The Electroreduction of the Disulphide Bonds of Insulin and other Proteins

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PART 1. POLAROGRAPHIC STUDIES

Simple disulphides give cathodic waves at the dropping-mercury electrode which are due to their reduction to the corresponding thiols. The polarographic reduction of cystine has been studied by a number of authors (Brdicka, 1933*b*; Roncato, 1934; Kolthoff & Barnum, 1941; Kolthoff, Stricks & Tanaka, 1955). In acid solution it gives a single wave with a pronounced maximum, and in neutral or alkaline solution a two-step wave. Kolthoff *et al.* (1955) suggested that the first part of the wave is kinetic and is due to the formation of mercury cysteinate at the surface of the mercury drop:

This is then reduced:

the overall reaction being:

The mechanism proposed for the reduction of GSSG (Stricks & Kolthoff, 1952) is different:

although the overall reaction is the same.

A different type of wave has been observed at more negative potentials in the presence of low concentrations of thiols and disulphides. Heyrovsky & Babicka (1930) observed a wave at -1.6 v in an ammonia-ammonium chloride buffer containing a trace of protein, i.e. at 0.2 v more positive than that due to the reduction of the NH_4^+ ion. They showed that this wave is due to the electroreduction of hydrogen catalysed by protein on the surface of the mercury drop. A similar effect was observed by Brdicka (1933*a*) when he used serum to suppress the maximum that the cobalt wave gives in ammoniacal solution. The maximum was suppressed, but a new double wave was produced. Brdicka (1933*a*) found that, of all the amino acids, only cystine and cyteine gave rise to this type of wave. The height of the wave was several hundred times as great as that due to the cathodic reduction of the SS group of cystine which showed that it must be a catalytic wave. Since the height of the cysteine catalytic wave was half the height of the

corresponding cystine wave at the same concentration, Brdicka (1933*a*) concluded that the SH group is responsible for the wave (at the potential at which the catalytic waves are observed SS groups will be reduced to SH).

Previous polarographic studies with proteins have been concerned with the catalytic waves, usually in the presence of cobalt (Millar, 1951, 1953*a, b*; Ledvina, 1956; Kalous & Pavlicek, 1962). Although some SS groups must necessarily have been reduced as catalytic waves were observed there have been no reports of cathodic waves due to this reduction. The explanation for this discrepancy must lie in the large difference in the heights of the two types of wave. The present authors had occasion to study polarograms of insulin at high sensitivity and observed waves with limiting currents of about $0.1\text{ }\mu\text{A}$, whereas the currents reported for catalytic waves are about $10\text{--}100\text{ }\mu\text{A}$. The present paper deals with the behaviour of the cathodic waves observed with insulin and other proteins containing SS groups.

EXPERIMENTAL

Polarographic measurements. The dropping-mercury indicator electrode was used, together with the saturated calomel reference electrode to which all potentials are referred. Current-voltage curves were recorded on a Radiometer type PO 4 recording polarograph. To observe the insulin waves with sufficient accuracy the polarograph had to be used with full-scale deflexions of between 0.10 and $1.0\text{ }\mu\text{A}$. In this current range the magnitude of the condenser current is sufficient to give current-voltage curves of the buffer solutions a high slope which renders interpretation of any reduction waves very difficult. To overcome this condenser-current compensation (Kolthoff & Lingane, 1952) was used, usually to the extent of $0.2\text{ }\mu\text{A/v}$.

Unless otherwise stated all current-voltage curves were recorded at 25° and at a mercury column height (uncorrected, see below) of 42.5 cm . Under these conditions the characteristics of the capillary in 0.1 N-HCl on open circuit were: m , 2.4 mg./sec. ; t , 3.1 sec. Measurements were also made at different mercury pressures. This is defined as the height of the mercury column less the back pressure due to the interfacial tension at the surface of the drop (Meites, 1955*a*).

The capacity of the polarographic cell was 3.5 ml. It incorporated a water jacket through which water at any

desired temperature could be circulated; all observations were at 25° unless otherwise stated. Oxygen was removed from the solutions by bubbling nitrogen. Frothing was prevented by a ring of silicone grease on the inside of the cell.

MATERIALS

Insulin. Crystalline (zinc) ox insulin (batch no. CBI 3714B) was purchased from Boots Pure Drug Co. Ltd., Nottingham, and stored over saturated CaCl_2 solution at 2–3° which maintains a relative humidity of 40%. Under these conditions the moisture content of the insulin remains constant at 9.9% (Cecil & Loening, 1960). Solutions were made up by weight in 0.1 N-HCl. The millimolar extinction coefficient at 277 μm was then found to be 6.13 and this value was used to check the concentrations of solutions during experiments. Solutions of zinc-free insulin were prepared by dialysis against 0.1 N-HCl.

Chymotrypsin. Crystalline chymotrypsin (lot no. 23 544) was obtained from the Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex. Solutions were made up in 1.0 mN-HCl and concentrations estimated from the extinctions at 282 μm . The extinction coefficient, $E_{1\text{cm}}^{1\%}$, was taken as 20.0, which is the value quoted by Wilcox, Cohen & Tan (1957) for chymotrypsinogen (see Chervenka, 1957).

