Born, G. V. R., Dawes, G. S. & Mott, J. C. (1955). *J. Physiol.* **130**, 191.
Bruce, H. M. & Parkes, A. S. (1956). *J. Anim. Tech. Ass.* **7**, 54.
Cahill, G. F., Ashmore, J., Earle, A. S. & Zottu, S. (1958). *Amer. J. Physiol.* **192**, 491.
Cori, G. T., Closs, J. O. & Cori, C. F. (1933). *J. biol. Chem.* **103**, 13.
Cotlove, E. (1954). *Amer. J. Physiol.* **176**, 396.
Coxon, R. V. & Robinson, R. J. (1959). *J. Physiol.* **147**, 487.
Drury, D. R., Wick, A. N. & Almen, M. C. (1956). *Amer. J. Physiol.* **186**, 361.
Engel, F. L., Winton, M. G. & Long, C. N. H. (1943). *J. exp. Med.* **77**, 387.
Feller, D. D., Strisower, E. H. & Chaikoff, I. L. (1950). *J. biol. Chem.* **187**, 571.
Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
Frohman, C. E., Orten, J. M. & Smith, A. H. (1951). *J. biol. Chem.* **193**, 277.
Gey, K. F. (1956). *Biochem. J.* **64**, 145.
Gould, R. G., Sinex, F. M., Rosenberg, I. N., Solomon, A. K. & Hastings, A. B. (1949). *J. biol. Chem.* **177**, 295.
Green, H. N. & Stoner, H. B. (1950). *The Biological Action of the Adenine Nucleotides.* London: H. K. Lewis and Co. Ltd.
Hers, H. G. (1957). *Le Métabolisme du Fructose.* Bruxelles: Arscia.
Huggett, A. St G. & Nixon, D. A. (1957). *Lancet*, ii, 368.
Ingle, D. J. & Nezamis, J. E. (1949). *Amer. J. Physiol.* **159**, 95.
Kornberg, H. L., Davies, R. E. & Wood, D. R. (1952). *Biochem. J.* **51**, 351.
LePage, G. A. (1948). *Cancer Res.* **8**, 193.
Lindenbaum, A., Schubert, J. & Armstrong, W. D. (1948). *Analyt. Chem.* **20**, 1120.
Manery, J. F. (1954). *Physiol. Rev.* **34**, 334.
Neish, W. J. P. (1953). *Rec. Trav. chim. Pays-Bas*, **72**, 105.
Robinson, R. J. & Coxon, R. V. (1957). *Nature, Lond.*, **180**, 1279.
Rosenthal, S. M. & Millican, R. C. (1954). *Pharmacol. Rev. Lond.*, **6**, 489.
Saifer, A. & Gerstenfeld, S. (1958). *J. Lab. clin. Med.* **51**, 448.
Shipley, R. A., Baker, N., Incefy, G. E. & Clark, R. E. (1959). *Amer. J. Physiol.* **197**, 41.
Snedecor, G. W. (1946). *Statistical Methods*, 4th ed. Ames, Iowa: Iowa State College Press.
Somogyi, M. (1930). *J. biol. Chem.* **86**, 655.
Stoner, H. B. (1954). *Brit. J. exp. Path.* **35**, 487.
Stoner, H. B. (1958*a*). *Brit. J. exp. Path.* **39**, 251.
Stoner, H. B. (1958*b*). *Brit. J. exp. Path.* **39**, 635.
Stoner, H. B. & Threlfall, C. J. (1954). *Biochem. J.* **58**, 115.
Stoner, H. B. & Threlfall, C. J. (1960). *The Biochemical Response to Injury*, p. 105. Oxford: Blackwell Scientific Publications Ltd.
Stoner, H. B., Threlfall, C. J. & Green, H. N. (1952). *Brit. J. exp. Path.* **33**, 131.
Threlfall, C. J. & Stoner, H. B. (1954). *Quart. J. exp. Physiol.* **39**, 1.
Threlfall, C. J. & Stoner, H. B. (1957). *Brit. J. exp. Path.* **38**, 339.
Wynn, V. (1956). *Clin. Sci.* **15**, 297.

Biochem. J. (1960) **76**, 146

The Reaction of the Disulphide Groups of Insulin with Sodium Sulphite

By R. Cecil and U. E. Loening*
Department of Biochemistry, University of Oxford

(*Received* 7 *December* 1959)

The reaction of simple disulphides with sulphite has been studied in some detail in recent years. Stricks & Kolthoff (1951) showed that, with cystine and oxidized glutathione, the equilibrium

$$RS{\cdot}SR + SO_3^{2-} \leftrightharpoons RS{\cdot}SO_3^- + RS^-$$

is dependent on pH, as this affects the relative proportions of $RS^- + RSH$ present. Cecil & McPhee (1955) and McPhee (1956) studied the kinetics of the reaction of sulphite with a number of simple disulphides. They observed that under the conditions used reaction took place with SO_3^{2-} but not with HSO_3^- ions. The net charge on the disulphide molecule was found to have a considerable effect on the rate of reaction with SO_3^{2-} ion, the positively charged compounds reacting rapidly and the negatively charged ones slowly. All disulphides investigated reacted to completion under suitable conditions.

In the present work, the behaviour of disulphide bonds in insulin has been studied in the light of the earlier investigations. Insulin was chosen as a suitable protein for various reasons. At the time the work was started it was the only protein in which both the arrangement of amino acids and the position of the disulphide bonds were known (Ryle, Sanger, Smith & Kitai, 1955). Fig. 1, showing the structure of the molecule, is reproduced from this paper. It will be seen that there are three disulphide bonds, two joining the A and B chains and

* Present address: Department of Botany, University of Edinburgh.

