CXXXVIII. THE THIOL-DISULPHIDE SYSTEM.

I. COMPLEXES OF THIOL-ACIDS WITH IRON.

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THE addition of iron salts to alkaline solutions of a variety of thiol acids leads to the immediate production of intense red or violet colours. These fade more or less rapidly but may repeatedly be regenerated by bringing the faded solutions into contact with oxygen. This characteristic of thiol acids has long been familiar and has generally been held to indicate the existence of complexes of RSH^1 with both ferric and ferrous ions. The colour has been attributed to an unstable ferric complex which suffers spontaneous autoreduction at the expense of RSH to a colourless, freely autoxidisable ferrous complex. It is assumed, in other words, that these complexes constitute an oxidation-reduction system reversible with respect to the state of oxidation of the iron, the oxidation of RSH being merely an accessory and irreversible change.

The significance of such a hypothesis to the chemical and the biological activity of the RSH — RSSR system is obvious. The high rates at which oxygen oxidises the ferrous complex and RSH reduces the ferric complex provide a simple basis for the definition of the important catalytic influence of traces of iron on the oxidation of RSH and suggest that it may be these which control the interesting relations of glutathione² to tissue respiration. This argument

¹ The symbols RSH and RSSR are employed as general descriptive terms for the thiol and disulphide components, respectively, in the systems discussed.

² The question of the chemical constitution of glutathione has been re-opened since this paper was written. It now appears that those preparations from natural sources, of a material to which we have been accustomed to give the name "glutathione" and with which the chemical and biochemical behaviour of "glutathione" have been established, were chemically impure. It appears that the chief thiol constituent of this material is a tripeptide [Hopkins, 1929], some of whose biochemical activities differ from those of the impure product [Dixon and Meldrum, 1929]. Such a situation, of course, demands a re-investigation of the biological function of this substance. In the meantime, however, the facts of "glutathione respiration" and the dominant part which the thiol group plays in these phenomena remain unchallenged. In the present paper the name "glutathione" is retained, without structural implications, for the description of those phenomena involving the thiol group which have been disclosed with the aid of impure glutathione.

was first developed by Warburg, whose view seems to be that the essential biological rôle of the thiol group in such compounds as glutathione is the provision of a suitable vehicle for the exhibition of the catalytic activity of iron. The matter is of some importance since the quantitative establishment of the above contention would undoubtedly lead to a reconciliation of many conflicting views of the mechanism of biological oxidation-reduction.

In spite of these implications, however, no quantitative attempt appears to have been made to arrive at the chemical composition of these hypothetical complexes or at the conditions of their formation and reaction. In the present paper such an attempt is described. The data assembled will, in a later communication, be extended towards a quantitative definition of the main kinetic relations of the RSH—RSSR system and of the catalytic influence of iron upon these. Since the completion of the present work there have been published two reports by Michaelis and his colleagues [Michaelis and Barron, 1929, 2; Michaelis and Yamaguchi, 1929] of attempts to arrive at the nature of these iron-thiol complexes by the study of the more stable cobalt-thiol complexes. These interesting studies are discussed in the text.

QUALITATIVE OBSERVATIONS.

Discussion will be facilitated by the preliminary description of orienting experiments which serve to define the general relations of iron to RSH—RSSR. These observations actually relate specifically to thioglycollic acid. They may, however, be extended to such substances as thiolactic acid, cysteine and glutathione with only minor modifications affecting equilibrium constants, rates of reaction and relations to p_H . It must be emphasised, at the outset, that the critical requirement of all this work is the rigorous exclusion of oxygen from all reagents and apparatus. This was achieved by thorough deaeration with purified nitrogen.

1. When, under these conditions, ferric ions are added to a solution of RSH buffered within the p_H range 6–11, a deep red colour instantly forms. This proceeds to fade at a rate which increases rapidly with $[H^+]$ between these limits of p_H . At p_H 7 the change is almost instantaneous. The coloured solution, moreover, is not only subject to spontaneous fading, but may be instantly decolorised by such soluble reducing agents as titanous chloride and sodium hydrosulphite. In all cases the colour may be instantly restored by admission of oxygen or by soluble oxidising agents, *e.g.* iodine or methylene blue. The cycle of fading and regeneration may be repeated indefinitely until such time as iron begins to separate from solution. Examination of the solution at this point shows that almost the whole of the RSH has been converted into RSSR.

2. The tendency of ferric ions to form the red colour diminishes rapidly as the p_H is raised above 10. Instead, there is an increasing tendency for the immediate precipitation of the added iron by the anions present.

3. When ferric ions are added to a solution of RSH buffered at a p_H more acid than 4, a transient blue flush replaces the red flush observed when the p_H is raised to about 6. The one colour merges gradually into the other as the reaction is modified between these limits, the colour change appearing to be centred very approximately at p_H 5. The colourless solutions which succeed the blue colour in acid solution yield no colour with oxygen or other oxidising agents. When the acid mixture is made alkaline no colour appears if sufficient care has been taken to prevent the intrusion of traces of oxygen. The familiar red colour is, however, produced as soon as an oxidising agent is added.

4. When ferrous ions are added to RSH no colour appears in either acid or alkaline solution. The mixture behaves towards oxidising agents exactly like the solutions resulting from the decoloration of ferric-RSH mixtures.

5. Apart from the effect of p_H , the most important factors controlling the stability of the red colour are the concentrations of ferric iron and of RSH. Owing to the spontaneous fading of the red colour it is not possible to obtain a precise measure of any stoichiometric relations which may govern the influence of these functions. If, however, we choose a p_H , *e.g.* 10, at which fading is relatively slow, it is possible to arrive at some approximate relations of interest. When solutions of ferric iron in RSH at this p_H are progressively diluted there is, at first, no loss of colour greater than that which can be attributed to dilution. When the concentration of RSH has been reduced to about $0.005 M$ —the figure varies somewhat with p_H , increasing rapidly above 10—there is a fairly sudden diminution in colour intensity and a simultaneous separation of a precipitate containing iron. The same critical point is obtained if to a suspension of ferric iron in buffer solution increasing amounts of RSH are added.

