tri-*n*-propylamine and guanidine picrates were measured photographically in the near infrared using ordinary microscope and camera equipment.

5. Red ammonium picrate is evidently not a distinct polymorph but simply a slightly contaminated form of the yellow salt. The optical properties and crystal habit of the two materials are not significantly different.

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[CONTRIBUTION NO. 459 FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF PITTSBURGH]

The Kinetics of the Reaction between Ascorbic Acid and Oxygen in the Presence of Copper Ion

BY ETHEL SILVERBLATT,[1] A. L. ROBINSON AND C. G. KING

The oxidation of ascorbic acid (vitamin C) by molecular oxygen is catalyzed with remarkable activity by copper ion. Many fundamental aspects of the reaction have not been clarified in reasonable degree, however. Among recent papers, the studies of Schummer,[1a] Dekker and Dickinson[2] and Hand and Greisen[3] are especially pertinent to the present investigation.

The reaction is of interest, too, because the most active and widespread natural catalysts for the same reaction in plants are formed reversibly from copper ion and protein.[4,5,6]

The present paper deals with (a) qualitative and quantitative evidence for the formation of hydrogen peroxide in the oxidation, (b) correlations between the total oxygen consumption and the production of hydrogen peroxide and dehydroascorbic acid, and (c) the kinetics and mechanism of the first oxidative step.

Procedure

Manometric Studies.—A Warburg respirometer with Pyrex glassware was used for the determination of oxygen consumption. All water used for crystallization of reagents or preparing solutions was triply distilled from Pyrex stills, the last distillation being from a conductivity type (Yoe) still. The vitamin solution, 0.3 ml., was placed in the side-arm of the Warburg vessels. Phosphate buffer was used to adjust the *p*H of the solutions above 4; sulfuric acid was added for the lower range. 5.35×10^{-5} *M* copper sulfate and 6.6×10^{-3} *M* ascorbic acid were studied over a *p*H range of 6.3 to 3.0. The reaction was stopped by the addition of 3 ml. of 4% metaphosphoric acid. The solution was washed into an Erlenmeyer flask with 2% metaphosphoric acid and titrated at once with 2,6-dichlorobenzenoneindophenol as described by Bessey and King.[7] After a short initial period, the oxidation of ascorbic acid invariably consumed oxygen in excess of that required for conversion to dehydroascorbic acid. The excess increased with decreasing *p*H values, indicating that dehydroascorbic acid was being oxidized further or that a significant amount of hydrogen peroxide was being formed.

Recoveries of 96 to 100% of the ascorbic acid were obtained regularly by hydrogen sulfide reduction after oxidation by either oxygen or iodine, showing that the oxidation was not proceeding appreciably beyond the dehydroascorbic acid stage.

The presence of "active oxygen" was demonstrated by (a) the release of iodine from potassium iodide, (b) oxidation of Fe^{++} to Fe^{+++} ion, and (c) a modification of these two effects by the addition of catalase. The addition of 0.1 ml. of purified liver catalase solution quickly destroyed the capacity of the solutions to give positive tests for the oxidation of either I^- or Fe^{++}. Tests in Warburg vessels, comparing excess oxygen uptake with the oxygen liberated by catalase, and checked against the liberation of iodine from I^-, gave values in close agreement, considering the small quantities and techniques involved (*e. g.*, 5.19 ± 0.75 cu. mm. of oxygen liberated, against 5.65 cu. mm. calculated from the titration). We believe that these results justify acceptance of the postulate that hydrogen peroxide is formed in the catalytic oxidation of ascorbic acid, and that it is relatively stable under the conditions of the experiment.

(1) The present paper is based upon a thesis presented by one of the authors (E. S.) to the Graduate School of the University of Pittsburgh, June, 1940.

(1a) Schummer, *Biochem. Z.*, **304**, 1 (1940).

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

(3) Hand and Greisen, *ibid.*, **64**, 358 (1942).

(4) McCarthy, Green and King., *J. Biol. Chem.*, **128**, 455 (1939).

(5) Stotz, *Proc. Am. Soc. Biol. Chem.*, **9**, c (1940).

(6) Lovett-Janison and Nelson, THIS JOURNAL, **62**, 1409 (1940).

(7) Bessey and King, *J. Biol. Chem.*, **103**, 698 (1933).

