,4,6-trinitrochlorobenzene and dinitrobenzene sulphate to give TNP and DNP proteins respectively. Guinea pigs were primed to these antigens and the T cells tested for cross-reactive responses. It was shown that most TNP reactive cells could also react to DNP-proteins, while a substantial percentage of DNP reactive cells failed to be stimulated by TNP derivatives. One possible

explanation for this result was that DNP-specific sites on the T cell receptors may be unable to accomodate the larger TNP group. In an attempt to further measure hapten recognition by T cells, non-stimulatory DNP modified conjugates were used to block proliferative responses, both in vivo and in vitro. Little or no inhibition of the response was found in either situation (Janeway et al, 1976b).

Haptenation of insulins with TNP has given some evidence of a separation of the regulatory mechanisms governing proliferative and antibody responses. In this system antibody responses may be regulated by antigen specific suppressor cells, T cell proliferative responses were not. It is possible therefore that proliferative and antibody responses may be regulated at different stages of T cell activation (Flory, Wallace, Oettel and Chain 1989).

Haptens have also been shown to modify self proteins to render them immunogenic to syngeneic T cells. BALB/c immunoglobulins were derivatised with 4-hydroxy-3-nitrophenyl acetyl (NP) and used to immunise syngeneic BALB/c mice. Primed T cells were demonstrated by in vitro proliferation assays. In agreement with the results found in guinea pigs, distinct specificities were created by coupling NP to different BALB/c monoclonal antibodies, or by attachment via a linker. Therefore T cells were

recognising a determinant of the hapten plus a part of the protein carrier (Birkoff et al, 1982).

In summary, haptenated proteins provide a good antigen system for studying epitope analysis. Production of monoclonal T cells that corecognise a single defined determinant, such as a hapten, in association with Class II MHC antigens allows direct biological definition of this interaction. The hapten can elicit T cell responses while the carrier molecule provides the Ir gene restriction. By separating the two processes in this way both the epitope and agretope can be studied.

Chapter 2:1
Materials and Methods: Cellular.

Animals: Mice were obtained from the Imperial Cancer Research Fund breeding unit, and kept under SPF conditions. The strains used were C57BL/B10ScSn ($H\text{-}2^b$), $B10D_2$ ($H\text{-}2^d$), BALB/c ($H\text{-}2^d$), and CBA ($H\text{-}2^k$).

Antigens: Beef insulin, pork insulin, oxidised bovine A chain and B chain were obtained from Sigma (St.Louis, U.S.A.). Human insulin was supplied by Lilley in the form of Humulin S. Desoctapeptide insulin pork (DOP) was prepared by Alistair McLeod at the Crystallography Department, Birkbeck College, London.

Heat-killed Mycobacteria tuberculosis (H37RA) was obtained from Difco (Detroit, U.S.A.).

Ovalbumin and bovine serum albumin were purchased from Sigma (St.Louis U.S.A.).

Haptenation: haptenated antigens were prepared by dissolving 70 mg of 1,3,5 - trinitrobenzenesulphonic acid in 1 ml 0.1 M sodium bicarbonate (pH 8.2) just prior to use. An aliquot was added slowly to insulin (10 mg\ml) in 0.1 M sodium bicarbonate (pH 8.2) to give a molar ratio of 3:1 (TNBS:protein). The reaction was allowed to proceed for two hours at room temperature in the dark. TNP-protein was separated from unreacted TNBS on a Sephadex G25 (Pharmacia) column (15 x 6 cm), pre-equilibrated in sodium bicarbonate buffer (pH 8.2). The major coloured band was collected and lyophilised.

The absorbance of a sample of TNP-insulin was measured

at 280nm and 340 nm in a Pye Unicam SP8-400 spectrophotometer. The concentration of protein was calculated by the equation;

$$\text{mg\ml} = \frac{OD_{280} - 0.34 \times OD_{340}}{1}$$

where 1 is the molar absorption coefficient of insulin.

The molar concentration of insulin was calculated as;

$$\text{moles\litre} = \frac{\text{mg\ml protein}}{6 \times 10^3}$$

taking 6×10^3 as the molecular weight of insulin.

The molar concentration of TNP was calculated as;

$$\text{moles\litre} = \frac{OD_{340}}{1.14 \times 10^4}$$

where 1.14×10^4 is the molar absorption coefficient of TNP.

Using this method of coupling, ratios (TNP : insulin) obtained were 1 : 1 + 20%. (Mishell and Shigii, 1980).

Immunisation Protocol: For injection into animals antigen was prepared at a concentration of 2 mg\ml and emulsified 1:1 with Complete Freund's Adjuvant, using a sonic probe (Sonifier B-12, Branson Sonic Power Co. USA), whilst keeping the sample on ice. 50ug was injected subcutaneously at the base of the tail. Ten days later the animals were sacrificed and the inguinal and para-aortic lymph nodes were collected.

Media.

Iscove's Modified Dulbecco's Medium (IMDM); solid form obtained from Gibco, UK., with the addition of penicillin (100U\ml), streptomycin (100ug\ml), indomethacin (1ug\ml), sodium selenite (5ng\ml), ferric chloride (0.3ug\ml), 2-mercaptoethanol (5×10^{-5}M), and sodium pyruvate (1mM).

Roswell Park Memorial Institute 1640 (RPMI); supplemented with glutamine, penicillin (100U\ml), streptomycin (100ug\ml) and buffered with sodium bicarbonate obtained from ICRF.

Minimal Essential Medium (MEM); obtained from ICRF.

Proliferation Assays.

Foetal calf serum(FCS) was obtained from Gibco UK. and before use was incubated at 56°C to destroy complement. Normal mouse serum(NMS) was prepared by collecting blood from B10ScSn mice and allowing it to clot for 1 hour at 37°C, then 1 hour at 4°C. The serum was pooled, 2-mercaptoethanol was added (final conc. 5×10^{-5}M) and the serum dialysed for 48 hours against several changes of saline. More mercaptoethanol was then added(same final conc.) and the serum stored at -20°C.(Chain et al, 1987).

Standard Assay: (Corradin et al, 1977) used for cell lines and clones. Cells from culture were harvested and washed twice in MEM, counted and made up to the

appropriate concentration,(1-2 x 10^6) in IMDM containing 5% FCS and 50ul added to each well in a round-bottomed 96-well plate. Syngeneic spleens were collected, dissociated to a single cell suspension through a nylon mesh, washed twice in MEM, and made up to a concentration of 1 x 10^7\ml, in IMDM -5% FCS. The cells were then irradiated (3000 rads from a ^{60}Co source and 50ul were added to each well. 100 ul of antigen at appropriate concentration(IMDM - 5% FCS) were added to the correct wells, and the plate incubated in 5% CO_2 at 37 c in a humidified incubator (LEEC) for three days. The cells were then pulsed with 1uCi (^3H) thymidine for 16 hours, harvested onto glass filter paper, and counted in a liquid scintillation counter.

Modified Proliferation Assay (Chain et al, 1987): (see Appendix 1) was used for primary lymph node assays as this method greatly decreases background incorporation of thymidine.

Lymph nodes from primed mice were collected and dissociated into a single cell suspension by passage through a nylon mesh. The cells were washed twice in MEM counted on an Improved Neubauer haemocytometer, then made up to final concentration ($1x10^7$) in IMDM containing 1% NMS.

50ul of cells were added per well (round -bottomed, Nunc UK.) giving a concentration of 5 $x10^5$. Antigens prepared at the appropriate concentrations were added in 50ul of the same medium. the plates were incubated, as

above for three days. On the third day 100ul of IMDM medium containing 5% foetal calf serum was added to all wells. The contents of each well were resuspended by pipetting 100ul up and down four or five times, and 100ul of contents of each well were transferred to 100ul of IMDM containing a source of IL-2 (see below) in wells of a 96-well plate (flat-bottomed, Nunc, U.K.). The second plates were incubated a further two days, and proliferation was measured by adding 1 uCi (^{3}H)thymidine (Amersham) for 16 hours, harvesting the cells onto glass filter paper in a cell harvester (Dynatech UK) and counting incorporated thymidine in a liquid scintillation counter (LKB).

Antigen Specific T cell Lines: lymph node cells from antigen primed mice were cultured at 2 x 10^6\ml in the presence of antigen(100ug\ml) for three days in 5% CO_2,37° c. At the end of this period the viable cells were harvested on Ficoll-Hypaque, (stock solutions; 14% Ficoll 32.8% Hypaque. Mix 12 parts Ficoll stock with 5 parts Hypaque stock, autoclave and store in the dark), to remove dead cells, debris and excess antigen, then cultured for a further seven days in the presence of an exogenous source of IL-2. On the seventh day the cells were harvested, washed twice in MEM to remove IL-2, and either used for assay or put back on antigen to repeat the cycle. For restimulation the cells were set up at 2 x 10^5\ml and cocultured with sygeneic irradiated spleen

cells, 5 x 10^6\ml and antigen.

Preparation of T cell Clones: a stock mixture containing 5 x 10 ^{6}\ml irradiated(3000 rads from ^{60}Co source), syngeneic spleen cells, antigen at 200 µg\ml and IL-2 at 10%. The cells to be cloned were prepared at various concentrations to give final numbers of 300, 30, 3, and 0.3 per well, and were mixed 1:1 with the stock. 20 µl were then added to each well on a 60 well Terasaki plate. The plates were incubated in 5% CO_2 at 37°C for one week. The plates were then checked for positives which were collected and expanded on antigen\feeder cells - IL-2 cycles as described above. A plate containing feeder cells only was used as a negative control.

IL-2 Sources. two sources of exogenous IL-2 have been used, a recombinant form, which was a gift from Dr. M. Feldmann, Sunley Research Centre, Charing Cross Hospital, London., used at a concentration of 0.05 ug\ml, and supernatent from a gibbon cell-line MLA-144.

Each batch of supernatant was tested on the IL-2 dependant cell line CTLL, in a 24 hour assay and proliferation measured by thymidine incorporation. On the basis of these assays the concentration at which each batch of supernatant was determined.

Spleen Adherent Cells for use as Feeders. spleens were collected from mice of the appropriate H-2 haplotype and prepared as a single cell suspension in RPMI containing

5% FCS, at a final dilution of 5 ml per spleen. 5 ml aliquots of cells were then added to tissue culture dishes (90 mm, single vent, Becton Dickson, USA) and swirled around to cover the surface. The dishes were incubated at 37°C for 2 hours, the medium removed, and the dishes washed 4 times in 5ml of RPMI-5%FCS to remove the non-adherent cells. A further 5 ml of medium was added and the dishes left for 16 hours. The medium containing cells which had become non-adherent during the incubation was collected, the cells washed twice in RPMI/5%FCS and set up at the appropriate concentration in IMDM - 5% FCS for use in proliferation assays. SAC were routinely used at 5×10^4.

Antibodies: the antibody TIB120 (monomorphic anti-mouse Class II MHC), HB38 (IgM anti-I-A^b) and HO13.4 (anti-Thy 1.2) were obtained from ATCC. 3.168.8.1 (anti-CD8), H40-481.3 (anti-I-A^k) and HI29 (anti CD4) were a kind gift from Dr. F.W.Fitch, University of Chicago, USA.

Identification of the Site of Haptenation by High Pressure Liquid Chromatography. (see Appendix 2)

Chromatography was carried out on a Varian 5000 liquid chromatograph unit, with a C18 uBondapak reversed phase cartridge in a Z module holder (Waters, Milford MA. USA.). Absorbance was measured at 280nm. For analysis of insulin and it's analogues, the aqueous phase used was 0.1M phosphate buffer (pH3), while for separation of TNPylated and dansylated amino acids the aqueous phase was 0.1% trifluoroacetic acid.

Acetonitrile was the organic modifier in all cases. Details of gradients and flow rates are given for each separation in Appendix 2.

Dansylation of Insulins.

Samples of protein were reconstituted in a small volume of 0.1M sodium bicarbonate buffer (final concentration approximately 5mg\ml) and 0.1 ml of 0.25% dansyl chloride (Sigma UK.) in acetone was added. The reaction was left in the dark at room temperature for 1 hour, then the solvent was removed in a rotary evaporator (Savant UK).

If the protein was to be used directly for analysis then it was redissolved in 0.1% TFA to be run on the HPLC, alternatively it was hydrolysed for analysis of individal amino acids.

Acid Hydrolsis.

Proteins were hydrolysed in 6M hydrochloric acid for 16 hours at 110°C, in sealed evacuated hydrolysis tubes (Sterilin, UK.). Hydrolysates were dried in a rotary evaporator and stored at -20°C until required, and reconstituted in 0.1% TFA before running on the HPLC.

TNP and dansyl amino acid standards were obtained from Sigma, with the exception of TNP-glycine which was synthesised by reacting equimolar amounts of TNBS and glycine in 0.1M sodium bicarbonate (pH 8.2).

Materials and Methods: Molecular Biology.

Culture of plac 289\PI.

The bacteria were grown on a YT agar plate, (0.8% w\v tryptone, 0.5% w\v yeast extract, 0.5% w\v NaCl, 1.5% w\v agar, and 25 ug\ml ampicillin), and a single colony was picked up on a loop and used to inoculate a flask. The medium used was Minimal Garland's Medium;

2.8 mM	K_2HPO_4
12.8 mM	KH_2PO_4
11.5 mM	Na_2SO_4
1.0 mM	Citric acid.H_2O
1.2 mM	$MgCl_2.6H_2O$
50 uM	$MnCl_2.4H_2O$
90 uM	$FeCl_3.6H_2O$
100 uM	$CaCl_2.2H_2O$

This was kept as a 10 x stock and for use, 1.0% w\v tryptone, 0.5% w\v yeast extract and 1.0% w\v sodium succinate was added, and the medium autoclaved. After cooling the following filter sterilised solutions were added; 50% w\v monosodium glutamate to give 1% final concentration, and 100 mg\ml ampicillin to give a final concentration of 25 ug\ml.

Flasks were shaken at 250 rpm in a 37°C chamber.

After 4 hours incubation isopropyl B-D-thiogalactopyranoside was added to give a final concentration of 3 mM. After a further 18 hours incubation, the cells were harvested by centrifugation, 10,000rpm for 10 mins.(Beckman UK). The cell pellet was stored at -20°C until required.

Disruption of Cells and Extraction of Proinsulin Fusion Protein.

The cell pellet was resuspended in 5 ml 50 mM Tris-HCl, 5 mM EDTA pH 8.0. 1 mg\ml of lysozyme and 100 ug\ml DNAase were added and left for 1 hour at room temperature. The lysate was then sonicated x 4, for 20 seconds, keeping the sample on ice and allowing it to cool down between each sonication. The sample was then transferred to a 50 ml ultracentrifuge tube,and the tube filled with 50 mM Tris-HCl, 0.2 M NaCl pH 8.0, and spun for 10 mins. at 10,000 rpm. The pellet was resuspended in Tris-NaCl and stored at -20°C. (Dr S. Cockle, pers. com.)

SDS-Polyacrylamide Gel Electrophoresis.(Laemmli).

Gels were run either on BRL.(Cambridge, U.K.) apparatus,(plate sizes 19x19.5cm and 16x19.5cm), or the Mini-Protean (Bio-Rad U.K.) system (plate sizes 8.3x10.2 and 7.3x10.2). All gels were 10% final acrylamide concentration using a discontinuous buffer system. The

resolving gel was prepared by adding 10ml acrylamide stock solution (30% acrylamide, 0.8% bisacrylamide), 3.75 ml resolving gel buffer (3M Tris-HCl pH 8.8), 0.3ml 10% sodium dodecyl sulphate (SDS), 1.5ml of 1.5% ammonium persulphate, 14.45ml of water and 15ul of N,N,N',N'- tetramethylethylenediamine (TEMED). This mixture was poured into the gel tank and when polymerised the stacking gel was added containing 2.5 ml acrylamide stock, 5.0ml stacking gel buffer (0.5M Tris-HCL pH 6.8), 0.2ml 10% SDS, 1.0ml of 1.5% ammonium persulphate, 11.3ml water, and 15ul of TEMED. When this mix was poured the appropriate comb was inserted and the gel left to polymerise.

Samples of lysed bacteria containing human proinsulin as a fusion protein, were solubilised by boiling in a loading buffer containing 0.0625M Tris-HCl pH 6.8, 4M urea, 2% w/v 2-mercaptoethanol, 2% SDS, 10% glycerol and 0.002% bromophenol blue, before loading onto the gel.

The gel was run in a buffer containing 0.025M Tris, 0.192M glycine pH 8.3. at 25-30 mA for BRL gels, or 40 mA for the Bio-Rad system.

Pre-stained standards (36-180,000 Sigma U.K.),or unstained low molecular weight standards (10-100,000 Bio-Rad), were run on each gel as markers.

Gels were stained in Coomassie Blue R250 (0.1%) in water:methanol:glacial acetic acid (5:5:2 by volume) for 4 hours and excess stain was removed by washing in 12.5% methanol: 10% acetic acid until protein bands were

clearly visible.

Western Blotting

Gels were blotted in either a Trans-Membrane Blot™ Cell, or the Mini Trans Blot Electrophoretic Transfer Cell (Bio-Rad U.K.), as follows. The press was opened, one half submerged in transfer buffer,(20mM Tris, 150mM glycine, 200ml methanol, make up to 1 litre with distilled water),and a pre-soaked fibre pad placed on the press. Pre-soaked 3MM filter paper followed by the gel were placed on top of the fibre pad, and a section of nitrocellulose membrane filter, 0.45um, (Schleicher + Scheull, West Germany) was added ensuring that there were no bubbles between paper and gel. A second piece of filter paper and a second fibre pad were placed on top in that order and the press closed. The press was placed in the tank with the nitrocellulose sheet towards the anode, and the tank filled with transfer buffer. Blots were run at 250mA overnight in the large transfer tank, and at 100mA for 1 hour in the mini tank.

Blots were either stained using 0.1% amido black (naphthelene black, BDH UK) in 45% methanol, 10% acetic acid, to visualise all transferred protein bands, or with an anti-insulin antibody (Sigma UK) and peroxidase labelling to visualise the insulin band.

Peroxidase staining on nitrocellulose blots to identify specific bands was carried out as follows; non-

specific binding was first blocked with 3% bovine serum albumin in Tris buffered saline pH 7.4, (TBS, 0.9% NaCl in 10mM Tris) for 1 hour at room temperature. The guinea pig anti-insulin antibody, (Sigma U.K.) 1 :1000, in 3% BSA in TBS containing 100mM lysine and 0.05% Tween, was added and incubated, with gentle shaking, for one hour at room temperature. Excess antibody was removed with five 20 minute washes in 100ml of TBS -0.05% Tween, then the second layer anti-guinea pig IgG biotin conjugate (Sigma U.K.) 1:100, in TBS-Tween was added for 1 hour at room temperature with gentle shaking. The blot was washed as above and the third layer strepavidin peroxidase (Sigma U.K.) 1:100 in TBS-Tween was used to bind the enzyme to the antibody. After a final series of washes positive bands were visualised by adding 4-chloronaphthol (24mg) dissolved in methanol, made up to 40ml with TBS and 16ul of hydrogen peroxide until bands appeared, then the reaction was stopped by washing in TBS.

