Müller, R. H., Garman, R. L. & Droz, M. E. (1943). *Experimental Electronics*, p. 223. New York: Prentice Hall.
Rimington, C. (1940). *Biochem. J.* **34**, 935.
Rimington, C. & Rowlands, I. W. (1941). *Biochem. J.* **35**, 736.
Rimington, C. & Rowlands, I. W. (1943). *Nature, Lond.*, **152**, 355.
Rimington, C. & Rowlands, I. W. (1944). *Biochem. J.* **38**, 54.
Siebert, F. B. & Atno, J. (1946). *J. biol. Chem.* **163**, 511.
Sørensen, M. (1936). *Biochem. Z.* **287**, 140.
Sørensen, M. & Haugaard, G. (1933). *Biochem. Z.* **260**, 247.
Staub, A. M. & Rimington, C. (1948). *Biochem. J.* **42**, 5.
Weehuizen, F. (1907). *Chem. Zbl.* I, S. 134.

Fractionation of Oxidized Insulin

By F. SANGER (Beit Memorial Fellow), *Biochemical Laboratory, University of Cambridge*

(*Received* 18 *May* 1948)

From the determination of the terminal residues of insulin it was suggested that the submolecule of molecular weight 12,000 is made up of four open peptide chains bound together by disulphide linkages (Sanger, 1945). Two of these chains have glycine and two have phenylalanine as terminal residues. By the action of performic acid it is possible to split insulin into its separate polypeptide chains by conversion of the cystine to cysteic acid residues (Sanger, 1947). The object of the present work was to separate the chains in a pure form, and the first problem was to find a suitable analytical method to follow the course of fractionation. Electrophoretic analysis gave rather variable results; this was largely due to the low molecular weight of the fractions which led to rapid spreading of the boundary by diffusion and did not allow preliminary dialysis. Later the method of end-group assay (Sanger, 1945; Porter & Sanger, 1948) was used in an attempt to obtain fractions containing only glycine or only phenylalanine terminal residues. This method of characterization does not necessarily indicate complete homogeneity, but the fractions are at least representative of the two types of chains in the insulin molecule. In this paper the preparation and some preliminary observations on the properties of two fractions are reported; the one, fraction *A*, containing only glycine terminal residues and no basic amino-acids; the other, fraction *B*, containing 97% phenylalanine terminal residues.

EXPERIMENTAL

Oxidation of insulin

The method was essentially that of Toennies & Homiller (1942), who showed that within 1 hr. cystine consumes the theoretical 5 atoms of oxygen, and that after 2 hr. a more general oxidation of a number of amino-acids takes place. It was thus desirable to use a time of oxidation which was the minimum required to split the S–S bridges completely. Using paper chromatography (Consden, Gordon & Martin, 1946*b*) it was found that the reaction with free cystine was complete in 5 min., but with insulin 15 min. were required. The method finally used was as follows: 0·25 g. insulin was dissolved in 9 ml. formic acid, 1 ml. 30% (w/w) H_2O_2 was added, and the mixture allowed to stand for 15 min. at room temperature. Water (10 ml.) was added and the mixture evaporated *in vacuo* to a small volume (1–2 ml.). The oxidized protein was then precipitated by a large volume of acetone, centrifuged, washed with acetone till free of formic acid and dried in air; yield, 0·25 g.

Fractionation of the oxidized insulin

Of the various fractionation procedures tried, the most satisfactory were the conventional methods of precipitation by alteration of pH or addition of salt or ethanol. In the salting-out procedures, however, it was difficult to remove the salt since the peptides were too small to be dialyzed. This was overcome by using a volatile salt, ammonium acetate, which could be removed by evaporation.

Freshly oxidized insulin (1 g.) was dissolved in 12 ml. 0·1 M-NH_3, and 0·1 M-acetic acid was added to bring the pH of the solution to 6·5. The precipitate was centrifuged down and used in the preparation of fraction *B*. The solution was used for the preparation of fraction *A*.

Fraction A. The above solution was brought to pH 4·5 with 0·1 M-acetic acid and, after removal of the precipitate (fraction *M*), was taken almost to dryness *in vacuo*. It was then transferred to a centrifuge tube with a minimum volume (about 3 ml.) of water, and an equal volume of 50% (w/v) ammonium acetate, which had been brought to pH 5·5 with glacial acetic acid, was added. This brought about the separation of a small precipitate which was discarded. The water and ammonium acetate were then removed by leaving the solution in a high vacuum over H_2SO_4 and NaOH till it reached constant weight. The residue was a white powder; yield, 0·25–0·32 g.

