taken into account when considering possible configurations for the polypeptide chain in this area. It is interesting that the ring is the same size as the similar disulphide ring found in oxytocin (Tuppy & Michl, 1953; du Vigneaud, Ressler & Trippett, 1953) and vasopressin (Acher & Chauvet, 1953; du Vigneaud, Lawler & Popenoe, 1953), which suggests that it may have a possible structural or biological significance.

SUMMARY

1. Insulin was subjected to partial hydrolysis with chymotrypsin, with a crude pancreatic extract and with acid under conditions in which the disulphide bonds were stable.

2. Cystine-containing peptides in the hydrolysates were separated and their structure determined after oxidation to cysteic acid peptides. Paper ionophoresis at high potential gradients in pyridine-acetic acid buffers was found useful for the separations.

3. From the structure of the cystine peptides the distribution of the disulphide bonds of insulin was deduced and is shown in Table 10.

A.P.R. is indebted to the Medical Research Council for a Scholarship for training in research methods.

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The Structure of Pig and Sheep Insulins

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Insulins from a variety of different animal species show the same biological activity (Scott & Fisher, 1940) and immunological behaviour (Wasserman & Mirsky, 1942). They have the same crystalline form and mixtures of different insulins behave as a single substance in the phase-rule solubility test (Lens & Evertzen, 1952). However, in a preliminary chemical study of pig and sheep insulin it was shown that, whereas the general structure was similar, there were certain differences in individual amino acid residues (Sanger, 1949*b*). Harfenist & Craig (1952) have recently analysed the different insulins, and found differences in the contents of the amino acid residues given in Table 1. No differences were found for the other amino acids.

The complete sequence of amino acids in cattle insulin has recently been determined in this laboratory (Sanger & Tuppy, 1951*a*, *b*; Sanger & Thompson, 1953*a*, *b*), and the present paper describes similar studies on pig and sheep insulins. Since the larger part of the molecule was the same for all three species it was justifiable not to determine the amino acid sequence unequivocally in each case, but only to identify lower peptides embodying each residue in the molecule. Where

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these were identical with the corresponding peptides from cattle insulin it could be assumed that they were derived from identical sequences. Any differences in the original insulins were readily evident from the peptides produced.

Table 1. *Analysis of different insulins (Harfenist & Craig, 1952)*

Results expressed as amino acid residues/molecule of insulin.

Amino acid	Cattle insulin (mol.wt. 5734)	Sheep insulin (mol.wt. 5704)	Pig insulin (mol.wt. 5778)
Serine	2·89	2·07	2·79
Threonine	0·97	0·96	1·77
Glycine	3·94	4·70	3·94
Alanine	2·91	2·99	2·17
Valine	4·68	4·80	3·68
Isoleucine	0·66	0·69	1·54

After splitting the insulins into the glycyl (fraction *A*) and phenylalanyl (fraction *B*) chains by oxidation (Sanger, 1949*a*) each chain was hydrolysed with enzymes. The resulting large peptides were fractionated by paper ionophoresis and subjected to complete hydrolysis. Some were also subjected to partial acid hydrolysis to give small peptides, which were separated on paper chromatograms. In this way it was possible to obtain peptides representing all of the residues in the insulin chains.

Peptides with reference numbers starting *A* and *B* refer to those obtained from cattle insulin and described by Sanger & Thompson (1953*a*, *b*) and Sanger & Tuppy (1951*a*, *b*).

MATERIALS

Various preparations of pig insulin have been used throughout this work:

(1) Crystalline pig insulin obtained from Dr J. Lens of N. V. Organon, Holland, as used by Lens & Evertzen (1952).

(2) Crystalline pig insulin, N. V. Philips-Roxane, Weesp, Holland.

(3) Novo pig insulin (Novo Terapeutisk A/S, Copenhagen), several-times recrystallized.

(4) Crystalline insulin (pig pancreas), batch no. E178, Allen and Hanburys Ltd. and The British Drug Houses Ltd.

(5) A freeze-dried preparation of pig insulin obtained from Eli Lilly Company, Indianapolis, U.S.A.

In most of the work reported in this paper sample 2 was used, though some of the earlier preliminary experiments were carried out with sample 5. The other samples were used only in the experiment recorded in Fig. 5.

The sheep insulin used was crystalline insulin (sheep pancreas) batch E73, Allen and Hanburys Ltd. and The British Drug Houses Ltd.

The cattle insulin was batch 9011G (six-times recrystallized), Boots Pure Drug Company (Nottingham).

METHODS

Oxidation

Oxidation and fractionation of the oxidized insulin was carried out as described for cattle insulin (Sanger, 1949*a*). The fractions were found to behave similarly except that the fraction *A* of pig insulin was largely insoluble in 25% (w/v) ammonium acetate and was precipitated during the final stage of the purification. This precipitate, which contained no detectable phenylalanyl *N*-terminal residue, was used as fraction *A* in most of the following work. The fractions *A* and *B* from pig and sheep insulins are referred to below as P*A*, P*B*, and S*A*, S*B*, respectively.