Plasmid Preparations.

Bulk prep.: 300 ml of bacteria, or cells plus M13 supernatant was grown as an overnight culture,then spun at 8000rpm for 10 mins. The supernatant was discarded and the pellet resuspended in 6 ml of ice cold 25%

sucrose, 0.05 M tris pH 8.0.

1.2 ml of 0.05 M tris pH 8.0, 0.01 M EDTA, 5 mg\ml lysozyme were added, and the mix gently rocked for 5 mins.

2.4 ml of 0.25 M tris pH 8.0, 0.25 M EDTA were added and rocked gently for 5 mins.

9.6 ml of 0.05 M tris pH 8.0, 0.01 M EDTA ,2% Triton X100, were added and rocked gently for 10 mins.

The lysate was then spun at 18,000rpm for 1 hour at 4°C The supernatant was collected and 1 gm\ml of caesium chloride, 2.5 ml ethidium bromide (5mg\ml stock) were added, and the plasmid banded at 38,000 rpm at 20°C for 20 hours (VTI 50 Beckman UK). The banded DNA was collected under UV light, and the ethidium bromide removed by extracting with butan-2-ol. The DNA was precipitated by adding 3 volumes of distilled water:1 volume of DNA and 8 volumes of ethanol. The sample was stored at -20°C overnight and then spun to collect the DNA,which was resuspended in TE buffer (10 mM tris-HCl,pH 8.0,1 mM EDTA.) and stored at -20°C.

Mini-Prep: 10 ml of bacteria containing plasmid, or cells plus M13 supernatant, was grown overnight at 37°C. The cells were harvested by spinning at 2500 rpm. for 10 mins.,then resuspended in 200 µl of 25 mM tris pH 8.0, 50 mM glucose, 10 mM EDTA. 400 µl of freshly prepared 1% SDS in 0.2 M NaOH was added ,then 200 µl 3 M potassium acetate pH 4.8. The lysate was vortexed vigorously, and spun for 10 mins. at 10,000 rpm. The supernatant was

collected into an eppendorf tube (Sarstedt, UK), which was filled up with 60 % isopropanol, vortexed and spun for 5 mins. at 10,000 rpm . The supernatant was removed and the pellet washed in 70 % ethanol, dried, resuspended in TE buffer, and stored at -20°C.

Isolation of the Human Proinsulin Gene fragment from plasmid plac 289\PI.

plac 289\PI was prepared as above and 1 µl of DNA (1 - 5 µg) was incubated for 1 hour, at 37°C, with 1 µl EcoRI, (10 units), 1 µl Bam HI (12 units), (both from Bethesda Research Laboratories, USA.) and 1 µl 10 x high salt buffer, 100 mM Tris pH 7.4, 100 mM $MgCl_2$, 1 mg\ml bovine serum albumin, 10 mM dithiothreitol, (BRL. USA.). 1 µl of DNAase free RNAase (Sigma, UK) was added and the mixture was made up to 10 µl with distilled water.

After incubation the cleaved DNA was run on a 1% agarose gel (Pharmacia UK.) with an aliquot of the uncut DNA and a HindIII of lambda DNA digest as standards. The gel was prepared by dissolving 1 gm of agarose in 100 ml of TAE buffer (1.6 M Tris, 0.8 M sodium acetate.$3H_2O$, 40 mM EDTA, adjust to pH 7.2 with acetic acid) in a microwave oven. When the agarose had cooled to 60°C it was poured in a gel former (Bio-Rad UK.) and allowed to set, with a well-forming comb in place. The gel was then placed in the electrophoresis tank, the comb removed, and covered with TAE buffer. The samples to be run were

mixed with 10% v\v loading buffer (50% glycerol, 1% bromophenol blue, 1% xylene cyanol in TAE buffer, and loaded into the wells. The gel was run at 120 V until the dye front reached the bottom edge, then the gel was removed from the tank and stained in a solution of ethidium bromide (5 µg\ml in TAE) for 10 mins. The unbound ethidium bromide was washed out in several changes of water and the DNA bands visualised using a UV transilluminator(UVP Inc. UK.).

If the insulin gene fragment was detectable a larger aliquot of plasmid DNA was digested and the cleaved sample was run on a 1% gel prepared using low melting point agarose(Sigma UK.). After the DNA had been visualised the insulin gene band was carefully cut out using a fresh scalpel blade, placed in an eppendorf tube with 300 µl of TE buffer, and incubated in a 65°C water bath until the agarose had dissolved. An equal amount of water-saturated phenol (Rathburn Chemicals, UK.) was added, the mixture vortexed and spun at 10,000 rpm for 5 mins. The upper, aqueous phase was carefully removed and the phenol extraction repeated until protein could no longer be seen at the interface between the two phases, then a chloroform extraction was performed in the same manner, and the aqueous phase was again collected. The DNA was precipitated by adding 10% v\v 3 M sodium acetate pH 7.0, to give a final concentration of 0.3 M, and 2.5 volumes of ice-cold ethanol. The tube was placed either on dry ice for 20 mins.,or at -20°C

for 16 hours. The sample was then spun at 10,000 rpm for 15 mins. at 4°C, and the supernatant carefully discarded without disturbing the pellet. Excess salt was removed by washing in 500µl of 80% ethanol, and spinning at 4°C for 2 mins. The supernatant was again discarded, the pellet air-dried, and finally resuspended in TE buffer to be stored at -20°C.

Ligation of Insulin Gene Insert into Linearised M13 Vector.

M13mp19 was cut with EcoRI and BamHI to generate a cloning site in the polylinker region with ends compatible with the insert, and purified from a low melting point agarose gel by the method described above. The ligation was performed using 1 µl of linear M13 vector (2 - 5 µg DNA), 1 - 3 µl of insert DNA (a four - fold molar excess to vector DNA), 2 µl of 10 x T4 DNA ligase buffer (0.5 M Tris pH 7.4, 0.1 M $MgCl_2$, 0.2 M dithiothreitol, 10 mM ATP pH 7.0, 100 µg bovine serum albumin), 1 µl T4 DNA ligase (Pharmacia UK.), in a total volume of 20 µl and incubated for 16 hours at 14°C. Ligations were stored at -20°C until required.

Preparation of Competent host Cells.

Colonies of JM101 cells were picked from a minimal essential medium plate with a flame sterilised loop and used to inoculate 50 ml of 2 x TY medium, (16 gm bacto

tryptone (Difco UK.), 10 gm yeast extract (Sigma UK.) and 5 gm NaCl in 1 litre of distilled water). The cells were incubated at 37°C with shaking until the O.D. at 600 nm was approximately 0.3, indicating that they were in log phase growth, then were spun at 2500 rpm for 5 mins. The medium was discarded and the cell pellet resuspended in 20 ml of 50 mM calcium chloride and incubated on ice for 20 mins. The cells were then spun again, at 2500 rpm for 5 mins. and resuspended in 4 ml of 50 mM CaCl. The competent cells were either used immediately or stored at 4°C for up to 36 hours before use.

Transfomation of JM101 Cells with Recombinant M13 DNA.

5 µl of ligation mix was added to 300 µl of competent JM101 cells, and were incubated on ice for 40 mins. The cells were then heat shocked at 42°C for three mins and returned to the ice bath. A mixture containing 40 µl of 100 mM IPTG, 40 µl of X-gal, and 200 µl of a 1:100 dilution of a fresh overnight culture of JM101 cells was prepared and added to each ligation. The heat shocked competent cells and 4 mls of molten (42°C) H top agar, (10 gm tryptone, 8 gm NaCl, 8 gm agar per litre), were then added, the tube mixed by gently shaking, and poured immediately onto a prewarmed (37°C) H plate, (10 gm tryptone, 8 gm NaCl, 12 gm agar), and left to set. The plates were inverted and incubated overnight at 37°C.

Control plates included, uncut M13 - 1 ng\plate, cut M13 - 10 ng\plate, and cut and religated M13 - 10 ng\ml as a ligation control.

The plates were examined the following day for blue (no insert) or white (insert present) plaques.

Preparation of Single-Stranded DNA.

1.5 ml of 2 x TY medium in a 25 ml universal (Sterilin UK.) was inoculated with 100 µl of an overnight culture of JM101 cells, and a single white plaque picked from an agar plate. The tubes were shaken at 37°C for 5 hours, the medium was transferred to a microcentrifuge tube, and spun at 10,000 rpm for 5 mins. The supernatant was carefully poured off into a fresh tube, respun, and decanted into a fresh tube to eliminate contamination with cells. The cells were kept for preparation of double - stranded DNA,(see above). Supernatants in this form could be stored at 4°C for several weeks.

To prepare SS DNA 1.5 ml of 2 x TY medium was inoculated with 100 µl of JM101 cells and 20 µl of M13 supernatant, incubated at 37°C for 5 hours and the cells removed. 200 µl of 20% polyethylene glycol in 2.5 M NaCl was added to the supernatant and left for 15 mins. at room temparature before spinning at 10,000 rpm for 5 mins. The supernatant was discarded, all traces of PEG removed and the pellet resuspended in 200 µl of TE buffer. 200 µl of water saturated phenol was added, the

sample vortexed and spun at 10,000 rpm for 3 mins. The upper aqueous phase was collected into a fresh microcentrifuge tube, 20 µl of 3 M sodium acetate and 440 µl of ethanol were added and the sample incubated at -20°C for 16 hours to precipitate the DNA. The DNA was collected by centrifuging at 10,000 rpm for 15 mins. at 4°C. The pellet was washed in 1 ml of cold ethanol, to remove any salt, which was then carefully decanted, and the pellet left to dry. The pellet was finally resuspended in 20 µl of TE buffer and stored at -20°C.

Site - Directed Mutagenesis.

Oligonucleotides containing the desired mutant sequence were synthesised by the ICRF, Clare Hall. The oligonucleotides were 5 - phosphorylated by adding 2.5 µl of the oligonucleotide stock solution, (5 O.D.units\ml) to 3 µl of 10 x kinase buffer, 1 M Tris - HCl pH 8.0, 100 mM $MgCl_2$, 70 mM dithiothreitol, 10 mM ATP, 2 units of T4 kinase. The mixture was incubated at 37°C for 15 mins., then 70°C for 10 mins. to destroy the enzyme.

All mutations were prepared using the oligonucleotide - directed in vitro mutagenesis system from Amersham.

The phosphorylated oligo and a single stranded DNA template (M13 mp18) were annealed and the mutant DNA strand was synthesised using Klenow fragment and T4 DNA ligase, and a nucleotide mix containing thiolated dCTP.

Any remaining single - stranded (non-mutant) DNA was removed by filtration and the double - stranded DNA collected by ethanol precipitation.

As the mutant strand contains thiolated dCTP it is resistant to cleavage by the restriction enzyme NciI, whereas the non - mutant strand will be nicked. Exonuclease III was then added and the non - mutant strand was digested, leaving the mutant strand which was repolymerised using DNA polymerase 1 and T4 ligase. The mutated double - stranded DNA was then used to transform competent JM101 cells.

Screening of M13 Plaques with a Radiolabelled Probe to Detect Mutants.

Labelled probes were prepared by incubating 0.5 µg of mutant oligonucleotide, 15 units of T4 kinase, 200 µCi of τ-^{32}P-ATP, in kinase buffer, for 45 mins. at 37°C. The sample was then incubated at 65°C for 5 mins. to inactivate the enzyme.

Columns were prepared by packing 1 ml syringe barrels with G-50 Sephadex and washing through several column volumes of wash buffer, 50 mM NaCl, 0.1% SDS in TE buffer, and stopping the flow with 100 µl left on top. Before loading the probe sample the column was spun at 1600 g for 2 mins., in a 15 ml Falcon tube, the sample was loaded with 400 µl of wash buffer and the column was placed in a new 15 ml tube and spun again at 1600 g for

2 mins. This procedure separates labelled sample from unincorporated ^{32}P-ATP. The eluent containing the labelled probe was collected from the Falcon tube and stored at -20°C until required.

DNA was lifted from agar plates containing plaques, which had been air-dried for 30 mins. at room temperature by placing a nitrocellulose filter,(0.45µm Schleicher and Scheull, West Germany) onto the agar surface. Holes were punched asymmetrically through the filter into the agar with an 18-gauge needle to allow reorientation after hybridisation, and the filters left in place for 20 mins. to absorb DNA from the plaques. The filters were carefully peeled off, left to dry at room temperature for 30 mins., then sequentially immersed for 1 min. in the following solutions, (a)0.2M NaOH, 1.5M NaCl (b)2x SSC (20x stock is, 3M NaCl 0.3M Na_3citrate.$2H_2O$ adjust to pH 7.0 with 1M HCl), 0.4M Tris pH 7.4 (c) 2x SSC. The filter was then dried at room temparature, plaque side up, for 1 hour, then dried in a vacuum oven at 80°C for 2 hours.

The dried filter was then placed in a plastic bag and 15ml of hybridisation buffer added (50% formamide, 5x SSC, 0.5M phosphate buffer pH 6.4, 0.5 mg\ml salmon sperm DNA, 1x PM (10% Ficoll, 10% BSA, 10% PVP), 0.1% SDS). The labelled oligonucleotide probe was added to give a concentration of 5 ng\ml, air was expelled, and the bag was sealed, and incubated at 42°C for 16 hours.

The filter was removed from the bag and washed three times in 250ml of 2x SSC for 30 mins. each wash, then blotted dry on Whatman 3MM paper. When fully dry the filter was wrapped in clear plastic (Saranwrap, Genetic Research Instrumentation, UK) and exposed to X-ray film (XAR-5 film, Kodak USA), with intensifying screens, at -70°C for 24 hours, or until positive signals were detected.

Sequencing DNA from Positive Plaques.

Plaques identified as positive by oligonucleotide hybridisation were selected and single stranded DNA was obtained. This was then used as a template for sequencing using the chain termination method (Sanger,F., et al, 1977). The enzyme Sequenase™ (Tabor,S., and Richardson,C.C., 1987) was used in kit form obtained from Cambridge Biosciences UK. The method involves annealing an oligonucleotide primer (a universal M13 oligo that binds upstream of the polylinker sequence) to the single-stranded DNA template. Synthesis is initiated at this site but is terminated by incorporation of 2 , 3 ,-dideoxynucleoside 5 -triphosphates(ddNTP) which lack the 3 -OH group necessary for chain elongation. Mixtures of deoxynucleotide triphosphates(dNTP) and one of the four ddNTPs are prepared and termination of enzyme catalysed polymerisation at each site where the ddNTP is

incorporated will occur. By preparing four separate reactions each with a different ddNTP and incorporating a labelled dNTP(α-^{35}S dATP, Amersham UK) labelled chains of various lengths could be identified and the sequence determined.

To visualise the chains the reaction was terminated by the addition of a stop solution (95% formamide, 20mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF) and the chains separated by high resolution electrophoresis. The gel was prepared by dissolving 7M ultrapure urea (Sigma UK) in 7.5ml of acrylamide stock (38g acrylamide, 2g bisacrylamide) and 5ml of 10xTBE buffer (1M Tris, 1M boric acid and 20mM EDTA), made up to 50ml with water, and warmed to 42°C. When the urea had dissolved 300µl of freshly-made 10% ammonium persulphate and 50µl of TEMED (NNN'N'-tetramethylethylenediamine) were added and the gel poured. A BRL S2 model gel tank was used to give gel dimensions of 40 x 32 x 0.04cm.

When the gel had polymerised it was pre-run for 1 hour at the running current 35mA to increase the gel temperature to prevent the DNA from renaturing. Prior to loading the DNA samples are heated in a 95°C water bath for 3 mins., and then 2.5µl were loaded into the appropriate lane. The gel was run at 35mA until the dye front had reached the bottom edge.

The plates were then carefully separated and the gel immersed in a 10% acetic acid, 10% methanol solution for

15 mins. to remove the urea, then the gel was transferred to a sheet of Whatman 3MM filter paper and dried, at 80°C for 1 hour. The dried gel was then exposed to X-ray film (RX film, Fuji Photo Film Co. Japan) for 24 hours at -70°C.

Chapter 3:1.

Proliferative responses to insulin in different inbred mouse strains.

Previous reports have shown that inbred MHC congenic mouse strains have different response patterns to insulin. To confirm that pattern H-2^b (B10ScSn) mice were primed with BI and after eight days the lymph nodes were removed and tested in a proliferative assay with response being measured by labelled thymidine incorporation. The cells responded to the priming antigen BI, but not to PI or HI (Table 3). In contrast, cells from H-2^d mice primed with PI responded to PI, and also BI and HI (Table 3). F1 mice (b x d) primed with PI responded to both PI and BI (Table 3). When tested H-2^b mice never responded to PI (data not shown).

The results confirm previous studies, and show that H-2^b mice are non-responders to PI. An important region of the insulin molecule involved in T cell activation in these mice must be the A-chain loop, since this is the only difference in primary amino acid sequence between PI, and BI which does elicit a response in H-2^b mice.

H-2^d mice responded to all three insulin variants tested and therefore must share a common epitope. This epitope cannot, therefore include the A-chain loop region, where BI differs from both PI and HI, nor the C terminal amino acid on the B chain as HI differs from

both BI and PI.

Responder x non-responder F1 mice recognise both BI and PI. In these animals the response to PI is inherited as a dominant trait, and is presumably restricted by the parental $H\text{-}2^d$ molecules, or perhaps a hybrid d/b MHC molecule. Dominant responsiveness is characteristic of Ir gene phenomena and also argues against suppression being involved in the non-responsiveness to PI in $H\text{-}2^b$ mice, since suppressor effects are generally inherited as a dominant non-responder phenotype. The data confirms previous studies of the response patterns to insulin variants in inbred mouse strains.

The term primary assay used in the following text and tables denotes an assay on cells immediately after their removal from the lymph nodes of primed mice, as opposed to cells from lines or clones.

Unless otherwise stated, cells were used at 5×10^5/well in primary assays, while cell lines were tested at 5×10^4/well, with sygeneic irradiated spleen cells at 5×10^5 as feeder cells.

Table 3.

H-2 ^{b} mice primed to BI.

	Antigen Concentration. (µg\ml)		
	100	10	1
BI	107.8 ± 6.4	24.0 ± 2.4	12.0 ± 1.2
PI	18.4 ± 1.6	11.2 ± 0.7	8.9 ± 0.7
MED	8.0 ± 0.4		

H-2^{d} mice primed with PI.

	Antigen Concentration (µg\ml)		
	100	10	1
BI	18.4 ± 1.2	14.2 ± 5.4	9.4 ± 1.7
PI	23.3 ± 1.1	17.2 ± 1.6	9.6 ± 2.5
HI	19.1 ± 4.2	17.1 ± 2.3	15.4 ± 1.7
Medium	5.4 ± 1.8		

Proliferative response of F1 (b x d) mice primed to PI to haptenated and unhaptenated insulins.

	Antigen Concentration (µg\ml)		
	100	10	1
BI	19.9 ± 0.5	10.3 ± 1.2	7.9 ± 1.0
PI	18.2 ± 2.7	11.8 ± 3.1	6.5 ± 1.4
Medium	3.9 ± 0.5		

All results cpm x 10^{3}.

