THE FREE ENERGY
OF THE
SULFHYDRYL - DISULFIDE OXIDATION - REDUCTION SYSTEM
AND
ITS PHYSIOLOGICAL SIGNIFICANCE

Thesis by

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In Partial Fulfillment

of the

Requirements for the Degree of

Doctor of Philosophy

CALIFORNIA INSTITUTE OF TECHNOLOGY

Pasadena, California

1934

IV. SUMMARY

Complete thermodynamic data are presented concerning two organic sulfhydryl-disulfide oxidation-reduction systems (cysteine: cystine and β-thiolactic acid: ββ'-dithiodilactic acid). These data include heat capacity measurements on the crystalline substances from 90° to 298° K, heat of combustion determinations, solubility measurements and determinations of the ionization constants of dissociable groups in the molecules.

The method of calculating from these data the reduction potential for any conditions of concentration, pH, and temperature is discussed. The results of such calculations for several sets of conditions are submitted.

The relationship of the sulfhydryl-disulfide system to the process of cell division is discussed and experimental evidence is submitted which shows that sulfhydryl substances have no direct connection with the process of cell division.

The role of oxidation-reduction systems in intracellular metabolism is discussed. From the available experimental data relating to the function of -SH substances in living tissue, an hypothesis of the general function of these substances is developed and discussed.

I. INTRODUCTORY

The purpose of the investigation described here is the determination of the magnitude of the free energy change for the reaction

$$2\ RSH \rightarrow RSSR + 2\ H^{+} + 2\ E^{-}$$

and the application of this, and the oxidation potential derived from it to the problem of the biological significance of the sulfhydryl-disulfide oxidation-reduction system. The substances chosen for investigation were the amino acid cysteine, its disulfide oxidation product cystine, β-sulfhydryl propionic acid (β-thiolactic acid), and its oxidation product $\beta\beta'$-dithiodilactic acid. This choice was made because of the close chemical relationship of these substances to one another and to the naturally occurring sulfhydryl substance, glutathione.

Biological oxidations may be defined to include those reactions occurring in vivo which lead either to the combination of oxygen with a metabolite or the removal of hydrogen from a metabolite. These two processes (oxidation and dehydrogenation respectively) are so closely related that a discussion of one almost necessarily involves the other. Defined in this way biological oxidations are the general animal energy source and the larger part of all intermediary metabolism.

It is implied in the above definition that the oxidation of a given metabolite in vivo may proceed by a number of steps, each step involving a dehydrogenation or an oxidation. Substances which

can be reversibly oxidized or reduced in a biological system are a significant part of the mechanism for accomplishing such step-wise oxidations. This biologically reversible oxidation and reduction does not imply that these substances are necessarily reversibly oxidized in pure solution at an inert electrode.

Evidence that will be discussed later has shown that the naturally occurring sulfhydryl substance glutathione (glutamyl-cysteinyl-glycine) is reversibly oxidized in vivo to the corresponding disulfide. Since the isolation of this substance by Hopkins in 1921, several groups of investigators have been studying various aspects of sulfhydryl-disulfide systems, using glutathione, cysteine, thioglycolic acid, thiolactic acid, and related compounds.

Several attempts by different investigators to measure the oxidation potential have yielded anomolous results. The usual potentiometric methods in which measurements are made of the EMF of an inert electrode, immersed in a solution containing known amounts of oxidized and reduced substance, has generally been used. In certain cases the procedure was the titration in the electrode vessel, of a solution of the reduced substance with an oxidizing agent. The results of these experiments are briefly discussed in the following paragraphs.

The derivation of the equations expressing oxidation potentials will not be set forth here. The final equation for a reversible oxidation reaction of the type

$$RSSR + 2\,H^{+} + 2\,E^{-} \rightleftharpoons 2\,RSH$$

is

$$E = E_{o} - \frac{RT}{2F}pH - \frac{RT}{2F}\ln\frac{(RSH)^{2}}{(RSSR)} \qquad (1)$$

where (RSH) and (RSSR) are the concentrations of the reacting molecular species, not the total concentrations.

Dixon and Quastel (1923) were the first to attempt to measure the cysteine-cystine potential. They found that the potential obtained with a given solution was inconsistent. Platinum, gold plated platinum, and massive gold electrodes gave potentials which were neither in agreement with one another nor reproducible. However, during a given titration experiment, the values obtained were sometimes consistent and could be expressed by the equation

$$E = E_o - \frac{RT}{F} pH - \frac{RT}{F} \ln (RSH) \qquad (2)$$

where (RSH) is the concentration of the sulfhydryl substance. The potential was shown to be wholly independent of the concentration of the corresponding disulfide oxidation product (RSSR).

Michaelis and Flexner (1928) conducted a very careful investigation of this system by potentiometric methods. They were able to obtain reproducible potentials at platinum, gold plated platinum, and mercury electrodes, but they showed that minute traces of oxygen had a relatively enormous effect in changing the potential in the positive direction. Their results are likewise expressed by equation (2) above.

A contradiction of the above results appeared when Ghosh et al (1932) using a mercury electrode found that the equation (1) above expressed the cysteine-cystine potential under their experimental conditions. The only significant difference between their method and that of previous workers was in the preparation of the cysteine used. A solution of cystine was electrolysed against the

mercury electrode in order to reduce some of the cystine to cysteine. At the conclusion of the electrolysis the reduction potential was determined against the same mercury electrode, and the solution was then analysed for its cysteine content.

This apparent anomaly was explained when Green (1933) performed a similar experiment, but electrolysed the cystine solution against one mercury electrode and measured the reduction potential against another. The "clean" mercury electrode gave potentials agreeing with those of Michaelis and Flexner while the electrode previously used for electrolysis gave potentials in agreement with those obtained by Ghosh. This and other similar experiments led Green to the conclusion that electrolysis results in the deposition within the mercury of some cysteine or cystine complex which in some manner determines the apparent "reversibility" of the system at the electrode.

It is clear from the discussion above that the behavior of the sulfhydryl-disulfide system at a noble metal electrode is not the same as that of the usual oxidation-reduction systems. Ultimately it may be possible to explain its irreversible behavior at the electrode but it is not clear how the potentials measured can even then be related to biological systems in which the oxidation is certainly reversibly.

Fortunately there are other methods of obtaining information equivalent to that yielded by the potentiometric measurement of oxidation potentials. The free energy change of a reaction is related to the potential given when that reaction takes place reversibly at an electrode by the expression

$$-\Delta F = E n F \qquad (3)$$

When $-\Delta F$ for a given set of conditions is known, the potential E can be calculated. However, the potential is only a convenient way of expressing the free energy change of the reaction, the latter being the quantity which is of primary importance and utility.

As listed by Parks and Huffman (1932) there are four methods of determining ΔF for a given reaction. One of these, the measurement of the EMF of a galvanic cell, has been tried several times in the case of the sulfhydryl → disulfide reaction, always with unsatisfactory results. This method must be discarded, in the case in question, unless a cell can be devised in which the reaction can be caused to take place reversibly. A second method, the calculation of ΔF by a combination of suitable chemical equations for which the free energy data is known, must be discarded in the present case because of the lack of such free energy data for organic sulfur compounds. A third method involving the measurement of equilibrium constants is unsatisfactory for the same reason as the first, namely, the difficulty of obtaining reversibility in pure solution.

The remaining method is more laborious than the others but is apparently the only one applicable to the case in hand. It involves the determination of the heat capacity down to low temperatures of the crystalline substances and the combination of this with other data to calculate ΔF by means of the third law of thermodynamics. In the following paragraphs the data required will be enumerated and the method of calculation indicated.

The third law of thermodynamics states that at the absolute zero of temperature the entropy of pure crystalline substances becomes zero. At any other temperature the entropy of the same crystalline substance is determined by the expression

$$S_T = \int C_p \, d \ln T \qquad (4)$$

If the heat capacity (C_p) be known from $T = 0$ to $T = T_1$, the entropy S_{T_1} can be calculated by the integration of the graph of C_p vs. ln T. It is not necessary to determine the heat capacity of all substances to temperatures in the neighborhood of absolute zero. Extrapolation formulae which give results of sufficient accuracy (Kelley, Parks, and Huffman, 1929) can be applied to the heat capacity curves below 90° K. The extrapolation formula used for the compounds considered here was of the form

$$C_p = (A + BT) \, C_p^\circ \qquad (5)$$

where

$$B = \frac{C_{p(120)} - 1.2137 \, C_{p(90)}}{351}$$

and

$$A = \frac{C_{p(120)} - 1404 \, B}{11.70}$$

C_p° is the molal specific heat of the standard substance.

Equations (4) and (5) make possible the calculation of the entropy at any temperature T from heat capacity data over the range 90° K to T. If T lies above the melting point or some other transition,

the entropy is increased by the entropy of the fusion or transition

$$\Delta S_F = \frac{\Delta H_F}{T_F} \qquad (6)$$

where ΔH_F is the heat absorbed in the fusion and T_F is the fusion temperature.

The entropy of formation of the compound (ΔS) at a temperature T is the difference between the entropy of the compound and the entropies of the elements forming the compound. Thus for the compound $C_aH_bO_c$,

$$\Delta S = S_{(compound)} - a\,S_{(c)} - \frac{1}{2}\,b\,S_{(H_2)} - \frac{1}{2}\,c\,S_{(O_2)} \qquad (7)$$

The entropy of formation ΔS is related to the free energy of formation of the compound from the elements by the expression

$$\Delta F = \Delta H - T\,\Delta S \qquad (8)$$

The quantity ΔH is the heat of formation of the compound from the elements in their standard states. ΔH can be calculated from the heat of combustion of the compound, provided the heats of formation of the products of combustion are known. The steps involved in the determination of ΔH are outlined below.

a) Measurement of the heat envolved when a known quantity of the substance is caused to burn in the bomb calorimeter. The products of the combustion must be determined or assumed on the basis of previous experience.

b) Correction of this measured heat quantity to give the value for the heat evolution of the corresponding reaction occurring at constant temperature and a constant pressure of one atmosphere (Q_p).

c) Calculation of the heat of formation from this value with the aid of data on the heats of formation of the products of the combustion. If the combustion reaction is

$$A + n\,O_2 \rightarrow b\,B + c\,C + d\,D$$

the heat of formation of A is

$$\Delta H_A = Q_p + b\,\Delta H_B + c\,\Delta H_C + d\,\Delta H_D \qquad (9)$$

where ΔH_B, ΔH_C, ΔH_D are the heats of formation of the products of the combustion.

The free energy change for the reaction

$$RSSR_{(s)} + H_{2(g)} \rightarrow 2\,RSH_{(s\text{ or }l)}$$

is simply the difference in the free energies of formation of the products and the reactants. The free energy change for the reactions in aqueous solution with all substances at given concentrations or partial pressures can be calculated with the aid of (1) the solubility in water of the substances involved in the reaction and (2) the dissociation constants of any dissociable groups present in the molecule.

Before developing a general formula applicable to any concentration of reactants or products it is convenient to calculate a quantity $\overline{\Delta F}$, the free energy change when all reactants and products are present in the solution at unit concentrations. One molecular species of RSSR and RSH must be arbitrarily designated as the reactant

and product respectively. Choice is made of the forms present in the greatest amount at neutrality. In the four compounds studied these forms are the single negative ion for β-thiolactic acid, the double negative ion for $\beta\beta'$-dithiodilactic acid, and the zwitter ion forms of the amino acids, cysteine and cystine:

$$HS - CH_2 - CH_2 - COO^- \quad \text{and} \quad \begin{matrix} S - CH_2 - CH_2 - COO^- \\ | \\ S - CH_2 - CH_2 - COO^- \end{matrix}$$

$$HS - CH_2 - \overset{NH_3^+}{\underset{|}{CH}} - COO^- \quad \text{and} \quad \begin{matrix} S - CH_2 - \overset{NH_3^+}{\underset{|}{CH}} - COO^- \\ | \\ S - CH_2 - \underset{NH_3^+}{\underset{|}{CH}} - COO^- \end{matrix}$$

The calculation of $\overline{\Delta F}$ can now be accomplished by evaluating the concentration of each of these ions in a saturated aqueous solution, assuming the laws of perfect solutions and applying the equation

$$\Delta F = NRT \ln \frac{c_2}{c_1} \qquad (10)$$

to the evaluation of the free energy change accompanying the change from saturated solutions to unit concentrations. In the case of β-thiolactic acid, which is miscible with water in all proportions, equation (10) becomes

$$\Delta F = NRT \ln x \qquad (11)$$

where x is the mol-fraction of RSH at which the concentration of the anion is unity. $\overline{\Delta F}$ is not changed by changes in pH, and is a constant at a given temperature.

A general formula for the free energy change in terms of the total concentration of the reductant and oxidant, the hydrogen ion activity, and the dissociation constants can be readily developed through the simultaneous solution of the mass action equations expressing the dissociation constants and the substitution of the result in the expression

$$\Delta F = \overline{\Delta F} - RT \ln c_1 - 2 RT \ln (H^+) + 2 RT \ln c_2 \qquad (12)$$

where c_1 and c_2 are the concentrations of the reacting molecular species of RSSR and RSH respectively.

The final equation in the case of β-thiolactic acid for the free energy change for the reaction

$$RSSR + 2 H^+ + 2 E^- \rightarrow 2 RSH$$

is

$$\Delta F = \overline{\Delta F} - RT \ln \frac{(C_{ss})}{(C_{sh})^2} - RT \ln \frac{K_4 K_5}{(K_1)^2}$$

$$+ RT \ln \frac{(H^+)^2 + (H^+) K_4 + K_4 K_5}{(H^+)^2 + (H^+) K_1 + K_1 K_2}^2 \qquad (13)$$

where K_1 and K_2 are the first and second dissociation constants respectively of the sulfhydryl compound, C_{sh} is the total concentration of all forms, dissociated and undissociated, of the sulfhydryl compound, K_4 and K_5 the dissociation constants of the disulfide,

C_{ss} the total concentration of the disulfide, and (H^+) the hydrogen ion activity. The dissociation constants are the true constants, not K' values.

The equation expressing the cysteine-cystine free energy change is the following

$$\Delta F = \overline{\Delta F} - RT \ln \frac{C_{ss}}{(C_{sh})^2} - RT \ln \frac{K_4 K_5}{(K_1)^2} + RT \ln \frac{(H^+)^4 + (H^+)^3 K_4 + (H^+)^2 K_4 K_5 + (H^+) K_4 K_5 K_6 + K_4 K_5 K_6 K_7}{[(H^+)^3 + (H^+)^2 K_1 + (H^+) K_1 K_2 + K_1 K_2 K_3]^2} \quad (14)$$

The reduction potential is related to the free energy change by the formula

$$-\Delta F = E n F \quad (15)$$

The general expression for the potential is derived from equations (13) and (14) by simply dividing both sides by the factor - 2F. For the β-thiolactic acid system the equation becomes

$$E = \overline{E} + \frac{RT}{2F} \ln \frac{C_{ss}}{(C_{sh})^2} + \frac{RT}{2F} \ln \frac{K_4 K_5}{(K_1)^2} - \frac{RT}{2F} \ln \frac{(H^+)^2 + (H^+) K_4 + K_4 K_5}{[(H^+)^2 + (H^+) K_1 + K_1 K_2]^2} \quad (16)$$

and for the cysteine-cystine system the corresponding equation is

$$E = \bar{E} + \frac{RT}{2F} \ln \frac{C_{ss}}{(C_{sh})^2} + \frac{RT}{2F} \ln \frac{K_4 K_5}{(K_1)^2} \tag{17}$$

$$- \frac{RT}{2F} \ln \frac{(H^+)^4 + (H^+)^3 K_4 + (H^+)^2 K_4 K_5 + (H^+) K_4 K_5 K_6 + K_4 K_5 K_6 K_7}{\left[(H^+)^3 + (H^+)^2 K_1 + (H^+) K_1 K_2 + K_1 K_2 K_3\right]^2}$$

II. PRESENTATION OF DATA

1. Heat capacity measurements.

The method and apparatus used for the heat capacity measurements have been described by Parks (1925). Briefly, the apparatus consisted of a metal calorimeter of about 50 cc. capacity, wound with a heating coil and containing a thermocouple for temperature measurements. The calorimeter, filled with the substance under investigation, was suspended within a heavy copper block, the temperature of which could be controlled by a heating coil. The copper block and calorimeter were sealed in a metal container which was then evacuated, and the container was surrounded with a cooling bath (liquid air, solid CO_2—alcohol, or ice-water depending upon the temperature at which measurements were being made). The electrical measurements involved were made by means of a White double potentiometer and a suitable galvanometer.

The heat capacity of the calorimeter and its contents was calculated from determinations of the temperature rise resulting from the input to the calorimeter of a measured quantity of electrical energy.

β-thiolactic acid for the heat capacity determinations was prepared according to the synthesis reported by Biilmann (1905 and 1906) from β-iodopropionic acid and potassium ethyl xanthate. The β-thiolactic acid was purified by repeated vacuum distillations at a pressure of about 2 mm. of mercury in a special type of distilling flask resembling a Hickman molecular still. It was not necessary in this flask to heat the liquid to its boiling point since evapo-

ration from the liquid surface gave a sufficiently rapid distillation at a temperature ten to twenty degrees below the boiling point at this pressure. The final product used for the heat capacity determinations was nearly water-white and had an apparent melting point of 16.5° C. determined by following the temperature of a quantity of the substance during its freezing in a bath at 10° to 12° C. Biilmann reported a melting point of 16.8° C.

