*Polarographic Investigations of Vitamin C. III. Reduction Waves of the Condensation Products of Dehydro-L-ascorbic Acid and its Related Compounds with o-Phenylenediamine. Application to the Simultaneous Determination of L-Ascorbic Acid, Dehydro-L-ascorbic Acid and their Related Compounds**

BY Tamotsu WASA, Masanosuke TAKAGI and Sôzaburo ONO

(Received September 19, 1960)

It has been previously reported[1-3] that the reduction waves of dehydro-L-ascorbic acid (DAA), dehydroreductic acid(DRA), mesoxalaldehyde(MOA) and alloxan are all typically kinetic in nature and the limiting currents are very small, compared with their expected diffusion currents. These reduction waves are therefore not suited for the analytical purpose.

In order to obtain the diffusion currents corresponding to the above named substances, the experiments have been carried out under conditions where the substances may form condensation products with *o*-phenylenediamine (OPD). The diffusion controlled reduction waves have been obtained, which are characteristic of each substance.

In the present paper, the authors describe the polarographic behavior of these condensation products and some considerations are made on their molecular structures. Further, as an application of these reduction waves, a method of the simultaneous determination of L-ascorbic acid(AA), DAA and their related compounds is presented.

Experimental

Materials.—AA, reductic acid (RA), triose-reductone (TR) and alloxan were the same specimens as those used for the previous works[1-3]. For the preparation of aqueous solutions of DAA, DRA and MOA, 1 ml. of 10^{-2} M aqueous solution of each of the above endiols, that is, AA, RA and TR, was taken in a 10 ml. volumetric flask and 1 ml. of 2×10^{-2} M aqueous solution of silver nitrate was added. After the oxidation of the endiol with silver nitrate had been completed, the volumetric flask was filled up to 10 ml. with appropriate buffer solution (pH below 4) and the precipitate of silver was filtered off and the filtrate was used as 10^{-3} M aqueous solution of the oxidized form, that is DAA, DRA and MOA, respectively. The solution was used as a stock solution for the experiments with more dilute concentrations of the oxidized form. For the stock solutions containing both the endiol and its oxidized form, the above endiols were treated with an equimolar amount of silver nitrate, consequently the filtrates of the resulting mixtures were expected to contain both the endiol and its oxidized form in equal amouts. To check the concentrations of the endiol and its oxidized form in the above prepared solution, polarograms were sometimes taken with the standard solution containing only the endiol of known concentration, and by comparing both the limiting current obtained with the standard solution and that obtained with the above stock solution, corrections were made, when necessary.

For DAA, the preparation according to Pecherer[4] was also used, but it was only for the qualitative purpose because of its less degree of purity, compared with the above preparation.

2,3-Diketogulonic acid (DGA) was prepared as barium salt according to Penney and Zilva[5].

---

* This paper is the thesis submitted by Tamotsu Wasa for the Degree of Master the University of Osaka Prefecture.

[1] S. Ono, M. Takagi and T. Wasa, *J. Am. Chem. Soc.*, 75, 4369 (1953).

[2] S. Ono, M. Takagi and T. Wasa, This Bulletin, 31, 356 (1958).

[3] S. Ono, M. Takagi and T. Wasa, ibid., 31, 364 (1958).

[4] B. Pecherer, *J. Am. Chem. Soc.*, 73, 3827 (1951).

[5] J. R. Penney and S. S. Zilva, *Biochem. J.*, 39, 1 (1945).

Dialuric acid was prepared according to Tipson⁶⁾ by reducing alloxan with hydrogen sulfide.

Stock solutions of alloxan, dialuric acid and DGA were prepared by dissolving the specimens directly into buffer solutions.

OPD was a commercial product of analytical grade and was checked to give no reduction waves under the present experimental conditions, unless the oxidized form of the endiols existed.

Buffer solutions.—Walpole's buffer for pH 0.5 to 5.0, McIlvain's buffer for pH 2.2 to 8.0 and the mixtures of disodium hydrogen phosphate with sodium hydroxide for pH above 8, were used, investigating the effect of pH and, at the same time, checking the effect of the components of the buffer solutions.

Apparatus and Procedure.—The polarograph used was the same one as that used for the previous works¹⁻³⁾. The characteristics of the capillaries used were, a) $m=2.652$ mg./sec., $t=3.09$ sec., b) $m=1.987$ mg./sec., $t=2.85$ sec. at open circuit, at 25°C, when the height of the mercury reservoir was 60 cm.

For the temperature control by using a thermostat, an electrolytic cell with potassium nitrate saturated agar bridge, was used. The accuracy of the temperature control was ±0.05°C. As the reference electrode, a normal calomel electrode was used. The cell resistance was less than 500 ohm.

The experimental procedure is as follows: a given volume (usually 5 to 8 ml.) of the sample solution diluted with a given volume of a buffer solution is taken in an electrolytic cell and the cell is kept in the thermostat of a given temperature for about 20 min., while the dissolving oxygen in the solution is removed by bubbling nitrogen gas. After the removal of dissolving oxygen, a given volume of OPD solution of known concentration, which had been separately freed from oxygen, is taken and added into the electrolytic cell. The cell is then kept in the thermostat for a given time, during which the bubbling of nitrogen gas is still continued, and after the given period, polarograms are taken.

Results

Reduction Waves of the Condensation Products of DAA with OPD.—*Effect of the Concentration of OPD.*—As the first attempt to see the polarographic behavior of the condensation products of DAA with OPD, the concentration of DAA was kept constant (approximately 1×10^{-3} M) and the concentration of OPD was changed in the acetate buffer solution of pH 3.6. Polarograms were taken at about one hour after the addition of OPD into the electrolytic solution. For this experiment, no thermostat was used and the room temperature was about 20°C. The polarograms are shown in Fig. 1, from which it can be seen that polarograms of the condensation products of DAA with OPD show three different reduction waves and the ratio of the limiting currents of the three is changed, depending on the concentration of OPD. The limiting currents of the three waves have also been observed to change with time. The half-wave potentials of the three waves at pH 3.60 are −0.26, −0.44 and −0.67 V. (vs. N. C. E.), respectively. Fig. 2 shows the relationships between the limiting currents, the concentration of OPD and the time elapsed after the addition of OPD. As seen from Fig. 2, when the concentration of OPD is equal to or less than that of DAA, the third wave prevails and the ratio of the three waves remains almost unchanged with time even after 24 hr., while with increasing concentration of OPD, the growing velocities of the first and the second wave increase at the expense of the third wave. The total height of the three waves has been observed to increase for the first one hour and remain almost constant for a further 24 hr.