Trypsin. A sample (lot DK 2671) was obtained from the Armour Pharmaceutical Co. Ltd. Solutions were made up in 1.0 mN-HCl and concentrations measured from the extinctions at 280 μm by taking $E_{1\text{cm}}^{1\%}$ to be 14.4 (Davie & Neurath, 1955).

Bovine serum albumin. A sample (fraction V; batch no. CA 0520) was purchased from the Armour Pharmaceutical Co. Ltd. Solutions were made up by weight in 1.0 mN-HCl on the basis of a moisture content of 9% (dry-weight determination).

Haemoglobin. Solutions of adult human carboxyhaemoglobin were prepared as described by Allison & Cecil (1958).

Ribonuclease. A sample (batch no. R 81 B-249) was purchased from the Sigma Chemical Co., St Louis, Mo., U.S.A. Solutions were made up in water and concentrations measured from the extinctions at 278 μm , by taking ϵ to be 9800 (Sela, Anfinsen & Harrington, 1957). The number of SS bonds/molecule was found to be 3.7 instead of the theoretical value of 4.0. The method of determination and the reason for the low values are discussed by Cecil & Wake (1962).

Mercury. This was purified by the method of Wichers (1942).

Guanidine hydrochloride. Stock solutions (approx. 7 M) were prepared by adding conc. HCl at 0° to solid guanidine carbonate (AnalaR grade; British Drug Houses Ltd.) to give approx. pH 3. The solutions were boiled to remove CO_2 and stored at 2°.

Buffer solutions. The following solutions were used: pH 1, 0.1 N-HCl; pH 7.1, 0.075 M- Na_2HPO_4 -0.025 M- NaH_2PO_4 -0.1 M-KCl; pH 9.2, 0.1 M- $\text{Na}_2\text{B}_4\text{O}_7$ -0.1 M-KCl.

RESULTS

Insulin

The molecular weight is 5733 and the molecule consists of two peptide chains which are linked by two SS groups. There is a third, intrachain, SS

group between residues 6 and 11 (referred to below as the 6–11 intrachain group) in the A chain (Ryle, Sanger Smith & Kitai, 1955).

Polarography at pH 1. A single cathodic wave with $E_{\frac{1}{2}} - 0.25\text{v}$ was observed (Fig. 1). The wave height increased linearly with the concentration of insulin until a limit was reached at approx. 12 μM (Fig. 2). The variation of wave height with the pressure of mercury above the capillary (h) was measured at two concentrations of insulin, one

Fig. 1. Current-voltage curve of insulin at pH 1. Details are given in the text. A, 0.1 N-HCl; B, 8.2 μM -insulin in 0.1 N-HCl. The condenser-current compensation was 0.2 $\mu\text{A}/\text{v}$.

Fig. 2. Variations in the height of the polarographic waves of various proteins with concentration at pH 1. Details are given in the text. ●, Insulin; ▲, trypsin; ○, ribonuclease; △, chymotrypsin; □, bovine serum albumin.

above and one below the limiting concentration. The results, given in Table 1, show that below the limiting concentration the current varied as $h^{\frac{1}{2}}$, whereas above it varied as h . This indicates that in the range of concentration where there is a linear current:concentration relationship the current is diffusion-controlled, but that when the limiting concentration is reached the current is limited by adsorption of the protein on to the mercury surface.

The temperature coefficient of the wave height at all concentrations was $1.2\%/1^\circ$ in the range $0-40^\circ$. The wave was unaffected by the presence of zinc: this is to be expected since insulin does not bind zinc at pH 1.

Polarography at pH 7.1. Two cathodic waves were observed with zinc-free insulin which are referred to as waves I and II (Fig. 3). At low insulin concentrations only wave I, with $E_{\frac{1}{2}} - 0.65$ v, was observed. The height of this wave increased linearly with concentration until a limit was reached at approx. $12\mu\text{M}$, at which concentration wave II, with $E_{\frac{1}{2}} - 1.02$ v, appeared. As the insulin concentration was further increased the height of wave I remained constant whereas that of wave II increased until a limit was reached at approx. $25\mu\text{M}$ (Fig. 4).

The effect of mercury pressure on the heights of waves I and II was similar to that observed on the wave at pH 1. The height of both waves varied as $h^{\frac{1}{2}}$ below the limiting concentration of insulin (equals diffusion control), but above this it varied as h (equals adsorption control).

Temperatures above 25° affected the two waves differently. The $E_{\frac{1}{2}}$ of wave I changed from -0.65 v at 25° to -0.63 v at 37° , whereas that of

wave II changed from -1.02 v to -0.88 v at these temperatures. The temperature coefficient of the height of wave I was $2\%/1^\circ$ below the limiting concentration but was zero above this concentration. The height of wave II had a temperature coefficient of $-3.5\%/1^\circ$ in both regions of concentration.