$\beta\beta'$-dithiodilactic acid was prepared by oxidizing β-thiolactic acid with the theoretical quantity of iodine. This substance was purified by repeated recrystallization from water. The melting point by the capillary method was 155-156° C. with slight decomposition. Titration with sodium hydroxide using phenolphthalein for the indicator in the hot solution required (average of two determinations) 0.3 per cent more than the calculated amount of alkali.

Cysteine was prepared from cysteine hydrochloride (Hoffmann-La Roche) according to the procedure of Du Vigneaud et al. (1930), by dissolving cysteine hydrochloride in absolute ethyl alcohol and precipitating cysteine by exact neutralization of the HCl with a solution of ammonia in absolute alcohol. The product was perfectly white and had the following nitrogen content as determined by micro-Kjeldahl:

Nitrogen found	11.72	11.53	11.63%
Nitrogen calculated			11.58%

The cystine provided by Hoffmann-La Roche was found to be of sufficient purity to justify its use for the heat capacity measurements without further purification. The nitrogen content by micro-Kjeldahl was:

Nitrogen found 11.67%
Nitrogen calculated 11.66%

In the formol titration, the cystine required 0.2 per cent less than the calculated amount of NaOH.

The specific heat capacities of these four substances are given in Tables I, II, III, and IV, and are shown graphically in Figures 1, 2, 3, and 4.

The entropies were calculated from the heat capacity data by graphical integration of the curve of C_p against ln T and by the application of the extrapolation formula (equation 5) to the region 0 to 90° K. Table V shows the entropies of the compounds at 298.1° K. The case of β-thiolactic acid is complicated by the fusion of the material below 25° C. The calculation of the part of the entropy of this compound due to heat capacity is made by graphical integration of the heat capacity curves extrapolated to the temperature chosen as the melting point, namely 291.8° K. The heat input corresponding to the increasing heat capacity in the region 250 to 260° K (see Figure 1) which is due to some pre-melting, and the heat required for the fusion are combined to give the heat of fusion. This was found to have the value 1676 calories per mol, corresponding to an entropy change of 5.43 entropy units per mol.

The values of the specific heats seldom deviate more than 0.1 per cent from a smooth curve, and the values given may be taken as precise to one part in 1000. The use of the extrapolation formula in the calculation of the entropies introduces an uncertainty of about ± 0.1 entropy units according to data given by Kelley, Parks, and Huffman (1929).

Table I.

Specific heat of β-thiolactic acid

Temp. °K.	C_p per gram	Temp. ° K	C_p per gram
85.3	0.1313	203.7	0.2567
88.9	0.1359	212.3	0.2666
93.9	0.1415	215.1	0.2693
100.3	0.1484	220.4	0.2755
107.9	0.1559	229.9	0.2883
119.3	0.1678	236.2	0.2956
134.4	0.1823	243.9	0.3112
149.4	0.1988	245.2	0.3127
164.7	0.2147	251.6	0.3223
167.3	0.2175	254.8	0.3252
179.7	0.2307	258.8	0.3435
187.0	0.2388	299.8	0.4576+
194.9	0.2469	304.5	0.4603+
200.0	0.2542	309.9	0.4622+

+liquid state

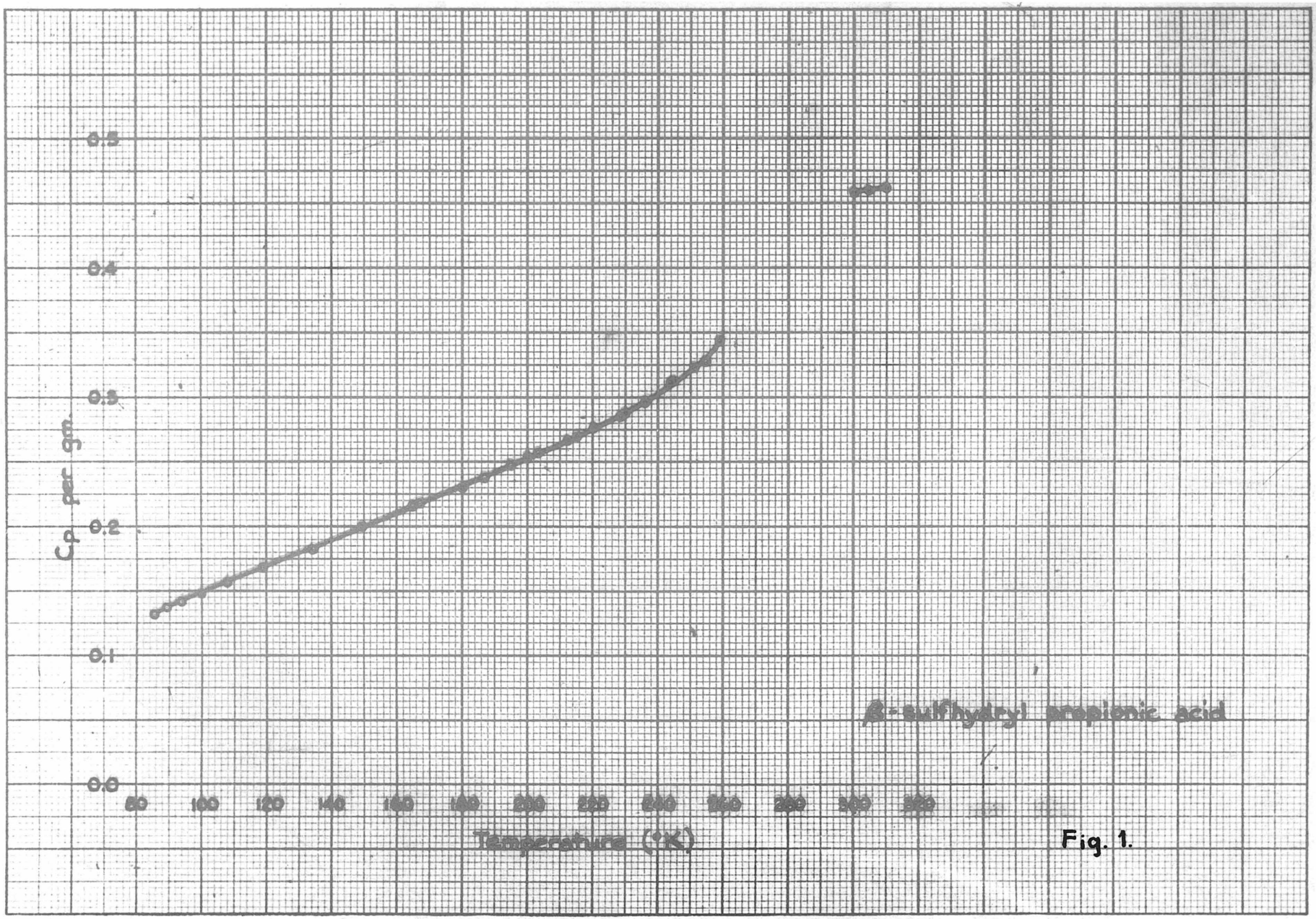

0.5
0.4
0.3
0.2
0.1
0.0
C_p per gm.
β-sulfhydryl propionic acid
Temperature (°K)

Fig. 1.

Table II.

Specific heat of $\beta\beta'$-dithiodilactic acid.

Temp. ° K.	C_p per gram	Temp. ° K	C_p per gram
84.9	0.1100	188.5	0.1912
89.2	0.1141	200.0	0.1998
94.1	0.1184	220.0	0.2151
99.6	0.1231	240.1	0.2298
105.3	0.1279	260.1	0.2449
111.7	0.1334	277.1	0.2574
120.0	0.1397	282.0	0.2611
129.7	0.1476	290.1	0.2675
144.6	0.1588	290.2	0.2671
159.0	0.1693	296.6	0.2719
180.6	0.1856	296.8	0.2724

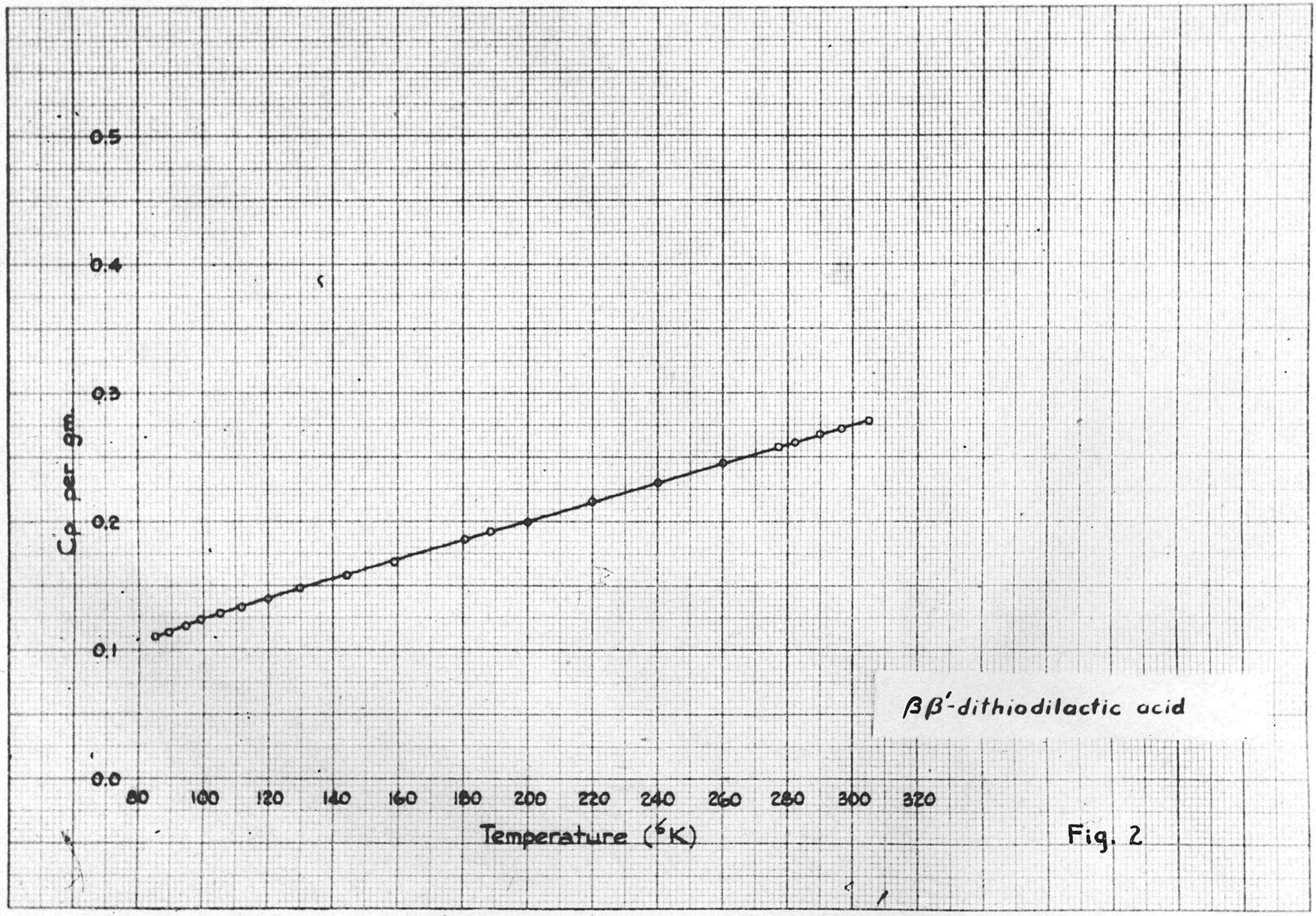

0.5
0.4
0.3
0.2
0.1
0.0
Cp per gm.
80
100
120
140
160
180
200
220
240
260
280
300
320
Temperature (°K)
ββ'-dithiodilactic acid

Fig. 2

Table III.

Specific heat of l-cysteine

Temp. °K.	C_p per gram	Temp. °K.	C_p per gram
85.1	0.1143	199.9	0.2218
90.4	0.1206	220.4	0.2403
97.2	0.1280	240.2	0.2592
105.9	0.1368	260.7	0.2802
119.3	0.1492	275.6	0.2960
134.5	0.1632	282.8	0.3041
149.6	0.1771	290.0	0.3121
165.2	0.1908	0.297.6	0.3203
179.8	0.2033		

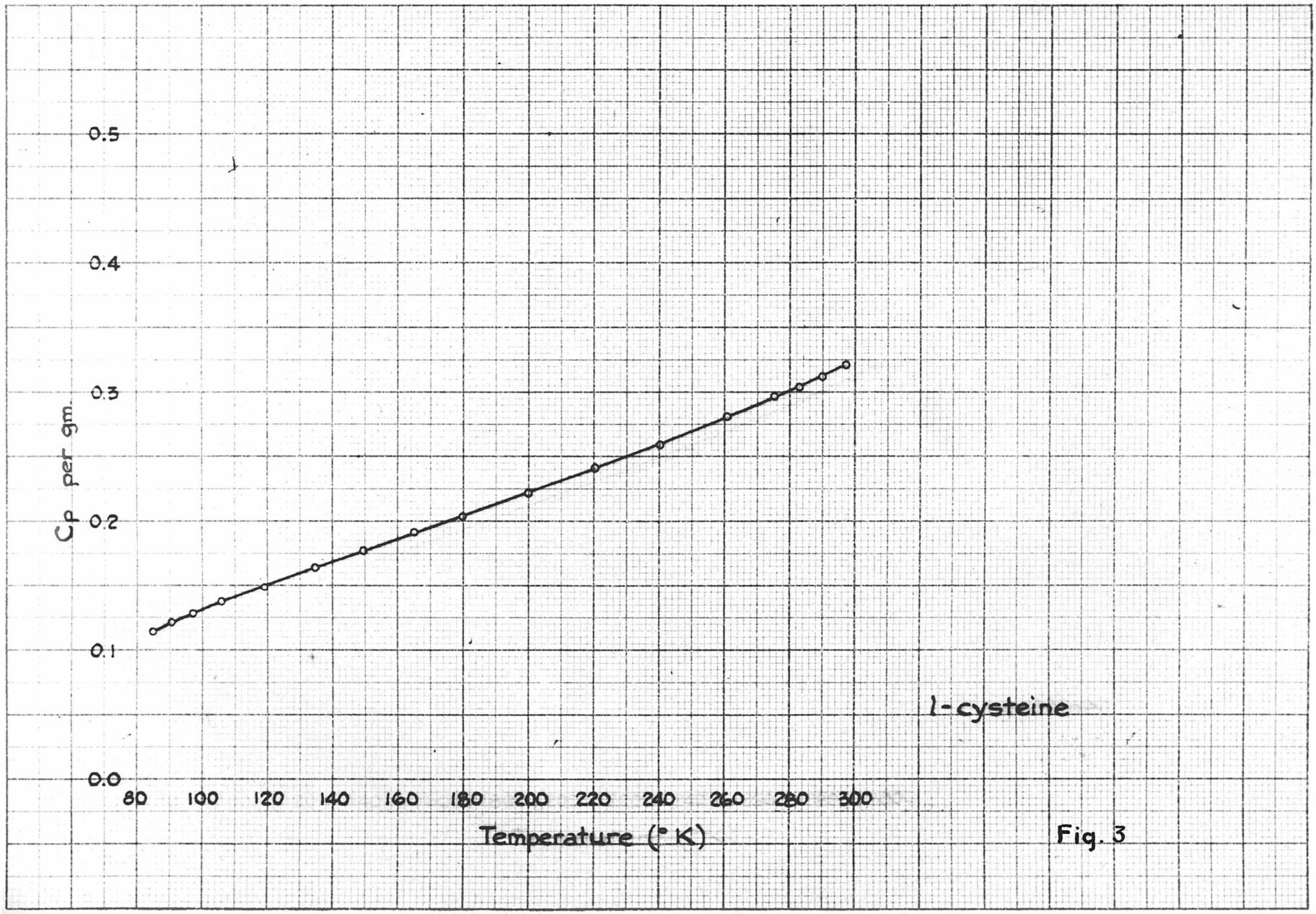

0.5
0.4
0.3
0.2
0.1
0.0
Cp per gm.
80
100
120
140
160
180
200
220
240
260
280
300
Temperature (° K)
l-cysteine

Fig. 3

Table IV.

