The Sequence of Amino Acids in Insulin Isolated from Islet Tissue of the Cod (*Gadus callarias*)

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1. *S*-Aminoethylcysteinyl derivatives of the A and B chains of cod insulin were prepared from the individual *S*-sulpho chains. 2. Studies on small peptides derived from the *S*-aminoethylated peptide chains by treatment with trypsin allowed the amino acid sequences in the region of the cysteinyl residues of the A and B peptide chains to be defined. 3. The six amide groups in cod insulin were located by complete digestion of small peptides from the A and B chains with aminopeptidase followed by amino acid analyses. 4. The results, together with previous studies on the oxidized A and B chains, define the sequences of the 51 amino acids that constitute cod insulin.

The isolation and properties of cod insulin together with evidence for the sequential order of 39 of the 51 constitutive amino acids were described by Grant & Reid (1968). In these studies, amino acid sequences were derived from the structure of small peptides obtained from the oxidized A and B chains. The undefined sequences in both chains contained cysteic acid and were comprised of consecutive amino acid sequences in the interior of two relatively large peptides that were not susceptible to further specific cleavage by trypsin or by other procedures. These peptides prepared from AE* derivatives of the A and B chain respectively have now been hydrolysed by trypsin at AE-cysteinyl bonds. The sequence of the resulting peptide fragments, together with the location of amide groups, permits the definition of the complete amino acid sequences of both the A and the B chain of cod insulin.

MATERIALS

Phenyl isothiocyanate, *N*-ethylmorpholine, trifluoroacetic acid and β -mercaptoethanol were obtained from Kodak Ltd., Kirkby, Liverpool. Ethyleneimine was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., and was stored over solid KOH at 4°. Amino acids, DNS chloride and all other chemicals used (AnalaR grade) were obtained from British Drug Houses Ltd., Poole, Dorset.

*Abbreviations: AE, *S*-aminoethyl; DNS, 1-dimethylaminonaphthalene-5-sulphonyl; AEC, *S*-aminoethylcysteine; Asx and Glx refer to residues that could be either aspartic acid or asparagine and either glutamic acid or glutamine respectively.

Sephadex G-25 and G-75 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Carboxypeptidase A (di-isopropyl phosphorofluoridate-treated; batch 7AA), pepsin (batch PM712), trypsin (batch 7FB), preparations of carboxypeptidase B (di-isopropyl phosphorofluoridate-treated; batch 6079) and leucine aminopeptidase (di-isopropyl phosphorofluoridate-treated; batch 61C) were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Chymotryptic activity of the trypsin was minimized by treatment with *L*-1-chloro-4-phenyl-3-toluene-*p*-sulphamidobutan-2-one as described by Kostka & Carpenter (1964).

METHODS

Isolation and purification of cod insulin. Cod insulin was isolated and purified as described by Grant & Reid (1968).

Preparation and separation of the S-sulpho A and B chains of cod insulin. The $\text{Na}_2\text{S}_4\text{O}_6$ was prepared from $\text{Na}_2\text{S}_2\text{O}_3$ and iodine, as described by Gilman, Philips, Koelle, Allen & St John (1946). Urea was purified by passing an 8M solution of AnalaR-grade urea over a bed of Amberlite MB-1 cation- and anion-exchange resin. Cod insulin (10mg.) was dissolved in 0.5ml. of 0.2M-potassium phosphate-8M-urea buffer, pH 7.5; Na_2SO_3 (12mg.) and $\text{Na}_2\text{S}_4\text{O}_6$ (7mg.) were added to this solution and the reaction was allowed to proceed at 37° for 3hr. The reaction mixture was acidified with acetic acid, and the *S*-sulpho A and B chains were then separated by applying the entire reaction mixture to a column (80cm. $\times$ 1.3cm.) of Sephadex G-75 that had been equilibrated with aq. 50% (v/v) acetic acid (Varadani, 1966). Elution with the same solvent was maintained at 10ml./hr. by the use of an LKB proportionating pump. The column effluent was monitored at 276m μ . The separation of the *S*-sulpho A and B chains of cod insulin from each other and from salts and urea is shown in Fig. 1. Fractions containing the *S*-sulpho chains were pooled and freeze-dried.