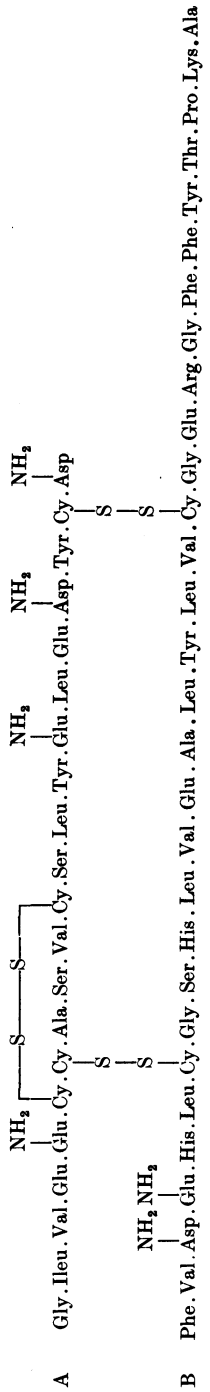

Fig. 1. Structure of insulin as given by Ryle, Sanger, Smith & Kitai (1955).

the third forming an internal ring between residues 6 and 11 in the A chain. There are no free thiol groups.

No attempt has been made to study the kinetics of these reactions in this preliminary survey. There are difficulties due to the presence of more than one reacting group on the same molecule and also to the fact that the reaction products are often insoluble. Because of this, only the extent of the reaction was studied under different conditions.

The phrase 'extent of reaction' requires some explanation. Three reactions have to be taken into account:

$$RS{\cdot}SR + SO_3^{2-} \leftrightharpoons RS{\cdot}SO_3^- + RS^-, \quad (1)$$

$$RS^- + H^+ \leftrightharpoons RSH, \quad (2)$$

$$SO_3^{2-} + H^+ \leftrightharpoons HSO_3^-. \quad (3)$$

At neutral pH most of the thiol formed is in the un-ionized form (pK_{SH} is normally in the region 8–10) and reaction (1) goes to completion. As the pH is increased above neutrality ionization of the thiol takes place and the equilibrium position of reaction (1) is displaced to the left. This is shown by allowing the reaction to reach equilibrium and acidifying to approx. pH 2 (which freezes the equilibrium) before estimating the amount of thiol formed (Cecil & McPhee, 1955). If the titration is carried out at the reaction pH, then removal of thiol from the system (as mercaptide) causes the reaction to 'follow' the titration and go to completion.

If the pH is reduced below neutrality the ratio of HSO_3^- to SO_3^{2-} ions increases (3); the rate of reaction falls rapidly and it may be difficult to reach equilibrium.

Very large differences have been observed in the second-order rate constants of positively and negatively charged disulphides with SO_3^{2-} ions and it is possible for a positively charged disulphide to react to completion under conditions under which a negatively charged compound would remain virtually intact. This could apply to a protein molecule containing several disulphide bonds of different properties.

A short account of some of this work has already appeared (Cecil & Loening, 1957).

EXPERIMENTAL

Reagents

Insulin. Crystalline ox insulin was obtained from Boots Pure Drug Co. Ltd., Nottingham. Two batches were used, nos. 4245 LL and 9011 G, with identical results. They were stored at 2–3° in a vessel containing a saturated solution of $CaCl_2,6H_2O$, which gives a relative humidity of 40% (*Handbook of Chemistry and Physics*, 1951–52). The moisture content of insulin stored in this way was found to

be 9·9%. Solutions (mM–0·1 mM) were made up in 5 mM-HCl as required.

Phenylmercuric hydroxide. Solutions were prepared and analysed as described by Allison & Cecil (1958).

Nitric acid. Dilute solutions were prepared according to Cecil (1950).

Other reagents. These were of normal reagent grade.

Reaction conditions

Insulin (50–500 μM) was allowed to react with Na_2SO_3 at 37° over the range pH 2·5–9·5. The concentration of Na_2SO_3 was such that the molar ratio Na_2SO_3:insulin was 100 or greater. Since Na_2SO_3 was always present in large excess it was also used as the buffer over the range pH 6·5–7·5 ($pK_2 = 7$, Tartar & Garretson, 1941), dil. HCl or HNO_3 being added to obtain the required pH. Phthalate was used between pH 2·5 and 6·5 and borate between pH 6·5 and 9·5. The concentration of both these buffers in the reaction mixtures was approximately 10 mM.

One source of error was the loss, by aerobic oxidation, of the insulin-SH groups formed; O_2 was therefore excluded either by the use of Thunberg tubes flushed with N_2 or by passing a slow stream of N_2 through the reaction mixture. In certain cases the reaction was carried out in the presence of an excess of phenylmercuric hydroxide, which itself prevented oxidation of SH groups.

Analytical methods

The extent of reaction of insulin with Na_2SO_3 was determined by measuring the amount of SH produced. This was done by amperometric titration with phenylmercuric hydroxide in the absence of O_2 with the dropping-mercury indicator electrode. The reasons for using this reagent to estimate SH groups in proteins are discussed by Allison & Cecil (1958) and Cecil & McPhee (1959*a*). The apparatus used was described by Allison & Cecil (1958).

Titrations were carried out over the range pH 2–9. They were followed by plotting the first reduction wave of phenylmercuric hydroxide (Benesch & Benesch, 1951). The potentials used, which are always referred to the saturated-calomel electrode, varied from −0·15 v at pH 2 to −0·5 v at pH 9. The correct potential under each set of conditions was determined by plotting the relevant current–voltage curves (see Figs. 2 and 3).

Below pH 5 KNO_3 (0·2 M) was used as the supporting electrolyte because of the insolubility of phenylmercuric chloride in acid solution. For the same reason chloride was excluded from the reaction mixtures. Above pH 5 KCl (0·2 M) was used. If KNO_3 was used between pH 5·5 and 8 trouble was experienced, owing to a flattening of the first reduction wave of phenylmercuric hydroxide. This was thought to be due to a separation of the adsorption and diffusion components of this reduction wave (see Benesch & Benesch, 1951).

Errors due to non-specific binding of phenylmercuric hydroxide by insulin (i.e. by groups other than SH) was prevented by the high concentration of Na_2SO_3 present. This forms a complex with phenylmercuric hydroxide which is easily reducible at the dropping-mercury electrode (see discussion in Cecil & McPhee, 1959*a*).

