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The Polarographic Behavior of Several α-Keto Acids in Buffered Solutions Containing *o*-Phenylenediamine, and Its Analytical Application

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Such α-keto acids as glyoxylic (I), pyruvic (II), α-ketobutyric (III), mesoxalic (IV), oxalacetic (V), and α-ketoglutaric acid (VI) condense with *o*-phenylenediamine (OPD) to form two kinds of products, depending on the pH values of the reaction mixtures. The condensation product formed at pH values lower than 4 gives a well-defined reduction wave ($E_{1/2}$ *vs.* SCE at pH 2.0: −0.490 V for I, −0.587 V for II, −0.592 V for III, −0.181 V for IV, −0.590 V for V, and −0.576 V for VI) at a potential more positive than that of free α-keto acid, while the other product, formed at pH values between 4 and 8, gives an oxidation wave ($E_{1/2}$: −0.1 — −0.2 V *vs.* SCE for all α-keto acids at pH 6.0). From the present polarographic investigations, it was deduced that the condensation product giving the reduction wave is probably 2-hydroxyquinoxaline derivatives and that the product giving the oxidation wave is benzimidazoline derivatives. A linear relation was obtained between the wave height for the reduction of the condensation product and the concentration ($(0.5—20)\times10^{-4}$ M) for each α-keto acid under appropriate conditions. The reduction wave of the condensation product was used for the determination of II in a commercial product containing II as an impurity.

Since the first work by Shikata and Shoji[1] (1928) on the reduction wave of α-keto acids at a dropping mercury electrode, systematic studies of the polarographic behavior of pyruvic acid and related compounds in buffered solutions have been made by several authors;[2-6] this reduction wave has also been used for the determination of α-keto acids in biological materials.[7] However, the reduction wave is partially reaction-controlled in nature, especially for glyoxylic acid and mesoxialic acid; the limiting current varies intricately with the temperature and with the pH value of the electrolytic solution. For analytical use of this reduction wave, therefore, the experimental conditions must be strictly controlled.

On the other hand, the methods of the chromatographic separation[8,9] and the fluorometric determination[10] of α-keto acids utilizing the 2-hydroxyquinoxalines (HQ)[11] which are formed by the condensation of α-keto acids with *o*-phenylenediamine (OPD) have been developed. The polarographic behavior of several HQ derivatives and a method for the determination of α-keto acids after the paper-chromatographic separation of their HQ's were also reported by Brockelt and Pohloudek-Fabini.[12]

The present authors have previously reported on the polarographic behavior of various carbonyl compounds in buffered solutions containing an excess of OPD, and on the application of the polarograms of the condensation products to the determination of each carbonyl compounds.[13-18]

The present paper will describe the polarographic behavior of several α-keto acids, *i.e.* glyoxylic acid (I), pyruvic acid (II), α-ketobutyric aicd (III),

1) M. Shikata and K. Shoji, *Nippon Nogei Kagaku Kaishi*, **4**, 475 (1928).
2) O. H. Müller and J. P. Baumberger, *J. Amer. Chem. Soc.*, **61**, 590 (1939).
3) R. Bridička, *Collect. Czech. Chem. Commun.*, **12**, 212 (1947).
4) S. Ono, M. Takagi, and T. Wasa, *ibid.*, **26**, 141 (1961).
5) M. Takagi, This Bulletin, **34**, 905 (1961).
6) M. Takagi, *Bull. Univ. Osaka Pref., Ser., B*, **11**, 177 (1961).
7) M. Brezina and P. Zuman, "Polarography in Medicine, Biochemistry, and Pharmacy," Revised English Ed., Interscience, New York (1958), p. 269.
8) D. J. D. Hocknhull and G. D. Floodgate, *Biochem. J.*, **52**, 38 (1952).
9) A. Harjanne, *Suomen Kemistilehti, B*, **28**, 37 (1955).
10) J. E. Spikner and J. C. Towne, *Anal. Chem.*, **34**, 1468 (1962).
11) O. Hinsberg, *Ann.*, **292**, 242 (1896).
12) G. Brockelt and R. Pohloudek-Fabini, *Scintica Pharmaceutica*, **31**, 94 (1963).
13) T. Wasa, M. Takagi, and S. Ono, This Bulletin, **34**, 518 (1961).
14) S. Musha, T. Wasa, and T. Naito, *ibid.*, **39**, 1902 (1966).
15) T. Wasa and S. Musha, *ibid.*, **40**, 1617 (1967).
16) T. Wasa and S. Musha, *ibid.*, **40**, 1624 (1967).
17) T. Wasa and S. Musha, *ibid.*, **41**, 1578 (1968).
18) T. Wasa and S. Musha, *Bull. Univ. Osaka Pref., Ser. A*, **17**, 139 (1968).