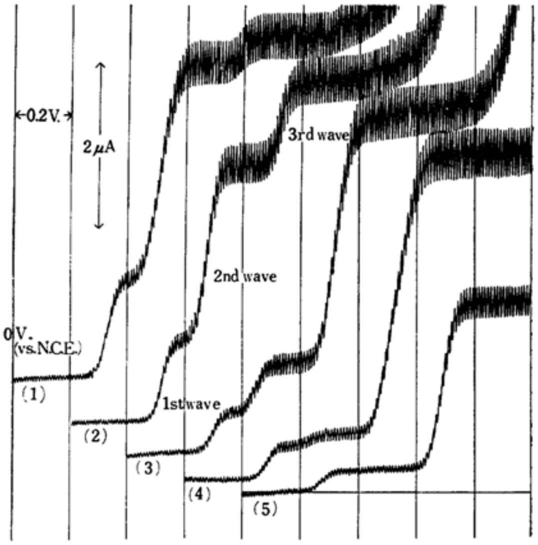


Fig. 1. Polarograms of the condensation products of DAA with OPD. Concn. of DAA: 1×10^{-3} M. Concn. of OPD: (1) 2×10^{-2} M, (2) 1×10^{-2} M, (3) 2×10^{-3} M, (4) 1×10^{-3} M, (5) 5×10^{-4} M. Capillary (b). pH 3.6, temp. 20°C.
Polarograms were taken at one hour after the addition of OPD.

Effect of pH.—Fig. 3 shows the limiting currents of the three waves at one hour after the addition of OPD at 25°C, when the concentrations of DAA and OPD were kept constant and only the pH of the electrolytic solution was changed. Under the present experimental conditions, the limiting current of the third wave reached its maximum value in pH between 3 and 4, and decreased with further increasing pH. The first wave was observed in all the pH range studied and increased

---

6) R. S. Tipson, "Organic Syntheses", Coll. Vol. 33, J. Wiley & Sons, New York (1953), p. 3.

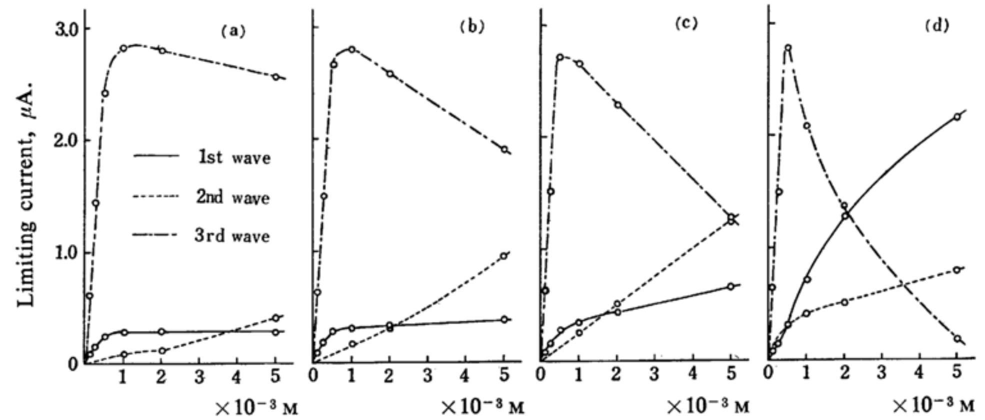


Fig. 2. Effects of the concentration of OPD and the condensation time on the reduction waves of the condensation products of DAA with OPD.
Concn. of DAA: 5×10^{-4} M in acetate buffer of pH 3.59, temp. 25°C, capillary (a).
Polarograms were taken a) at one hour after the addition of OPD, b) 3 hr., c) 6 hr., and d) 25 hr., respectively.

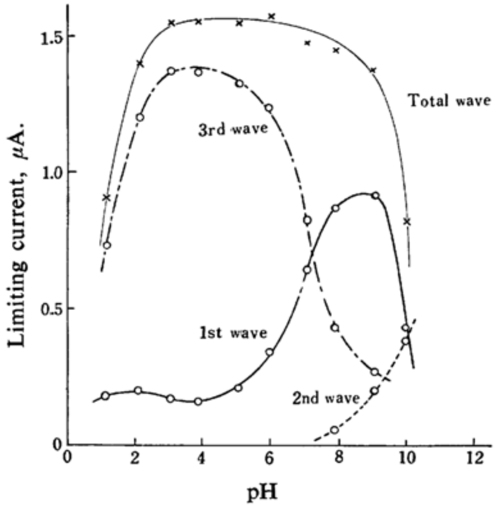


Fig. 3. Effects of pH on the reduction waves of the condensation products of DAA with OPD.
Concn. of DAA: 2.5×10^{-4} M, concn. of OPD: 1×10^{-3} M, temp. 25°C. Polarograms were taken at one hour after the addition of OPD. Capillary (a).

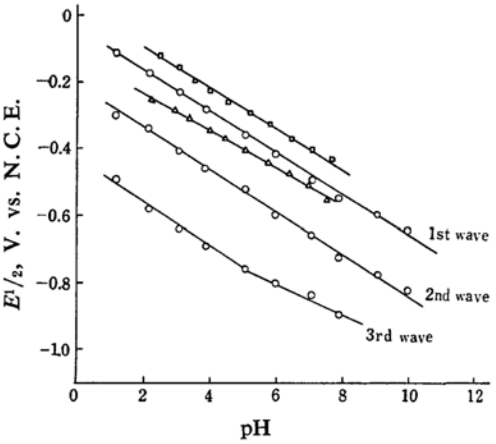


Fig. 4. pH-Dependence of the half-wave potentials of the condensation products of DAA, DRA and alloxan with OPD.
Temp. 25°C.
□ DRA △ alloxan ○ DAA

rapidly in pH above 4, showing its maximum value in pH near 9. The second wave was hardly observed in pH below 7, but increased rapidly in pH above 7.

The fact that the total height of the three waves was decreased with decreasing pH in acid range, may be related with the rates of the condensation reactions, while another decrease in alkaline range may be due to the instability of DAA.

