The Effect of Chemical Modifications Induced in Insulin on the Reactivity of the Interchain Disulphide Bonds towards Sodium Sulphite

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The reactivity of the three disulphide bridges of insulin towards sodium sulphite was studied by amperometric titration of the liberated thiol groups. In the native, acetylated or succinylated molecule two bridges react at pH 7, but in the methylated or phenylcarbamoylated molecule only one bridge reacts. All three bridges react in all derivatives in 8M-urea or at pH 9. Loss in biological activity parallels the loss in reactivity of one of the bridges during methylation. It is suggested that change in reactivity of the S-S bonds reflects the occurrence of a conformational modification of the protein. The possibility is discussed that the unusually high reactivity of the S-S bonds in native insulin depends strictly on the integrity of the native molecule, suggesting that S-S bonds are in some way involved in the hormone's mode of action.

Recognition of a possible effect of specific substitutions on the ability of the insulin disulphide bridges to be cleaved by sodium sulphite began when we found that iodination beyond a substitution of about 4-5 atoms of iodine/monomer unit of mol. wt. 5734 caused one of the insulin interchain S-S bonds to be unreactive towards sodium sulphite at pH 7 (Rosa, Massaglia, Pennisi, Cozzani & Rossi, 1967). In native insulin the reaction attains an equilibrium value corresponding to the splitting of both interchain S-S bonds, whereas the intrachain S-S bond linking residues A₆ and A₁₁ in the A chain remains intact (Cecil & Loening, 1960). To establish whether such an effect is specifically related to the iodination or even occurs as a consequence of other substitution processes, we have studied the effect of esterification (with methanol), acylation (with phenyl isocyanate), acetylation and succinylation on the reactivity of the insulin S-S bonds towards sodium sulphite. The behaviour of desalanine-desasparagine-insulin and of its methylated derivative has also been investigated.

EXPERIMENTAL

Reagents

Pig insulin (10× crystallized) was supplied by Novo Research Institute, Copenhagen, Denmark (lot S23267). The moisture content was determined by heating to constant weight and was found to be 5.01%. Zinc-free insulin was prepared by the procedure of Sluyterman (1955). The material so obtained contained less than 0.04% of zinc,

as calculated by the EDTA-titration method of Flaschka (1952).

Phenylmercuric hydroxide was prepared and titrated potentiometrically as described by Allison & Cecil (1958).

All other chemicals were of reagent grade and twice-distilled water was used throughout.

Preparation of substituted insulins

Acylation (with phenyl isocyanate). Insulin was acylated by the procedure of Sidney & Wormald (1934). A 0.1 g. sample of pig insulin was dissolved in 12.5 ml. of water with a trace of N-NaOH; 12.5 ml. of 0.2M-NaHCO₃, pH 8.5, was then added. The solution was cooled to 0° and treated under continuous stirring with 1.25 ml. of phenylisocyanate. The mixture was shaken well for about 20 min., and the pH was then adjusted to 6.5. The mixture was successively extracted with four 20 ml. portions of diethyl ether. The clear solution was dialysed against four changes of twice-distilled water with its pH adjusted to 8 with a few drops of 0.01N-NaOH. The solution was dialysed and then freeze-dried; the acylated insulin was stored at 4° until used.

Esterified insulin. Insulin was methylated by the procedure of Mommaerts & Neurath (1950). Acid methanol was prepared by passing dry HCl gas into methanol; after a suitable time-interval the acid concentration was determined and the solution was brought to 0.1N-HCl by adding methanol.

(a) Fully methylated insulin. Insulin contains 6 carboxyl groups/monomer unit of mol. wt. 5734; fully esterified insulin was prepared as follows. A 0.5 g. sample of pig insulin was suspended in 50 ml. of the acid methanol solution and stirred in a closed vessel at 25 ± 1° for 24 hr. The hydrochloride of methyl-insulin was precipitated by addition of anhydrous diethyl ether and then dried under vacuum and stored at 4°. The methoxyl content was

Table 1. *Analytical data of insulin derivatives*

Approximate extents of reaction are indicated in parentheses, in terms of percentages of residues affected.

Expt. no.	Reaction and conditions	Protein groups involved	Biological activity (% of original)
1	Esterification (methanol-0.125 N-HCl, 25°, reaction time 24 hr.)	Carboxyl (> 95%)	< 3*
2	Esterification of acetyl-des-Ala-des-Asn-insulin (methanol-0.125 N-HCl, 25°, reaction time 24 hr.)	Carboxyl (> 95%) Amino (> 84%)	< 3*
3	Acylation (phenyl isocyanate, 0°, pH 8.5, reaction time 30 min.)	Amino (> 95%) Phenolic (< 10%)	< 5
4	Acetylation (acetic anhydride, 0°, reaction time 1 hr.)	Amino (> 83%) Phenolic (< 15%)	40 ± 17†
5	Succinylation (succinic anhydride, 0°, pH 8, reaction time 30 min.)	Amino (> 90%)	31 ± 8
6	Removal of C-terminal residues (acetyl-des-Ala-des-Asn-insulin) (see the Experimental section for conditions)	Amino (> 84%) C-Terminal Ala (> 95%)‡ C-Terminal Asp (100%)‡	13 ± 3

* The product was insoluble from pH 4.5 to pH 10-12, and was therefore assayed in suspension.

