almost all the cytoplasmic granules visible under the phase microscope. Glucuronidase activity was distributed over granules of all sizes.

2. Making the preparation 0·1 M with respect to sodium chloride led to slight agglutination and much easier sedimentation of the granules and the associated enzyme activity.

3. Buffering the preparation to pH 4–6 with acetate led to pronounced agglutination of the granules, which then sedimented after 15 min. at 1500 *g*. Adjusting the pH to within these limits with acid alone led not only to agglutination of the granules, but to precipitation of part of the water-soluble enzyme. The latter effect was overcome by adding electrolyte, as in buffered preparations, or by warming.

4. Incubation in acetate buffer, pH 5·2, led to extraction of the enzyme from the granules. This did not occur on incubating an untreated homogenate, or after pH adjustment with acid alone.

5. Shaking a homogenate in a tissue disintegrator, or repeated freezing and thawing, caused disintegration of the granules and release of the associated enzyme to the solution. Homogenizing the tissue in acetone followed by resuspension in water had the same effect, but release of the enzyme was not seen when the acetone-dried powder was resuspended in acetate buffer.

6. Treatment with the surface-active agents, Teepol XL and Triton X-100, brought the granular enzyme into solution with disappearance of particulate matter from the preparation.

7. Glucuronidase was inhibited non-competitively by the anionic Teepol XL, but not by the non-ionic Triton X-100. Inhibition by Teepol was at first reversed by adding inactive protein, but later became irreversible.

The authors wish to express their gratitude to their colleague, Mr F. Baker, for instruction in the use of the phase microscope, and to the Rohm and Haas Company for gifts of Triton A-20 and X-100. Contact with this firm was made through the courtesy of Dr H. N. Glassman. One of us (P.G.W.) is in receipt of a grant from the Agricultural Research Council.

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Degradative Studies on the 'Core' Resulting from Chymotryptic Digestion of Insulin

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(*Received* 23 *January* 1951)

Previous work on the action of enzymes on insulin (Butler, Dodds, Phillips & Stephen, 1948) has shown that chymotryptic digestion releases a number of peptide fragments which are soluble in 0·25 N-trichloroacetic acid, leaving a 'core', with a molecular weight of approximately 5000, which is precipitated. Some properties of this core have already been described (Butler, Phillips, Stephen & Creeth, 1950).

The core can be oxidized by the method used by Sanger (1949*a*) for insulin, but application of Sanger's fractionation procedure did not separate the glycyl and phenylalanyl chains as in the original insulin. Difference in solubility at pH 6·5, which in oxidized insulin distinguishes the chains, does not occur among the oxidation products of the core, presumably because of the decreased length of the chains and the removal from the phenylalanyl chains of a large proportion of the basic amino-acids by enzymic action.

The oxidation products of the dinitrophenyl derivative of the core or the dinitrophenyl or benzoyl

derivatives of the oxidized core can, however, be fractionated because of the difference in their solubility, which seems to depend on the relative content of sulphur (present as cysteic acid residues). With the exception of one, the dinitrophenyl

METHODS

Preparation of derivatives and fractionation procedures

The fractionations described below are summarized in Fig. 1.