Reduction and aminoethylation of the S-sulpho chains. The freeze-dried preparations of the S-sulpho A and B chains were reduced and aminoethylated essentially as described by Raftery & Cole (1966). The S-sulpho A or B chain (5mg.) was dissolved in 0.5ml. of deionized 8M-urea, and 5 μ l. (72 μ moles) of 2-mercaptoethanol, 25 μ l. of EDTA solution (containing 2.0mg. of the disodium salt/ml.) and 150 μ l. of 3M-tris, pH 8.6, were added. The mixture was kept at room temperature for 6hr., and aminoethylation was effected by the addition of ethyleneimine; a total of 30 μ l. (0.58m-mole) was added in three equal portions at intervals of 10min. After a total reaction time of 45min., the entire mixture was applied to and eluted from a column (80cm. $\times$ 1.3cm.) of Sephadex G-25, with 0.2N-acetic acid for the A chain and 0.5M-NH₃ for the B chain. AE chains were eluted in the void volume of the column and the appropriate fractions were pooled and freeze-dried.

Electrophoresis. Peptides were examined by electrophoresis in pyridine-acetic acid-water (25:1:225, by vol.), pH 6.5, on Schleicher and Schuell electrophoresis strips (42cm. $\times$ 4cm.; no. 2042) at 300v/strip for 4–5hr.

Quantitative amino acid analysis. This was carried out on a Technicon AutoAnalyzer as described by Grant & Reid (1968). In this system AEC was eluted between NH₃ and lysine. Asparagine and glutamine were eluted together, with the same retention volume as serine.

Amide estimation. Approx. 0.1 μ mole of peptide was dissolved in 100 μ l. of water; 10 μ l. of 25mM-MgCl₂, 10 μ l. of 0.5M-tris buffer, pH 8.5, and 5–10 μ l. of aminopeptidase (12.6mg./ml.) were added, and the mixture was incubated at 40° for 6hr. The reaction was stopped by the addition of 1.0ml. of 0.2M-citrate buffer, pH 2.2, containing 0.1 μ mole of norleucine/ml., and the entire reaction mixture was subjected to amino acid analysis.

Asparagine and glutamine were determined by the difference between the aspartic acid or glutamic acid content of a complete acid hydrolysate and that found in a comparable aminopeptidase digest (Tables 1 and 2).

Other methods. Procedures of partial acid hydrolysis with acetic acid, Edman degradation, N-terminal analysis, enzymic digestions, paper chromatography and detection of peptides after chromatography and electrophoresis were as described by Grant & Reid (1968).

RESULTS

The S-sulpho A and B chains of cod insulin were separated on a column of Sephadex G-75 (Fig. 1). Subsequently, the individual chains were reduced and treated with ethyleneimine as described in the Methods section to form the AE derivatives. The overall yield of each chain was about 80% of the theoretical value; this contrasts with the maximum yield of about 50% for the oxidized chains obtained by treatment of cod insulin with performic acid (Grant & Reid, 1968). Both AE chains migrated as single components on electrophoresis at pH 4.5, and the results of amino acid analyses (Tables 1 and 2) were in agreement with the corresponding values obtained by Grant & Reid (1968) for the oxidized chains. In this connexion it was found essential to remove salts and residual reagents after

aminoethylation of the B chain, by gel filtration with 0.5M-ammonia as solvent. In early experiments, 0.2N-acetic acid was used as solvent and resulted in a marked decrease in the methionine content of the peptide. Under the acidic conditions, it is presumed that residual ethyleneimine had reacted with the methionine residue to form a stable sulphonium salt. This reaction of methionine has been studied (Schroeder, Shelton & Robberson, 1967) and is known to be dependent on pH.

The evidence for amino acid sequences of both chains shown in Fig. 3, except for the nature of the residues at positions A5–A11 and B6–B10 and the location of certain amide groups, was given by Grant & Reid (1968).

Amino acid sequences of the A chain

The AE derivative of the A chain was digested with pepsin, and peptides AP1 and AP2 (Fig. 3) were separated by gel filtration on Sephadex G-25 under conditions described by Grant & Reid (1968). The amino acid compositions of these peptides are given in Table 1.

