Summary

It has been shown that pure pyrophosphoric acid produces the orthophosphoric acid esters of thiamine as well as of 4-methyl-5-hydroxyethyl thiazole, and that metaphosphoric acid or phosphorus pentoxide calculated to give metaphosphoric acid is necessary to form the pyrophosphoric acid ester of thiamine (cocarboxylase). Thiamine sulfuric acid ester has been prepared as well as the pyrophosphoric acid ester of 4-methyl-5-hydroxyethyl thiazole. The latter ester was condensed with the pyrimidine portion of cocarboxylase to give a product with cocarboxylase activity. A similar condensation was carried out with the pyrimidine portion and chlorothiazole in the presence of silver pyrophosphate, the isolated reaction product showing cocarboxylase activity.

Absorption of Oxygen by Glutathione in Alkaline Solutions. I. Kinetics of the Reaction at pH 9 to 11

BY M. B. YOUNG AND H. A. YOUNG

It has been shown[1] that, in the presence of a copper salt as catalyst and at a pH below approximately 8.5, a solution of glutathione is oxidized by oxygen from the mercaptan to the disulfide, that the rate of the reaction increases with incrase in pH, and that the factor determining the rate is the concentration of the dissociated ion.

It has also been shown[2] that as the pH is increased beyond 8.5, the reaction becomes more complex both with regard to the products formed and to the interpretation of the reaction rates. From approximately pH 9 to 11, the volume of oxygen absorbed per mole of glutathione is slightly greater than that calculated to change the mercaptan to the disulfide. The absorption, however, comes to a definite and abrupt end and there is no indication of a further reaction involving the formation of an oxidation product higher than the disulfide. Colorimetric tests with titanium sulfate have shown this excess absorption to be caused by the production of small amounts of hydrogen peroxide, which appears to be quite stable, particularly at pH 9, even in the presence of the copper catalyst. As the pH increases beyond 11, the reaction involving the disulfide formation

$$2GS^- + \tfrac{1}{2}O_2 + H_2O = GSSG + 2OH^- \quad (a)$$
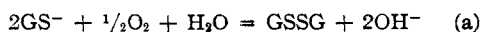
is followed by another reaction involving oxygen and the disulfide produced n (a) and indicated by a continued slow increase in oxygen absorption. This follow reaction increases in rate as hydroxide

(1) Carl M. Lyman and E. S. G. Barron, J. Biol. Chem., 121, 275 (1937).
(2) M. B. Young, H. A. Young and Max Kleiber, THIS JOURNAL, 63, 1488 (1941).

ion increases while the rate of reaction (a) remains essentially constant until finally all discontinuity in the resulting curve is lost. The volume of oxygen absorbed indicates that the principal product formed is the salt of the sulfinic acid of glutathione and that the follow reaction is

$$GSSG + \tfrac{3}{2}O_2 + 2OH^- = 2GSO_2^- + H_2O \quad (b)$$
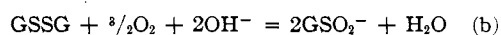
Again the oxygen absorbed is a few per cent. high, possibly caused by the formation of more hydrogen peroxide or some sulfonic acid.

The reaction has been studied over the pH range 9 to 13.3 with glutathione concentration 0.00110 to 0.00880 molar, copper sulfate concentration 0 to 25 × 10^{-6} molar and oxygen pressure 0.2 to 1.0 atm. The results of three exemplary experiments are shown in Fig. 1 in which approximately equal amounts of glutathione, 5 cc. of 0.00220 molar solution in the presence of copper sulfate of approximately 5 × 10^{-6} mole per liter, were oxidized by oxygen at 1 atmosphere at hydroxide ion concentrations of 10^{-5}, 0.0431, and 0.171 mole per liter, respectively. The lines A and B indicate the calculated volumes of oxygen to change the mercaptan to the disulfide and to the sulfinic acid, respectively. There is a distinct break in curve 2 near A.

The present publication is concerned with an attempt to explain the kinetics of the simplest of the reactions, i. e., the oxidation of the mercaptan to the disulfide as it occurs at pH 9 to 11.