Enzymic hydrolyses

The treatment of the fractions with proteolytic enzymes was carried out as described in previous papers (Sanger & Tuppy, 1951*b*; Sanger & Thompson, 1953*b*). The hydrolysates were fractionated by ionophoresis under toluene in the apparatus of Michl (1951) (see Ryle, Sanger, Smith & Kitai, 1955). Approx. 10 mg. of hydrolysate were fractionated on a 18 cm. wide sheet of Whatman no. 3 filter paper. After ionophoresis and drying, marker strips were cut out and tested with ninhydrin (Toennies & Kolb, 1951), the Sakaguchi reaction as modified by Jepson & Smith (1953) and by the following modification of the Pauly test.

Pauly test on filter paper. Equal volumes of 5% (w/v) $NaNO_2$ and of 1% (w/v) sulphanilic acid in 10% (w/v) HCl were mixed and applied to a clean strip of no. 3 filter paper on a glass plate, so that the paper was wet all over but no puddles were formed. The strip to be tested was then pressed on to the wet strip with another glass plate so that the reagent was evenly blotted on to it. It was removed and allowed to dry in air for 10 min. It was then treated in the same way with a solution of 20% (w/v) Na_2CO_3. Histidine peptides give a bright orange red, tyrosine peptides a brownish red. This test is more sensitive than the one used previously (Sanger & Tuppy, 1951*a*), although rather more tedious. The above 'blotting' technique has been found to be a convenient method of testing papers with water-soluble reagents.

Peptides studied. Peptides P*A*p5 and S*A*p5 were prepared by ionophoresis in 0·2N acetic acid of pepsin hydrolysates of the corresponding oxidized insulin as described by Sanger, Thompson & Kitai (1955).

Partial acid hydrolysis of peptides from enzymic hydrolysates

Material eluted from an ionophoretic fractionation of 10–20 mg. of one of the oxidized fractions was subjected to partial hydrolysis in 12N-HCl for 3 days at 37°. After removal of HCl the small peptides were fractionated by two-dimensional chromatography on a sheet of Whatman no. 4 filter paper using phenol–3% aq. NH_3 and *n*-butanol–acetic acid–water (4:1:5, by vol.) as solvents. The spots were located by spraying with 0·025% ninhydrin in *n*-butanol and their amino acid composition determined after complete hydrolysis in the usual manner (Sanger & Tuppy, 1951*a*).

RESULTS

N-*Terminal residues*

By the method of forming 2:4-dinitrophenyl (DNP) derivatives (Sanger, 1945) it was shown that both insulins contained glycine and phenylalanine as *N*-terminal residues.

The phenylalanyl chains (fractions P*B*, S*B*)

Peptic hydrolysates (P*B*p and S*B*p). Fig. 1 shows an ionophoretic separation of the peptic hydrolysates of the *B* fractions of cattle (*B*p), pig (P*B*p) and sheep (S*B*p) insulin. It can be seen that the hydrolysates appear indistinguishable, indicating at least that the distribution of charged groups and of groups attacked by pepsin are the same for all three fractions. In fact, their structures were found to be identical. For this reason only the results with fraction P*B* will be described, since fraction S*B* was investigated by essentially the same methods and gave the same results. Table 2 shows the amino acids given on hydrolysis of the various peptides from P*B*p and the structure of the corresponding peptides from cattle insulin, or, if the peptide had not previously been encountered, the presumed structure.

Peptides P*B*p7 and P*B*p10 were subjected to further partial hydrolysis with acid. The results are given in Tables 3 and 4. The R_F values on the chromatograms were the same as those for the corresponding peptides from cattle insulin, which are also listed in the Tables.

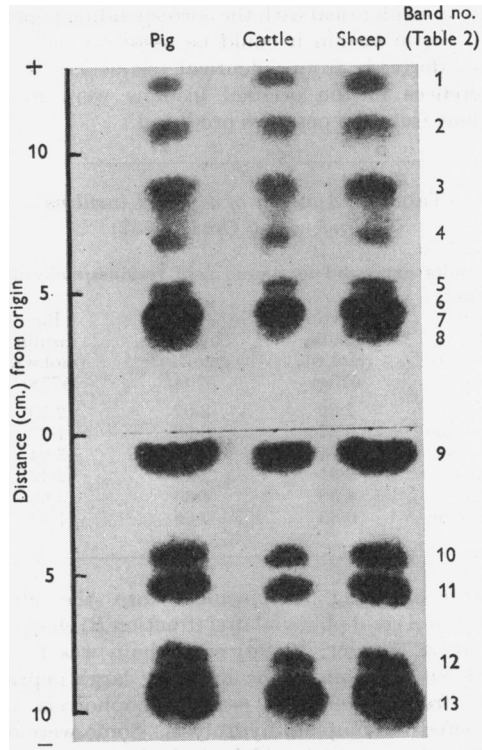


Fig. 1. Ionophoretic separations of peptic hydrolysates of *B* fractions of cattle (*B*p), pig (P*B*p) and sheep (S*B*p) insulins. Pyridine–acetate buffer (pH 6·5); 28 v/cm.; 3 hr. (cf. Ryle *et al.* 1955). Coloured with ninhydrin.