Peptide AP1. Peptide AP1 (0.2 μ mole) was digested with trypsin (enzyme/substrate ratio 1:100) at pH 8.1 for 3hr. at 37°, and the digest was separated into four bands on paper chromatography with butan-1-ol-acetic acid-water as solvent. Two of the bands, with *R_F* values 0.27 and 0.62, after elution from the paper, migrated as single components on electrophoresis at pH 6.5. Amino

Fig. 1. Separation of the S-sulpho A and B chains of cod insulin on a column (80cm. $\times$ 1.3cm.) of Sephadex G-75 in aq. 50% (v/v) acetic acid. The flow rate was 10ml./hr. and the fraction volume 1.6ml. Subsequently the fractions within the horizontal bars were pooled and freeze-dried.

Table 1. *Amino acid composition and properties of A chain of insulin and of derived peptides*

Experimental details are given in the text. All peptides were hydrolysed for 22 hr. Values are uncorrected for hydrolytic losses or incomplete hydrolysis of the isoleucine-valine bond. The mobilities of peptides are expressed relative to a lysine marker on electrophoresis at pH 6.5. ND, Not determined.

		Amino acid composition (ratios)									
Peptide ... Amino acid	A chain	AP1	AP2		AP1 _{T1}		AP1 _{T2}	AP1 _{T3}	AP1 _{T4}	AP1 _{T5}	AP1 _{T6}
Asp	4.99	2.00	2.97	1.03*	1.00	1.00*	—	—	0.74	1.00	—
Ser	—	—	—	—	—	—	—	—	—	—	—
Glu	2.03	0.98	1.00	—	1.07	—	—	—	—	1.04	—
Pro	0.97	0.96	—	—	—	—	—	0.96	—	—	1.00
Gly	1.00	1.00	—	—	1.02	1.09*	—	—	—	1.03	—
Ala	—	—	—	—	—	—	—	—	—	—	—
Val	0.83	0.91	—	—	0.80	0.98*	—	—	—	0.87	—
Met	—	—	—	—	—	—	—	—	—	—	—
Ile	1.72	1.64	—	—	0.80	0.80*	—	—	1.04	0.81	—
Leu	1.00	—	0.97	0.96*	—	—	—	—	—	—	—
Tyr	0.94	—	0.73	0.85*	—	—	—	—	—	—	—
Phe	1.00	0.96	—	—	—	—	—	—	1.00	—	—
Lys	—	—	—	—	—	—	—	—	—	—	—
His	1.04	1.07	—	—	—	—	—	1.00	—	—	1.00
Arg	1.09	1.02	—	—	—	—	—	1.00	—	—	0.97
AEC	4.13	3.10	1.10	1.02*	0.85	0.93*	1.00	1.10	—	1.62	2.02
Total residues	21	14	7	—	6	—	1	4	3	7	5
Yield (% of theoretical)	80	92	78	—	62	—	16	25	62	16	71
Mobility of peptide	ND	ND	0		0		0.70	0.97	Acidic	0.70	0.83
Terminal amino acids	$\left\{ \begin{array}{l} N- \\ C- \end{array} \right.$	Gly	Gly	Asp		Gly	—	His	Asp	Gly	AEC
		Asn	Phe	Asn		AEC	—	AEC	Phe	AEC	AEC

* Values obtained after complete digestion of the peptide with aminopeptidase.

acid analyses indicated that these components (Table 1) were peptides API_{T1} and API_{T4} respectively. The other two peptide bands with R_F values 0.05 and 0.18 each separated into two components on electrophoresis at pH 6.5. Amino acid analyses of these four components (Table 1) indicated that the material with R_F 0.18 was composed of free AEC (peptide API_{T2}) and a peptide (peptide API_{T5}), and the material with R_F 0.05 was composed of peptides API_{T3} and API_{T6} (Fig. 3).

Peptide API_{T1}. Amino acid analyses indicated that this peptide was composed of the first six amino acid residues from the *N*-terminal end of the A chain (Table 1 and Fig. 3). Comparison of the amino acid analyses obtained after acid hydrolysis and after aminopeptidase digestion (Table 1) indicated that the peptide contained one residue of aspartic acid and one residue of glutamine. This conclusion is in agreement with the lack of mobility of the peptide on electrophoresis at pH 6.5. The specificity of trypsin indicated that AEC was the *C*-terminal amino acid. These results, together with

previous studies (Grant & Reid, 1968), indicated that the sequence was Gly-Ile-Val-Asp-Gln-AEC.