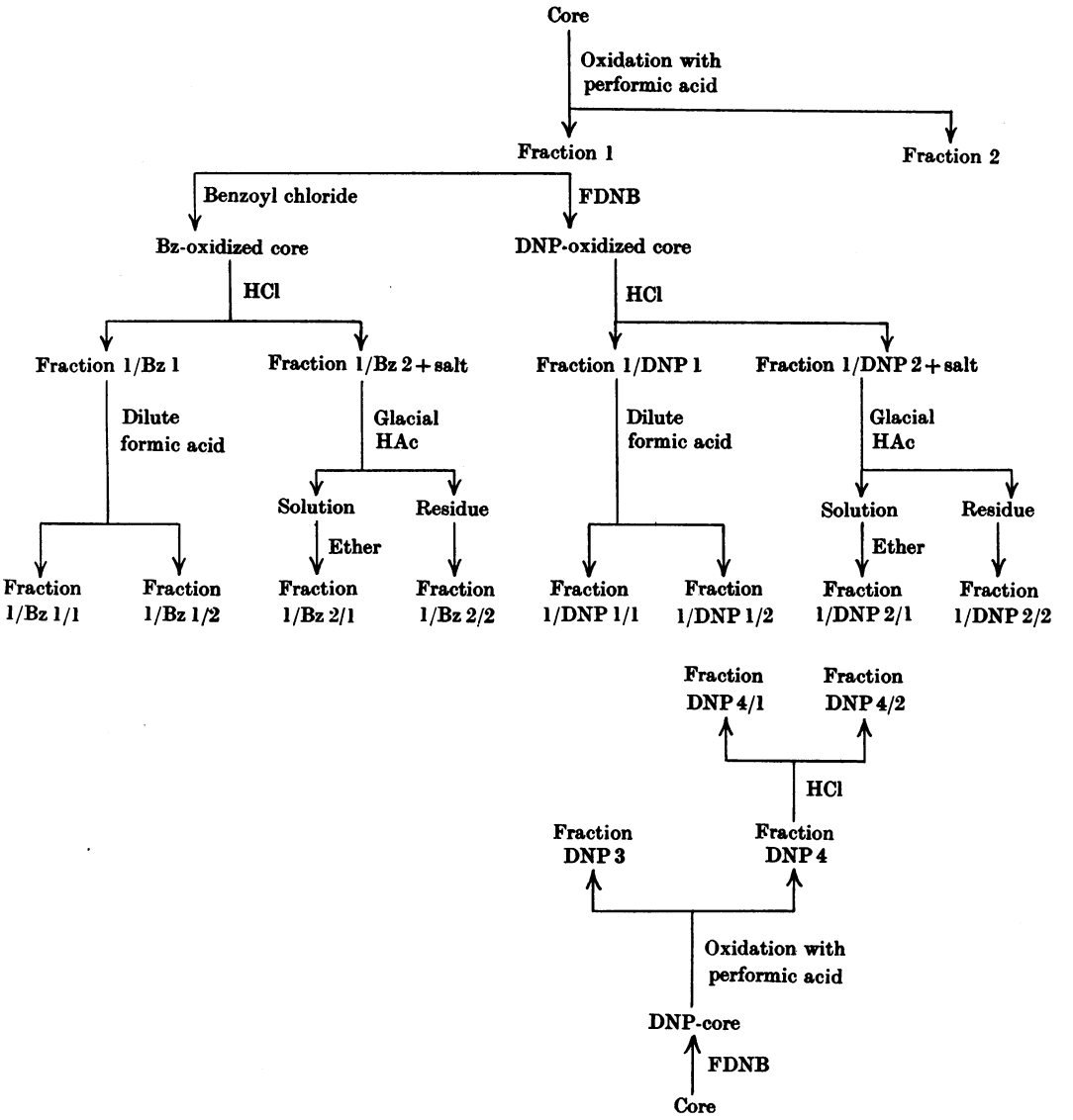

Fig. 1. Fractionation of the insulin core after oxidation and treatments with fluorodinitrobenzene (FDNB) and benzoyl chloride. For details see text.

fractions were still complex, as indicated by the multiplicity of their end groups. The one which appears to be simple has been more thoroughly investigated.

Oxidation of the dinitrophenyl-(DNP-) core. DNP-core (0·5 g.) (Butler *et al.* 1950) was treated with 18 ml. of 90% (v/v) aqueous formic acid and 2 ml. of H_2O_2 (100 vol.). The solid was broken up, and after standing for 1 hr. it had all

dissolved. Water (40 ml.) was added, which precipitated a bright-yellow solid. This was centrifuged down, washed with water and dried (wt. 160 mg., fraction DNP3). The supernatant liquid was combined with the washings and concentrated *in vacuo* in a water bath below 50°. Finally the concentrated solution was taken to dryness in a desiccator (wt. 313 mg., fraction DNP4).