Specific heat of l-cystine

Temp. °K.	C_p per gram	Temp. °K.	C_p per gram
85.6	0.09675	179.7	0.1786
91.3	0.1034	199.9	0.1946
97.1	0.1091	220.0	0.2094
105.8	0.1177	240.3	0.2238
119.3	0.1300	260.3	0.2381
135.0	0.1432	276.0	0.2500
150.0	0.1556	282.8	0.2551
164.7	0.1669	290.0	0.2608
		297.3	0.2666

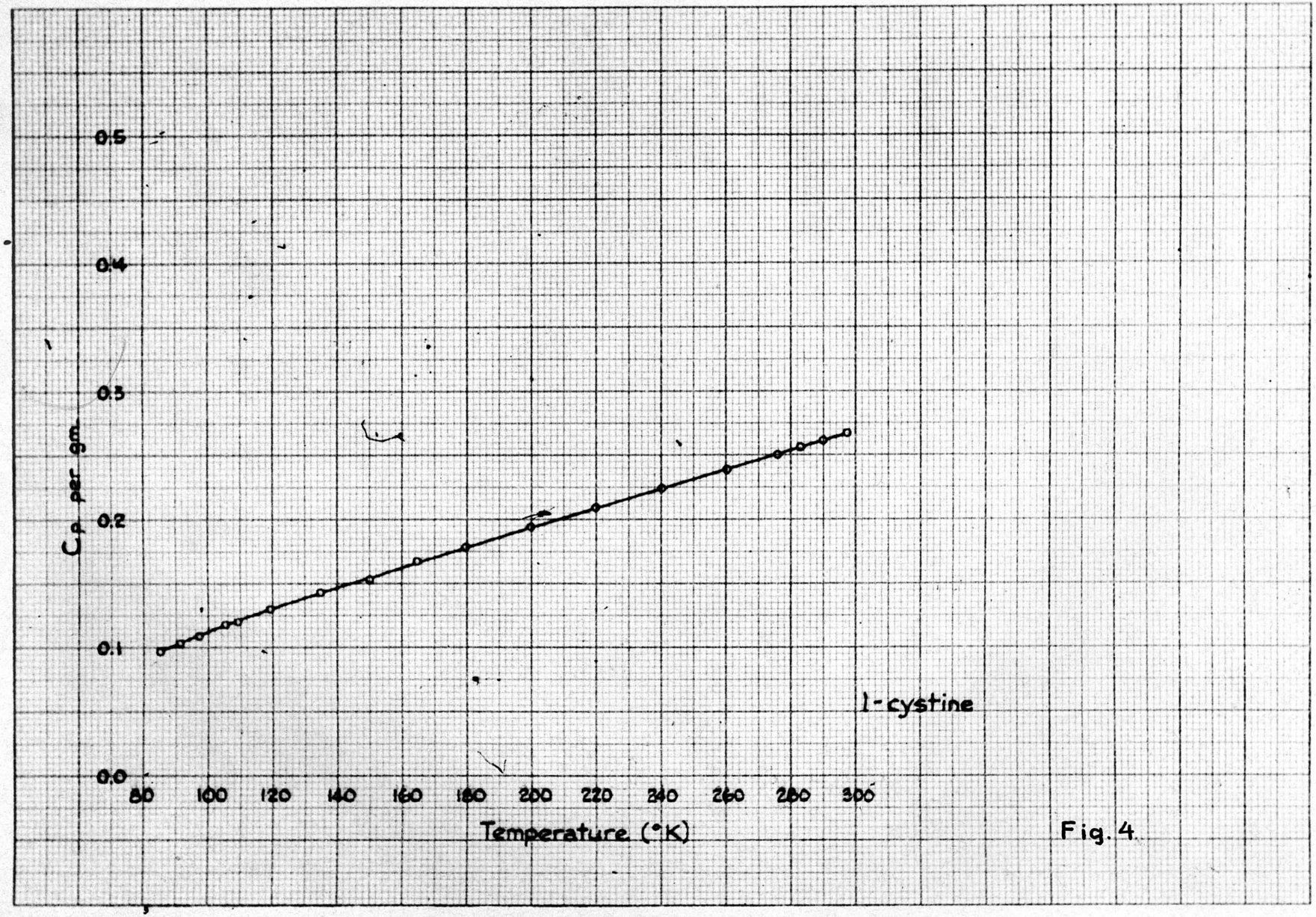

0.5
0.4
0.3
0.2
0.1
0.0
C_p per gm
80
100
120
140
160
180
200
220
240
260
280
300
Temperature (°K)
l-cystine

Fig. 4.

Table V.

Entropies of the compounds
(E.U. per mol)

Substance	$S_{90°}$	$\Delta S_{90°-298.1°}$	$S_{298.1°}$
β-thiolactic acid	11.90	34.37	46.27
ββ'-dithiodilactic acid	20.18	45.28	65.46
l-cysteine	11.71	28.85	40.56
l-cystine	18.99	49.51	68.50

2. Heats of combustion.

The apparatus used for measurements of heats of combustion was similar to that described by Dickinson (1914). It consisted of a constant temperature jacket surrounding a stirred calorimeter which contained the Parr bomb. The temperature of the calorimeter was followed by means of a platinum resistance thermometer connected to a Mueller bridge. The times at which predetermined temperatures were attained were recorded on a chronograph.

The charge of the bomb was ignited by means of a filter paper fuse, ignited by a small loop of #34 platinum wire brought to incandescence. The electrical energy required for this was controlled by a motor driven mechanical switch. Several measurements with a recording oscillograph showed that this electrical energy input was constant at 1.4 ± 0.1 calories.

The combustion of sulfur containing compounds requires a procedure differing in several details from that usually employed for other organic compounds. Complete combustion of the sulfur to SO_3 was obtained when atmospheric nitrogen was allowed to remain in the bomb. The one gram of water usually added to the bomb before admitting the oxygen had to be omitted since it led to widely different concentrations of sulfuric acid in the different parts of the bomb after the combustion, and introduced an uncertainty of about fifty calories in the heat to be attributed to dilution of the sulfuric acid formed in the combustion. All the substances under discussion here, except β-thiolactic acid, were burned with an auxiliary substance, oil, to insure successful ignition.

The gas from each combustion was discharged through a scrubbing column containing a known quantity of standard NaOH. The contents of the column were analysed for total acid absorbed by back titration, and for nitrous acid by a colorimetric method (Treadwell and Hall, 1924). Analysis for sulfur (Eschaka, 1927) always showed negligibly small quantities present in the gas absorbate. Nitric acid was obtained by difference, on the assumption that only nitrous and nitric acids were present.

The emptied bomb was taken apart and thoroughly washed. The washings were analysed for total sulfate (Eschaka, 1927), nitrous acid (Treadwell and Hall, 1924), and for total acid by titration, using bromcresol green as indicator. As above, nitric acid was obtained by difference, assuming sulfuric and nitrous acids to be the only other acids present.

The heat evolved in the calorimeter was calculated according to the method given by Dickinson (1914). This value was then corrected to give the heat for the corresponding isothermal process at the standard temperature, 25° C. From this quantity were subtracted the heat of formation of the sulfuric acid (Roth, Grau, and Meichsner, 1930) and its heat of dilution to the concentration existing in the bomb at the end of the combustion (Brönsted, 1910). The necessary data on the heat capacity of sulfuric acid solutions were obtained from the measurements of Birons (1905).

The analyses of the gas absorbate tended to show that approximately equal quantities of nitrous and nitric acids were discharged from the bomb when the pressure was released. The assumption was therefore made that N_2O_4 was the only nitrogen compound leaving the bomb in the gas phase. It was also assumed that no

nitrogen compounds except nitrous and nitric acids remained in the bomb. The quantities of nitrogen compounds formed were always relatively small and the total correction for their heats of formation seldom exceeded five calories. The quantities of materials to be burned were chosen so that the total heat evolution in a combustion was 6000 to 8000 calories. An error of 25 per cent in the correction applied for the nitrogen compounds introduces an insignificant error in the heat of combustion.

The materials used for heat of combustion measurements were subjected to more extensive purification than for the heat capacity determinations. The β-thiolactic acid was dried in contact with anhydrous calcium chloride and redistilled. Cystine and ββ'-dithiodilactic acid were repeatedly crystallized from water. Cysteine was prepared from a pure sample of the hydrochloride by the method previously used (Du Vigneaud et al. 1930). The sulfur analyses (by combustion in the bomb) are given in Table VI. The sulfur content always appeared to be somewhat low. However, combustion of pure (twice distilled) sulfur with benzoic acid or oil in the bomb under the same conditions gave correspondingly low figures. Thus it seems highly probable that the sulfur content of these compounds is very near the theoretical. The heat of combustion of both cystine and ββ'-dithiodilactic acid remained the same after recrystallization. The ash content of the four compounds was in all cases insignificant.

The heat of combustion data are shown in Table VII. The values calculated for combustion to gaseous CO_2, gaseous N_2, liquid H_2O and solid S are given in column 2. Column 3 shows the values corresponding to combustion to give aqueous H_2SO_4 in which the mol

Table VI

Sulfur content of substances used in heat of combustion measurements.

Substance	S found %	S calculated %
β-thiolactic acid	30.01 30.02 30.18 30.10	30.21
ββ'-dithiodilactic acid	30.21 30.22 20.11 30.18	30.50
l-cysteine	26.24 26.22 26.20	26.47
l-cystine	26.45 26.25 26.19 26.51	26.69
Sulfur + benzoic acid	31.34 46.19	31.60 46.37

Table VII.

Heats of combustion

(15° calories per gram at 25° C.)

Substance	Combustion to give: S (solid)	$H_2SO_4 \cdot nH_2O$
β-thiolactic acid	3538.1	
	3529.9	
	3535.2	
	3527.9	(N = 2.0)
Av.	3532.8	4812.5
ββ′-dithiodilactic acid	3203.7	
	3208.8	
	3206.6	(N = 2.0)
Av.	3206.4	4498.4
l-cysteine	3249.7	
	3250.2	
	3250.9	(N = 3.5)
Av.	3250.3	4392.0
l-cystine	3008.1	
	3008.4	
	3009.8	
	3008.6	
	3007.1	(N = 2.7)
Av.	3008.1	4150.2

ratio (N) of water to H_2SO_4 has the value indicated. N is given in each case a value that approximates that in the actual experiment. The data indicate that the accuracy of the determinations is about $\pm$ 5 calories per gram or approximately one part in 1000.

The heats of formation of these four compounds have been calculated (equation 9) and are shown in Table VIII; the heat data used in the calculations are given in Table XII.

3. Solubility data.

β-thiolactic acid is miscible in all proportions with water at 25° C. The solubilities of the three remaining compounds at 25° and 37° C. are presented in Table IX. The solubilities of l-cystine are those of Dalton and Schmidt (1934); the remainder are approximate values determined by a technique similar to that used by Dalton and Schmidt (1933). Solubility tubes were shaken in an air bath at the temperature indicated for 48 to 72 hours and the solution was then filtered at the temperature of the bath. Densities were determined by weighing a 5 cc. pycnometer filled with the solution. The quantity of solid in a weighed quantity of solution was determined by evaporating the solution to dryness in an oven held at 85° to 90° C. Preliminary experiments showed that the two substances under investigation could be recovered quantitatively from their solutions by this method of evaporation. In the determinations of the solubility of l-cysteine, the tubes containing the amino acid were half filled with freshly boiled water and the air was then displaced by hydrogen to prevent oxidation during the period of shaking. The solubility figures given are averages of two or more separate experiments in which the difference

Table VIII.

Heats of formation

(15° calories per mol at 25° C.)

Substance	$\Delta H_{298.1°}$
β-thiolactic acid	-111,600
$\beta\beta'$-dithiodilactic acid	-231,180
l-cysteine	-127,090
l-cystine	-250,900

Table IX.

Solubility in water
(mols per 1000 grams of water)

Substance	25° C.	37° C.
$\beta\beta'$-dithiodilactic acid	0.00637	0.0126
l-cysteine	0.228	0.404
l-cystine	0.000544	0.000770

was less than six per cent. Errors of this magnitude produce negligible errors in the calculation of the free energy of the substance in solution.

4. Dissociation constants.

The dissociation constants of β-thiolactic acid and l-cysteine were determined by electrometric titration. The titration vessel contained two Moloney electrodes supplied with electrolytic hydrogen, and was closed with a rubber stopper having holes for a calibrated burette, an agar-KCl bridge, and a hydrogen exit. The entire apparatus was assembled in a constant temperature closet held to 25° C. EMF measurements were made with a Leeds and Northrup type K potentiometer in conjunction with a suitable galvanometer. Alternate readings were made on the two electrodes until steady values which agreed with one another were obtained. Electrodes which drifted or failed to agree were replaced before continuing the titration. The data for one such titration is submitted in Table X to show the agreement obtained in the pK values in the course of a single titration.

The two disulfide compounds could not be titrated at the hydrogen electrode since platinum black and hydrogen reduce them to the corresponding sulfhydryl substances. The glass electrode was used for the titration of ββ'-dithiodilactic acid and gave satisfactory approximations of its dissociation constants. The type K potentiometer in conjunction with a high sensitivity galvanometer was used for this work. The glass electrode was calibrated before use against buffers which had been calibrated against the hydrogen electrode. The pK' values calculated for this titration were constant

Table X.

Electrometric titration of l-cysteine COOH group

25° C. 1.040 millimol cysteine;
initial volume 25.0 cc. of 0.208 m. KCl
Std. HCl = 0.07496 n.

cc HCl added	pH observed	pK' calculated	pK calculated
2.00	2.874	1.92	1.80
4.00	2.552	1.92	1.80
5.02	2.443	1.92	1.80
6.01	2.359	1.92	1.80
7.03	2.278	1.92	1.80
8.10	2.207	1.91	1.79
9.01	2.153	1.90	1.78
10.02	2.100	1.90	1.78
11.08	2.053	1.91	1.79
12.06	2.009	1.90	1.78
13.09	1.972	1.89	1.77
14.07	1.934	1.89	1.78
16.09	1.875	1.89	1.78
18.05	1.825	(1.84)	(1.73)
20.09	1.779	1.89	1.78
22.10	1.742	1.89	1.78
24.01	1.706	(1.87)	(1.76)

for the second COOH group to ± 0.01 units, and for the first COOH group to ± 0.03 units.

Dissociation constants lying within two pK units of one another were calculated by a method of approximations. The dissociation constants obtained from the titration curve were used to calculate the concentrations of the various molecular species present at each point of the titration. From these the corresponding constants were calculated. These calculated constants were then assumed and a new calculation made which in most cases yielded values of satisfactory accuracy.

The apparent dissociation constants (pK') are shown in Table XI. The pK' values for l-cystine are from Cannan and Knight (1927). The last column in the table gives the pK values calculated on the basis of certain assumptions regarding the activity coefficients. The activity coefficients of neutral molecules (unionized, or zwitter ions) were taken as unity. The activity coefficients of singly charged ions were calculated by the simplified Debye-Huckle equation (Clark, 1928)

$$- \log \gamma_i = \frac{0.5 \; z_i^2 \sqrt{\mu}}{1 + 0.33\sqrt{\mu}}$$

For divalent ions the term z_i^2 was replaced by a term v^z (Simms, 1928) which is equal to z_i^2 when the charges of the divalent ion coincide in position, and is less for "long" ions in which the charges are separated by a distance of several angstroms.

The justification for these activity corrections is seen in the pK values of l-cysteine, which was titrated twice in solutions

Table XI.

Dissociation constants.

Substance	Temp. °C.	Ionic strength	Group	pK'	pK
β-thiolactic acid	25	0.03	COOH	4.26	4.31
	25	0.05	SH	10.21	10.50
ββ'-dithiodilactic acid	23	0.001	1-COOH	3.84	3.85
	"	0.003	2-COOH	5.02	5.15
l-cysteine	25	0.17	COOH	1.90	1.79
	"	0.03	"	1.85	1.79
	"	0.17	NH_2	8.27	8.42
	"	0.03	"	8.30	8.36
	"	0.17	SH	10.35	10.88
	"	0.03	"	10.57	10.82
l-cystine	30	0.02	1-COOH	1.0[a]	1.0[a]
	"	"	2-COOH	1.7	1.7
	"	"	1-NH_2	7.48	7.53
	"	"	2-NH_2	9.02	9.30

[a]Values assumed from titration curve of Cannan and Knight (1927).

of different ionic strengths. The COOH and SH groups for which the pK' values were somewhat different in the two titrations show pK values which are in fairly good agreement.

5. Calculation of results.

The data presented in the preceding sections makes possible the calculation of the free energies of the substances under discussion, and the free energy change of the oxidation reaction under any conditions of concentration not far removed from 25° C.
The steps involved in such a calculation are briefly as follows:

(1) Calculation of the entropies of formation of the crystalline compounds at 298.1° K. from the known entropies of the elements and the entropies of the compounds derived from heat capacity measurements. If for example the formula of the compound is $C_aH_bO_c$, then

$$\Delta S_{298} = S_{(\text{compound, } 298)} - a\,S_{(C,\ 298)} - \tfrac{1}{2}\,b\,S_{(H_2,\ 298)} - \tfrac{1}{2}\,c\,S_{(O_2,\ 298)}.$$

(2) Calculation of the free energies of formation at 298.1° K. from the heats of formation derived from heats of combustion measurements and the entropies

$$\Delta F = \Delta H - T\,\Delta S$$

(3) Calculation of the free energies of formation at any other desired temperature (near 298° K.) with the assumption that ΔH is a constant in this temperature interval, using the expression

$$\frac{\Delta F_2}{T_2} - \frac{\Delta F_1}{T_1} = \int_{T_1}^{T_2} -\frac{\Delta H}{T^2}\, dT$$

(4) By the use of solubility data and ionization constants, calculation of the standard free energy change in solution for the reaction.

The heats of formation and entropies of the elements used in these calculations are given in Table XII. The entropy of sulfur is from the "International Critical Tables". All other values are from a table given by Borsook and Huffman (1933). Table XIII gives the entropies and free energies of formation of the crystalline substances calculated according to the method outlined above.