Fig. 3. Current-voltage curve of insulin at pH 7.1. Details are given in the text. A, $0.075\text{M-Na}_2\text{HPO}_4-0.025\text{M-NaH}_2\text{PO}_4-0.1\text{M-KCl}$; B, $19.2\mu\text{M-insulin}$ in the same solution showing waves I and II. The condenser-current compensation was $0.2\mu\text{A/v}$.

Fig. 4. Variation in height of the polarographic waves of insulin with concentration at pH 7.1. Details are given in the text. ●, Wave I; ○, wave II.

Table 1. Variation of the height of the polarographic wave of insulin at pH 1 with mercury pressure

Details are given in the text.			
h (cm.)	i (μA)	$10^3 \times i/h$	$10^3 \times i/h^{\frac{1}{2}}$
With $8.2\mu\text{M-insulin}$			
25.5	0.0488	1.914	9.663
27.5	0.0518	1.884	9.879
31.5	0.0538	1.708	9.585
33.5	0.0564	1.684	9.745
42.5	0.0636	1.496	9.757
45.5	0.0656	1.442	9.725
51.5	0.0708	1.375	9.865
With $38\mu\text{M-insulin}$			
25.5	0.0576	2.259	11.41
27.5	0.0609	2.215	11.61
31.5	0.0711	2.257	12.67
33.5	0.0738	2.204	12.76
42.5	0.1008	2.371	15.45
45.5	0.1029	2.261	15.25
51.5	0.1242	2.412	17.31
54.5	0.1265	2.321	17.14
57.5	0.1320	2.295	17.41
63.5	0.1510	2.378	18.95

Different results were obtained in the presence of zinc (P. D. J. Weitzman, unpublished work).

Polarography at pH 9.2. These results were similar to those at pH 7.1 except that the two waves were closer together, wave I having $E_{\frac{1}{2}} - 0.77$ v and wave II $E_{\frac{1}{2}} - 0.9$ v. Wave I reached its limiting height at an insulin concentration of approx. $13 \mu\text{M}$ and wave II at approx. $30 \mu\text{M}$. The same relation between mercury pressure and wave height was observed.

When the temperature was raised from 25° to 45° the two waves merged into a single wave with $E_{\frac{1}{2}} - 0.75$ v. The effect of raising the temperature at pH 7.1 was to shift wave II to a more positive potential without affecting wave I, and it is likely that a similar shift of wave II was being observed at pH 9.2, which resulted in a coincidence of the two waves.

Effect of surface-active substances on the insulin waves. Kolthoff & Barnum (1941) found that surface-active substances shifted the cathodic wave of cystine to more negative potentials, presumably by competing for the surface of the mercury drop. Since the dependence of the insulin waves on concentration and on mercury pressure suggest that adsorption plays a part in the electrode process, the effect of some surface-active substances has been examined.

The addition of increasing amounts of gelatin or Triton X-100 to insulin solutions at acid or neutral pH was found to depress, and finally eliminate, the insulin waves. The effect of octan-2-ol at pH 7.1 was to eliminate wave II but to leave wave I unaltered.

Effect of sulphite on the insulin waves. Cecil & Loening (1960) have shown that the two interchain SS groups react with sulphite alone but that the 6-11 intrachain group only reacts with sulphite in the presence of a heavy-metal salt. These reactions have been used to determine whether the polarographic waves involved the reduction of some or all the SS groups.

At pH 7 sulphite itself gives rise to a polarographic wave in the same region as the insulin wave. At pH 1, however, the sulphite wave (due to the reduction of sulphur dioxide) does not start until -0.3 v, and so it is possible to observe the presence or absence of an insulin wave with $E_{\frac{1}{2}} - 0.25$ v.

The reaction with sulphite was carried out under the conditions used by Cecil & Loening (1960). A sample of the reaction mixture was introduced into the polarographic cell together with sufficient 0.5 N-hydrochloric acid to decrease the pH to 1. After deoxygenation in the usual way the current-voltage curve was recorded. No insulin wave was observed, suggesting that these waves are caused by the reduction of the two interchain SS bonds.

Polarography of reduced insulin. Insulin was reduced at a stirred-mercury cathode at pH 1, under conditions which resulted in the reduction of the two interchain SS bonds only (Part 2 of the present paper). Samples were removed at various stages of the reduction and current-voltage curves recorded at pH 9.2 (Fig. 5). They show a progressive decrease in the height of the cathodic wave as the reduction proceeds. This is accompanied by the appearance of an anodic wave due to the oxidation of the SH groups formed.

Application of the Ilkovic equation. The limiting current in a diffusion-controlled wave can be defined by the Ilkovic equation:

$$i_d = 607nD^{\frac{1}{2}}Cm^{\frac{2}{3}}t^{\frac{1}{6}}$$

where n is the number of electrons involved in the reaction, D is the diffusion constant of the reacting substance, C is the millimolar concentration of the reacting substance, m is the flow of mercury through the capillary (mg./sec.) and t is the drop time (sec.). This equation can be used to determine n for the insulin waves provided that the concentra-

Fig. 5. Effect of electroreduction on the current-voltage curve of insulin. 0.5 mM-Insulin in 0.1 N-HCl was reduced at a mercury cathode. Details are given in the text. Samples were taken at specified times and diluted with 0.1 M- $\text{Na}_2\text{B}_4\text{O}_7$ - 0.1 M-KCl to give a final concentration of $8.2 \mu\text{M}$. A, 0.1 M- $\text{Na}_2\text{B}_4\text{O}_7$ - 0.1 M-KCl; B, $8.2 \mu\text{M}$ -insulin in the same solution at zero time; C, $8.2 \mu\text{M}$ -insulin in the same solution after 30 min. reduction; D, $8.2 \mu\text{M}$ -insulin in the same solution after 2 hr. reduction.