Further fractionation of fraction DNP4. Fraction DNP4 (78 mg.) was dissolved in 5 ml. of water and 3 ml. of 0·1N-NaOH (resulting pH approx. 9·5). A small amount of insoluble material was filtered off and rejected. 2·3N-HCl (0·5 ml.) was added, which brought the pH to 1, and then a drop of 6N-HCl. On standing for a few minutes a precipitate formed, was centrifuged down and dried (wt. 15 mg., fraction DNP4/1). The supernatant liquid was discarded as it contained such a large amount of salt.

Fractionation of the oxidized core. Oxidized core (350 mg.) (Butler *et al.* 1950) was dissolved in 10 ml. of 0·1N-NaOH to give final pH approx. 8. A clear solution was produced. The pH was adjusted to 4·5 by the addition of 0·1N-HCl (5 ml.) and the resulting flocculent precipitate was centrifuged down, washed with water and dried (wt. 11 mg., fraction 2). The supernatant was neutralized and evaporated to dryness in a desiccator (wt. of dry residue 358 mg., fraction 1). This contained salt. Analyses showed N (Kjeldahl), 9·7 (Dumas, by Weiler and Strauss, Oxford), 10·9%. Assuming the N content of the core to be about 15%, that of the oxidized core (containing eight —SO_3H groups/5000 mol.wt.) should be about 14%. This suggests that fraction 1 contains about 260 mg. peptide material.

DNP derivative of fraction 1. Fraction 1 (323 mg.), still containing about 80 mg. NaCl, was dissolved in 10 ml. of water. 250 mg. $NaHCO_3$ were added, followed by a solution of 0·5 ml. of 1-fluoro-2:4-dinitrobenzene (FDNB) in 10 ml. ethanol. The mixture became bright yellow on shaking. It was left to stand overnight, and then evaporated to dryness. The orange residue was extracted three times with ether to remove excess FDNB, dissolved in warm water and acidified with a few drops of 6N-HCl. A bright-yellow precipitate immediately formed, which was centrifuged down, washed successively with water, ethanol and ether and dried (wt. 210 mg., fraction 1/DNP1). It was obvious that this did not represent the total reaction product, so the yellow supernatant liquid and the washings were neutralized and evaporated to dryness in a desiccator (wt. of dry residue 328 mg., fraction 1/DNP2). This was found to contain only 4% of N, which indicates a salt content of 70%, assuming the true N content of the peptide to be about 14%. The total weight of DNP products recovered, therefore, was 210 mg. insoluble material and about 95 mg. soluble.

A preparation of fraction 1/DNP2 containing about 315 mg. peptide was treated with glacial acetic acid. Ether was added to the yellow solution and a yellow precipitate was produced (wt. when dry 143 mg., fraction 1/DNP2/1). The residue which was insoluble in glacial acetic acid contained 80 mg. of peptide (fraction 1/DNP2/2). This amounted to about 13% of the total DNP products obtained from this preparation.

Further fractionation of fraction 1/*DNP*1. Fraction 1/DNP1 (77 mg.) was dissolved in 3 ml. 90% (v/v) formic acid and water (10 ml.) was added. An immediate precipitate was produced. This was centrifuged down, washed with water and dried (wt. 28 mg., fraction 1/DNP1/1). The supernatant liquid was evaporated to dryness (wt. of dry residue 34 mg., fraction 1/DNP1/2).

Benzoylation of fraction 1. Fraction 1 (400 mg.) was dissolved in water (20 ml.) with 1 g. of $NaHCO_3$. 0·5 ml. benzoyl chloride was added in 0·1 ml. portions with vigorous shaking. The pH was maintained at 8·3. The solution frothed when shaken and a whitish solid formed in it. The mixture was left at room temperature for 2 hr. with frequent shaking. It was then acidified with 17·5 ml. of 0·5N-HCl to pH 2. A white solid was produced which was centrifuged down. It was extracted three times with ether to remove benzoic acid, etc. (wt. of solid 153 mg., fraction 1/Bz1). The supernatant liquid was neutralized to pH 7, and extracted with ether; after evaporation of the small amount of dissolved ether the remaining solution was freeze-dried (wt. 1·06 g., N 2·2%, fraction 1/Bz2). From this it was estimated that the solid contained approximately 180 mg. peptide, assuming a N content 13%, which allows for the addition of 10 benzoyl groups/mol.wt. 5000. Total benzoylated material recovered, therefore, was 333 mg., of which 46% was insoluble in dilute HCl and 54% soluble.