Table 2. *Peptides from peptic hydrolysate of fraction* P*B*

Peptide (Fig. 1)	Amino acids found on hydrolysis	Corresponding peptide from cattle insulin (Sanger & Tuppy, 1951*b*)
1	Glu, Val	Val.Glu (in *B*p1)
2	Glu, Ala, Val	Val.Glu.Ala (*B*p1)
3	Glu, Ala, Val, Leu	Val.Glu.Ala.Leu (*B*p2) (see footnote)
4	Glu, Ala, Val, Leu	See footnote
5	As for 6	See footnote
6	$CySO_3H$, Glu, Gly, Val, Leu, Phe, Arg	Leu.Val.$CySO_3H$.Gly.Glu.Arg.Gly.Phe (*B*p4)
7	$CySO_3H$, Glu, Gly, Tyr, Val, Leu, Phe, Arg	Tyr.Leu.Val.$CySO_3H$.Gly.Glu.Arg.Gly.Phe
8	As for 6	See footnote
9	All residues found in fraction B	Mixture of neutral peptides and amino acids
10	$CySO_3H$, Ser, Gly, Leu, His	His.Leu.$CySO_3H$.Gly.Ser.His.Leu (*B*p17)
11	$CySO_3H$, Asp, Glu, Ser, Gly, Val, Leu, Phe, His	Phe.Val.Asp.Glu.His.Leu.$CySO_3H$.Gly.Ser.His.Leu (*B*p3)
12	Thr, Ala, Tyr, Pro, Lys (Phe)	See footnote
13	Thr, Ala, Tyr, Pro, Lys	Tyr.Thr.Pro.Lys.Ala (*B*p15)

3. Partial acid hydrolysis on a small amount revealed the presence of [Ala, Leu] (P*B*p3a) corresponding to Ala.Leu (*B*1δ11).

4. This was present in only trace amounts, so analysis was not certain. It may contain tyrosine, in which case it could be Val.Glu.Ala.Leu.Tyr.

5 and 8. These were present in small amounts and probably differ from 6 and 7 by containing an extra residue on the *N*- or *C*-terminal position.

12. Present only in traces. In one experiment with P*B*p phenylalanine was detected, suggesting the structure Phe.Tyr.Thr.Pro.Lys.Ala, but there was not sufficient material to confirm this.

Table 3. *Peptides from partial acid hydrolysate of peptide* P*B*p7

Peptide	Amino acids found on hydrolysis	Corresponding peptide from cattle insulin
a	$CySO_3H$, Gly	$CySO_3H$.Gly (*B*1α1)
b	Glu, Gly	Gly.Glu (*B*1δ1)
c	$CySO_3H$, Glu, Gly, Val	Val.$CySO_3H$.Gly (*B*1α3) + free glutamic acid
d	$CySO_3H$, Val	Val.$CySO_3H$ (*B*1α4)
e	Glu, Arg	Glu.Arg (*B*1γ3)
f	Glu, Gly, Arg	[Glu, Arg, Gly] (*B*1γ2)
g	Gly, Arg	Arg.Gly (*B*2γ5)
h	$CySO_3H$, Val, Leu	Leu.Val.$CySO_3H$ (*B*1α8)
i	$CySO_3H$, Gly, Val, Leu	Leu.Val.$CySO_3H$.Gly (*B*1α7)
j	$CySO_3H$, Tyr, Val, Leu	Tyr.Leu.Val $CySO_3H$ (*B*2α21)
k	Gly, Phe	Gly.Phe (*B*1γ12)

Table 4. *Peptides from partial acid hydrolysate of peptide* P*B*p10

Peptide	Amino acids found on hydrolysis	Corresponding peptide from cattle insulin
a	$CySO_3H$, Gly	$CySO_3H$.Gly (*B*1α1)
b	$CySO_3H$, Leu	Leu.$CySO_3H$ (*B*1α6)
c	$CySO_3H$, Leu, His	His.Leu.$CySO_3H$ (*B*1γ4)
d	Ser, His	Ser.His (*B*2γ2)
e	Ser, Leu, His	Ser.His.Leu (*B*2γ7)
f	Leu, His	His.Leu (*B*2γ8)

Tryptic hydrolysates (P*B*t and S*B*t). Samples (30 mg.) of fractions P*B* and S*B* were treated for 24 hr. with trypsin at pH 8, and the water-soluble material subjected to ionophoresis at pH 6 on a 20 cm. wide strip of no. 3 filter paper for 3 hr. at 1500 v. Two ninhydrin-reacting bands were present, one (P*B*t1) had moved 6 cm. towards the cathode and was free alanine, the other (P*B*t2) had moved 17·5 cm. towards the cathode and gave on hydrolysis [Gly, Thr, Tyr, Phe, Pro, Lys]. It corresponded to the cattle peptide (*B*t2) Gly.Phe.Phe.Tyr.Thr.Pro.Lys, and was identical from both pig and sheep material. In order to determine the number of phenylalanine residues present samples of the hydrolysates were treated with fluorodinitrobenzene and assayed by the method of Levy (1954). It was found that there was approximately twice as much phenylalanine present as glycine or threonine.

The glycyl chain of pig insulin (fraction P*A*)

The results with fraction P*A* will be described as single experiments in which peptides derived from enzymic hydrolysates are subjected to further hydrolysis with acid, although in fact many of the peptides were obtained initially from direct acid hydrolysis of the fraction *A*.