† The biological test on acetylated insulin did not give reproducible results; the biological activity, however, was always lower than the value of 70% reported by Fraenkel-Conrat & Fraenkel-Conrat (1950).

‡ The data in parentheses give the percentages of C-terminal residues removed by the enzymic breakdown.

determined by the procedure of Cundiff & Markunas (1961), samples (20 mg.) of methyl-insulin being used in each analysis. The method is based on the conversion of the methoxyl groups into methyl iodide, which is distilled and collected in pyridine. The alkylpyridinium iodide thus formed is potentiometrically titrated with tetrabutylammonium hydroxide, with vanillin as reference standard. The methoxyl content of insulin esterified by the above procedure ranged from 5.8 to 6.2 methoxyl groups/mol. of insulin of mol. wt. 5734, in close agreement with the results reported by Mommaerts & Neurath (1950) (see Table 1).

(b) Insulins methylated to different extents. Insulin preparations containing less than 6 methoxyl groups/mol. were obtained by the procedure given above, but with shorter times for the esterification reaction. The methylation was carried out on a 1 g. sample of pig insulin suspended in 100 ml. of 0.1 N-HCl in methanol. At different time-intervals portions (20 ml.) were taken from the reaction mixture and the insulin methyl ester was precipitated as described above. The preparative conditions and the methoxyl contents of the various insulin samples are given in Table 1.

Acetylated insulin. Insulin was acetylated by the method of Fraenkel-Conrat & Fraenkel-Conrat (1950). A 1 g. sample of pig insulin was suspended in 20 ml. of 60% (w/v) sodium acetate solution; the suspension was kept at -30° and 1.5 ml. of acetic anhydride was added dropwise (1 drop every 2 min.) with continuous stirring. After being kept for 3 hr. the reaction mixture was diluted with 10 ml. of water and the pH was adjusted to 5.5 with 2N-acetic acid. The acetyl-insulin was separated by centrifuging, washed several times with cold water, and then treated with diethyl ether and dried in vacuum over P₂O₅. Ninhydrin colorimetric analyses at 570 mμ were performed to determine the free amino groups, solutions of the original insulin serving as standards containing 100% free amino groups.

Succinylated insulin. Insulin was succinylated by the

procedure given by Habeeb, Cassidy & Singer (1958) for the preparation of the succinyl derivatives of bovine serum albumin and lactoglobulin. Preliminary experiments showed that the extent of the reaction decreased with decreasing pH. At pH 7.5-8 succinylation involved specifically the free amino groups; the results of the Folin-phenol reagent test indicated that no detectable O-esterification of the tyrosine residues occurred. A 0.5 g. sample of crystalline insulin was dissolved in 12.5 ml. of water adjusted to pH 8.0; to this solution 12 moles of succinic anhydride/mole of free amino groups were gradually added, with continual magnetic stirring; the temperature was kept at 0° by an ice bath. The pH was maintained by the gradual addition of 1N-NaOH and was checked throughout the succinylation with a combined calomel-glass micro-electrode. After ½ hr. the succinylated insulin was precipitated by lowering the pH to 4.5 and separated by centrifuging. After exhaustive washing with water at pH 4.5, the succinyl-insulin was suspended in diethyl ether and then dried under vacuum over P₂O₅. Ninhydrin colorimetric analyses at 570 mμ were performed to determine, free amino groups, solutions of the native insulin serving as standards containing 100% free amino groups. O-Esterification of the tyrosine residues was checked with the Folin-phenol reagent method of Herriot, as modified by Miller (1942).

Desalanine - desasparagine - insulin. Acetyl-desalanine-desasparagine-insulin was prepared by the procedure of Nicol & Smith (1956). Acetyl-insulin (0.2 g.), prepared as reported above, was dissolved in 20 ml. of water with its pH adjusted to 7.8 with a drop of 0.01 N-NaOH. Carboxypeptidase A (supplied by Worthington Biochemical Corp., Freehold, N.J., U.S.A., as a suspension of crystals in water) was added (4 mg.) and the mixture was incubated for 20 hr. at 37°; the pH was kept constant during the incubation. At the end of the incubation the acetyl-desalanine-

desasparagine-insulin was precipitated by adjusting the pH to 4.2 and then separated by centrifuging and washed with cold water at pH 4.2. The white powder was then suspended several times in diethyl ether and finally dried under vacuum over P_2O_5 . Asparagine and alanine, which were released by enzymic breakdown of the insulin, were determined in the supernatant with a Beckman-Spinco amino acid analyser. The yield was 0.98 mole of asparagine and 0.93 mole of alanine/mole of insulin of mol. wt. 5734.