If, now, we choose a concentration of RSH which is sufficient for maximum colour production we may attempt the determination of the solubility of iron in it. A $0.1 M$ solution of RSH at p_H 10 was titrated with ferric chloride. The added iron remained in solution and the colour increased in intensity up to a point which corresponded very roughly with an iron: RSH ratio of 1:5. Further addition of iron merely resulted in the separation of a precipitate.

The behaviour of ferrous-thiol mixtures was rather different. Iron did not separate from such mixtures even at great dilutions of RSH.

6. Addition of cyanide to ferric-RSH mixtures led to a progressive discharge of the red colour. The resulting colourless solutions were not sensitive to oxygen.

7. RSSR does not appear to be capable of dissolving iron in alkaline solution and no colours are formed. RSSR was unable to oxidise the colourless ferrous-RSH solutions to the red colour.

The foregoing observations appear to give ample justification for the conclusion that both ferrous and ferric ions form complexes with RSH. The qualitative evidence presented suggests that the ferric complex dissociates into its components more readily than does the ferrous complex and is not

inconsistent with the probability that the composition of these complexes is determined by the usual co-ordination number of iron—six. The system ferrithiol-ferrothiol is freely reversible and attains rapid equilibrium with other reversible oxidation-reduction systems. Ferrithiol, moreover, suffers rapid autoreduction at the expense of RSH and ferrothiol is rapidly oxidised by oxygen in neutral and alkaline solution. The velocity of the former process is some direct function, while that of the latter may be some indirect function, of $[H^+]$. Cyanide enters into the composition of one or both of the complexes with modification in the oxidation-reduction behaviour of the system. The ferrithiol probably suffers a change of ionic charge in the neighbourhood of p_H 5. Above p_H 11 it is more or less completely dissociated into its constituents. There is no evidence that complex formation introduces any enhanced degree of reversibility into the more or less irreversible process $RSH \rightarrow RSSR$.

It should be mentioned that the above conclusions are contrary to those at which Lyons [1927] arrived as a result of observations on the reaction of iron with thioglycollic acid. Lyons expressed the view that the red colour is due to the coloured ion of a colourless ferrous complex. As Michaelis and Barron [1929, 2] have suggested, however, this erroneous conclusion arose through insufficient care being taken by Lyons to ensure that all trace of oxygen was excluded from the reaction mixtures which he regarded as being free from oxygen.

We have been unable to isolate in the solid state any of the complex compounds which may be present. Consequently we must be content to study their behaviour in systems in which the concentration of RSH and of iron are controlled. An attempt, on this principle, to give some quantitative definition of the kinetic and equilibrium relations which have been indicated above will now be made.

THE AUTOREDUCTION OF FERRITHIOL.

A series of velocity curves for this reaction at p_H values from 8 to 10.5 are given in Fig. 1 (p. 1249). Attempts to extend the experimental range of p_H failed owing to the great velocity of the reaction in solutions of greater $[H^+]$, and to the separation of a precipitate of iron beyond the more alkaline extreme. The method consisted in the determination at intervals of the amount of residual red colour (by titration with sodium hydrosulphite) in an oxygen-free reaction mixture originally constituted by adding a known amount of ferric ions to a buffered solution of RSH. Since no precipitates were observed in the reaction mixtures of any of the experiments quoted, we are entitled to assume that the whole of the iron which had been added had entered into complex formation. Indeed, the analysis of the electrometric data which is presented in a later section of this paper indicates definitely that the initial concentration of ferrithiol should have been equal to the amount of iron added. That is to say, we are entitled to assume a common origin to the several reaction curves. In such case it is clear that the reaction follows an unusual

course. The character of the curves is such as to suggest a process moving towards an equilibrium which is short of complete change of the initial reactant. This would imply that the reaction

was significantly reversible. Control experiments seem, however, to have excluded this explanation. Fig. 1 indicates that around p_{H} 10 the hypothetical reverse reaction should proceed at a rate comparable with that of the forward process. Nevertheless a ferrithiol solution which had been allowed to become completely decolorised at p_{H} 8 did not develop any colour during several hours after the p_{H} had been adjusted to 10 in the absence of oxygen. The addition of a high concentration of RSSR to a ferrothiol solution at the latter p_{H} did not lead to any development of colour.

Our observations are admittedly subject to some important sources of error. A more suitable choice of relative concentrations of reactants might have been made had the results of electrometric studies been then available. The temperature was not closely controlled. Moreover, in spite of the fact that the molar concentration of the buffer was adjusted to 25 times that of the iron, it may be that minor changes in p_{H} due to the progress of the reaction were not completely eliminated. Finally, there was a source of error which was not appreciated at the time. Michaelis and Barron [1929, 1] have shown that the oxidation of RSH by oxygen and by methylene blue is catalysed by mercury. It is probable, therefore, that its oxidation by ferrithiol is similarly catalysed. Now, the majority of our reaction mixtures were stored in protected burettes over mercury. We have, indeed, had occasion to suspect that in the mixtures which had been stored for long periods before titration, the layer of solution immediately adjacent to the mercury decolorised more quickly than the bulk of the solution. In spite of these sources of error, however, we are satisfied that the general form of the reaction curves is significant and, therefore, the description of them by means of an equation becomes a matter of convenience even if the latter is nothing more than an empirical correlation. We were, however, interested upon certain theoretical grounds, to test the validity of the relation

$$\frac{dx}{dt} = k \left(\frac{x}{a-x} \right)^2 \quad \text{.....(1)}$$

which may be integrated to

$$kt = 2aln \cdot \frac{a}{x} + \frac{x^2 - a^2}{x},$$

a being the initial concentration of ferrithiol and x the concentration remaining after time t . Table I indicates the degree to which a typical velocity curve conformed to this equation. If, for purposes of further analysis, we accept this order of reaction, the relation of k to p_{H} may be defined. The relation is displayed in Fig. 2. Within the experimental range of p_{H} this figure suggests that k varies with $[\text{H}^+]^2$. Further discussion of this reaction is postponed until a later communication.