A method of analysis was devised and carefully checked for the estimation of ascorbic acid, dehydroascorbic acid and hydrogen peroxide in the same solution. The technique was essentially as follows. Aliquot portions of 4.5×10^{-4} *M* ascorbic acid solution were partially oxidized by exposure to air in the presence of 2×10^{-5} *M* Cu^{++}. The reaction was stopped by adding an equal volume of *N* sulfuric acid, followed by titration of the residual ascorbic acid with 2,6-dichlorobenzenoneindophenol. 100 mg. of potassium iodide and 3 drops of a 3% solution of ammonium molybdate were then added. After five minutes, 1 ml. of 1% starch solution was added and the free iodine was titrated with 1×10^{-3} *M* $S_2O_8^=$.

Tests were made for interference by each of the reagents present and none was found to interfere significantly. Below *p*H 5 the $Cu^{++} \rightarrow Cu^{+}$ reaction with I^- could be corrected for uniformly (0.15 to 0.40 ml., depending on concentration) in 0.1 to 0.5 *N* sulfuric acid. Metaphosphoric acid was effective in combining with Cu^{++} (4) but was not so satisfactory as sulfuric acid in the present type of titrations, since the reaction between the peroxide and iodide was much faster in the latter solution. It is evident from the data in Table I that oxygen consumption alone could not be relied upon as a measure of ascorbic acid oxidation. From the curves in Fig. 1 it is evident that extrapolation of the curves could approach a ratio of 1:1 for dehydroascorbic acid and peroxide at zero time.

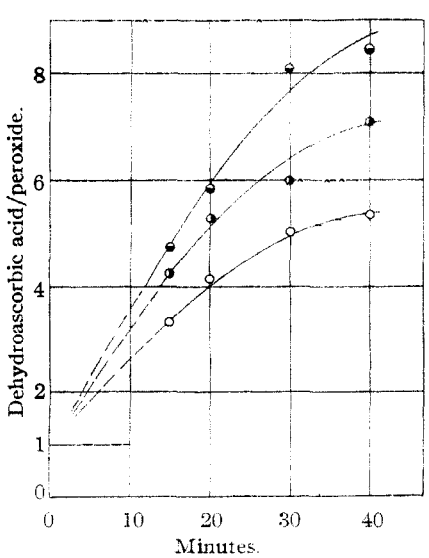

Fig. 1.—Ratio of equivalents of dehydroascorbic acid to peroxide: Ascorbic acid, 3.4×10^{-3} *M*; *p*H = 3.12; *T* = 35°; ○, 4.76×10^{-5} *M* $CuSO_4$; ◐, 9.53×10^{-5} *M* $CuSO_4$; ◒, 1.14×10^{-4} *M* $CuSO_4$.

TABLE I

COMPARISON OF THE TOTAL OXYGEN CONSUMPTION OF SOLUTIONS OF ASCORBIC ACID (IN THE PRESENCE OF Cu^{++}) WITH THE SUM OF THE OXYGEN EQUIVALENTS OF THE DEHYDROASCORBIC ACID AND PEROXIDE FORMED

Ascorbic Acid = 3.4×10^{-3} *M*, *T* = 35°, *p*H 3.12

Time, min.	Dehydro., O_2 equiv., cu. mm.	H_2O_2, O_2 equiv., cu. mm.	Sum O_2 equiv., cu. mm.	Total O_2 obs., cu. mm.
	$Cu^{++} = 4.76 \times 10^{5}$ *M*			
15	38.10	11.40	49.50	52.50
20	52.90	12.78	65.68	67.60
30	67.00	13.30	80.30	86.40
40	75.50	14.05	89.55	98.40
	$Cu^{++} = 9.53 \times 10^{5}$ *M*			
6	33.40	7.85	41.25	39.88
8	44.30	8.38	52.68	51.10
10	52.60	8.76	61.36	59.20
12	62.40	8.76	71.16	71.40
	$Cu^{++} = 11.43 \times 10^{5}$ *M*			
6	40.00	8.46	48.46	48.75
8	50.90	8.66	59.56	61.30
10	63.50	7.81	71.31	72.20
12	69.50	8.23	77.73	79.50

Kinetics.—After this work had been completed, Dekker and Dickinson[2] published a study of the cupric ion catalyzed oxidation of ascorbic acid at 25°. Although the two studies are in agreement in some respects, the conditions under which the two sets of specific rate constants were measured differed and there seem to be certain significant differences in the results obtained.