Table 2. *Application of the Ilkovic equation*

Details are given in the text.

Protein	pH	i_d (μA)	C (μM)	$10^7 D$	n
Insulin	1	0.059 ₈	8.2	16*	4.28
Insulin	7.1	0.052 ₈	9.8	10.03*	4.06
Ribonuclease	1	0.029 ₄	4	13.6†	4.8
Ribonuclease	9.2	0.004 ₄	2	13.6†	1.4
Chymotrypsin	1	0.02	2.45	10.2‡	6.1
Trypsin	1	0.025	2	9.40§	9.6
Bovine serum albumin	1	0.019	2	4.49	10.5

* Present paper.

† Rothen (1940).

‡ Schwert & Kaufman (1951).

§ Cunningham (1954).

|| R. A. Kekwick, quoted by Harrington, Johnson & Ottewill (1956).

tion of insulin lies in the range where the current is diffusion-controlled. The wave height, i_d , was measured graphically at the half-wave potential; m and t were also measured at this potential. Fredericq & Neurath (1950) have measured D for insulin at pH 2.6 over a range of concentrations and their value at zero concentration was taken to hold at pH 1. Creeth's (1952) data at pH 7.4 were extrapolated to a concentration of $10 \mu M$ and assumed to hold at pH 7.1. The results of these calculations with insulin and other proteins are given in Table 2. Since two electrons are required for the reduction of one SS group these calculations confirm that the insulin waves involve the reduction of two SS groups.

Reversibility of the electrode reaction. If the electrolytic reduction of a disulphide bond is thermodynamically reversible then the potential at the dropping-mercury electrode may be expressed by the equation (Kolthoff *et al.* 1955):

$$E = E_0 + 0.059 \log [H^+] + 0.0295 \log \left(\frac{i_d - i}{i^2} \right) + 0.0295 \log \left[\frac{f(RSH)^2}{f(RSSR)} \right]$$

where i_d represents the wave height, i and E the current and potential at any part of the curve, and $f(RSH)$ and $f(RSSR)$ are quantities which depend on the characteristics of the capillary and the respective diffusion constants.

From this equation three criteria of thermodynamic reversibility may be deduced.

(1) At any given pH a plot of E against

$$\log [(i_d - i)/i^2]$$

should give a straight line of slope 0.0295. Fig. 6 (curves A and B) shows the results obtained from two polarograms at pH 7.1. Both gave straight lines but the slopes were higher than the theoretical, namely 0.04.

(2) The half-wave potentials (i.e. when $i = i_d/2$) should change by 59 mv for a shift of 1 pH unit. The values of $E_{\frac{1}{2}}$ obtained at pH 1, 7.1 and 9.2

Fig. 6. Plots of $\log [(i_d - i)/i^2]$ against E . The data for curves A and B were derived from two current-voltage curves of insulin at pH 7.1. Details are given in the text.

were -0.25 , -0.65 and -0.77 v respectively, which correspond to a slope of 63 mv/pH unit.

(3) Since the equations given above hold in the presence of RSH as well as RSSR, it follows that both the oxidation and reduction processes should have the same half-wave potential. Fig. 5 shows polarograms of partially reduced insulin at pH 9.2, in which the anodic (SH) waves are continuous with the cathodic (SS) waves.

Ribonuclease

Ribonuclease consists of a single peptide chain of molecular weight 13700 which contains four SS groups. These form internal rings varying in size from 8 to 59 residues (Spackman, Stein & Moore, 1960).

The polarographic behaviour was similar to insulin. At pH 1 a single wave was observed with $E_{\frac{1}{2}} - 0.25$ v; the height of this wave reached a limiting value $0.042 \mu A$ at a concentration of approx. $9 \mu M$ (Fig. 2). At pH 9.2 two waves were

observed with $E_{\frac{1}{2}}$ -0.85v and -1.08v respectively, the second wave appearing after the first had reached its limiting height. These waves were less well-defined than those of insulin, and it was difficult to measure the variation of the height of each one with ribonuclease concentration. Instead the total current has been plotted against concentration (Fig. 7). The diffusion current reached a maximum of $0.021 \mu\text{A}$ at a concentration of approx. $5 \mu\text{M}$. Application of the Ilkovic equation (Table 2) shows that 2 SS bonds are reduced at pH 1 but only 0.7 at pH 9.2.

Chymotrypsin

Chymotrypsin has a molecular weight of 24500 and consists of three peptide chains (Meedom, 1956; Hartley, 1958). It contains five SS groups but it is not yet known for certain how many of these are interchain and how many intrachain (Cecil & Wake, 1962; Hartley, 1962).