Insulin, phenylmercuric hydroxide and the mercaptides of insulin that has reacted with sulphite are surface-active and tend to compete with one another in forming a layer on the surface of the mercury drops. The residual current (which depends on the capacitance of the mercury drops) may therefore change during the course of a titration as the relative proportions of these reagents change. A value taken from the buffer solution or buffer plus insulin may not be correct for the end of a titration. The intersection of the residual-current line and excess-reagent line (see Fig. 2) was taken, at which point all the insulin-SH groups had been

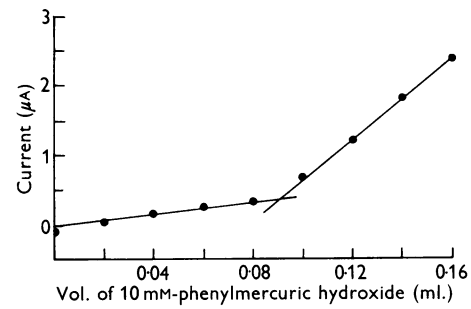

Fig. 2. Amperometric titration of an insulin–sulphite-reaction mixture with phenylmercuric hydroxide. The reaction mixture was as follows: insulin, 0·265 mM; Na_2SO_3, 28 mM; potassium hydrogen phthalate, 14 mM; H_2SO_4, 0·013 N; pH, 5·15. After reaction for 6 hr. at 37° a 3 ml. sample was acidified with 2 ml. of 0·1 N-H_2SO_4 to pH 1·85 and then titrated with 9·8 mM-phenylmercuric hydroxide at −0·1 v.

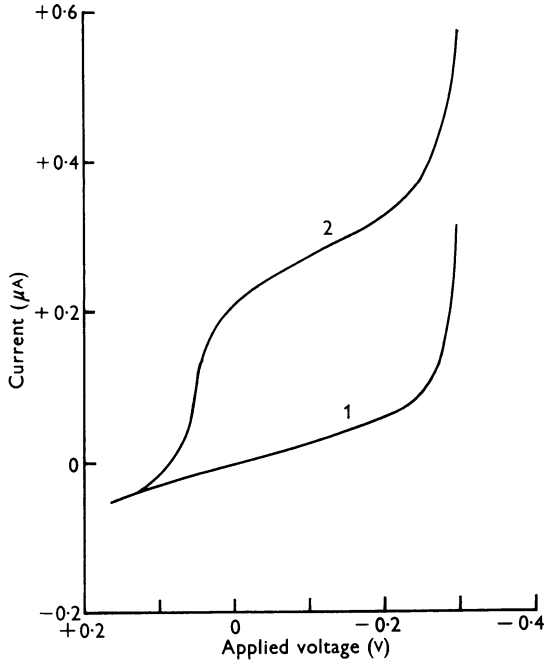

Fig. 3. Current–voltage curves of phenylmercuric hydroxide in the presence of an insulin–sulphite-reaction mixture. Curve 1 is that of the reaction mixture described in Fig. 2, acidified to pH 1·85, before titration. Curve 2 was taken at the end of the titration.

converted into mercaptide and there was no excess of phenylmercuric hydroxide. When the reaction was carried out in the presence of an excess of phenylmercuric hydroxide, the residual current had to be determined in a separate experiment.

Octan-2-ol, which is also surface-active, was originally used to prevent frothing when N_2 was bubbled through the solutions. It was found to cause errors, particularly at low pH, and its use was discontinued. Instead a ring of silicone grease was smeared round the top half-inch of the titration vessel.

RESULTS

Reaction of insulin with sulphite

The time course of the reaction was followed at a range of pH values at 37°. This was done in order to find the time required for complete reaction under various conditions. Samples from the reaction mixtures were removed at intervals, acidified with dilute nitric acid to approx. pH 2, and titrated with phenylmercuric hydroxide at −0·15 v. Typical results are given in Fig. 4.

Above pH 6·5 the reactions are rapid and true equilibrium is reached within about 20 min. Below pH 5·5 the reaction is slower and the plateau reached after 3–6 hr. is taken as the extent of reaction. It is probable that there is a further very slow reaction. Oxidation, which is difficult to prevent over long periods, tends to mask this. The plateau does not therefore represent a true equilibrium.

Insulin is sparingly soluble in the range of pH 3–6 and was sometimes present as a suspension. The products of reaction are also sparingly soluble and tended to precipitate during the reactions, particularly at low pH and high sulphite concentrations.

The extent of reaction, expressed as the number of S·S bonds/molecule of insulin, is shown in Fig. 5 (curve 1) as a function of pH. The reaction goes furthest at pH 6·5–7·0. Even so, only two-thirds of the S·S bonds react. Further experiments with a wide range of reaction times (4 min.–several hours) and of sulphite concentrations (100–270 moles of sulphite/mole of insulin) confirmed this result.

The fall in the extent of reaction above pH 7 is similar to that observed with simple disulphides, and the equilibrium position varies with the sulphite concentration. If the titrations were carried out at the same pH as the reaction then, as explained earlier, the reaction 'follows' the titration and an end point corresponding to two-thirds of the S·S bonds was obtained. Between pH 3 and 5·5, one-third of the S·S bonds were split. This result was independent of the sulphite concentration (above 100 moles of sulphite/mole of insulin) and was not influenced by the pH of titration between pH 1·8 and 6.

The most significant result of these experiments is that only two-thirds of the S·S bonds present reacted with sulphite under conditions in which the simple disulphides reacted completely. Conditions were therefore sought under which the remaining one-third of the S·S bonds would react.