Peptides API_{T3} and API_{T6}. Amino acid analyses indicated that these peptides differed only by one AEC residue (Table 1). Sequential Edman degradation indicated that the *N*-terminal sequence of peptide API_{T3} was His-Arg- and that of API_{T6} was AEC-His-Arg-. It was assumed that the residue adjacent to arginine was proline because a peptide bond between arginine and proline is known to be extremely resistant to the action of trypsin. The *C*-terminal amino acid of both peptide API_{T3} and peptide API_{T6} was deduced to be AEC from the known specificity of trypsin.

Peptide API_{T4}. Amino acid analyses (Table 1) indicated that this peptide was derived from the *C*-terminal end of peptide API. The sequence was established by Grant & Reid (1968).

Peptide API_{T5}. It was not possible to obtain this peptide completely free from traces of AEC. For this reason, the material was analysed before and after acid hydrolysis, and the amino acid analysis for peptide API_{T5} given in Table 1 is

corrected for this trace contaminant. Peptides $AP1_{T1}$ and $AP1_{T5}$ differed only by one AEC residue (Table 1), and it was concluded that peptide $AP1_{T5}$ contained -AEC-AEC as the C-terminal sequence.

Digestion of peptide AP1 with trypsin under the same conditions as those given above except that a higher ratio of trypsin to peptide was used (1:20, w/w) resulted in the formation of mainly peptides $AP1_{T1}$, $AP1_{T2}$, $AP1_{T3}$ and $AP1_{T4}$. Only trace amounts of peptides $AP1_{T5}$ and $AP1_{T6}$ were detected. This difference suggests that the rate of hydrolysis of the AEC-histidine bond is appreciably slower than that of either the AEC-AEC or the AEC-aspartic acid bond, and is in agreement with the conclusions made above on the amino acid sequence of peptide AP1.

Peptide AP2. The sequence of this peptide, which is composed of residues A15-A21 of the A chain, together with the identification of asparagine as the C-terminal amino acid residue, was given by Grant & Reid (1968). The location of other amide groups in this peptide was studied by comparison of amino acid analyses obtained after acid hydrolysis and after aminopeptidase digestion (Table 1). This comparison indicated that one aspartic acid, two asparagine and one glutamine residues were present per molecule of peptide AP2. This result is in agreement with the lack of mobility of peptide AP2 on electrophoresis at pH 6.5.

Electrophoresis of a chymotryptic digest of peptide AP2 (Grant & Reid, 1968) at pH 6.5 showed that the tripeptide Asx-Leu-Glx was acidic, Asx-Tyr was neutral and AEC-Asn was basic, a result in agreement with the location of amide groups shown in Fig. 3.

Amino acid sequence of B chain

The AE derivative of the B chain was hydrolysed in acetic acid and the hydrolysate separated by gel filtration into three peptide fractions and one fraction (fraction BHA4) containing free aspartic acid (Fig. 2). The peptide fractions BHA1, BHA2 and BHA3 were further purified by paper chromatography with butan-1-ol-acetic acid-water as solvent and gave R_F values 0.30, 0.73 and 0.44 respectively. The amino acid compositions are given in Table 2.

Peptide BHA1. Peptide BHA1 (1.0 μ mole) was digested with trypsin (enzyme/substrate ratio about 1:100) at pH 8.1 for 4 hr. The digest was separated into two bands (R_F 0.20 and 0.50) by paper chromatography with butan-1-ol-acetic acid-water as solvent. The separated peptides migrated as single components on electrophoresis at pH 6.5, and amino acid analysis indicated that these peptides were peptides $BHA1_{T1}$ and $BHA1_{T2}$ respectively (Table 2).

Fig. 2. Elution diagram of an acetic acid hydrolysate of the aminoethylated B chain (0.4 μ mole) on a column (80 cm. $\times$ 1.3 cm.) of Sephadex G-25 in 0.2N-acetic acid. The flow rate was 12 ml./hr. and the fraction volume 4 ml. A portion of each fraction was taken and measurements of E_{570} were carried out after alkaline hydrolysis and reaction with ninhydrin, as described by Grant & Reid (1968). —, $E_{232}^{1cm.}$; ----, $E_{570}^{1cm.}$. Subsequently the fractions within the horizontal bars were pooled.