Table I. *Rate of autoreduction of ferrithiol.*

0.0018 *M* Fe^{'''}. 0.0098 *M* RSH. 0.05 *M* carbonate buffer. p_H 9.0. Room temperature.
10 cc. samples titrated with 0.0042 *M* hydrosulphite.

$$kt = 2aln \cdot \frac{a}{x} + \frac{x^2 - a^2}{x}, a=1 \text{ and } x = \text{fraction of original titre.}$$

<i>t</i> (mins.)	Titre (cc.)	<i>x</i>	<i>k</i>
—	4.35	1.0	—
(1.25)	(1.40)	(0.32)	(0.4)
2.5	1.27	0.292	0.27
5.5	0.99	0.228	0.22
6.5	0.92	0.212	0.22
6.0	1.13	0.260	0.15
8.0	0.99	0.228	0.15
10.0	0.91	0.209	0.15
14.5	0.66	0.152	0.18
17.5	0.60	0.138	0.18
23.5	0.50	0.115	0.18
36.0	0.38	0.087	0.18
54.0	0.20	0.046	0.29

THE RATE OF AUTOXIDATION OF FERROTHIOL.

This is the reaction which, with the autoreduction of ferrithiol, must be assumed to control the catalytic efficiency of iron in the autoxidation of RSH. It is desirable, therefore, that there should be information on the rate of this reaction and on its variation with p_H . Unfortunately, it has not proved possible to make direct measurements of this velocity, since it is precisely within that range of p_H at which autoreduction of ferrithiol is most rapid that the autoxidation of ferrothiol proceeds at rates convenient for measurement. Only broad generalisations are possible.

Above a p_H of about 8 the autoxidation of ferrothiol, is, undoubtedly, very rapid. It even exceeds in rate the very rapid reverse process of autoreduction since solutions of RSH and iron maintain a deep colour when in equilibrium with partial pressures of oxygen as low as 10 mm. mercury. Below a p_H of 6, on the other hand, it is not possible to demonstrate any autoxidation of ferrothiol. It might be objected that this is due merely to the overwhelming rate at which the reverse reaction has been shown to proceed at this p_H . If this were the situation, however, it would follow that the autoxidation of RSH in the presence of iron should be almost instantaneous in acid solution. Actually, the catalytic influence of iron on the reaction diminishes rapidly as one passes to the acid side of neutrality. We must conclude, therefore, that the autoxidation of ferrothiol is some fairly high function of $[OH^-]$.

ELECTRODE POTENTIALS OF THE SYSTEM FERRITHIOL-FERROTHIOL.

Notation.

- [S] = the total concentration of RSH.
 [S'] = the total concentration of RSH not in combination with iron.
 [Fe''] = the total concentration of iron present in the system in the state of lower (ferrous) valency.
 [Fe'''] = the total concentration of iron present in the system in the state of higher (ferric) valency.

K'' = the dissociation constant of the ferrous complex.

K''' = the dissociation constant of the ferric complex.

k_a = the hydron dissociation constant of the thiol group of RSH.

k_b = the hydron dissociation constant of the trivalent cation of the ferric complex [Bronsted, 1923].

E_h = the observed potential referred to that of the normal hydrogen electrode.

E_0, E_1, E_2, E_3 , etc. = working electrode constants.

It is well known that solutions of "pure" thiol compounds will induce negative potentials at electrodes of gold, platinum and mercury. All observers are, however, agreed that the electrode behaviour of RSH is anomalous and capricious. Persistent and often irregular drifts are the rule, potentials are easily displaced by mild polarisation of the electrodes and may recover only slowly. Electrodes of different metals are inclined to record grossly different potentials and even "identical" electrodes will give concordant results only with strict standardisation of technique. At best, one does not attempt to record observations more accurately than to the nearest millivolt. Actual reproducibility of independent experiments is of a still lower order [cf. Michaelis and Flexner, 1928].

With the addition of ferric ions and the appearance of the red colour there is an immediate improvement in electrode response. The discrepancies between grossly discordant electrodes are greatly reduced and, generally, quite eliminated. Electrodes withstand polarisation well and recover rapidly. The potentiometer may be balanced confidently to the nearest 0.1 mv. Drifts may still persist but they are similarly recorded by different electrodes and are of a character to suggest changes in the system rather than idiosyncrasies of the electrodes. There can be little doubt that complex formation has induced a degree of electromotive activity previously absent.

In reaction mixtures in which we had reason to believe that the whole of the iron present was dissolved in the form of complexes, it soon became apparent that we were dealing with a system whose potential was determined by the ratio of the concentrations of ferrous and ferric iron. This was established in the usual way by varying the ratio while the p_H and the concentration of RSH were held relatively constant. Two methods were employed. In the first, ferrithiol was titrated with sodium hydrosulphite and in the second, ferric ions were added progressively to a solution of ferrothiol. Now, the application of these methods is complicated by the spontaneous autoreduction of the oxidant. When this is proceeding at a significant rate the experimentally established equilibrium mixture will be subject to continuous change and the determination of equilibrium potentials will be prejudiced by persistent negative drifts. Nor is the order of the reaction such that these drifts can be usefully extrapolated to zero time. The order of the reaction, on the other hand, is of such a character that it diminishes very rapidly with its own progress, and, as Fig. 1 indicates, the reaction above p_H 10 is virtually arrested before 50 %

of the ferrothiol has been reduced. By carrying out titrations at this p_H , therefore, it was possible to reproduce considerable sections of complete titration curves without the states of equilibrium being affected by the secondary reaction. Unfortunately, this situation only holds for thio-glycollic acid. The autoreduction of ferricysteine and of ferriglutathione is so

Fig. 1. Rate of autoreduction.
0.05 M buffer. 0.01 M RSH. 0.0018 M Fe''' .

Fig. 2. Relation of k to p_H .
Continuous line is: $\log k = 16.8 + \log [H^+]^2$.

much more rapid that it has not yet been possible to submit these to electrode analysis.

Within the titration range in which drifts were absent equilibrium potentials were always rapidly attained, remaining constant for long periods although adjusting themselves to changes in the system in a few minutes. The potentials of gold-plated platinum and bright platinum electrodes were usually within

a few tenths of a millivolt of one another. When the "poising" of the system was poor, however, there was some tendency for platinum electrodes to be displaced several millivolts negative to the gold electrodes. Since the latter exhibited the more consistent behaviour throughout, the data for these only will be reported.