The changes of the half-wave potentials of the three waves with changing pH are presented in Fig. 4, in which the half-wave potentials of the condensation products of DRA and alloxan with OPD are also shown. From Fig. 4, it can be seen that the first wave for DAA shows half-wave potentials close to those of DRA and alloxan, while the other two waves for DAA show considerably more negative half-wave potentials in all the pH range studied.

Effect of Temperature.—When the concentration of DAA was kept constant and the temperature was raised, the time required for the condensation was observed to be shortened depending on the concentration of OPD. Fig. 5 shows the relationships between the ratio of each of the three waves to the total, the concentration of OPD and the experimental

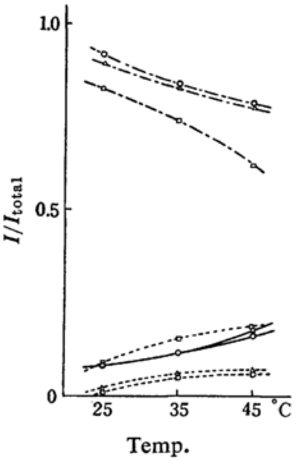


Fig. 5. Effects of temperature on the reduction waves of the condensation products of DAA with OPD.
Concn. of DAA: 2×10^{-4} M, pH 3.09.
Capillary (b).
Concn. of OPD: ○ 4×10^{-4} M, △ 1×10^{-3} M, □ 4×10^{-3} M.
Each wave height is expressed as its ratio to the total wave.
—— 1st wave ---- 2nd wave
-·-·- 3rd wave

temperature at one hour after the addition of OPD.

Effect of the Presence of AA. — It has been found that in pH between 3 and 4, the oxidation wave of AA is observed independently of the reduction waves of the condensation products of DAA with OPD in one polarogram, if OPD freed from oxygen is added into the electrolytic solution containing both AA and DAA, which has been freed from oxygen, as seen in Fig. 6. However here it should be noticed that, though AA has been known to be stable against the oxidation with atmospheric oxygen in such pH as that used for the present case, a considerable increase of the limiting current for DAA was observed at the expense of that for AA, unless the removal of oxygen from the electrolytic solution had been completed before the addition of OPD solution, which had been also separately freed from oxygen.

Reduction Waves of the Condensation Products of DRA and Alloxan with OPD.—A simple and well defined reduction wave was obtained, when DRA or alloxan was treated with OPD in acid buffer solutions in the same way as in the case of DAA, as seen in Fig. 7. The half-wave potentials of the reduction waves for alloxan and DRA at pH 3.60 are −0.32 and −0.19 V. (vs. N. C. E), respectively.

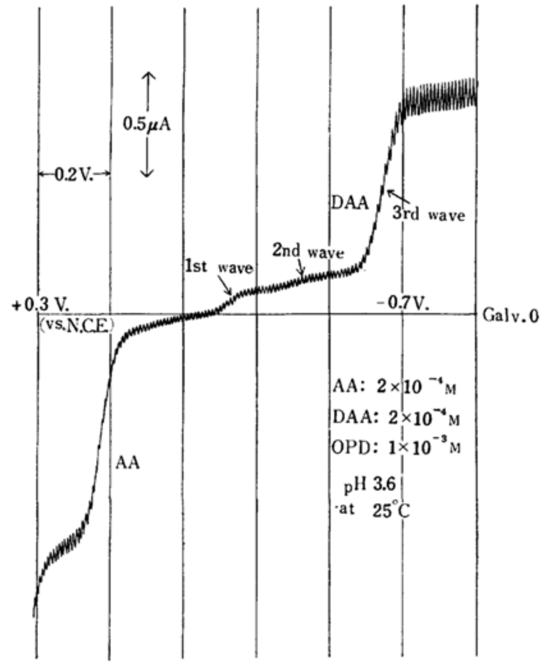


Fig. 6. A polarogram taken with an electrolytic solution containing both AA and DAA in equal amounts in the presence of excess OPD. The polarogram was taken at one hour after the addition of OPD. Capillary (b).

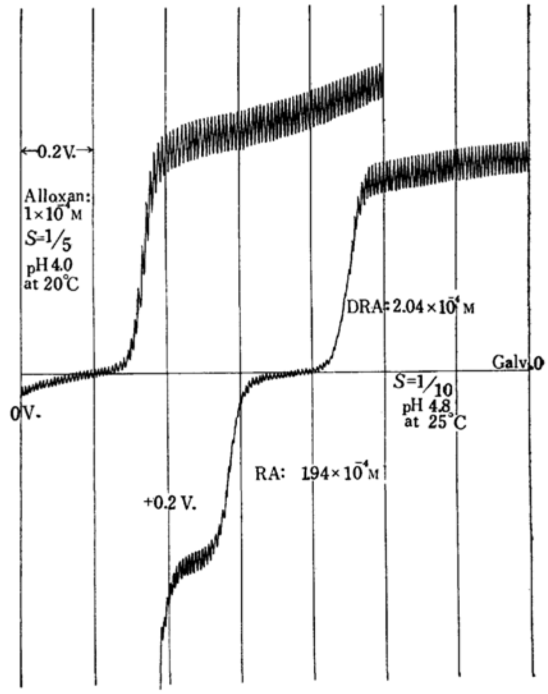


Fig. 7. A polarogram of the condensation product of alloxan with OPD and that of the condensation product of DRA with OPD, with the anodic wave of RA. Capillary (b).

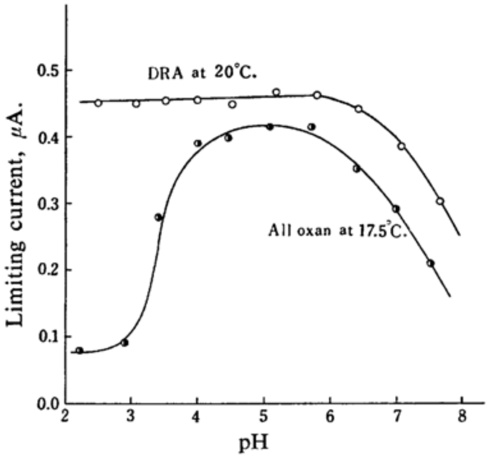


Fig. 8. Effect of pH on the reduction wave of the condensation product of alloxan with OPD and that of DRA with OPD. Concn. of alloxan and DRA: 1×10^{-4} M. Concn. of OPD: 1×10^{-3} M, capillary (b). The polarograms were taken at one hour after the addition of OPD.