The rate of release of free amino acids from peptide $BHA1_{T1}$ on treatment with a mixture of carboxypeptidase A and B was measured by amino acid analysis of portions of the digest over 3 hr. The results indicated that -His-Leu-AEC was the C-terminal sequence. The electrophoretic behaviour of peptide $BHA1_{T1}$ at pH 6.5 indicated that the peptide contained a glutamine rather than a glutamic acid residue. Attempts to confirm this conclusion more directly by amino acid analyses after complete digestion of the peptide with aminopeptidase were not successful. After digestion for 6 hr. only methionine, alanine and traces of proline could be detected. It is known that the rate of hydrolysis of prolyl bonds by mammalian aminopeptidase is relatively slow (Hill & Schmidt, 1962) and this direct approach was accordingly abandoned.

Peptide $BHA1_{T2}$ gave a yellow-brown colour with ninhydrin that suggested glycine as the N-terminal amino acid. This was confirmed by sequential Edman degradation of the peptide, which indicated that Gly-Ser-His- was the N-terminal sequence.

Peptide BHA2. The amino acid composition of this peptide (Table 2) indicated that it contained portions of two adjacent peptides whose amino acid sequences were established by Grant & Reid (1968).

Table 2. *Amino acid composition and properties of B chain of insulin and of derived peptides*

Experimental details are given in the text and in Table 1. ND, Not determined.

Peptide ... Amino acid	Amino acid composition (ratios)					
	B chain	BHA1	BHA2	BHA3	BHA1T ₁	BHA1T ₂
Asp	3.26	—	—	1.23	—	—
Ser	1.04	0.74	—	—	—	0.72
Glu	1.32	1.00	—	—	1.00	—
Pro	2.86	1.69	—	0.90	2.38	—
Gly	2.76	1.09	1.14	1.21	—	0.92
Ala	2.14	0.98	0.89	—	0.94	—
Val	1.83	0.86	1.00	—	—	0.92
Met	1.15	0.92	—	—	0.91	—
Ile	—	—	—	—	—	—
Leu	4.35	2.18	1.70	—	1.00	1.00
Tyr	1.58	—	0.87	0.82	—	—
Phe	2.00	—	—	1.96	—	—
Lys	1.25	—	—	1.11	—	—
His	1.60	2.01	—	—	1.18	1.23
Arg	1.11	—	—	1.00	—	—
AEC	1.56	0.76	0.70	—	0.80	—
Total residues	30	13	7	8	8	5
Yield (% of theoretical)	80	92	62	62	50	60
Mobility of peptide	ND	0.52	0.37	0.47	0.60	0.42
Terminal amino acids	<i>N</i> -Met	Met	Ala	Arg	Met	Gly
	<i>C</i> -Lys	Val	Gly	Lys	AEC	Val

Peptide BHA3. Treatment of this peptide for 6 hr. with aminopeptidase resulted in complete digestion, as indicated by amino acid analysis. One mole of asparagine was present per mole of lysine and no aspartic acid was detectable in the digest.

DISCUSSION

This study of amino acid sequences in cod insulin was facilitated by the observations that the arginine-proline bond in an AE peptide (peptide AP1, Fig. 3) was insensitive to trypsin, and that an AEC-histidine bond was hydrolysed at a slower rate than other AE-cysteinyll bonds in the same peptide. The insensitivity of the arginine-proline bond was not unexpected, since peptide bonds formed between basic amino acids and proline in various proteins have been found to be resistant to tryptic hydrolysis (Hirs, Moore & Stein, 1960; Leonis, Li & Chung, 1959; Harris & Roos, 1959; Edmundson, 1963; Piggot & Press, 1967). The slow hydrolysis of an AE-cysteinyll bond has not previously been reported, but the observed rate at an enzyme/substrate ratio about 1:100 (w/w) was almost certainly influenced by the basic nature of the amino acid residues in the immediate vicinity of the bond. For example, the presence of adjacent acidic or basic groups has been found to decrease the

the rate of hydrolysis of trypsin-sensitive bonds in haemoglobin (Schroeder, Shelton, Shelton, Cormick & Jones, 1963) and in ribonuclease (Plapp, Raftery & Cole, 1967). In addition, the ready hydrolysis of the AEC-AEC bond in the sequence -AEC-AEC-His- of peptide AP1 (Fig. 3) would result in the formation of a new peptide with a sequence beginning with AEC-His. It is reasonable to assume that an *N*-terminal AE-cysteinyll bond would be resistant to trypsin as are *N*-terminal arginyl and lysyl bonds in synthetic (Van Orden & Smith, 1954) and natural peptides (Hirs *et al.* 1960; Canfield, 1963).