Chapter 3:2.

TNP-Mycobacteria and TBI can prime a hapten dependent response.

Initially, as stated above, the object was to obtain hapten specific T cells,and modify the carrier protein to determine which sites are involved in T cell activation. Two strategies were employed to obtain a proliferative response to TBI in H-2^b mice. The first was to prime mice to TNP derivatised Mycobacteria tuberculosis, (H37 RA), which had been shown to prime a cross reactive response to a number of haptenated proteins in an analogous system in the guinea pig (Janeway et al, 1975). A similar result was obtained in the mouse (Table 4). These responses were largely hapten specific in the sense that unhaptenated proteins failed to stimulate a response in vitro. In this system, however, proliferation was rather low, and attempts to grow long term lines were unsuccessful.

The second strategy was to prime the animals with haptenated beef insulin (TBI). This gave a very strong proliferative response to TBI, and a weaker but significant response to BI. This could presumably just be carrier priming for a carrier response as TBI was the priming antigen. There was also a strong response to haptenated pork insulin (TPI), but not to PI alone. The initial interpretation of these results was that as hoped TBI primed a mixture of TNP-specific T cells,

which could recognise the TNP moiety on PI or BI, and BI specific cells, which recognise BI and TBI, but not PI or TPI.

It was also shown that mice primed to Mycobacteria alone gave no in vitro proliferation to any of the antigens tested, except PPD, ruling out the possibility of a cross reactive response with the haptenated forms of insulin.

Table 4 TNP-Mycobacteria and TBI prime a hapten dependent proliferative response

In vitro antigen	Concentration	Priming antigen: TNP-Mycobacteria	TBI	Mycobacteria
Medium alone	ug/ml	24.5 ± 1a	11.6 1	13.4 ± 0.8
TBI	100	36.2 ± 2*	206.7 ± 4*	9.0 ± 1
	10	27.0 ± 0.5	54.5 ± 8*	6.1 ± 1
TPI	100	43.1 ± 2*	167.2 ± 9*	19.3 ± 4.7
	10	29.0 ± 2	29.4 ± 2	N.T.
BI	100	26.5 ± 2	29.2 ± 2*	19.4 ± 0.8
	10	23.9 ± 6	12.4 ± 1	N.T.
PI	100	N.T.	9.7 ± 0.7	N.T.
	10	N.T.	14.0 ± 0.5	N.T.
PPD	50	53.5 ± 1*	N.T.	55.3 ± 1.1*

a Results are expressed as mean thymidine incorporated (cpm x 10^3 ± S.E.M.)
Values marked with an asterix are significantly greater than controls ($p < 0.01$)

Chapter 3:3.

Haptenation of pork insulin makes it immunogenic in H-2^b mice.

It was reasoned that, as PI is not recognised in H-2^b mice, priming H-2^b and H-2^d mice with TPI should give a population consisting predominantly of TNP-specific cells which could be readily cloned. In addition it would be possible on such a cell population to determine if there were any putative suppressor epitopes on PI, which could suppress TPI responses by a mechanism of linked suppression. TBI primed H-2^d mice gave good responses to both haptenated and unhaptenated insulins, and a similar pattern was seen when they were primed to TPI (Table 5). In most cases the response to the protein was increased when the hapten was added. This will be discussed further below.

H-2^b mice when primed to TBI responded to TBI, TPI and BI, but not to unhaptenated PI (fig 2, Table 6). When tested TPI primed mice gave an identical response (Table 7). The response to the haptenated proteins was as expected, with a heteroclitic response to TBI. However the unexpected finding was that TPI primed a cross-reactive response to unhaptenated BI.As BI could stimulate *in vitro* proliferation in cells from TPI primed mice both antigens must be recognised via the same epitope, and this cannot be the A-chain loop

region, as the two antigens differ at this site.

The *in vitro* response to BI was shown to be Ir gene restricted as it could be blocked by anti-Ia^b monoclonal antibodies (Table 8). This rules out the possibility of TNP having a non-specific mitogenic effect on the T cell population.

This finding of cross-reactivity between TPI and BI was the first indication that TNP was not acting simply as an epitope, since TNP epitopes were clearly not present on BI.

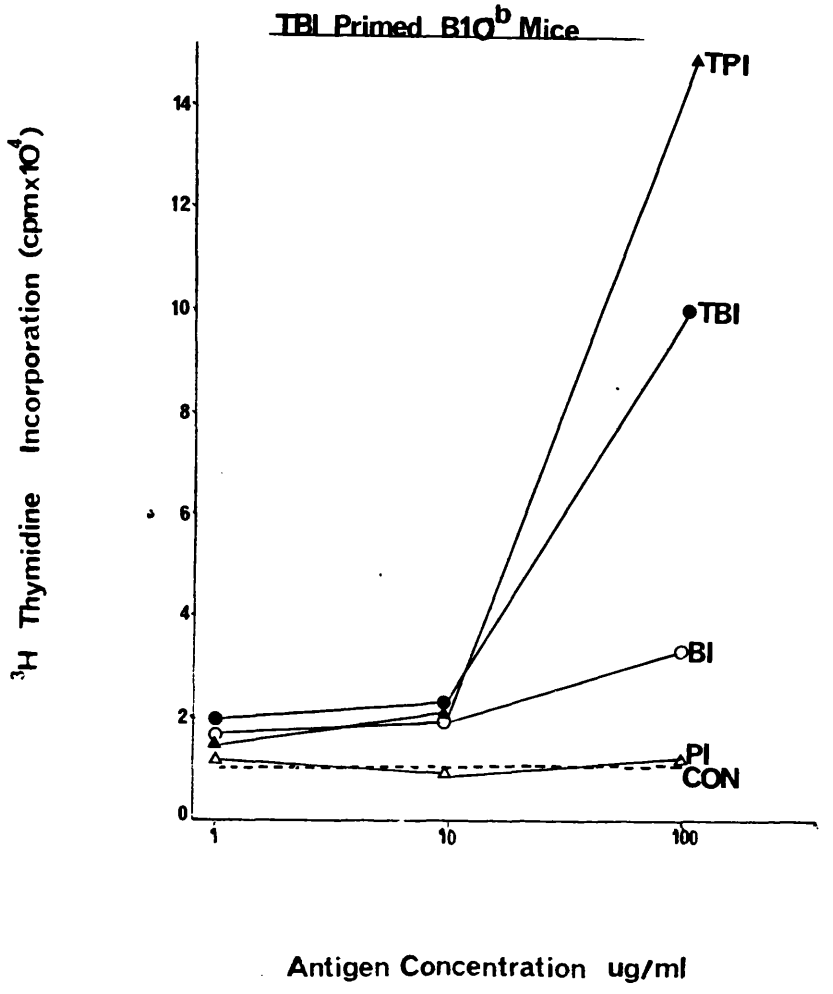

Fig. 2. H-2^{b} mice primed to TBI. A primary assay using normal mouse serum in modified proliferation assay. Con = medium only.

Table 5.

Proliferative responses of H-2^d mice to haptenated insulins.

Mice primed with TBI.

	Antigen Concentration (µg\ml)		
	100	10	1
TBI	52.6 ± 0.8	56.6 ± 8.9	NT
TPI	64.2 ± 10.5	52.5 ± 3.3	NT
BI	52.9 ± 0.5	43.5 ± 12.8	NT
PI	40.3 ± 2.7	46.7 ± 0.2	NT
Medium	19.9 ± 2.2		

Mice primed with TPI.

	Antigen Concentration.		
	100	10	1
TBI	18.5 ± 4.5	11.6 ± 2.3	11.2 ± 1.1
TPI	33.0 ± 8.9	16.7 ± 7.4	16.1 ± 1.3
BI	12.6 ± 1.0	11.6 ± 1.9	10.8 ± 1.4
PI	14.3 ± 2.3	13.0 ± 1.3	12.5 ± 1.2
Medium	7.2 ± 2.1		

All results as cpm x 10^3.

Table 6.

TNP increases the immunogenicity of beef and pork insulin.

Primary assay on H-2^b TBI primed mice.

Experiment 1.

	Antigen Concentration (µg\ml).		
	100	10	1
TBI	53.1 ± 1.2	36.4 ± 2.3	30.0 ± 1.9
TPI	52.5 ± 6.5	21.2 ± 2.7	25.7 ± 1.4
BI	39.4 ± 4.3	27.2 ± 0.4	27.2 ± 3.7
PI	23.8 ± 2.9	21.9 ± 1.7	20.4 ± 4.2
Medium	17.2 ± 2.9		

All results cpm x 10^3.

Experiment 2.

	Antigen Concentration. (100 µg\ml)
TBI	78.5 ± 6.1
TPI	107.0 ± 2.2
BI	37.7 ± 4.8
PI	0.8 ± 0.3
Medium	0.5 ± 0.2

All results cpm x 10^3.

Table 7.

TNP increases the immunogenicity of beef and pork insulins.

Experiment 3.

Primary assay on $H\text{-}2^b$ mice primed with TPI.

	Antigen Concentration. (100 µg\ml)
TBI	68.9 ± 17.3
TPI	58.8 ± 3.5
BI	33.1 ± 6.0
PI	0.9 ± 0.2
Medium	0.8 ± 0.3

All results cpm x 10^3.

Table 8.

TPI specific proliferative response can be inhibited by anti Class II antibodies.

H-2^b mice primed to TPI.

Concentration µg\ml	Antibody. TIB-120	HB38*	H40-481.3
20	27.7	24.7	71.8
10	21.9	31.9	51.9
5	17.9	40.4	48.8
2.5	17.2	40.5	41.7
Med	21.4		
TPI 100 µg\ml	41.8		

All results cpm x 10^3. SE were less than 15% of the means.

Antibodies: TIB-120 (anti Ia monomorphic)

HB38 (IgM anti Ia^b)

H40-481.3 (anti Ia^k)

Lymph node cells were incubated at 2.5 x 10^5/well in the presence of the appropriate antibody and TPI 100 µg\ml.

*HB38 was used as a supernatant and the concentrations given do not apply. The supernatant was used at dilutions 1, 1\2, 1\4, and 1\8.

All antibodies were obtained from ATCC.

Chapter 3:4.

Preparation of T cell lines.

Lymph nodes were collected from both TBI and TPI primed mice and a proportion used for a primary assay. The remainder were cultured in the presence of the appropriate antigen to establish cell lines (see Methods). The lines were assayed at the end of each antigen\IL-2 cycle to determine the response pattern. Both TBI and TPI primed lines could be maintained through several cycles, and it was shown that their antigenic specificity did not vary. PI never stimulated any of the lines when tested, and the cross reactive response to BI in TPI primed lines was stronger than in the primary assays (Tables 9, 10, Fig 3). The results shown are all after at least two cycles of Ag/IL-2 growth.

To examine the phenotype of the cells in the long term lines they were stained with a panel of monoclonal antibodies. A fluoroscein labelled second antibody was added and the cells examined by fluorescent microscopy (Table 11). The great majority of T cells present were shown to be $CD4^+$, with very few $CD8^+$. The few $CD8^+$ present could be due to cytotoxic T cells or to double positive CD^4/CD^8 cells, rather than suppressor cells. This result would argue that the low response PI response in these lines is not due to $CD8^+$ suppressor cells.

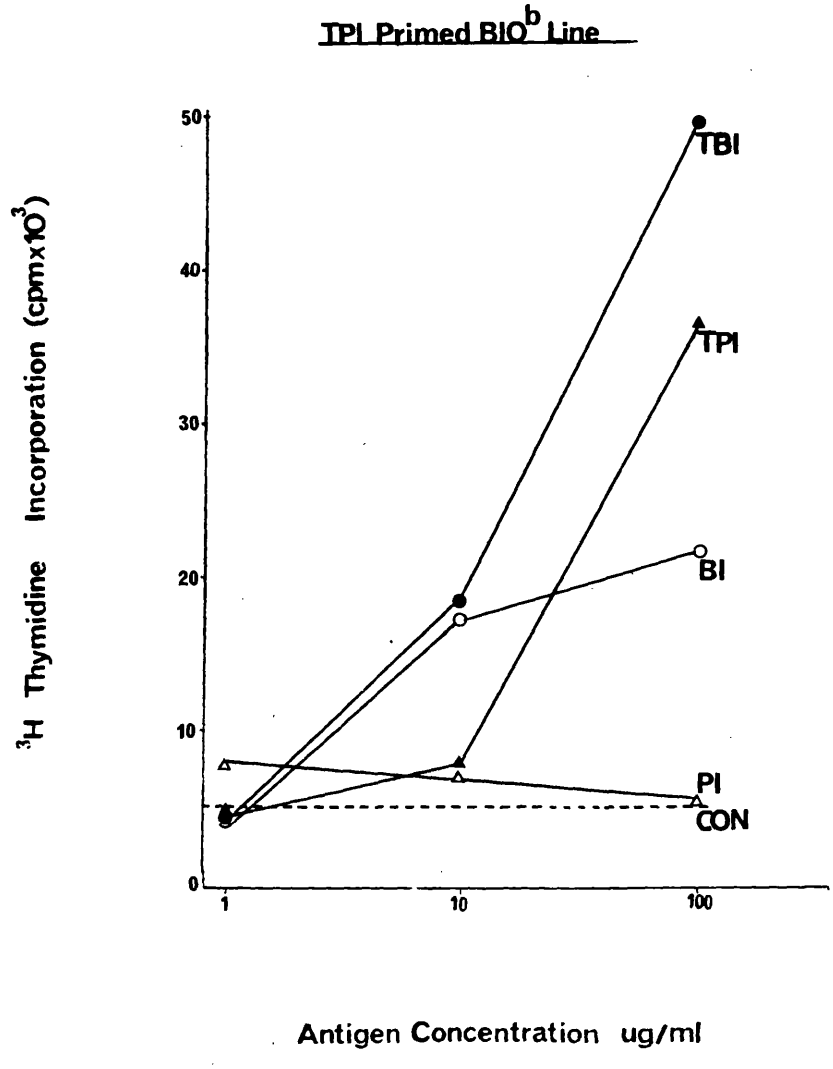

Fig. 3. H-2^{b} TPI primed cell line proliferation assay.

Table 9.

The pattern of proliferative responses to haptenated and unhaptenated insulins was maintained in cell lines derived from H-2^b mice primed with TPI.

Experiment 1.

	Antigen Concentration(μg\ml)		
	100	10	1
TBI	50.1 ± 8.0	18.2 ± 3.7	4.1 ± 0.9
TPI	36.2 ± 8.0	7.0 ± 2.7	4.4 ± 1.1
BI	22.9 ± 2.8	17.0 ± 5.1	4.3 ± 2.9
PI	7.6 ± 1.1	6.0 ± 2.0	3.4 ± 0.7
Medium	5.0 ± 2.1		

Experiment 2.

	Antigen Concentration (100 μg\ml)
TBI	5.0 ± 0.1
TPI	3.8 ± 0.1
BI	5.0 ± 0.7
PI	0.3 ± 0.02
Medium	1.0 ± 0.7

All results cpm x 10^3.

Experiment 2 - means and SE based on duplicate results.

Table 10.

The pattern of proliferative response to haptenated and unhaptenated insulins was maintained in cell lines derived from H-2^b mice primed with TBI.

	Antigen Concentration (µg\ml)		
	100	10	1
TBI	11.0 ± 0.2	8.0 ± 1.2	6.4 ± 0.6
TPI	6.7 ± 0.7	5.8 ± 0.02	5.5 ± 0.03
BI	5.3 ± 0.5	7.1 ± 1.5	6.1 ± 0.04
PI	2.8 ± 0	NT	NT
HI	1.8 ± 0.5	1.8 ± 0.2	1.6 ± 0
Medium	1.9 ± 0.2		

All results cpm x 10^3

Table 11.

Analysis of the phenotype of cells in TBI and TPI lines.

	% + ve	
Antibody	TBI	TPI
1.	86.5	76.0
2.	4.0	2.0
3.	70.5	71.0
4.	1.0	NT

Antibodies: 1. HO13.4 - anti Thy 1.2

2. 3.168 - anti CD8

3. H 129 - anti CD4

4. Control - no first antibody.

Cells at 3 x 10^5 were incubated with the first antibody for 30 mins, then washed twice in medium (serum-free).

Second layer - biotin anti rat for 30 mins., then wash twice in medium (serum-free)

Third layer - FITC- anti biotin for 30 mins. Wash twice then cells were counted by fluorescent microscopy.

200 cells from each group were counted.

Chapter 3:5.

Preparation of T cell clones from TBI and TPI lines.

T cell clones were produced in order to study the heterogeneity of the cell lines, and at a later date, for testing of insulin analogues obtained by mutagenesis. Cells were incubated with feeder cells and plated out by limiting dilution in Terasaki plates. After eight days cells from positive wells were collected and expanded.

An example of a clone obtained from a TBI primed line is given in Table 12. It shows a dose dependent response to TBI, and was also stimulated by TPI and BI at the highest concentration.

A panel of clones was derived from a TPI primed line (Table 13). Half of those tested, 3,6,7,9 and 10 gave the same pattern of reactivity as the primary assays and the lines, with no response to PI, but a cross reactive response to BI. The other clones also demonstrated some interesting results. Clones 1 and 2 gave a good example of the "autoreactive response" seen in some lines The cells respond not to antigen but to the syngeneic spleen cells used as feeder cells. Other clones, i.e. 8, appear to be TNP specific in that they do not respond to the unhaptenated insulins.

TBI and TPI primed H-2^{b+} T cell lines always gave a good response to TPI, but never to PI. The fact that no clon by the same cells.

There are several potential explanations for these

findings. One possibility is that the modification of PI by TNP induces a conformational change in the carrier molecule that mimics BI and increases affinity with either MHC or the T cell receptor.

A second mechanism is that the TNP moiety could interact with molecules on the surface of APC's in some non-specifi by the same cells.

There are several potential explanations for these findings. One possibility is that the modification of PI by TNP induces a conformational change in the carrier molecule that mimics BI and increases affinity with either MHC or the T cell receptor.

A second mechanism is that the TNP moiety could interact with molecules on the surface of APC's in some non-specific way, thereby allowing presentation of the antigen to the T cells.

A third possibility is that the TNP group is binding to the MHC antigen to form a new agretope allowing the presentation of a epitope common to both BI and PI to be recognised.

These possible mechanisms will be discussed in the following chapters.

Table 12.

T cell clone derived from a TBI line.

	Antigen Concentration (µg\ml)		
	100	10	1
TBI	13.8	6.7	1.8
TPI	9.7	0.7	0.7
BI	2.1	0.7	0.7
PI	0.4	0.8	0.7
Med	0.3		

T cell clones were set up at 1×10^4 cells/well, with syngeneic feeders cells at 5×10^5/well.

All results cpm x 10^3.

Table 13.

T cell clones derived from a TPI line.

