arginyl-A-chain-S-sulphonate was isolated in pure form by chromatography on DEAE-Sephadex and combined with bovine insulin B-chain-S-sulphonate. From the combination product 15% of arginyl-insulin was isolated. The crystallization from citrate buffer, pH 7, containing Zn^{2+} was nearly quantitative. A-1-lysyl-A0-arginyl-insulin, a non-crystallizable analogue that contains the last two amino acid residues of the proinsulin C-chain, had a biological activity of 20% and an immunoreactivity of 34%. It was prepared by chain lengthening of arginyl-A-chain-S-sulphonate with bisbutyloxycarbonyl-lysine trichlorophenyl ester, removal of the protecting groups by treatment with trifluoroacetic acid and combination of the isolated lysyl-arginyl-A-chain-S-sulphonate with B-chain-S-sulphonate.

The bovine insulin B-chain-S-sulphonate was also subjected to partial degradation in experiments similar to those described for the A-chain. One cycle of preparative Edman degradation yielded des-B1-phenylalanine-B-chain-S-sulphonate, from which a second cycle gave des-B1-phenylalanine-des-B2-valine-B-chain-S-sulphonate, characterized by electrophoresis and amino acid analysis (Herbertz, 1971).

For the preparation of [B1-*p*-iodophenylalanine]-insulin, two routes were followed. Both des-B1-phenylalanine-B-chain-S-sulphonate (route 1) as well as A1,B29-bisbutyloxycarbonyl-des-B1-phenylalanine-insulin (kindly given to us by Dr G. Geiger, Farbwerke Hoechst AG, Frankfurt-Hoechst, Germany) (route 2) were acylated with butyloxy-carbonyl-*p*-iodophenylalanine trichlorophenyl ester, the latter in dimethyl sulfoxide as solvent and *N*-methylmorpholine as a base. Cross-linking reactions (Brandenburg, 1971) were not observed. Deblocking and purification of the insulin derivative (method 2) gave [B1-*p*-iodophenylalanine]-insulin, which crystallized as spindles and possessed a biological activity of 60–70%.

The circular-dichroism spectra of all analogues (A. Wollmer, unpublished work) are significantly different from that of insulin, indicating that the loss of biological activity is due to changes of the three-dimensional structure of the molecule.

In conclusion, semisynthetic studies in the field of insulin chemistry look promising not only with transformations of the two-chain molecule but also with peptide synthesis involving the separated chains followed by chain combination. This latter operation seems not to be hampered by the fact that modified chains are combined.

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The Structure of Insulin in Relation to its Immunological Activity

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The identification of surface conformations on insulin to which antibodies bind is important in defining the interactions of insulin with other macromolecules. By comparing the immunologic activity of derivatives of insulin in the immune-haemolysis inhibition assay and in a radioimmune assay it has been possible to distinguish the determinants on insulin that are detected by the two assays. The elucidation of the secondary and tertiary structure of insulin (Blundell, Dodson, Dodson, Hodgkin & Vijayan, 1971) has permitted a more precise definition of such surface conformations.

The immunological studies concerned with the structure-function relationships of the insulin molecule that have been reported were concerned primarily with the use of insulin antisera prepared in strain 2 and strain 13 inbred guinea pigs. These antisera were tested for their ability to react with various derivatives of insulin containing known alterations in primary structure (Arquilla, Bromer & Mercola, 1969). These studies demonstrated that strain 2 antibodies react with determinants involving the free amino groups of insulin, whereas the strain 13 antibodies reacted preferentially with determinants involving the C-terminal portions of the A- and B-chains. They also indicated that insulin has a very tightly packed core, rich in

aromatic and hydrophobic amino acids, the integrity of which is important for the maintenance of all of the determinants on the insulin molecule. These studies demonstrated that a relationship exists between the intactness of the antigenic determinants and the biological activity of the various derivatives. The ability of insulin to aggregate was shown to be directly related to the maintenance of antigenic determinants on the surface of the molecule.

Recently we have been interested in derivatives with alterations at the *C*-terminal ends of the A-chain and of the B-chain, specifically des-A21-asparagine-insulin, des-B30-alanine-insulin and des-B23-B30-octapeptide-insulin. Both des-asparagine-insulin and des-octapeptide-insulin combine less well with strain 13 insulin antibodies than with strain 2 antibodies, and both derivatives have diminished biological activity. The circular-dichroism spectra in the near and far u.v. of des-octapeptide-insulin and des-asparagine-insulin are remarkably similar to one another, and both are distinct from that of insulin. The difference spectra obtained when each derivative is compared with insulin are also very similar. Consequently removal of asparagine-A21 by carboxypeptidase induces a conformational change that appears to be similar to the change in conformation resulting when the *C*-terminal octapeptide is removed from the B-chain by trypsin. This suggested that in native insulin asparagine-A21 may stabilize the folding back of the *C*-terminal octapeptide of the B-chain, possibly by an electrostatic interaction between asparagine-A21 and the dipeptide glutamylarginine at positions B21-B22, which are within Van der Waals radii of residue A21. When animals were immunized with des-octapeptide-insulin, des-asparagine-insulin and insulin and the antisera were tested for cross-reactivity (Table 1), des-asparagine-insulin reacted very poorly with anti-DOP (des-octapeptide-insulin) and similarly des-octapeptide-insulin re-

acted poorly with antibodies prepared against des-asparagine-insulin. These results indicated marked conformational differences on the surface of these two derivatives. Insulin was found to react to a greater degree with antibodies to des-asparagine-insulin than with antibodies to des-octapeptide-insulin, possibly because the *C*-terminal octapeptide of the B-chain may be exposed in des-asparagine-insulin and therefore immunogenic. On the other hand, because of aggregation, the *C*-terminal end of the B-chain may not be exposed when insulin is used as an antigen. These studies indicated that the circular-dichroism spectra apparently bear little, if any, relationship to the surface conformation in the vicinity of the antigenic determinants on insulin, des-octapeptide-insulin and des-asparagine-insulin.