mesoxalic acid (IV), oxalacetic acid (V), and α-ketoglutaric acid (VI), in various buffered solutions containing an excess of OPD, and on a method for determining such α-keto acids by using the polarograms of the condensation products.

Experimental

Materials and Reagents. The α-keto acids used were commercial products and were used without further purification: I, sodium glyoxylate monohydrate; IV, calcium mesoxalate trihydrate (Tokyo Kasei Kogyo Co.); II, sodium pyruvate (E. Merck AG.); III, α-ketobutyric acid; VI, α-ketoglutaric acid (Wako Pure Chem. Ind.), and V, cis-oxalacetic acid (Sigma Chem. Co.). The stock solutions (about 10^{-2} M) of α-keto acids, except for IV, were prepared by dissolving the chemicals in redistilled water; IV was dissolved in dilute hydrochloric acid. The HQ derivative corresponding to each of α-keto acids was prepared according to the method of Morrison[19] by condensing α-keto acid with OPD in dilute acetic acid. The HQ derivative of V was not obtained because of the β-decarboxylation[9] which is catalyzed by OPD, and the product obtained was found to be 2-hydroxy-3-methylquinoxaline by thin-layer chromatography. The stock solutions (about 10^{-2} M) of HQ's were prepared by dissolving the products in 50% ethanol or 50% ethanol containing an equimolar amount of sodium hydroxide. OPD was a product for chromatography from E. Merck AG.; the stock solution of about 0.1 M was prepared fresh daily by dissolving OPD in redistilled water or 0.5 N hydrochloric acid. As the buffer solution, the Britton-Robinson buffer (BR) was mainly employed. In the experiments for HQ's without OPD, 0.001% Triton X-100 was used as the maximum suppressor, but in the presence of an excess of OPD in the electrolytic solution no suppressor was added, since the condensation products showed no maximum. All the other chemicals were the same as those in previous works.[14-18]

Apparatus and Procedures. The apparatus and the experimental procedures for the polarographic investigations were almost the same as those reported in previous papers.[14-18] The characteristics of the capillary used were $m=1.96$ mg/sec and $t=3.97$ sec/drop in a buffer solution when the height of the mercury reservoir was 69 cm at 0 V *vs.* SCE. The accuracy of the temperature control was ±0.1°C. The final pH value of the polarographic solution was measured with a glass electrode pH-meter. The pK values of the authentic HQ derivatives were evaluated from the pH-titration curves for 10^{-2} M HQ's containing 50% ethanol and 1 M potassium chloride. The pH-titration was carried out coulometrically at a constant current of about 10 to 20 mA in a titration cell equipped with a platinum cathode, a silver anode, and a glass indicator electrode.[20]

The cyclic voltammetric experiments were carried out using a multipupose instrument employing solid-state operational amplifier circuitry and with a three-electrode configuration, similar to that reported by Goolsby and Sawyer.[21]

19) D. C. Morrison, *J. Amer. Chem. Soc.*, **76**, 4483 (1954).

20) J. J. Lingane, "Electroanalytical Chemistry," 2nd Ed., Interscience, New York (1958), p. 607.