The standard free energy change in solution $\overline{\Delta F}$ (i.e. the free energy change when all substances entering the reaction are at a concentration of unity) for the reaction

$$RSSR + 2\,H^+ + 2\,E^- \rightarrow 2\,RSH$$

is shown in Table XIV. The last column shows the corresponding potential $\overline{E}$, the molal electrode potential. The sign convention is that of Clark, i.e. the more powerful the reducing properties of the system the more negative the potential. The term E_o', the potential when the ln term in equations (16) and (17) involving concentration ratios becomes zero, has been calculated at various hydrogen ion activities, and its variation with pH is shown in Table XV and Figure 5. The values of E_o' for 37° C. are calculated with no correction for the effect of temperature change on the dissociation constants.

Table XII.

Thermal data used in calculations

Heats of formation

Substance	$\Delta H_{298.1}$
H_2O (liquid)	-68,310 cal.
CO_2 (gas)	-94,240
H_2SO_4 (aq.)	-125,790

Entropies of the elements

Element	$S_{298.1}$
C (graphite)	1.3
H_2 (gas)	31.23
N_2 (gas)	45.78
O_2 (gas	49.03
S (orthorhombic)	7.69

Table XIII.

Entropies and free energies of formation
(calories per mol)

Substance	ΔS (298.1 °K)	ΔF (298.1 °K)	ΔF (~~298.1~~ 310.1 °K)
β-thiolactic acid (liquid)	-108.0	- 79,400	- 78,100
$\beta\beta'$-dithiodilactic acid (cryst)	-211.9	-168,000	-165,500
l-cysteine (cryst)	-152.3	- 81,690	- 79,870
l-cystine (cryst)	-285.9	-165,700	-162,200

Table XIV.

Standard free energy change in solution ($\overline{\Delta F}$) for the reaction:

$$RSSR + 2\,H^{+} + 2\,E^{-} \rightarrow 2\,RSH$$

System	Temp. (°C)	$\overline{\Delta F}$ (calories)	$\overline{E}$ (volts)
β-thiolactic acid: ββ'-dithiodilactic acid	25	+ 2,170	- 0.047
	37	+ 2,270	- 0.049
Cysteine:cystine	25	-400	+ 0.009
	37	-680	+ 0.015

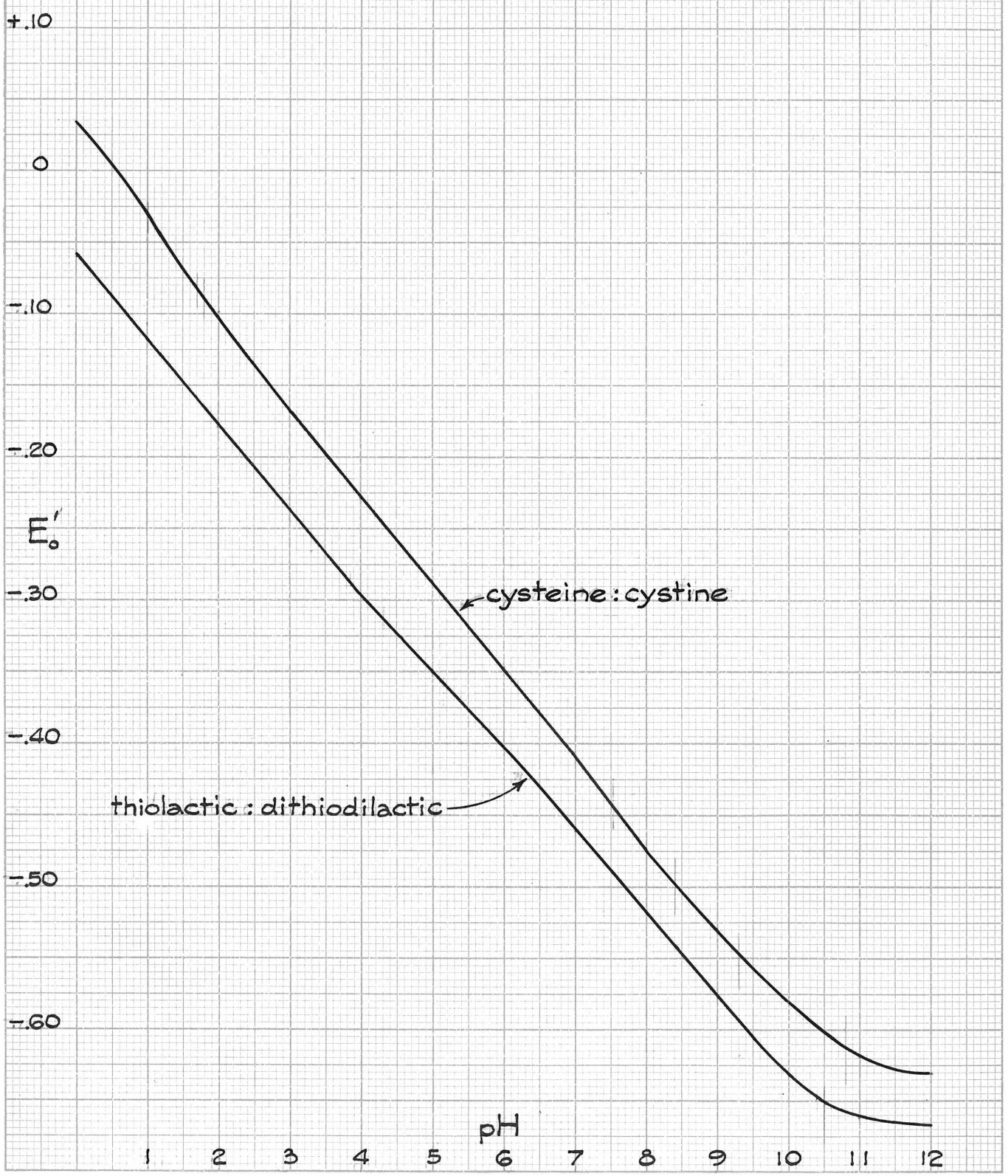
Table XV.

Relation between E'_o and pH at 25° C.

pH	E'_o (β-thiolactic acid)	E'_o (cysteine)
1.0	- 0.117	- 0.030
2.0	- 0.176	- 0.103
3.0	- 0.236	- 0.168
4.0	- 0.296	- 0.228
5.0	- 0.350	- 0.287
6.0	- 0.403	- 0.347
7.0	- 0.461	- 0.408
8.0	- 0.518	- 0.475
9.0	- 0.578	- 0.531
10.0	- 0.631	- 0.580
11.0	- 0.661	- 0.619
12.0	- 0.667	- 0.630

Fig. 5

Relation between E'_o and pH
25° C.



6. Discussion

The difference between the reduction potentials of β-thiolactic acid and l-cysteine under similar conditions is of the order of 50 millivolts. The correspondence of these potentials and the close chemical similarity of cysteine and glutathione makes it likely that the glutathione potential is approximately equal to that of cysteine. The similarity of the cysteine and glutathione potentials is further attested by the dye reduction experiments of Green (1933). At concentrations of 0.1 molal the two substances behave in an identical way toward the dyes tested, and at 0.01 molal the difference, if anything, is not more than 25 millivolts.

These dye experiments do present a serious discrepancy. On the basis of the thermodynamic potential derived above, cysteine should have reduced all the dyes used by Green, including the neutral red for which the E_o' value at pH 7 is - 0.340 volts. At pH 7 in a solution of 0.01 molal cysteine and 0.001 molal cystine, the reduction potential is - 0.346 volts. In Green's dye reduction experiments, a 0.01 molal solution of cysteine (cystine concentration not given) partially reduced cresyl violet (E_o' = - 0.167 volts at pH 7) and phenosafranine (E_o' = - 0.242 volts at pH 7) was not reduced.

The unreliability of dyes as indicators of oxidation-reduction potentials is attested by a variety of enzyme experiments from which two examples are given below. The acetaldehyde--acetic acid transformation which is catalysed by the Schardinger enzyme from milk has a free energy change sufficient to reduce neutral red, but in actual experiments neither neutral red nor Janus green B (E_o' = - 0.258 volts at pH 7) were reduced. Similarly the formate--bicar-

bonate transformation in which the free energy change corresponds to a potential of - 0.414 volts under the conditions of the experiment, should reduce all the dyes tried but failed to reduce rose indulin (E_0' = - 0.281 volts at pH 7) and neutral red, although safranine blue (E_0' = - 0.260 volts at pH 7) and Janus green B were reduced.

Here again the dye reduction experiments have failed to give the thermodynamic reduction potential. It is possible but improbable that the most negative dyes were reoxidized by a process liberating hydrogen from water, masking any slow reduction that may have taken place, but this explanation is not acceptable for cresyl violet and dyes above it which were not reduced by cysteine. Another explanation suggests itself, namely, that the two electron transfers involved in the dye reduction occur separately and the energy involved in the first is greater than that in the second. If the mechanism of the reduction of the dye by cysteine is such as to involve the two transfers separately, the energy requirement of the first transfer determines the reaction.

There is no assurance that we are dealing with equilibrium conditions in the dye reduction experiments, since the reactions were tried from one side; oxidized dye and sulfhydryl substance. The reverse reaction must be tried in all cases and must yield concordant results before the criterion of equilibrium is satisfied.

In vivo experiments indicate that the sulfhydryl--disulfide reaction is reversible and one is justified therefore in accepting the thermodynamic potential as characterizing the behavior of sulfhydryl substances with respect to biological systems.

The E'_o value of ascorbic acid as reported by Borsook and Keighley (1933) is + 0.066 volts at pH 7.0. The potential given above for cysteine requires that oxidized ascorbic acid be reduced by cysteine or glutathione. Its reduction by glutathione was observed by Szent-Gyorgyi (1928) who found that a solution of glutathione was completely oxidized in less than 90 minutes in vacuo in the presence of an excess of oxidized ascorbic acid.

III. PHYSIOLOGICAL APPLICATION

1. Distribution in nature.

An enormous literature on the subject of sulfhydryl substances has grown up since Hopkins (1921) first isolated the substance responsible for the nitroprusside test given by most animal and plant tissues and extracts. This substance, named "glutathione" by Hopkins has proven to be the tripeptide γ-glutamyl-cysteinyl-glycine. It is soluble in water, can be obtained crystalline, and forms insoluble salts with many heavy metals. Also it can be oxidized in solution with oxygen, or oxidizing agents to give among other products, a disulfide. This disulfide can be reduced with hydrogen and platinum black or with tin and HCl to the sulfhydryl compound.

The wide distribution of this substance in living material is one of the circumstances which has led to considerable curiosity regarding its function. Its chemical structure and properties indicate that it is probably concerned with biological oxidations, but no such connection has been demonstrated. Recent work has shown that glutathione is a coenzyme for glyoxalase and an activator for papain, cathepsin, and perhaps urease. This is discussed in another section of this paper. It should be pointed out here that none of the functions ascribed to this substance give it a physiological importance commensurate with its wide distribution in nature.

The glutathione content of many different kinds of material has been measured, most often by the iodine titration of Tunnicliffe (1925) or one of its many modifications, and in a few cases by colori-

metric methods. The utility of the iodine titration depends, of course, on the absence of all substances other than glutathione which react rapidly with iodine in dilute solution. The reaction assumed is the following

$$2\ GSH + I_2 \rightarrow GSSG + 2\ HI$$

Another reaction also occurs (see Bierich and Kalle, 1928), namely

$$GSH + 3\ I_2 + 3\ H_2O \rightarrow GSO_3H + 6\ HI$$

This reaction increases in importance as the solutions become more dilute, and explains the fact that the iodine titration often gives results that indicate too high a glutathione content.

It has been tacitly assumed by most investigators that no substances interfering with this iodine titration are present in appreciable quantities. The invalidity of this assumption has been shown by Birch and Dann (1933), who found that the presence of ascorbic acid in animal tissues may result in an iodine titration as much as 75% too high. Furthermore, very few investigators have taken the trouble to determine the oxidized (GSSG) glutathione content of their material. Methods for this depend on reduction of oxidized glutathione with sodium amalgam (Mason, 1931) or hydrogen (Litarzek et al. 1932) and subsequent determination of the total glutathione by titration. The determinations of the reduced glutathione in various tissues shown in Table XVI are subject to this criticism, and are submitted to show the distribution of glutathione in nature rather than to give exact data regarding its concentration in certain organs.

Table XVI.

Glutathione content of various materials.

(milligrams per 100 grams of fresh tissue)

Tissue	GSH	Method of analysis
blood (human)	30	I_2 titration, external nitroprusside indicator.
blood (Human) (male)	14.7-30.7 (av. 21.3)	Ferricyanide oxidation, estimate ferrocyanide produced colorimetrically as prussian blue
" " (female)	16.1-38.8 (av. 22.7)	
liver (guinea pig)	413 - 565	I_2 titration, starch indicator
lung " "	139 - 287	
kidney " "	202 - 234	
heart " "	126 - 166	
spleen " "	367 - 417	
testicle " "	134 - 194	
12 day chick embryo	32	I_2 titration, nitroprusside indicator
16 " " "	25	
20 " " "	18	
rat embryo		
0.07 - 0.8 gm.	60	
1 - 2 "	58	
3.9 - 4.7 "	44	
new born, (4.6 - 5 gm.)	36	
137 - 170 gm.	23	
blood (eel)	30 - 40	Method not given
liver "	65 - 90	
female organs (eel)	214 - 314	
blood (conger)	10	
liver "	35	
female organs (conger)	23	
liver (dog)	245 - 385	Add excess I_2 to trichloracetic extract, back titrate with $S_2O_3^=$
adrenal "	374 - 584	
thyroid "	143 - 293	
pancreas "	201 - 234	
ovaries "	143 - 324	
testicles"	255 - 266	

By way of generalizing in one statement the occurrence of glutathione in animals it may be said that organs (liver, kidney, adrenals, etc.) contain the most GSH (100 to 300 milligrams per 100 grams of tissue) and that muscle, skin, connective and adipose tissue contain the least (0 to 50 milligrams per 100 grams of tissue). Embryonic tissue contains in general, a higher concentration of glutathione than the corresponding adult structure.

2. Relation to cell division.

Introduction

It has been observed that in tissues in which cell division is occurring rapidly there is a relatively high concentration of glutathione. This has led several investigators to view that glutathione is the "stimulator" for cell division. The literature is confused with claims of positive and negative results of attempts to stimulate cell division with glutathione and a variety of other sulfhydryl substances.

Rapkine (1931) proposed the following theory regarding the role of glutathione in cell division: a) local denaturation of certain proteins as a result of protein catabolism sets free -SH groups; b) these groups reduce the oxidized (-SS-) glutathione in solution changing the reduction potential of these parts of the cell; c) the resulting stimulation of glycolysis provides the energy required for cell division.

The following evidence has been cited in support of this theory. Shearer (1922), Dulzetto (1931), and Rapkine (1931) found that fertilization is accompanied by an increase in the intensity of

the nitroprusside reaction. Rapkine found, in addition, an increase in -SH groups which reached a maximum at the time of cleavage. Hammett and his co-workers, in a long series of papers (1929, 1930, 1931, 1932) reported that lead ion slows or stops growth in root tips, presumably by combining with -SH groups; and that also the rate of cell division is increased by the addition of minute amounts of sulfhydryl or disulfide substances to the culture fluid (onion root tips and paramecium).

The work described in the following pages was undertaken in an effort to clarify this point. If the above hypothesis is correct, and carbohydrate metabolism and sulfhydryl groups are a fundamental part of the chemical mechanism of cleavage, interference with the operation of either should produce a marked change in the rate of division. Carbohydrate metabolism can be inhibited by iodoacetate or fluoride ions. Iodoacetate will also destroy reduced glutathione (Lohmann, 1933). The effect of these and other reagents on the rate of division of fertilized eggs was tested in the experiments described below. The results obtained are quite definite, namely, that glutathione may, by means of appropriate reagents, be partly or largely removed from a fertilized egg cell without stopping or even appreciably slowing the cell divisions. This renders the hypothesis that glutathione is the specific stimulator for cell division untenable, at least for the cell divisions of fertilized marine eggs.

Experimental

The eggs of Urechis caupo (Fisher and MacGinitie, 1928) were used in most of these experiments. In a few, sea urchin eggs (Strongylocentrotus purpuratus) were used. The test solutions were

prepared by dissolving the solid substance in fresh sea water or by adding not more than 5 drops of a sufficiently strong aqueous solution to 5 cc. of sea water, thus avoiding excessive dilution of the sea water. Concentrated solutions of iodoacetic acid and sodium lactate were titrated to pH 8, so that there would be no appreciable change in the pH of the sea water when these solutions were added in small amounts. For the prevervation of eggs and sperm for the short interval between extraction from the animal and use and in the control experiments, fresh filtered sea water was used.

In the first group of experiments, eggs were fertilized in a large dish, and 20 minutes later 3 or 4 drops (a few hundred eggs) were transferred to each of the dishes. When the development of the controls had proceeded to the desired stage, enough formaldehyde was added promptly to all dishes to stop development, and a census made of 1 to 200 eggs in each dish. In these and subsequent experiments unfertilized eggs were disregarded in order to exclude any effect of reagents on the fertilization process.

All possible sources of heat (sunlight or reading lamps) which might cause warming of some of the dishes of eggs and not others were excluded. The room temperature during the experiment probably varied less than 1°. The day-to-day variation was not more than 3° or 4°, remaining around 16° to 18° C.