A series of titrations, by both methods, was conducted at room temperature. They all conformed accurately to the rational equation (cf. (2)) for a ferrous-ferric system involving one equivalent of hydrogen. Since, however, subsequent work was standardised for a temperature of 30°, the results of these experiments will not be further utilised. In Table II the data for one titration at this temperature are recorded, the observed potentials being compared with those calculated from the equation

$$E_h = E_1 - 0.0601 \log \frac{[\text{Fe}''']}{[\text{Fe}'']} \quad \dots\dots(2).$$

It is of interest to note that the values of E_1 obtained at room temperature were not far removed from those found at 30°. It would seem that no large temperature correction is involved in the electrode equilibria. In passing, it should be pointed out that the agreement between the titrations with ferric iron and with hydrosulphite is the final justification for the use of the latter in the titration of ferrithiol.

Table II. *Titration at constant p_H .*

Initial system. $\begin{cases} 50 \text{ cc. } 0.1 M \text{ carbonate buffer containing } 0.030 M \text{ thioglycollic acid.} \\ 4.80 \text{ cc. } 0.0212 M \text{ ferrous sulphate in } 0.01 M \text{ HCl.} \end{cases}$
 Titrated with 0.0208 M ferric chloride in 0.01 M HCl.
 $E_1 = -0.3527.$ $p_H = 10.32.$ 30°.

Titre	0.0601 log $\frac{[\text{Fe}']}{[\text{Fe}''']}$	E_h	
		Calculated	Observed
0.20	+0.0850	-0.4377	-0.4472
0.31	0.0715	0.4242	0.4256
0.48	0.0600	0.4127	0.4140
0.62	0.0533	0.4060	0.4056
0.95	0.0422	0.3949	0.3951
1.32	0.0337	0.3864	0.3861
1.91	0.0239	0.3766	0.3764
2.71	0.0147	0.3674	0.3676
3.71	0.0065	0.3592	0.3589
4.75	+0.0001	0.3528	0.3535
5.74	-0.0048	0.3479	0.3484
6.76	-0.0091	0.3436	0.3436

Influence of $[H^+]$.

When, in a system containing a fixed ratio of ferrous and ferric iron, the concentration of RSH or the p_H was varied, it was found that the potential was also a function of each of these variables. The limits of the experimental p_H range were approximately 8 to 10.5. The reasons for these narrow limits have already been given. Within this range the potential was found to be governed accurately by the relation

$$E_h = E_2 + 0.0601 \log (k_a + [H^+]) \quad \dots\dots(3).$$

The data from two experiments illustrating this relation are found in Table III. Since the changes in p_H were effected titrimetrically they involved small progressive changes in $[S']$. An appropriate correction for this will be defined in a later section and is included in the table for the sake of precision.

Table III. *Relation of E_h to p_H .*

Exp. 1. Initial system. { 50 cc. 0.05 *M* boric acid—KCl containing 0.0091 *M* thioglycollic acid.
1.2 cc. 0.021 *M* ferrous-ferric ions.
Titrated with 0.2 *M* sodium hydroxide at 30°.

$E_0 = +0.1460$. $0.0601 \log \frac{[Fe'']}{[Fe''']} = +0.0381$. $K''' = 4 \times 10^{-3}$. $k_a = 8 \times 10^{-11}$ [Cannan and Knight, 1927].

Titre	$[S'] \times 10^3$	p_H	0.0601 log ($k_a + [H^+]$)	0.0601 log ($[S'] + K'''$)	E_h	
					Calculated	Observed
4.60	6.0	8.277	-0.4962	-0.1201	-0.2682	-0.2684
5.43	6.0	8.453	0.5067	0.1203	0.2785	0.2789
6.71	5.9	8.688	0.5203	0.1205	0.2919	0.2913
7.94	5.8	8.889	0.5318	0.1208	0.3031	0.3026
9.55	5.6	9.087	0.5428	0.1213	0.3136	0.3132
11.01	5.4	9.282	0.5533	0.1218	0.3236	0.3236
12.25	5.3	9.472	0.5629	0.1220	0.3330	0.3330
13.50	5.2	9.693	0.5730	0.1223	0.3428	0.3419
0.25 cc. Fe''' added, $0.0601 \log \frac{[Fe'']}{[Fe''']} = +0.0185$.						
—	4.7	—	0.5730	0.1239	0.3216	0.3204
0.50 cc. Fe''' added, $0.0601 \log \frac{[Fe'']}{[Fe''']} = +0.0010$.						
—	3.7	—	0.5730	0.1270	0.3010	0.3004

Exp. 2. Initial system. { 25 cc. 0.044 *M* thioglycollic acid.
1 cc. 0.021 *M* ferrous-ferric ions.
10 cc. 0.2 *M* sodium hydroxide.

Titrated with 0.2 *M* hydrochloric acid.

$0.0601 \log \frac{[Fe'']}{[Fe''']} = +0.0302$.

Titre	$[S'] \times 10^3$	p_H	0.0601 log ($k + [H^+]$)	0.0601 log ($[S'] + K'''$)	E_h	
					Calculated	Observed
0.00	27.6	10.633	-0.5993	-0.0902	(-0.3933)	-0.3911
0.52	27.3	10.440	0.5962	0.0905	0.3899	0.3899
1.00	26.9	10.270	0.5925	0.0908	0.3859	0.3859
1.50	26.5	10.093	0.5877	0.0910	0.3809	0.3815
1.99	26.2	9.930	0.5823	0.0913	0.3752	0.3760
2.51	25.8	9.726	0.5744	0.0916	0.3670	0.3673
3.00	25.5	9.467	0.5625	0.0920	0.3547	0.3539

Influence of the concentration of RSH.

In a rigid analysis of the relation of the concentration of RSH to the potentials the function employed should be the concentration of RSH actually existing as free molecules (or ions) in the system $[S']$, since it is this function which must determine the degree of association of the active complexes. The value of $[S']$ would be obtained from $[S]$ by subtraction of the concentration of RSH bound in the complexes. But, until the stoichiometric relations of complex formation could be presumed it was not possible to make the calculation. As a first approximation, therefore, we may be content to determine the variation of potential with $[S]$.