The composition of the precipitate of desalanine-desasparagine-insulin was also checked; this check was made on a sample that was submitted to total hydrolysis and then analysed with the amino acid analyser. The results showed that the final product contained about two-thirds of the aspartic acid + asparagine residues (found, 1.98 residues/mol.) and two-thirds of the alanine residues (found, 2.0 residues/mol.) of native insulin; in comparison with native insulin the lysine content was 0.9 residue/mol. (standardized on proline), showing that enzymic breakdown of the B chain had been limited to the alanine residue alone.

Methylated desalanine-desasparagine-insulin. The acetyl-desalanine-desasparagine-insulin was methylated by the procedure given above for the preparation of the fully methylated insulin. The methoxyl content of the esterified material was 5.89 methoxyl groups/mol. of insulin.

Reaction between the substituted insulins and sodium sulphite

The procedure given by Cecil & Loening (1960) was used. The reaction between insulin and Na_2SO_3 was carried out in a polarographic cell at pH 7 ± 0.1 and at $37 \pm 0.2^\circ$. When the equilibrium was reached the pH of the reaction mixture was adjusted to 2 and the extent of the reaction was measured by determining the amount of thiol formed. This was done at the dropping-mercury electrode by amperometric titration with phenylmercuric hydroxide; the titration had to be performed at pH 2 as the equilibrium reaction is 'frozen' at this pH value.

A 3 ml. sample of insulin solution at pH 7 (corresponding to approx. $0.8\text{--}1 \mu\text{mole}$ of insulin) was used in each experiment; each sample was diluted with 1 ml. of a 6.0 M- KNO_3 solution and made air-free in the polarographic cell by bubbling N_2 . Then 1 ml. of an air-free Na_2SO_3 solution was added to give an Na_2SO_3 /insulin molar ratio about 200. The reaction mixture was kept at $37 \pm 0.2^\circ$ for the time required to reach equilibrium. When not stated otherwise the reaction time was 120 min. At the end of the reaction the pH was adjusted to 2 with 10 M- H_2SO_4 , the dropping electrode and the reference electrode were applied to the polarographic cell and the titration was carried out with a 10 mM-phenylmercuric hydroxide solution. The dropping-mercury electrode was polarized at -0.12 v against the saturated-calomel electrode; this was connected to the cell through a saturated KNO_3 salt bridge to avoid the presence of Cl^- ions, which interfere in the titrations at pH 2.

Control experiments on the native insulin were performed with the same cell and electrode set before and after each titration of a substituted insulin sample. Five samples of the substituted insulin solution and five of an insulin blank were alternately titrated in each run. The titrations on the native insulin were used to standardize the results of each set of experiments internally. In some experiments the reaction between insulin and Na_2SO_3 was studied at pH 7

or at pH 9 in the presence of a slight excess of the titration reagent (3.3–5 moles of phenylmercuric hydroxide/mole of insulin). In this case the titration was carried out at the same reaction pH, with the same apparatus and procedure as described above with the exception that the supporting electrolyte was KCl instead of KNO_3 ; the reference calomel electrode was directly connected to the polarographic cell and the dropping-mercury electrode was polarized at -0.5 v against the saturated-calomel electrode. The intersection of the residual-current line and the excess-of-reagent line was taken as the final titration point. The residual current line was determined in separate experiments. In some experiments the reaction between insulin and sulphite was carried out in the presence of 8 M-urea at pH 7, and the titration was carried out at pH 2 as described above. More details on the titration procedure can be found in the original paper of Cecil & Loening (1960).

Sucrose-density-gradient centrifugation

Linear 5–20% (w/v) sucrose gradients in 0.05 M-tris-HCl buffer, pH 7.5, were prepared by the method of Martin & Ames (1961). Some experiments were carried out in 0.05 M-sodium acetate buffer, pH 5. The labelled insulin sample (approx. $100 \mu\text{g.}$, corresponding to about $0.02 \mu\text{C}$ of ^{125}I) in 0.1 ml. of 0.05 M-tris-HCl buffer, pH 7.5, or of 0.05 M-acetate buffer, pH 5, was layered on top of a 5 ml. gradient and centrifuged at 38000 rev./min. for 16 hr. at 4° in the swinging-bucket rotor SW 39 of the Spinco model L preparative ultracentrifuge (Beckman Instruments Inc., Spinco Division, Munich, Germany).

The bottom of the centrifuge tube was punctured with a hypodermic needle and the contents were collected serially in 44 fractions (7 drops in each). Each fraction was diluted with 1 ml. of water and the radioactivity was measured in a well-type scintillation counter for ^{125}I or by liquid-scintillation counting for ^{14}C .

Bioassay of the substituted insulins

The biological activity of the substituted insulins was evaluated by the rat epididymal fat-pad assay. In each run five to ten assays were carried out on each insulin sample, and the activity was determined by comparison with a blank insulin standard solution.