A single wave was observed at pH 1 with $E_{\frac{1}{2}}$ -0.24v. The current reached a maximum of $0.027 \mu\text{A}$ at a concentration of approx. $4 \mu\text{M}$ (Fig. 2). At pH 9.2, in the range of concentration in which insulin and ribonuclease were studied, chymotrypsin gave only a small wave at -0.8v.

Fig. 7. Variation in height of the polarographic waves of ribonuclease with concentration at pH 9.2. Details are given in the text.

At much higher concentration ($60 \mu\text{M}$) a composite wave was observed which appeared to consist of two waves with $E_{\frac{1}{2}}$ approx. -0.55v and -0.8v. Application of the Ilkovic equation to the data at pH 1 (Table 2) showed that three SS groups were being reduced.

Trypsin

Trypsin consists of a single peptide chain of molecular weight 23500 and contains six SS groups (Neurath, 1957).

A single wave was observed at pH 1 with $E_{\frac{1}{2}}$ -0.25v. The current reached a maximum of $0.062 \mu\text{A}$ at a concentration of approx. $30 \mu\text{M}$, the change of slope being more gradual than that observed with the other proteins (Fig. 2). At pH 9.2 two waves, with $E_{\frac{1}{2}}$ -0.59v and -0.85v at low concentration, were observed, but these merged into a single wave with $E_{\frac{1}{2}}$ -0.75v as the concentration was increased. The current continued to increase with increasing concentration over the range investigated (up to $55 \mu\text{M}$). No sign of a limiting value was observed.

Bovine serum albumin

Bovine serum albumin has a molecular weight of 66000 and consists of a single peptide chain (Hunter & McDuffie, 1959) with 17 SS groups (Kolthoff, Anastasi & Tan, 1958).

A single wave was observed at pH 1 with $E_{\frac{1}{2}}$ -0.26v. The current reached a maximum of $0.02 \mu\text{A}$ at a concentration of approx. $2.5 \mu\text{M}$ (Fig. 2). At pH 9.2 a very small wave was observed at -0.8v at a concentration of $30 \mu\text{M}$. It was not investigated further.

Haemoglobin

Human adult haemoglobin has a molecular weight of 64500 (Hill, Koningsberg, Guidotti & Craig, 1962); it contains six SH groups but no SS groups (Allison & Cecil, 1958). Haemoglobin is denatured in acid solution and so observations were confined to pH 9.2. No waves were observed up to a concentration of 0.13 M .

PART 2. PREPARATIVE ELECTROREDUCTION

This work was started as one means of investigating the nature of the reaction responsible for the polarographic waves given by insulin and other proteins (Part 1 of the present paper). The intention was to reproduce, as nearly as possible, the conditions used for the polarographic reduction on a preparative scale and then to analyse the products formed. The groups most likely to be involved in this type of reduction are disulphide and a number

of papers have already been published on the reduction of disulphides at a mercury cathode.

Dohan & Woodward (1939) used a stirred-mercury cathode for the electroreduction of GSSG, and Benesch & Benesch (1957) used the same method for the reduction of cystine, homocystine and GSSG. Both sets of workers found that the reduction gave the corresponding thiols in quantitative yield. Hata & Matsushita (1953) used this

method for the reactivation of oxidized papain. Markus (1960) reduced insulin at a mercury cathode at pH 8.5 and found that two SS groups appeared to be reduced more rapidly than the third.

All the experiments quoted above were carried out at comparatively high potentials (10v or greater). In the present work the potentials were controlled in the same range as those used in the polarographic studies and the reductions were carried out in acid solution to prevent the simultaneous occurrence of SH-SS exchange reactions.

EXPERIMENTAL

Electroreduction. The apparatus used for electroreduction consisted of separate anode and cathode compartments connected by a salt bridge. Both compartments had a layer of mercury, diam. approx. 3 cm., on the bottom, which in each case was stirred with a small glass paddle. The anode compartment was filled with saturated KCl solution at the beginning of each experiment. The cathode compartment contained the substance to be reduced, dissolved in 5 ml. of 0.1 N-HCl; it was sealed to the atmosphere and oxygen removed with a stream of nitrogen. The salt bridge, internal diam. 1 cm., contained 2% agar in saturated KCl.

A 100 Ω potentiometer was connected across a 4v accumulator and the potential controlled manually. It was measured with a Radiometer type 22 pH-meter, used as a millivoltmeter connected between the cathode and a calomel reference electrode mounted in the cathode compartment (Lingane, 1953).