Fraction 1/Bz2 (1·06 g.) was treated with about 15 ml. of glacial acetic acid which had been dried over $CaSO_4$. The residue was filtered off and treated twice more with acetic acid. An equal volume of ether was added to the acetic acid solution, giving a white precipitate which was centrifuged down, washed with ether twice and dried (wt. 111 mg., fraction 1/Bz2/1). The insoluble residue weighed 842 mg., and contained 0·75% N, i.e. approximately 45 mg. peptide material (fraction 1/Bz2/2).

Further fractionation of fraction 1/*Bz*1. Fraction 1/Bz1 (144 mg.) was dissolved easily in 9 ml. of 90% (v/v) formic acid. Water (25 ml.) was added. A white precipitate was immediately formed, which was centrifuged down, washed with water and dried (wt. 65 mg., fraction 1/Bz1/1). The supernatant liquid and washings were concentrated in a water bath at about 50° *in vacuo*, and finally taken to dryness in a desiccator (wt. 60 mg., fraction 1/Bz1/2).

End-group determinations

DNP derivatives of the fractions were hydrolysed by refluxing for 8 or 12 hr. with 6N-HCl in an oil bath at 130–140°, and the end groups were determined by the method of Mills (1950). The acid solution was extracted with approximately equal volumes of ether four times, and the ether extracts were washed once with 6N-HCl to remove traces of free amino-acids which might have been carried over, then evaporated to dryness in a small test tube. A few drops of a saturated solution of $Ba(OH)_2$ were added to the residue and the tube was placed in a boiling-water bath for 0·5–1 hr. Excess Ba was precipitated as $BaCO_3$ by the addition of a small piece of solid CO_2 and centrifuged off. The supernatant liquid was drawn off in a micropipette, evaporated to dryness and the residue applied to a two-dimensional paper chromatogram, which was developed by the method of Consden, Gordon & Martin (1944).

Where the presence of *N*-terminal glycine was suspected, this had to be determined separately, because DNP-glycine is not stable to prolonged hydrolysis, nor is glycine efficiently regenerated by hydrolysis of DNP-glycine with $Ba(OH)_2$. Consequently, the hydrolysis procedure of Sanger (1949*b*) was used. A sample of the acid hydrolysate was withdrawn after 2 hr. and extracted with ether. The ether extract after evaporation was run on a series of buffered silica columns. First, it was run on silica moistened with 0·25M-sodium phosphate buffer (pH 8), using a water-saturated solution

(10%, v/v) of *n*-butanol in $CHCl_3$ as moving phase. Secondly, it was run on silica buffered with 0·5 M phosphate (pH 8), using a water-saturated solution (33%, v/v) of ether in ethyl methyl ketone. Any likely bands were compared with those given by authentic DNP-glycine on similar columns; the material from them was then mixed with DNP-glycine and, if no separation occurred on one of these columns, the unknown was taken to be DNP-glycine.

Enzymic digestions of fractions

Crystalline pig pepsin was obtained from Parke Davis and Co. Ltd., papain from May and Baker Ltd. and pancreatin from British Drug Houses Ltd. Papain and pancreatin were used without further purification, but the papain was activated with HCN according to the method of Anson (1938).

Solutions of the DNP fractions (approximately 5 mg./ml.) were prepared by dissolving in water with the addition of drops of 0·1 N-NaOH to pH 8–8·5 for pancreatin, or pH 5 for papain, or with the addition of 0·1 N-HCl to pH 2 for pepsin digests. The enzymes were used in concentrations of approximately 5×10^{-4} unit/ml. digest at 25° for at least 24 hr., and in some cases 48 hr.

RESULTS

DNP fractions from the core and oxidized core

In five preparations from two different preparations of core, the amount of insoluble product, fraction DNP3, expressed as percentage by weight of the original DNP-core, maintained a fairly uniform proportion, as shown in Table 1.