Clone No	Antigen (100 µg\ml) TBI	TPI	BI	PI	Med
1.	5936	5827	6562	1432	4756
2.	5065	2900	5761	4187	4815
3.	4102	4508	3366	541	1048
4.	998	887	2245	447	531
5.	2877	1409	1749	1283	345
6.	3139	6361	2316	452	330
7.	11072	13150	1332	482	484
8.	7200	4728	1413	2575	2337
9.	6898	10827	2096	685	706
10.	11422	17725	5066	3776	3813

T cell clones were set up at 1 x 10^4\ well, with antigen at 100µg\ml and syngeneic feeder cells at 5 x10^5\well. Data has been corrected for SE.

Chapter 3:6.

Proliferative responses to TNP on other proteins and different haptens on insulin.

To confirm that a large proportion of the cells in these T cell lines were unable to recognise TNP independently of the carrier molecule, a set of proteins were haptenated with TNP (Table 14). None of the three proteins tested gave a response in either the haptenated or unhaptenated form. This demonstrated that the TNP group alone was not being recognised by the T cell lines.

In a converse experiment insulin was modified by different haptens (Table 15). Oxazalone and fluoroscein both augmented the BI response though not to the same degree as TNP. In contrast, while oxazalone-PI gave a good response, fluoroscein-PI was a poor stimulator by comparison.

These results also provide evidence that the T cell receptor is not recognising the TNP group. Modification by either TNP or oxazalone appears to convert PI from a non-responder to a responder by a mechanism that does not involve the T cell receptor directly as two different haptens could give the same effect. It must also be considered that the same side chains may be presented to the T cell on both oxazalone and TNP. The finding that fluoroscein-PI was non-stimulatory could be explained by the difference in size of the three haptens (Fig 4). Fluoroscein is a much larger group than either

oxazalone or TNP and therefore if the hapten group is involved in MHC binding fluoroscein may be too large to achieve this. As BI has, a natural agretope for H-2^b, haptens in this system may not be directly involved in antigen\MHC interactions. They may help to stabilise this binding, but in this case the size of the hapten is not as important .

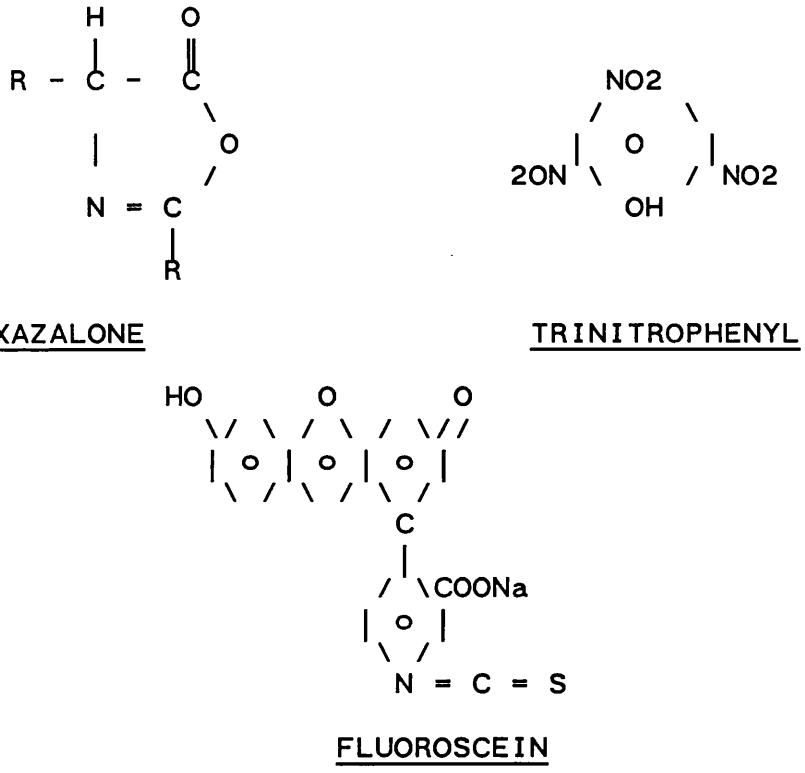

Fig 4 : Structures of the haptens used in this study.

Table 14.

Response of a TBI line to TNP bound to other proteins.

	Antigen Concentration. (µg\ml)		
	100	10	1
TBI	21.4 ± 1.5	6.3 ± 1.2	1.65 ± 0
TPI	24.5 ± 2.9	3.4 ± 0.6	2.2 ± 0.02
TBSA	1.9 ± 0.5	1.7 ± 0.2	1.7 ± 0.01
TOVA	2.3 ± 0.9	1.6 ± 0.5	1.1 ± 0.01
TKLH	2.6 ± 0.5	1.6 ± 0.4	1.7 ± 0.2
BI	5.6 ± 0.2	3.9 ± 3.6	2.7 ± 1.2
PI	0.7 ± 0.2	1.1 ± 0.7	1.4 ± 0.1
OVA	1.2 ± 0.3	1.0 ± 0.6	1.4 ± 0.2
KLH	0.9 ± 0.02	0.8 ± 0.2	1.2 ± 0.4
Medium	0.9 ± 0.08		

All results as cpm x 10^3.

Table 15.

The effect of different haptens on beef and pork insulins.

	Antigen Concentration. (100 µg\ml)
TBI	71.6
TPI	43.4
BI	35.3
PI	0.5
BI - ox	45.0
BI - fl	54.7
PI - ox	25.0
PI - fl	7.1
Med	0.8

ox = oxazalone fl = fluoroscein

Chapter 3:7. The role of suppressor cells in PI non-responsiveness.

As discussed in the introduction, non-responsiveness to PI in H-2^b mice has been ascribed to suppression. This hypothesis holds that I-A^b MHC antigen in complex with PI preferentially stimulates a population of suppressor inducer cell, which in turn activate a $CD8^+$ subpopulation of suppressor cells.

To investigate this mechanism H-2^b mice were primed to TPI, and analysed *in vitro* against a panel of monoclonal antibodies and complement (Table 16). Anti-Thy 1 completely abolished the proliferative response, while anti-CD4 antibodies partially inhibited the response as expected. However anti-CD8 antibody never enhanced the proliferative response and usually inhibited it. A possible explanation of this phenomenon may be that *in vivo* activated $CD8^+$ cells are depleted by bystander killing, or that the $CD8^+$ cells were proliferating and the antibody/complement treatment inhibited this response. However the data in Table 11 shows only a small number of $CD8^+$ cells are present in the cell lines and this would argue against the second possibility.

These experiments have been repeated by two students in the lab., as part of their BSc projects, and the same results were obtained.

In addition the cell lines and clones described earlier maintained the pattern of non-responsiveness to PI under conditions where no evidence for $CD8^+$ suppressor cells could be found.

This provides strong evidence that suppression is not a major regulatory mechanism involved in proliferative non-responsiveness to PI, though studies on antibody production have shown a different picture (Flory, Wallace, Oettel and Chain, 1989).

Table 16.

Analysis of phenotype of the cells proliferating to haptenated and unhaptenated insulins.

Antigen µg\ml		Antibody Med	αCD8	αCD4	αThy 1
TBI	1	5.1	2.4	1.9	1.9
	10	13.5	5.1	5.8	0.2
	100	33.4	19.9	14.6	2.6
TPI	1	3.7	2.2	4.3	0.7
	10	8.4	2.8	3.7	1.6
	100	40.6	29.6	16.3	1.4
BI	1	3.8	1.4	3.0	1.6
	10	4.0	1.8	4.4	1.4
	100	6.6	2.4	4.2	2.7
PI	1	2.0	1.3	3.6	1.5
	10	3.9	3.3	4.8	0.8
	100	5.9	3.2	6.5	2.6
	MED	4.0	2.1	3.9	0.3

All results cpm x 10^3. SE were within 15% of the means.

Antibodies are listed in the Methods section.

T cells were enriched on nylon wool and 1 x 10^7 cells incubated in 10-25 µg of antibody for 45 mins. at 4°C. Cells were then pelleted by centrifugation and resuspended in a 1\10 dilution of rabbit complement (Buxted UK.) for 30 mins at 37c. A final concentration 5 x 10^4 cells\well was used

Chapter 3:8.

Different sites of haptenation affect the immunogenicity of TPI.

To answer the question of whether the effect of modification of PI, was specific or due to non-specific " stickiness " to antigen presenting cell surface it was necessary to determine the site of haptenation. In addition, previous reports have shown the that TNP group preferentially binds to B_{29} lysine (Li, 1956). If this was the same for our haptenated insulins it would be difficult to envisage a mechanism by which haptenation on that site could affect the A-chain loop region.

The question was answered by the use of reversed phase high pressure liquid chromatography (see Appendix 2). It was shown that our method of haptenation preferentially linked the TNP group to the glycine at A_1, not B_{29} lysine. The important factor was the pH at which the reaction was carried out. The initial experiments by Li were at pH 11, whereas we haptenated at pH 8.4.

Both haptenated forms were prepared and tested in vitro on H-2^b mice primed to BI (Table 17). The results show that for TBI, and particularly TPI, the A_1 Haptenated forms are more potent stimulators than the B_{29} forms. The effect of haptenation therefore depends on the site at which the TNP molecule is attached and is not a random event due to non-specific binding to cell surface receptors.

Table 17.

Different sites of haptenation affect the immunogenicity of TPI.

Experiment 1. H-2^b mice primed with BI.

	Antigen Concentration (μg\ml)		
	100	10	1
TBI mix	108.8 ± 9.0	92.9 ± 9.8	27.8 ± 10.6
TBI-A1	102.2 ± 1.2	59.1 ± 8.2	17.4 ± 3.8
TBI-B29	79.1 ± 7.5	39.6 ± 1.7	14.6 ± 3.9
TPI mix	81.3 ± 7.6	17.7 ± 10.1	6.0 ± 0.9
TPI-A1	78.1 ± 5.4	34.9 ± 1.7	10.6 ± 2.1
TPI-B29	28.8 ± 5.7	7.0 ± 0.2	6.7 ± 0.4
Medium	6.4 ± 3.8		

Experiment 2. H-2^b mice primed to BI.

	Antigen Concentration (μg\ml).		
	100	10	1
TPI-A1	73.6 ± 2.5	45.8 ± 2.8	28.0 ± 2.2
TPI-B29	48.3 ± 3.1	20.2 ± 1.2	15.4 ± 0.6
BI	39.2 ± 1.6	38.3 ± 0.6	23.2 ± 0.8
PI	19.8 ± 2.8	16.1 ± 1.1	15.1 ± 0.6
Medium	14.0 ± 1.9		

TPI-A1 was prepared as in the methods section in 0.1 M sodium bicarbonate pH 8.4.
TPI-B29 was prepared in 0.1 M sodium carbonate pH 11.
The isomers were separated by reverse phase HPLC.
The mix sample is also prepared at pH 8, but not run on HPLC and will contain a small amount of B 29 isomer.

Chapter 3:9.

Proliferative responses to desoctapeptide insulin.

Desoctapeptide insulin (DOP) is prepared by treatment with trypsin which cleaves the last eight amino acids from the B chain. It is known that this region is involved in dimer formation and in binding to the insulin receptor (Pullen, 1976).

When tested F1 (b x d) mice primed to PI, gave a very strong dose-dependent response to DOP. The stimulation was also shown to be a conventional MHC class II restricted response by inhibition with a monoclonal anti-Ia antibody (Table 18).

The data clearly demonstrates that the eight carboxy terminal amino acids on the B chain are not required for T cell activation in H-2^d mice.

One possible explaination for the initial strong response to DOP may be that without those eight amino acids dimer formation is virtually abolished leaving a large remainder of the B chain visible. Native insulins would, at the conditions of the assay, be able to form dimers which could shield potential T cell epitopes.

Table 18.

The effect of removal of the eight carboxy terminal amino acids on the B chain (desoctapeptide DOP) of insulin on PI primed F1 (b x d) mice.

Experiment 1.

	Antigen Concentration (µg\ml)		
	100	10	1
BI	18.2 ± 0.3	11.5 ± 1.2	5.5 ± 1.4
DOP	111.3 ± 6.5	33.2 ± 1.6	4.3 ± 1.9
Medium	4.5 ± 0.8		

Primary assay in NMS.
All cpm x 10^3.

Experiment 2. -the above cells were grown as a line and tested on BI and DOP with and without α-Ia.

	Antigen Concentration (µg\ml)		
	100	10	1
BI	39.3 ± 3.1	31.9 ± 4.2	29.4 ± 2.5
BI + αIa	5.0 ± 0.8	3.6 ± 0.4	3.5 ± 0.4
DOP	29.1 ± 1.5	27.8 ± 7.2	26.5 ± 3.5
DOP + αIa	5.2 ± 0.3	3.1 ± 0.2	3.3 ± 0.7
Medium	3.0 ± 0.06		

All results cpm x 10^3.

α Ia was TIB - 120 at concentration 1 : 100.

Chapter 3:10.

Proliferative responses to haptenated and unhaptenated single chains of insulin.

In H-2^d mice it has been reported that the B chain of insulin alone can stimulate T cell proliferative responses. Therefore both the epitope and the agretope must be present on that chain. To test this result H-2^d and H-2^b mice were primed to the single chains of insulin both in haptenated and unhaptenated forms. In all experiments shown the A-chain used was the porcine variant.

H-2^b TNP-B chain primed T cells were shown to be very strongly stimulated *in vitro* by TBI and to a lesser degree by TPI. TNP-A chain primed T cells responded only at the highest concentrations of TBI and TPI. The unhaptenated chains did not appear to prime aresponse in H-2^b mice (Table 19).

H-2^d B chain primed T cells gave good *in vitro* proliferative responses to TBI and TPI, and to BI and PI at the highest concentrations (Table 20) Only TBI and TPI stimulated TNP-B chain primed T cells to a significant degree. The results to *in vitro* stimulation with TNP-A chain suggest that it may be mitogenic as elicits a good response from B chain primed T cells but

not from BI primed T cells.

Taken together these results confirm that H-2^d recognise the B chain alone, and suggest that the epitope and agretope are both present on this chain.

In H-2^b mice, neither of the single chains elicit a response, therefore it would seem that both chains are required. The two most likely explanations are that either the epitope is on one chain and the agretope on the other, or that one or both of the sites are made up of residues from both chains.

The strong responses of TNP-B chain to TBI may be due to the TNP group acting as an agretope, and presenting an epitope on the B chain, or an agretope on the B chain presenting the TNP group. As the response to TPI is much lower the first explanation would be favoured.

Table 19

H-2^b mice primed with single insulin chains

Priming Ag	Ag in vitro ug/ml											
	TBI			TPI			BI			PI		
	100	10	1	100	10	1	100	10	1	100	10	1
TNP-B (3.5)	89.6	74.5	43.7	19.5	10.8	3.5	2.6	2.1	2.0	3.4	2.5	3.9
B (1.8)	10.6	1.9	0.2	2.7	1.7	0.4	0.6	0	0.3	0.8	7.3	0
TNP-A (4.2)	19.6	0.6	3.2	11.2	3.7	1.8	2.1	2.3	0.2	5.1	1.3	1.8
A (0.6)	8.0	1.2	0.4	3.4	2.0	1.1	0.7	0.6	0.8	2.0	1.6	0.9
	A chain			TNP-A			B chain			TNP-B		
	100	10	1	100	10	1	100	10	1	100	10	1
TNP-B	3.4	2.6	1.2	10.5	0.1	0	2.7	0	0	12.3	4.2	0.7
B	0.4	0	0	0	0	0	0	0	0	0.3	0	0
TNP-A	2.9	1.8	0.1	0	0	0	0.7	1.7	0	0	0	0
A	1.7	0.6	1.8	1.5	0	0.3	0.9	0.4	0.5	0.8	0.3	0

All results cpm X 10^3. Means corrected for appropriate medium control. Control in brackets.
SE. within 15% of means.

Table 20

H-2^d mice primed with single insulin chains.

Priming Ag	Ag in vitro ug/ml											
	TBI			TPI			BI			PI		
	100	10	1	100	10	1	100	10	1	100	10	1
TNP-B (3.9)	26.4	6.3	2.0	14.2	1.5	0.9	2.7	0.3	1.1	2.8	0	0.2
B (7.5)	26.2	13.1	9.3	23.1	6.6	3.2	8.3	6.2	4.5	7.3	1.2	1.2
TNP-A (2.4)	18.7	5.7	1.6	12.6	1.1	0.7	1.6	2.3	2.1	1.7	0.5	0
A (5.5)	17.5	2.8	0	16.5	1.5	0.5	1.1	0.5	0	3.5	0	0
	A chain			TNP-A			B chain			TNP-B		
	100	10	1	100	10	1	100	10	1	100	10	1
TNP-B	1.0	1.2	0.1	18.4	3.5	0.8	4.1	1.7	0	5.1	1.3	0.4
B	4.7	0	2.3	18.2	5.2	0.2	3.6	1.4	0.6	3.9	0.4	0
TNP-A	3.4	0.2	0	14.3	1.3	0	1.0	0.3	0	0.6	0.1	0
A	1.9	0.5	0	14.0	2.0	0	0.9	0	0	1.3	0	0

All results cpm X 10^3. Means corrected for appropriate medium control. Control in brackets. SE. within 15% of means.

Chapter 3:11.

Site-directed mutagenesis of human proinsulin.

As stated previously, insulin variants differ by only a few amino acids. To assess the role of these particular residues in T cell activation, hybrid insulins were produced by site-directed mutagenesis.

The expression plasmid plac 289/PI containing the human proinsulin coding sequence fused, through a met codon, to a 5' -sequence of the β-galactosidase gene, was obtained for Dr Steven Cockle, Connaught Research Institute, Ontario, Canada, (Fig 5). The gene product, BHPIP-289/PI (Biosynthetic Human Proinsulin Precursor), comprised a 289-amino acid leader ending in a Met residue, attached to the proinsulin sequence. The composite gene was under the control of a wild-type *lac* promoter which can be repressed by glucose via the cAMP receptor protein (CRP). This is the inhibition of transcription of genes for enzymes needed in catabolism of lactose, and other energy-yielding substrates, when the more efficient energy source glucose is available. cAMP stimulates the initiation of transcription of many operons in bacteria, and although the mechanism is unknown, the presence of glucose causes a decrease in the level of cAMP. Due to this the growth medium specified lacks sugars.

The plasmid does not have a functional *lac* repressor gene, but could be grown in *E.Coli* strains such as JM101 which is an overproducer of the *lac* repressor. The proinsulin could therefore be induced by isopropylthio-

β-galactosidase (IPTG). This operates by interacting with the repessor protein and inhibits its binding to the operator.

An ampicillin resistance gene present in the plasmid provided a positive selection mechanism during growth. The bacteria were grown in medium containing ampicillin and therefore only those containing the plasmid would replicate.

The bacteria were grown in Minimum Garlands Medium and induced with IPTG. The bacteria were disrupted by sonication and cell lysates were run on SDS-PAGE to identify the presence of proinsulin (Fig 6). To confirm that the band was indeed proinsulin, proteins were also transferred by Western blotting to nitrocellulose, and stained with anti-insulin antibody (Fig 7).