Estimation of thiol groups. The reduction of the disulphide groups was followed by estimating the number of SH groups formed. Cysteine concentrations were measured by potentiometric titration with HgCl_2 in acid solution (Cecil, 1955). The concentrations of protein SH groups were measured by amperometric titration with phenylmercuric hydroxide as described by Allison & Cecil (1958) and Cecil & Loening (1960). Since the reductions were carried out in 0.1 N-HCl it was necessary to raise the pH before titration to 8-9 so as to avoid precipitation of phenylmercuric chloride (Cecil & McPhee, 1959). Proteins in which the SS groups have been reduced are very susceptible to reoxidation. In this they resemble denatured haemoglobin (Allison & Cecil, 1958). The technique of Allison & Cecil (1958), involving two titrations, was used to avoid errors from this cause. The first titration was carried out in the usual way and used to give the value of the residual current at the end point, although this latter could be as much as 10% low. In the second titration a small excess of phenylmercuric hydroxide was added to the buffer before the sample so that the SH groups present were immediately converted into mercaptide. The titration was continued to give an excess-of-reagent line which was extrapolated back to the residual-current line obtained in the first titration.

Estimation of disulphide groups. Cecil & Wake (1962) made a distinction between the SS groups in a protein that would react with sulphite at pH 7 and those that would not. Their technique was used to help identify those SS groups that had been reduced and those that had not. The total

number of SS groups/molecule was determined by reaction with sulphite in the presence of 3M-guanidinium chloride (Cecil & Wake, 1962).

RESULTS

Cystine

Cystine was used as a test substance to ensure that quantitative reduction could be achieved with the apparatus described. The diffusion current of cystine in 0.1N-hydrochloric acid reaches its maximum height at -0.7v (Kolthoff & Barnum, 1941), and the reduction should therefore be carried out at a potential more negative than this; -0.9v was used. The cathode compartment of the cell was filled with 5 ml. of 1.0 mM-cystine solution in 0.1N-hydrochloric acid and a stream of nitrogen passed for 10 min. to remove oxygen. The current was then switched on and the potential adjusted to -0.9v . The current was initially 2 mA but fell to 0.1 mA after 30 min. A sample of the solution was then withdrawn and shown to contain 99% of the theoretical quantity of cysteine.

Insulin

The molecular weight is 5733 and the molecule consists of two peptide chains which are linked by two SS groups. There is a third intrachain SS group between residues 6 and 11 in the A chain.

The electroreduction was carried out in the same way as with cystine, the concentration of insulin being 0.5 mM. As an additional precaution a current-voltage curve was plotted manually by using the reduction cell (Fig. 8). It resembled closely that observed with the dropping-mercury

Fig. 8. Current-voltage curve of insulin at pH 1 with a stirred-mercury cathode. The course was plotted manually by using the electroreduction apparatus described in the text. The concentration of insulin was 0.5 mM.

electrode (Part 1 of the present paper), except, of course, that the current was much greater.

The best results were obtained when the reduction was carried out at -1.35 v; it was followed by withdrawing samples and titrating the SH groups formed with phenylmercuric hydroxide. Fig. 9 shows that 3.9 SH groups were formed after $1\frac{1}{2}$ hr., no further reduction taking place after 7 hr. The product was insoluble; no SH groups could be detected in the solution. Reaction with sulphite alone at pH 7 gave no further increase in SH content, but reaction in the presence of 3M-guanidinium chloride gave a further 0.64 SH group/molecule (the reason for the low recovery of the third SS group is not known).

The reduction was repeated at a potential of -1.8 v and, after $1\frac{1}{2}$ hr., a soluble product was obtained. This was found to contain 6 SH groups/molecule, indicating that, at this potential, all three SS groups had been reduced. As a further confirmation the reduction was carried out at -1.35 v for 5 hr. and then at -1.8 v for a further 2 hr. The white precipitate, which had formed initially, redissolved and the final product was again shown to contain 6 SH groups/molecule.

The absorption spectra of fully reduced insulin and untreated insulin were compared and found to be identical. This, and the fact that the polarographic waves can be accounted for by the reduction of the disulphide bonds, suggest that no other groups are involved in the reaction.

Ribonuclease

Ribonuclease consists of a single peptide chain of molecular weight 13700 and contains four SS

Fig. 9. Time-course of the electroreduction of insulin at -1.35 v. The reduction was carried out as described in the text. Samples were removed at intervals and analysed for SH groups.

groups. The reduction was carried out at a potential of -2.0 v, the concentration of ribonuclease being 0.2 mM. After 2 hr. a clear solution was obtained and analysis of the SH groups showed that all four SS groups had been reduced.

DISCUSSION

The polarographic waves described in Part 1 of the present paper are clearly different from the catalytic waves that have been observed by other workers in the presence of proteins. Not only are the currents about 1000-fold smaller, but these waves occur under conditions in which the catalytic waves do not, e.g. in acid solution.

The half-wave potentials of the waves given by insulin are comparable with those given by reduction of the SS groups of cystine and GSSG (Stricks & Kolthoff, 1952; Kolthoff *et al.* 1955). This, combined with the fact that insulin does not possess other groups that are reducible in this range of potential, suggested that SS groups might be responsible for the waves observed. This idea has been confirmed by examining insulin, which has had the two interchain SS groups split (a) by reaction with sulphite, and (b) by electrolytic reduction. In both cases the polarographic waves were eliminated. This evidence also shows that the 6-11 intrachain group is not involved in the reaction.