Table XVII shows that cysteine in concentrations from 10^{-5} to 10^{-7} molal is without effect on the rate of cell division. If any stimulation of cell division had occurred there would have been a much higher percentage of eggs in the eight-cell stage and correspondingly less in the four-cell stage than in the controls. The last column shows that also on the basis of the total number of

Table XVII

The effect of cysteine and cupric ion on the rate of cell division

Solution	Stage of cell division				Total No. of divisions per 100 eggs.
	1 cell per cent	2 cell per cent	4 cell per cent	8 cell per cent	
1. Sea water	3	0	46	51	245
2. Cu acetate 6×10^{-7} m.	1	0	41	58	256
3. Cysteine 6×10^{-5} m.	2	0	65	33	229
4. Sea water	3	0	81	16	210
5. Cysteine 6×10^{-6} m.	4	0	51	45	237
6. Cysteine 6×10^{-7} m.	6	0	53	41	229
7. Sea water	3	0	83	14	208

Urechis caupo. Batch of eggs inseminated, transferred to test solutions at 20 minutes; formaldehyde added to all dishes at 200 minutes.

divisions cysteine is without effect.

The effect of cupric ion is also shown in Table XVII. Copper is the most likely impurity in glutathione preparations, and it was obviously necessary to know whether this ion produces an effect on the rate of division before engaging on any experiments with glutathione. The data show that this ion in the concentration in which it is likely to occur has no marked effect.

Table XVIII is a summary of the results obtained with iodoacetate, fluoride, and lactate. It is clear that these poisons for carbohydrate metabolism do not stop or retard cell division in Urechis eggs. The concentration of the iodoacetate used in dish no. 2 (m/100) is much greater than that which completely inhibits carbohydrate metabolism in muscle, yet there is no slowing of the division rate. From Rapkine's hypothesis complete failure in the development was to be expected. These results similarly fail to confirm the contention of Hammett (1932) that glutathione accelerates cell division, because the concentration of iodoacetate used must have removed most of this substance, and should therefore have decreased the rate of division.

The addition of lactate to some of the test dishes likewise produced no effect on the rate of division. The ineffectiveness of lactate, shown in in Table XVIII, is in accord with the absence of any inhibitory effect of the specific poisons for carbohydrate metabolism.

Table XIX shows the results of iodine titrations (method of Tunnicliffe) on trichloracetic acid extracts of eggs allowed to develop in sea water, and in a sea water solution of iodoacetate (m/100). In each case the eggs were allowed to develop 25 minutes in sea water before adding the requisite amount of iodoacetate solution.

Table XVIII.

The effect of iodoacetate, fluoride, and lactate ions on the rate of cell division

Solution	Stage of cell division: 1 cell per cent	2 cell per cent	4 cell per cent	8 cell per cent	Total No. of divisions per 100 eggs
1. Sea water	4	5	89	2	189
2. Iodoacetate m/100	2	1	97	0	195
3. Iodoacetate m/1000	8	2	90	0	182
4. Sea water	3	2	93	2	194
5. Iodoacetate m/100 Lactate 0.1 per cent	4	2	93	1	191
6. Iodoacetate m/1000 Lactate 0.1 per cent	8	1	88	3	186
7. Sea water	11	0	78	11	189
8. Fluoride m/100 (?)	6	5	83	6	187
9. Fluoride m/1000	5	4	90	1	187
10. Sea water	3	3	89	5	196
11. Fluoride m/100 (?) Lactate 0.1 per cent	5	7	65	23	206
12. Fluoride m/1000 Lactate 0.1 per cent	1	1	95	3	200
13. Sea water	3	3	91	3	194
14. Lactate 0.1 per cent	3	2	94	1	193

Urechis caupo. Batch of eggs inseminated, transferred to test solutions at 20 minutes; formaldehyde added to all dishes at about 2 1/2 hours after insemination. In dishes nos. 8 and 11 a precipitate of some insoluble fluoride formed. The concentration of fluoride in these dishes is therefore less than the value given.

Table XIX.

Effect of iodoacetate on the glutathione content of developing eggs.

(Glutathione calculated in mg. per cent weight of eggs)

Sea water	Iodoacetate m/100
72.5	29.9
78.0	30.4

Urechis caupo. Eggs fertilized and allowed to develop 25 minutes in sea water. Sufficient concentrated reagent then added to bring final concentration of egg suspension to the value given at the head of the column. Weight of eggs is wet weight.

Two hours after fertilization the eggs were centrifuged, weighed, and ground with 10 per cent trichloracetic acid. The filtered extract was promptly titrated with m/100 iodine solution. The eggs exposed to iodoacetate showed by titration less than half the glutathione in the controls, which showed that the iodoacetate had penetrated the egg and combined with a large fraction of its glutathione. Titrations of eggs exposed in a similar way to a 25 mg. per cent glutathione solution did not show as clearly that this substance enters the egg because of the high concentration in the egg and the low concentration outside. A larger concentration in the solution could not be used without retarding the development of the eggs, which in itself is evidence that glutathione enters the eggs (Table XXI).

The results in Table XX show that incidental variations in pH could not have been a factor in the results obtained. Samples of sea water titrated with acid or alkali to approximately the pH indicated (determined colorimetrically) and 5 cc. of each sample were placed in watch glasses. The pH of normal sea water is about 8.1. To these dishes, 3 or 4 drops of fertilized eggs were added, containing 200 or 300 eggs. They displayed remarkable resistance to the pH changes, eggs at pH 10 developing nearly as fast as the controls.

Table XXII is a summary of some experiments in which the eggs were placed in dishes containing test solutions before fertilization. In general, the results were the same as when the various inhibitors were added 20 minutes after insemination. Glutathione at 10^{-4} and 10^{-6} molal (3 and 0.03 mg. per cent) retarded the division slightly. (Glutathione is present in sea urchin eggs to about 20 mg. per cent (Rapkine, 1931). No analyses are available for Urechis eggs).

Table XX.

The effect of changes of pH on rate of division

	Solution	Stage of cell division: 1 cell Per cent	2 cell Per cent	4 cell Per cent	8 cell Per cent	Total no. of divisions per 100 eggs
1.	Sea water	3	7	56	34	221
2.	pH 10.0	5	3	91	1	188
3.	pH 9.5	4	2	92	2	192
4.	pH 9.0	3	3	93	1	192
5.	Sea water	4	2	91	3	193
6.	pH 8.5	4	5	78	18	205
7.	pH 7.5	2	1	94	3	198
8.	pH 7.0	1	0	99	0	198
9.	Sea water	4	0	86	10	202

Urechis caupo. Fresh sea water adjusted approximately to the pH indicated with NaOH or HCl. Eggs transferred to these solutions 20 minutes after insemination; formaldehyde added about 2 1/2 hours after insemination.

Table XXI.

Effect of glutathione on rate of division

Solution	Stage of cell division			Total number of divisions per 100 eggs
	1 cell	2 cell	4 cell	
1. Sea water	0	0	100	200
2. Glutathione 30 mg. per cent	1	22	77	176
3. Sea water	0	3	97	197

Urechis caupo. Eggs inseminated in fresh sea water; transferred to solution of reagent 20 minutes after insemination.

Table XXII.

Effect of exposing eggs to various reagents during fertilization and subsequent development on the rate of division.

Solution	Stage of cell division: 1 cell per cent	2 cell per cent	4 cell per cent	Total number of divisions per 100 eggs	Fertilized per cent
1. Sea water	2	6	92	190	100
2. pH 10	0	3	97	197	100
3. pH 7	9	30	61	152	100
4. Sea water	1	1	98	197	100
5. Iodoacetate m/100	1	17	82	181	97
6. Fluoride m/100 (?)	2	12	86	184	100
7. Sea water	2	14	84	182	99
8. Glutathione 10^{-4} m.	17	28	55	138	100
9. Glutathione 10^{-6} m.	8	23	69	161	100
10. Sea water	3	11	86	183	100
11. Lactate 0.1 per cent	26	50	24	98	42

First four columns based on a census of eggs in which only those which have become fertilized are taken into account.

Urechis caupo. Eggs placed in reagents for 5 minutes, inseminated in situ and allowed to develop in the solution. Same quantity of sperm suspension used in each.

The slight inhibitory effect of glutathione may have been on the fertilization process, rather than on cell division. In 0.1 per cent lactate, only about half the eggs become fertilized, and these develop more slowly than the controls. From the observations of Tyler and Schultz (1932 and from the negative results obtained with lactate previously, the slowing is probably to be attributed to interference with fertilization rather than division processes.

The results with glutathione find verification in another experiment in which the eggs were fertilized in fresh sea water, and allowed to develop 20 minutes before addition to the reagent. In the presence of 30 mg. per cent of GSH there was about a 10 per cent decrease in the number of divisions per 100 eggs, as is shown in Table XXI.

After these observations that inhibition of carbohydrate metabolism had no effect on the rate of cell division, it was obviously interesting to observe the effect, if any, of an inhibition of the total respiration. For this purpose cyanide was employed. A large batch of eggs was inseminated and allowed to develop for 5 to 45 minutes. The eggs were then distributed among a number of Syracuse dishes, 100 or more to each. Every fifth dish contained fresh sea water. The others contained 5 cc. of various test solutions freshly made up in sea water. The dishes were inspected from time to time to observe whether division had occurred in a large fraction of the eggs. In these experiments it was found that NaCN at 0.001 molal retarded the first division of the fertilized eggs for an hour or more, when the eggs were placed in the solution 5 minutes after insemination. Nevertheless they eventually reached an advanced

stage of development, though of course later than the controls. This concentration of cyanide was then used with the addition of various oxidation-reduction dyes to test if these agents could replace the function of whatever essential system was poisoned by cyanide, and also whether the characteristic reduction potential of the dye is the determining factor in its utility. In each case where a dye was used, a control experiment with the dye alone was made to determine whether the dye in question was toxic at the concentration in which it was used. The indophenols, catachol, and rose indulin were found to be toxic at concentrations of 0.005 per cent. Similar results were obtained with sea urchin eggs. NaCN, 0.0002 molal, delayed the first cleavage a half hour when the eggs were placed in the test solution 20 minutes after insemination; but even in 0.001 molal cyanide the eggs ultimately developed to an advanced stage.

Table XXIII is a summary of these experiments. The results with the toxic dyes are not included. A plus sign indicates that a large fraction of the eggs had completed the first cleavage at the time of inspection. According to Newby (1932), when the egg reaches the sixty-four-cell stage, cilia develop on the prototroch and start to beat, causing the egg to rotate. This rotary motion was taken as a criterion of advanced development. In Table XIII a plus sign in the column headed "movement" indicates that the eggs were rotating at the time of inspection.

From Table XXIII it appears that indigo disulfonate, indigo tetrasulfonate and methylene blue have contributed to overcoming the inhibition due to cyanide, while 2, 4-dinitrophenol, indigo monosulfonate and anthroquinone sulfonate are ineffective. The reduction

Table XXIII.

Effect of cyanide and cyanide plus dye on time of first cleavage in fertilized eggs.

Solution	Inspected for cleavage			Movement (several hrs)
	65 min.	85 min.	120 min.	
1. Sea water	+	+	+	+
2. m. dinitrophenol (0.001 per cent)	-	+	+	+
3. NaCN and m-dinitrophenol (0.001 per cent)	-	-	-	-
4. NaCN	-	-	+	-
5. Sea water	+	+	+	+
6. Anthroquinone sulfonate	+	+	+	+
7. NaCN and anthroquinone sulfonate	-	-	-	-
8. Indigo disulfonate	+	+	+	+
9. NaCN and indigo disulfonate	-	-	+	+
10. Sea water	+	+	+	+
11. Indigo tetrasulfonate	+	+	+	+
12. NaCN and indigo tetrasulfonate	-	-	+	+
13. Methylene blue	+	+	+	+
14. NaCN and methylene blue	-	+	+	-
15. Indigo monosulfonate	-	+	+	+
16. NaCN and indigo monosulfonate	-	-	-	-

Urechis caupo. Eggs inseminated in large dish. Distributed among dishes 5 minutes later (about 100 per dish). Final concentration of NaCN when present is 0.001 molal; final concentration of dye is 0.005 per cent, except where otherwise indicated.

potentials (E'_o) of these dyes at pH 8.0 are:

Anthroquinone B-sulfonate,	- 0.278 v.
Indigo monosulfonate,	- 0.205
Indigo disulfonate,	- 0.167
Indigo tetrasulfonate,	- 0.083
Methylene blue,	- 0.020
2, 4-dinitrophenol	+ 0.4 (+)

The effective dyes range between - 0.17 and - 0.02 volts; there is clearly an upper and a lower limit.

Discussion

The observations described above are not the first to be reported which have failed to confirm Hammett's conclusion that sulfhydryl is the key group in cell division. Morgulis and Green (1931) failed to obtain significant stimulation of regeneration with sulfhydryl in the worm Podarke obscura. These authors also pointed out that the effects which Hammett obtained with Paramecia and with mitotic counts on root tips are insignificant. Gaunt (1931), working with the eggs of the snails Physa and Limnae, found that cysteine as well as alanine retarded development as judged by hatching time. In other words the apparent stimulation by cysteine in Hammett's experiments may have been really a lesser degree of inhibition by cysteine than by the amino acid which was used as a control.

The experiments described above provide further evidence against the hypothesis that -SH is the natural growth hormone. First, the addition of SH groups did not stimulate cell division and secondly, removal of SH groups by iodoacetate (Lohmann, 1933, and Schroeder, Woodward, and Platt, 1933) left the rate of cell division unchanged.

The reported stimulation of cell division with -SH compounds has always been the result of the addition of a small amount of -SH to a system already containing a relatively large amount of this group. Destruction of a portion of the existing -SH groups should then have produced an opposite and much larger effect.

Further, the observations that cell division proceeds at an undiminished rate in the absence of carbohydrate metabolism, as shown by the fluoride and iodoacetate experiments, indicate that carbohydrate metabolism is not essential for cell division. It may be argued that carbohydrate metabolism may be very different in these eggs as compared with yeast or muscle. The above experiments leave this unanswered.

The experiments with dyes on cyanide inhibited eggs do suggest that some special type of metabolism is concerned in cell division. Himwich and his co-workers (1933) have found that methylene blue lowers the R.Q. of excised tissue and of the intact animal. In the presence of both cyanide and methylene blue the R.Q. is markedly depressed, indicating that a different kind of metabolism is taking place. Thus, it seems that addition of a dye with appropriate reduction potential to cyanide inhibited eggs restores an essential process which allows division to proceed, even though at a somewhat slower rate than that found in eggs in a normal environment. Rapkine (1929) obtained similar results in an analogous experiment in which he found that sea urchin eggs can develop in the absence of oxygen if methylene blue is present.

The energy requirements for the increase in surface which takes place in cell division is very small compared to the total respiration, as the following computation shows. The surface tension

of the water-air interface is 72.75 dynes/cm. at 20° C. The measurements of Harvey (1931) and Cole (1932) indicate that the tensions in the cell surface are much less than this, of the order of 1.0 to 2.0 dynes/cm. The formation of 1 sq. cm. of new water-air surface requires 72.75 dyne centimeters of work, or approximately 1.74×10^{-6} calories. An increase of surface of 10^6 sq. cm. would require only 1.74 gm. calories. If the volume of a chick embryo is taken as 50 cc., and it is assumed that it is a solid mass of spherical cells whose average volume is one-tenth that of a human red blood cell, their total surface would be 2.4×10^5 sq. cm., which is certainly a maximum value. The energy used in basal metabolism during the development of the embryo is about 23,000 calories (Needham, 1931). It is clear that measurements of total oxygen consumption and of the over all respiratory quotient during cell division cannot be resolved to yield the component responsible for cell division.

(The experimental work described in this section has been published in the Jour. Cell. and Comp. Physiol. 4:127 (1933)).

3. Mediators.

A mediator may be defined as a substance which takes part in two reactions, in one of which it undergoes a change in which its free energy content increases and in the other in which it returns to its initial state with a corresponding decrease in its free energy content. The simplest example is a substance which is alternately reduced and oxidized as illustrated in the following equations:

$$A H_2 + M \rightarrow A + M H_2 \qquad (1)$$

$$B + MH_2 \longrightarrow BH_2 + M \qquad (2)$$

where M and MH_2 represent oxidized and reduced mediator respectively. Either or both of these reactions may require enzymes in order to proceed at a reasonable rate. The sum of these reactions is:

$$AH_2 + B \longrightarrow BH_2 + A \qquad (3)$$

The mediator is a catalyst in the sense that its presence facilitates the occurrence of a reaction which results in no permanent change in the mediator.

In order that both reaction (1) and (2) may occur, the free energy change under the experimental conditions of concentration, temperature, etc., must be negative in each case. It follows that the free energy decrease for the dehydrogenation of AH_2 must be greater than that for the dehydrogenation of MH_2, and the dehydrogenation of MH_2 must involve a greater free energy decrease than the dehydrogenation of BH_2. This relationship can be stated algebraically as follows:

$$-\Delta F_{(AH_2 \rightarrow A)} > -\Delta F_{(MH_2 \rightarrow M)} > -\Delta F_{(BH_2 \rightarrow B)}$$

In other words, the oxidation potential of the mediator must lie between the potentials of the other two pairs of compounds.

Since the oxidation potential in any reversible system is a function of the ratio of the concentrations of the oxidized and reduced substance, any reversible oxidation-reduction system should be capable of acting as mediator in any reaction. However, in order for a reaction of this kind to proceed at an appreciable rate, the concentrations of both the oxidized and reduced forms of the mediator

must be appreciable. This limitation imposes a certain degree of specificity in a mediator with reference to the reactions in which it may function.