RESULTS

Reaction of substituted insulins with sodium sulphite at pH 7. Among the substituted insulins we have studied, the methyl esters of both native and desalanine-desasparagine-insulin are sparingly soluble at pH 7, whereas the others are completely soluble. To be sure that the results obtained with esterified insulins were not affected by the low solubility of the products, some special experiments were carried out, which are reported in a separate section.

The time-course of the reaction between sodium sulphite and the substituted insulins that are soluble at pH 7 (acyl-insulin, acetyl-insulin, succinyl-insulin and desalanine-desasparagine-insulin) was followed for up to 250 min. This was done in a

single experiment for each substituted insulin, 50mg. of the product being allowed to react with sodium sulphite at pH7 under the experimental conditions given above. Samples from the reaction mixture were removed at intervals and acidified to pH2, and the free thiol groups were titrated. The results are shown in Fig. 1. For all the insulin samples the equilibrium was reached in about 40–50min. For acetyl-, succinyl- and desalanine-desasparagine-insulin the equilibrium value corresponded to two-thirds of the S·S bonds split/mol.; therefore these substituted insulins showed the same behaviour as native insulin (see Table 2).

Insulin acylated with phenyl isocyanate showed a different behaviour, since the equilibrium value,

Fig. 1. Time-course of the reaction between fully methylated ($\square$) or fully acylated ($\triangle$) insulin and Na_2SO_3 at pH7 and 37° . The time-course of unsubstituted native standard insulin ($\circ$) is also shown. The concentrations of insulin and Na_2SO_3 were 0.185mM and 36mM respectively. The titrations were carried out at pH2 with 1mM-phenylmercuric hydroxide.

which was attained in about 40min., corresponded to one-third of the S·S bonds split/mol. Some experiments with a greater excess of sodium sulphite or prolonged reaction times were carried out in an attempt to check the dependence of the equilibrium value on the reaction conditions; the results of these experiments confirmed that at pH7 only one-third of the S·S bonds reacted with sodium sulphite in the acylated insulin (Fig. 1). Since both methylated insulin and methylated desalanine-desasparagine-insulin are sparingly soluble at pH7, they were present as a suspension in the reaction mixture with sodium sulphite. The time-course of the reaction was therefore followed in separate experiments. For both esterified insulins the reaction with sodium sulphite was rapid and an equilibrium value corresponding to the splitting of one-third of the S·S bonds was attained in 30–40min. (see Fig. 1). Some experiments were carried out to establish whether the equilibrium value was affected or not by the low solubility of the methyl esters; the reaction was carried out with a greater excess of sodium sulphite (up to 2000 moles/mole of insulin) and lower insulin concentrations. No effect was found on the equilibrium value. The results are summarized in Table 2.

Reaction of substituted insulins with sodium sulphite in the presence of urea or of an excess of phenylmercuric hydroxide. A series of experiments similar to those described above were carried out with the difference that the solvent was 8M-urea instead of water. It was found that the time required to reach the equilibrium value was about 10–20min.; all the substituted insulins behaved as native insulin, 2.4–2.5 S·S bonds/mol. being split (see Table 2). The substituted insulins were also allowed to react with sodium sulphite at pH7 or at pH9 in the presence of an excess of phenylmercuric hydroxide (2.6–3.5 moles/mole of insulin). It was

Table 2. S·S bond titrations on insulin derivatives

Native insulin was assumed to have mol.wt. 5734; for substituted insulins the mol.wt. was corrected on the basis of the analytical data reported in Table 1. HOPhHg, Phenylmercuric hydroxide.

Conditions	...	...	S-S bonds reacting/mol.			
			Reaction at pH7, titration at pH2	Reaction at pH7, excess of HOPhHg, titration at pH7	Reaction at pH7, 8M-urea, titration at pH2	Reaction at pH9, excess of HOPhHg, titration at pH9
Insulin derivative						
Native insulin			1.87 ± 0.08	2.57 ± 0.10	2.44 ± 0.12	3.00 ± 0.10
Fully methylated insulin			1.00 ± 0.06	1.65 ± 0.10	2.33 ± 0.11	2.61 ± 0.11
Acetylated insulin			2.09 ± 0.14	2.65 ± 0.21	2.44 ± 0.16	2.89 ± 0.08
Succinylated insulin			1.99 ± 0.15	2.59 ± 0.16	2.45 ± 0.18	2.97 ± 0.09
Acetylated des-Ala-des-Asn-insulin			1.79 ± 0.06	2.40 ± 0.12	2.41 ± 0.15	2.92 ± 0.11
Methylated des-Ala-des-Asn-insulin			0.94 ± 0.02	1.60 ± 0.09	2.36 ± 0.10	2.76 ± 0.20
Acylated insulin (phenylcarbamoyl-insulin)			0.99 ± 0.05	1.96 ± 0.11	2.38 ± 0.14	3.01 ± 0.10