Fraction B. The material precipitated at pH 6·5 from 1 g. oxidized insulin was washed with 10 ml. 0·01 M-acetic acid, dissolved in 5 ml. 0·1 M-HCl and 40 ml. absolute ethanol added. This brought about the separation of a precipitate (fraction *X*) often in the form of a gel, which was centrifuged off and washed well with 80% ethanol. The combined

supernatant solution and washings were taken almost to dryness *in vacuo* and precipitated by a large volume of acetone; yield, 0·14–0·21 g. The yield of fraction *X* was 0·1–0·2 g. Various other methods were studied as a means of fractionating the oxidized insulin, but none was found more suitable than the above procedure.

Fraction *A* could be prepared satisfactorily by ionophoresis. Using ammonium acetate buffer, according to method C 1 of Consden, Gordon & Martin (1946*a*), it moved towards the anode as a discrete band, and could be identified by applying the Pauly diazo reaction to a print. After removal of the ammonium acetate *in vacuo* and elution with water the fraction was shown to contain only glycine terminal residues. Fraction *B* moved only slowly at various pH values and formed badly tailing bands indicating that adsorption on the gel had taken place. It was difficult to elute from the gel, and only a small yield was obtained of material containing only phenylalanine terminal residues.

The method of adsorption analysis (Tiselius, 1947) could be used as an analytical method (Tiselius & Sanger, 1947), but it was not found possible to use elution or displacement analysis to fractionate the material.

Using paper chromatography (Consden, Gordon & Martin, 1944), it was difficult to find a sufficiently sensitive colour test that could be applied to the paper. A weakly positive ninhydrin test was given by fraction *B*, as by insulin, but was not given by fraction *A*. The most satisfactory test was the Pauly reaction, which is given by histidine and tyrosine residues. Using this test and relatively high concentrations of the peptides it was found that they formed fast-moving tailing spots with phenol and collidine, and did not move with butanol or butanol-acetic acid.

Properties of the fractions

Fraction A. Fraction *A* is the most soluble material in the oxidized insulin. It is not precipitated at any pH value in low salt concentrations, or by any concentrations of sodium chloride or ammonium acetate, but it can be precipitated by high concentrations of ammonium sulphate.

The material, prepared as described above, contained 2·12 % NH_3 bound in salt linkage presumably to the sulphonic acid groups. Excluding this N, the N content of the dry protein was 12·80 % and the amide N 15·15 % of the total N. End-group analysis showed almost only glycine and less than 1 % phenylalanine terminal residues. The 2:4-dinitrophenyl (DNP) derivative which was prepared in the usual manner was slightly soluble in acid. Excess 1:2:4-fluorodinitrobenzene was removed as follows: The reaction mixture was taken to a small volume *in vacuo*, and the residue suspended in water and extracted well with ether. On acidification the DNP derivative was partly precipitated. Both the precipitate and the solution yielded only DNP-glycine on hydrolysis.

Paper chromatography (Consden *et al.* 1944) of a hydrolysate showed the following amino-acids to be present: leucine, isoleucine, valine, tyrosine, alanine, glycine, serine and glutamic, aspartic and cysteic acids. The following, though present in insulin, were absent from fraction *A*: lysine, arginine, histidine, threonine and phenylalanine. No proline spot could be detected, but its absence is not absolutely certain, as the ninhydrin test is not so sensitive as with other amino-acids. The absence of arginine and histidine was confirmed by the methods of Macpherson (1946) and of lysine by the absence of N^5-DNP-lysine in a hydrolysate of the DNP derivative. The absence of threonine was demonstrated by Mr M. W. Rees using the periodate technique (Rees, 1946). A very rough analysis was carried out by a method similar to the 'spot-dilution' technique of Polson, Mosley & Wyckoff (1947). All the amino-acids present could be separated using phenol-0·3 % NH_3-coal gas as solvent; standard solutions of amino-acids were run parallel with the hydrolysate of fraction *A* and the colour and size of the spots compared. The results are shown in Table 1. They are expressed as the number of residues of amino-acids/mol. of mol. wt. 2500, assuming fraction *A* to be homogeneous. While this is probably not true, the figures do give an approximate estimate of the amino-acid distribution within the limits of the method.

Table 1. *Amino-acid composition of fraction A*

Amino-acid	No. of residues/mol. of mol. wt. 2500	Amino-acid	No. of residues/mol. of mol. wt. 2500
Leucine	3	Glycine	1
Isoleucine	1	Serine	2
Valine	2	Glutamic acid	4
Tyrosine	2	Aspartic acid	2
Alanine	1	Cysteic acid	4

In developing a new method for the determination of sedimentation constants of substances of low molecular weight in the ultracentrifuge, Gutfreund & Ogston (1948) studied a rather cruder preparation of fraction *A* in which the ammonium acetate precipitation was omitted. They considered it to be relatively homogeneous and to have a mol. wt. of 2900.