Procedure

With the exception of the variant under investigation, rates of reaction were measured with the following concentrations of reactants ascorbic acid, 4.5×10^{-4} *M*; cupric sulfate, 2×10^{-5} *M*; hydrogen ion, 3.8×10^{-4} *M*; oxygen, 1.039×10^{-3} *M*. The temperature was 35°. The hydrogen ion concentration was adjusted with sulfuric acid and checked against standard buffers with a Beckman *p*H meter at the beginning and at the end of each experiment. The complete oxidation of the highest concentration of ascorbic acid used caused a rise of 0.08 *p*H unit in solutions originally adjusted to *p*H 3.42. However, since the oxidation was not generally carried to completion, the *p*H increase in the course of an experiment was about 0.02–0.04. Since the specific rate constant was sensitive to change of *p*H, a drift of the constants toward higher values might be expected during the course of an experiment, but in most cases this effect was less than the experimental errors.

0.3 ml. of copper sulfate made by dilution of a standard solution (6.29×10^{-3} *M*) was added to 250 ml. of sulfuric acid of desired strength in a liter Erlenmeyer flask. The solution was saturated with oxygen or air at a temperature above 35° chosen to give the desired oxygen concentration, covered with a two-inch layer of pure mineral oil and

placed in the thermostat at 35°. After temperature equilibrium was established, the required amount of ascorbic acid solution, whose concentration was checked by titration, was introduced through the oil layer and the contents of the flask were mixed by shaking. After about one minute, 25 ml. of the reaction mixture was withdrawn and immediately mixed with 5 ml. of 0.6 *N* sulfuric acid. This time was taken as the arbitrary "zero" for each experiment. Ascorbic acid and peroxide were then determined as described above.

Pseudo-unimolecular specific rate constants were calculated from

$$k = \frac{2.303}{t} \log \frac{(H_2A)_0}{(H_2A)_t} \qquad (1)$$

Results.—In a number of experiments good specific rate constants were obtained. In other cases a drift of the k values was observed; the drift was always toward higher values of k as the reaction proceeded. The rate constants obtained when the concentrations of cupric sulfate, ascorbic acid, hydrogen ion and oxygen were varied independently are shown in the curves. In view of the low concentration of ascorbic acid involved in the determinations, a variation of 10% in k during the course of a run was considered to be within the experimental error. In runs where drift was noted the k values given are average values.

Details of typical runs are given in Table II. The figures in parentheses refer to a check run under identical conditions. Numerous duplicate experiments showed similar agreement.

TABLE II

DETAILED RESULTS OF REPRESENTATIVE EXPERIMENTS

Experiment 2: $CuSO_4 = 2 \times 10^{-6}$ *M*, $H^+ = 3.8 \times 10^{-4}$ *M*, $O_2 = 1.04 \times 10^{-3}$ *M*, $H_2A = 4.5 \times 10^{-4}$ *M*.

t, min.	$(H_2A) \times 10^4$	k, min.$^{-1}$
0	4.35 (4.12)	
6	3.67 (3.79)	0.024 (0.028)
9	3.42 (3.54)	.027 (.025)
12	3.16 (3.29)	.027 (.025)
15	2.89 (3.05)	.027 (.025)
18	2.76 (2.83)	.025 (.025)
21	2.55 (2.63)	.025 (.025)

Experiment 6: $CuSO_4 = 10^{-5}$ *M*[a]

0	4.18	
3	3.44	0.065
6	2.80	.067
9	2.26	.069
12	1.82	.069
15	1.49	.069
18	1.25	.067

[a] Other reactant concentrations same as Experiment 2.

The results of six experiments with solutions containing no copper sulfate gave $k = 0.00168 \pm 0.00014$ min.$^{-1}$. This rate is less than 10% of the slowest rate measured.

To test the homogeneous nature of the reaction, experiments were carried out in which short lengths of Pyrex tubing (flask surface increased five-fold) were added to the reaction mixture. Rates were also measured in flasks coated with Gulf Petrowax, Gulf Paraffin and Atlantic Parawax. In all cases the rate constants checked well with values obtained in untreated flasks. Addition of 1.5 to 2 g. of Pyrex glass wool increased the reaction velocity by about 100%; this was probably due to hydrogen ion adsorption and consequent increased pH of the solution. The reaction, therefore, seems definitely homogeneous.

