OXIDATION-REDUCTION POTENTIALS OF ASCORBIC ACID

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Green (1), in studying the potentials of ascorbic acid by means of metallic electrodes, found that the potential was independent of the concentration of either of the reactants and merely a function of the pH; i.e., the system did not fulfill the thermodynamic conditions for reversibility. The contradictory evidence obtained by other workers (2, 3) using metallic electrodes made the significance of all these results extremely uncertain. In a study,¹ in progress in this laboratory, of the potentials in sulfhydryl-disulfide systems in which a similar situation exists, a method of determining the potentials which obviated the employment of metallic electrodes was adopted. This approach, involving the use of oxidation-reduction indicators of known potentials, has been employed by Quastel and Whetham (4) in their studies on the succinic acid-dehydrogenase-fumaric acid system, and by Conant (5) in the case of the hemoglobin-methemoglobin system. In the present experiments the extent of color change was determined photometrically.

This general method can be employed to decide the question whether the ascorbic acid system is thermodynamically reversible or not. It was found that in the reaction between ascorbic acid and certain indicators decolorization of the dye proceeded until a stationary value was reached. In order to determine whether this value might be considered a true equilibrium, the "potential" of the ascorbic acid system was calculated on the assumption that thermodynamic reversibility actually did exist.

* W. J. Gies Fellow in Biological Chemistry, 1932-33.
1 The results of this work, which is nearly complete, will be reported in the near future.
Assuming the reaction, ascorbic acid + oxidized dye $\rightleftharpoons$ dehydroascorbic acid + leuco dye, to be reversible, then

$$\frac{[D_r]}{[D_o]} \times \frac{[A_o]}{[A_r]} = K$$

where $D_o$ and $D_r$ are the oxidized and reduced forms of the dye, and $A_o$ and $A_r$ are the oxidized and reduced forms of ascorbic acid.

Since the oxidation and reduction involve the loss or gain of 2 hydrogen atoms, the characteristic potential depends on the pH of the medium. By keeping the pH constant the last term of the equation

$$E_h = E_o + \frac{RT}{nF} \ln \left( \frac{[S_o]}{[S_r]} \right) - \frac{RT}{nF} \text{pH}$$

can be merged with the constant $E_o$ to give a new constant $E'_o$ which varies only with the pH.

At equilibrium, the potentials of both partial oxidation-reduction systems are the same, and therefore

$$E'_o(d) + 0.03 \log \frac{[D_o]}{[D_r]} = E'_o(a) + 0.03 \log \frac{[A_o]}{[A_r]}$$

or

$$\log \frac{[D_o]}{[D_r]} \times \frac{[A_o]}{[A_r]} = \log K = \frac{E_o(a) - E_o(d)}{0.03}$$

where $E'_o(a)$ and $E'_o(d)$ are the characteristic potentials of the ascorbic acid and dye systems respectively. Since 2 equivalents participate in the reaction, the factor 0.03 must be applied.

Therefore, if the system is reversible, different indicators will give equilibria which, at the same pH, will yield the same potential value for the system under investigation. Also, if the ratio of concentrations of the reactants is changed, equilibrium relationships should be disturbed to the extent required by these mass law considerations. To complete the proof that these stationary values are true equilibria, it is necessary also that the oxidized form of the ascorbic acid react with the leuco form of the dye used. In suitable concentration ratios equilibria should be obtained which give the same potential for the ascorbic acid system as is obtained from the reverse reaction.
For these experiments the principal requisite is the complete absence of molecular oxygen from the reaction. To achieve this, a modified Thunberg tube was used and the exclusion of O₂ effected by alternately evacuating and filling the tube with pure N₂ and by maintaining a slightly positive pressure of N₂ during the course of the experiment. The nitrogen was passed over Cu heated to 650° and the buffer was deaerated, kept under pure N₂, and swept with N₂ before it was added to the vessels under anaerobic conditions. The buffer was introduced after the tube containing the dye and the ascorbic acid had been evacuated and flushed with N₂. The total volume of the buffered solution was 10 cc. The tube was kept under water in a thermostat regulated at 25°.