Table 1. *Yield of insoluble fraction from oxidation of DNP-core*

Preparation	Core	Fraction DNP3 (%)
1	C16B	43
2	C16B	32
3	C16B	29
4	C16B	40
5	C15B	29

Benzoylated fractions from the oxidized core

Yields of the fractions, which were almost the same in three different preparations are shown in Table 2.

Table 2. *Yields of benzoylated fractions from oxidized core*

(Percentages by wt. of aggregate wt. of fractions.)

	Insoluble			Soluble		
Benzoylation	Bz1→Bz1/1 + Bz1/2			Bz2→Bz2/1 + Bz2/2		
1	43	26	17	57	—	—
2	46	24	22	54	39	15
3	45	27·5	17·5	55	42	13

Benzoylation of the core followed by oxidation was not successful.

The four benzoyl fractions, Bz1/1, Bz1/2, Bz2/1, Bz2/2, are assumed to correspond to the four DNP fractions, DNP1/1, DNP1/2, DNP2/1, DNP2/2.

Analysis of fractions

DNP fractions 1/DNP1,1/DNP2/1,1/DNP1/1,1/DNP 1/2, DNP 3 and DNP 4 and benzoyl fractions 1/Bz2/1, 1/Bz1/1, 1/Bz1/2 were analysed for S and N by Drs Weiler & Strauss. Results corrected for ash and moisture content are given in Table 3, where corresponding DNP and benzoyl fractions are shown side by side.

From these results it can be seen that the solubility which in the case of the DNP fractions follows the order

$$\begin{matrix}1/\text{DNP}2/1\\ \text{DNP}4\end{matrix} > 1/\text{DNP}1/2 > \begin{matrix}1/\text{DNP}1/1\\ \text{DNP}3\end{matrix}$$

and in the case of benzoyl derivatives

$$1/\text{Bz}2/1 > 1/\text{Bz}1/2 > 1/\text{Bz}1/1$$

seems to depend on the amount of S present in the fraction.

Cysteic acid in two benzoyl fractions, 1/Bz2/1, 1/Bz2/2, was determined by the method of Naftalin (1948) on paper chromatograms run in butanol-acetic acid mixture. 1/Bz2/1 contained 6·0% S and 1/Bz2/2, 5·9% by this method.

Terminal amino-acids

The *N*-terminal amino-acids of the cores from which these fractions were derived were glycine, valine and leucine. The distribution of these end groups amongst the DNP fractions can be seen in Table 4.

It will be seen that only in the case of fraction 1/DNP2/2 is the fraction completely homogeneous as shown by its end

Table 3. *Analysis of DNP and benzoyl fractions of oxidized core*

DNP fraction	S (%)	N (%)	Benzoyl fraction	S (%)	N (%)
1/DNP1	2·47	13·9	—	—	—
	1·93	15·1	—	—	—
1/DNP2/1	3·87	14·5	1/Bz2/1	5·22	11·65
				5·30	12·17
				5·02	10·20
1/DNP1/1	1·65	14·3	1/Bz1/1	1·46	12·98
				1·41	12·49
				2·48	12·56
1/DNP1/2	2·52	15·2	1/Bz1/2	2·77	10·39
				3·49	10·64
DNP3	1·51	17·1	—	—	—
DNP4	4·03	14·2	—	—	—
	3·66	13·3	—	—	—

group. Other fractions were apparently mixtures of chains in which one terminal amino-acid might predominate over the others, as for instance glycine does in the soluble fractions.

Chromatography on starch and alumina columns and on paper caused some further separation, but did not lead to products with single end groups.

Table 4. *Terminal amino-acids of DNP fractions*

(+, present; ++, very marked; −, weak; o, absent.)

Fraction	Glycine	Valine	Leucine
1/DNP1	+	++	+
1/DNP2	+	+	−
DNP3	+	++	−
DNP4	++	−	?
1/DNP2/1	+	+	+
1/DNP2/2	++	o	o

Distribution of amino-acids in fractions

Benzoyl fractions. These fractions were examined by paper chromatography after hydrolysis with 6N-HCl. Results are shown in Table 5.