found (see Table 2) that about 1·5 S·S bonds/mol. reacted at pH 7 in esterified or acylated insulin in spite of the presence of the excess of phenylmercuric hydroxide, which certainly favours the shift of the reaction towards the right by blocking the thiols formed. Native insulin under the same conditions gave $2·57 \pm 0·10$ (10) S·S bonds split/mol., in agreement with the results reported by Cecil & Loening (1960). Acetyl-insulin, succinyl-insulin and acetyl-desalanine-desasparagine-insulin behaved as native insulin. When the reaction with sodium sulphite was carried out at pH 9 in the presence of an excess of phenylmercuric hydroxide, the uptake of phenylmercuric hydroxide corresponded exactly to the reaction of 3 S·S bonds/mol. with sodium sulphite in all the substituted insulins. One may therefore conclude that at pH 9 or in the presence of 8M-urea the failure of 1 S·S bond/mol. to react with sodium sulphite disappeared in both methylated and acylated insulin.

The following experiment was carried out on acylated and methylated insulin. The substituted insulins were allowed to react with sodium sulphite at pH 7 under the experimental conditions given above. The pH was then adjusted to 2 and solid urea was added to the reaction mixture to give a concentration of approx. 8M. After 1 hr. at 4° the solution was titrated with phenylmercuric hydroxide. The amount of thiols formed was found to correspond to the splitting of one-third of the S·S bonds. This result shows that only 1 thiol group/mol. is actually liberated when the substituted insulins react with sodium sulphite at pH 7; this excludes the possibility that a part of the thiol groups is in some way masked, e.g. through the polymerization of the reaction products. It should be mentioned that such a polymerization, if it occurs, cannot involve the oxidation of the thiols formed, since the reaction mixture of native insulin and sodium sulphite (at pH 7) showed no appreciable loss of the titratable thiol when maintained at pH 2 and at 4° for 10 hr.

Biological activity of the substituted insulins. As expected on the basis of the data already reported in the literature, acylated insulin (Fraenkel-Conrat & Fraenkel-Conrat, 1950), fully methylated insulin (Mommaerts & Neurath, 1950) and acetyl-desalanine-desasparagine-insulin (Nicol & Smith, 1956) were found to have lost their biological activity. As far as acetylated insulin is concerned, the measurement of the biological activity gave no reproducible results; all samples (five) that have been tested retained 30–60% of their original activity. The same was found with succinylated insulin. The results are reported in Table 1.

Sedimentation of acylated and methylated insulins in sucrose density gradients. The possibility that both methylated and acylated insulins are grossly

Fig. 2. Sucrose density gradient of methylated insulin (6 methoxyl groups/mol.; labelled with ^{14}C at $0·5 \mu\text{C}/\text{mg.}$) (—). Lysozyme (····) and adenosine deaminase (----) were used as standards. Assuming for lysozyme and adenosine deaminase mol.wt. 17200 and mol.wt. 35000 respectively, the mol.wt. of methylated insulin was about 10000. Under the same conditions native insulin gave mol.wt. about 30000.

Fig. 3. Sucrose density gradient of acylated insulin (fully acylated; labelled with ^{125}I at $2 \mu\text{C}/\text{mg.}$) (—). Lysozyme (····) and adenosine deaminase (----) were used as standards. Assuming for lysozyme and for adenosine deaminase mol.wt. 17200 and mol.wt. 35000 respectively, the mol.wt. of acylated insulin was about 11000. Under the same conditions native insulin gave mol.wt. about 30000.

aggregated was checked by the sucrose-gradient technique (Martin & Ames, 1961), with adenosine deaminase and lysozyme as standards. Adenosine deaminase activity was determined by the method of Kalckar (1947) and lysozyme was assayed by following the decrease in turbidity at $450\text{m}\mu$ of a suspension of *Micrococcus lysodeikticus* cells.

Both substituted insulins gave a single symmetrical peak (Figs. 2 and 3); the molecular weight, as evaluated by comparison with the standards, appeared to be lower than that of native insulin. However, it must be emphasized that an accurate determination of the actual molecular weight of the substituted insulins was beyond the scope of the present work. The demonstration that both acylated and methylated insulins have the same degree of homogeneity as native insulin rules out the possibility that aggregation phenomena might account for the titration results (see the Discussion section). Methylated and acylated insulins were labelled respectively with ^{14}C and ^{125}I as reported below.

Reaction of insulins methylated to different extents with sodium sulphite at pH 7. With the esterification procedure reported in the Experimental section we were not able to prepare methylated insulins containing less than 2 methoxyl groups/mol. This can probably be ascribed (Mommaerts & Neurath, 1950) to the higher rate of esterification of α -carboxyl groups in comparison with the side-chain carboxyl groups of aspartic acid and glutamic acid residues. On the other hand, esterification at lower acid concentrations and for more prolonged reaction times was avoided because of the danger of the occurrence of side reactions; Mommaerts & Neurath (1950) found that prolonged esterification involves the hydrolysis of the amide groups.