Fig. 8 shows the relationship between the limiting current and pH, when the concentrations of DRA, alloxan and OPD were kept constant and polarograms were taken at one hour after the addition of OPD at 20°C. The limiting current of the condensation product of alloxan with OPD shows its maximum value in pH between 4 and 6 under the present experimental conditions. In pH above 6 the splitting of the reduction wave into two waves was observed. The limiting current of the condensation product of DRA with OPD reached its maximum value very rapidly in pH below 6, and in pH above 6 the rate of the condensation reaction was observed to decrease with increasing pH, but no splitting of the wave was seen as in the case of alloxan.

The pH-dependence of the half-wave potentials of the above two waves for alloxan and DRA is as already presented in Fig. 4.

When the electrolytic solution contained both DRA and its reduced form RA, polarograms of both the anodic wave of RA and the cathodic wave of the condensation product of DRA with OPD were obtained in one polarogram as in the case of DAA, if the removal of oxygen from the solution was complete. On the contrary, when OPD was added into the electrolytic solution containing the reduced form of alloxan, i. e., dialuric acid, the oxidation of dialuric acid was observed to be greatly accelerated and no stable polarograms of its anodic wave were obtained even with a careful removal of atmospheric oxygen.

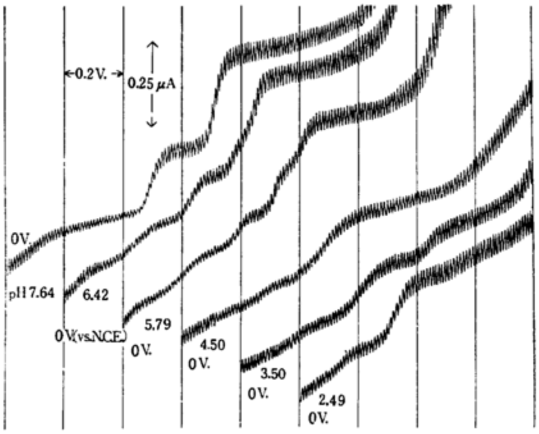


Fig. 9. Polarograms of the condensation products of MOA with OPD. Concn. of MOA: 1×10^{-4} M. Concn. of OPD: 1×10^{-3} M, temp. 25°C, capillary (b). Polarograms were taken at one hour after the addition of OPD.

Reduction Waves of the Condensation Products of MOA with OPD. — The condensation products of MOA with OPD showed no well defined waves in acid pH and with increasing pH two reduction waves were observed to be more distinct, as seen in Fig. 9. The reduction waves in acid pH were somewhat complicated and the limiting current was considerably small, compared with the above three cases. No further experiments have been made with MOA.

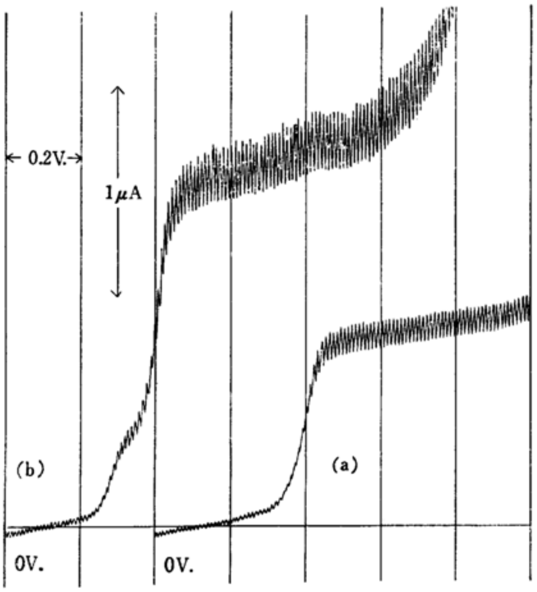


Fig. 10. Polarograms of the condensation products of DGA with OPD. Concn. of DGA: 4×10^{-4} M. Concn. of OPD: 1×10^{-3} M. Capillary: $m=1.261$ mg./sec., $t=2.84$ sec. Polarograms were taken at 128 min. at 25°C (a) and at 6 days at about 15°C (b), after the addition of OPD. pH 3.44.

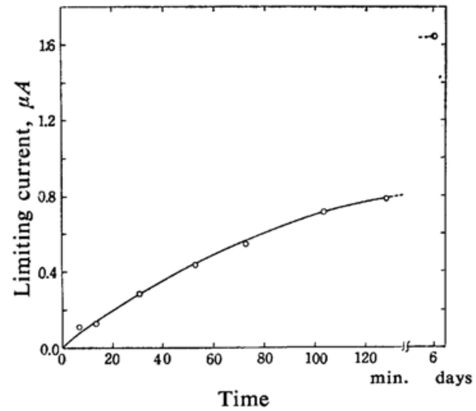
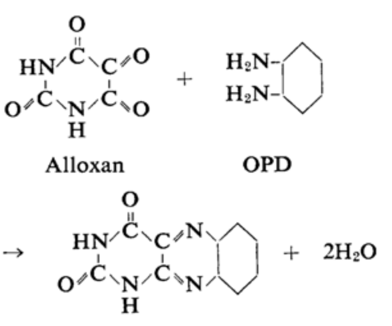


Fig. 11. Change of the limiting current of the reduction wave of the condensation product of DGA with OPD. The experimental conditions are the same as for Fig. 10. The limiting current after 6 days is that for the total wave in Fig. 10 (b).

Reduction Waves of the Condensation Products of DGA with OPD. — When OPD was added into the electrolytic solution containing DGA, a well defined reduction wave ($E_{1/2}=-0.40$ V. vs. N. C. E. at pH 3.44) was observed as seen in Fig. 10. However, the time required for the condensation was much longer than those required for the cases of DAA, DRA and alloxan, as seen in Fig. 11. After the same electrolytic solution was left for several days at room temperature, a more positive reduction wave was observed ($E_{1/2}=-0.27$ V. vs. N. C. E. at pH 3.44).

Discussion

According to Kühling⁷⁾ and Archibald⁸⁾, the most probable condensation product of alloxan with OPD in acid pH may be considered to be alloxazine and the reaction may be written as



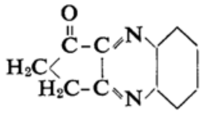
Alloxan OPD

Alloxazine (I)

(1)

On the other hand, alloxazine has been polarographically studied by Koide⁹⁾ and the half-wave potential obtained is practically identical with that of the present case.