Table 5. *Qualitative amino-acid composition of the benzoyl fractions*

(+, present; o, absent; −, faint; ?, doubtful.)

	Fractions			
Amino-acid	1/Bz1/1	1/Bz1/2	1/Bz2/1	1/Bz2/2
Cysteic acid	+	+	+	++
Aspartic acid	+	+	+	−
Glutamic acid	+	+	+	+
Serine	+	+	+	+
Glycine	+	+	+	+
Alanine	+	+	+	+
Valine	+	+	+	+
Leucine isomers	+	+	+	+
Tyrosine	+	+	+	+
Phenylalanine	?	?	?	o
Threonine	o	o	o	o
Histidine	+	+	+	o
Arginine	?	o	?	o
Lysine	?	o	?	o
Proline	o	o	o	o

According to these results, threonine and proline are absent from all fractions. The presence of such amino-acids as phenylalanine, arginine and lysine was doubtful. The main conclusion to be reached was that the fraction 1/Bz2/2 contained neither basic amino-acids, threonine nor phenylalanine.

DNP fractions. Of the DNP fractions examined, 1/DNP2/2 was the only simple fraction, containing only one end group, DNP-glycine. A two-dimensional chromatogram of the acid hydrolysate showed the presence of the following amino-acids: cysteic, aspartic and glutamic acids, serine, alanine, valine and leucine isomers. Glycine, phenylalanine, threonine and basic amino-acids were absent. Tyrosine would be present as *O*-DNP-tyrosine, which is not easily distinguished on paper chromatograms.

The molecular weight of fraction 1/DNP 2/2

The average molecular weight of this peptide fraction 1/DNP2/2 was determined by Mr G. L. Mills by a method to be published later, involving the reduction of the nitro groups in DNP-amino-acids by titanous chloride. He estimated the amount of DNP-glycine present in an acid hydrolysate after its separation by paper chromatography. Allowing for 60% breakdown of the DNP-glycine during hydrolysis for 8 hr. in 6N-HCl (a correction given by Sanger, 1945), he found that 1 g. residue of glycine would be present in 2000 g. There should be two DNP groups present, one terminal and one on a tyrosine residue; in this case therefore the mol.wt. of the unsubstituted peptide is approximately 1700.

This is more than was at first thought, when it was considered that the core was composed of four chains having mol.wt. about 1200 each. The figure is confirmed by other determinations. For example, cysteic acid determined by Naftalin's method gave 2 g. residues/2000 g. for 1/DNP2/2, and 3 g. residues/1600 g. for 1/Bz2/2, the corresponding benzoyl-fraction. Therefore the mol.wt. of this fraction 1/DNP2/2 can be taken as 1700–2000.

The action of enzymes on DNP fractions

The treatment of some DNP fractions with enzymes such as pepsin, papain and pancreatin showed that DNP-peptides can be split off and extracted from the acidified digest into ether. The ether-soluble material is probably not a single peptide, particularly as these fractions are known to be complex, containing more than one end group. However, the fact that they are soluble in ether shows that they must contain a DNP group, derived either from histidine, tyrosine

Table 6. *Composition of peptides extracted by ether from DNP fractions digested with enzymes*

	Composition of peptides split off by		
Fraction	Papain	Pepsin	Pancreatin
1/DNP1	Glutamic acid	Glutamic acid	Glutamic acid
	Serine or glycine	Serine, Glycine (?)	Glycine
	Alanine	Alanine	Alanine
	Leucine and/or isoleucine	Leucine and/or isoleucine	Leucine and/or isoleucine
	Valine	Valine	Valine
		Aspartic acid (?)	Aspartic acid
1/DNP2/1	—	Aspartic acid, Alanine, Leucine: Very faint	—
1/DNP2/2	None	None	Glutamic acid, leucine, isoleucine, valine, DNP-glycine

or the terminal amino-acid. The amino-acid composition of these mixed peptides was determined by paper chromatography after acid hydrolysis, and is shown in Table 6.