A few measurements were made at 25°. For the concentrations used in Experiments 6 and 7, k_{35}/k_{25} was found to be 4.0 and 4.8, respectively. Because of different experimental conditions, it is not easy to compare the values of our specific rate constants with those of Dekker and Dickinson. A rough extrapolation of our Experiments 18–21 to $H^+ = 5.58 \times 10^{-3}$ (this is the hydrogen ion concentration in Experiment 5 of Dekker and Dickinson) gives $k_{35} = 0.016$ and, using the temperature coefficient noted above, $k_{25} \approx 0.004$ min.$^{-1}$. To compare with the k_{25} of Dekker and Dickinson, this value should be multiplied by $(H^+)^2/(Cu^{++})$; this gives 0.006 min.$^{-1}$. Considering the uncertainties involved in this approximate calculation, the agreement is fair.

In Fig. 2 are plotted the k values for Experiments 1–9 against the concentration of copper sulfate. Again considering the sensitivity of the reaction to traces of other catalysts and promoters, the results are adequately represented by a straight line. It should be noted that the copper sulfate is not necessarily all present in the reaction mixture as Cu^{++}. This point will be considered in the next section.

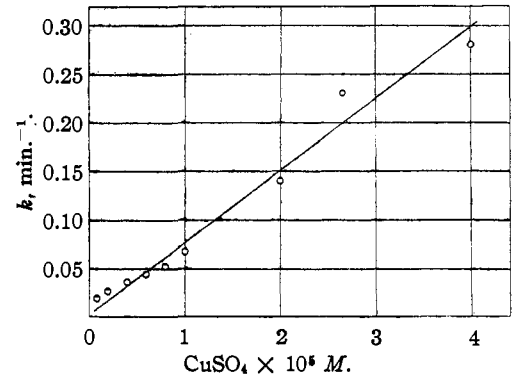

Fig. 2.—Dependence of specific rate constants on concentration of $CuSO_4$ at 35°.

In Fig. 3 are plotted the k values for Experiments 10–17 against the concentration of ascorbic acid. It is evident that the rate constants obtained in these experiments show a strong dependence on the initial concentration of the ascorbic acid. Experiments 18–28 also indicate that the reaction rate is considerably influenced by the concentrations of hydrogen ion and oxygen. This dependence is shown in Fig. 4. Empirically all the rate constants can be expressed by

$$k \approx \frac{k'(O_2)^{0.4}(CuSO_4)}{(H_2A)^{0.5}(H^+)^{0.7}} \qquad (2)$$

The amounts of peroxide formed during typical experiments are shown in Figs. 5 to 7. The amount of peroxide accumulating during these runs was less than the concentration of dehydroascorbic acid produced by oxidation of the ascorbic acid. This might be attributed to decom-

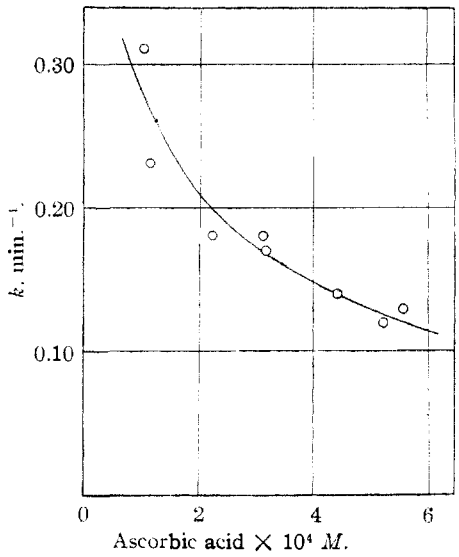

Fig. 3.—Dependence of specific rate constants on concentration of ascorbic acid at 35°.

position of the peroxide, catalyzed by cupric ion[8]; a somewhat lower stability of the peroxide in the presence of ascorbic acid was also indicated. In experiments in which the oxidation of ascorbic acid to dehydroascorbic acid was carried to completion, the limiting ratio of dehydroascorbic acid to peroxide rose from 1.2 in the absence of cupric ion to 2.5 in 2.7×10^{-5} M copper sulfate. This ratio changed from 1.7 to 2.3 as the ascorbic acid concentration was changed by regular increments from 1.05×10^{-4} M to 4.2×10^{-4} M.