. Two methods were followed. In one, the total concentration of iron in all forms was varied simultaneously with $[S]$. In the other, the latter only was subjected to wide variation. Since the two methods indicated the same relation of E_h to $[S]$ we may conclude that the potential is not any important function of the total concentration of iron within the experimental limits of 0.002 to 0.0001 M . The results are summarised graphically in Fig. 3.

Fig. 3. Relation of E_1 to $[S]$.

Inset: Relation to p_H of the points of intersection of the two slopes.

The interpretation of the complex relation of E_1 to $[S]$ which is displayed in the figure is facilitated by a description of the visible changes in the system accompanying the tracing of one of the curves. As $[S]$ is diminished the potential first follows the "0.06 slope" represented by the right-hand arm of each curve. Within the range of $[S]$ covered by this section of the curve there is no diminution in the intensity of the red colour greater than that which can be attributed to the accompanying dilution. Consequently—if the assumption be permitted that the red colour is the active ferrithiol—the amount of oxidant is not undergoing change. The reasonable inference, therefore, since this section of the curve is defined by

$$E_h = E_s - 0.0601 \log [S] \quad \text{.....(4)}$$

is that the concentration of active ferrothiol is proportional to $[S]$. That is to

say, ferrothiol must be substantially dissociated to the extent of one molecule of RSH per atom of iron.

As $[S]$ is further diminished a point is reached at which a change in direction of the curve is inaugurated. The $E_1 : [S]$ relation changes sign and, in experiments which are at favourable p_H values, ultimately attains a slope which may be described fairly accurately by

$$E_h = E_4 + 0.0601 \log [S]^5 \quad \dots\dots(5).$$

Accompanying this inflection in each curve there is an abrupt diminution in intensity of colour. A diffuse precipitate appears with the change in sign of the curve and almost all colour has disappeared by the time the full slope of the left-hand arm is attained. Now, to attempt the potentiometric determination of equilibrium conditions in a system containing a solid phase is always a hazardous undertaking. The observations relating to these lower ranges of $[S]$ have not, therefore, the same validity as those corresponding to the systems in which no precipitate was present. Nevertheless, the potentials do represent equilibria in so far as they are definitely balanced potentials which do not vary importantly with time. Moreover, the curves are reversible in the sense that they display the same relations to $[S]$ when traced by the progressive dilution of a concentrated iron-thiol system as by titration of a dilute solution of iron-thiol with RSH. We are satisfied that equation (5) does, in fact, correctly reflect the changes in $[S]$ which accompany the dissociation of ferrithiol, although ignorance of the complex phase relations of iron in alkaline buffer solutions prohibits any attempt to embrace this equation in a general electrode equation. Combining the influence of equations (4) and (5) (both being operative at the lower values of $[S]$) we arrive at the conclusion that the concentration of active ferrithiol varies with $[S]^6$. This may be taken to confirm the suggestion that the composition of ferrithiol and, by inference, of ferrothiol, is determined by a co-ordination number of six.

Two other points may be observed in Fig. 3. The displacement of the right-hand arm of the curve with p_H is merely the graphic expression of the relation which has been established in Table III and is defined by equation (3). The displacement of the left-hand arm is not capable of such precise definition. Its significance, moreover, is confused by the unknown phase relations of iron which are involved in it. But it will be seen that this section of the curve moves with p_H towards more negative potentials at a rate greater than does the other arm. At the same time it encroaches more and more on higher values of $[S]$. If, as is suggested, this part of the curve betrays the influence of $[S]$ upon the dissociation of active ferrithiol, then the tendency for the latter process must increase as the p_H rises. These varied effects of p_H are, probably, open to several interpretations, but it seems that the one which best accommodates them all is that it is the undissociated thiol molecule whose concentration determines the concentration of ferrithiol and of ferrothiol. This conclusion may be supported by the curve inset in Fig. 3. Approximate determinations of the points of intersection of the two slopes of each curve

were made graphically. The values of these points on the $\log [S]$ ordinate were then plotted against p_H . The curve resulting can be shown to be determined by a function of $k_a + [H^+]$.

If we accept the suggestions which have been advanced above as to the constitution of the complexes present in the experimental systems calculation can, then, be made of $[S']$. It will later be made clear that $5 [[Fe''] + [Fe''']]$ is a fairly close approximation to the concentration of bound RSH. We then have

$$[S] = [S'] + 5 [[Fe''] + [Fe''']] \quad \text{.....(6).}$$

If, now, $[S']$ replace $[S]$ in Fig. 3 a relation is disclosed whose influence was not manifested by the less rigid treatment. Two curves in Fig. 3 are reproduced in Fig. 4 with this alteration. The difference between the relation to $[S]$ and to $[S']$ can be reconciled by the equation

$$E_h = E_5 - 0.0601 \log (K''' + [S']),$$

K''' being a constant with a value of 4×10^{-3} .

In Table IV this equation is applied to the calculation of theoretical potentials for comparison with those actually observed. The agreement is highly satisfactory.

Table IV. *Relation of E_h to $[S']$.*

Initial system.		$\begin{cases} 25 \text{ cc. boric acid—KCl buffer.} \\ 1 \text{ cc. } 0.090 \text{ } M \text{ thioglycollic acid.} \\ 1 \text{ cc. } 0.021 \text{ } M \text{ ferrous-ferric ions.} \end{cases}$				
Titrated with 0.090 <i>M</i> thioglycollic acid at 30°.						
$E_0 = +0.1460. \quad 0.0601 \log \frac{[Fe'']}{[Fe''']} = +0.0473. \quad K''' = 4 \times 10^{-3}. \quad k_a = 8 \times 10^{-11}.$						
Titre	p_H	$[S'] \times 10^3$	0.0601 log $([S'] + K''')$	0.0601 log $(k_a + [H^+])$	E_h	
					Calculated	Observed
0.50	8.404	0.9	-0.1385	-0.5035	-0.2663	-0.2667
1.00	—	2.4	0.1317	0.5036	0.2732	0.2742
1.55	—	4.2	0.1254	0.5037	0.2796	0.2800
2.70	8.407	7.4	0.1168	0.5038	0.2883	0.2885
4.50	—	12.1	0.1078	0.5040	0.2975	0.2970
8.00	8.413	19.9	0.0975	0.5042	0.3080	0.3076
11.50	—	26.3	0.0913	0.5044	0.3144	0.3144
16.50	8.420	33.6	0.0856	0.5046	0.3203	0.3204
24.00	8.423	41.9	0.0810	0.5048	0.3251	0.3249