Experimental

The experiments were carried out in the Warburg apparatus at 37°. Four cc. of glutathione

solution was placed in the main compartment of the reaction flask and 1 cc. of a mixture of sodium hydroxide and copper sulfate in the side arm. Oxygen at the desired pressure was used to saturate the solutions and to fill the gas space above (about 10 cc.). After the establishment of temperature equilibrium, the sodium hydroxide–copper sulfate mixture was mixed with the glutathione[3] and the rate of oxygen absorption measured at constant volume. Since the process of mixing necessitated the removal of the apparatus from the thermostat for a few seconds, readings taken during the first minute are considered to be in error.

The apparatus was shaken at a rate of 180 oscillations per minute. Increasing the rate to 320 showed no increase in the rate of oxygen absorption; decreasing it to 100 showed a slight decrease. During several test experiments, the reaction was stopped before completion by adding sufficient sulfuric acid, and the concentration of the unoxidized glutathione determined iodometrically following the procedure of Woodward and Fry.[4] Even in the faster experiments, the total reducing power of the glutathione solution checked the oxygen consumption within 3%. In view of the fact that more than enough oxygen is dissolved in the solution initially to oxidize all of the glutathione, it is assumed that these experiments indicate that equilibrium had been established throughout between the oxygen of the gas phase and that of the solution. Changing the glass surface within the reaction flask by adding glass beads had no effect upon the reaction rate.

C. P. quality chemicals were used throughout without further purification. As was to be expected in a reaction sensitive to traces of catalyst, there was some variation in going from one source of chemical to another, particularly the sodium hydroxide. A bottle of Mallinckrodt heavy metal-free product was finally obtained and used consistently. The water was doubly distilled in Pyrex glass. Various samples of glutathione obtained from different establishments (Eimer and Amend, Paul–Lewis Laboratories) were reasonably reproducible. By using the same supply of reactants, any series of experiments performed within a period of a month or so proved to be re-

(3) Under the conditions of the experiments the suspension of copper hydroxide dissolved immediately in the glutathione forming a glutathione-copper complex of some kind. For simplification Cu^{++} will refer to Σ Cu in whatever form it may be.

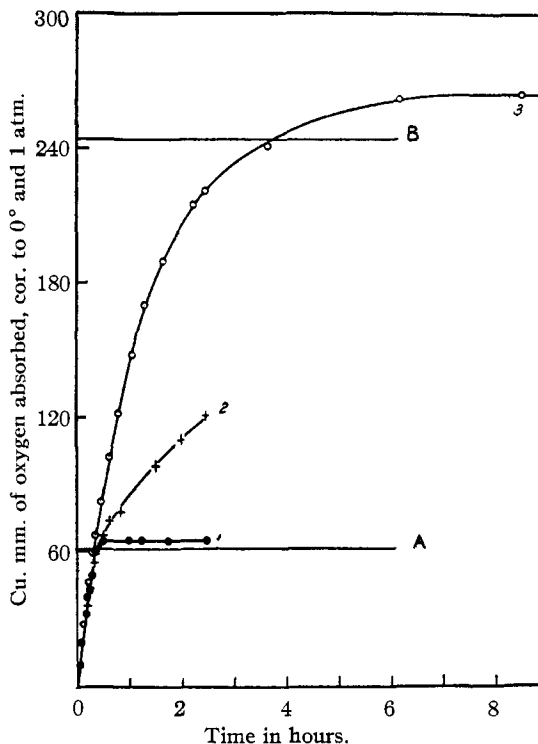(4) Gladys E. Woodward and Edith G. Fry, *J. Biol. Chem.*, **97**, 465 (1932).

Fig. 1.—Absorption of oxygen at 37° and 1 atm. pressure by glutathione in alkaline solutions.

producible; and when going from one source of glutathione to another, while the absolute rates varied somewhat, the shapes and general form of the absorption curves were always the same.

No buffers were added to the reaction system.