Analogously to the case of alloxan, the condensation product of DRA with OPD may be assumed to be



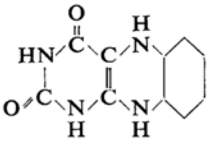
(II)

Since the limiting current of each of the above condensation products is observed to be almost equal to the anodic limiting current of the corresponding reduced form, i. e., RA or dialuric acid, of the same concentration, a two-electron transfer has been supposed to exist for the electrode reaction, referring to the two-electron transfer for the electrode oxidation of RA, dialuric acid and many other endiols¹⁰⁾. The slight difference between the cathodic and the corresponding anodic limiting current can be well understood as due to their diffusion coefficients.

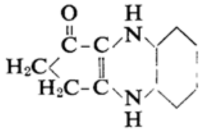
The mechanisms of the electrode reactions of the two substances may possibly be assumed to be expressed as follows.

$$(\mathrm{I}) + 2\mathrm{H}^+ + 2e \rightarrow (\mathrm{III}) \qquad (2)$$

$$(\mathrm{II}) + 2\mathrm{H}^+ + 2e \rightarrow (\mathrm{IV}) \qquad (3)$$



(III)



(IV)

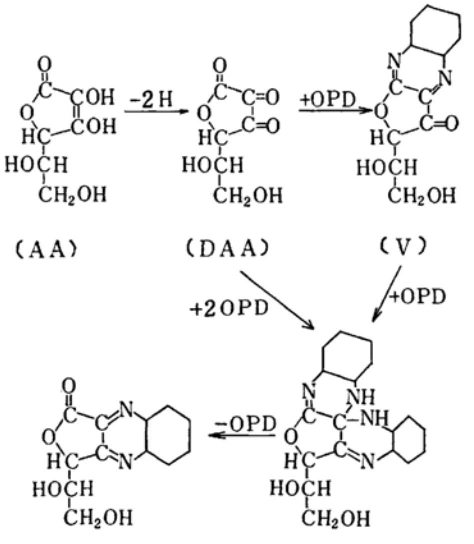
In contrast with DRA and alloxan, at least three different types of the condensation products for DAA should be considered, which would correspond to the three waves obtained (see Fig. 1).

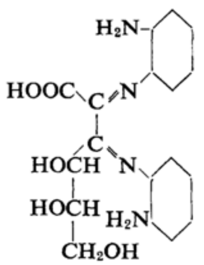
Erlbach and Ohle¹¹⁾ studied the condensation products of iso-DAA and DAA with OPD. Hasselquist¹²⁾ investigated the condensation products of OPD, aniline, etc., especially in respect to the characteristics of the lactone ring of the products. Ogawa¹³⁾ also studied this kind of condensation of DAA with OPD for the purpose of the determination of total vitamin C, in which he measured the fluorescence of one of the condensation products of DAA with OPD, referring to those of iso-DAA studied by Erlbach and Ohle¹¹⁾.

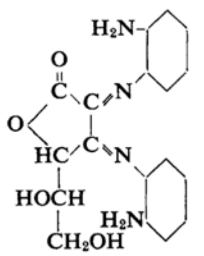
---

7) O. Kühling, *Ber.*, **24**, 2364 (1891).
8) R. M. Archibald, *J. Biol. Chem.*, **158**, 347 (1945).
9) S. Koide, a private communication to the authors.

10) R. Brdicka and P. Zuman, *Collection Czechoslov Chem. Communs., Suppl.*, **15**, 766 (1950).
11) H. Erlbach and H. Ohle, *Ber.*, **67**, 555 (1934).
12) H. Hasselquist. *Arkiv Kemi*, **4**, 369 (1952).
13) S. Ogawa, *J. Pharm. Soc. Japan* (*Yakugaku Zasshi*), **73**, 54, 59, 94, 309, 316, 324 (1953).




(VIII)


(IX)

The mechanism of the condensation reactions in the present experiments may be profitably considered with the reaction diagram which was given by Ogawa for DAA and is analogous to the one originally presented for the condensation of iso-DAA with OPD by Erlbach and Ohle. According to Erlbach and Ohle, and also Ogawa, OPD reacts stoichiometrically with DAA to transform to V, when the concentration of OPD is equal to or less than that of DAA, and V corresponds to the product whose fluorescence was utilized by Ogawa for the determination of total vitamin C. From the polarograms in Fig. 1, it would be reasonable to say that the product corresponding to the third wave may be identical with the one described as V, since the third wave is much larger than the other two waves and considerably stable for a relatively long period, under conditions similar to those described for V by the above authors. Further the above authors' diagram shows that when the concentration of OPD is sufficiently greater than that of DAA, VI and VII are produced and their final equilibrium ratio is about 1/3. Since our polarographic results showed that when an excess of OPD to DAA was used, the first and the second wave increased with time at the expense of the third wave and the final ratio of the second wave to the first was found to be about 1/3, the first and the second wave may be considered to correspond to the reductions of the products described as VII and VI, respectively, if a two-electron transfer is assumed for both the first and the second wave. However, according to Hasselquist⁽¹²⁾, when freshly prepared DAA was treated with excess OPD, VIII was produced instead of IX. This he could obtain by treating VIII with various solvents. He also observed the separation of one mole of OPD from VIII or IX in about 1 N hydrochloric acid solution.

As the reduction wave first obtained with DGA in our experiments seemed to be identical with the second wave for DAA (see Fig. 10), the possibility that VI in the above diagram can be replaced with VIII, may be considered. Further, the appearance of a more-positive reduction wave in the case of DGA after a long period, may suggest the conversion from VIII to VII, though the results are not sufficient for full discussion.

On the other hand, there still remain some difficulties, in interpreting the difference between the half-wave potentials of the three waves for DAA: V and VII would be expected to be reduced at very near potentials to each other. It seems therefore necessary to study further on the configuration of the product corresponding to the third wave. The reduction wave due to the condensation product of an oxidation product of AA (probably DGA) with ammonia studied by Zuman⁽¹⁴⁾ seems also to be interesting in comparison with the present case.