The value of K''' has been confirmed by all available data on systems not much more alkaline than p_H 9.5. Its physical significance is uncertain. We may tentatively regard it as the primary dissociation constant of ferrithiol controlling the degree of dissociation of the first RSH molecule in the complex. As such its value on the $[S']$ scale should be a function of k_a and should increase rapidly above p_H 10. Unfortunately, the influence of K''' is not clearly manifested above p_H 10, owing to the fact that, as we have seen, the $[S]$ range of complete instability of ferrithiol encroaches more upon higher values of $[S]$ as p_H is increased. Above p_H 10 the separate effect of K''' is submerged in the more general dissociation.

The several variables which have been disclosed may now be assembled in a general electrode equation which will read

$$E_h = E_0 - 0.0601 \log \frac{[\text{Fe}^{2+}]}{[\text{Fe}^{3+}]} - 0.0601 \log \frac{[\text{S}'] + K'''}{k_a + [\text{H}^+]} \quad \dots\dots(7).$$

This is applicable only to systems containing no solid phase of iron, within the p_{H} limits 8–10.5 and within the limits of $[\text{S}']$ which vary with p_{H} but are roughly 0.005 to 0.1 M .

It is this equation which has been employed for the calculation of theoretical potentials in Tables II–IV. The least satisfactory part of this has been the quantitative evaluation of E_0 . Normally this would have been established with precision from the data of a titration at constant p_{H} and $[\text{S}]$. It was, however, pointed out at the beginning of this section that such titrations could

only be conducted in the neighbourhood of p_{H} 10. Now, as has just been shown, it is at such a p_{H} that the absolute magnitude of $[\text{S}'] + K'''$ is uncertain. The derivation of E_0 from experiments below p_{H} 9 in which a predetermined ratio of ferrous and ferric ions was added to a RSH solution is equally unsafe. This is due to the difficulty of ensuring that a given ferrous-ferric ratio retains that ratio precisely when added to a deaerated solution of RSH. Traces of oxygen introduced with the burette tip or momentary local concentration effects leading to local autoreduction are two factors liable to modify an experimental ratio. The procedure which has been adopted has been to assign to E_0 the value 0.1460 because this fits closely the majority of the data. Such observations as disagreed indicated lower values, the lowest being 0.1380. With E_0 fixed experimental values of $\log \frac{[\text{Fe}^{2+}]}{[\text{Fe}^{3+}]}$ were then adjusted to give the best alignment with E_0 . The magnitude of this correction was, generally, small.

In conclusion, allusion must be made to one point in the electrode analysis. It is surprising that in a series of experimental systems it should have been possible to replace the rational term $[S'] + K'''$ by the empirical term $[S']$ without serious distortion of the data. This was of the nature of a fortunate coincidence. It happened that within those rather narrow ranges of $[S]$ in which K''' was a significant term the experimental values of $5 [[Fe''] + [Fe''']]$ approximated closely to 4×10^{-3} . The small differences between $[S]$ and $[S'] + K'''$ were still less evident in the logarithmic terms.

CHEMICAL DESCRIPTION OF THE SYSTEM.

It is always dangerous to translate thermodynamic relations into chemical structures. Yet if we are to define systems such as iron-cysteine, iron-glutathione, etc., which are not amenable to extensive electrometric analysis, some attempt to generalise the system in broad chemical terms must be made.

The detailed analysis which has been presented is consistent with the following scheme. The reversible potentials are controlled by the oxidation-reduction equilibrium:

The concentrations of these active reactants are determined by the equilibria:

and

The further dissociation of the complexes with liberation of more RSH molecules, and the remaining hydron dissociations of the complex cations, may be ignored since they do not influence the behaviour of the system within the experimental ranges of $[S']$ and p_H within which equation (7) holds. The nature of X , the component which has been included to complete the co-ordination ring, is unknown. It might be a molecule of water by analogy with the aquopentacyano-ferroates of Hofmann [1900], or a carboxyl group of one of the co-ordinated RSH molecules. In any case, this hypothesis of a potential vacancy in the co-ordination ring of iron-thiol complexes allies them closely with a number of other co-ordinated iron compounds [cf. Manchot, 1912], and even, on the basis of Conant's views [1923], with haemoglobin and related substances. Possibly, the peculiar relations of RSH with carbon monoxide, oxygen, hydrocyanic acid, hydrogen sulphide, and perhaps, the "fixed SH" of tissues [Hopkins and Dixon, 1922], may be due to preliminary co-ordination of these molecules in the vacant position of ferrothiol.

The spontaneous autoreduction of ferrothiol may be described as an intramolecular process in which an electron passes from a co-ordinated RSH

molecule to the iron atom. The free radicle of RSSR which results then dissociates from the complex and polymerises to RSSR. The ferrous complex is then reconstituted by co-ordination of a free RSH molecule

This complex set of equilibria is probably less complex than the situation which obtains in the system RSH—Fe. It does, however, give a plausible description of the catalytic influence of iron upon the oxidation of RSH, and it does not appear necessary as yet to complicate the situation by the consideration of further variables.

It remains to add that the theoretical equation which may be derived for the hypothetical system

may be reduced to equation (7) provided that we make certain reasonable assumptions. These are, that K'' is very great compared with $[S']$, and that both complexes are completely dissociated as bivalent cations within the experimental range of p_{H} . The former assumption is implicit in the treatment which has been accorded the electrode data. The latter receives support from the colour change of ferrithiol in the neighbourhood of p_{H} 5. This may correspond with the third basic constant of the oxidant.