The amino acid sequences and the locations of amide groups of cod insulin now found, together with the sequences determined previously (Grant & Reid, 1968), permit the definition of the complete structures of both peptide chains of cod insulin (Fig. 4). Partial amino acid sequences of the insulin of other fishes such as bonito (Kotaki, 1962, 1963), bonito and tuna (Satake, Tanaka & Haniu, 1967), angler-fish (Humbel & Crestfield, 1965) and toad-fish (Smith, 1966) show a marked similarity to that of cod. In particular, when comparison is made with the sequences and sequential numbering of the residues of the ox B chain it is necessary to align the B chain of cod as shown in Fig. 4, since fish insulins such as cod, angler-fish and toad-fish all contain

Fig. 3. Newly proposed amino acid sequences and the location of amide groups in AE chains of cod insulin. These are shown in **bold type**. Evidence for the other sequences was given by Grant & Reid (1968). The vertical lettered arrows indicate peptide-bond cleavage by trypsin (T), pepsin (P), or on hydrolysis by acetic acid (HA). The horizontal bars designate peptides isolated and analysed as described in the text. The symbols → indicate *N*-terminal analyses by the Edman procedure; ← ← represent *C*-terminal analysis with carboxypeptidase A and B.

A chain	
Cod	Gly-Ile-Val-Asp-Gln-Cys-Cys-His-Arg-Pro-Cys-Asp-Ile-Phe-Asp-Leu-Gln-Asn-Tyr-Cys-Asn
Ox	—Glu—Ala-Ser-Val—Ser-Leu-Tyr-Gln—Glu— 4 8 9 10 12 13 14 15 17
B chain	
Cod	Met-Ala-Pro-Pro-Gln-His-Leu-Cys-Gly-Ser-His-Leu-Val-Asp-Ala-Leu-Tyr-Leu-Val-Cys-Gly-Asp-Arg-Gly-Phe-Phe-Tyr-Asn-Pro-Lys
Ox	Phe-Val-Asn—Glu—Glu—Thr—Ala 0 1 2 3 13 21 27 30

Fig. 4. Comparison of amino acid sequences of the A and B chains of cod and ox insulin. The sequences of the ox chains are identical with that of cod except in the positions indicated.

an additional *N*-terminal residue. At the same time a residue corresponding to B30 of the ox chain is not present. Methionine is the *N*-terminal residue of the B chain of both toad-fish (Smith, 1966) and cod (Grant & Reid, 1968) insulin. Apart from the β -chains of sheep (Boyer, Hathaway, Pascasio, Bordley & Orton, 1967) and ox haemoglobin (Schroeder, Shelton, Shelton, Robberson & Babin,

1967), there are no previous reports of *N*-terminal methionine residues in other specific proteins of vertebrate tissues. In bacteria, however, an *N*-terminal methionine residue is present in the A protein of tryptophan synthetase (Yanofsky, Drapeau, Guest & Carlton, 1967) and in other proteins of *Escherichia coli* (Waller, 1963). These observations may be connected with the role of

N-formylmethionyl-transfer-RNA as a chain initiator in bacterial protein synthesis (Clark & Marcker, 1965; Bruton & Hartley, 1968), where the formyl group rather than the *N*-formylmethionine residue is cleaved from the nascent protein (Weissbach & Redfield, 1967).