The peptide obtained by peptic digestion of fraction 1/DNP 1 contained *N*-terminal glycine. This was of interest because 1/DNP 2/2, which originally contained terminal glycine, was unaffected by pepsin, and 1/DNP 2/1 only very slightly so. It suggests, therefore, that the glycyl chains of the core may be of two different kinds, one present in fraction 1/DNP 1 and the other in fractions 1/DNP 2/1 and 1/DNP 2/2. This means that one type must be derived from the phenylalanyl chains of the original insulin as there is only one glycine residue present in the glycyl chains thereof (Sanger, 1949*a*).

Pancreatin digested both 1/DNP 1 and 1/DNP 2/2. The peptide from 1/DNP 2/2 contained the amino-acids valine, glutamic acid, leucine and isoleucine, also DNP-glycine; the leucine isomers were separated on a paper chromatogram in 2-dimethylpropan-1-ol in the presence of diethylamine (Work, 1949). The sequence of these amino-acids was not determined, but it appears likely that they are derived from a peptide similar to that isolated by Sanger (1949*b*) from the glycyl chains of insulin, DNP-glycylisoleucylvalylglutamylglutamic acid.

DISCUSSION

The core of the insulin molecule left after digestion with chymotrypsin contains 60–70 % of the original cystine (Butler *et al.* 1950). It is regarded as having the same form as the original insulin molecule, consisting of four peptide chains of relatively shorter lengths, held together by three or four disulphide bridges.

Fig. 2 shows the possible structures of the core derived from two of the structures of insulin proposed by Sanger (1948). The closed system for the core has been disregarded because it is thought that after oxidation it would not lead to the fractionation which occurs, because of the equal number of cysteic acid residues which each chain would contain.

Rupture of these disulphide linkages by oxidation with performic acid no longer permits ready separation of two dissimilar types of chain as in oxidized insulin. This is possibly due to the removal of most of the arginine and lysine from the phenylalanyl chains and to their decreased length, which would render them more soluble. The glycyl and phenylalanyl chains of oxidized insulin have four and two cysteic acid residues respectively. In the oxidized core the chains should now contain one, two or three cysteic acid residues.

Conversion of the disulphide linkages to sulphonic acid residues by oxidation markedly influences the solubility of the chains, which is further increased by the masking of the basic groups with groups such as dinitrophenyl or benzoyl. The mixture can then be separated easily into four main fractions.

This distinction is not clear-cut, as all DNP fractions except one contain more than one end group, signifying more than one type of chain. This may mean that chains with less sulphur are digested down to a shorter length, thereby increasing their solubility to that of longer chains with more sulphur. There are, of course, other factors which undoubtedly influence the solubility as well, which are as yet undetermined. Moreover, sulphur analyses of the fractions isolated according to this difference in solubility show that the more soluble the fraction, the higher is its sulphur content.

Further separation of these fractions by chromatography did not resolve the peptides present, although in some cases it diminished the predominance of one component in favour of another. Nor did chromatography on paper, starch or alumina produce any fraction with a single end group.

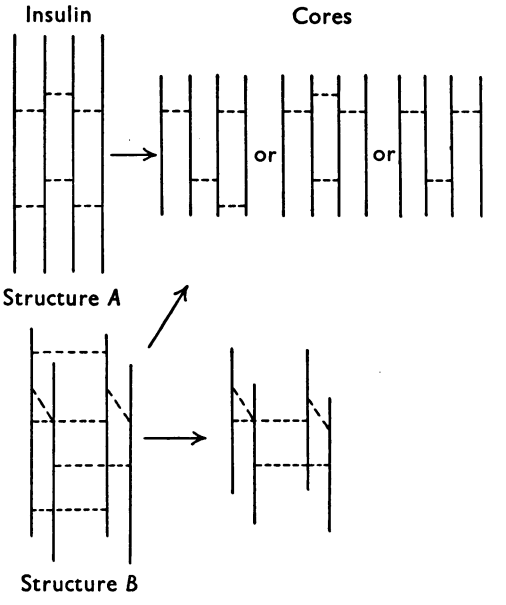

Fig. 2. Possible structures of insulin and the cores. Vertical lines represent the peptide chains and the dotted horizontal lines the disulphide bridges.