Reference has been made to the recent studies of the complexes formed between cysteine and cobalt [Michaelis and Barron, 1929, 2, and Michaelis and Yamaguchi, 1929]. It appears that cobalt forms with cysteine a cobaltous complex which is almost colourless in dilute solution. This substance is very readily oxidised to a reddish brown product, in which, as is definitely indicated by the evidence of these authors, RSH and Co are present in a ratio of 3 : 1. Since the oxidation involves two equivalents of hydrogen for each atom of cobalt, Michaelis and Yamaguchi believe that, in the coloured product, not only has cobalt been oxidised to the cobaltic state but also that one of the combined RSH molecules has lost one equivalent of hydrogen. If we understand their argument, the process may be represented as follows:

or

according to whether the undissociated RSH or the thiol anion be regarded as the group co-ordinated.

Michaelis and Barron have suggested that their oxidised cobalt complex is comparable to the coloured ferrithiol complex, and suppose that the difference is merely that the co-ordination complex "which is intermediary, unstable,

and inaccessible therefore for chemical analysis in the case of iron, is stable and suitable for chemical analysis in the case of cobalt." We believe the two compounds to be distinct in respect of both the number of co-ordinated thiol groups and of the number of equivalents of oxygen necessary to create them from the reduced complexes. Our titrations indicate unequivocally that each atom of ferrous iron required only one equivalent to produce the active coloured oxidant. But apart from these distinctions, there are many points in common in the structural formulations which Michaelis and Yamaguchi have adopted for cobalt-cysteine and which we have suggested for iron-thioglycollic acid.

One point in the work of Michaelis and Barron requires further discussion. They showed that systems of cobalt and cysteine developed intensely negative potentials at an inert electrode. The reproducibility of these potentials appears however to have been poor, and no detailed study of them was made. The oxidation of the cobaltous-cysteine was, however, followed electrometrically, and as a result the authors arrive at the conclusion that the progress of the reaction varies with the nature of the oxidising agent. When ferricyanide was employed there was evidence of the removal of the two equivalents separately. In other words, there was evidence of a stable intermediate. When, on the other hand, indophenol was employed as oxidising agent there was no evidence of this stepwise oxidation to the cobaltic dithiol product. The authors suggest that cobaltous cysteine may be oxidised in two individual steps by ferricyanide, because the latter only requires a single equivalent of hydrogen per molecule on reduction. Indophenol, they argue, can only oxidise by accepting a pair of equivalents owing to the quinonoid structure of the molecule.

To this conclusion we cannot subscribe. The distinction between the progress of the reaction in the two cases which is indicated by the dissimilar electrode titration curves, is, after all, merely a thermodynamic abstraction. It can carry no definite implications as to the molecular processes involved. If the titration curves are significant, they rest upon the assumption that the sole purpose of the oxidising agent is to alter the ratio of the oxidant to reductant in the system. In that case there is no thermodynamic basis for distinguishing between the two oxidising agents. Therefore a difference between the types of curve traced in the two cases must be attributed to secondary reactions induced by the presence of one or other of the titrating agents. It is appropriate, therefore, to question whether ferricyanide was a happy choice of reagent for titrating, without complication, the co-ordination complexes of cobalt. Be that as it may, it must be pointed out that the systems which Michaelis and Barron titrated with ferricyanide and indophenol respectively were very different. In the indophenol titrations the whole of the cobalt was present as the cysteine complex, but in the titration with ferricyanide there was only sufficient cysteine present to co-ordinate one-half of the cobalt. Such a difference would be expected to have an important effect on the titration curve.

THE ELECTRODE BEHAVIOUR OF RSH WITHOUT ADDED IRON.

Reference has already been made to the anomalous electrode behaviour of solutions of "pure" thiol compounds. A great deal of attention has been devoted to this problem since the first observations of Dixon and Quastel [1923]. These workers found that inert electrodes of gold or platinum in solutions of cysteine or glutathione developed potentials which were approximately defined by

$$E_h = E_0 - 0.06 \log \frac{[S]}{[H^+]}$$

The least satisfactory character of this equation was, as has already been described, the wide variation in the absolute magnitude of E_0 in different experiments. Dixon [1927] extended the equation to the behaviour of the mercury electrode. He made the plausible suggestion, though on quite inadequate data, that a more comprehensive relation is given by

$$E_h = E_0 - 0.06 \log \frac{[S]}{[k_a + [H^+]]}$$

As a matter of fact, our own observations, which need not be reported in detail for the present argument, on the mercury-thioglycollic acid electrode have established the validity of the modified equation with some precision. The further study of the problem has been greatly advanced by the work of Michaelis and Flexner [1928] who have established the conditions of technique appropriate to the most reproducible results.

The fundamental peculiarity of the thiol electrode is that the potentials are independent of the concentration of RSSR; that is to say, they are not a function of the concentration of the presumed oxidant of RSH. Dixon and Quastel made the suggestion that this might be due to the fact that the immediate oxidant which was in equilibrium with RSH was a hypothetical intermediate whose concentration was independent of RSSR. In a later paper, however, Dixon [1927] abandoned this difficult assumption for a general hypothesis of electrode mechanism which, based upon kinetic considerations of hydrogen transport and Wieland's theory, was avowedly designed to explain the anomalies of the thiol electrode. The theory of Dixon has not been found acceptable by other students of the problem, and, since it was a kinetic theory unsupported by kinetic evidence, it need not be further discussed here. Michaelis and Flexner were unable to arrive at any satisfactory explanation of the thiol electrode.

The results which have been reported in the present paper upon the electromotive activity of the thiol-iron complexes naturally raise the question whether these may not be responsible for the behaviour of so-called pure solutions of RSH. No confident answer can be given. There is a superficial resemblance between the electrode equation for the iron-thiol system and the equation of Dixon. But the highly complex relation of the iron system to the concentration of RSH indicates that the behaviour of the two electrodes is very different. It seems quite clear that the thiol electrode does not behave

merely as a system in which the ratio $\frac{[\text{Fe}^{''}]}{[\text{Fe}^{'''}]}$ has been held constant by the experimental technique employed. At the same time, it is not possible to deny that the influence of the dissociation of the complexes upon the potentials might be neutralised by changes in the ferrous-ferric ratio taking place even in the absence of oxygen. This situation might well arise as the result of experimental modifications of the system reacting, through the various equilibria of complex formation, upon the velocity of autoreduction. However this may be, there seems little advantage at present in adopting an explanation of the thiol electrode which is no less artificial and abstract than that originally suggested by Dixon and Quastel.