The reaction of methylated insulins and sulphite was studied in a series of experiments similar to those described in the preceding section. The results are reported in Table 3. Esterification of about two-thirds of the initially available carboxyl groups/mol. of insulin caused one-third of the insulin S-S bonds to become unreactive towards sodium sulphite. The titrations of esterified insulin samples containing less than 3.5 methoxyl groups/mol. were not very reproducible when compared with the results obtained with insulin esterified to a greater extent. An insulin sample containing 2.75 methoxyl groups/mol. was used in some experiments with higher concentrations of sodium sulphite (up to 1000 moles/mole of insulin). It was found that if the reaction was prolonged far beyond the time required to attain an apparent equilibrium the amount of titratable thiol increased. Further, if the sodium sulphite/insulin molar ratio was increased to 2000, we found that two-thirds of the S-S bonds were split. On the other hand, when the same experiments were carried out on an insulin sample containing 6 methoxyl groups/mol., we found that neither an excess of sodium sulphite nor a more prolonged reaction time caused a shift in the equilibrium value. In our opinion the anomalous results obtained with insulins with a lower methoxyl

content could be accounted for by hydrolysis of the methyl esters occurring during the relatively long stay in the reaction vessel (in the experiments at prolonged reaction times) or due to the high concentration of sodium sulphite.

Esterification and biological activity. The biological activity of esterified insulins at different methoxyl contents was measured by the fat-pad assay. The results are reported in Table 3. As is shown by the s.d. values, the accuracy of the bioassay was rather poor and the results must be accepted within a confidence limit of about 20%. The results of the bioassay prove that complete loss of the biological activity occurs at an average degree of esterification corresponding to 3-4 methoxyl groups bound/mol. Mommaerts & Neurath (1950) reported that esterification of about two-thirds of the initially available carboxyl groups has practically no effect on the activity of the hormone. In spite of a careful check of the experimental conditions we were not able to explain the discrepancy between our results and those reported by Mommaerts & Neurath (1950). The rate of esterification observed in our experiments was in good agreement with that reported by Mommaerts & Neurath (1950), but they reported neither the procedure used for the biological-activity measurement nor the accuracy of their results, making impossible any comparison.

However, the noteworthy point as far as the present work is concerned is that the failure of the S-S bonds to react in esterified insulin and the biological inactivation appear to be correlated, as both occur within the same range of extent of esterification (see Table 3).

Paper electrophoresis of the reaction mixture of methylated and acylated insulin with sodium sulphite at pH 7. It was important to establish whether the results obtained with acylated and methylated insulin reflect the occurrence of a mixture of products averaging 1 unreactive S-S bond/mol. This would imply, to account for the titration data, that one-half of the original insulin molecules were still intact after the reaction with sulphite at pH 7. Therefore paper electrophoresis was used in an attempt to identify intact methylated or acylated insulin possibly present among the products of the reaction with sodium sulphite. To facilitate the work, labelled insulins were used. Fully methylated insulin was prepared from [^{14}C]methanol (code no. CMM-87, supplied from CEA-CEN-SORIN) by the procedure given above (2mc of [^{14}C]methanol gave 10 μC of insulin esterified at 5.5 methoxyl groups/mol., 0.5 $\mu\text{C}/\text{mg.}$). Acylated insulin was labelled with ^{125}I by the procedure given by Rosa *et al.* (1967) for the iodination of native insulin; the labelled acylated insulin contained 0.2 atom of $^{125}\text{I}/\text{mol.}$ (2 $\mu\text{C}/\text{mg.}$); such an iodination level does not

Table 3. S·S bond titrations and biological activity of insulin methylated to different extents

Biological activities are expressed as percentages of the activity of native insulin standard. When not stated otherwise, the conditions for the reaction with sulphite and for the titration were those given in the text.

Expt. no.	Esterification time	S·S bonds reacting/mol.	Biological activity(%)	Methoxyl groups/mol.	Sulphite/insulin molar ratio
1	—*	1.83±0.08	93.6±10	—	200
2	<5 min.	1.36±0.04	38.9±3.4	2.13±0.04	200
3	<5 min.	1.31±0.04	3.9±0.3	2.75±0.18	200
4	<5 min.	1.84±0.05	3.9±0.3	2.75±0.18	600
5	—†	1.94±0.05	100	—	600
6	<5 min.	1.97±0.05	3.9±0.3	2.75±0.18	1000
7	—†	2.17±0.06	100	—	1000
8	<5 min.	1.12±0.03	4.1±0.2	3.21±0.18	200
9	5 min.	1.08±0.03	4.6±0.3	3.46±0.07	200
10	10 min.	1.13±0.06	14.1±3.2	3.59±0.07	200
11	2 hr.	1.03±0.06	2.8±0.3	5.01±0.17	200
12	13 hr.	1.04±0.03	<1.0	4.91±0.15	200
13	24 hr.	1.00±0.06	<1.0	6.0±0.20	200
14	24 hr.	1.11±0.06	<1.0	6.0±0.20	1000
15	24 hr.	1.21±0.05	<1.0	6.0±0.20	2000

* Blank experiment: insulin was treated for 24 hr. with 0.125 N-HCl at 25° in the absence of methanol, and then separated and purified as reported in the text.