Experimental Results

Within the chosen pH ranges, two types of absorption curves were encountered. In Fig. 2, the curve represents an autocatalytic reaction with its maximum rate occurring at about 75% completion. This type of reaction is always associated with an excess absorption of oxygen. In Fig. 3, the curve represents a reaction which has its maximum rate at zero time. If excess oxygen absorption occurs at all, it is to a much slighter degree. In both figures line A represents the theoretical volume of oxygen required to convert the mercaptan to the disulfide. All kinds of intermediate curves may be obtained, depending upon experimental conditions. Factors tending to produce the first type are high values of the ratio of copper ion to glutathione, high oxygen pressure, and high pH. Those tending to produce the latter are conversely low values of the ratio of copper ion to glutathione, low oxygen pressure, and low pH.
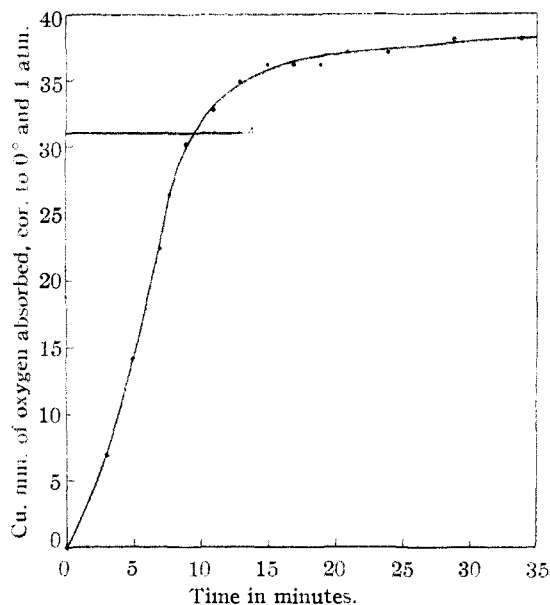
Fig. 2.—Absorption of oxygen by glutathione: GS^- = 0.00111 M; Cu^{++} = 4.63 × 10⁻⁶ M; initial pH, 10.8 (3.07 equivalents NaOH); O_2, 1 atm.

Tests with titanium sulfate indicated that hydrogen peroxide often accumulated during the course of the reaction. In several cases, the
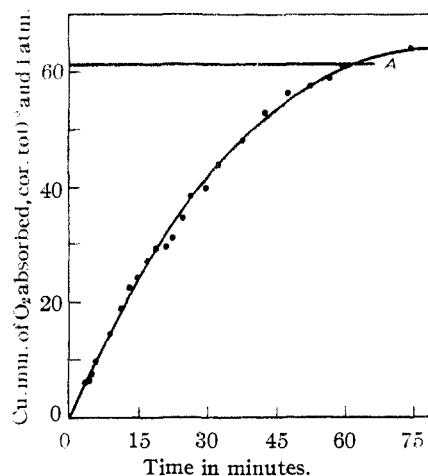

Fig. 3.—Absorption of oxygen by glutathione: GS^- = 0.00220 M; Cu^{++} = 4.63 × 10⁻⁶ M; initial pH, 9.4 (2.05 equivalents NaOH); O_2, 0.2 atm. (air).

itially added to the reaction mixture. The copper sulfate and glutathione concentrations are 5.0 × 10⁻⁶ and 0.00220 molar, respectively. As is shown, the rate increases with increase in base until about 3.5 equivalents are present at a pH of approximately 11. As more base is added, there is actually a slight decrease, accompanied, however, by

TABLE I

COMPARISON BETWEEN OBSERVED AND CALCULATED HYDROGEN PEROXIDE CONCENTRATION

GS⁻	OH⁻ equiv.	Cu⁺⁺ × 10⁶	O₂	Calcd. H₂O₂/G₂S₂	Found H₂O₂/G₂S₂	Found H₂O₂	Calcd. H₂O₂
0.00223	3.35	2.52	1	0.08	0.07	0.8 × 10⁻⁴	0.8 × 10⁻⁴
.00223	3.35	10.08	1	.24	.18	.2 × 10⁻⁴	2.4 × 10⁻⁴
.00220	1.50	10.08	1	.20	.1	1 × 10⁻⁴	2.6 × 10⁻⁴
.00220	1.50	10.08	0.5	.20	.1	1.0 × 10⁻⁴	2.0 × 10⁻⁴
.00220	1.50	10.08	0.2	.16	.05	5 × 10⁻⁵	18 × 10⁻⁵
.00198	1.50	10.08	1	.14	.15	1.5 × 10⁻⁴	1.4 × 10⁻⁴
.00100	1.65	10.08	1	.16	.18	1.5 × 10⁻⁴	1.6 × 10⁻⁴
.00397	1.65	10.08	1	.04	.07	1.5 × 10⁻⁴	0.8 × 10⁻⁴

amount of hydrogen peroxide formed was determined colorimetrically by comparison with standard hydrogen peroxide solutions to which titanium sulfate had been added. The results are shown in Table I. Although the experimental error in such colorimetric determinations is high, the results indicate that the excess oxygen consumption can be satisfactorily explained by the presence of hydrogen peroxide. They also illustrate the fact that the faster the reaction, the greater the accumulation of hydrogen peroxide.