One of the conditions which seems to make mediators necessary is the separation of the enzyme centers where reactions (1) and (2) above can take place. The mediator supplies the mechanism whereby the transfer of electrons takes place from the locus of the first reaction to that at which the second reaction may occur (Borsook and Schott, 1933; and Borsook and Keighley, 1933).

The whole of intracellular metabolism may be pictured as a vast complex of reactions, requiring in some cases mediators as well as enzymes. The reactions involving mediators depend for their continuation on the presence of both the oxidized and reduced forms, and likewise the activity of certain enzymes depends on the state of oxidation of certain mediators. This picture gives a hint of one of the ways in which the many chemical processes of metabolism are coordinated to meet changing conditions. This conception that enzymes may be activated and inactivated by oxidation-reduction systems has appeared from a number of quarters in the last year or so and a summary of this work is presented in the next section.

It should be pointed out that in a given heterogeneous system in which several mediators are present, reactions may be in progress which involve two or more of the mediators. The reaction rates may be such that a dynamic steady state exists in which these mediators are present in finite concentrations in both their oxidized and reduced forms. Such a heterogeneous system may be considered to have simultaneously several oxidation potentials, one with respect to each of the mediators present in both forms.

The above concept of the manner in which oxidizable-reducible substances control the processes in living material is a useful working hypothesis. It explains simply the results of experiments like those of Himwich et al. (1933) with methylene blue and Hall, Field, and Sahyun (1933) with dinitrophenol. With several different kinds of tissue Himwich observed that the addition of methylene blue had a marked effect in decreasing the respiratory quotient. The explanation is simply that, since the dye is reversibly oxidized and reduced, its addition to the cell supplies the cell with an abnormal amount of a certain mediator and therefore changes the balance of processes in such a way that reactions using oxygen but producing water instead of carbon dioxide are more active, while the CO_2 producing reactions become relatively slower. Other workers have observed in similar experiments with methylene blue an increase in oxygen consumption. The above hypothesis also explains the observations of Field et al. that dinitrophenol increases oxygen consumption and decreases the respiratory quotient.

4. Function of sulfhydryl substances.

Although disulfides appear not to be reduced at an inert metal electrode, they are readily reduced by living tissues, tissue extracts and tisue residues (Hopkins and Elliott, 1931). In the case of liver this reducing mechanism is destroyed by heating the tissue to 50° C. There remains after heating to 100° another mechanism which can slowly reduce the disulfide, which is not extracted from the tissue even by several washings with hot water. This was called by Hopkins the "thermostable tissue residue". The mechanism

of this reduction is not understood, but it is possible that one of the agencies responsible is the -SH group of the protein. The thermostable residue gives a positive nitroprusside test and this reaction may be made to disappear by aeration in the presence of added glutathione. In such an experiment the oxygen uptake is several fold greater than would be predicted on the basis of the sulfhydryl groups present in the protein and the added glutathione.

There are several other systems capable of reducing oxidized glutathione. Hopkins (1925) showed that unsaturated fats and fatty acids, when aerated with reduced glutathione, became oxidized. These results imply the alternate oxidation of GSH by oxygen and its reduction by the fat or fatty acid. In the course of some experiments with the glucose dehydrogenase of Harrison (1933) it was found that the coenzyme preparations were able to reduce cystine in the absence of oxygen. Meldrum (1932) found that oxidized glutahtione is reduced in intact erythrocytes, especially in the presence of added glucose or other hexose.

This reversibility of glutathione in vivo satisfies the first requirement of a mediator. It may be assumed with some justification that the potential of glutathione is approximately the same as that derived above for cysteine. On this basis we may calculate the potential due to glutathione in liver tissue as an example. The concentration of -SH glutathione is approximately 200 milligrams per 100 grams of tissue, or 0.0065 mols per 1000 grams. The -SS- glutathione may be taken as about one tenth this amount, or 0.00065 mols per 1000 grams. The cysteine potential at these concentrations and at pH 7.0 is -0.390 volts. The corresponding free energy change is + 17,980 calories. Oxidized glutathione can serve as hydrogen

acceptor for "half reactions" in which the free energy decrease is greater than this figure. As examples of such reactions, in which there is a sufficient free energy change, formic acid to bicarbonate ion, acetaldehyde to acetic acid, and glucose to gluconic acid may be submitted.

5. Relation of sulfhydryl substances to enzymes.

These intracellular oxidation-reduction systems may also possess another function--that of activating or inactivating enzymes, according to the physiological state of the whole cell, in the following manner: assuming that a given enzyme is active only when a certain labile group in the molecule is in an oxidized state, and that this group is subject to reduction or oxidation by a mediator system, whether this enzyme is active or not will depend on whether the mediator is chiefly in the oxidized or in the reduced state. Thus the speed of the enzymatic reaction, which does not necessarily involve oxidation or reduction processes, but which is proportional to the amount of active enzyme, is determined by the state of oxidation of the mediator system. This conception has appeared from a number of quarters in the last year or so and a summary of this work is presented below.

Salaskin and Solowjew (1931) have shown that liver arginase which has been inactivated by aeration is reactivated by the addition of cysteine in the absence of oxygen. Edlbacher et al. (1932) was able to activate arginase preparations with cysteine at pH 9.4 , but found this substance inhibitory at neutrality.

Willstätter and Grassmann (1924) showed that papain could be activated by HCN. Maschmann and Helmert (1933 and 1934) showed that papain is activated by many sulfhydryl substances, different ones producing different degrees of activation. In these experiments and the ones described below with cathepsin, the effects of glutathione, cysteine, and β-thiolactic acid were always approximately equal and generally different from those of the α-sulfhydryl acids tested. Ascorbic acid alone was inhibitory, but ascorbic acid with iron led to activation. Similar experiments by the same investigators on cathepsin showed that glutathione, cysteine, and β-thiolactic acid activate the enzyme while α-sulfhydryl acids are without effect or inhibitory.

Bersin (1933) obtained striking results with papain preparations which had been inactivated by oxidation. He found that activation could be produced with glutathione, certain organic arsenic derivatives, and irradiation with ultraviolet light. Ultraviolet light was found to reduce ββ'-dithiodilactic acid in solution. It seems likely therefore that the labile groups in papain, the oxidation of which results in inactivation, are sulfhydryl.

Hellerman, Perkins, and Clark (1933) have investigated the activation and inactivation of urease. Their evidence indicates that the inactivation of urease by oxygen or by oxygen with metals is due to an oxidation of -SH groups in the emzyme molecule. Such inactivated preparations are almost completely reactivated by H_2S or HCN and partially reactivated by cysteine. H_2S is a stronger reducing agent than cysteine by about 50 millivolts.

The role of glutathione as a coenzyme has been demonstrated clearly by Jowett and Quastel (1933) in the transformation of methyl glyoxal into lactic acid by glyoxalase. This reaction involves no oxidation or reduction. The evidence indicates that glutathione forms a compound with methyl glyoxal which is subsequently split by the enzyme to give lactic acid and unchanged glutathione.

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APPENDIX I.

Calculation of heats of combustion of sulfur compounds

The heat evolved in the combustion calorimeter, corrected for the heat interchange with the environment was calculated from the time - temperature record of the run by Dickinson's (1914) method. The calculation from this heat quantity of the heat of combustion of the pure substance at constant temperature to give gaseous CO_2 and N_2, liquid H_2O and solid sulfur is presented in the following paragraphs.

The heat for the actual combustion process ($-\Delta u_B$) taking place at a constant temperature t_H is given by the expression

$$-\Delta u_B = S_B(t_2 - t_1) + S_I(t_H - t_1) + S_F(t_2 - t_H) + (n\Delta H)_{H_2O} \quad (1)$$

where S_B is the effective heat capacity of the calorimetric system, $t_2 - t_1$ is the observed temperature rise, corrected for heat interchange with the environment, S_I the heat capacity of the bomb contents before the combustion, S_F the heat capacity of the bomb contents at the end of the run, and $(n\Delta H)_{H_2O}$ the heat absorbed in the vaporization of sufficient water to saturate the bomb at the partial pressure of water vapor over sulfuric acid of the concentration formed in the combustion. The quantity $S_B(t_2 - t_1)$ is calculated from the time - temperature record as noted in the first paragraph. t_H is the standard temperature, 25° C. S_I and S_F are calculated from a knowledge of the initial and final contents of the bomb respectively.

Since the compound, with oil when necessary, the filter paper fuse, and oxygen and nitrogen gas were the only materials

initially present in the bomb in the combustions of sulfur containing compounds, the quantity S_I is given by the expression

$$S_I = 5.01(n_{O_2} + n_{N_2})_I + \Sigma m C_p \qquad (2)$$

where n_{O_2} and n_{N_2} are the mols of oxygen and nitrogen respectively, and $\Sigma m C_p$ is the tital heat capacity of the material to be burned. The term S_F is given by the expression

$$S_F = 5.01(n_{O_2} + n_{N_2})_F + (m C_p)_{H_2SO_4} + 6.93 n_{CO_2} \qquad (3)$$

where $(n_{O_2} + n_{N_2})_F$ is the number of mols of nitrogen and oxygen after the combustion, $(m C_p)_{H_2SO_4}$ is the number of grams of aqueous sulfuric acid formed multiplied by the specific heat of a solution of the same concentration, and n_{CO_2} is the number of mols of gaseous CO_2 at the end of the combustion. The calculation of S_F is simplified by first calculating the coefficients a, b, c, d, and e for the effective formula $C_aH_bO_cN_dS_e$ of the total combustible material in the bomb before ignition (substance + oil + cellulose).

The part of the total heat evolution $(-\Delta u_B)$ due to the combustion in the bomb of the substance under investigation to give solid sulfur, gaseous CO_2 and liquid H_2O is

$$Q_v = -\Delta u_B - \Delta H_{(oil)} - \Delta H_{(cell)} - \Delta H_{EIt} - \Delta H_{NO} - \Delta H_{H_2SO_4}$$

where $\Delta H_{(oil)}$ and $\Delta H_{(cell)}$ are the heat evolution due to the combustion of the quantities of oil and cellulose respectively used in the combustion, ΔH_{EIt} is the electrical energy input, in calories

to the firing loop, ΔH_{NO} is the heat evolved in the formation of the nitrogen oxides and acids subsequently found by analysis, and $\Delta H_{H_2SO_4}$ is the heat evolved in the formation from sulfur, oxygen and water of the calculated quantity of H_2SO_4 at the concentration calculated for the materials burned. The cellulose fuses used were cut from ashless filter paper and stored over $CaCl_2 \cdot 6H_2O$. This salt gives a humidity approximating that in the room and facilitated accurate weighings by reducing the loss or gain of water during exposure of the fuse to the air. Combustion of the same paper stored for several days in this atmosphere gave the average value 3961 calories per gram. The oil used as an auxiliary substance was found to contain 0.2 per cent sulfur. The empirical formula was assumed to be CH_2 for calculations involving the composition of the oil.

A calculation of the results from one of the cystine combustions is submitted below to show the magnitude of the quantities involved and to give a convenient form for performing such calculations.

$$S_B(t_2 - t_1) = 8882.6 \text{ cal.}$$

$$t_H - t_1 = 1.62^\circ \text{ C.}$$

$$t_2 - t_H = 1.18^\circ \text{ C.}$$

$$(n_{O_2} + n_{N_2})_I = 0.477 \text{ mols}$$

$$C_p \text{ (cystine)} = 0.267 \text{ cal/gm}$$

$$m \text{ (cystine)} = 1.8543 \text{ gm.}$$

$$C_p \text{ (oil)} = 0.495 \text{ cal/gm}$$

$$m \text{ (oil)} = 0.1085 \text{ gm.}$$

$$\Sigma\, m\, C_p = 0.549 \text{ cal/}^\circ$$

$$S_I = 2.94 \text{ cal/}^\circ$$

$$S_I(t_H - t_1) = 4.76 \text{ cal.}$$

m (cellulose) = 0.0050 gm.

For the formula $C_aH_bO_cN_dS_e$

$a = 0.0542$

$b = 0.1084$

$c = 0.0311$

$d = 0.0154$

$e = 0.01545$

$m_{(H_2SO_4)} = 2.21$ gm.

$N\left(\frac{H2O}{H_2SO_4}\right) = 2.52$

$m\ C_{p(H_2SO_4)} = 1.21$ cal/°

$(n_{O_2} + n_{N_2})_F = 0.396$ mols

$n_{CO_2} = 0.0542$ mols

$S_F = 3.57$ cal/°

$S_F(t_2 - t_1) = 4.22$ cal.

$(n\ \Delta H)_{H2O} = 0.31$ cal

$-\Delta u_B = 8892.0$ cal.

$\Delta H_{(cell)} = 19.8$ cal.

$\Delta H_{(oil)} = 1176.1$ cal.

$\Delta H_{(EIt)} = 1.4$ cal.

N_2O_4 found in gas = 0.000065 mols

HNO_2 found in bomb = 0.00043 mols

HNO_3 found in bomb = 0.0000

$\Delta H_{(NO)} = -2.4$ cal.

$\Delta H_{(H_2SO_4)} = 2116.0$ cal.

$Q_v = 5581.0$ cal.

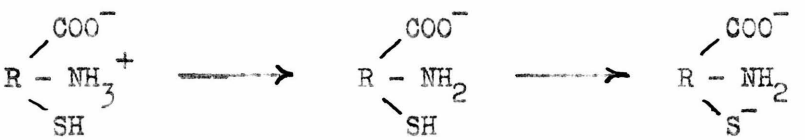
$= 3009.8$ cal/gm

APPENDIX II.

Dissociation Constants

Tables XXIV to XXIX show the titration data for l-cysteine, β thiolactic acid, and ββ'dithiodilactic acid, and the calculation from this data of the dissociation constants involved in the titrations. The method of calculating the overlapping constants of l-cysteine and ββ' dithiodilactic acid is described in detail in the following paragraphs.

It is convenient to consider the cysteine zwitter ion as a dibasic acid dissociating in alkaline solution in two steps to give two hydrogen ions, as illustrated in the following diagram



These forms of the molecule are designated by x, y, and z respectively, and their concentrations by the symbols (x), (y), and (z). The total concentration of cysteine (t) is the sum of the concentrations of these three forms.

$$(t) = (x) + (y) + (z) \qquad (1)$$

The dissociation constants are given by the expressions

$$\frac{(y)[H^+]}{(x)} = K_2' \qquad (2)$$

$$\text{and} \quad \frac{(z)[H^+]}{(y)} = K_3' \qquad (3)$$

where $[H^+]$ is the hydrogen ion activity. Simultaneous solution of equations (1), (2), and (3) gives the following expressions for (y) and (z)

$$(y) = \frac{(t)\ K_2'\ (H^+)}{(H^+)^2 + K_2'(H^+) + K_2'K_3'} \qquad (4)$$

and

$$(z) = \frac{(t)K_2'K_3'}{(H^+)^2 + K_2'(H^+) + K_2'K_3'} \qquad (5)$$

The ionic strength of the solution is

$$\mu = \frac{1}{2}\left\{(y) + 3.3\,(z) + b + 2\,c + (OH^-)\right\} \qquad (6)$$

where b is the concentration of base added in the titration, c is the concentration of KCl and (OH^-) the hydroxyl ion concentration. A satisfactory approximation of μ is obtained by substituting in equation (6) the approximate values (y') and (z') calculated from equations (4) and (5) using the approximate values of K_2' and K_3' determined from a plot of the titration data, and substituting for the hydroxyl ion concentration (OH^-) its activity $[OH^-]$ observed during the titration.

The hydroxyl ion activity coefficient is calculated from the above values of μ by the Debye - Hückle equation:

$$-\log \gamma_i = \frac{0.5\ v_i^2 \sqrt{\mu}}{1 + 0.33\sqrt{\mu}} \qquad (7)$$

The hydroxyl ion concentration (OH^-) is simply the observed activity $[OH^-]$ divided by this activity coefficient. Accurate values of (y) and (z) are calculated according to the equations below:

$$(y) = b - (OH^-) - 2(z') \qquad (8)$$

and

$$(z) = \frac{1}{2}\left\{b - (OH^-) - (y')\right\} \qquad (9)$$

The uncertainty of (z') introduces an error into (y) which is negligible in the region where (z') is small, and similarly the error in (z) caused by the uncertainty in (y') is negligible where (y') is small. The quantity (x) is obtained by difference (equation (1)). The values of K_2' and K_3' are most simply calculated from the logarithmic forms of equations (2) and (3):

$$pK_2' = pH - \log (y) + \log (x) \qquad (10)$$

and

$$pK_3' = pH - \log (z) + \log (y) \qquad (11)$$

In case the calculated values pK_2' and pK_3' show poor agreement with the values assumed for the calculation of (y') and (z') it is necessary to repeat the calculation of the latter quantities using better values of K_2' and K_3'.

The true dissociation constants are

$$pK_2 = pK_2' - \log \gamma_y \qquad (12)$$

and

$$pK_3 = pK_3' - \log \gamma_z + \log \gamma_y \qquad (13)$$

The term $-\log \gamma_z$ is calculated from the Debye - Hückle equation as noted on page 21, replacing v_i^2 by 3.3, its value for "long" ions having about 7 A° separation between the charges.

The assumptions implied in the above discussions are: (a) the zwitter ion makes no appreciable contribution to the ionic strength of the solution; (b) the zwitter ion has an activity coefficient of unity; and (c) the activity coefficients of the other ions are determined with no appreciable error by the simplified Debye - Hückle equation. The ionization constant of water at 25° C. was taken as 1.005×10^{-14}.