The shape of the tube (Fig. 1) permitted changes in color intensity to be followed quantitatively by means of the Pulfrich step photometer, the filter corresponding most closely to the maximum absorption of the dye being used. The transmission of each tube filled with distilled water had been previously determined and the values read on the photometer correspondingly corrected. Beer's law was assumed to hold throughout.

In the experiments in which the dehydroascorbic acid reacted with the leuco dyes, an apparatus was required which permitted reduction of the dye and transfer of the leuco form into the reaction vessel without oxidation by air. This need was satisfactorily met by Kühnau's vacuum pipette (6). In these experiments the buffer was introduced anaerobically into the tube already containing the dehydroascorbic acid and the leuco dye, prepared by reduction.
Potentials of Ascorbic Acid

with H₂ in the presence of platinized asbestos, was added last. A measure of the efficiency of this method is given by the observation that a solution of leuco brilliant alizarin blue changed its transmission by only 1.5 per cent when kept under the above conditions, without ascorbic acid, for a period of 1 week.

The ascorbic acid used in these experiments was prepared by Dr. Szent-Györgyi. A stock solution was prepared by dissolving 17.6 mg. in 10 cc. of 0.1 N HCl giving a 0.01 M solution. This was kept under nitrogen.

The solution of oxidized ascorbic acid was prepared by oxidizing 8.8 mg. of ascorbic acid with the theoretical amount (1.00 cc.) of 0.1 N iodine; an equal amount of 0.1 N AgNO₃ was added, the AgI centrifuged off, and the supernatant solution made up to 25 cc. The concentration of this solution was 0.002 M.

The dyes employed were recrystallized commercial samples of indigotetrasulfonate and indigodisulfonate. These were made in stock solutions of 0.001 M concentration. For both of these indicators Filter S-61 of the step photometer was the most suitable. This filter transmits maximally at 620 mμ. The tetrasulfonate absorbs most strongly at 590 mμ and the disulfonate at 610 mμ, as determined by means of the König-Martens spectrophotometer. In the case of the former dye the matching of intensities is not entirely satisfactory; Filter S-57 (transmitting maximally at 570 mμ) was even less so. However, with practise the colorimetric comparison of intensities could be reproduced with a precision of 0.2 per cent in the transmission value.

Precautions were taken to prevent the access of light to the dye either in the stock solution or in the reaction vessel so as to minimize errors due to photochemical processes. Control experiments with indigodisulfonate and brilliant alizarin blue kept very well under the conditions used in these studies, showing no measurable change in transmission after 2 weeks. The dye solutions were made up freshly before each set of experiments.

Results

The calculation of the potentials for the ascorbic acid system from the equilibrium values obtained with the two dyes yields

* The author wishes to express his gratitude to Dr. Szent-Györgyi, as well as to Dr. L. Kast of the Josiah Macy, Jr., Foundation, who transmitted the material.
results which, for a given pH, were identical within the precision of the method. The adherence to the theoretical relationships given above is shown also by the independence of the potential from changes in the concentration ratios. The data are given in Table I. The pH dependence curve is found to follow a 0.06 slope between pH 5.5 and 7.5 (Fig. 2).

The thermodynamic reversibility of the system is further shown by the reaction of dehydroascorbic acid with leucoindigotetrasulfonate in a concentration ratio of 1:1 at pH 7.3. Here