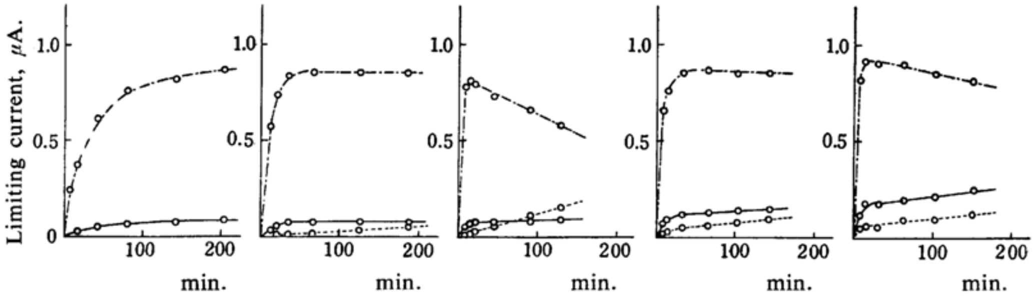
However, as the present results are insufficient for a complete revision of the diagram, for the time being, only the importance of the third wave for DAA will be emphasized; the wave is characteristic of DAA and the separation of the half-wave potential from those of the condensation products of other related compounds is considerably large in the wide pH range.

Though the electrode reaction corresponding to each wave can not be discussed in detail, a two-electron transfer for each wave is readily understood by comparing the limiting currents with the anodic limiting current of AA of the same concentration, as for the cases of alloxan and DRA.

As for the condensation products of MOA with OPD, though several types of the reaction may be supposed, discussions are not made on this problem, since the polarograms of the products themselves were not well defined and moreover the limiting currents in acid pH

---

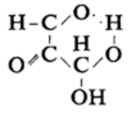
14) P. Zuman, *Collection Czechoslov Chem. Communs., Suppl.*, 15, 839 (1950).



(i)　(ii)　(iii)　(iv)　(v)

Fig. 12. Time-dependences of the limiting currents of the condensation products of DAA with OPD. Concn. of DAA: 2×10^{-4} M. Concn. of OPD: i) 4×10^{-4} M; ii), iv), v) 1×10^{-3} M; iii) 4×10^{-3} M. Temp: i), ii), iii) 25°C; iv) 35°C; v) 45°C. pH 3.6. Capillary (b).
—— 1st wave　---- 2nd wave　—·— 3rd wave

range were considerably smaller than those of other related substances. These facts may be qualitatively interpreted partly by the hydration of carbonyl group and partly by the hydrogen bonding in hydrated MOA, one of whose possible structures may be X.



(X)

Application to the Simultaneous Determination of AA, DAA and their Related Compounds. —As described above, the reduction waves of the condensation products of the oxidized forms of the endiols with OPD are readily obtained, and especially the third wave for the condensation product of DAA with OPD is so characteristic of DAA, that an application of these polarographic phenomena to the simultaneous determination of AA, DAA and their related compounds has been attempted.

In order to establish the most practical conditions for the determination of DAA by utilizing the third wave, the following additional experiments have been carried out. Since the third wave has been found to be comparatively stable in acid pH range (pH 3～5), when the concentration of OPD is low (see Fig. 2), the change of the limiting currents of the condensation products of DAA with OPD has been investigated for the first one hour after the addition of OPD, by changing the concentration of OPD in a limited low concentration range and the temperature for the condensation also in a practical range. The data are presented in Fig. 12. From such experiments as the above, for the concentration of OPD, the temperature and time for the condensation, 1～2×10^{-3} M, 25°C and 50～60 min., respectively, may be recommended. The linearity of the limiting current of the third wave with the concentration of DAA has also been tested, as shown in Fig. 13, for which the stock solution containing both AA and DAA in equal amounts, was used, consequently

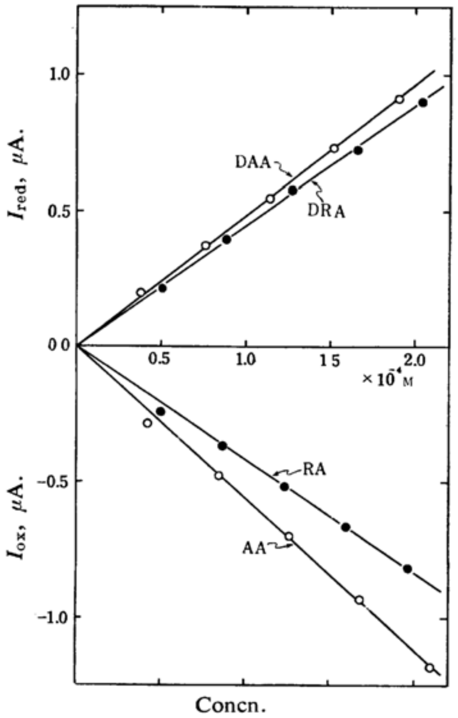


Fig. 13. Linear relationships between the limiting currents and the concentrations for AA and DAA or for RA and DRA, when the electrolytic solutions contain AA and DAA, or RA and DRA. Concn. of OPD: 2×10^{-3} M. Temp. 25°C, pH: 3.6 for AA and DAA, 4.8 for RA and DRA. Capillary: (a) for AA and DAA, (b) for RA and DRA. Polarograms were taken at one hour after the addition of OPD for AA and DAA. I_{red}: the cathodic limiting current, I_{ox}: the anodic limiting current.

the linearity of the limiting current of the oxidation wave with the concentration of AA could be seen simultaneously, without any interference with that of the third wave for DAA, under the present experimental conditions.

For RA and DRA, experiments similar to those for AA and DAA have been carried out. The linearity of the limiting currents with the concentration of RA and DRA, respectively, is presented in Fig. 13. In this case, the time required for the condensation was not considered strictly, since the growth of the reduction wave of the condensation product of DRA with OPD has been observed to be much faster than that of the third wave for DAA, and moreover the limiting current has been seen to be quite stable for a long period.

For dialuric acid and alloxan, a linearity of the limiting current with the concentration for alloxan similar to those for DAA or DRA, was obtained, when the electrolytic solutions contained no dialuric acid (Fig. 14). However, as already stated, dialuric acid is very readily oxidized to alloxan, when OPD is added into the electrolytic solution even under conditions where the atmospheric oxygen is excluded from the solution as carefully as possible. Consequently the oxidation wave of dialuric acid could not be obtained with the reduction wave of the condensation product of alloxan with the OPD in one polarogram.