Effect of Hydroxide Ion.—The variation of reaction rate with hydroxide ion is shown in Fig. 4, where the observed maximum rates are plotted against the equivalents of sodium hydroxide in-

the appearance of the follow reaction involving the formation of the sulfinic acid—as referred to earlier.
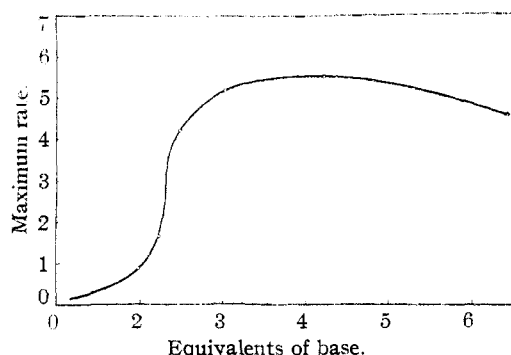

Fig. 4.—Variation of maximum rate with added base.

In 1929 Pirie and Pinhey[5] obtained a titration curve for the then recently discovered glutathione using a product obtained from yeast. For the dissociation of the —SH group, they chose a pK of 9.62. Except for a slight horizontal displacement, we have reproduced quite closely their titration curve using our commercial supply of glutathione at 0.00220 M and including copper sulfate at concentrations comparable to those in the rate experiments (see Fig. 5). The pH values were determined with a Coleman glass electrode, readings being taken immediately after mixing of the glutathione and base. The shaded points represent values obtained with Cu^{++} concentrations of 10×10^{-6} mole per liter; the open circles 5×10^{-6} mole per liter. The three circled points at high pH values have been corrected for errors resulting from glass electrode determinations at the sodium ion concentrations involved. Following the interpretation of Pirie and Pinhey, a value of 10.0 has been chosen as representing the pK of the reaction GSH = $GS^- + H^+$.
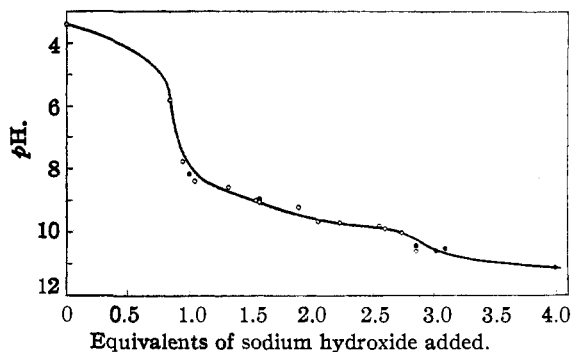
Fig. 5.—Titration curve of glutathione at 25°.

Considering the above results, it is seen that the maximum reaction rate is obtained at the pH where the transformation of GSH to GS^- becomes complete. We have therefore concluded, as did Lyman and Barron,[1] that GS^- is the primary reactant and have looked to the rate results at pH 11 for an explanation of the reaction system. Also, since at this pH, the autocatalytic nature of the absorption curve persists, the effect of the OH^- produced in (a) in transforming any GSH to GS^- as the reaction proceeds cannot be considered a complete explanation for this characteristic.

Effect of GS⁻ Concentration, pH 11.— Figure 6 shows four experiments in which

(5) Norman W. Pirie and Kathleen Goodwin Pinhey, *J. Biol. Chem.*, **84**, 321 (1929).

Cu^{++} was maintained at 5×10^{-6} molar, O_2 at 1 atm., and GS^- varied from 0.00110 to 0.00884 molar. This series of experiments indicates that the effect of GS^- is very slight, since the maximum rates are largely independent of the GS^- concentration. Although not clearly seen on the small plot, the greater the GS^- concentration the nearer the origin to the maximum rates occur.