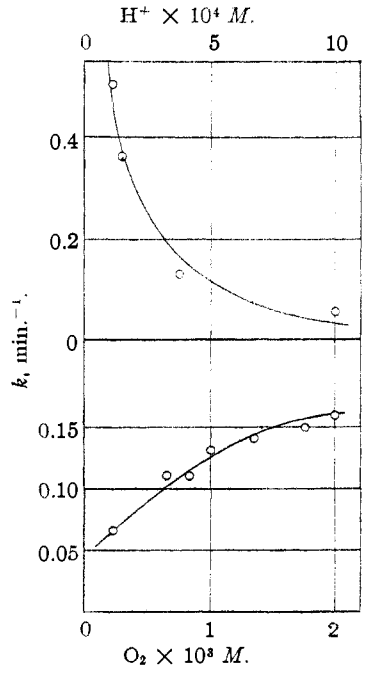

Fig. 4.—Dependence of specific rate constants on hydrogen ion and oxygen concentrations at 35°.

(8) Wieland and Franke, *Ann.*, **473**, 289 (1929).

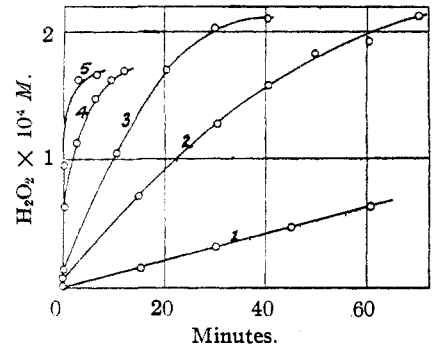

Fig. 5.—Formation of H_2O_2 in experiments with varying $CuSO_4$ concentration: Curve 1, no added $CuSO_4$; curve 2, 8×10^{-7} M; curve 3, 2×10^{-6} M; curve 4, 2×10^{-5} M; curve 5, 5×10^{-5} M.

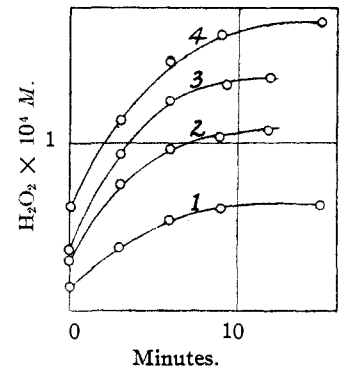

Fig. 6.—Formation of H_2O_2 in experiments with varying ascorbic acid concentration: Curve 1, 1.05×10^{-4} M; curve 2, 2.10×10^{-4} M; curve 3, 3.15×10^{-4} M; curve 4, 4.20×10^{-4} M.

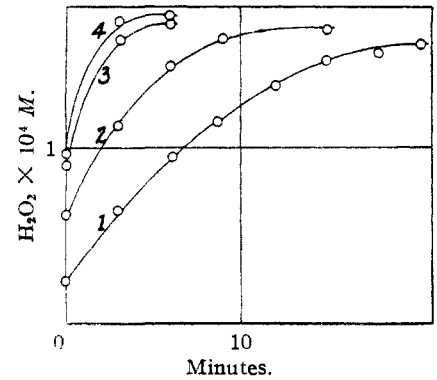

Fig. 7.—Formation of H_2O_2 in experiments with varying hydrogen ion concentration: Curve 1, 10^{-3} M; curve 2, 3.8×10^{-4} M; curve 3, 1.2×10^{-4} M; curve 4, 1.07×10^{-4} M.

Discussion

The good pseudo-unimolecular rate constants obtained in a number of experiments and their dependence on reactant concentrations suggest a mechanism in which an essentially constant con-

centration of cupric ion, controlled by balanced reactions, was maintained in each experiment. The specific reaction rate constants show a much greater dependence on initial reactant concentrations than was observed by Dekker and Dickinson,[2] and whereas all their experiments show an increase of k during the course of the reaction, which they attributed to the accumulation of hydrogen peroxide and its effect upon the oxidation of the ascorbic acid, our k values do not show this increase when $k = 0.12$ min.$^{-1}$.