Reaction of insulin with sulphite in the presence of urea

A series of experiments similar to those described above were carried out with the difference that the solvent was 6 M-urea instead of water. It was found that the reaction time required to reach a plateau at any given pH was approximately one-quarter of that required in the absence of urea, and that the products of reaction were soluble. The results are given in Fig. 5 (curve 2). It will be seen that a maximum of 2·4–2·5 S·S bonds react. If the titrations were carried out at the reaction pH, an uptake of phenylmercuric hydroxide corresponding to

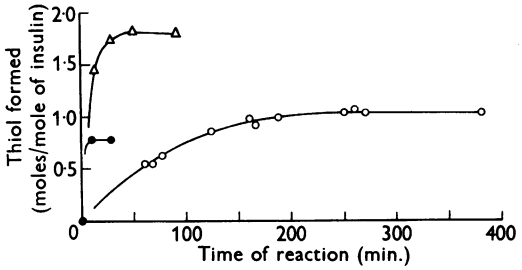

Fig. 4. Time course of the reaction between insulin and sulphite at 37° and at different values of pH. Three typical examples are given at different points in the pH range. The concentrations of insulin and Na_2SO_3 were 0·26 and 28 mM respectively with other substances as follows: ○, pH 4·5, 14 mM-potassium hydrogen phthalate and 0·017 N-H_2SO_4; △, pH 6·9, 0·01 N-H_2SO_4; ●, pH 9·0, 14 mM-$Na_2B_4O_7$.

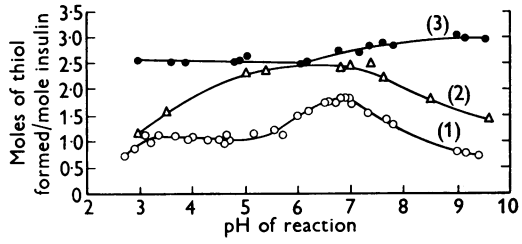

Fig. 5. Extent of reaction between insulin and sulphite under various conditions. The time course of the reactions at different values of pH was determined as shown in Fig. 4 and the maximum extent of reaction plotted against pH. Curve 1 (○), reaction with sulphite alone; curve 2 (△), reaction with sulphite in the presence of 6 M-urea; curve 3 (●), reaction with sulphite in the presence of phenylmercuric hydroxide. A small excess of phenylmercuric hydroxide (3–5 moles of phenylmercuric hydroxide/mole of insulin) was used.

2·8 S·S bonds was obtained. It is clear therefore that all three S·S bonds are involved in the reaction in the presence of urea. Similar results were obtained in the presence of 2 M-guanidine.

Reaction in the presence of excess of phenylmercuric hydroxide

These experiments were originally started in an attempt to prevent SH–S·S-exchange reactions. It was then found that the reaction went further in the presence of phenylmercuric hydroxide than in the presence of sulphite alone.

The compositions of the reaction mixtures were the same as those for insulin and sulphite alone except that a small excess of phenylmercuric hydroxide (i.e. > 3 moles/mole of insulin) was added. It was important to add the phenylmercuric hydroxide to the insulin before the sulphite for reasons given below.

The titrations were carried out at the reaction pH since there was no question of a shift in equilibrium. Since there was always some excess of phenylmercuric hydroxide present at the end of the reaction the value of the residual current was found from a separate experiment. For this an insulin–sulphite reaction mixture containing slightly less than one equivalent of phenylmercuric hydroxide was used (see under Analytical methods). If the excess of phenylmercuric hydroxide at the end of the reaction was small, further increments were added and the excess reagent line was extrapolated back to the residual current line. If a large excess of phenylmercuric hydroxide was present it was back-titrated with cysteine or homocysteine.

The results at different values of pH are given in Fig. 5 (curve 3). At pH 9 the uptake of phenylmercuric hydroxide corresponded exactly to the reaction of all three S·S bonds with sulphite but between pH 3 and 6 the reaction takes 24 hr. and the number is reduced to 2·5. If insulin is allowed to react with sulphite at pH 7–9 for a few minutes before adding the phenylmercuric hydroxide only 2·4–2·6 S·S bonds react. Presumably SH–S·S interchange reactions are rapid at this pH and a different reaction product results.

It was necessary to establish that the increased uptake of phenylmercuric hydroxide at pH 9 was due to reaction of the S·S bonds with sulphite and not phenylmercuric hydroxide. Heavy-metal ions, such as Ag^+ and Hg^{2+} ions, react with simple disulphides according to the equation (representing the metal ion as M^+):

$$2\ RS{\cdot}SR + 3\ M^+ + 2H_2O \rightarrow 3\ RSM + RSO_2H + 3H^+.$$

Thus 1 RS·SR ≡ 1·5 RSM (Cecil & McPhee, 1957, 1959*b*), whereas if the disulphide is first split by sulphite and the thiol formed reacts with heavy-metal ion then 1 RS·SR ≡ 1·0 RSM. If the two reactions are comparable in rate then the possibility of a mixed end point exists. R. Cecil (unpublished work) has found that phenylmercuric hydroxide reacts with some simple disulphides.

Insulin (0·1 mM) and phenylmercuric hydroxide (mM), buffered with phosphate at pH 7 or borate at pH 9, were incubated at 37° for periods up to 3 hr. Samples were removed at intervals and acidified with dilute nitric acid. The free phenylmercuric hydroxide present was estimated by potentiometric titration with potassium bromide (Allison & Cecil, 1958). These experiments showed that there was no detectable reaction of phenylmercuric hydroxide with the S·S bonds of insulin.

There remained the possibility that the reactivity of some of the insulin S·S bonds towards phenylmercuric hydroxide might be increased after others had reacted with sulphite. If this were so, the reaction of phenylmercuric hydroxide with an insulin–sulphite mixture would be dependent on its initial concentration. Experiments in which the amount of phenylmercuric hydroxide used was varied between 3 and 5 moles/mole of insulin showed that the uptake was constant. It may be assumed therefore that the S·S bonds react with sulphite and that the only function of the phenylmercuric hydroxide is to react with the SH groups so formed.

It was reported earlier that when insulin had been allowed to react with sulphite at pH 9 and titrated with phenylmercuric hydroxide at the same pH only two S·S bonds reacted. The reason for the difference between this result and those reported in this section lies in the fact that the concentration of free phenylmercuric hydroxide during a titration is small and the time it is in contact with insulin is short, when compared with the experiments just described.

Resolution of the reaction products of insulin and sulphite

The evidence obtained so far is that two-thirds of the S·S bonds of insulin react with sulphite under the same conditions as do simple disulphides, whereas the remaining third reacts only in the presence of phenylmercuric hydroxide, urea or guanidine. The simplest assumption is that two of the three S·S bonds present in insulin react normally with sulphite and that, for some reason, the third bond does not. Referring again to Fig. 1, two of the bonds act as covalent links between the A and B chains and there is no obvious reason why they should not react with sulphite. The other bond forms part of a closed-ring structure and to that extent differs from the simple disulphides studied by Cecil & McPhee (1955) and McPhee (1956), which were all open-chain compounds.