One fraction, however, which amounts to about 12 % of the whole core, has been obtained both in benzoyl and dinitrophenyl preparations of the oxidized core, and appears to have a single *N*-terminal amino-acid, glycine. The fraction contains no basic amino-acids, and a relatively high proportion of sulphur. It has a molecular weight of 1700–2000.

The glycyl chains of insulin have a molecular weight of 2750 (Sanger, 1949*b*), so that only about seven amino-acid residues have been split from the intact chain to form the new glycyl fraction. If all chains of the core were of approximately the same length, their molecular weight would be about 1200. Presumably some chains with less sulphur are shorter to compensate for this long one. The disulphide bridges in the original molecule probably provide a barrier to the action of the enzyme so that

the chains with a greater number of these are not digested down so far as those with fewer.

The action of pancreatin on this peptide has revealed that it terminates in a peptide containing DNP-glycine, isoleucine, valine and glutamic acid, which Sanger has described as a *N*-terminal peptide sequence from the glycyl chains of insulin. It also contains an additional leucine residue. The fraction is therefore probably derived from the *N*-terminal end of the original glycyl chains and digestion by the enzyme apparently occurs from the other end of the chain.

This fraction 1/DNP 2/2 is unaffected by other enzymes such as pepsin and papain; this distinguishes it from the glycyl chains in another complex fraction 1/DNP 1, which are digested with pepsin. This suggests that not all the glycyl chains in the core are alike. Unless rearrangements are brought about by the enzymes the latter glycyl chains must therefore come from the phenylalanyl chains of insulin. The chains having valyl or leucyl end groups may be derived from either type of original chain.

SUMMARY

1. The dinitrophenyl derivative of the 'core' resulting from chymotryptic digestion of insulin has been oxidized with performic acid, and three fractions, which differ in solubility, have been isolated.

2. The core itself has been oxidized, and the main product after treatment with fluorodinitrobenzene or benzoyl chloride has in each case been separated into four fractions.

3. These benzoyl or dinitrophenyl fractions have been analysed for sulphur.

4. The dinitrophenyl fractions, with the exception of one, are complex, having a variety of *N*-terminal amino-acid residues.

5. One dinitrophenyl fraction, about 13 % of the total product, has a glycyl end group, a molecular weight of about 2000, and a high cysteic acid content. A terminal peptide, yielding on acid hydrolysis *N*-dinitrophenylglycine, isoleucine, valine, glutamic acid and leucine, has been split from it by pancreatin.

I acknowledge with pleasure the interest of Prof. E. C. Dodds, F.R.S., in this work, and helpful advice from Dr J. A. V. Butler. I am also grateful for discussions with Dr D. M. P. Phillips, and for the molecular-weight determinations carried out by Mr G. L. Mills. Work reported in this paper formed part of a thesis submitted to the University of London for the Ph.D. degree.

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The Spectrophotometric Determination of Ascorbic Acid in Tissue Extracts, Particularly those of the Walnut (*Juglans regia*)

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(*Received* 27 *January* 1951)

Although ascorbic acid shows a well defined absorption band in the ultraviolet region of the spectrum, this property has received little attention for the determination of the vitamin in tissue extracts. The reasons for this would seem to be that the band is in a region where many other substances interfere, that the position of the band varies with pH (Karrer, Salomon, Morf & Schöpp, 1933) and that beyond certain limits the intensity of absorption is not directly proportional to the concentration (Herbert, Hirst, Percival, Reynolds & Smith, 1933).

Previously described spectrophotometric methods of assay have depended upon the measurement of absorption, before and after the destruction of ascorbic acid, by oxidation in the presence of copper (Johnson, 1936) or by irradiation (Chevallier & Choran, 1937). These methods are not applicable to extracts which contain a second reducing agent which absorbs in the same region as the ascorbic acid. Loureiro (1936) suggested that the shift with pH of the absorption curve might be utilized for determining ascorbic acid, but gave few details.