Results and Discussion

When α-keto acid was treated with OPD in buffered solutions of various pH values by the procedure described above, all the α-keto acids examined show a well-defined reduction wave at a potential more positive than that of the original acids at pH values below 4 (Fig. 1a), while there was a well-defined oxidation wave in the pH range between 4 and 8 (Fig. 1c). The relation between

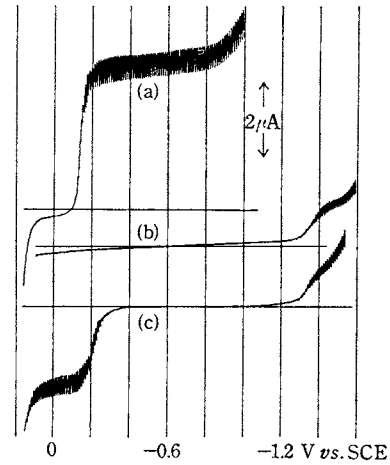


Fig. 1. Polarograms of IV with or without OPD at 25°C.
Mesoxalic acid: 5×10^{-4} M; condensation: 60 min
a) with 5×10^{-2} M OPD in 0.25 N HCl
b) without OPD in BR buffer (pH 6.6)
CO with 4×10^{-2} M OPD in BR buffer (pH 6.4)

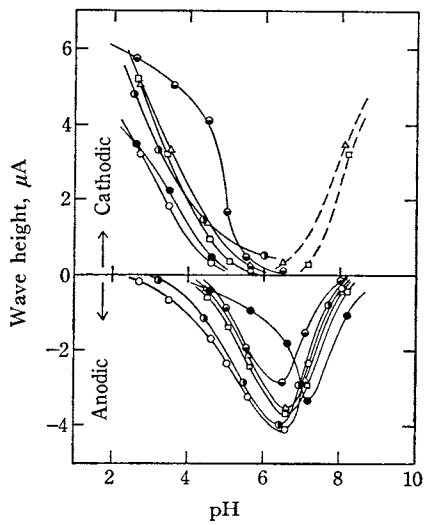


Fig. 2. Effect of pH on the condensation reaction of α-keto acids (about 1×10^{-3} M) with OPD (4×10^{-2} M) at 25°C.
Condensation: 30 min; ● I, □ II, ○ III, ◑ IV, △ V, and ◒ VI.

21) A. D. Goolsby and D. T. Sawyer, *Anal. Chem.*, **39**, 411 (1967).

the heights of the waves and the pH values of reaction media are presented in Fig. 2. The ill-defined reduction wave obtained at pH values above 7 (Fig. 2, dashed line) seems to be due to the reduction of the α-keto acid which had not reacted, because the half-wave potentials are in rough accord with those for free acids. Consequently, it is evident that two kinds of the products were produced by the condensation reactions of α-keto acid with OPD, depending on the pH values of the reaction mixtures.

Reduction Wave of the Condensation Product. Figure 2 shows that the reduction wave rapidly increases in height with a decrease in pH in the range below 4. The effect of the concentration of hydrochloric acid on the condensation reaction of α-keto acid with OPD was examined in the range from 0.05 to 2 N; the results obtained are shown in Fig. 3. The wave height, which is equivalent to the amount of the condensation product, had its maximum value in the range of 0.1 to 1 N hydrochloric acid. The same effect was also observed with sulfuric acid instead of hydrochloric acid.

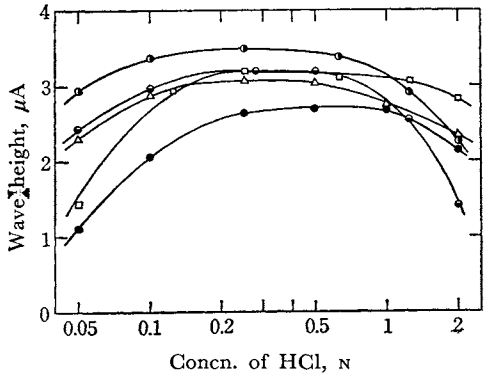


Fig. 3. Effect of the concentration of HCl on the condensation reaction at 25°C.
α-Keto acid: 5×10^{-4} M; OPD: 4×10^{-2} M; Condensation: 30 min
●—I, □—II, ◑—IV, △—V, and ◒—VI