If indeed it is the two inter-chain bonds that react with sulphite at pH 7 then the insulin molecule would be split into the two chains. It should then be possible to resolve the reaction products into two fractions corresponding to the two chains. One fraction should contain a disulphide bond, which will react with sulphite plus phenylmercuric hydroxide.

It is important, in any experiment designed to test this hypothesis, that the amount of SH–S·S interchange should be kept to a minimum. This was done by using a high concentration of sulphite and a short reaction time. The composition of the reaction mixture used was as follows: insulin, 0·18 mM; HCl, 0·04 M; Na_2SO_3, 0·1 M; pH 6·9. The sulphite and insulin + HCl solutions were each heated to 37° before mixing. The reaction was complete (i.e. two S·S bonds were split) in 4 min., after which time the pH was lowered to 5 by the addition of acetic acid. A precipitate formed which was separated by centrifuging.

The A and B chains each contain two tyrosine and no tryptophan residues (see Fig. 1) and so should have the same molar absorption at 280 mμ. This provides a simple method of determining the distribution of protein between the precipitate and supernatant.

The filtrate contained a high concentration of sulphite and the absorption due to this was determined in a separate experiment. The figures in a typical experiment were as follows: supernatant, E 1·105; control (i.e. reaction mixture less insulin), E 0·683; therefore for soluble protein fraction, E is 0·422. The precipitate was dissolved in 0·01 M-sodium borate, the volume being the same as the original reaction mixture. The absorption at 280 mμ was 0·675. Thus approximately 40 % of the insulin appeared in the filtrate and 60 % was precipitated. The precipitate was not washed as this caused a loss of protein. Comparison of the absorption value of the fractions with that of the original insulin gave a recovery figure of 110 %, showing that the error caused by sulphite adhering to the precipitate was small.

In some experiments 2 moles of phenylmercuric hydroxide/mole of insulin were added after acidification in order to prevent oxidation of the SH groups. This did not affect the results.

These experiments showed that there were two fractions and so these were examined further.

Analysis of the thiol groups in the reaction products

The supernatant and precipitate were separated by centrifuging and analysed immediately. Separate experiments were done with each fraction to avoid delay and minimize loss of SH due to oxidation. Washing of the precipitate was omitted for the same reason.

The supernatant was titrated (at pH 5) with phenylmercuric hydroxide at −0·35 v after the addition of KCl to 0·1 M. The precipitate was suspended in 4 ml. of a solution of the same composition and titrated with phenylmercuric hydroxide in the same way.

One mole of insulin gave 0·6–0·7 mole of SH group in the supernatant and 1·0–1·1 mole in the precipitate. These proportions are similar to those obtained by ultraviolet absorption. This suggests that each fraction contains one SH group and that some of the soluble fraction is carried down with the precipitate.

Analysis of the disulphide bonds in the reaction products

The remaining S·S bonds will only react with sulphite plus phenylmercuric hydroxide at pH 9.

Two equivalents of phenylmercuric hydroxide were added to the reaction mixture after acidification to prevent oxidation of SH groups (this is rapid at pH 9).

Supernatant (3 ml.) was added to the following in the polarographic cell, which was kept at 37°: 0·5 ml. of 0·6 N-NaOH, 0·1 ml. of saturated KCl, 150 mg. of $Na_2B_4O_7$. N_2 was bubbled through for 10 min., after which time the current at −0·45 v was noted. 0·01 M-Phenylmercuric hydroxide (0·1 ml.) was then added. The current at −0·45 v (corresponding to the first reduction wave of phenylmercuric hydroxide) remained constant with time, indicating that there was no reaction. It was therefore concluded that there was no S·S present in the soluble fraction.

The precipitate was dissolved in the following solution, previously deoxygenated, in the polarographic cell at 37°; 3 ml. of 0·1 M-$Na_2B_4O_7$, 1 ml. of 0·2 M-Na_2SO_3, 0·1 ml. of saturated KCl. The current at −0·45 v was noted and 0·2 ml. of 0·01 M-phenylmercuric hydroxide added. The reaction was followed by observing the fall in the current, at −0·45 v with time. It was complete in 40 min. at 37°. The excess of phenylmercuric hydroxide at the end of the reaction was calculated from the current at −0·45 v. The uptake corresponded to 0·85–0·95 bond/mole of protein in different experiments.

It appears therefore that the precipitate contains the whole of the residual S·S.

End-group analyses of the reaction products

The two protein fractions were allowed to react with 1-fluoro-2:4-dinitrobenzene in an ethanol–water solution of bicarbonate (Sanger, 1945). After hydrolysis the dinitrophenyl (DNP)-amino acids were separated by paper chromatography (Blackburn & Lowther, 1951). DNP-Gly and DNP-Phe [for definitions of these abbreviations see *Biochem. J.* (1957), **66**, 6] were run on the papers as

markers. After drying the paper at 105°, the spots were cut out and eluted by soaking in 3 ml. of methanol + 1 drop of conc. HCl. The solutions were clarified by centrifuging and the absorption at 360 mμ was measured. No attempt was made to measure absolute quantities since a knowledge of the relative amounts of DNP-Gly and DNP-Phe was all that was needed.

Insulin. Control experiments were carried out with insulin to see whether the correct ratios of DNP-Gly and DNP-Phe were obtained with the procedure adopted. DNP-insulin was prepared according to Sanger (1945). It was hydrolysed with 6N-HCl in a sealed tube at 105° for 8 hr. The hydrolysate was diluted with water and extracted with ether. After evaporation of the ether the extract was taken up in a small amount of methanol and loaded on to the paper. Two spots corresponding to DNP-Gly and DNP-Phe (together with a trace of 2:4-dinitrophenol) were obtained. The absorptions at 360 mμ were: DNP-Gly, 0·120 and DNP-Phe, 0·118. This experiment was repeated a number of times and the ratio of the two terminal amino acids was always 1:1 within ± 5%, indicating that the destruction of DNP-Gly during hydrolysis is negligible under these conditions.