Of particular interest are the immunological and biological properties of derivatives of insulin with alterations in the *C*-terminal of the A-chain, specifically at tyrosine-A14 and tyrosine-A19. Recently we have prepared [mononitrotyrosine]-insulin and iodo-insulin demonstrated 6.7 and [moniodotyrosine]-insulin, both of which have approximately 70% biological activity. Mononitro-insulin and iodo-insulin demonstrated 6.7 and 3.8% immunological activity respectively in the immune-haemolysis inhibition assay, and 37.7 and 58.4% respectively in the radioimmune assay (Table 2). Since moniodo-insulin is the indicator in the radioimmune assay, it is probable that most functional antibodies in this assay are involved with determinants not in proximity to tyrosine-A14 or tyrosine-A19.

When proinsulin was tested for cross-reactivity in the two immunological assays, it was found to be 25-fold more reactive in the radioimmune assay than in immune-haemolysis inhibition (Table 2). This suggested that the connecting peptide may physically interfere with the binding of antibodies to determinants in the region of tyrosine-A14 and

Table 1. *Immunological cross-reactivity of des-A21-asparagine-insulin, des-B23-B30-octapeptide-insulin and insulin*

The percentage inhibition was calculated at 70, 50 and 30% lysis. The variations represent the greatest differences in percentage inhibition at these three extents of lysis. The degree of inhibition with the homologous antigen was assumed to be 100% and the percentage inhibition by the two heterologous antigens was calculated by comparison on a picomolar basis.

Inhibitor	Insulin*	% inhibition in immune-haemolysis system	
		Des-asparagine-insulin*	Des-octapeptide-insulin*
Insulin	100	14.8-15.7	2.8-4.1
Des-asparagine-insulin	4.5-5.1	100	3.1-4.4
Des-octapeptide-insulin	2.5-3.7	3.3-4.8	100

* Refers to the antiserum and coated erythrocytes used in the respective immune-haemolysis system.

Table 2. *Immunological activity of derivatives of insulin measured by immune-haemolysis inhibition and radioimmune assays*

The percentage activity in the immune-haemolysis inhibition assay was determined from the number of picomoles of inhibitor needed to produce 50% inhibition. The percentage activity in the radioimmune assay was determined from the number of picomoles needed to give a C_0/C_1 ratio 1.5. Insulin was considered to be 100% active in both systems.

Insulin derivative	Immunological activity (%)	
	Radioimmune assay	Immune-haemolysis inhibition assay
Insulin	100	100
Moniodo-insulin	58.4	3.9
Mononitro-insulin	37.7	6.7
Proinsulin	26.3	0.06*
Des-asparagine-insulin	3.5	0.17
Des-octapeptide-insulin	2.0	0.08

* Calculated at 20% inhibition of immune haemolysis.

tyrosine-A19 that are not detected by antibodies in the radioimmune assay.

The data *in toto* suggest that most antibodies that are functional in the immune-haemolysis assay combine with determinants that are dependent on the tertiary relationship between the C-terminal portions of the two chains. The slightly greater immunological activity in the radioimmune assay is interpreted to mean that antibodies functional in the radioimmune assay are directed to determinants less involved with the two C-termini. Alterations in the regions of the N-terminus of the B-chain have little influence on either immune assay (Brandenburg & Ooms, 1968; Arquilla *et al.* 1969). It is possible that relatively few antibodies in insulin antisera are directed to residues in the C-terminus of the B-chain. This region is involved in the dimer site and may not be exposed when insulin is used as an antigen.

Of interest in these studies is the fact that both mononitro- and moniodo-insulin retain considerable biological activity compared with other derivatives with the same degree of immunological alteration (Table 2). This suggests that the regions of tyrosine-A14 and possibly tyrosine-A19 are not involved directly in the areas of the insulin molecule that are essential for biological activity.

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Structural Relationships among Growth Hormones and Prolactins

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The pituitary growth hormones and prolactins comprise a group of protein hormones with similar structural features, homologous amino acid sequences and, in some species, overlapping biological properties. There is considerable species specificity within the group. Relationships between these hormones will be considered here in the light of information currently available about their primary structures.

An amino acid sequence for human growth hormone was first proposed by Li, Liu & Dixon (1966), but has since been modified (Niall, 1971; Niall, Hogan, Sauer, Rosenblum & Greenwood, 1971). The hormone possesses a single polypeptide chain (molecular weight about 20000), though there is some tendency for the chains to aggregate. Bovine and ovine growth hormones normally exist as dimers, with a molecular weight of about 45000. These can dissociate under various conditions (Andrews & Folley, 1963, and many subsequent reports). Studies on the primary structure, particularly at the N- and C-termini, have shown that bovine growth-hormone preparations contain two types of polypeptide chain, but that these are very similar and may differ only by the presence or absence of a single N-terminal alanine residue (Wallis, 1969).