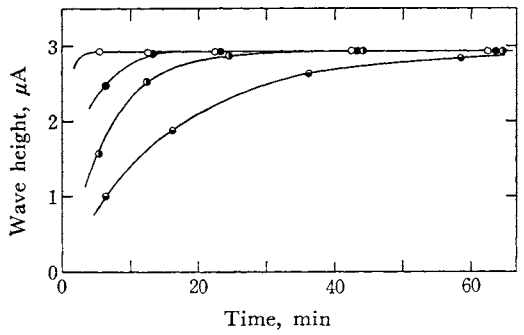


Fig. 4. Effect of the concentration of OPD at 25°C.
α-Ketoglutaric acid: 5×10^{-4} M; OPD: ○—8×10^{-2}, ●—4×10^{-2}, ◑—2×10^{-2}, and ◒—8×10^{-3} M

Figure 4 shows the relation between the wave height of the condensation products and the time elapsed after the addition of VI to the electrolytic solutions containing OPD in various concentrations in 0.25 N hydrochloric acid. With the increase in the concentration of OPD, the growing velocity of the reduction wave increased. A similar effect was also observed for all the other α-keto acids; the time required to reach the maximum value of the limiting current was 20—60 min with the concentrations of OPD higher than 2×10^{-2} M. Moreover, the saturation wave height obtained for each α-keto acid was found apparently to correspond to that of the authentic HQ of the same concentration.

The pH dependence of the wave heights of the condensation products, which were prepared by condensing α-keto acids with OPD in 0.25 N hydrochloric acid and by then neutralizing them with 1 N sodium hydroxide, is given in Fig. 5. The same pH-dependence was obtained for all the α-keto acids other than IV ; the limiting current remains almost constant at pH values lower than 9, while with an increase in pH above 9 a new second reduction wave (curve b), whose half-wave potential of about 100 mV more negative than that of the first wave (curve a), grows at the expense of the latter, which disappears at a pH value of about 11. For the condensation product of IV, a more complicated behavior was observed, as may be seen in Fig. 5 (dashed line): the reduction wave split into two waves at a pH value of about 2.

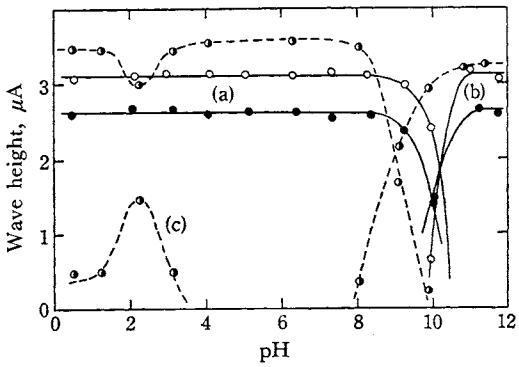


Fig. 5. pH-Dependence of the reduction waves of the condensation products (about 5×10^{-4} M) at 25°C.
—●— I, —○— II (III, V, and VI), --◑-- IV; (a)—1st wave, (b) and (c)—2nd wave

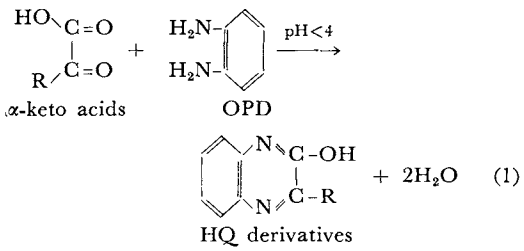
The half-wave potentials of the condensation products are listed in Table 1 as a function of the pH value, together with the pK value. The condensation products for each α-keto acid prepared in acidic media of pH lower than 4 were identical in polarographic behavior with the corresponding authentic HQ's obtained by Morrison's method;[19]

Table 1. Half-wave Potentials and dissociation constants (pK) for the condensation products at 25°C