Soluble fraction. The supernatant contained a large amount of sulphite which had to be destroyed before reaction with 1-fluoro-2:4-dinitrobenzene. This was done by the addition of a slight excess of 30% (w/v) hydrogen peroxide. Bicarbonate and ethanol were then added and the reaction with 1-fluoro-2:4-dinitrobenzene carried out in the usual way. The DNP derivative is soluble over the whole pH range and so cannot be recovered, as with insulin, by precipitation at pH 5. Instead the solution was dried *in vacuo* and the solid extracted with ethanol in which the DNP derivative is, rather surprisingly, quite soluble. The ethanolic extract was dried *in vacuo* and extracted with ether to remove dinitrophenol. It was then dissolved in 0·5 ml. of 6M-HCl and hydrolysed for 8 hr. at 105°.

Chromatography showed DNP-Phe but no DNP-Gly. To confirm this another run was done with the paper more heavily loaded. The DNP-Phe spot had an absorption at 360 mμ of 1·25 and there was still no detectable DNP-Gly.

It appears therefore that the soluble fraction must consist almost entirely of B chain. This accords with the absence of any reaction for S·S bonds.

Precipitate. The precipitate was washed twice with water and the DNP derivative prepared in the same way as DNP-insulin. Chromatographic analysis of the hydrolysate gave the following absorptions at 360 mμ: DNP-Gly, 0·220; DNP-Phe, 0·550.

The other results suggest that 60–70% of the B chain is in the filtrate and the precipitate should therefore contain all the A chain and the remainder of the B chain. It was to be expected therefore that the ratio DNP-Gly:DNP-Phe would be at least 2:1. Instead the reverse was found.

It was thought that this discrepancy might be due to the presence of free SH groups in the reaction products. Accordingly, similar experiments were carried out with DNP-insulin to which cystine and cysteine (approx. 20%, w/w) were added before hydrolysis. The extinctions at 360 mμ with added cystine were 0·156 for DNP-Gly and 0·048 for DNP-Phe; those with added cysteine were 0·125 and 0·045 respectively. In both cases there appears to have been a loss of DNP-Phe, whereas, with the A chain, there appears to have been a loss of DNP-Gly. The quantitative aspects of end-group analysis must therefore be interpreted with caution.

Relative rates of reaction of the inter- and intra-chain disulphide bonds

It has been shown that, when insulin reacts with sulphite, the two inter-chain S·S bonds are split. There is the possibility that, in the presence of phenylmercuric hydroxide, when the reverse reactions are inhibited, the 6–11 intra-chain bond might react as fast as, or faster than, the inter-chain bonds. If this were so, then the insulin would not split into two components after only two bonds were split.

The composition of the reaction mixture was: insulin, 0·185 mM; HCl, 0·03M; phenylmercuric hydroxide, 0·37 mM (2 moles/mole of insulin); Na_2SO_3, 0·05M; pH, 6·9. The reaction was continued until all the phenylmercuric hydroxide had been taken up, 7 min. at 37°, when it was stopped by acidification with acetic acid to pH 5. The precipitate formed was separated by centrifuging and dissolved in a volume of 10 mM-$Na_2B_4O_7$ equal to that of the original reaction mixture. The absorption at 280 mμ was: supernatant, 0·280; control (i.e. reaction mixture less insulin), 0·210; soluble fraction, 0·070; precipitate in 10 mM-$Na_2B_4O_7$, 0·980. Since only 7% of the protein remained in the supernatant (as compared with 30–40% after reaction with sulphite above) it is likely that one of the two inter-chain bonds is intact. Reaction must therefore have taken place with one inter-chain bond and with the intra-chain bond.

DISCUSSION

One of the objects of this work was to find the extent to which the S·S bonds of insulin resemble those of the simple disulphides. The behaviour of two S·S bonds of insulin above pH 7, where the extent of reaction decreases as the pH is increased,

is similar to that found by Stricks & Kolthoff (1951), Cecil & McPhee (1955) and McPhee (1956) with all the simple disulphides investigated.

The behaviour at pH 3–5, where reaction is effectively confined to one S·S bond, can also be accounted for by reference to work with simple disulphides. McPhee (1956) showed that the differences in second-order rate constants between positively and negatively charged disulphides was sufficiently large to allow for complete reaction of a positively charged compound under conditions where a negatively charged compound would be almost unaffected. These studies did not extend below pH 4·5 but Cecil (1960) has since studied the behaviour of the positively charged compound, 2:2′-diaminodiethyl disulphide (cystamine), at pH 3–5. This compound reacted slowly with HSO_3^- at low pH in phthalate buffer, but not in formate. Whether or not insulin reacts with HSO_3^- ions can only be determined by kinetic studies. It may be tentatively concluded, however, that one of the insulin S·S bonds has a positively charged environment.

The difference between insulin and the simple disulphides lies in the complete failure of one of the S·S bonds to react with sulphite. Since it has been shown that insulin splits into the A and B chains after reaction with sulphite it follows that the inter-chain S·S bonds have reacted and the 6–11 intra-chain bond in the A chain has not.

This bond has been shown to react with sulphite in the presence of urea, guanidine and phenylmercuric hydroxide. It is possible to explain these facts on the assumption that the configuration of the 6–11 ring is stable and that this stability depends on hydrogen bonding and not on the S·S bond. This would have the effect of shifting the equilibrium of reaction (1) to the left; i.e. of increasing the rate of the reverse reaction relative to the forward reaction. The effect of urea and guanidine is explained by a reduction in the stability of the ring due to interference with the hydrogen bonding. This would reduce the rate of the reverse reaction. Phenylmercuric hydroxide, on the other hand, combines with the SH groups to form undissociated mercaptide and so must inhibit the reverse reaction.