Effect of Cu^{++} Concentration and Oxygen Pressure, pH 11.—The effect of varying Cu^{++} concentration is shown in Fig. 7. In all of these experiments GS^- was kept constant at 0.00220 mole per liter and the oxygen pressure at 1 atmosphere. It is observed that small amounts of Cu^{++} ion influence the reaction rate tremendously and that t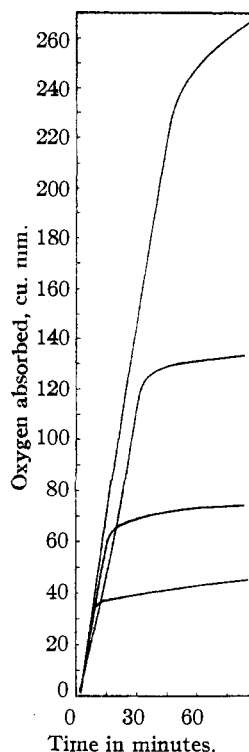he reaction becomes more autocatalytic as Cu^{++} increases. The residual reaction in 1 is presumably caused by traces of catalyst in the reactants. Again, line A represents the theoretical oxygen consumption. When these autocatalytic curves are plotted on a large scale, it is noticeable that the maximum slope of the curve is nearly proportional to the Cu^{++} concentration. This fact is seen in Fig. 8 where maximum slopes are plotted against Cu^{++} concentration at three different oxygen pressures.

The above observation suggests that as the Cu^{++} increases, a catalyst is formed, the concentration of which controls the rate of oxygen consumption and which is determined by Cu^{++} concentration. Further, in the very rapid runs, the indication is that the steady state concentration of this intermediate is not reached until the reaction is nearly over; but when Cu^{++} is very low, the steady state is reached quite early in the experiment.

Fig. 6.—Absorption of oxygen by glutathione at various GS^- concentrations: $Cu^{++} = 5 \times 10^{-6}$ M; pH, 10.9 (3.38 equivalents NaOH); O_2, 1 atm. Beginning with lowest curve, $GS^- = 0.00111$, 0.00220, 0.00440, 0.00884 M, respectively.
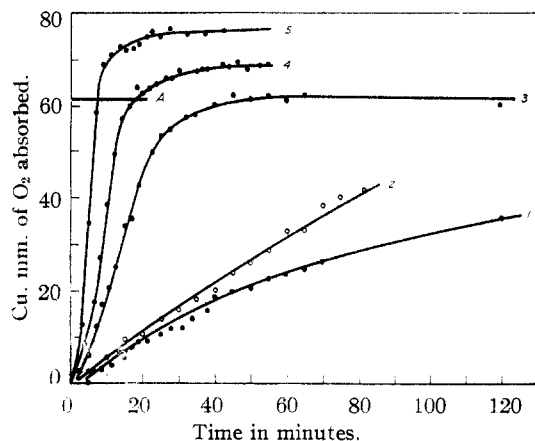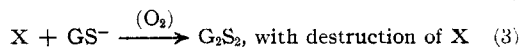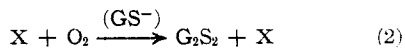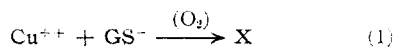
Fig. 7.—Absorption of oxygen by glutathione at various concentrations of Cu^{++}: $GS^- = 0.00220\ M$; initial pH 10.9 (3.42 equivalents NaOH); O_2, 1 atm.; $Cu \times 10^6$ in 1, 2, 3, 4, 5 is 0, 0.504, 2.52, 5.04, 10.08 M, respectively.

The effect of changes in oxygen pressure is also shown in Fig. 8. At very low Cu^{++} concentration, the reaction rate is independent of oxygen pressure but as Cu^{++} increases, this is no longer true. If the maximum rates of Fig. 8 are plotted against the square roots of the oxygen pressures for the three highest Cu^{++} concentrations, a series of lines passing through the origin is obtained. These indicate that, neglecting any effects of absorption or diffusion, the maximum reaction rate at high Cu^{++} concentration varies as the one-half power of the oxygen pressure. During the course of any experiment the oxygen pressure remains essentially constant as less than 1% of the total oxygen available is used.