In a future communication we hope to return to the question of the thiol electrode both from the electrometric and kinetic points of view. The object will be to suggest the advantage of attributing to the system reversible equilibria not conditioned by the presence of casual traces of one metal or another. With such a system ferro-ferrithiol, thiol-mercury thiol [cf. Barron, Flexner and Michaelis, 1929] or any other reversible system present may be considered to be in simultaneous equilibrium. It then becomes merely a matter of stoichiometric or kinetic predominance to which system we choose to assign responsibility for the phenomena.

EXPERIMENTAL DETAIL.

Autoreduction of ferrithiol. A solution of thioglycollic acid in a deaerated carbonate buffer (0.1 *M*) of suitable p_{H} was prepared and stored in the reservoir of a burette protected from oxygen. The p_{H} of an aliquot portion was determined by means of the hydrogen electrode. A known volume of the remainder was transferred to another vessel under pure nitrogen, a known amount of standard deaerated ferric chloride solution was added at zero time and the mixture returned to the reservoir. At suitable intervals, samples were removed and titrated with a standard solution of sodium hydrosulphite. The most careful protection of the reaction mixture from all traces of oxygen during preparation, storage and titration was essential for reliable results. In those cases in which it was desired to make determinations after only short reaction times, individual mixtures of iron and RSH were made in the actual titration vessel and titrated after the desired interval. The concentrations of the components were, approximately, 0.05 *M* buffer, 0.01 *M* RSH and 0.0018 *M* iron. Owing to the difficulty of conducting such operations within a thermostat, the experiments were carried out at room temperature (13–16°).

The sodium hydrosulphite was standardised by means of a standard solution of ferric iron prepared from ferrous ammonium sulphate and its equivalent of potassium permanganate. Methylene blue served as indicator. In the titration of ferrithiol no added indicator was necessary. The justification for the employment of hydrosulphite to titrate ferrithiol has been given in the discussion of the electrode studies. It may be added that the fall in titration

value was parallel with the decay of the intensity of the red colour. Moreover, in those cases in which the rate of the reaction was slow, the initial titrations were equal to the total amount of ferric iron originally added. The only reaction likely to confuse the titration would be the oxidation of ferrothiol or hydrosulphite by RSSR. Control experiments established the absence of these reactions under the experimental conditions.

Electrode titration at constant p_H and constant $[S]$. Two methods were followed. 1. A standard solution of ferric chloride was added to a buffered deaerated solution of RSH in the electrode vessel. Autoreduction was allowed to proceed until its velocity, as indicated by the absence of further potential drifts, became insignificant. The titration of the residual ferrithiol with sodium hydrosulphite was then commenced. The end-point of this titration was identified with the disappearance of the last trace of red colour. With this determined and a knowledge of the amount of ferric iron originally added the ratio $\frac{[Fe'']}{[Fe''']}$ at each step in the titration could be calculated. 2. In the second method a known volume of a standard solution of ferrous sulphate was added to a buffered, deaerated solution of RSH. The mixture was then titrated with ferric chloride. Equilibrium potentials were always attained within 2-5 minutes of each change in the system and remained constant for long periods. With the progress of the titration and the consequent increase in $\frac{[Fe''']}{[Fe'']} = \frac{x}{a-x}$, a point was ultimately reached at which significant negative drifts of potentials suddenly became apparent. These were undoubtedly due to the magnification of the rate of autoreduction as the ratio of ferrithiol to ferrothiol increased. The first appearance of these drifts terminated the titration since the nature of the velocity curve discouraged attempts to extend the titration by a series of hurried observations.

Relation of E_h to p_H . A 0.01 *M* solution of RSH in 0.05 *M* boric acid buffer was adjusted to a p_H of about 8 and deaerated. A mixture of ferrous and ferric ions in known proportions was added and the solution then titrated with deaerated sodium hydroxide. Alternatively, a solution adjusted to about p_H 11 was titrated with hydrochloric acid. Periodic determinations of the equilibrium potentials were made. The corresponding changes in p_H were determined by means of a hydrogen electrode titration in which all conditions, except the presence of the iron, were duplicated.

Relation of E_h to $[S]$. Two methods were followed. 1. To a dilute buffered solution of RSH was added a known ratio of ferrous and ferric ions. The mixture was then titrated with a concentrated solution of RSH identically buffered. Equilibrium potentials were determined after each addition. Incidental minor changes in p_H were controlled by a duplicate titration at the hydrogen electrode (the iron only being omitted from the system). 2. A concentrated (0.1 *M*) buffered solution of RSH was progressively diluted with the pure buffer solution and the changes in potential determined. Changes in p_H were not, in this case, controlled and this may account for the

small discrepancies between observed and calculated potentials during the first three dilutions. This would arise were the pure buffer and the buffered RSH not identical in p_H .

SUMMARY.

1. Both ferrous and ferric ions form soluble complexes with certain thiol acids. The ferrithiol complex suffers spontaneous autoreduction to the corresponding ferrothiol. A preliminary study has been made of the rate of this reaction and of its relation to p_H . The rate of autoxidation of ferrothiol has been considered.

2. Ferrithiol-ferrothiol constitutes a reversible oxidation-reduction system which is electromotively active. The equilibrium potentials of the system have been determined and an electrode equation has been established which defines the electrode behaviour within a limited range of p_H and concentration of thiol acid.

3. Arising out of these studies certain suggestions have been made as to the nature of the complexes. These have been elaborated to a tentative structural description of the various equilibria of importance in the system thiol acid-iron.

4. The purpose of these studies is to provide a quantitative basis for the interpretation of the catalytic influence of iron on the thiol-disulphide system and the anomalous electrode activity of thiol compounds.

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