<table>
<thead>
<tr>
<th>Indicator</th>
<th>pH</th>
<th>Ratio, ascorbic acid to dye</th>
<th>Time required</th>
<th>Equilibrium concentration of dye</th>
<th>$E'_b$ (d)</th>
<th>$E'_b$ (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indigotetrasulfonate</td>
<td>5.74</td>
<td>2:1</td>
<td>18</td>
<td>18.0 (82.0)</td>
<td>+0.021</td>
<td>+0.006</td>
</tr>
<tr>
<td></td>
<td>6.28</td>
<td>5:1</td>
<td>4</td>
<td>3.5 (96.5)</td>
<td>-0.012</td>
<td>-0.036</td>
</tr>
<tr>
<td></td>
<td>6.66</td>
<td>2:1</td>
<td>15</td>
<td>10.7 (89.3)</td>
<td>-0.030</td>
<td>-0.055</td>
</tr>
<tr>
<td></td>
<td>6.67</td>
<td>1:1</td>
<td>15</td>
<td>25.5 (74.5)</td>
<td>-0.030</td>
<td>-0.055</td>
</tr>
<tr>
<td></td>
<td>7.31</td>
<td>2:1</td>
<td>5</td>
<td>6.8 (93.2)</td>
<td>-0.059</td>
<td>-0.091</td>
</tr>
<tr>
<td>Indigodisulfonate</td>
<td>5.65</td>
<td>10:1</td>
<td>6</td>
<td>60.4 (39.6)</td>
<td>-0.048</td>
<td>-0.001</td>
</tr>
<tr>
<td></td>
<td>6.53</td>
<td>20:1</td>
<td>3</td>
<td>39.4 (60.6)</td>
<td>-0.100</td>
<td>-0.060</td>
</tr>
<tr>
<td></td>
<td>7.07</td>
<td>20:1</td>
<td>1.8</td>
<td>38.1 (61.9)</td>
<td>-0.130</td>
<td>-0.091</td>
</tr>
<tr>
<td></td>
<td>7.24</td>
<td>10:1</td>
<td>1.2</td>
<td>55.1 (44.9)</td>
<td>-0.140</td>
<td>-0.098</td>
</tr>
</tbody>
</table>

The dye concentration was $5 \times 10^{-4}$ M; the concentration of ascorbic acid varied—molarity is implied in the ratios, ascorbic acid to dye; temperature 25°; phosphate buffers employed.

The equilibria were attained very rapidly, the final transmissions being reached in about 10 minutes. Five such experiments were run, but owing to the difficulty in excluding all traces of $O_2$, the reproducibility desired could not be attained. The average of the corrected transmissions was $40.5 \pm 2.3$ per cent. From the transmission of the oxidized form of the dye the percentages of the dye reactants were found to be 33.1 per cent oxidized and 66.9 per cent reduced. On calculation from this value the potential of the ascorbic acid system at pH 7.30 is found to be $-0.078$ volt, approximately 15 millivolts more positive than that obtained.
from the reverse direction. This difference, which involves no serious discrepancy, is probably due to imperfect exclusion of $O_2$.

It should be noted that there is a great difference in the rates with which equilibrium is reached from opposite sides. The influence of catalysts in the reduction of the dyes has not been investigated, so that the precise kinetics of the decolorization cannot justifiably be calculated. It is clear, however, that the reaction proceeds more slowly with decrease in pH.

![Figure 2. Potentials of ascorbic acid.](image)

**DISCUSSION**

These results are consistent with the qualitative observations made by Green on the decolorization of a series of indicators but differ sharply from his electrometric findings. The incomplete state of our knowledge concerning the details of electrode proc-
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esses frustrates attempts at interpretation. However, the fact that under certain conditions ascorbic acid behaves reversibly casts considerable doubt as to whether the electrometric results throw light on its rôle in biological reactions.

The growing interest in the rôle of oxidation-reduction phenomena in biological systems makes necessary the development of new techniques for the study of potentials as well as reaction kinetics in which oxidation-reduction substances take part. It is possible that the method outlined above can contribute towards filling this need. An attempt is now being made to apply this method to extracts of endocrine organs containing reducing substances.

SUMMARY

1. A method is described for the determination of the oxidation-reduction potentials of reversible systems by the use of dyes.
2. This method when applied to ascorbic acid shows that this system behaves reversibly between pH 5.5 and pH 7.5.
3. The potential of ascorbic acid at pH 7 is \(-0.081\) volt.
4. The rate of oxidation of leucoindigotetrasulfonate by dehydroascorbic acid is much greater than in the reverse reaction.

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BIBLIOGRAPHY

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