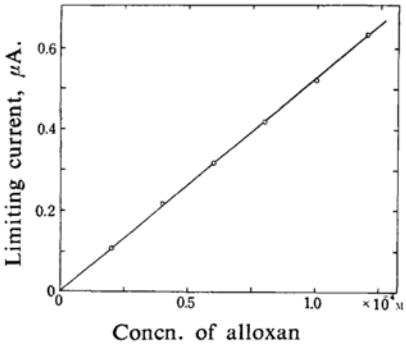


Fig. 14. Linear relationship between the limiting current of the condensation product of alloxan with OPD and the concentration of alloxan. Concn. of OPD: 2×10^{-3} M, temp. 20°C, pH 4.0. Capillary (a). Polarograms were taken at one hour after the addition of OPD.

Simultaneous Determination of AA and DAA in Natural Products.—For the determination of AA and DAA in natural products by using the present polarographic method, the following procedure has been used; 10 g. of the sample and about 1 g. of quartz sand are taken in a mortar and 40 ml. of 2% metaphosphoric acid aqueous solution is added into it. The mixture is crushed and ground, and then filtered or centrifuged. The filtrate or the supernatant is expected to be an extract, in which AA and DAA in the starting material are diluted five times. Now, in a run, 2 ml. of the extract is taken in an electrolytic cell and 7 ml. of McIlvain's buffer of pH 4.7 is added. The electrolytic cell is immersed in a thermostat of 25°C and the oxygen dissolving in the electrolytic solution is chased out by bubbling nitrogen gas for 20 min. One milliliter of 2×10^{-2} M OPD aqueous solution which has been separately freed from oxygen by bubbling nitrogen gas is added, and the electrolytic cell is kept in the thermostat for a further 50 min. during which time the bubbling of nitrogen gas is still continued. After that, a polarogram is taken in the usual way (A). In another run, 2 ml. of the extract is taken in an electrolytic cell and 6.4 McIlvain's buffer pH 4.7 and 0.6 ml. of the standard solution containing both AA and DAA of 1×10^{-3} M, are added. After that, the common procedure with that of the former run is carried out and a polarogram is taken (B). Comparing the polarograms (A) and (B), AA and DAA can be calculated with the anodic and the cathodic limiting currents, respectively. When *A* and *B* represent the values of the limiting currents of the polarograms (A) and (B) respectively, the contents

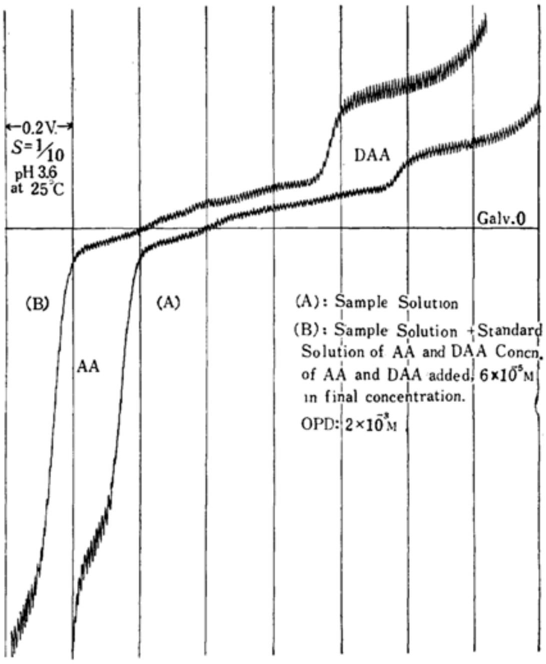


Fig. 15. Polarograms for the simultaneous determination of AA and DAA in radish leaves by means of polarographic OPD method.

C of AA and DAA in the natural material may be calculated as follows.

$$C_{\mathrm{AA}} = A_{\mathrm{ox}}/(B_{\mathrm{ox}} - A_{\mathrm{ox}}) \times 1.056 \times 25 \quad (\text{mg. }\%)$$

and

$$C_{\mathrm{DAA}} = A_{\mathrm{red}}/(B_{\mathrm{red}} - A_{\mathrm{red}}) \times 1.044 \times 25 \, (\text{mg. }\%)$$

where suffix ox and red represent the anodic and the cathodic limiting currents, respectively. In Fig. 15, an example of the polarograms obtained with radish leaves is presented. Some of the analytical results obtained by using the present method are listed in Table I, together with those obtained by the 2, 4-dinitrophenylhydrazine method[15-18]. The results by both methods are in good agreement with each other.

TABLE I. AA AND DAA IN NATURAL PRODUCTS DFTERMINED BY MEANS OF POLAROGRAPHIC OPD METHOD

Sample	AA mg. %	DAA mg. %	Note
Radish leaves	102.8	20.6	
	106.5	17.3	DNP method*
Spinach	58.6	5.5	
	57.8	9.8	DNP method*
Mizuna	68.9	8.3	
Mandarin orange	28.1	5.1	

* Determined by 2, 4-dinitrophenylhydrazine method.

In the above described OPD method, although the addition of the standard solution into the sample solution was utilized, the calibration curve may also be used, with which the procedure may be more simplified, when a number of samples are treated.

Determination of AA in the Presence of One of the Other Endiols. — When two or more endiols are in a sample solution, their oxidation waves are not suited for the analytical purpose, since their half-wave potentials are usually very close to each other[10]. In such cases, the present OPD method may be recommended, especially for the determination of AA coexisting with other endiols. As a demonstration of this method, the following procedure has been employed by the present authors; a given volume of the sample solution (usually 1 ml.) is taken in a 10 ml. volumetric flask and an excess of silver nitrate aqueous solution is added. After the oxidation of the endiols has been completed, the excess of silver ions is precipitated by adding a sodium chloride solution. Then 1 ml. of 1×10^{-2} M OPD solution is added and the volumetric flask is filled up to 10 ml. with an appropriate buffer solution. The mixture is filtered and the filtrate is taken in an electrolytic cell. The cell is kept at 25°C for 50 min., during which oxygen is chased out by bubbling nitrogen gas, and after that polarograms are taken in the usual way. Examples of the polarograms taken with the solutions containing DAA and one of the oxidized forms of different endiols are presented in Fig. 16. As seen from the Fig. 16, the simultaneous determination of AA and DRA is possible. The figure may also indicate that AA can be determined even in the presence of triose-reductone, though the method itself is not suitable for the determination of the latter substance.