The calculation of the overlapping constants of $\beta\beta'$ dithiodilactic acid is accomplished in an analogous manner. The data and calculations are shown in table XXIX. x, y, and z refer to the undissociated molecule, the anion, and the divalent anion respectively. Since this titration takes place in acid solution, equation (6) becomes

$$\mu = \frac{1}{2}\left\{(y) + 2.7\,z + (H^+) + b\right\} \qquad (14)$$

where the coefficient 2.7 replaces v_i^2 for this "long" ion in which the separation of the charges is taken as equal to 12 A°. As shown by the results, these constants lie very near to one another, making their estimation from a graphical representation of the titration data difficult, and making two approximations necessary before the calculations given in the table were made.

In tables XXVI and XXVII giving the data and calculations for titrations of the carboxyl group of cysteine, (w) represents the concentration of the positive ion $R\begin{smallmatrix}\diagup\ \mathrm{COOH}\\ -\ \mathrm{NH_3^+}\\ \diagdown\ \mathrm{SH}\end{smallmatrix}$ and (x) that of the zwitter ion. In table XXVIII, (w) is the concentration of the undissociated acid $R\begin{smallmatrix}\diagup\mathrm{COOH}\\ \diagdown\mathrm{SH}\end{smallmatrix}$, (x) the concentration of the anion $R\begin{smallmatrix}\diagup\mathrm{COO^-}\\ \diagdown\mathrm{SH}\end{smallmatrix}$ and (z) that of the divalent anion $R\begin{smallmatrix}\diagup\mathrm{COO^-}\\ \diagdown\mathrm{S^-}\end{smallmatrix}$.

The EMF in tables XXIV to XXVIII is that of the saturated calomel half cell against the hydrogen electrode corrected to a hydrogen pressure of one atmosphere.

Table XXIV.

l-cysteine: titration of NH_3^+ and SH groups. (Dissolved 0.1176 gm. pure cysteine in 25 cc 0.208 m KCl; titrated with 0.07748 n NaOH; hydrogen electrode; 25° C.)

NaOH added cc	Total vol cc	EMF	pH	Conc. cysteine (all forms) (t)	OH^- activity	(y')	(z')	(b)	μ	$-\log_{OH}$	(OH^-) conc.	(y)	(z)	(x)	pK'_2	pK'_3	$-\log_z$	pK_2	pK_3
0.00	25.0	.4999	4.297	.0388	$.0_9200$	----	----	----	.208	.198	0_9316	----	----	----	----			----	
1.98	27.0	.6872	7.464	.0360	$.0_6261$	$.0_2486$	----	.00568	.198	.195	0_6409	0_2567	----	0314	8.21			8.41	
4.01	29.0	.7122	7.886	.0335	$.0_6773$	.0106	----	.01071	.189	.191	0_5120	01064	----	0228	8.22			8.41	
6.01	31.1	.7296	8.181	.0313	$.0_5152$	.0149	$.0_31$	.01502	.182	.186	0_5233	01484	----	0164	8.22			8.41	
8.01	33.0	.7467	8.470	.0294	$.0_5296$	.0187	$.0_32$	.01881	.176	.184	0_5451	01859	----	0107	8.23			8.41	
10.04	35.0	.7656	8.789	.0277	$.0_5618$	.0214	$.0_35$	.02223	.171	.182	0_5939	02121	----	0050	8.16			8.34	
12.01	37.0	.7899	9.200	.0262	$.0_5159$	.0224	$.0_315$	.02515	.167	.180	0_4240	02213	0_2150	0026	8.27	10.37	-.720	8.45	10.91
14.03	39.0	.8179	9.674	.0249	$.0_4475$	.0202	$.0_238$	.02787	.163	.178	0_4715	02012	0_2384			10.39	-.713		10.92
16.09	41.1	.8386	10.024	.0236	$.0_3106$	.0165	$.0_270$	.03033	.161	.177	0_3159	01627	0_2695			10.39	-.708		10.92
18.07	43.1	.8550	10.301	.0225	$.0_3200$	.0124	.0100	.03249	.159	.176	0_3300	01229	0_2995			10.39	-.704		10.91
20.05	45.1	.8703	10.560	.0215	$.0_3365$	$.0_287$	.0127	.03445	.157	.175	0_3546	0_2850	01270			10.39	-.700		10.91
22.08	47.1	.8865	10.834	.0206	$.0_3686$	$.0_256$	.0151	.03632	.156	.174	0_2102	0_251	0150			10.36	-.699		10.89
24.01	49.0	.9026	11.106	.0198	$.0_2129$	$.0_233$	.0166	.03797	.154	.174	0_2193	0_228	01662			10.33	-.695		10.86
26.07	51.1	.9184	11.373	.0190	$.0_2237$	$.0_218$	.0172	.03953	.152	.173	0_2353	0_216	01720			10.34	-.692		10.86

approximations: $pK'_2 = 8.22$
$pK'_3 = 10.40$

Table XXV

l-cysteine: titration of NH_3^+ and SH groups. (Dissolved 0.1635 gm. cysteine in 25 cc. H_2O; titrated with 0.07748 n NaOH; hydrogen electrode; 25°C.

NaOH added cc	Total vol cc	EMF	pH	Conc. cysteine (all forms). (t)	OH^- activity	(y')	(z')	(b)	μ	-log $_{OH}$	(OH^-) conc.	(y)	(z)	(x)	pK'_2	pK'_3	-log $_z$	pK_2	pK_3
0.00	25.0	.4971	4.249	.05400	0_9178	----	----	----	----	.0008	----	----		----	----			----	
1.00	26.0	.6590	6.987	.05192	0_7975	$0_2$2405	----	$0_2$298	$0_2$269	.0255	----	$0_2$2979		04894	8.20			8.23	
2.00	27.0	.6805	7.350	.05000	$0_6$225	$0_2$538	$0_5$140	$0_2$574	$0_2$557	.0364	----	$0_2$5738		04426	8.24			8.28	
3.00	28.0	.6940	7.579	.04821	$0_6$388	$0_2$782	$0_5$910	$0_2$830	$0_2$808	.0436	----	$0_2$8282		03993	8.26			8.30	
5.00	30.0	.7123	7.888	.04500	$0_6$789	01271	$0_4$302	01291	0129	.0548	----	01285		03215	8.29			8.34	
7.09	32.1	.7258	8.116	.04206	$0_5$135	01689	$0_4$683	01711	0171	.0633	----	01697		02509	8.29			8.35	
9.03	34.0	.7379	8.321	.03971	$0_5$216	02055	$0_3$134	02058	0208	.0698	$0_5$2	02041		01930	8.30			8.37	
11.03	36.0	.7481	8.494	.03750	$0_5$344	0235	$0_3$243	02374	0241	.0742	$0_5$4	02325		01425	8.28			8.35	
13.01	38.0	.7629	8.744	.03553	$0_5$577	0260	$0_3$452	02653	0271	.0787	$0_5$7	02562	00026	00965	8.32	10.74	.313	8.40	10.97
15.04	40.0	.7797	9.028	.03375	$0_4$110	0283	$0_3$940	02912	0303	.0823	$0_4$13	02724	00041	00610	8.38	10.85	.329	8.46	11.10
17.04	42.0	.7998	9.368	.03214	$0_4$240	0277	$0_3$200	03143	0329	.0858	$0_4$29	02740	00185	00289	8.39	10.54	.342	8.48	10.80
19.04	44.0	.8175	9.667	.03068	$0_4$528	0257	$0_2$409	03352	0365	.0904	$0_4$65	02527	00388	00153	8.45	10.48	.360	8.54	10.75
21.09	46.1	.8370	9.997	.02928	$0_3$100	0221	$0_2$675	03544	0400	.0940	$0_3$124	02182	00661	00085		10.52	.376		10.80
23.11	48.1	.8509	10.232	.02807	$0_3$171	01845	$0_2$940	03723	0435	.0972	$0_3$214	01822	00929	00056		10.53	.390		10.82
25.06	50.1	.8621	10.421	.02695	$0_3$264	01496	01183	03876	0466	.1000	$0_3$333	01477	01174			10.52	.399		10.82
27.06	52.1	.8729	10.604	.02591	$0_3$405	01162	01418	04025	0495	.1035	$0_3$514	01138	01406			10.55	.413		10.86
29.15	54.2	.8842	10.795	.02491	$0_3$626	$0_2$870	01620	04168	0523	.1070	$0_3$801	$0_2$848	01609			10.52	.426		10.84
31.10	56.1	.8955	10.986	.02406	$0_3$971	$0_2$620	01784	04296	0546	.1088	$0_2$123	$0_2$605	01777			10.52	.433		10.84
33.05	58.1	.9067	11.175	.02324	$0_2$150	$0_2$416	01904	04408	0564	.1103	$0_2$193	$0_2$407	01900			10.51	.442		10.84

approximations: $pK'_2 = 8.30$

$pK'_3 = 10.52$

Table XXVI

l-cysteine: titration of COOH group (Dissolved 0.1035 gm. cysteine in 25 cc 0.07496 n. HCl; added 15.2 cc H_2O, titrated with 0.07748 n NaOH; hydrogen electrode; 25° C.)

cc NaOH added	Total vol	EMF	pH	conc. cysteine (all forms) (t)	Ionic strength (μ)	(H^+) conc.	(w)	(x)	pK'	$\log \gamma_{COOH}$	pK
0.00	40.2	.3408	1.606	.02126	.0466	.02808	.01854	.00272	(2.44)	-0.06	(2.38)
5.08	45.3	.3477	1.723	.01886	.0414	.02133	.01134	.00752	1.90	-0.06	1.84
10.07	50.3	.3585	1.906	.01699	.0373	.01399	.00776	.00923	1.83	-0.06	1.77
15.16	55.4	.3735	2.159	.01542	.0338	.00778	.00485	.01057	1.82	-0.06	1.76
20.05	60.3	.3980	2.574	.01417	.0311	.00299	.00303	.0114	(2.01)	-0.06	(1.95)
22.09	62.3	.4203	2.951	.01372	.0301	.00167	.00094	.01205	1.84	-0.06	1.78
23.08	63.3	.4434	3.341	.01350	.0296	.00051	.00085	.01265	(2.17	-0.06	(2.11)

Table XXVII

l-cysteine: titration of COOH group. (Dissolved 0.1260 gm. cysteine in 25 cc 0.208 m KCl; titrated with 0.07496 n. HCL; hydrogen electrode; 25° C.)

cc HCl added.	Total vol	EMF	pH	conc. cysteine (all forms) (t)	ionic strength (μ)	(H^+) conc.	(w)	(x)	pK'_1	$-\log\sqrt{}_w$	pK_1
1.00	26.0	.4190	2.929	.0400	.203	.00151	.00137	.0386	1.48	.13	(1.35)
2.00	27.0	.4158	2.874	.0385	.198	.00172	.00383	.0347	1.92	.12	1.80
4.00	29.0	.3967	2.552	.0359	.190	.00357	.00667	.0291	1.92	.12	1.80
5.02	30.0	.3903	2.443	.0347	.186	.00458	.00796	.0267	1.92	.12	1.80
6.01	31.0	.3853	2.359	.0335	.182	.00553	.00900	.0245	1.92	.12	1.80
7.03	32.0	.3805	2.278	.0321	.179	.00665	.00982	.0223	1.92	.12	1.80
8.10	33.1	.3763	2.207	.0314	.175	.00781	.01053	.0209	1.91	.12	1.79
9.01	34.0	.3731	2.153	.0306	.173	.00883	.01103	.0196	1.90	.12	1.78
10.02	35.0	.3700	2.100	.0297	.170	.00995	.01151	.0182	1.90	.12	1.78
11.08	36.1	.3672	2.053	.0288	.167	.01100	.01201	.0168	1.91	.12	1.79
12.06	37.1	.3646	2.009	.0280	.165	.01222	.01215	.0158	1.90	.12	1.78
13.09	38.1	.3624	1.972	.0273	.162	.01332	.01243	.0149	1.89	.12	1.77
14.07	39.1	.3602	1.934	.0266	.160	.01440	.01257	.0140	1.89	.11	1.78
16.09	41.1	.3567	1.875	.0253	.156	.01650	.01285	.0124	1.89	.11	1.78
18.05	43.1	.3537	1.825	.0241	.152	.01855	.01284	.0123	1.84	.11	(1.73)
20.09	45.1	.3510	1.779	.0231	.149	.0204	.01299	.0101	1.89	.11	1.78
22.10	47.1	.3488	1.742	.0221	.146	.0223	.01287	.0092	1.89	.11	1.78
24.01	49.0	.3467	1.706	.0212	.143	.0242	.01253	.0087	1.87	.11	(1.76)

Table XXVIII

β-thiolactic acid: titration of COOH and SH groups.
(Dissolved 0.206 gm. β thiolactic acid in 25 cc H_2O; titrated with 0.07748 n NaOH; hydrogen electrode; 25° C.)

cc NaOH added	Total vol	EMF	pH	conc. RSH (all forms) (t)	Ionic strength ( )	(H^+) conc.	(OH^-) conc.	(w)	(x)	(z)	pK_1'	pK_2'	$-\log \gamma_x$	$-\log \gamma_z$	pK_1	pK_2
0.00	25.0	.4140	2.844	.07280	..0014	.00015		.07265	.0015		4.53		.01		(4.54)	
1.09	26.1	.4310	3.132	.06973	.0033	.00008		.06642	.00331		4.43		.02		(4.45)	
2.07	27.1	.4428	3.331	.06716	.0060	.00005		.06119	.00597		4.34		.03		(4.37)	
3.97	29.0	.4589	3.603	.06276	.0106	.00003		.05212	.01064		4.29		.04		4.33	
6.07	31.1	.4713	3.813	.05852	.0151	.00002		.04338	.01514		4.27		.04		4.31	
8.02	33.0	.4812	3.980	.05515	.0188	.00001		.03631	.01884		4.27		.05		4.32	
10.04	35.0	.4900	4.129	.05200	.0222	.00001		.02976	.02224		4.26		.05		4.31	
11.98	37.0	.4984	4.271	.04919	.0251	.00001		.02409	.02510		4.25		.06		4.31	
14.04	39.0	.5072	4.420	.04667	.0279			.01878	.02789		4.25		.06		4.31	
16.07	41.1	.5170	4.586	.04428	.0303			.01399	.03029		4.25		.06		4.31	
18.04	43.0	.5278	4.768	.04233	.0325			.00982	.03251		4.25		.06		4.31	
20.07	45.1	.5425	5.017	.04035	.0345			.00587	.03448		4.25		.06		4.31	
21.99	47.0	.5660	5.414	.03872	.0363			.00247	.03625		4.25		.06		4.31	
22.51	47.5	.5788	5.631	.03832	.0367			.00160	.03672		4.27		.06		4.33	
23.08	48.1	.6012	6.010	.03784	.0372			.00066	.03718		4.26		.06		4.32	
23.58	48.6	.6990	7.663	.03745	.0397				.03731	.00014		10.09	.07	.32		(10.34)
24.08	49.1	.7258	8.573	.03707	.0386				.03614	.00093		10.16	.07	.32		(10.41)
25.48	50.5	.7883	9.173	.03604	.0411	.00001			.03300	.00304		10.21	.07	.33		10.47
27.00	52.0	.8051	9.457	.03500	.0436	.00003			.02980	.00520		10.22	.07	.34		10.49
29.02	54.0	.8200	9.709	.03370	.0468	.00006			.02582	.00788		10.22	.07	.35		10.50
31.02	56.0	.8306	9.888	.03250	.0496	.00009			.02217	.01033		10.22	.08	.36		10.51
33.02	58.0	.8400	10.047	.03138	.0524	.00013			.01868	.01270		10.21	.08	.37		10.51
35.04	60.0	.8480	10.183	.03033	.0549	.00018			.01559	.01474		10.21	.08	.38		10.51
37.04	62.0	.8560	10.318	.02935	.0572	.00025			.01266	.01669		10.20	.08	.39		10.51
39.07	64.1	.8640	10.453	.02839	.0593	.00034			.00980	.01850		10.18	.08	.40		10.50
41.06	66.1	.8750	10.639	.02753	.0612	.00052			.00745	.02008		10.21	.08	.40		10.53
43.05	68.1	.8842	10.795	.02673	.0630	.00075			.00523	.02150		10.18	.08	.41		10.51
45.07	70.1	.8948	10.974	.02596	.0647	.00113			.00324	.02272		10.13	.08	.42		10.46
47.04	72.0	.9054	11.153	.02528	.0662	.00171			.00165	.02363		10.01	.09	.42		(10.34)
48.06	73.1	.9110	11.248	.02490	.0667	.00213			.00099	.02391		9.87	.09	.42		(10.21)

Table XXIX

ββ'-dithiodilactic acid, first and second COOH groups. (Dissolved 0.0336 gm. ββ'-dithiodilactic acid in 50 cc H_2O; titrated with 0.07102 n NaOH; glass electrode; 25° C.)

cc NaOH added	Total vol	EMF	pH	Conc. RSSR (all forms) (t)	(b)	(y')	(z')	Ionic strength ( )	(H^+) conc.	(x)	(y)	(z)	pK'_4	pK'_5	$-\log_y$	$-\log_z$	pK_4	pK_5
0	50.0	.1529	3.39	.00320	0	.00063	.00002	.0003	.00041	.00252	.00096		3.81		.01		3.82	
0.14	50.1	.1565	3.46	.00319	.000199	.00071	.00002	.0003	.00035	.00238	.00107		3.81		.01		3.82	
0.41	50.4	.1660	3.62	.00317	.000579	.00092	.00004	.0005	.00024	.00209	.00135		3.81		.01		3.82	
0.86	50.9	.1810	3.87	.00314	.00120	.00130	.00010	.0009	.00014	.00161	.00172	.00015	3.84	5.02	.01	.04	3.85	5.05
1.55	51.6	.2005	4.20	.00310	.00215	.00173	.00027	.0016	.00007	.00096	.00206	.00035	3.87	5.02	.02	.05	3.89	5.05
2.07	52.1	.2136	4.43	.00307	.00282	.00187	.00050	.0022	.00004	.00060	.00209	.00057	3.89	5.02	.02	.06	3.91	5.06
2.60	52.6	.2270	4.66	.00304	.00351	.00181	.00083	.0030	.00002		.00202	.00088		5.02	.03	.07		5.06
2.93	52.9	.2349	4.79	.00302	.00394	.00170	.00105	.0035	.00002		.00179	.00108		5.01	.03	.08		5.06
3.59	53.6	.2561	5.16	.00298	.00476	.00120	.00174	.0048	.00001		.00125	.00172		5.02	.03	.09		5.08
4.21	54.2	.2957	5.83	.00295	.00552	.00038	.00256	.0062	---		.00039	.00253		5.02	.04	.10		5.08
4.71	54.7	.5542																

approximations: $pK'_4 = 3.75$

$pK'_5 = 5.02$

APPENDIX III.