Partial hydrolysis of peptide P*A*p5. Peptide P*A*p5 derived from 25 mg. pig insulin was subjected to partial acid hydrolysis and the hydrolysate fractionated by paper chromatography in the usual manner. Fig. 2 is a diagram of the chromatogram, and in Table 5 are given the analyses of the various spots.

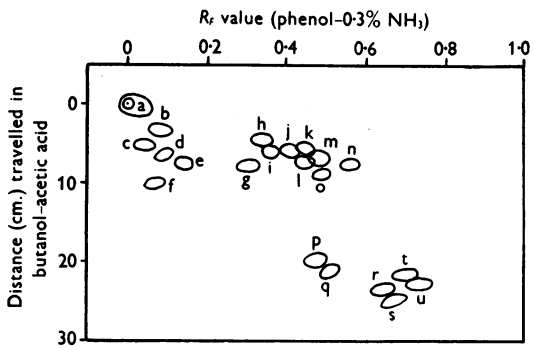


Fig. 2. Chromatogram of partial acid hydrolysate of peptide P*A*p5. The material was applied in the position marked ⊙ and run first in phenol–0·3% NH_3 and then in butanol–acetic acid (solvent front moved approx. 40 cm.) (see Table 5).

Chymotryptic hydrolysate. (P*A*c) Fraction P*A* (10 mg.) was treated with chymotrypsin (0·5 mg.) at pH 7·5 and the hydrolysate subjected to ionophoresis at pH 6·5 for 3 hr. using 1000 v. The bands were distributed as shown in Fig. 3 and their composition is given in Table 6. Peptides 1, 2 and 3 were subjected to partial acid hydrolysis. Peptide 1 gave rise to the same peptides that were obtained from P*A*p5 (Table 5) together with [Ser, Tyr, Leu] corresponding to *A*1a19 (Ser.Leu.Tyr) and [$CySO_3H$, Asp] presumably due to the presence of $CySO_3H$.Asp (*A*c1) in band P*A*c1. The peptides detected in the hydrolysate of P*A*c2 are listed in Table 7. With the exception of a, b and d the same peptides were obtained from P*A*c3.

The glycyl chain of sheep insulin (fraction S*A*)

Partial hydrolysis of peptide S*A*p5. In Fig. 4 and Table 8 are shown the results obtained with a partial acid hydrolysate of peptide S*A*p5 prepared

Table 5. *Peptides from partial acid hydrolysate of peptide* P*A*p5

Peptide (Fig. 2)	Amino acids found on hydrolysis	Strength	Corresponding peptide from cattle insulin
a	$CySO_3H$	×	Glu.$CySO_3H$.$CySO_3H$
	Glu	×	
	Ala	?	
b	$CySO_3H$	× × ×	Cysteic acid
c	$CySO_3H$	×	Glu.$CySO_3H$ (*A* 1α1)
	Glu	×	
d	$CySO_3H$	×	—
	Thr	×	
e	$CySO_3H$	?	$CySO_3H$.Ala (*A* 1γ3)
	Ala	?	
f	Glu	×	Glu.Glu (*A* 1β4)
g	Glu	× × ×	Free glutamic acid
h	$CySO_3H$	×	Ser.Val.$CySO_3H$ (*A* 1ζ2)
	Ser	×	
	Val	×	
i	Ser	× ×	Free serine
j*	Gly	× × ×	Free glycine
k*	$CySO_3H$	× × ×	—
	Ser	× × ×	
	Thr	× × ×	
	Ileu	× × ×	
l*	$CySO_3H$	× × ×	—
	Ser	× × ×	
	Ileu	× × ×	
m*	Thr	× ×	Free threonine
n	Ala	×	Free alanine
o	$CySO_3H$	×	—
	Ileu or Leu	×	
p	Glu	× ×	Gly.Ileu.Val.Glu.Glu (*A* 1a15)
	Gly	×	
	Val	×	
	Ileu	×	
q	Glu	× ×	Ileu.Val.Glu.Glu (*A* 1a16)
	Val	×	
	Ileu	×	
r	Glu	×	Gly.Ileu.Val.Glu (*A* 1η3)
	Gly	×	
	Val	×	
	Ileu	×	
s	Glu	×	Ileu.Val.Glu (*A* 1η4)
	Val	×	
	Ileu	×	
t	Ser	× × × ×	Ser.Leu (*A* 1ι7)
	Leu	× × × ×	
u	Leu + Ileu	× × ×	Free leucine and isoleucine

* Spots j, k, l and m overlapped considerably on this chromatogram, but could be obtained pure by developing longer with both solvents. Peptides k and l were in fact first obtained pure from a fraction of cysteic acid peptides prepared by ionophoresis in 0·2N acetic acid of a partial acid hydrolysate of fraction P*A* (as in Expt. *A* 2 of Sanger & Thompson, 1953*a*).

in the same way as the partial hydrolysate of P*A*p5 (above).