† Blank experiments carried out on native insulin.

affect the reactivity of insulin towards sodium sulphite (Rosa *et al.* 1967). A 5 mg. sample of each product was allowed to react with sodium sulphite at pH 7 under the standard conditions given above; after 120 min. the pH was adjusted to 3 with acetic acid, solid urea was added to give a final concentration of 8 M and nitrogen was bubbled for 60 min. A 50 μ l. sample of the reaction mixture was submitted to paper electrophoresis with 8 M-urea–20% (v/v) acetic acid buffer, pH 3.2, on Whatman no. 1 paper for 16 hr. at 4–6 v/cm.; the paper strip was developed by radioautography with Ferrania 3N films. A major spot, accounting for 85–90% of the total radioactivity of the strip, was found on the starting point, with a ‘tail’ towards the cathode. The original acylated or methylated insulins gave, under the same conditions, a single and rather well-defined spot in the cathodic region, at about 8–10 cm. from the starting point. These results exclude the possibility that intact insulin molecules are present among the reaction products in an amount that would account for the titration results. In separate experiments, methylated or acylated insulins were added to the reaction mixture at pH 3 in 8 M-urea in an amount sufficient to increase the total radioactivity by about 50%. Paper electrophoresis proved that, when intact acylated or methylated insulin is present among the reaction products, it can be easily detected.

DISCUSSION

When native insulin reacts with sodium sulphite at pH 7, only the interchain S·S bonds are split

(Cecil & Loening, 1960); in iodinated insulin one of the interchain bonds is unreactive towards sodium sulphite (Rosa *et al.* 1967). The purpose of the present work was to establish whether such an effect is limited to iodination alone, or whether other substitution processes interfere with the ability of the interchain insulin bonds to react with sodium sulphite. The results obtained can be summarized as follows: acetylation, succinylation and removal of the C-terminal residues (alanine and asparagine in pig insulin) do not modify the behaviour of the interchain S·S bonds in insulin, as far as the reaction with sodium sulphite is concerned; esterification (with methanol) and acylation (with phenyl isocyanate) have the same effect as that previously found with iodination, since only one-third of the S·S bonds in insulin react with sodium sulphite. There is a large body of direct and indirect evidence against the possibility that this result reflects the occurrence of a mixture of products averaging 1 unreactive S·S bond/mol. To account for the titration data this would imply that one-half of the original insulin molecules were still intact in the reaction mixture; the electrophoretic analysis gives evidence that this is not the case.

Further, the sedimentation patterns of both methylated and acylated insulin suggest that no aggregation occurs in these products in comparison with the native insulin. On the other hand, as reported by Rosa *et al.* (1967), the reactivity of the interchain S·S bonds seems to be unaffected by the state of aggregation, since fibrous insulin is split by sodium sulphite at pH 7 into its component chains. In addition, the reaction with sodium sulphite of

methylylated and acylated insulins appears to attain a true equilibrium, which cannot be shifted towards the right either by prolonging the reaction time or by increasing the sodium sulphite/insulin molar ratio. The alternative possibility might be that polymeric material, in which the S-S bonds are inaccessible to the SO_3^{2-} ions, is formed by thiol-disulphide interchange during the reaction with sodium sulphite, as soon as some thiol groups are liberated. This possibility is ruled out by the results of the titrations at pH 7 in the presence of an excess of phenylmercuric hydroxide, which blocks the thiol groups formed. The titration results are unchanged, even in conditions where the thiol-disulphide interchange is certainly inhibited. The experimental results therefore seem to be consistent with the conclusion that each monomer unit in methylylated, and acylated insulin actually contains an unreactive S-S bond.

As far as methylation is concerned, this effect is to be considered as a side consequence of the esterification of the carboxyl groups, since no other reactions occur when treatment of insulin with methanolic hydrochloric acid is carried out under the experimental conditions we have used (Mommaerts & Neurath, 1950; Fraenkel-Conrat & Fraenkel-Conrat, 1950).

The actual extent of esterification at which the effect occurs on the single insulin molecule cannot be predicted on the basis of our results, in the absence of any possibility of evaluating the actual intermolecular distribution of the methoxyl groups. Since the insulin molecule contains 6 carboxyl groups (4 side-chain carboxyl groups and 2 terminal carboxyl groups) available for the esterification, it is theoretically possible for a given methylylated insulin sample to be constituted of different molecular species containing from 0 to 6 methoxyl groups/mol. Mommaerts & Neurath (1950) suggested that α -carboxyl groups are esterified at a much higher rate than the side-chain carboxyl groups; this is consistent with the difficulty we have found in the preparation of insulin samples containing less than 2 methoxyl groups/mol. If this were true it would imply that esterification of the α -carboxyl groups does not affect the S-S bonds' reactivity, since the failure to react with sodium sulphite occurs only when the extent of esterification increases from 2.3 to 3.2 methoxyl groups/mol. of insulin. The effect on the S-S bonds' reactivity would therefore seem to be related to the esterification of one of the side-chain carboxyl groups. As far as acylation is concerned, no data are available on the dependence of the S-S bonds' reactivity on the extent of the reaction with phenyl isocyanate, since only the fully acylated derivative has been tested. The relationship between the biological activity and the extent of esterification is of some interest, although