Purification of insulin from cell lysates was a long and complex procedure. Previous studies have reported that proteins bound to nitrocellulose are capable of stimulating a proliferative response in primed T cells (Lamb et al, 1987). To test whether this method would work in the proinsulin system antigens were spotted onto discs of nitrocellulose cut with a hole punch, and placed in wells with F1 (b x d) T cells primed to PI (Table 21). Though the results were variable as shown by the high SE. it was demonstrated that primed T cells could be stimulated in this way. The results were better in most cases when 100 µg of protein were added to the

disc, but were still stimulatory at 20 µg. Bacterial cell lysate containing proinsulin was shown to give a positive result, but this could be due to some non-specific proliferation caused by bacterial cell proteins. To confirm that proinsulin could stimulate primed T cells a bacterial cell lysate was separated on a polyacrylamide gel and blotted onto nitrocellulose. The region of the blot containing proinsulin was identified and cut into small strips which were placed in wells of a 96-well plate. Primed T cells were added and a standard proliferation assay performed (Table 22). The results show that proinsulin bound to nitrocellulose can stimulate primed T cells. This method was therefore a possible assay system for any mutated insulins produced by site-directed mutagenesis (see below).

The plasmid was purified, cut by restriction enzyme digest using EcoRI and Bam HI. The fragment incorporating the proinsulin gene was identified on a 2% low melting point agarose gel (Fig 8). The band was cut out from the gel and the DNA purified by phenol/ethanol extraction.The purified fragment was then ligated into the cloning vector M13.

M13 is a filamentous coliphage which contains a circular single-stranded DNA molecule. The filamentous phages only infect strains of bacteria harbouring F pili, although the exact mechanism by which the phage genome gets inside the cell is unknown. Replication of phage DNA does not result in host cell lysis. Rather,

infected cells continue to grow and divide, albeit at a slower rate than uninfected cells, and extrude infective viral particles. The single-stranded phage DNA enters the cell and is converted to the double-stranded replicative form (RF). The RF multiplies rapidly until approximately 100 RF molecules are formed inside the cell. Replication of the RF then becomes asymmetric due to the accumulation of a viral-encoded single-strand specific DNA binding protein. This prevents synthesis of the complimentary strand, and only viral single strands are synthesised. These progeny single strands are released from the cell as filamentous particles.

There are a number of advantages of using single-stranded cloning vectors. The RF form can be purified and manipulated in vitro just like a plasmid. Secondly, both RF and single-stranded DNA will transfect competent E.Coli cells to form either plaques or infected colonies. Thirdly, the size of the phage particle is governed by the size of the viral DNA and therefore there are no packaging constraints (Messing et al, 1981). Finally, with phages it is very easy to determine the orientation of the insert (Barnes et al, 1980).

The proinsulin gene fragment was ligated into M13mp19 which included the correct polylinker to give the corrct orientation. The phage molecule was cut with EcoRI and Bam HI to generate a cloning site in the polylinker region with ends compatible with the insert. The

fragment was ligated into the cut M13, and the ligated phage used to transform competent JM101 cells. Plaques were harvested from an agar plate, and RF DNA was prepared from the cells (Fig 9). Single-stranded DNA was purified from the supernatant and to confirm the presence of the proinsulin gene was sequenced (Figs. 10, 11).

Site- directed mutagenesis was performed on the single-stranded DNA using oligonucleotides containing a single base mismatch. Initially, three mutations were attempted, A_8 threonine to alanine, A_{10} isoleucine to valine, and both changes together which, in effect, alters human insulin to beef insulin at the A chain loop region. The latter mutation was to be used as a positive control in functional studies.

Agar plates containing white (positive) plaques were tested for positive mutations by hybridisation with labelled oligonucleotides (Fig 12). Positives were picked and sequenced. Both the A_8 and the double mutation were obtained (Fig 13), however no plaques containing the A_{10} mutation have yet been identified.

M13 containing the mutated proinsulin gene fragments were grown in competent cells, and the fragment excised with restriction endonucleases. Attempts were made to ligate the insert back into plasmids, in order to produce mutant proinsulins for functional studies. Both pKK and plac 289/PI, with the original proinsulin gene fragment removed, were used but no positive colonies

were found.

Due to a lack of time at the end of my studentship I could not pursue this work further. However it is hoped that it may be continued by another student in the near future.

Table 21.

Proliferative responses of F1 (b x k) PI primed T cells to antigens spotted onto nitrocellulose discs.

	Antigen Concentration. 100	20
TBI	162.3 ± 57.1	44.2 ± 5.2
BI	67.4 ± 4.4	56.1 ± 16.2
PI	53.4 ± 19.5	51.8 ± 12.2
Lysate	75.6 ± 31.7	50.4 ± 20.4
MED z	23.4 ± 4.7	

Cells were added at 5x 10^5\well.

All counts cpm x 10^3.

Discs were added to the wells, then antigen was spotted directly onto them. The plates were incubated in UV light for 30 mins. to sterilise the filters, before the cells were added.

Table 22.

H-2^d TBI primed T cells cultured with human proinsulin on nitrocellulose strips.

	Section Number.							
Band	1.	2.	3.	4.	5.	6.	7.	8.
1.	13.1	12.5	9.3	2.7	4.5	2.6	1.5	1.2
2.	11.7	7.2	5.9	11.9	6.8	11.4	7.0	5.7
3.	3.4	15.3	12.7	12.9	15.3	23.7	33.5	11.2
cells + nitrocellulose	1.2							

The nitrocellulose band containing human proinsulin was first cut lengthwise into three thin bands. Each band was then cut into eight small squares, and each square into a well.

Cells were added at 5 x 10^5, and cultured in a standard proliferation assay.

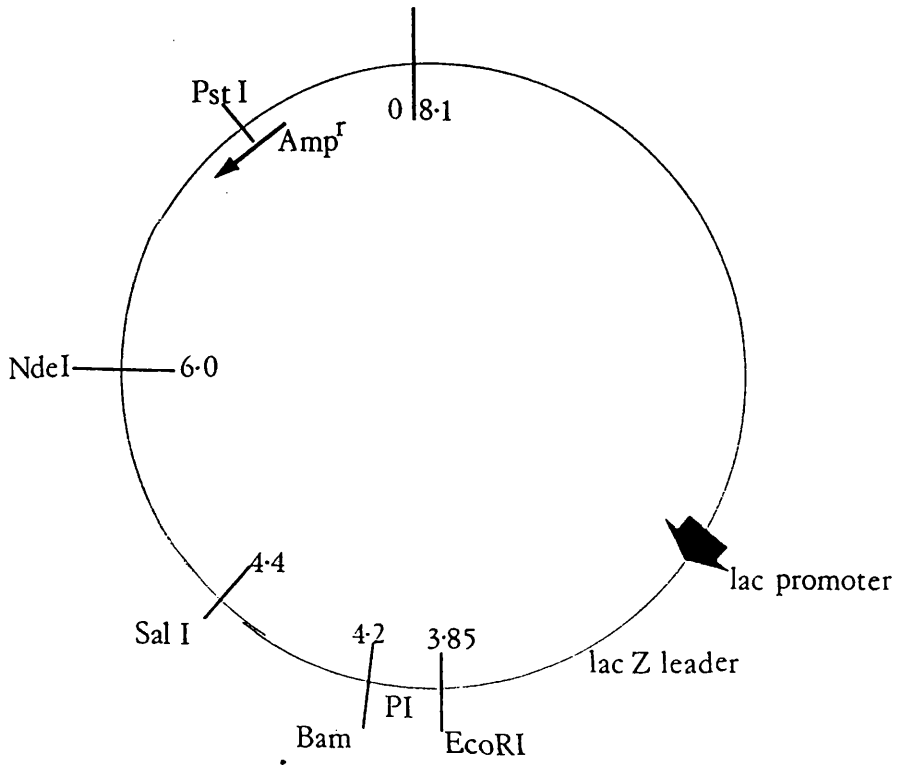

Fig. 5. Restriction map of plasmid plac 289\PI.

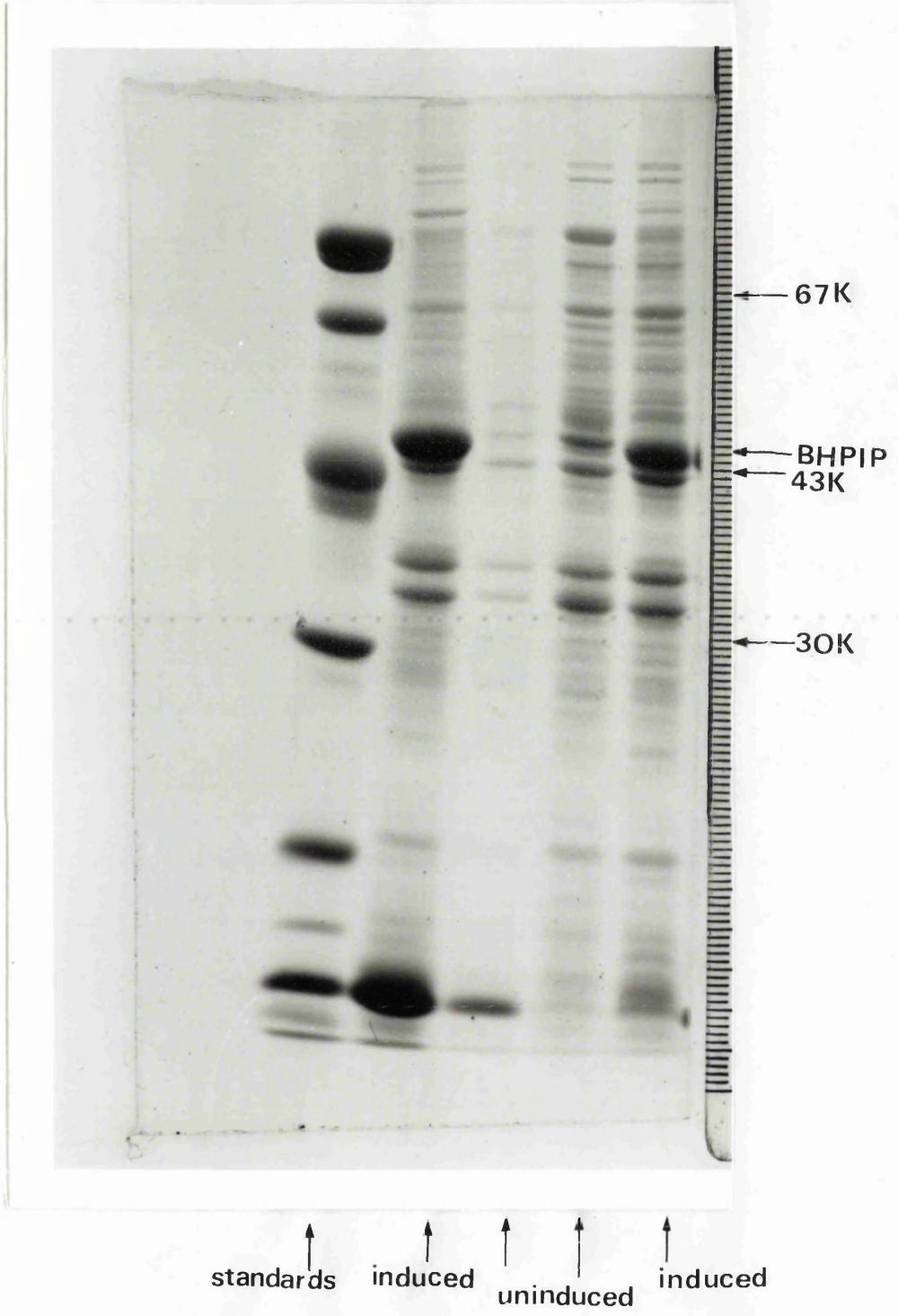

Fig. 6. SDS-PAGE showing induction of BHPIP in JM103 cells.

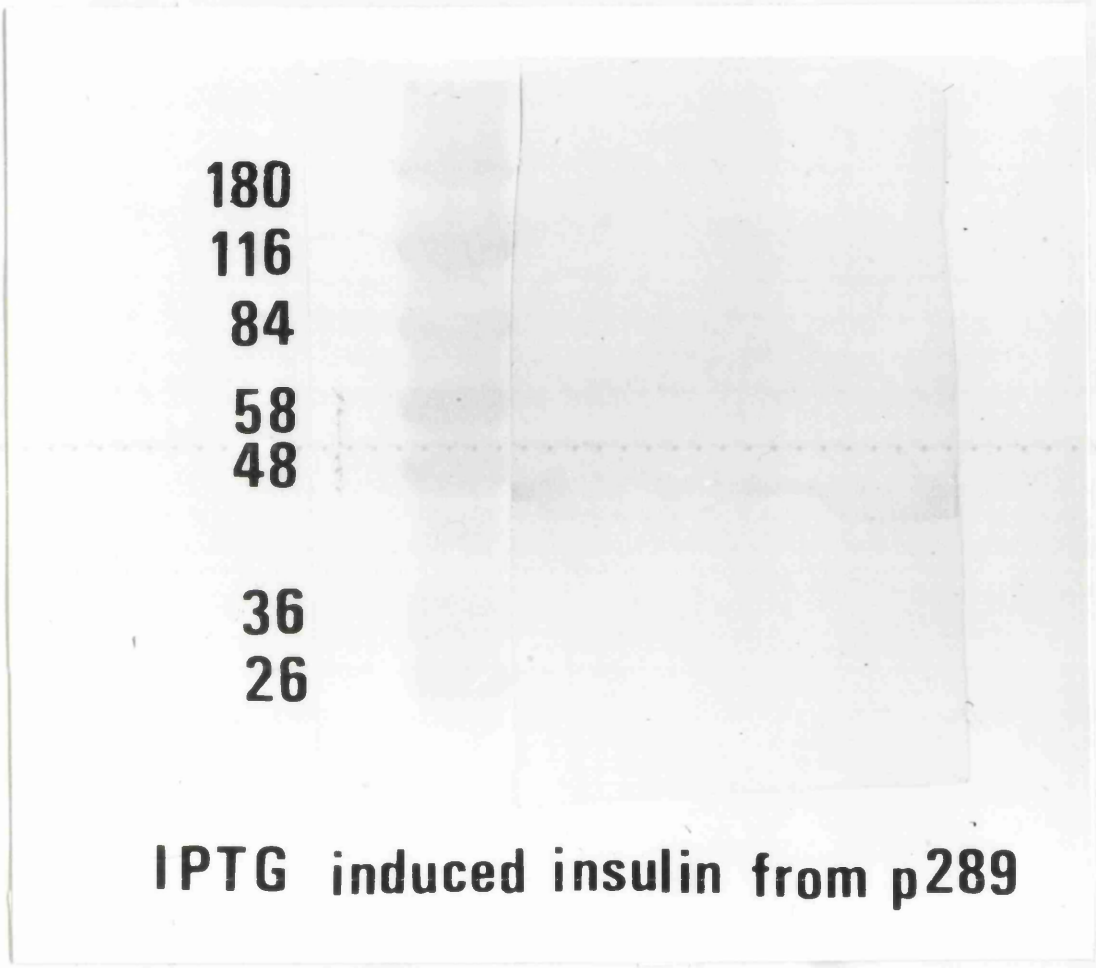

Fig. 7. Western blot showing staining of BHPIP with anti-insulin antibody.

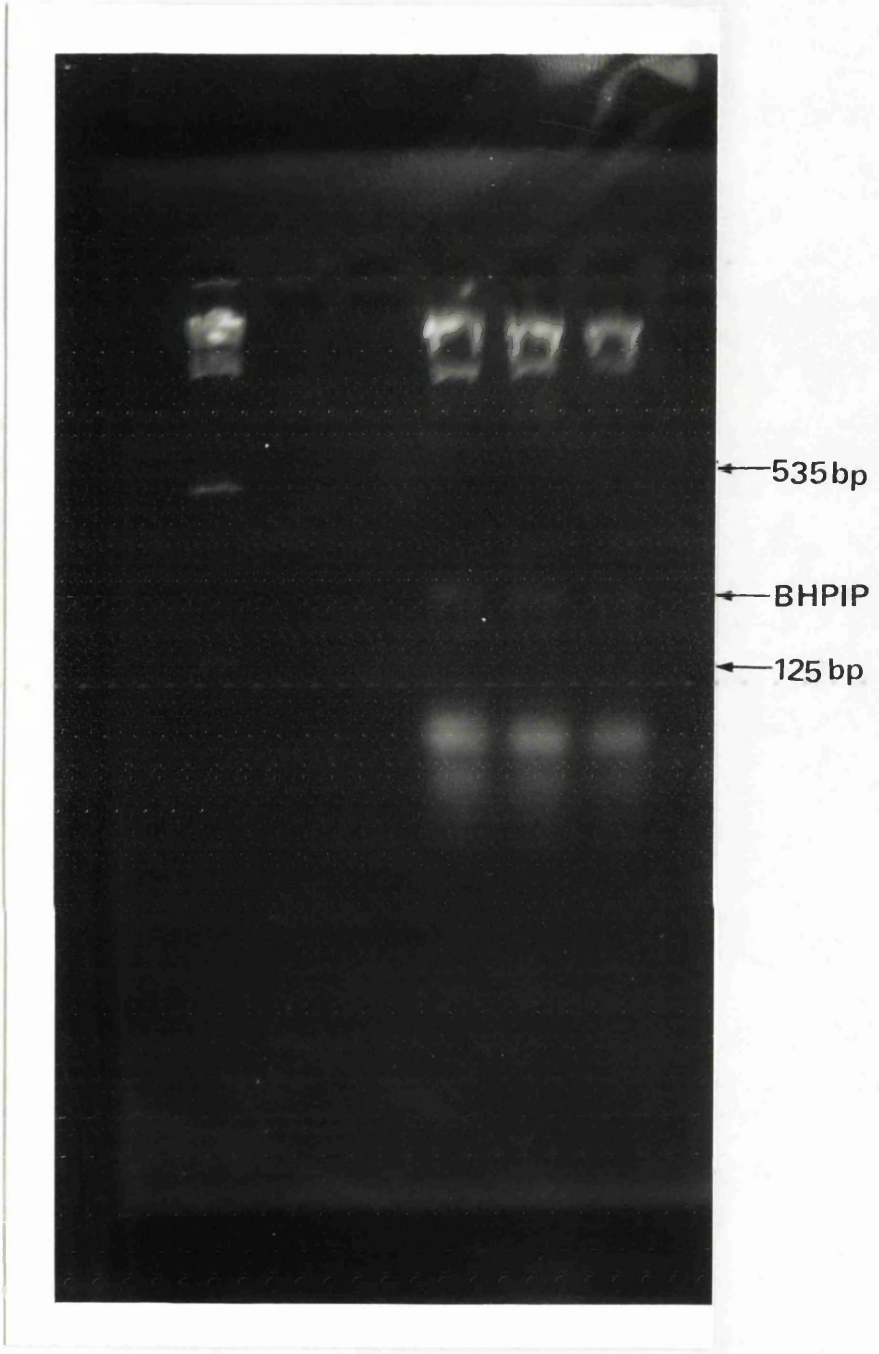

Fig. 8. BHPIP gene fragment separated on 2% agarose gel. Standards are lambda DNA cut with Hind III.