The reason why only 2·5 S·S bonds react with sulphite plus phenylmercuric hydroxide between pH 3 and 6 is obscure. The products of SH–S·S-exchange reactions, obtained by allowing insulin to react with sulphite alone for a time before adding phenylmercuric hydroxide, behave similarly. Kolthoff, Anastasi & Tan (1959) have observed a similar phenomenon with bovine-serum albumin.

The rate of the forward reaction of the 6–11 bond with sulphite was compared with that of the two inter-chain bonds by allowing only two S·S bonds to react with sulphite in the presence of phenylmercuric hydroxide. In the absence of phenylmercuric hydroxide under these conditions the two inter-chain bonds react and the insulin is split into the A and B chains. In the presence of phenylmercuric hydroxide, however, the insulin does not split into two components after two bonds have reacted. It follows that the 6–11 bond and one inter-chain bond, probably that carrying a positive charge, must have reacted.

It appears therefore that, given suitable conditions, the 6–11 S·S bond is highly reactive and, moreover, is probably not required to maintain the configuration of the ring. Lindley's (1955) observation that the 6–11 bond alone is reduced by lithium thioglycollate at pH 5 is consistent with this view. These facts, together with the similarity of the 6–11 ring to those of oxytocin and vasopressin (Tuppy, 1953; du Vigneaud, Ressler & Trippett, 1953; Popenoe & du Vigneaud, 1954), suggest that this reactive S·S bond might be intimately concerned in the biological action of insulin.

If the 6–11 S·S bond is essential to the biological activity of insulin, it can only be one of several different groups so involved; thus it has long been known that insulin is inactivated if the tyrosine residues are modified (Harington & Neuberger, 1936; Stern & White, 1937).

Fisher and Zachariah (see Addendum) found that the fraction that was precipitated at pH 5 after reaction with sulphite showed greatly decreased insulin-like activity. Since this contained all the A chain and some of the B chain, it appears that the separated chains are probably without biological activity.

The properties of the separated A and B chains are interesting. Fractionation based on the difference in solubilities was all that was attempted in order to avoid loss of material and errors due to oxidation of SH groups. It was established on the basis of SH, S·S and end-group analysis that the soluble fraction consisted of almost pure B chain and the precipitate of all the A chain, together with 20–30% of B chain. The inter-chain S·S bonds react in such a way that each chain carries a total of one SH group and presumably one $-S{\cdot}SO_3^-$ group. It has not yet been established whether this represents one SH and one $-S{\cdot}SO_3^-$/chain molecule or whether there is a mixture of products which average one group/chain. The fact that some B chain is associated with the A chain suggests that there may be a mixture in which one of the B-chain components is less soluble than the remainder. The S·S bond in the 6–11 ring of the separated A chain maintains its unique properties and will only react with sulphite in the presence of phenylmercuric hydroxide.

The relative solubilities of the two chains require comment. Sanger (1949) found that, when prepared from insulin by oxidation with performic acid, the A chain was soluble over the whole pH range, whereas the B chain was precipitated at pH 6·5. In this case, all the sulphur was in the form of SO_3^- ion. When insulin is split by reaction with sulphite at pH 7, the A chain can be precipitated at pH 7 whereas the B chain is soluble over the whole pH range. The DNP-substituted B chain, when prepared in this way, is soluble in ethanol.

Kolthoff, Anastasi & Tan (1958, 1959) found that the S·S bonds in bovine-serum albumin show many similarities to the 6–11 bond of insulin. There are 17 S·S bonds in the molecule. In the native protein, none of these reacts with sulphite. When the protein is denatured with urea or guanidine, the extent of reaction is dependent on pH, but at pH 4–6 reaction is virtually complete. If the native protein was allowed to react with sulphite in the presence of a small excess of mercuric chloride all the S·S bonds reacted.

Hunter & McDuffie (1959) reduced bovine albumin with thioglycollate in the presence of both urea and sodium dodecylsulphate, under which conditions all the S·S bonds were reduced. They found that the molecular weight of the reduced protein was the same as that of the native protein. It appears likely therefore that the molecule consists of one long peptide chain with a number of intra-chain S·S bonds. Thus the behaviour of the S·S bonds of bovine albumin with sulphite could be explained on the same basis as that of the 6–11 S·S bond of insulin.

The same type of structure has been demonstrated for lactogenic hormone (Li, Cole & Coval, 1957; Cole, Geschwind & Li, 1957; Li, 1957) and for ribonuclease (Moore & Stein, 1958; Anfinsen, 1958; Hirs, Stein & Moore, 1958). Bailey (1957) and Bailey & Cole (1959) have studied the reaction of both insulin and ribonuclease with sulphite, but only in the presence of 8 M-urea and an oxidizing agent. Under these conditions all the disulphide bonds react to give *S*-sulphonate. No studies have been reported on the reaction of lactogenic hormone with sulphite.

The S·S bonds of keratin show a similar type of behaviour to those of serum albumin. Approximately half of the S·S bonds present react with sulphite at pH 5 (see review by Lindley, 1959). If the reaction is carried out in the presence of Hg^{2+} ion and urea (Leach, 1959) then all the bonds react.

The explanation proposed for the behaviour of the 6–11 S·S bond of insulin accounts for most of the observed facts and could apply to S·S bonds in other proteins. Until this is established, however, the possible effects of other factors, such as the influence of side chains, must not be ignored.

SUMMARY

1. The reaction of the disulphide bonds of insulin with sulphite has been studied under a range of conditions.

2. Two of the three disulphide bonds react with sulphite in a manner comparable with simple disulphides. Under suitable conditions they react to completion.

3. One of these two disulphide bonds reacts with sulphite at low pH and is likely to have a positively charged environment.

4. If insulin is allowed to react with sulphite under conditions in which two disulphide bonds are split, but thiol–disulphide-exchange reactions are kept to a minimum, the reaction product can be resolved into the A and B chains. This establishes that it is the two inter-chain disulphide bonds that have reacted.

5. The intra-chain bond in the A chain will only react with sulphite in the presence of urea, guanidine or phenylmercuric hydroxide. An explanation for this behaviour is suggested which could also apply to apparently unreactive disulphide bonds in other proteins.