The following mechanism is essentially that suggested by Dekker and Dickinson; they have indicated the possibility of formally equivalent mechanisms.

$$Cu^{++} + H_2A \rightleftharpoons CuA + 2H^+ \quad (3)$$

$$CuA \longrightarrow Cu^+ + \text{intermediate} \quad (4)$$

$$\text{Intermediate} + O_2 \longrightarrow \text{dehydroascorbic acid} \quad (5)$$

$$Cu^+ + O_2 + H^+ \longrightarrow Cu^{++} + HO_2 \dashrightarrow H_2O_2 \quad (6)$$

If (4) is the rate determining step, it is readily shown, neglecting activity coefficients, that

$$-d(H_2A)/dt = k_4K_3(Cu^{++})(H_2A)/(H^+)^2 \quad (7)$$

But the concentration of (Cu^{++}) is not necessarily that of the added cupric sulfate. Neglecting the concentration of (CuA)

$$(Cu^{++}) + (Cu^+) = Cu_{total} \quad (8)$$

Assuming $d(Cu^+)/dt = 0$, the steady state concentration of (Cu^+) is given by

$$(Cu^+) = \frac{k_4K_3(Cu^{++})(H_2A)}{k_6(H^+)^2(O_2)} \quad (9)$$

and from (8) and (9) the steady state concentration of (Cu^{++}) is

$$(Cu^{++}) = \frac{Cu_{total}\, k_6(H^+)^2(O_2)}{k_6(H^+)^2(O_2) + k_4K_3(H_2A)} \quad (10)$$

Introducing (10) into the rate equation (7) an expression is obtained which formally seems to correspond to the experimental equation (2) with regard to the dependence of the k values on concentrations of cupric sulfate, ascorbic acid, oxygen and hydrogen ion.

Dekker and Dickinson found that an empirical term $k'(Cu^{++})^{0.79}$ (H_2O_2) added to (7) accounted for the drift of their rate constants. If the accumulation of hydrogen peroxide is responsible for the increased reaction rate during the course of an experiment, we might expect to observe this same drift of k values in the experiments which showed the highest concentrations of peroxide. Reference to Figs. 5, 6 and 7 shows that for changing cupric sulfate and hydrogen ion concentrations this prediction is confirmed, but for variation of ascorbic acid concentration, drift of the k values is observed in those experiments where the smallest concentrations of peroxide were detected. There is the further discrepancy that no significant drift was detected in experiments where $k \leqq 0.12$ min.$^{-1}$ although in all such experiments appreciable concentrations of peroxide were present. Perhaps part of the failure of the two sets of experiments to correspond is due to the different working conditions. In any case, the effect of peroxide on the reaction velocity, though possibly significant in some of our experiments, is apparently negligible in others. If, as Dekker and Dickinson suggest, a slow forming complex, $CuH_2O_2^{++}$, reacts rapidly with ascorbic acid, the increased temperature of our experiments, as well as other unknown factors, might influence unfavorably the formation of this complex.

Dekker and Dickinson have made suggestions with reference to the constitution of the intermediate compound formed by the decomposition of the CuA (4). These suggestions are consistent with the Michaelis theory of reversible two-step oxidation.[9]

Summary

The kinetics of the oxidation of ascorbic acid catalyzed by cupric ion have been studied at 35°. Under conditions such that $k \leqq 0.12$ min.$^{-1}$ the reaction rate was directly proportional to the concentrations of cupric ion and ascorbic acid but the k values depended in a rather complex manner upon the initial concentrations of ascorbic acid, hydrogen ion and oxygen. This variation is explained in terms of a steady state concentration of cupric ion determined by a mechanism similar to that suggested by Dekker and Dickinson.

Although appreciable concentrations of hydrogen peroxide accumulated during the course of the reaction a drift of the rate constants toward higher values as the reaction proceeded was observed only when $k \geqq 0.12$ min.$^{-1}$. Dekker and Dickinson attributed this drift to the effect of the peroxide on the oxidation of the ascorbic acid; this drift was not observed in a number of our experiments.

PITTSBURGH, PENNSYLVANIA RECEIVED APRIL 8, 1942

(9) Michaelis and Schubert, *Chem. Rev.*, **22**, 437 (1938).