The potency of cod insulin as judged by bioassays with rat epididymal fat pad (Grant & Reid, 1968) and the effect on the blood glucose concentration of guinea pigs (Dr F. Webb, personal communication) is of the same order as that found in the mouse-convulsion procedure. In two different preparations, the potency of cod insulin by the mouse-convulsion bioassay was determined to be 11.5 i.u./mg. (95% probability limits 10.2–12.8) (Grant & Reid, 1968) and 15.2 i.u./mg. (95% probability limits 12.9–18.0) (Dr F. Webb, personal communication) compared with a standard preparation of bovine insulin with a potency of 25 i.u./mg. However, insulin isolated by an unspecified procedure from cod caught in North American waters has been reported to have a potency of about 20 i.u./mg. in the same bioassay system (Wilson & Dixon, 1961; Falkmer & Wilson, 1967). Apart from differences in technique and in insulin standards, the possibility cannot be completely excluded that there are two types of cod insulin. Alternatively, the values reported here may be minimal owing to an inhibitory effect on the bioassay procedure of some chemical modification of the molecule that occurred during the isolation procedure. The analytical results make the latter possibility unlikely except perhaps for an oxidation of the *N*-terminal methionine to the corresponding sulfoxide (Grant & Reid, 1968).

Most mammalian insulins, such as those of ox, sheep, pig, horse and whale, differ only in the nature of amino acids at positions 8, 9 and 10 of the A chain (Brown, Sanger & Kitai, 1955; Harris, Sanger & Naughton, 1956). A comparison of the sequences of cod and ox insulins (Fig. 4) shows that there are nine differences between the A chains and eight between the B chains. These substitutions do not effect a dramatic decrease in the activity of cod insulin, and it seems that the essential features for biological activity are largely present in the invariant residues common to both insulins. It is generally presumed that the nature of these residues confers the correct configuration of the molecule, and the invariant location of cysteine residues of all insulins, together with the lack of biological activity of reduced chains of ox insulin (Dixon & Wardlaw, 1960), indicates that the inter- and intra-chain disulphide bonds have an essential but not exclusive role in this respect. Other features, such as a *C*-terminal asparagine or aspartic acid residue in the A chain and the nature of the heptapeptide after arginine in the B chain of ox insulin,

are also necessary for a complete expression of biological activity (Carpenter, 1966).

The existence and nature of a catalytic centre in the insulin molecule has often been discussed. Reiser (1966) attempted to implicate a histidine-serine interaction from a comparison of the proteolytic action of insulin with the insulin-like activity of chymotrypsin and from the effects of photo-oxidation on the enzyme-like and hormonal activities of ox insulin. He concluded that there was some significance in the somewhat similar relationships between interchain disulphide bridges, histidine residues and serine residues in insulin and chymotrypsin, in particular the normally invariant histidine residues at positions 5 and 10 of the B chain in relation to the serine residues at positions A9 and A12 of ox insulin. The serine residue at position A12 is replaced by aspartic acid in cod insulin, and further support for this important but speculative concept may be the substitutions in other insulins of very low biological activity. For example, the insulin of guinea pig, with about one-quarter of the biological activity of ox insulin, has only one histidine residue in the B chain, the histidine residue at position B10 being replaced by aspartic acid, and the serine residue at position A12 is replaced by a threonine residue (Smith, 1966). Moreover, the insulin of the hag-fish [2 i.u./mg. by epididymal-fat-pad assay (Weitzel, Stratling, Jurgen & Martini, 1967); 0.8–1.13 i.u./mg. by mouse-hemidiaphragm assay (Falkmer & Wilson, 1967)] contains only one histidine residue and no serine in the amino acid analysis of the B chain (Weitzel *et al.* 1967).

The most profound differences between cod and ox insulin (Fig. 4) are at positions 8–10 and 12–15 of the A chain and at positions 0, 1, 2 and 3 of the B chain. These substitutions may affect the biological activity of cod insulin when tested in mammalian systems, and they probably account for the very poor antigenic reaction of cod insulin with antiserum prepared against ox insulin; on a weight for weight basis, cod insulin has an apparent activity of less than 0.2 i.u./mg. in the double-antibody immunoassay procedure for mammalian insulins (Dr F. Webb, personal communication). Wilson, Aprile & Sasaki (1967) showed that the ability of either peptide fragments derived from ox insulin or similar synthetic peptides to induce passive cutaneous anaphylaxis in the guinea pig is consistent with the main antigenic loci of bovine insulin being contained in peptides that comprise residues 10–21 of the A chain and residues 1–8 of the B chain. At the same time another antigenic locus must be contained by residues 8, 9 and 10 of the A chain, since mammalian insulins that differ only in these positions can be distinguished immunochemically (Berson & Yalow, 1959).

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