the poor solubility of methylylated insulin makes it necessary to consider with caution the reliability of the biological test. The failure of one-third of the interchain S-S bonds to react with sodium sulphite and the biological inactivation seem to occur at the same 'critical' extent of esterification. A similar relationship was found with iodination (Rosa *et al.* 1967), where the solubility of insulin was not affected at all by the substitution process. Further, acylation, which leads to a complete loss of the biological activity, also affects the S-S bonds' reactivity.

Acetylation and succinylation are more difficult to place in this context, since the bioassay gave too variable results; the residual activity of acetyl-insulin is lower than one would expect on the basis of the data reported by Fraenkel-Conrat & Fraenkel-Conrat (1950) (see Table 1). However, it is noteworthy that both processes neither affect the S-S bonds' reactivity nor lead to the abolition of the biological activity. Therefore, as far as the substitution processes we have studied are concerned, it would seem that the failure to react of one interchain S-S bond is always accompanied by complete inactivation.

In the attempt to explain the effect of the substituent groups on the S-S bonds' reactivity, two mechanisms can be envisaged: there can be a direct interfering effect such as steric hindrance or modification of the charge distribution; alternatively, there can be an indirect effect due to a change in protein conformation. The direct type of effect occurs in simple organic disulphides, where steric configuration and charged structure are the most important factors governing the rate of reaction with sodium sulphite. However, any attempt to explain the observed effect in terms of direct steric hindrance must take into account that the substitution reactions affecting the reactivity of the S-S bonds involve sites having very different locations inside the molecule. On the other hand, the hypothesis of a direct charge effect is inconsistent with the finding that acetylation, which abolishes the positive charge of the amino groups, and succinylation, which introduces a negative charge, do not affect the reactivity of the S-S bonds. Further, a complete failure to react, as that observed in substituted insulins, does not occur in simple disulphides as a consequence of an unfavourable charged structure.

The indirect type of effect, that is the occurrence of a conformational rearrangement of the molecule, seems to be consistent not only with the different location of the 'key sites', but also with the finding that the loss in biological activity and that in reactivity of the disulphide bridges parallel each other. It is not surprising that the chemical behaviour of the S-S bonds may be affected by a

conformational change of the molecule, when their unique structural role in insulin is considered. However, any attempt to suggest a plausible mechanism through which a conformational rearrangement could influence the S·S bonds' reactivity is, at the present time, largely speculative. This could occur, for instance, through the enlargement of the hydrophobic region of the molecule, since alcohols and detergents (A. Massaglia, unpublished work) allow the iodinated insulin to be completely split by sodium sulphite, or through a new and more stable spatial arrangement of the sulphur atoms. Beychok (1966), on the basis of circular-dichroism studies, has suggested that the disulphide dihedral angles of some of the insulin S·S bonds could be altered by as much as 25° from the characteristic 90° value of simple open disulphides; a 'normalization' seems to occur in alkaline solution (Beychok, 1966), where the extent of helical folding decreases. An attractive working hypothesis consistent with our results can be derived by considering that a more favourable spatial arrangement of an S·S bond may decrease its reactivity towards the nucleophilic attack (Fava, Iliceto & Camera, 1957).

The high reactivity towards sodium sulphite and thiols at pH 7 confers on insulin a peculiar position among the proteins, most of which are unreactive in the native form (Cecil & McPhee, 1959; Cecil, 1963; Davidson & Hird, 1967). This behaviour could be inherent to the biological function of insulin since a metabolic regulator is unlikely to be effective unless it is not only rapidly made available but also rapidly destroyed. There is some evidence in favour of the hypothesis that the reductive cleavage of the disulphide bridges is the initial step of insulin degradation, thus representing the rapid inactivating reaction required by an efficient regulation mechanism. In addition, it has been suggested that the mode of action of the hormone could involve attachment to the cell membranes by thiol-disulphide exchange reactions. The finding that selective substitutions confined to a few definite sites inhibit the splitting of insulin by sodium sulphite suggests that the high reactivity of the S·S bonds is strictly dependent on the integrity of the native molecule.

The availability of the disulphide bridges to nucleophilic attack could be viewed as one of the

basic properties of the molecule that the native conformation has to secure. Viewed against this background, the finding that the biological activity follows the same fate as the disulphide reactivity could imply that both properties depend to the same extent on the preservation of the native structure, thus adding further evidence in favour of the hypothesis that the availability of the S·S bonds plays a role in the biological function of the hormone.

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