α-Keto acid		$-E_{1/2}$, V *vs.* SCE							pK*
	pH: (1 N HCl)	2.0	4.0	6.0	8.0	10.0	11.0	
I	R_1	0.313	0.490	0.637	0.782	0.880	0.976		(9.08)
	R_2						1.076	1.097	
	Ox				0.12	0.22			
II	R_1	0.439	0.587	0.745	0.876	0.987	1.060		9.8
	R_2						1.160	1.188	
	Ox				0.11	0.18			
III	R_1	0.438	0.592	0.739	0.880	1.002	1.050		
	R_2						1.143	1.180	
	Ox			0.11	0.12	0.17			
IV	R_1	0.108	0.181	0.363	0.548	0.728			3.2, 9.5
	R_2	0.78	0.92			1.015	1.080	1.146	
	Ox			0.24	0.24	0.24			
V	R_1	0.436	0.590	0.739	0.880	0.993	1.065		
	R_2						1.160	1.180	
	Ox				0.11	0.18			
VI	R_1	0.428	0.576	0.695	0.827	0.982	1.065		5.4, 10.1
	R_2						1.240	1.276	
	Ox				0.14	0.21			

R_1: 1st reduction wave; R_2: 2nd reduction wave; Ox: oxidation wave.
* in 1 M KCl containing 50% ethanol.
( ) A. Albert and J. N. Phillips, *J. Chem. Soc.*, **1956**, 1294.

the present results are also in accordance with the observation by Brockelt and Pohloudek-Fabini,[12] when the pH values of their electrolytic solutions are corrected, taking into account the alcohol error caused by the 50% ethanol addition. As has been reported by Hinsberg,[11] the reaction may be written as:



The limiting current for each condensation product at pH values lower than 9 or higher than 11 was the diffusion-controlled current, since the wave height varied in proportion to the square root of the height of the mercury reservoir. By comparing the wave height for the HQ derivatives with that for dimethylquinoxaline[15] at the same concentration, a two-electron reduction was deduced for the reduction wave of HQ.

A cyclic voltammogram of 2-hydroxy-3-methylquinoxaline with a hanging mercury drop electrode is given in Fig. 6 (a); it is typical of the cyclic voltammetric behavior exhibited by the HQ derivatives examined: the electron-transfer step exhibits no reversal peak upon scan reversal about 160 mV past the cathodic peak. Though this result seemingly suggests that the electron transfer is irreversible, a small a. c. peak was observed at the potential crresponding to the d. c. wave for each HQ derivative over all the pH range studied (pH 0—12), the peak height being about one-tenth of that for dimethylquinoxaline at the same concentration. From both of these results, the electrode reaction of HQ derivtives, though a more complete explanation of the mechanism must await further investigation, may be deduced to be a reversible two-electron transfer, followed by a fast chemical reaction.[22]

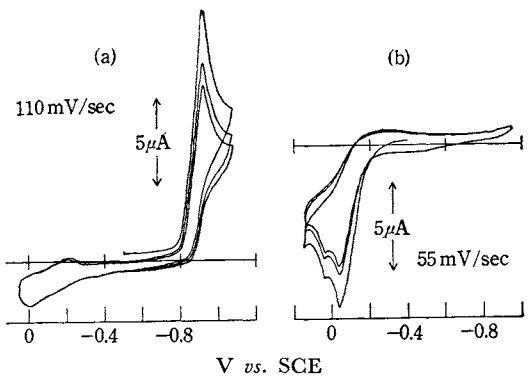
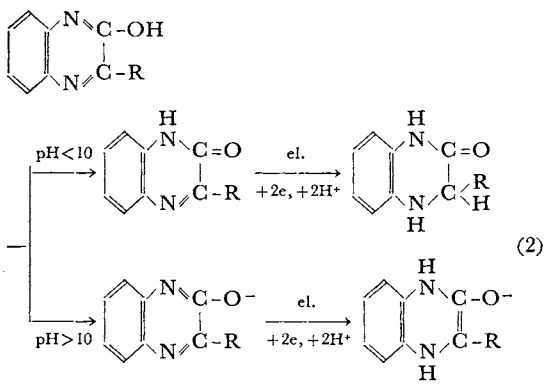


Fig. 6. Cyclic voltammograms of the condensation products of II with OPD at 25°C.
a) cathodic peak of 2-hydroxy-3-methylquinoxaline (5×10^{-4} M) in BR buffer (pH 6.0)
b) anodic peak of II (5×10^{-4} M) in BR buffer (pH 6.0) containing 2×10^{-2} M OPD; condensation: 30 min

22) Joe L. Sadler and Allen J. Bard, *J. Amer. Chem. Soc.*, **90**, 1979 (1968).