Chymotryptic hydrolysate (S*A*c). Hydrolysis of fraction S*A* with chymotrypsin yielded peptides similar to those obtained from fraction P*A* (Fig. 3), and partial hydrolysis of bands S*A*c2 and S*A*c3 produced the same peptides as P*A*c2 and P*A*c3 respectively (Table 7). Partial hydrolysis of S*A*c1 gave rise to the same peptides as S*A*p5 (Table 8) together with Ser.Leu.Tyr and $CySO_3H$.Asp.

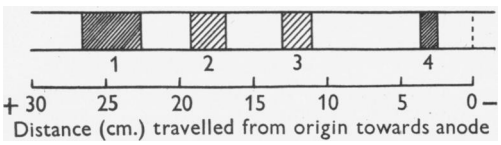


Fig. 3. Ionophoresis of chymotryptic hydrolysate of fraction *A* of pig insulin (P*A*c): pyridine–acetate buffer, pH 6·5; 28 v/cm.; 3 hr.; bands located by ninhydrin reaction (see Table 6).

summarized in Table 9, where the peptides identified in the present work are indicated by underlining the corresponding sequence in the formula for cattle fraction *B*. All these peptides were in fact derived from peptic hydrolysates of fraction *B* (P*B*p and S*B*p). The reference numbers refer to those used in Tables 2–4.

In order to show that a particular position in the chain is occupied by the same amino acid residue in the pig or sheep as in the cattle insulin, we have considered it necessary to detect the amino acid in a characteristic small peptide. Such a peptide must have a sequence which only occurs once in the chain and must contain only one residue of the amino acid concerned. This does not give an absolute proof of the structure, but it would be an extreme coincidence if any difference were not detected. In the following discussion only the pig insulin will be considered. The results with the sheep were identical.

Table 6. *Peptides from chymotryptic hydrolysate of fraction* P*A*

Peptide (Fig. 3)	Amino acids found on hydrolysis	Corresponding peptide in cattle insulin
1	$CySO_3H$, Asp, Glu, Ser, Gly, Thr, Ala*, Tyr, Val, Leu, Ileu	Mixture of *A*c4, *A*c2, *A*c1
2	$CySO_3H$, Asp, Glu, Tyr, Leu	Glu.Leu.Glu.Asp.Tyr.$CySO_3H$.Asp (*A*c3)
3	Asp, Glu, Tyr, Leu	Glu.Leu.Glu.Asp.Tyr (*A*c5)
4	Ser, Tyr, Leu	Ser.Leu.Tyr (*A*c6)

* Alanine present only in trace amounts.

Table 7. *Peptides from partial acid hydrolysate of peptide* P*A*c2

Peptide	Amino acids found on hydrolysis	Corresponding peptide from cattle insulin (Sanger & Thompson, 1953*a*)
a	$CySO_3H$, Asp	$CySO_3H$.Asp (*A* 1α2)
b	$CySO_3H$, Asp, Tyr	(Asp).Tyr.$CySO_3H$.(Asp) (*A* 1a4, *A* 1a5)
c	Asp, Glu	Glu.Asp (*A* 1β2)
d	$CySO_3H$, Tyr	Tyr.$CySO_3H$ (*A* 1a7)
e	Asp, Glu, Leu	Leu.Glu.Asp (*A* 1δ5)
f	Asp, Glu, Tyr	Glu.Asp.Tyr (*A* 1a11)
g	Asp, Tyr	Asp.Tyr (*A* 1a9)
h	Glu, Leu	Glu.Leu.Glu (*A* 1δ6)
i	Glu, Leu	Leu.Glu (*A* 1ε7)
j	Glu, Leu	Glu.Leu (*A* 1ε8)

DISCUSSION

The phenylalanyl chains

Fig. 1 shows that the ionophoretic separations of the peptic hydrolysates of the *B* fractions from cattle, pig and sheep insulins are identical, suggesting that their structures are the same. This is confirmed by the fact that all the small peptides found on partial hydrolysis of the pig and sheep material had also been obtained from cattle insulin. These results are

Position 1 is occupied by phenylalanine, since this is the *N*-terminal residue determined by the DNP method. Peptide P*B*p11 (Table 2) differs from peptide P*B*p10 in containing a single residue of phenylalanine, valine, aspartic and glutamic acids. These are the first four residues of the chain of cattle insulin and must therefore also be the first four of fraction P*B*. If the relative order of the residues was different, it would not have been detected, but this would seem to be rather an unlikely coincidence.

Positions 5–11 are well covered by the small peptides from partial hydrolysis of P*B*p10 (Table 4). It is interesting to note that the structure of peptide P*B*p10 could not have been determined from the peptides isolated, since the simpler structure Ser.His.Leu.$CySO_3H$.Gly would in fact fit the results.

Residues 12–15 are found in small peptides from the peptic hydrolysate, and residues 16–24 in peptides from the partial hydrolysate of P*B*p7. A characteristic peptide present in large amounts in the peptic hydrolysate is P*B*p13, which represents residues 26–30. Each residue occurs only once in the peptide, which is the same for cattle and pig insulin, provided there is no reversal of order. This leaves only residue 25 unaccounted for. However, that

this is phenylalanine may be concluded from the fact that peptide P*B*t2 contains only the expected amino acids and has twice as much phenylalanine as glycine or threonine. It may thus be concluded that the phenylalanyl chains of pig and sheep insulins are identical with the phenylalanyl chain of cattle insulin.