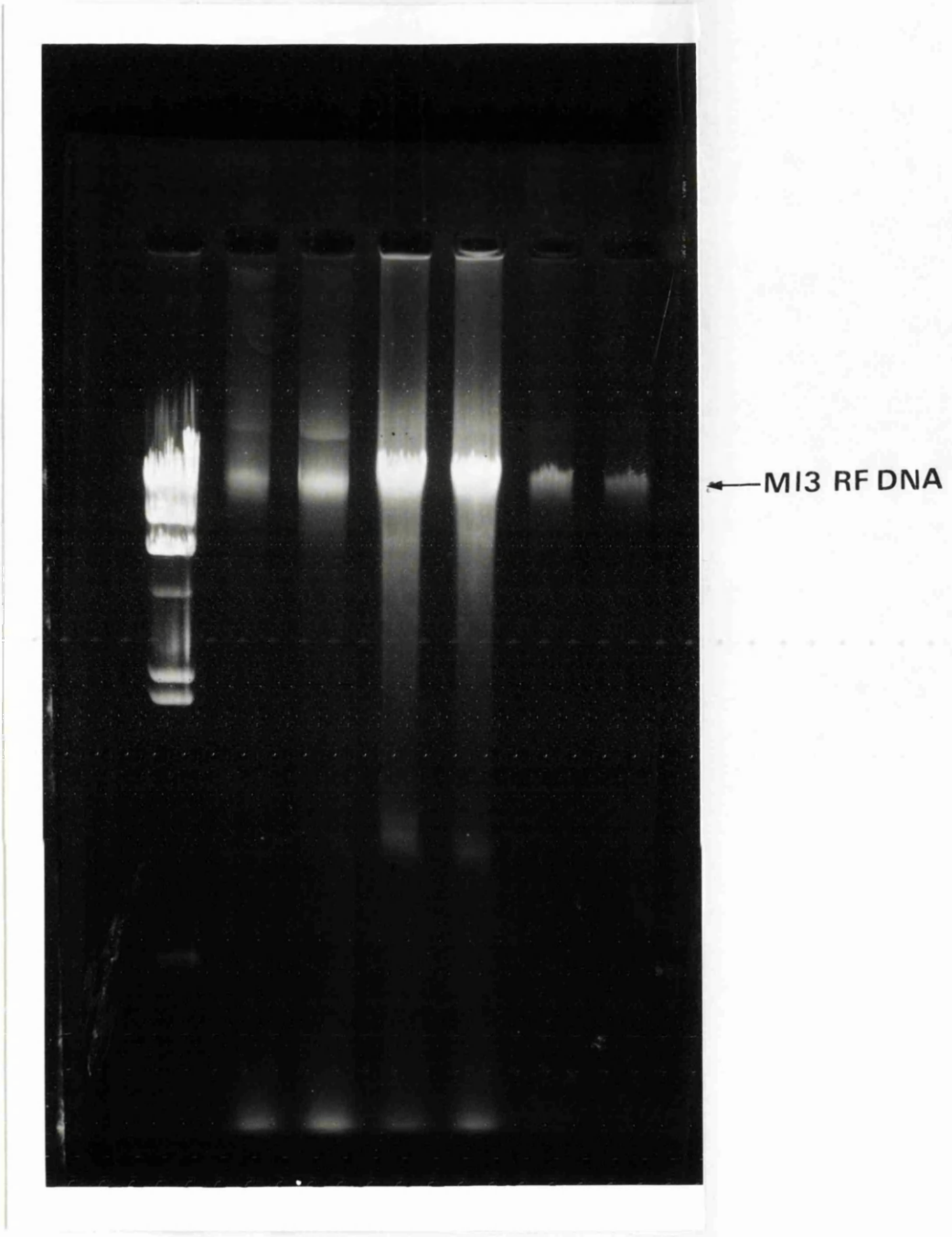

Fig. 9. RF DNA from positive M13 plaques containing the BHPIP gene fragment.

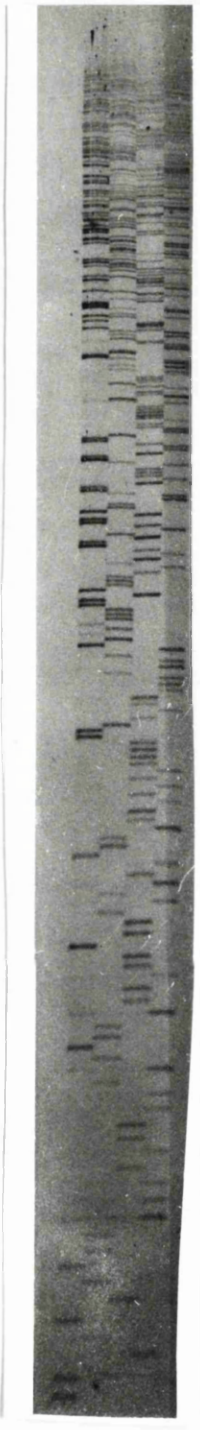

Fig. 10. Autoradiograph of sequencing gel showing human proinsulin.

Fig 11.

Sequence of human proinsulin gene in phage Mins 7

TTT	GTG	AAC	CAG	CAC	CTT	TGT	GGT	TCT	CAC	CTG
Phe	Val	Asn	Gln	His	Leu	Cys	Gly	Ser	His	Leu
GTG	GAG	GCT	CTG	TAC	CTG	GTG	TGT	GGG	GAA	CGT
Val	Glu	Ala	Leu	Tyr	Leu	Val	Cys	Gly	Glu	Arg
GGT	TTC	TTC	TAC	ACA	CCC	AAG	ACC|	CGT	CGT	GAA
Gly	Phe	Phe	Tyr	Thr	Pro	Lys	Thr|	Arg	Arg	Glu
GCT	GAA	GAC	CTT	CAA	GTG	GGT	CAA	GTT	GAA	CTT
Ala	Glu	Asp	Leu	Gln	Val	Gly	Gln	Val	Glu	Leu
GGT	GGG	GGT	CCT	GGT	GCG	GGT	TCT	CTT	CAA	CCT
Gly	Gly	Gly	Pro	Gly	Ala	Gly	Ser	Leu	Gln	Pro
TTG	GCT	CTC	GAG	GGA	TCA	CTT	CAA	AAG	CGT|	GGC
Leu	Ala	Leu	Glu	Gly	Ser	Leu	Gln	Lys	Arg|	Gly
ATT	GTG	GAG	CAG	TGC	TGC	ACC	AGC	ATC	TGC	TCC
Ile	Val	Glu	Gln	Cys	Cys	Thr	Ser	Ile	Cys	Ser
CTC	TAC	CAA	CTG	GAA	AAT	TAC	TGC	ACC		
Leu	Tyr	Gln	Leu	Glu	Asn	Tyr	Cys	Asn		

Reading from the 5 prime to 3 prime from sequencing gel of single stranded DNA from plaque Mins 7, see Fig .

| denotes the three regions of proinsulin in the order B chain : C peptide : A chain.

Fig. 12. Autoradiograph of hybridisation of A_8 mutant with labelled oligonucleotide.

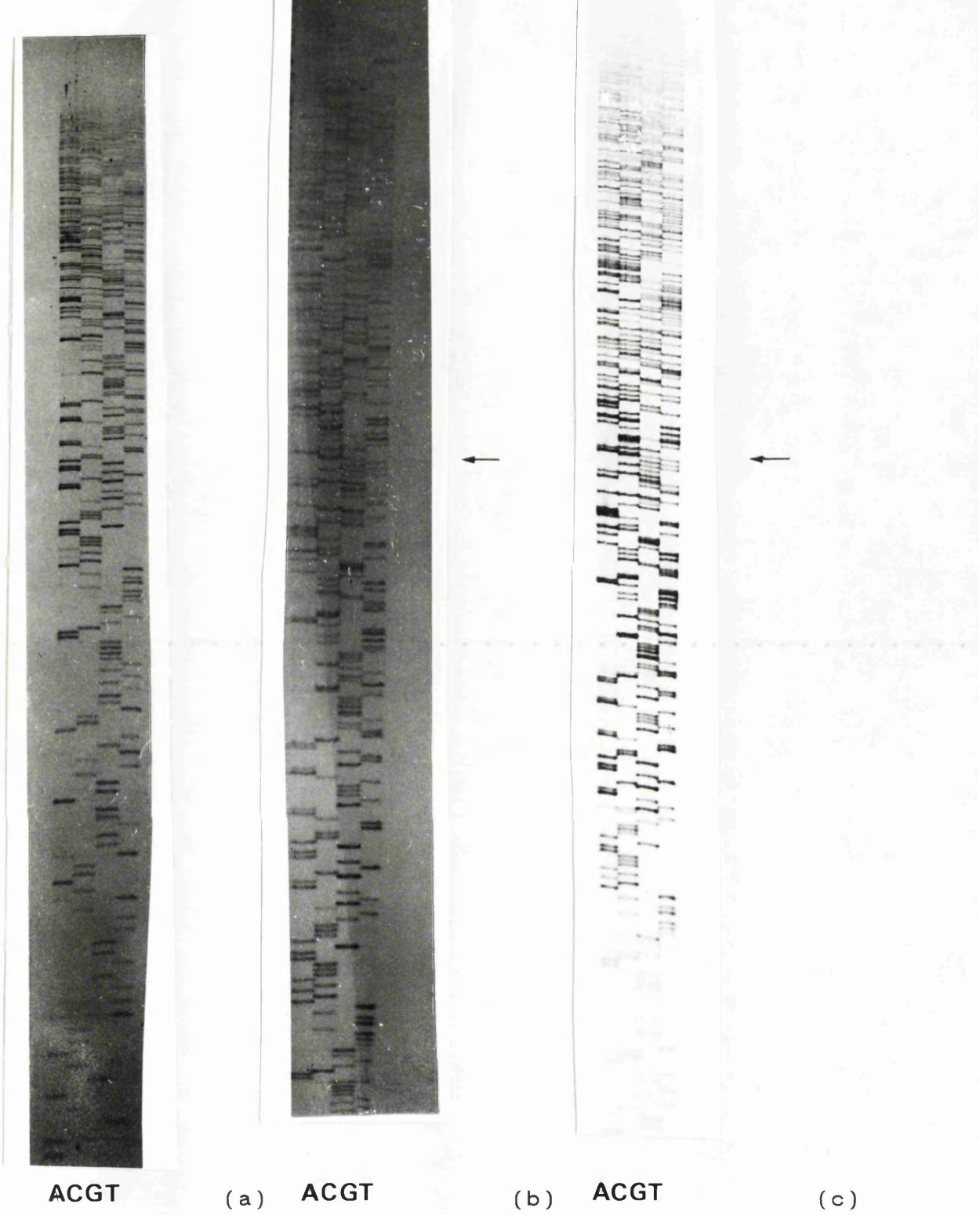

Fig. 13. Autoradiographs showing the (a) the sequence of native insulin (b) the A_8-A_{10} mutant (c) the A_8 mutant. ⟵ = site of mutation.

Chapter 4.

Discussion.

The initial objective of this project was to prepare TNP-specific T cell clones from mice primed to haptenated insulins. However, at an early stage, it was noted that adding a TNP group onto pork insulin made the protein immunogenic in H-2^b mice, while the unhaptenated pork insulin remained non-immunogenic.

There were several possible explanations for this result, and experiments designed to test these mechanisms form the basis of this thesis.

Initially, it was shown H-2^b mice responded to beef insulin but not to pork insulin, as has been reported by other groups (Rosenwasser et al, 1979, Reske-Kunz et al, 1982), (Table 3). However, it was shown that on haptenation with a TNP group pork insulin becomes immunogenic for H-2^b mice (Fig 2, Table 7).

Following this result it was necessary to identify whether the TNP moiety was binding to either the T cell receptor and being recognised by it, or was perhaps involved in binding to the H-2^b MHC molecule. TNP was conjugated to other poteins such as bovine serum albumin and keyhole limpet hemocyanin and these conjugates were tested for their ability to stimulate TNP-insulin primed T cells (Table 14). It was shown that none of these conjugates gave a response much above the relevant unconjugated protein, particularly when compared to the response to TPI. This is strongly suggestive that the

TNP group is not being recognised by the T cell receptor, at least not as a single entity. This would support the results of Clayberger et al, 1983, that the large majority of T cells primed by hapten-carrier antigens are conjugate-specific. The failure of other TNP-protein conjugates to stimulate TNP-insulin primed T cells may be due to the T cell receptor recognising not only the hapten group but also few surrounding amino acids (Janeway et al, 1976a). The other haptenated proteins may not have produced an identical site which could be recognised by T cells primed to TNP-insulin. However that other haptens such as oxazalone, when used to modify pork insulin, could also render it immunogenic in H-2^b mice (Table 15) argues strongly that the hapten group is not involved in a specific epitope recognised by T cells.

A more important finding was that the epitope recognised by TPI primed T cells is also present on BI, as the cross-reactive response between TPI and BI shows (Table 7). This response was maintained in cell lines (Tables 9 & 10, Fig. 3), which were cultured for up to four months at a time, and T cell clones (Table 13). The proliferative response of H-2^b mice to TPI was also shown to be MHC resticted by blocking with anti-I-A^b antibody but not anti- I-A^k antibody (Table 8). These results show that the epitope shared by BI and TPI, and recognised by T cells primed to either antigen, cannot

include the A chain loop region as at this site BI and PI differ (Table 1). Rather it would appear that in H-2^b mice the A chain loop and the TNP group are involved in binding of the antigen to the MHC. The studies on DOP insulin show also that the eight carboxy terminal amino acids on the B chain are not involved in the T cell response to insulin (Table 18).

Numerous studies have demonstrated that most T cell antigen receptors do not recognise the native conformation of protein molecules. Synthetic peptides, cleavage products and denatured proteins are all capable of stimulating T cells in the absence of native conformation (Chesnut et al, 1980, Thomas et al, 1981). Rosenwasser et al, 1979, showed that in H-2^d mice primed with several variants of insulin the B chain alone contains both the epitope and the agretope. Using haptenated and unhaptenated single chains of insulin I was able to confirm this finding in H-2^d mice (Table 20). However, in similar experiments with H-2^b mice unhaptenated single chains did not prime T cells, suggesting that elements from both chains are required to elicit a response (Table 19). However, when TNP-B chain primed mice were stimulated <u>in vitro</u> with TBI and to a lesser extent TPI, a good response was shown (Table 19). This would suggest that the epitope recognised is on the B chain of insulin,and that the TNP is acting as an agretope.

Studies have suggested that a peptide forms an α helix

with one side in contact with an MHC molecule and the other with the TcR (Allen et al, 1987). Insulin includes three helical regions A_2-A_8, A_{13}-A_{20} and B_9-B_{19}. it would therefore be possible for a helical region on the A chain to form one binding site and a second region on the B chain to form the other.

Several physical factors such as the site of linkage, and the chemical bond formed have been shown to affect the immunogenicity of a hapten carrier system. Covalent linkage of the hapten to the protein carrier has been shown to be more effective in stimulating an antibody response to the drug chlorohexidine linked to KLH than a simple electrostatic linkage (Layton G.T. et al, 1987). The immunogenicity of this system was related to the degree of conjugation of hapten to carrier, with the covalent linkage inducing a T cell dependant response while the electrostatic linkage could not (Layton et al, 1986). Reports also show that the site of coupling is important, where the drug amino chloro-quinoline was linked to KLH. Specific antibodies were produced but it was shown that they reacted best to compounds which were substituted on the exocyclic amino group. At this site a bridge was formed between the hapten and the carrier which gave a 1000-fold increase in affinity (Freier et al, 1986).

To determine the site of haptenation in the TNP-insulin system the haptenated antigen was investigated by HPLC

(see Appendix 2). The results clearly showed that insulin was being haptenated on the amino group of glycine at position 1 on the A chain. The possibility existed that the TNP moiety was not acting specifically but that it was increasing uptake of the insulin by non-specific binding to some surface receptor on the APC. To test this insulin was haptenated at different pH, pH 8 which bound the TNP to the A1 site, and pH 11 which bound the TNP to the B29 site (Li, 1956). It was shown that B_{29} TPI was a much less potent antigen to BI primed T cells than was A_1 TPI (Table 17). This implies that the effect of TNP is not a global one on the immunogenicity of the whole molecule, but depends on the precise relative positions of the TNP group and other structures on the carrier.

The possible binding of TNP-insulin to insulin receptors on either T cells or APCs can also be discounted. Unstimulated T lymphocytes do not express detectable levels of insulin receptor on their surface, and only reach measurable levels after stimulation by antigen or mitogen (Helderman et al, 1977, Helderman et al, 1978). In addition insulin was shown to be only a minor growth factor to T lymphocytes, acting in concert with transferrin to stimulate progression of cells stimulated by IL-2 through the G1 and S phases of activation (Snow, 1985). This effect has been classically detected as an augmentation of proliferation in response to mitogen or antigen challenge (Kumagai et

al, 1981, Snow et al, 1981). Insulin receptors are present on cells of the moncyte series, including macrophages, but the presence of insulin has been shown to have no obvious effect on the cellular metabolism (Schwartz et al, 1975). Therefore taken together with the results demonstrating that the response of H-2^b T cells to TPI can be blocked with anti-I-A^b monoclonal antibody (Table 8), and the differential responses to insulin haptenated at different sites it is unlikely that the effect of haptenation is non-specific. Conversely, the TNP group has an important and specific role to play in the trimolecular structure formed by the T cell receptor, TPI and the MHC molecule. There are several possible explanations of the finding that the addition of a TNP group to pork insulin can alter it's immunogenicity in H-2^b mice. Studies by Jensen and Kapp (1984, 1986) have suggested a role for T suppressor cells in non-responsiveness of PI in H-2^b mice. They found that selective irradiation of PI primed cell lines elicited good anti-PI antibody responses in secondary <u>in vitro</u> assays. The possibility existed therefore that the TNP group may have been masking a suppressor cell epitope on PI, thereby freeing the helper response from restraint. However in our system no evidence for suppression was found. When the phenotype of the cell lines was tested $CD8^+$ cells were virtually absent (Table 11). In addition, treatment of primed

cells with anti CD8 antibody did not enhance the PI response, which would be expected if suppression was involved (Table 16). Taken together with the fact that PI stimulated lines always failed to grow and that TPI and BI stimulated lines did not respond to PI in the absence of $CD8^+$ cells, suppresion was not a major factor in the response to haptenated PI.

A second possible explanation is that TNP could be modifying the conformation of PI in such a way that it mimics BI at the A chain loop region, and is therefore recognised. It has been shown that modifying groups linked to A1 can alter the helical region A2-A8, however this has a relatively minimal effect on the A-chain loop region itself (Chothia et al, 1983). It has the effect of elongating the helical structure, but the movement demonstated was insufficient to allow PI to mimic BI at this region. The A-chain loop region is held in a semi-rigid structure due to the close proximity of cysteine residues which form the disulphide bonds. The possiblity that the TNP group bound to A_1 glycine could physically interact with the A-chain loop can be discounted due to the distance involved (Dr S.P. Wood, personal communication). The question of the physical effect of haptenating insulin can only be fully answered by crystallising the structure, but in the light of present knowledge this explanation would seem unlikely.