From the facts that the pH values, at which the height of the first wave is equal to that of the second wave, were found to be near the pK values (Table 1) for the hydroxyl group of each HQ and that HQ derivatives were proved by the spectrophotometric study[23] to be present in the aqueous solution largely as the amide form, it may be concluded that the first and the second reduction wave of HQ are due to the reduction of the undissociated tautomeric amide form and the dissociated enol form respectively. The electrode reaction may be written as:



(2)

Oxidation Wave of the Condensation Products. The pH-dependence of the oxidation waves of the condensation products of α-keto acids with OPD (shown in Fig. 2 and Table 1) was, in general, very similar to that observed for monoaldehydes:[16,17,24] the wave heights, which are equivalent to the amount of the condensation product, attain their maximum value in the pH range between 6 and 7.5, and then decrease beyond this pH region. Figure 7 shows the change in the anodic limiting current in buffered solutions (pH: 7.17, 6.10, and 5.30) containing II and OPD with the time elapsed after the addition of OPD. At pH values higher than 6 the condensation reaction was slow (curve b). On the other hand, at pH values lower than 6 the condensation product was unstable (curve c), though the condensation reaction itself was very rapid. At a pH value of about 6 the reaction of every α-keto acid with OPD attained its equilibrium state within 20—60 min after the addition of OPD and the condensation products were comparatively stable (curve a). The oxidation wave was also diffusion-controlled.

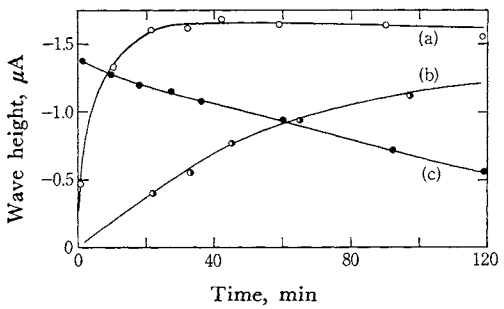


Fig. 7. Change of the oxidation wave for 5×10^{-4} M II with time in BR buffer containing 4×10^{-2} M OPD at 25°C.
a) pH 6.10, b) pH 7.17, and c) pH 5.30

A representative cyclic voltammogram of the condensation product is shown in Fig. 6 (b): the electron-transfer step exhibits no cathodic peak upon scan reversal. Moreover, from the facts that no a. c. polarogram was observed with the condensation product and that no reduction wave was obtained with the oxidation product prepared by aeration through the solution containing the condensation product, the electrode reaction may be considered to be irreversible.

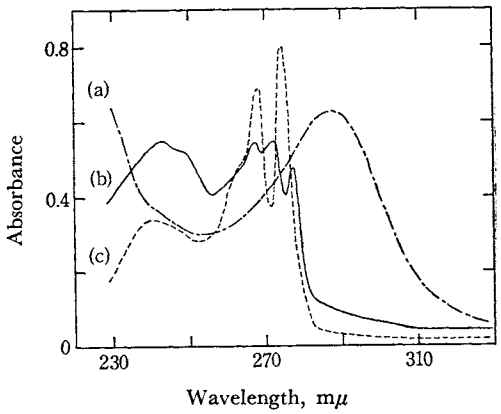
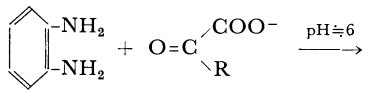


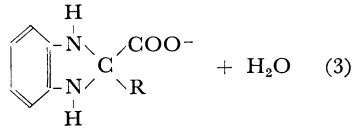
Fig. 8. UV-absorption spectra of the oxidation products corresponding to I (b), II (c), and IV (a) in BR buffer (pH 6.0).
α-Keto acid: 2×10^{-3} M; OPD: about 1×10^{-4} M
Spectrum was recorded for the condensation product after complete oxidation with air.