Rate Law and Mechanism

The foregoing observations that GS^- is the reactive species, that the rate of the autocatalytic reaction is independent of GS^- concentration and dependent upon Cu^{++} concentration together with what is known about the usual role of cupric ion as a catalyst in oxygen reactions suggest the following series of reactions

$$Cu^{++} + GS^- \xrightarrow{\ (O_2)\ } X \qquad (1)$$

$$X + O_2 \xrightarrow{\ (GS^-)\ } G_2S_2 + X \qquad (2)$$

$$X + GS^- \xrightarrow{\ (O_2)\ } G_2S_2, \text{ with destruction of } X \quad (3)$$

Parentheses indicate that the substances are used in the reaction but do not enter into the rate law. In other words, each of the above incomplete equations indicates the substances entering into the rate-determining step of a series of two or more reactions.
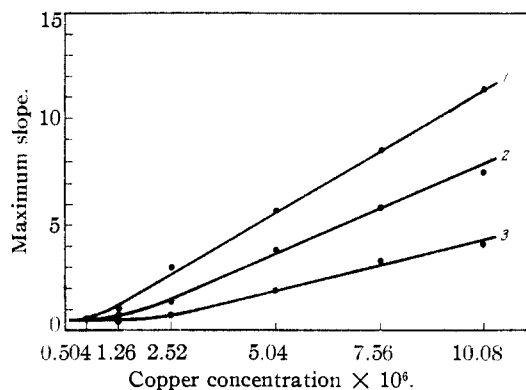

Fig. 8.—Variation of maximum slope with Cu^{++} concentration at pH 11.0: O_2 pressures in 1, 2, 3 are 1, 0.5, 0.2 (air) atm., respectively.

Proceeding in the usual manner, and remembering the experimental facts regarding oxygen pressure, the following rate expressions are obtained

$$-\frac{d[GS]}{dt} = k_1(Cu^{++})(GS^-) + k_2(X)(O_2)^{1/2} + k_3(GS^-)(X) \quad (4)$$

$$-\frac{d[X]}{dt} = k_1(Cu^{++})(GS^-) - k_3(X)(GS^-) \quad (5)$$

$$-\frac{d[O_2]}{dt} = -\frac{d(GS^-)}{dt}(2.8 \times 10^4) \quad (6)$$

Equation 6 is obtained assuming that the only reduction product of oxygen is hydroxide ion.

When the catalyst, X, is in a steady state, then

$$X = k_1(Cu^{++})/k_3 \quad (7)$$

When the concentration of Cu^{++}, and consequently X, is relatively high, the maximum rates occur near the end of the experiment, after the concentration of GS^- has been greatly decreased. Under these conditions, the contributions of reactions 1 and 3 to the rate of absorption of oxygen are negligible, and the rate expression may be written

$$-\frac{d[GS^-]}{dt} = k_2(X)(O_2)^{1/2} = k_2\frac{k_1}{k_3}(Cu^{++})(O_2)^{1/2} \quad (8)$$

and

$$-\frac{d[O_2]}{dt} = k_1\frac{k_2}{k_3}(Cu^{++})(O_2)^{1/2}(2.8 \times 10^4) \quad (9)$$

Using the values of maximum rates and Cu^{++} concentration of Fig. 8, a value of 40 is obtained for the function k_1k_2/k_3 at an oxygen pressure of one atmosphere.

The solution to the system of differential equations (4), (5), (6) is elusive. However, by using the method of successive approximations, it is possible to approximate the oxygen consumption as a function of time. By assuming $k_1 = k_3 = 200$ and k_2 between 30 and 40, it was possible to repro-

duce reasonably well the experimental data so far obtained at pH 11. The variation of k_2 is considered to be within the range of experimental error. Figure 9 shows the comparison between observed and calculated curves for three cases. Although the agreement is not perfect, it seems sufficiently good to encourage the view that the main features of the autocatalytic reaction are represented by equations (1), (2) and (3). The results of varying the oxygen pressure indicate, however, that the catalytic reaction is probably more complicated than as represented in equation (2). A possible explanation of the one-half power for the oxygen pressure would be the establishment of a rapid reversible equilibrium involving oxygen.