At pH 1 a single wave was observed with insulin, but at pH 7.1 and 9.2 a second wave appeared after the first had reached its limiting height. The height of wave I, at all values of pH, was shown to be directly proportional to the insulin concentration until a limiting height was reached at approx. $12\mu\text{M}$. Below $12\mu\text{M}$ therefore the current must be limited by the rate of diffusion of insulin and above it by the amount of insulin that can be adsorbed on the surface of the mercury drop (the properties of adsorption waves are summarized by Meites, 1955b). This is confirmed by the fact that, at insulin concentrations below $12\mu\text{M}$, the current was proportional to $h^{\frac{1}{2}}$ but above $12\mu\text{M}$ to h (h is the pressure of mercury above the capillary).

The characteristics of wave II are similar to those of wave I. It appears after wave I has reached its limiting height and, in turn, reaches a limiting height approximately equal to that of wave I at approximately double the insulin concentration. The explanation suggested for these observations is that, when wave I has reached its limiting height and the surface of the mercury drop is saturated with insulin, a second layer of insulin starts to form on top of, or interwoven with, the first layer. This layer in turn becomes saturated at approximately double the insulin concentration required for the first layer,

The stability of this second layer is less than that of the first. Thus addition of a surface-active substance such as octan-2-ol causes wave II to disappear without affecting wave I. This difference in stability is also reflected in the temperature coefficients. Wave I has a temperature coefficient of $+2\%/1^\circ$ below the limiting concentration (i.e. when the current is diffusion-controlled) and zero above it (i.e. when the current is adsorption-controlled), showing that the first insulin layer is unaffected by temperature in the range $25-37^\circ$. The value for wave II is $-3.5\%/1^\circ$ in both regions of concentration, suggesting both that the second layer tends to break down as the temperature is raised and also that reduction can only take place via adsorption. The half-wave potential of wave I is scarcely affected by raising the temperature from 25° to 37° (i.e. from -0.65 v to -0.63 v); that of wave II changes from -1.02 v to -0.88 v suggesting that the actual process of reduction is easier at the higher temperature.

Analysis of wave I for insulin showed that the reduction process was thermodynamically reversible within reasonable limits. The plot of $\log [(i_d - i)/i^2]$ against E gave a straight line of slope 0.04 . Although this differs from the theoretical of 0.0295 it is the same as that found for cystine by Kolthoff *et al.* (1955). The half-wave potential changed by 63 mv/pH unit as compared with the theoretical value of 59 mv . Stricks & Kolthoff (1952) obtained a value of 59 mv for the reduction of GSSG, and Moret (1961) a value of 65 mv for diphosphothiamine disulphide. An important criterion is that the anodic and cathodic waves of partially reduced insulin have the same half-wave potential.

The results with other disulphide proteins were similar to those obtained with insulin. At pH 1 all gave single waves, with half-wave potentials in the range -0.24 to -0.26 v , which reached limiting heights as the protein concentration was increased (Fig. 2).

The behaviour at pH 9.2 was more varied. Ribonuclease gave two waves similar to these obtained with insulin. Chymotrypsin and bovine serum albumin gave small ill-defined waves under

the same conditions. Trypsin gave two waves at low concentration but, as the concentration was increased, these merged into one which showed no sign of reaching a limiting height below $55\mu\text{M}$. Pechère, Dixon, Maybury & Neurath (1958) have shown that trypsinogen undergoes a large change in configuration when the SS groups are split. The structure of trypsin differs only slightly from that of trypsinogen (Neurath, 1957) and this change of configuration may well result in the SS groups becoming more susceptible to reduction. Haemoglobin, which contains six SH groups but no SS groups, does not give a polarographic wave under these conditions.

Fig. 2 shows that the limiting heights of the waves observed at pH 1 occur at widely differing protein concentrations. If the limit in wave height is due to saturation of the mercury surface with protein molecules then the limiting current should depend on the number of reducible SS groups/molecule and inversely on the molecular weight. The number of reducible SS groups/molecule was obtained by application of the Ilkovic equation (Table 2). The limiting current was then divided by the ratio (number of reducible SS groups/molecule)/(molecular weight). The values for the different proteins are remarkably consistent (see Table 3), suggesting that this is the right explanation.

Cecil & Wake (1962) studied the reaction of these and other disulphide proteins with sulphite. They found that some intrachain SS groups failed to react with sulphite, whereas the interchain groups reacted normally. There is some correspondence between the number of SS groups reduced at the dropping-mercury electrode and the number that react with sulphite at pH 7 (Table 4). However, this correspondence may be fortuitous since the two reactions depend on different factors. The reactivity of an intrachain SS group towards sulphite is thought to depend on the stability of the ring structure of which it forms a part (Cecil & Wake, 1962), whereas the ease of reduction appears to depend on the position of the SS group relative to the mercury surface. This in turn depends on the configuration of the protein in a surface film.