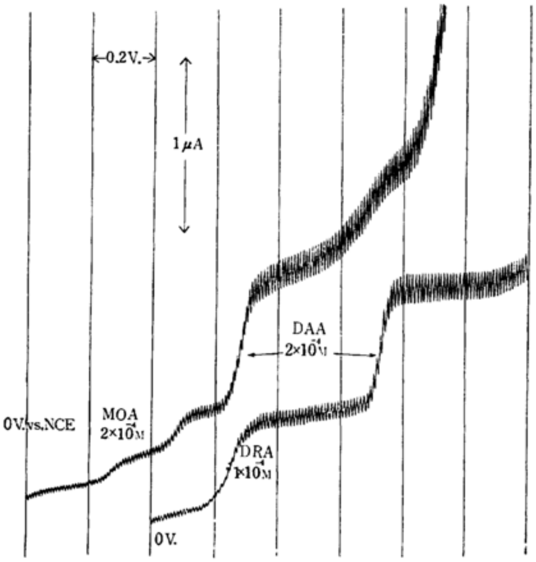


Fig. 16. Polarograms taken with the electrolytic solutions containing DAA and MOA (a) or DAA and DRA (b). Concn. of OPD: 1×10^{-3} M, pH 4.2. Capillary: $m = 1.261$ mg./sec. $t = 2.84$ sec, temp. 25°C.

Comparison with Other Determination Methods. —In the determination of vitamin C by using either one of the previously reported methods i. e. 2, 6-dichlorophenolindophenol method[19], usual polarographic method[20], colorimetric method[15-18], etc., either AA or DAA can be directly determined. Therefore, in order to determine both AA and DAA, either one of the two forms should be converted into the other in a preliminary treatment. Further, when other endiols exist in the same sample

---

[15] J. H. Roe and C. A. Kuether, *J. Biol. Chem.*, **147**, 399 (1943).

[16] J. H. Roe and M. J. Oesterling, ibid., **152**, 511 (1944).

[17] J. H. Roe, M. B. Mills, M. J. Oesterling and C. M. Damron, ibid., **174**, 201 (1948).

[18] J. Teruuchi, *Kitasato Arch. Experimental Med.*, **23**, 37 (1950).

[19] J. Tillmans: *Z. Unters. Lebensm.*, **60**, 34 (1930); **63**, 1, 21, 241, 267, 276 (1932); See also H. v. Euler and H. Hasselquist, "Die Reduktone", Stuttgart, Enke (1950).

[20] See, for example, M. Brezina and P. Zuman, "Die Polarographie in der Medizin, Biochemie und Pharmazie", Akademische Verlagsgesellschaft, Leipzig (1956), p. 370.

solution, the contents of AA and DAA are in most cases overestimated, since endiols have closely resembling chemical properties; all of the endiols reduce 2, 6-dichlorophenolindophenol and the half-wave petentials of the oxidation waves of the endiols are very close to each other. Also the colorization of the oxidized forms of the endiols with 2, 4-dinitrophenylhydrazine resembles one another.

In recent years, Ogawa$^{13)}$ reported his fluorometric determination method of vitamin C, in which he measured the fluorescence of the condensation product of DAA with OPD described as V. However, as reported in his paper, the presence of TR causes the overestimation of vitamin C. Moreover, in order to obtain a satisfying reproducibility of the results, stricter experimental conditions seem to be required$^{21)}$ than those recommended by Ogawa.

In our polarographic OPD method, firstly, for the simultaneous determination of AA and DAA, no need for the conversion of one of the two forms into the other may be emphasized. Secondly, when two or more endiols are in the same sample solution, there is still a possibility of determining these endiols separately as described above. Of course, the method may not be applicable to all of the endiols, but it is interesting that the third wave for DAA is considerably more negative than the reduction waves corresponding to the oxidized forms of the endiols tested, and even MOA (or TR) and DGA may not seriously interfere with the determination of AA and DAA.

Summary

The polarographic behavior of dehydro-L-ascorbic acid (DAA), dehydroreductic acid (DRA), alloxan, mesoxalaldehyde (MOA) and 2, 3-diketogulonic acid (DGA) in acid buffer solutions containing *o*-phenylenediamine (OPD), has been investigated.

DAA shows three diffusion controlled reduction waves, whose half-wave potentials ($E_{1/2}$) at pH 3.60 are -0.26, -0.44 and -0.67 V. (vs. N. C. E.), respectively. The limiting currents of the three waves depend on the time after the addition of OPD into the electrolytic solution, the concentration of OPD. pH and the temperature. When the electrolytic solution contains both AA and DAA, the oxidation wave of L-ascorbic acid (AA) can be obtained independently of the three waves for DAA in one polarogram.

DGA shows two reduction waves whose $E_{1/2}$ at pH 3.44 are -0.27 and -0.40 V., respectively. The $E_{1/2}$ of the first wave is very close to that of the first wave for DAA and the second wave seems to be identical with the second wave for DAA, but the velocities of the condensation reactions of DGA with OPD are much slower than those of DAA with OPD.

Alloxan and DRA show single well defined reduction waves, whose $E_{1/2}$ at pH 3.60 are -0.32 and -0.19 V., respectively. The oxidation wave of reductic acid (RA) can be obtained with the reduction wave for DRA in one polarogram, while the oxidation wave of dialuric acid decreases very fast when alloxan and OPD are added into the electrolytic solution even after the careful removal of the atmospheric oxygen.

MOA shows a somewhat complicated behavior. In acid pH, the waves are not well defined and are considerably smaller than those of the above substances of the same concentration.

Discussions are made on the structures of the condensation products corresponding to their reduction waves.

Since the linear relationship between the limiting current and the concentration has been obtained, for DAA (the 3rd wave), DRA and alloxan, these polarographic phenomena have been applied to the simultaneous determination of AA and DAA, and to that of RA and DRA and further to that of AA and RA. The method is also applicable to the determination of alloxan, but not to those of MOA (or TR) and DGA, none of which, however, seriously interfere with the determination of DAA(or AA) by means of this polarographic OPD method.

Laboratory of Biophysical Chemistry
College of Agriculture
University of Osaka Prefecture
Sakai, Osaka

---

21) M. Taki, a private communication to the authors.