A third possibility for the affect of haptenation of PI is that the TNP group is not directly involved in the

response but that it may affect the processing of the antigen (Shastri N. et al, 1984, Buchmuller Y. et al, 1982). This was demonstrated in studies on hen egg lysozyme specific T cell clones which were found to be more sensitive to an antigenic analogue, pheasant lysoyme, in proliferation assays. When the clones were stimulated by cyanogen bromide-cleaved peptides from the antigens known to contain determinants recognised by the clones, the heteroclitic response was lost and the clones responded equally to both peptides. The authors suggested that amino acids differences between the two analogues, but distant to the T cell epitopes recognised by the T cell clones, could have an affect on differential processing of the relevant antigenic peptides (Shastri et al, 1984). While this remains a possible explaination for the data presented in this thesis it would appear that the site of haptenation (A_1) is very close to the determinant sites (A_8-A_{10}), and therefore is more likely to be involved in the response than in any alteration to processing of the antigen. The question of processing of insulin will be discussed later.

The explantion for the above data that I favour is that the TNP molecule is enhancing the interaction between PI and MHC antigens. Increased binding to the T cell receptor can be discounted by the result that other haptens, such as oxazalone, could also alter the

immunogenicity of PI. In addition TPI primed H-2^b T cells respond to BI in it's unhaptenated form and this would argue against a role for TNP binding to the T cell receptor. An enhanced interaction with MHC allows the presentation of an epitope, common to both BI and PI, to be presented to the T cell receptor in the correct orientation. The finding that haptenic groups can act as agretopes have been reported by other groups. Using the simple immunogen L-tyrosine azobenzenearsonate and a series of analogues it was shown that the epitope included the arsonate group and elements in the side chain of tyrosine. The agretope was defined as being the planar structure of the azo-linked aromatic rings (Godfrey et al, 1984, Morita et al, 1986). In addition, the linking of photoreactive moieties to immunogenic peptides of hen egg lysozyme was shown to increase the binding affinity of the peptides to Class II molecules (Luescher et al, 1988).

In both cases described above the hydrophobicity of the groups binding to MHC appear to be important. TNP, due to the presence of the benzene ring, is also somewhat hydrophobic, although the NO_2 groups are hydrophilic. The epitope model proposed by Rothbard and Taylor stresses the requirement of hydrophobic residues. At the A chain loop on BI, both A_8-alanine and A_{10}-valine are hydrophobic. In PI, only the A_{10}-isoleucine is hydrophobic therefore the binding to MHC may not be of an affinity high enough to present the epitope in the

correct orientation. The TNP group, due to it's close proximity to this region, could enhance that affinity, through it's hydrophobic regions, allowing presentation. Evidence has shown that only a few amino acids need to differ to affect antigen presentation (Adorini et al, 1988). Mutant Ia^{bm12} only differs from the parent Ia^{b} molecule at three amino acid sites (McIntyre et al, 1984). Beef-insulin specific T cell clones from C57BL/6 mice could only respond to antigen presented on B6 ($H\text{-}2^{b}$) APC, but not B6.C ($H\text{-}2^{bm12}$) APC (Spinella et al, 1987). Therefore only a small alteration in either the MHC molecule or presumably the antigen can lead to different response characteristics.

That the A chain loop region was involved in the interaction of PI A chain and Class II molecules has recently been demonstrated in $H\text{-}2^{b}$ x $H\text{-}2^{k}$ F1 mice (Plachov et al, 1988). The authors state that potency differences between insulin variants, in these mice, were attributable to the interaction of the proteins with I-A molecules. This would support our hypothesis that the A chain loop region is acting as an agretope in $H\text{-}2^{b}$ mice, and that the difference seen in the responses to PI and BI are due to the affinity of the interaction. Plachov also implicated the glutamic acid residue at position A_4 as playing a crucial role in PI recognition. The current hypothesis on the need for antigen processing suggests that denaturation of an antigen will

expose sites necessary for binding to either the T cell receptor and/or the appropriate MHC molecule (Babbit et al 1985, Berkower et al 1986, Chain et al 1988). A_4 glutamate and aspragine at position B_3, are the only differences between PI and mouse insulin in close proximity to the A chain loop region and could therefore have a role to play either as part of the agretope or as part of the epitope. A comprehensive study of processing and presentation of insulin has shown that a disulphide linked peptide A_1-A_{14}\B_7-B_{15} was immunogenic for both H-2^d PI and H-2^b BI specific hybridomas. Further removal of residues resulted in a loss of responsiveness. These results would appear to eliminate the B_3 residue from being involved in T cell recognition, but the A_4 site was clearly necessary. (Naquet et al, 1987). This would seem to support our data (Chapter 3:10) that in H-2^b restricted responses to beef and haptenated pork insulin both chains of the protein are required, and that desoctapeptide insulin is recognised, therefore the eight terminal amino acids on the B chain are not required for immune recognition (Table 18).

As stated above, if both chains are required there are two possible explanations. One is that the epitope exists on one chain and the agretope on the other, the second is that elements of one or both sites are situated on different chains, to give conformational determinants. While conformational epitopes have been shown in antibody responses they remain a contentious

issue in T cell recognition. They were reported in influenza haemagglutinin as epitopes which were destroyed by processing in vitro, (Mills et al, 1986), and previously for insulin (Glimcher et al, 1983). However the data from Naquet would suggest that limited proteolysis may be necessary for presentation of insulin to T cells. This may not involve the lysosomal compartment of the antigen presenting cell, rather association may take place at another site such as the plasma membrane or a post Golgi storage region (Buus et al, 1986). An intermediate vesicle also containing Ia molecules has been suggested as an alternative fate for antigen rather than entering lysosomes (Werdelin, 1986, Unanue et al, 1987,). In this vesicle the antigen will undergo processing by proteolytic enzymes until it has been altered enough to bind to Ia. Once bound no further processing can occur and the Ia\Ag complex is transported to the cell surface (Donermeyer et al, 1989). In the case of insulin, limited proteolysis in an intermediate vesicle may not cleave the disulphide bonds necessary to hold the two chains together, but could expose sequestered epitopes, or that the removal of certain residues will allow the remainder of the molecule to assume a different conformation necessary for recognition by T cells.

Antigen interactions with MHC, as discussed above, have been the subject of many studies. Virtually all the

residues in the A_1-A_{14}\B_5-B_{17} peptide are situated within amphipathic helical regions, and this structure would therefore support the model proposed by De Lisi et al, 1985. The glutamic acid residue at position A_4 clearly has an important role in the immune response to pork insulin in that it is the only amino acid in this peptide that differs from mouse insulin. Together with positions A_8 and A_{10} in the A chain loop A_4 would appear to hold the answer to the question of non-responsiveness of PI in H-2^b mice. Peptides made from the individual A or B chains regions may have provided some information on this question, but due to the conformational nature of the insulin molecule as an antigen it was felt that another approach was required.

Oligonucleotide directed mutagenesis of the insulin gene should provide further information for identifying residues involved in the epitope and agretope regions. The only naturally occurring class II mutant is the B6.CH-H-2^{bm12} mouse and this mutation has provided valuable information into the stucture and function relationship of class II molecules and antigen (Kanamori et al, 1985, Spinella et al, 1987.). A point mutation in I-A^k α chain generated by ethyl methanesulphonate abolished the mutant's ability to present antigen to two of a large panel of antigen-specific I-A^k restricted T cell hybridomas (Griffith et al, 1987). Site directed mutagenesis has been used to construct mutants of I-A^b α chain (Cohn et al, 1986) and HLA-A2 in human studies

(Hogan et al, 1988a, Hogan et al, 1988b). This data provided evidence of serological and functional regions of the MHC molecules used. It was decided that analysis of the insulin protein could be studied in this way and determine the role of individual amino acids in the binding of the antigen to both MHC and the T cell receptor.

To this end I have already prepared an A_8 threonine to alanine hybrid. The addition of a hydrophobic residue at this site may increase the immunogenicity of human insulin in H-2^b mice, by providing an extra MHC binding residue and increasing the affinity of the interaction. In addition, a hybrid containing mutations at both A_8 and A_{10} has been prepared, effectively changing human insulin to beef insulin at the A chain loop region. It is hoped that further mutations can be carried out at the A_4 and B_3 sites to determine their role in T cell responses. This method should provide the opportunity to investigate the fine specificity of the T cell clones we have produced.

In conclusion, I have shown that the binding of a simple hapten group, at a specific site, can change a protein from being non-immunogenic to being immunogenic in H-2^b mice. The hapten group is not involved directly in T cell receptor binding, but may enhance the affinity of binding between the protein and MHC molecules. This could be of significant importance in fields outside

insulin immunobiology. For example, in autoimmune disease, where small organic groups may bind to self-proteins allowing them to be recognised by the immune system. In addition, hapten modification may have a role to play in vaccine design, where a small weakly immunogenic peptide may be modified to increase it's effectiveness. This approach is currently being investigated with peptides from papilloma virus in this laboratory.

Appendix 1.

Proliferation Assays.

Antigen dependent T cell activation is commonly assayed by measuring proliferation of the T cell population in response to antigenic challenge (Schwartz, et al, 1975, Corradin et al, 1977). This assay involves incubating primed T cells with antigen for 72 hours, then pulsing with (^{3}H) thymidine for 16 hours, harvesting the cells onto filter paper and counting incorporated thymidine in a scintillation counter. Although this assay is convenient and fairly rapid, the signal to noise ratio is often low. This is especially true in lymph node assays and seems to depend critically on the batch of foetal calf serum (FCS) used. The modification described below relies on minimising the background signal by carrying out incubations in homologous normal mouse serum (NMS), and amplifying the initial antigen-driven signal by expansion of the activated cells in IL-2.

Both the FCS assay and the NMS assay are described in the Methods section, and will not be repeated here. Table 23 shows the results of a comparison assay on H-2^b mice primed to BI. It is clearly shown that the signal to noise ratio is greatly altered. The background proliferation was decreased while the responses to antigen were substantially increased.

The effects were shown to be influenced by several factors. The response was optimal using around 5 x 10^5

cells/well, although clear responses could be obtained over a wide range of cell numbers. The incubation time in NMS was critical, with an optimum of 72 hours. Any less and a high background could still be detected presumably due to cells activated to express IL-2 receptors *in vivo*. At longer incubation times, the antigenic response was diminished, possibly due to a non-specific toxic effect of NMS, since numbers of viable cells also fell. A final factor involved in the response was the incubation time in exogenous IL-2. Proliferation began to rise after 24 hours and continued to do so for several days. Assays were routinely pulsed after 48 hours but this was somtimes varied after microscopic examination of the cultures (Chain et al, 1987).

In the more conventional FCS assay, antigen triggers the production of IL-2, as well as IL-2 receptor expression (Tse et al, 1980). It is this endogenous IL-2 which drives the proliferation of activated cells. Indeed it is likely that it is this Il-2 production which is the specific step in this assay, and many bystander cells expressing IL-2 receptors (perhaps from *in vivo* activation) participate in the measured proliferation (Augustin et al, 1979, Tse et al, 1980, Petit et al, 1986). In the modified assay exogenous IL-2 is added to the cultures, therefore the assay is a measure only of antigen dependent IL-2 receptor

expression. The expression of these receptors seems to require T cell receptor/antigen binding to take place, but, in contrast to the production of IL-2, does not require the cross-linking of the T cell receptor (Meuer et al, 1984). This may explain the greater sensitivity of the assay at lower antigen concentrations, or with weaker antigens. However, it is important to realise that a different marker of T cell activation is being assayed, and that the two events of receptor expression and lymphokine production may not always occur in parallel.

In conclusion, the NMS assay offered a significant improvement in the signal to noise ratio in the antigen system studied. It was routinely used in all primary proliferation assays described in this thesis.

This work was done in conjunction with Drs. B.M. Chain and P.W. Askenase, and Ian McCafferty who worked as a project student in the laboratory. However the data presented is my own.

Table 23.

Comparison of primary proliferation using the standard foetal calf serum assay and the modified normal mouse serum assay.

$H\text{-}2^b$ mice primed with BI.

	Antigen Concentration (µg\ml)					
	FCS			NMS		
	100	10	1	100	10	1
BI	35.0	18.7	19.9	107.8	24.0	12.0
PI	21.5	17.3	18.1	18.4	11.2	8.9
HI	17.7	15.3	16.6	12.0	10.6	10.3
Medium	18.5			8.0		

All results cpm x 10^3.

Appendix 2.

Determination of the site of haptenation by reversed phase high performance liquid chromatography (HPLC).

Reversed phase chromatography involves a non-polar stationary phase, which binds sample components to it's surface by hydrophobic selectivity. A polar mobile phase elutes the different components at different concentrations dependent on how strongly it is bound.

The stationary phase consisted of C_{18} alkyl hydrocarbons chemically bonded to silica particles to form the column packing.

A radial compression separation system was used, as pressure along the radial axis of the column containing the packing decreases void space within the bed and gives a more homgeneous packed structure.

The organic component of the mobile phase was, in all cases, acetonitrile, while the aqueous component varied with requirements. The mobile phase can be run either as a linear gradient, or at a constant concentration known as isocratic elution.

Insulin variants with minimal differences in structure can be separated by reverse phase HPLC (McLeod et al, 1984). Insulin modified by haptenation with TNP eluted at a higher concentration than the native insulin due to the hydrophobicity of the hapten group. TNP-insulin consistantly gave a single component when separated on a linear with 0.1M phosphate buffer, pH 3.6 as the aqueous solvent (Fig 14a). When this major peak was collected

and run under isocratic conditions only one component could be detected (Fig 14b). This confirmed previous reports that TNP-insulin consists predominantly of a single haptenated form.

Both insulin and TNP-insulin were dansylated and the free amino groups separated at isocratic conditions, and readily identified with commercial dansyl amino acid standards (Fig. 15). The three dansylated amino acids of interest in this study (glycine A_1, lysine B_{29}, and phenylalanine B_1) were found in the acid hydrolysates of dansylated insulin. Identical dansylation of TNP-insulin showed an almost total absence of dansyl-glycine, suggesting that this amino acid is protected from dansylation by prior modification by TNP.

In order to confirm this finding a preparation of TNP-insulin was first fractionated according to the protocol shown in Fig. 14. The samples corresponding to the major peak were collected, pooled and then subjected to the same analysis as described for the unfractionated sample (Fig. 16). As shown dansylation and hydrolysis of this material revealed that both B_{29} lysine and B_1 phenylalanine could be detected, but dansyl glycine was absent. The agreement between the results obtained on analysis of the unseparated TNP-insulin and the major peak obtained after chromatography confirms that TNBS reacts predominantly with A_1 glycine.

To confirm directly this result, an acid hydrolsate of

TNP-insulin was analysed for the presence of TNP-glycine. This analysis was greatly simplified by the fact that TNP groups absorb at 280 nm, unlike most free amino acids. The TNP amino acids were identified by comparison to E-TNP-lysine and TNP-glycine (Fig. 17). The results unequivocally identified the presence of TNP-glycine, but not TNP-lysine in the hydrolysate.

The results of this study show that, in the reaction conditions used, TNBS reacts predominantly with the α-amino group of A_1 glycine. The only published studies of nitrophenyl derivatives of insulin are those of Li, and Keck et al, which both follow the same protocol. Under these conditions (pH 11.5, 24hrs) DNBS reacts predominantly with the B_{29} lysine group. We did not follow this protocol since the insulin molecule may undergo structural modifications at these high pH values.

Analogues with substituent groups on the A_1 amino group have been studied extensively to determine their biological, spectroscopic and structural properties (Freissen et al, 1976, Chothia et al, 1983). As well as the ability of insulin to form dimers, addition of bulky groups to this region of the molecule has been shown to affect the three-dimensional structure of the insulin monomer, particularly the helical region A_2-A_8. Comparison of the different monomer conformations found in different crystal forms has shown that changes affecting this helix are associated with relative

movements of other regions of the molecule (Chothia et al, 1983), including conformations of the A_8-A_{10} region. Studies using computer modelling of TNP modified insulin are in progress to try to predict these changes and to relate them to changes in immunological recognition.

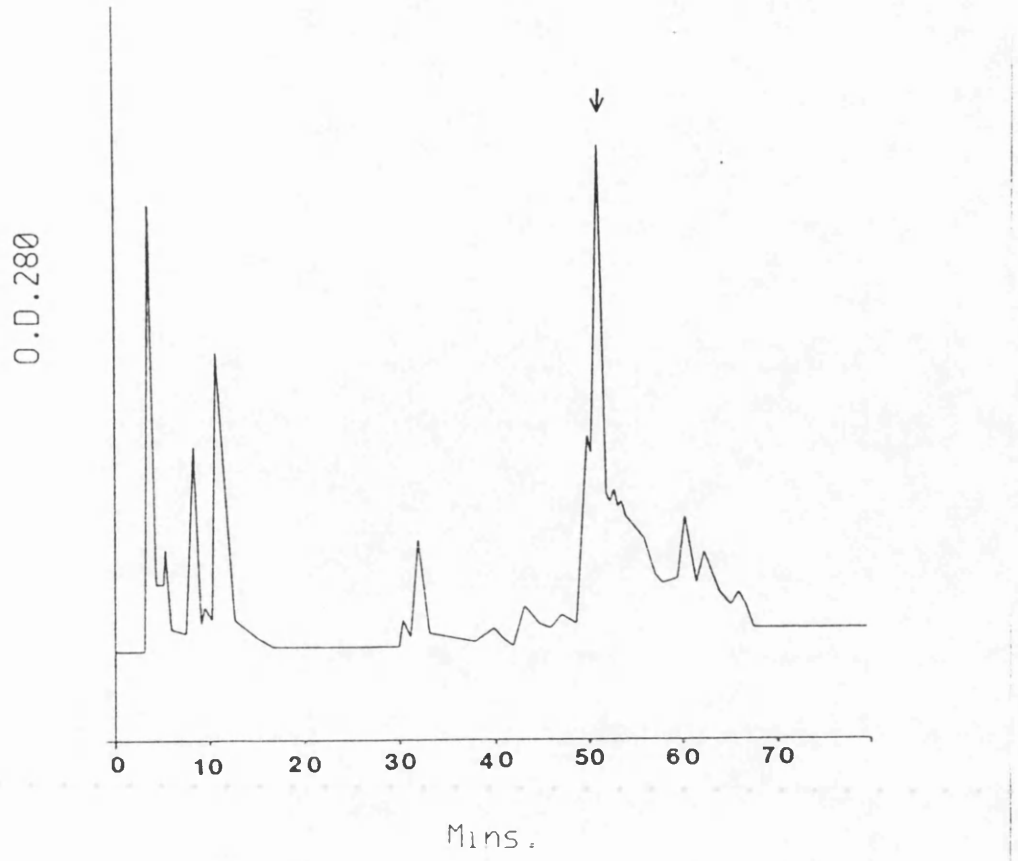

Fig 14. (a) Preparative separation of insulin derivatives by reaction with TNBS. TNP-insulin was prepared as described in Methods, and separated by reverse phase chromatography on a C18 column.

Aqueous solvent- sodium phosphate (0.1M, pH 3.6).

Organic solvent- acetonitrile.