The glycyl chain of pig insulin

The results with the *A* fractions are summarized in Table 10. Small peptides were obtained to cover the whole of the chain and establish that positions 1–7 and 11–21 are occupied by the same amino acids in pig and cattle insulin.

Ignoring for the present trace amounts, certain peptides which had been found in cattle material

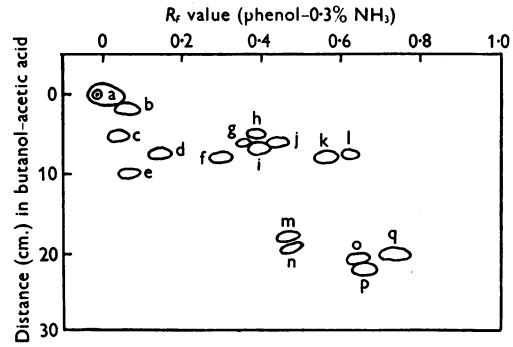


Fig. 4. Chromatogram of partial acid hydrolysate of peptide S*A*p5. Conditions as in legend to Fig. 2 (see Table 8).

Table 8. *Peptides from partial acid hydrolysate of peptide* S*A*p5

Peptide (Fig. 4)	Amino acids found on hydrolysis	Strength	Corresponding peptide from cattle insulin
a	$CySO_3H$	× × × ×	Mixture (*A* 2γ1, *A* 2δ1, etc.)
	Glu	× ×	
	Ala	× ×	
b	$CySO_3H$	× × ×	Free cysteic acid + $CySO_3H$.$CySO_3H$.Ala (*A* 2δ1)
	Ala	×	
c	$CySO_3H$	× ×	Glu.$CySO_3H$ (*A* 1α1)
	Glu	× ×	
d	$CySO_3H$	× ×	$CySO_3H$.Ala (*A* 1γ3)
	Ala	× ×	
e	Glu	×	Glu.Glu (*A* 1β4)
f	Glu	× × ×	Free glutamic acid
g	Ser	×	Free serine
h	$CySO_3H$	×	Ser.Val.$CySO_3H$ (*A* 1ζ2)
	Ser	×	
	Val	×	
i	$CySO_3H$	× × × ×	—
	Gly	× × × ×	
	Val	× × × ×	
j	Gly	× ×	Free glycine
k	Ala	× × ×	Free alanine
l	Gly	× ×	—
	Ala	× ×	
m	Glu	× ×	Gly.Ileu.Val.Glu.Glu (*A* 1a15)
	Gly	×	
	Val	×	
	Leu or Ileu	×	
n	Glu	× ×	Ileu.Val.Glu.Glu (*A* 1a16)
	Val	×	
	Leu or Ileu	×	
o	Glu	×	Gly.Ileu.Val.Glu (*A* 1η3)
	Gly	×	
	Val	×	
	Leu or Ileu	×	
p	Glu	×	Ileu.Val.Glu (*A* 1η4)
	Val	×	
	Leu or Ileu	×	
q	Ser	× × ×	Ser.Leu (*A* 1ι7) + leucine and isoleucine
	Leu or Ileu	× × × ×	

Table 9. *The structure of the phenylalanyl chains of pig and sheep insulins*

In the reference numbers, P*B* or S*B* is omitted, only the suffixes being given.