Fraction B. Fraction *B* is readily precipitated near pH 6 and also by low concentrations of salt. Thus a solution in 0·03 M-HCl is precipitated by 1·5 % NaCl. Electrophoretic analysis at pH 8 showed one main boundary and a trace of a faster moving component. On ionophoresis at pH 7 it appeared to be slightly more basic than unchanged insulin, in spite of its cysteic acid content, suggesting a high content of basic amino-acids.

End-group analysis showed about 97 % phenylalanine and about 3 % glycine terminal residues. It has not yet been possible to obtain a preparation completely free of glycine terminal residues. Paper chromatography showed the presence of all the amino-acids that are found in insulin, that is to say,

all the commonly occurring amino-acids except tryptophan, methionine and hydroxyproline.

In the ultracentrifuge (Gutfreund & Ogston, 1948) this preparation appeared to be less homogeneous than fraction *A* and to have a mean mol. wt. 7000.

DISCUSSION

By a variety of methods, including precipitation at pH 6 or with 3–30 % NaCl or ionophoresis at pH 7, it is possible to separate the oxidized insulin into two crude fractions: an acidic fraction *A*, with glycine as terminal residues and a basic fraction *B*, with phenylalanine as terminal residues. This does suggest that there are essentially two types of peptide chains in insulin, although the different chains of each type are probably not identical. Since the yield of the pure fraction *A* is usually greater than 25 % of the oxidized insulin, it follows that, if there are two glycyl chains in insulin, then they must both be present in this fraction, so that, besides having the same terminal residue and no basic amino-acids, they must also both be free from threonine and phenylalanine and have the same molecular weight and electrophoretic mobility.

Besides fractions *A* and *B*, the only other significant fractions are fractions *X* and *M*. It seems likely that both of the latter contain essentially the same material, since by using a lower pH for the initial precipitation, the yield of fraction *X* is increased at the expense of fraction *M* and vice versa. The sum of the yields of the two fractions is 20–30 % of the oxidized insulin, and they contain about equal amounts of the two terminal residues. By repeating the ethanol precipitation on either *X* or *M* a certain amount of fraction *B* can be prepared from them, but it seems probable that they consist chiefly of a mixture of incompletely oxidized and over oxidized insulin. Fraction *X* tends to darken on standing, suggesting the presence of oxidation products of tyrosine. The yield of these fractions is greater if an oxidation time of 5 min. is used instead of 15 min., although it is not possible to lower the yield by further oxidation. While the possibility cannot be excluded that these fractions contain another chain of the insulin molecule, it seems likely that all the chains are actually represented in the purified fractions *A* and *B*. Thus it appears that the insulin submolecule is built up of two types of peptide chains, a basic type and an acidic type. It is possible that this particular structure may account for some of the properties of insulin.

SUMMARY

Two fractions have been prepared from insulin that has been oxidized by performic acid:

A, an acidic fraction containing glycine as terminal residues and no arginine, histidine, lysine, phenylalanine or threonine.

B, a basic fraction containing phenylalanine as terminal residues and all the amino-acids present in insulin.

I wish to express my thanks to Mr M. W. Rees for the threonine analysis and to Prof. A. C. Chibnall for his advice and encouragement.

REFERENCES

Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.

Consden, R., Gordon, A. H. & Martin, A. J. P. (1946*a*). *Biochem. J.* **40**, 33.

Consden, R., Gordon, A. H. & Martin, A. J. P. (1946*b*). *Biochem. J.* **40**, 580.

Gutfreund, H. & Ogston, A. G. (1948). *Biochem. J.* **44**, 163.

Macpherson, H. T. (1946). *Biochem. J.* **40**, 470.

Polson, A., Mosley, V. M. & Wyckoff, R. W. G. (1947). *Science*, **105**, 603.

Porter, R. R. & Sanger, F. (1948). *Biochem. J.* **42**, 287.

Rees, M. W. (1946). *Biochem. J.* **40**, 362.

Sanger, F. (1945). *Biochem. J.* **39**, 507.

Sanger, F. (1947). *Nature, Lond.*, **160**, 295.

Tiselius, A. (1947). *Advanc. prot. Chem.* **3**, 67.

Tiselius, A. & Sanger, F. (1947). *Nature, Lond.*, **160**, 433.

Toennies, G. & Homiller, R. P. (1942). *J. Amer. chem. Soc.* **64**, 3054.