Table 3. *Dependence of the limiting wave height at pH 1 on the reducible disulphide content and the molecular weight*

Protein	Details are given in the text.		
	Reducible SS groups/molecule		
	mol.wt. (A)	i_{lim}^* (μA) (B)	B/A ratio
Insulin	$2/5733 = 3.48 \times 10^{-4}$	0.096	276
Trypsin	$5/23500 = 2.13 \times 10^{-4}$	0.062	291
Ribonuclease	$2/13700 = 1.46 \times 10^{-4}$	0.042	284
Chymotrypsin	$3/24500 = 1.23 \times 10^{-4}$	0.027	219
Bovine serum albumin	$5/66000 = 0.76 \times 10^{-4}$	0.02	263

Table 4. *Number of disulphide groups/molecule in various proteins (a) that react with sulphite at pH 7 and (b) that are reduced at the dropping-mercury electrode*

Details are given in the text. The values for the sulphite reaction are taken from Cecil & Wake (1962).

Protein	No. of SS groups reacting with sulphite at pH 7	No. of SS groups reduced at the dropping-mercury electrode at pH 1
Insulin	2	2 (2 at pH 7.1)
Ribonuclease	0.5	2 (0.7 at pH 9.2)
Chymotrypsin	3.1	3
Trypsin	5.2	5
Bovine serum albumin	0	5

The work on electroreduction shows that, at potentials of up to -1.35 v, two SS groups/molecule of insulin are reduced. The failure of the remaining SS group to react with sulphite, except in the presence of 3M-guanidinium chloride, shows that the two groups reduced are those connecting the A and B chains (Cecil & Wake, 1962). This is confirmed by the fact that the polarographic wave is not given by insulin that has been reduced at -1.35 v or by insulin in which the two interchain SS groups have been split by reaction with sulphite.

The reduction of the intrachain group in the A chain does not take place at -1.35 v even after several hours but can be achieved by raising the potential to -1.8 v. The fact that the absorption spectrum is unchanged by this procedure is evidence that the tyrosine residues have not been affected.

The solubilities of the various insulin derivatives, in which some or all the SS groups have been split, are interesting. Sanger (1949) found that, after oxidation of all three SS groups with performic acid to give six SO_3^- groups, the A chain was soluble over the whole pH range but that the B chain was precipitated at pH 6. Cecil & Loening (1960) found that, after reaction of two SS groups with sulphite to give two SH groups and two SSO_3^- groups, the B chain was soluble over the whole pH range but that the A chain was precipitated at pH 5. This work shows that, after reduction of two SS groups to give four SH groups, both A and B chains are insoluble at pH 1 but that after reduction of all three SS groups, to give six SH groups, both chains are soluble.

The experiments with ribonuclease show that complete reduction of the SS groups can be achieved in the absence of an unfolding agent. The reaction with thiols requires the presence of 8M-urea before it will go to completion (White, 1961; Anfinsen & Haber, 1961); similarly the reaction with sulphite requires the presence of 3M-guanidinium chloride (Cecil & Wake, 1962).

It would appear therefore that electroreduction has certain advantages as a method for the complete reduction of disulphide proteins. These may be summarized as follows:

(1) The reduction can be carried out in acid solution in which both the reoxidation of SH groups and SH-SS exchange reactions are minimized.

(2) The reaction is specific for disulphides and can easily be followed by analysing the SH groups formed.

(3) The addition of other reagents, e.g. reducing agents or unfolding agents, is avoided.

SUMMARY

1. The polarographic behaviour of five proteins containing SS groups and one containing SH groups has been studied by using the dropping-mercury electrode.

2. The disulphide proteins all gave a single cathodic wave at pH 1 which was shown to involve the reduction of some of the SS groups. The number of SS groups involved with each protein was calculated from the Ilkovic equation.

3. The height of these waves increased linearly with protein concentration until a limit was reached. The limiting current, and the concentration at which it occurred, varied between proteins and was shown to be related to the number of reducible SS groups/molecule and the molecular weight.

4. At pH 7.1 and 9.2 a second cathodic wave was observed in some cases. This appeared after the first wave had reached its limiting height and was also shown to involve the reduction of SS groups.

5. The behaviour of both types of polarographic wave has been explained in terms of the adsorption of protein on to the surface of the mercury drop.

6. Haemoglobin, which contains SH groups but no SS groups, does not give a polarographic wave under these conditions.

7. Insulin and ribonuclease were reduced at a stirred-mercury cathode.

8. At -1.35 v only the interchain SS bonds of insulin were reduced but at -1.8 v all three bonds were reduced.

9. At -2.0 v all four SS bonds of ribonuclease were reduced.

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Determination of Oxo Steroids as Coloured Salts of their Girard Derivatives

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A group of analytical methods is based on the observation that salts formed between strong acids and bases of relatively high molecular weight are extractable from aqueous solutions by chlorinated hydrocarbons (cf. Prudhomme, 1938; Auerbach,

1943; Lehman & Aitken, 1943; Jones, 1945; Barr, Oliver & Stubbings, 1948; Roy, 1956). In aqueous solution the equilibrium:

(A^+ and B^- being large ions, a^+ and b^- small ions) is shifted to the right by the extraction of A^+B^- with a chlorinated hydrocarbon. Consequently B^- may be extracted from its aqueous solution by the addi-

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