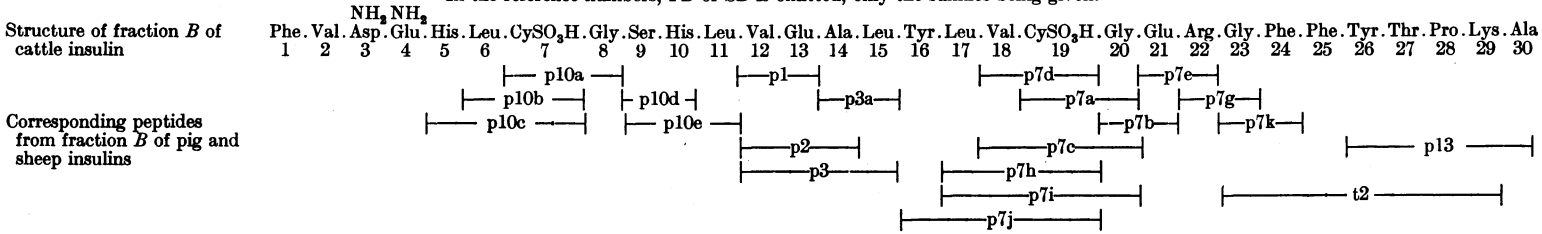
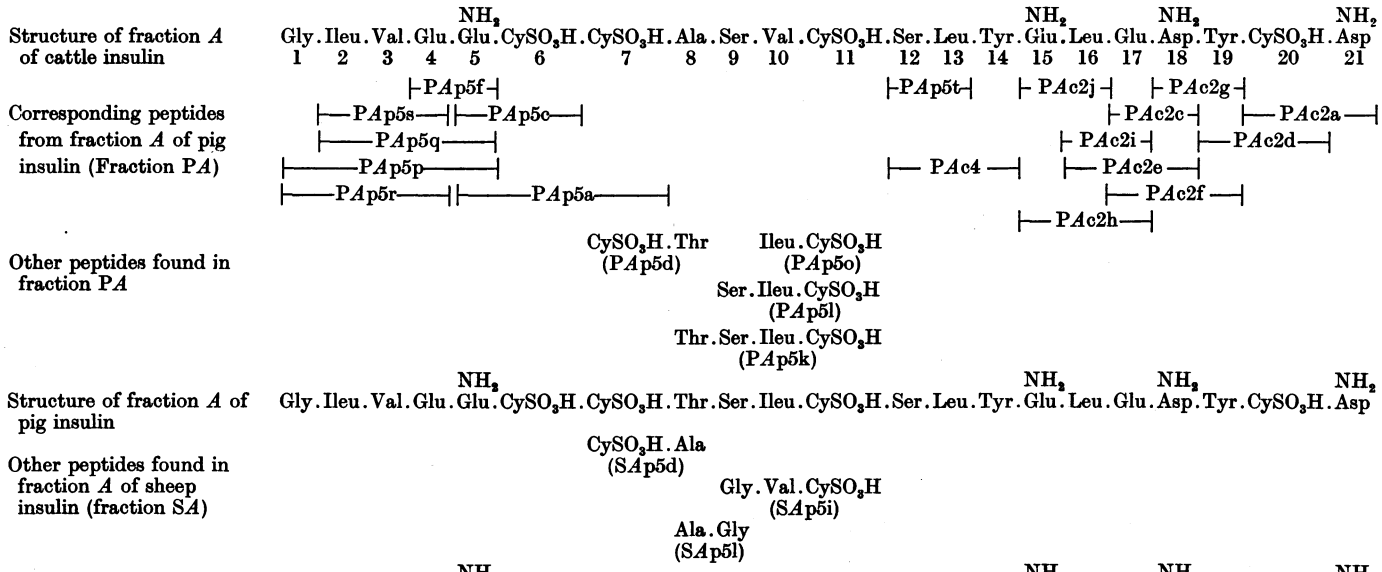


Table 10. *The structure of the glycyl chains of pig and sheep insulins*



were absent from pig insulin. These were $CySO_3H$.Ala, Val.$CySO_3H$ and Ser.Val.$CySO_3H$. In their places are found [$CySO_3H$, Thr] (P*A*p5d), [$CySO_3H$, Ileu] (P*A*p5o), [$CySO_3H$, Ser, Ileu] (P*A*p5l) and [$CySO_3H$, Ser, Thr, Ileu] (P*A*p5k). These sequences can only be explained if the amino acid sequence in positions 7–11 is $CySO_3H$.Thr.Ser.Ileu.$CySO_3H$ and the structure of the peptides is as shown in Table 10. Theoretically the reverse order is possible but such a structure would not give large amounts of [$CySO_3H$, Ser, Ileu] and [$CySO_3H$, Ser, Thr, Ileu] owing to the lability of bonds involving the amino groups of hydroxyamino acids. In this connexion it is interesting that the Thr.Ser bond found here is considerably more stable than the Ala.Ser bond which was never found intact after acid hydrolysis of cattle material.

The glycyl chain of sheep insulin

The sequence of amino acids in positions 1–7 and 11–21 is the same in the sheep insulin as for the cattle and pig insulin. $CySO_3H$.Ala (S*A*p5d) is also found, as it was found in cattle material, so that position 8 is occupied by alanine. The main difference between the sheep and cattle fraction *A* is that Ser.Val.$CySO_3H$ is virtually absent and in its place is found [$CySO_3H$, Gly, Val] (S*A*p5i), whose structure is thus most probably Gly.Val.$CySO_3H$. This is confirmed by the presence of the new peptide Ala.Gly (S*A*p5l). The sequence in positions 7–11 is thus $CySO_3H$.Ala.Gly.Val.$CySO_3H$.

General

Whereas the main peptides produced on partial hydrolysis of pig or sheep insulin were those listed in Table 10 which were characteristic of the particular species, it was always found that small amounts of the peptides characteristic of cattle insulin were also present. Thus, for instance, Ser.Ileu.$CySO_3H$ was obtained in high concentrations from fraction P*A*, but a small amount of Ser.Val.$CySO_3H$ was present in spot P*A*p5h. Similarly, preparations of fraction P*A* or P*A*p5 always contained some alanine.

To test if this was characteristic of all preparations of pig insulin peptide P*A*p5 was prepared from a number of different samples of pig insulin; the peptides were hydrolysed and the amino acids present separated on a phenol chromatogram. The results are shown in Fig. 5. All samples do in fact contain alanine, though in varying amounts. Sample 1, from which alanine is almost absent, was prepared by Lens & Evertzen (1952) and is probably the most rigorously purified. The other samples were commercially produced. Most of the work reported here was done with sample 2, which contains the most alanine of any. It seems probable that this phenomenon is due to contamination of the preparations with small amounts of cattle insulin. On the other hand, it is possible that the pig and sheep do actually produce more than one type of insulin. This effect has never been observed with cattle insulin. Within the limits of the methods used there is no threonine in the cattle fraction *A* or peptide *A*p5 and neither Ser.Ileu.$CySO_3H$ nor Gly.Val.$CySO_3H$ have been found, so that it would be strange if cattle produce a single unique insulin and pigs and sheep produce more than one.