Derivation of free energy and electrode equations

Given the free energy change (ΔF_1) for the reaction:

$$RSSR_{(s)} + H_{2(g,\ 1\ atm.)} \longrightarrow 2\ RSH_{(s\ or\ l)} \qquad (1)$$

the free energy change for the same reaction occurring in solution is derived below.

The solution of one mol of cysteine or cystine in an infinite quantity of its saturated solution is accompanied by a free energy change of zero. The free energy change for the reaction

$$H_{2(1\ atm.)} \longrightarrow 2\ H^{+}_{(1\ atm.)} + 2\ E^{-} \qquad (2)$$

is taken as zero. Therefore, the free energy change ΔF_1 for reaction (1) above is also the free energy change for the reaction

$$CSSC_{(sat.\ sol.)} + 2\ H^{+}_{(1m)} + 2\ E^{-} \longrightarrow 2\ CSH_{(sat.\ sol.)}$$

The concentration of the molecular species of cystine chosen as the reactant ($^{\pm}CSSC^{\pm}$) is evaluated from the solubility of cystine and its dissociation constants. The solubility of l-cystine at 25° C. is 0.000544 mols per 1000 grams of water, or 0.00542 mols per liter. In the saturated solution of cystine the approximate hydrogen ion concentration is obtained from the expression

$$\frac{(^{\pm}CSSC^{-})(H^{+})}{(^{\pm}CSSC^{\pm})} = K_6$$

or

$$\frac{c\gamma^2}{1-\gamma} = K_6 = 10^{-7.53}$$

where γ is the fraction of the zwitter ion ($^{\pm}CSSC^{\pm}$) dissociated to give the negative ion ($^{\pm}CSSC^{-}$), and c is the solubility of cystine. The solution of this expression gives the values

$$\gamma = 0.0037$$

and $$pH = 5.70.$$

At this pH the dissociation of the second amino group ($pK_7 = 9.30$) is negligible as is the association of the first ($pK_4 = 1.0$) and second ($pK_5 = 1.7$) carboxyl groups. The concentration of the zwitter ion is

$$c(1-\gamma) = 0.000540 \text{ mols/liter}$$

Similarly, for cysteine

$$\frac{(^{-}CSH)(H^{+})}{(^{\pm}CSH)} = K_2 = 10^{-8.36}$$

or $$\frac{c\gamma^2}{1-\gamma} = K_2$$

The solubility of cysteine is 0.2275 mols per liter at 25° C. Solution of this expression gives the values

$$\gamma = 0.00014$$

and $$pH = 4.5$$

The concentration of the zwitter ion product ($^{\pm}CSH$) is

$$c(1-\gamma) = 0.2275$$

The dissociation of the other groups of cysteine ($pK_1 = 1.79$) and ($pK_3 = 10.82$) introduces no appreciable correction to this concentration of the product, which may be taken as 0.228 mols per liter.

Assuming that these substances form perfect solutions, the free energy change accompanying the transfer of the reactant ($^{\pm}CSSC^{\pm}$) or the product ($^{\pm}CSH$) from its concentration in a saturated solution to a solution in which its concentration is one molal may be evaluated from the expression

$$\pm \Delta F = NRT \ln c$$

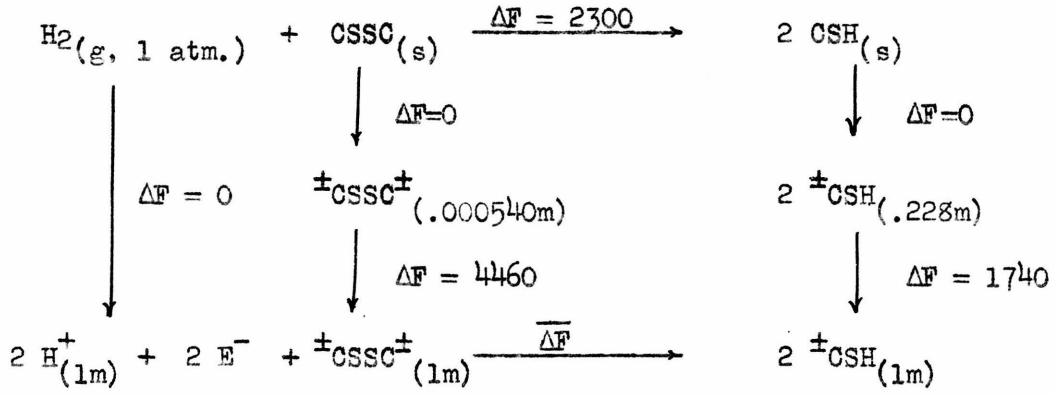
For the transfer of one mol of reactant ($^{\pm}CSSC^{\pm}$)

$$- \Delta F = - 4460 \text{ calories}$$

For the transfer of two mols of the product ($^{\pm}CSH$)

$$- \Delta F = - 1740 \text{ calories.}$$

The relationship of these quantities and the standard free energy change in solution $\overline{\Delta F}$ is shown in the following diagram:



Since the free energy change must be independent of the path,

$$\overline{\Delta F} = - 4460 + 2300 + 1700 = - 460 \text{ cal.}$$

The calculation of $\overline{\Delta F}$ for the $\beta\beta'$ dithiodilactic acid: β thiolactic acid transformation is quite analogous. The solubility of the reactant ("RSSR") is 0.00637 mols per liter. The concentration of the anion $RSSR^-$ in the saturated solution is obtained by the solution of the expression:

$$\frac{(RSSR^-)(H^+)}{(RSSR)} = K_4 = 10^{-3.85}$$

$$\text{or} \qquad \frac{c\gamma^2}{1-\gamma} = K_4$$

$$\text{from which} \qquad \gamma = 0.137$$

$$\text{and} \qquad pH = 3.06$$

The concentration of the anion $RSSR^-$ is 8.74×10^{-4}. The concentration of the reactant ($^-RSSR^-$) is obtained by solution of the expression

$$\frac{(^-RSSR^-)(H^+)}{(RSSR^-)} = K_5 = 10^{-5.06}$$

$$\text{from which} \qquad (^-RSSR^-) = 8.7 \times 10^{-6} \text{ mols/liter.}$$

The free energy change attending the transfer of one mol of this reactant from this concentration to a concentration of one molal is

$$-\Delta F = RT \ln c = 6920 \text{ cal.}$$

β thiolactic acid is miscible with water. It is necessary therefore to evaluate the free energy change for the transfer of pure β thiolactic acid into a solution in which the concentration of the anion product (^-RSH) is one molal. Assuming that this substance forms

perfect solutions, this free energy change is

$$-\Delta F = NRT \ln x$$

where x is the mol fraction of β thiolactic acid when the anion is present at a concentration of unity. Such a solution in pure water could never be realized, but the absence of data on solutions of RSH makes it necessary to evaluate the free energy change in this way.

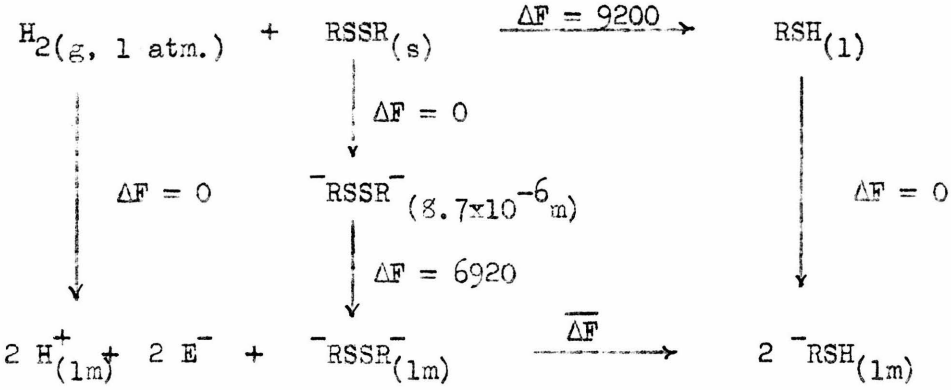
Since $$\frac{(\bar{R}SH)(H^+)}{(RSH)} = K_1 = 10^{-4.31}$$

and since $(\bar{R}SH)$ must be unity and hence (H^+) must be unity

$$(RSH) = 10^{4.31}$$

$$\text{and} \quad x = \frac{20500}{20556} = 1.0 \text{ (approx.)}$$

The free energy change for this transfer is negligible. The diagram below indicates the relationship of these quantities.



The free energy change in solution for any concentrations of hydrogen ion, reactant or product is given by the expression

$$\Delta F = \overline{\Delta F} - 2RT \ln (H^2) - RT \ln c_1 + 2RT \ln c_2$$

where c_1 is the concentration of reactant and c_2 the concentration of product. This expression, and the electrode equation derived from it

$$E = \bar{E} - \frac{RT}{F} \ln (H^+) - \frac{RT}{2F} \ln c_1 + \frac{RT}{F} \ln c_2$$

are more useful when the concentrations of reactants and products are expressed in terms of total concentration, hydrogen ion concentration and dissociation constant.

The successive dissociations of cystine may be abbreviated in the following way:

$$^{+}CSSC^{+} \longrightarrow {}^{\pm}CSSC^{+} \longrightarrow {}^{\pm}CSSC^{\pm} \longrightarrow {}^{-}CSSC^{\pm} \longrightarrow {}^{-}CSSC^{-}$$

The dissociation constants are

$$\frac{(^{\pm}CSSC^{+})(H^+)}{(^{+}CSSC^{+})} = K_4 \qquad \frac{(^{-}CSSC^{\pm})(H^+)}{(^{\pm}CSSC^{\pm})} = K_6$$

$$\frac{(^{\pm}CSSC^{\pm})(H^+)}{(^{\pm}CSSC^{+})} = K_5 \qquad \frac{(^{-}CSSC^{-})(H^+)}{(^{-}CSSC^{\pm})} = K_7$$

from which

$$(^{\pm}CSSC^{+}) = \frac{(^{\pm}CSSC^{\pm})(H^+)}{K_5}$$

$$(^{+}CSSC^{+}) = \frac{(^{\pm}CSSC^{+})(H^+)}{K_4} = \frac{(^{\pm}CSSC^{\pm})(H^+)^2}{K_4 K_5}$$

$$(^{-}CSSC^{\pm}) = \frac{(^{\pm}CSSC^{\pm})K_6}{(H^+)}$$

$$(^{-}CSSC^{-}) = \frac{(^{-}CSSC^{\pm})K_7}{(H^+)} = \frac{(^{\pm}CSSC^{\pm})K_6 K_7}{(H^+)^2}$$

The total concentration of cystine is

$$C_{ss} = ({}^{+}CSSC^{+}) + ({}^{\pm}CSSC^{+}) + ({}^{\pm}CSSC^{\pm}) + ({}^{-}CSSC^{\pm}) + ({}^{-}CSSC^{-})$$

substituting

$$C_{ss} = \frac{({}^{\pm}CSSC^{\pm})(H^{+})^{2}}{K_4K_5} + \frac{({}^{\pm}CSSC^{\pm})(H^{+})}{K_5} + ({}^{\pm}CSSC^{\pm}) + \frac{({}^{\pm}CSSC^{\pm})K_6}{(H^{+})} + \frac{({}^{\pm}CSSC^{\pm})K_6K_7}{(H^{+})^{2}}$$

solving for the reactant $({}^{\pm}CSSC^{\pm})$:

$$({}^{\pm}CSSC^{\pm}) = \frac{C_{ss}\,K_4K_5(H^{+})^{2}}{(H^{+})^{4} + (H^{+})^{3}K_4 + (H^{+})^{2}K_4K_5 + (H^{+})K_4K_5K_6 + K_4K_5K_6K_7}$$

A similar solution for the cysteine product $({}^{\pm}CSH)$ gives the expression

$$({}^{\pm}CSH) = \frac{C_{sh}K_1(H^{+})^{2}}{(H^{+})^{3} + (H^{+})^{2}K_1 + (H^{+})K_1K_2 + K_1K_2K_3}$$

Substituting these expressions for the reactant and product in the free energy equation

$$\Delta F = \overline{\Delta F} - 2\,RT \ln H^{+} - RT \ln c_1 + 2\,RT \ln c_2$$

gives the equation

$$\Delta F = \overline{\Delta F} - 2\,RT \ln H^+ - RT \ln \frac{C_{ss} K_4 K_5 (H^+)^2}{(H^+)^4 + (H^+)^3 K_4 + (H^+)^2 K_4 K_5 + (H^+) K_4 K_5 K_6 + K_4 K_5 K_6 K_7}$$

$$+ 2\,RT \ln \frac{C_{sh} K_1 (H^+)}{(H^+)^3 + (H^+)^2 K_1 + (H^+) K_1 K_2 + K_1 K_2 K_3}$$

collecting terms and simplifying, this equation becomes

$$\Delta F = \overline{\Delta F} + RT \ln \frac{(C_{sh})^2}{C_{ss}} + RT \ln \frac{K_1^2}{K_4 K_5}$$

$$+ RT \ln \frac{(H^+)^4 + (H^+)^3 K_4 + (H^+)^2 K_4 K_5 + (H^+) K_4 K_5 K_6 + K_4 K_5 K_6 K_7}{\left\{(H^+)^3 + (H^+)^2 K_1 + (H^+) K_1 K_2 + K_1 K_2 K_3\right\}^2}$$

This is equation (14) on page 11. The corresponding equation for the β thiolactic acid system is derived in a similar way.

It is noteworthy that the term 2 RT ln (H^+) in equation (12) does not appear in the final equation, since it is cancelled by an equal and opposite term which arises in the solution of the dissociation equations. The term cancels out in the derivation of the equations for the β thiolactic acid system also. The entire effect of the changes in pH on the free energy change are therefore included in the last term in equations (13) and (14)

APPENDIX IV.

Prediction of heats of reaction

Studies on the nature of the chemical bond have led Pauling (1932) to devise a map of the relative negativity of atoms with the aid of which predictions of the approximate heats of gas reactions may be made with an uncertainty in some cases of only one or two thousand calories. The use of this map for predictions for reactions in solution implies the assumption that the gas to solution phase change involves no large heat effect, or that the heat effect for the reactants is the same as that for the products.

The reastion

$$RSSR + H_2 \longrightarrow 2\ RSH$$

involves the breaking of one S:S bond and one H:H bond and the formation of two S:H bonds. The heat of reaction predicted by the electronegativity map has the value

$$\Delta H = -(2)(0.43)^2(23060) = -8500 \text{ calories.}$$

The heats of reaction for the cystine:cysteine and β thiolactic:$\beta\beta'$ dithiodilactic acid reactions are derived from the heats of formation of these substances and their heats of solution. The heats of solution of the cyrstalline substances are obtained from solubility data at two temperatures by solving the expression

$$\ln \frac{N_2'}{N_2} = \frac{-\Delta H}{R}\left(\frac{1}{T'} - \frac{1}{T}\right)$$

in which N_2' and N_2 are the mol fractions of solute in the saturated

solution at T' and T. These values of ΔH are:

1 mol l-cystine = 5050 cal.
2 mols l-cysteine = 8750 cal.
1 mol $\beta\beta'$ dithiodilactic acid = 10400 cal.

The heat of solution of β thiolactic acid is unknown and is taken equal to that of propionic acid, namely - 600 calories per mol or - 1200 calories for 2 mols. On this basis, the heats of reaction in solution are:

cystine:cysteine $\Delta H = -9400$ cal.

$\beta\beta'$ dithidilactic: β thiolactic acid $\Delta H = -3600$ cal.

The agreement of the predicted value is satisfactory, considering the uncertainties involved. The electronegativity map may be useful in certain biochemical problems for predicting the order of magnitude of the energy change in reactions between substances for which heat data is scanty or absent.