One of us (U.E.L.) is indebted to the Agricultural Research Council for a training grant and to the John Innes Horticultural Institute for support in the later stages of the work. We are grateful to Dr R. B. Fisher for many helpful discussions and to Miss Sybil Read for skilled technical assistance. This work was supported by a grant from the Rockefeller Foundation to the Department of Biochemistry, University of Oxford.

REFERENCES

Allison, A. C. & Cecil, R. (1958). *Biochem. J.* **69**, 27.
Anfinsen, C. B. (1958). In *Symposium on Protein Structure*, p. 223. Ed. by Neuberger, A. London: Methuen and Co. Ltd.
Bailey, J. L. (1957). *Biochem. J.* **67**, 21 P.
Bailey, J. L. & Cole, R. D. (1959). *J. biol. Chem.* **234**, 1733.
Benesch, R. & Benesch, R. E. (1951). *J. Amer. chem. Soc.* **73**, 3391.
Blackburn, S. & Lowther, A. G. (1951). *Biochem. J.* **48**, 126.
Cecil, R. (1950). *Biochem. J.* **47**, 572.
Cecil, R. (1960). *Biochem. J.* **76**, 156.
Cecil, R. & Loening, U. E. (1957). *Biochem. J.* **66**, 18 P.
Cecil, R. & McPhee, J. R. (1955). *Biochem. J.* **60**, 496.
Cecil, R. & McPhee, J. R. (1957). *Biochem. J.* **66**, 538.
Cecil, R. & McPhee, J. R. (1959*a*). *Advanc. Protein Chem.* **14**, 261.
Cecil, R. & McPhee, J. R. (1959*b*). *Advanc. Protein Chem.* **14**, 300.
Cole, R. D., Geschwind, I. I. & Li, C. H. (1957). *J. biol. Chem.* **224**, 399.
du Vigneaud, V., Ressler, C. & Trippett, S. (1953). *J. biol. Chem.* **205**, 949.
Handbook of Chemistry and Physics. (1951–52). 33rd ed. p. 2095. Cleveland, Ohio: Chemical Rubber Publishing Co.

Harington, C. R. & Neuberger, A. (1936). *Biochem. J.* **30**, 809.
Hirs, C. H. W., Stein, W. H. & Moore, S. (1958). In *Symposium on Protein Structure*, p. 211. Ed. by Neuberger, A. London: Methuen and Co. Ltd.
Hunter, M. J. & McDuffie, F. C. (1959). *J. Amer. chem. Soc.* **81**, 1400.
Kolthoff, I. M., Anastasi, A. & Tan, B. H. (1958). *J. Amer. chem. Soc.* **80**, 3235.
Kolthoff, I. M., Anastasi, A. & Tan, B. H. (1959). *J. Amer. chem. Soc.* **81**, 2047.
Leach, S. J. (1959). *Biochim. biophys. Acta*, **33**, 264.
Li, C. H. (1957). *J. biol. Chem.* **229**, 157.
Li, C. H., Cole, R. D. & Coval, M. (1957). *J. biol. Chem.* **229**, 153.
Lindley, H. (1955). *J. Amer. chem. Soc.* **77**, 4927.
Lindley, H. (1959). In *Sulphur in Proteins*, p. 33. Ed. by Benesch, R. *et al.* New York: Academic Press Inc.
McPhee, J. R. (1956). *Biochem. J.* **64**, 22.
Moore, S. & Stein, W. H. (1958). *Harvey Lect.* **52**, 119.
Popenoe, E. A. & du Vigneaud, V. (1954). *J. biol. Chem.* **206**, 353.
Ryle, A. P., Sanger, F., Smith, L. F. & Kitai, R. (1955). *Biochem. J.* **60**, 541.
Sanger, F. (1945). *Biochem. J.* **39**, 507.
Sanger, F. (1949). *Biochem. J.* **44**, 126.
Stern, K. G. & White, A. (1937). *J. biol. Chem.* **117**, 95.
Stricks, W. & Kolthoff, I. M. (1951). *J. Amer. chem. Soc.* **73**, 4569.
Tartar, H. V. & Garretson, H. H. (1941). *J. Amer. chem. Soc.* **63**, 808.
Tuppy, H. (1953). *Biochim. biophys. Acta*, **11**, 449.

ADDENDUM

Determination of the Insulin-like Activity of the A-chain of Insulin

By R. B. FISHER* and P. ZACHARIAH
Department of Biochemistry, University of Oxford

(*Received* 7 *December* 1959)

The procedure used to detect insulin-like activity depends on the observation that insulin, in very low concentration, increases the permeation of the rat heart by non-metabolized sugars.

The A chain of insulin was a sample supplied by Cecil & Loening (1960) prepared by reaction of insulin with sulphite. It probably contains approximately 20% of B chain as impurity.

The isolated rat heart was perfused, by techniques similar to those described by Fisher & Lindsay (1956), with Krebs-bicarbonate medium containing 30 mM-L-arabinose and 10 mM- raffinose, together with the material suspected of insulin-like activity. By the use of the raffinose as an indicator of extracellular water, it was then possible to determine the intracellular arabinose concentration.

After removal from the animal, the hearts were rendered insulin-free by perfusion for 45 min. with the sugar-free perfusate. They were then perfused for 15 min. with a medium containing the sugars and the sample to be tested. The arabinose penetration is expressed as the percentage of intracellular water in equilibrium with the perfusate after perfusion for 15 min. (Table 1).

Table 1

Sample	Concn. (μmM)	No. of expts.	Mean penetration of L-arabinose (%)
Nil	—	2	12·96
A chain	4	2	13·51
A chain	40	2	29·20
Insulin	4	2	39·21
Insulin	8	8	55·80

(8 μmM-Insulin is equivalent to 1 milliunit/ml.).

The insulin-like activity of the sample of A chain is certainly less than one-tenth of that of unchanged insulin.

* Present address: Department of Biochemistry, University of Edinburgh.

REFERENCES

Cecil, R. & Loening, U. E. (1960). *Biochem. J.* **76**, 146.
Fisher, R. B. & Lindsay, D. B. (1956). *J. Physiol.* **131**, 526.