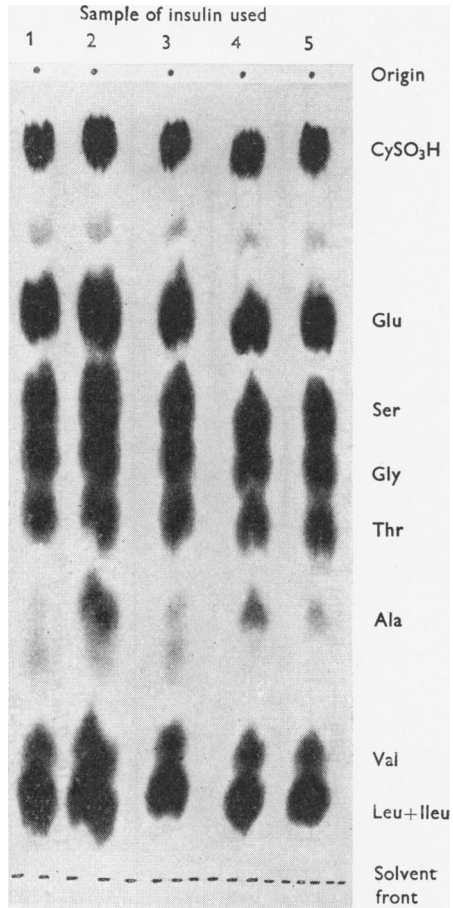


Fig. 5. Paper chromatograms on phenol–0·3% (w/v) aqu. NH_3 of hydrolysates of peptide P*A*p5 prepared from various samples of pig insulin. The chromatograms have been overloaded to show the presence of alanine.

It may, however, be noted that all the work on cattle insulin from this laboratory was done with insulin from the same source (six-times recrystallized insulin, Batches 9633B (Sanger & Tuppy, 1951*a*, *b*, and earlier papers) and 9011G (Sanger & Thompson, 1953*a*, *b* and later papers) from Boots Pure Drug Co., Nottingham). It is thus conceivable that if traces of insulins having a different structure

were present, they might have been lost during the recrystallizations. It is hoped to investigate this phenomenon further.

The above results are in qualitative agreement with the analyses of Harfenist & Craig (Table 1). The pig insulin differs from the cattle insulin in that an alanine and a valine residue are replaced by a threonine and an isoleucine residue respectively and the sheep insulin is the same as the cattle except that glycine replaces serine.

It is interesting that the only structural differences are in those residues which lie within the disulphide ring in insulin (Ryle *et al.* 1955). The fact that insulin is readily inactivated by any treatment that affects the disulphide bonds suggests that they may be concerned in its biological activity and it might seem that the part of the glycyl chain between positions *A*6–11, which contains three half-cystine residues, would be a physiologically important part of the molecule, perhaps the 'active centre'. However, it is clear from the present results that the activity of insulin is not dependent on the unique structure of the residues in these positions, since they can vary without any change in activity.

SUMMARY

1. The structures of pig and sheep insulin have been determined by subjecting them to partial hydrolysis and comparing the peptides produced with those obtained from cattle insulin.

2. The amino acid sequence in the phenylalanine chains is identical for all three insulins.

3. The glycyl chains are the same except for three residues in positions 8–10 inclusive. In the cattle insulin this sequence is Ala.Ser.Val, in the pig insulin Thr.Ser.Ileu and in the sheep insulin Ala.Gly.Val.

Some of the experiments reported here were carried out by advanced students during a postgraduate course in biochemistry. We wish to express our gratitude to them and to Dr J. Lens of N. V. Organon, Dr J. G. Faber of N. V. Philips-Roxane, Dr O. K. Behrens of the Lilly Research Laboratories and Mr F. A. Robinson of Allen and Hanburys Ltd. for samples of pig and sheep insulin.

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Component Fatty Acids of Indian Snake Oils

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Pollard & McLaughlin (1950) reported that the lobes of snakes were the main depots of fat which is characterized by its higher content of unsaturated fatty acids. They also observed in the depot fat of the cottonmouth moccasin (*Agkistrodon piscivorus*) the presence of arachidonic acid to the extent of 11%. The rest of the fat was largely saturated, and hence it did not resemble marine animal oils or higher land animal fats, where higher unsaturated acids are predominant.

Tsujimoto & Kobayaschi (1920) proved the presence of C_{20} and C_{22} unsaturated acids by forming their ether-insoluble bromo derivatives from python fat having an iodine value 80·3, while Gunstone & Paton (1953), using low temperature crystallization technique, confirmed that python fat was intermediate in character between higher land and marine animal fats.

In the present study additional work on the component fatty acids of Indian snake oils is reported.

EXPERIMENTAL

The snake oils from the python (*Python molurus*) and the diamond snake (*Python stinotis*) in the present study were kindly supplied to us by Dr J. N. Barowa of Victoria Gardens, Bombay. Both the oils were in a fresh state and