It will be observed from reactions (1), (2) and (3) that, when the concentration of X is low or when oxygen pressure is low, reaction (2) becomes less important, and a departure from the catalytic rate law would be expected. This condition is clearly realized at low oxygen pressures, Fig. 8, where the reaction rate becomes independent of oxygen pressure. Further, if undissociated glutathione can function in the destruction of the catalyst X, but not in its production, the rate law would be expected to fail at hydroxide ion concentration at which the concentration of GS⁻ was small compared to that of GSH. This assumption is borne out by the experiments at pH 9 (Table II) where the reaction rates become dependent upon the concentration of ΣGSH = GSH + GS⁻. Column 6 in Table II represents the approximate per cent. of the reaction completed when the maximum rate occurs.

TABLE II

VARIATION OF MAXIMUM RATE WITH ΣGSH AT pH 9

GSH, moles per liter	Cu⁺⁺(10^6), moles per liter	O_2 pressure, atm.	$H_2O_2(10^4)$ produced, moles per liter	Maximum slope, cu. mm./ minutes	% completion at maximum rate
0.00397	10.08	1	1.5	12.30	5
.00198	10.08	1	1.5	5.22	75
.00100	10.08	1	1.5	5.20	77
.00442	4.63	1		5.64	30
.00220	4.70	1		4.00	75

Hydrogen peroxide has been eliminated as the catalytic agent on the basis of experiments in which hydrogen peroxide, added to the reactants in molar concentrations varying from 1 to 20% of that of the glutathione, did not change the reaction rate. In this respect, the autoöxidation of glutathione differs from that of ascorbic acid in which hydrogen peroxide in the presence of copper ion has been shown to be a definite catalyst.[6]

(6) Dekker and Dickinson, THIS JOURNAL, **62**, 2165 (1940).
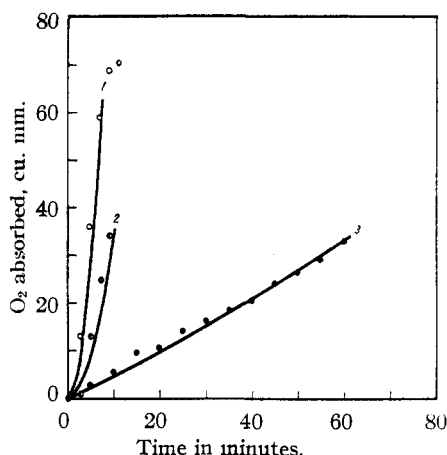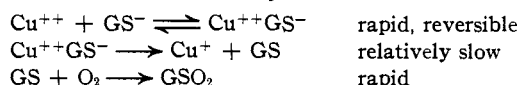

Fig. 9.—Comparison between experimental and calculated curves. The points are experimental; the lines are calculated. Initial pH is 10.9 (3.4 equivalents NaOH); O_2, 1 atm. In curves 1, 2, and 3 the concentrations of Cu⁺⁺ (10^6) are 10.1, 5.0, and 0.50 and of GS⁻ are 0.00220, 0.00110, and 0.00220 M, respectively.

Also we have concluded that HO is not the catalyst since, in the pH range considered, chloride ion in concentrations varying from 1 to 500% of that of the glutathione does not inhibit the reaction rate.[7]

In view of the widely recognized evidence that peroxy radicals often function as catalysts in the autoöxidation of organic substances, it seems reasonable to identify X as a radical of GS, probably GSO_2, and to write the following reactions as the initiators of the catalysis.

Cu⁺⁺ + GS⁻ $\rightleftharpoons$ Cu⁺⁺GS⁻	rapid, reversible
Cu⁺⁺GS⁻ $\longrightarrow$ Cu⁺ + GS	relatively slow
GS + O_2 $\longrightarrow$ GSO_2	rapid

While it is possible to write several series of reactions completing the mechanism, it seems advisable to await the interpretation of results at higher hydroxide ion concentrations before deciding upon the most probable reactions involved.

The authors are indebted to Prof. Max Kleiber for the use of the Warburg apparatus.

Summary

1. The rate of absorption of oxygen by glutathione in the pH range 9 to 11 and in the presence of copper sulfate catalyst has been measured. The effect of changes in copper sulfate and glutathione concentrations and oxygen pressure are presented.

2. A rate law and partial mechanism are proposed.

(7) Taube and Bray, ibid., **62**, 335 (1940).