The acetonitrile concentration was maintained at 25% for 10 mins. after loading the sample, then increased linearly to 75% over 60 mins. Flow rate = 1ml\min.

Arrow indicates peak collected for further analysis.

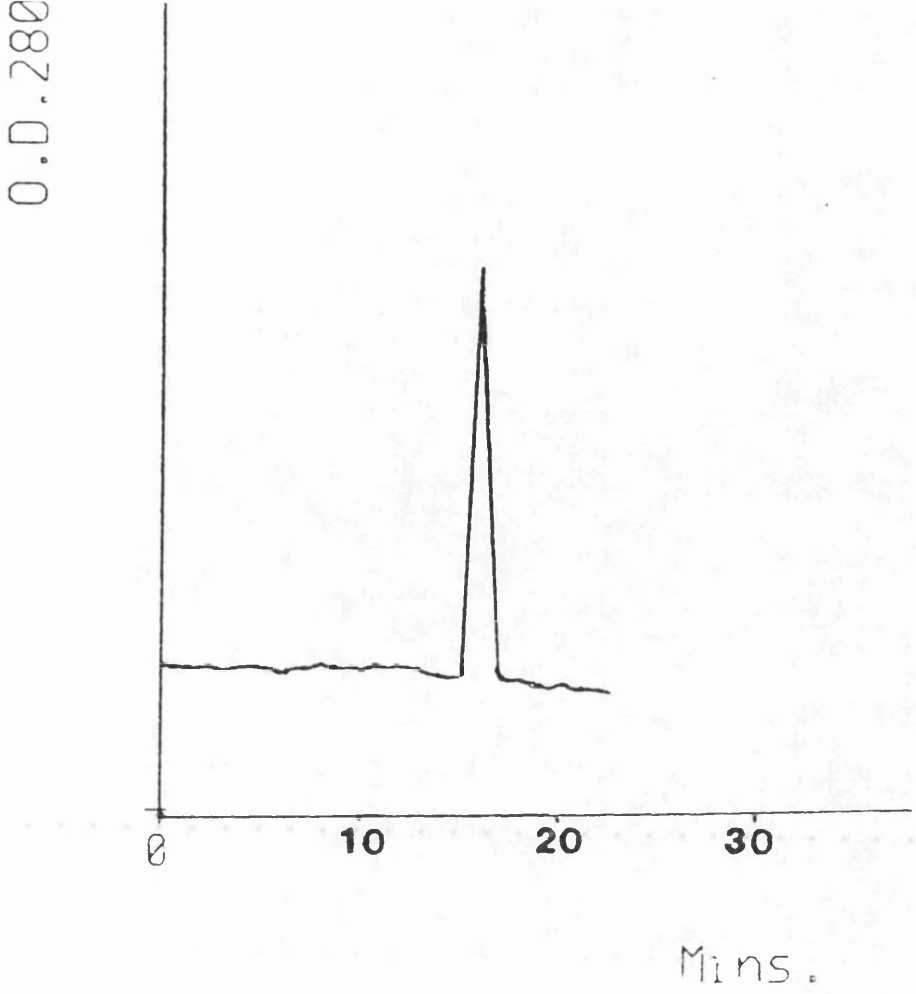

Fig 14 (b). Analysis of major peak collected as shown in 14 (a). This was carried out at isocratic elution conditions with acetonitrile at 36%.

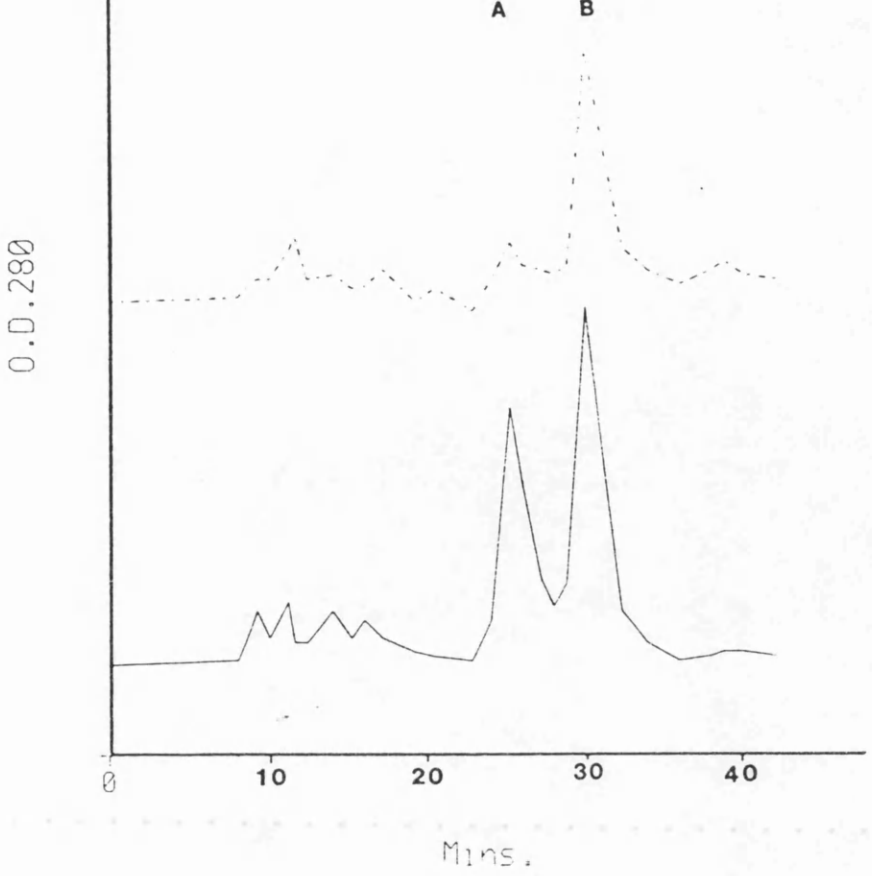

Fig 15: Analysis of free amine groups on insulin and TNP-insulin, by dansylation and hydrolysis as described in Methods. Dansyl amino acids were separated by reverse phase chromatography, and identified by comparison to dansyl amino acid standards. Aqueous solvent- 0.1% trifluoroacetic acid. Organic solvent- acetonitrile. Isocratic elution using (a) 5% acetonitrile. (b) 20% acetonitrile.

———— insulin

-------- TNP-insulin.

Dansyl standards. A - glycine. B - lysine.

C -phenylalanine

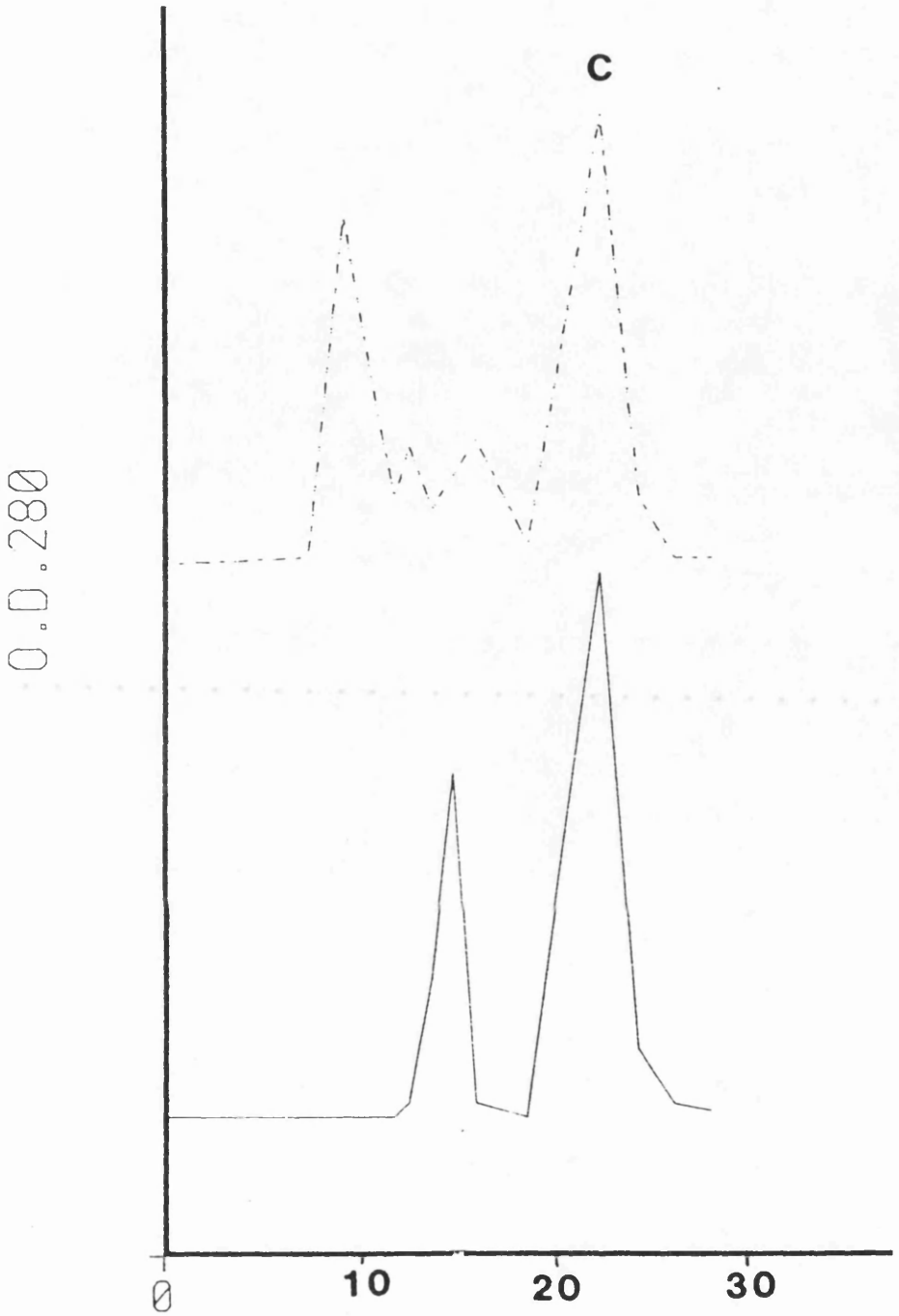

Fig 15B: See heading under Fig 15A

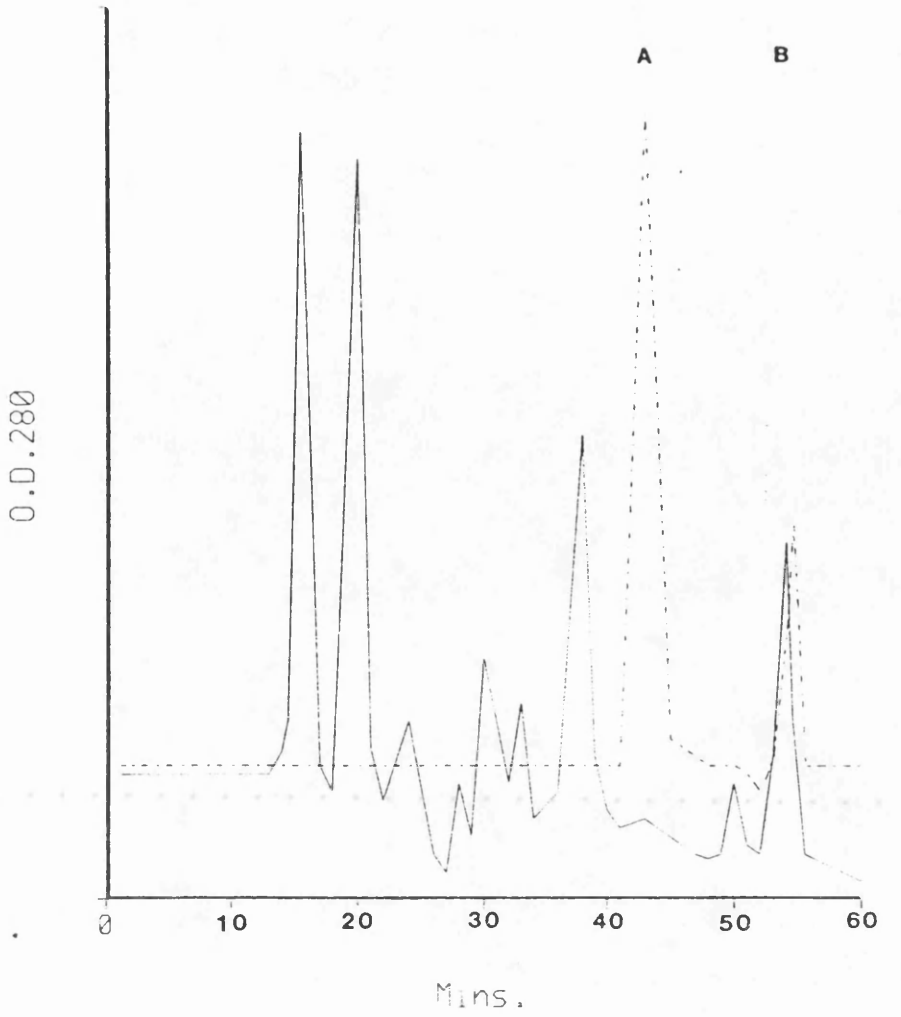

Fig 16: Analysis of free amino groups in purified TNP-insulin. The major peak identified in Fig.14a was collected, dansylated and hydrolysed. Dansyl amino acids were separated as for Fig.15., and identified by comparison to dansyl amino acid standards.

(a) 8% acetonitrile. (b) 26% acetonitrile.

———— TNP-insulin.

------- dansyl standards. A - glycine. B - lysine.
C - phenylalanine.

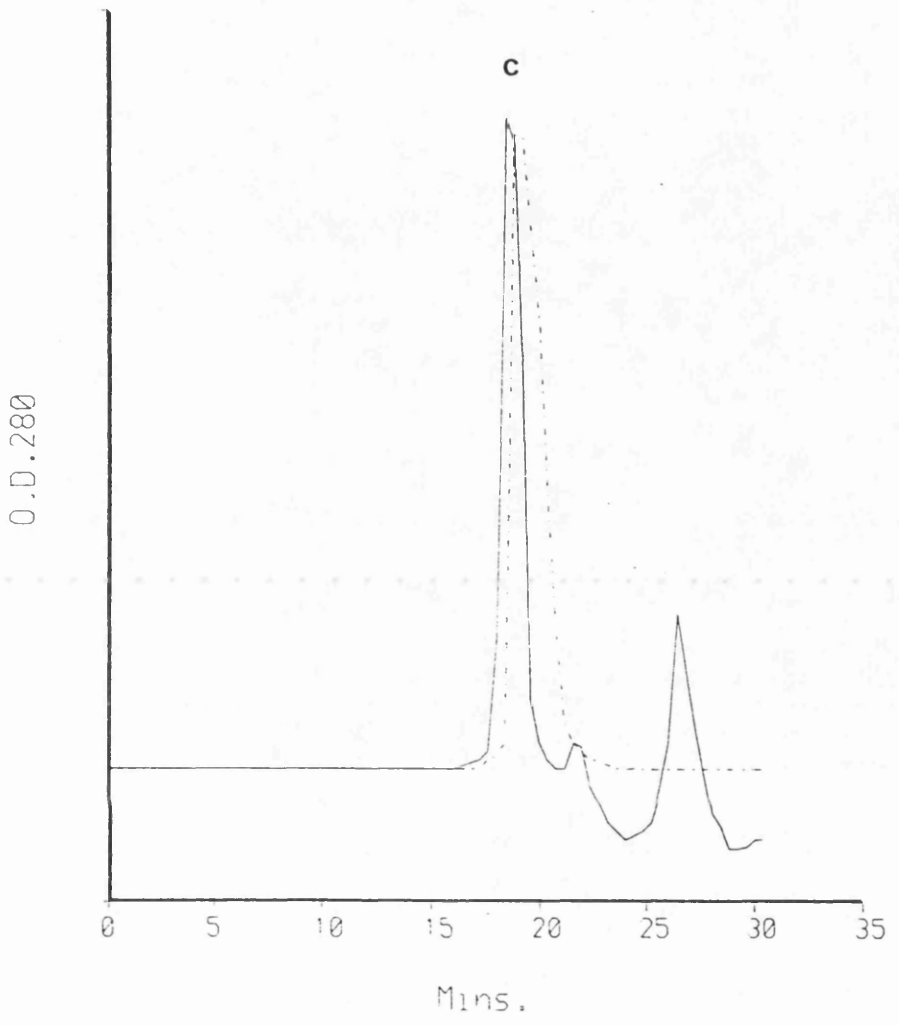

Fig 16B: See heading under Fig 16A

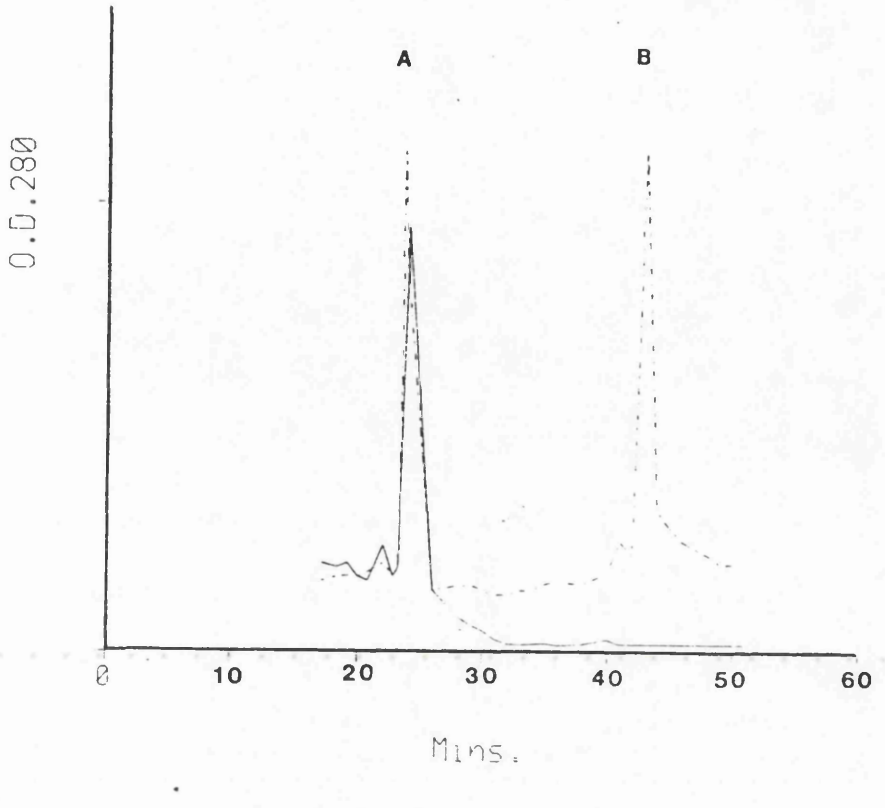

Fig 17: Direct identification of TNP-amino acids in TNP-insulin. TNP-insulin was hydrolysed, and the amino acids separated as in Fig.15 The elution profile of TNP-glycine and TNP-lysine is shown for comparison. Chromatography was carried out at 22% acetonitrile.

———— TNP-insulin.

------- TNP-standards. (a) glycine (b) lysine.

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