Figure 8 shows the UV-absorption spectra of the air-oxidation products. The absorption curves obtained for II, III, V, and VI were similar to each other, but they were entirely different from that for IV. The curves for I, II, and III were also identical with those of benzimidazole and the oxidation products from the condensation product of OPD with acetaldehyde and with propionaldehyde respectively. From the results described above, it may be concluded that the condensation products exhibiting oxidation waves are benzimidazolines and that their electro-oxidation products are benzimidazole derivatives, as has previously been reported for aldehydes[16,17] and ketones.[25] Their reactions may be expressed as follows:

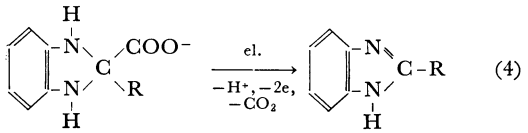
23) G. W. H. Cheeseman, *J. Chem. Soc.*, **1958**, 108.
24) M. Fedoroňko, J. Königstein and M. Bullová, *Chem. Zvesti*, **22**, 25 (1968).
25) R. C. Elderfield and J. R. McCarthy, *J. Amer. Chem. Soc.*, **73**, 975 (1951).



OPD α-keto acids



benzimidazolines



Analytical Application. From the results described above, the following conditions are recommended for the determintion of α-keto acids by using the reduction wave of the condensation products with OPD: 0.1—1 N for the concentration of hydrochloric acid, (2—4) × 10^{-2} M for the concentration of OPD, 25°C for the temperature, and 20—60 min for the condensation time. To avoid the precipitation of the condensation products, it is desirable to add ethanol (about 10% in volume) to the reaction mixture. Under these conditions, a linear relationship was obtained between the concentration of each α-keto acid in the range of (0.5—20) × 10^{-4} M and the wave height of the corresponding condensation product, as may be seen in Fig. 9.

Alcohols, carbohydrates, monocarbonyl compounds such as aliphatic aldehydes and ketones, and all carboxylic acids except α-keto acids did not interfere with the determination of α-keto acid. Since the condensation products of glyoxals[14,15] behave in a way similar to that of α-keto acids at the dropping mercury electrode, the presence of any glyoxal may interfere with the determination of α-keto acids.

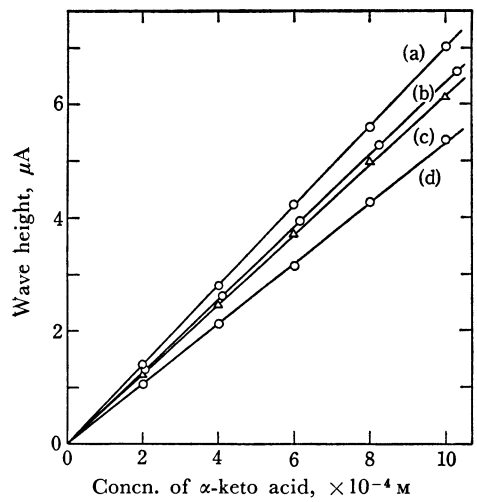


Fig. 9. Relationships between the wave heights (cathodic) of the condensation products and the concentrations of α-keto acids in 0.25 N HCl at 25°C. OPD: 5×10^{-2} M; condensation: 30 min a)—IV, b)—II (III and VI), c)—V, and d)—I

As an application, the determination of II in commercial products containing II as an impurity was carried out by means of the standard addition method; some of the analytical results obtained are presented in Table 2, together with those obtained by the direct polarographic method.[7] When no interfering substance is present in the sample, the results obtained by the two methods are in good agreement with each other. Even in the case of the sample containing a substance interfering with the direct polarographic method, the present polarographic OPD method is valid because the half-wave potentials of the condensation products shift to more positive potentials than that of free α-keto acid.

Table 2. The results of the determination of II in commercial products
OPD: 5×10^{-2} M, 0.5 N HCl, 25°C, 60 min

Sample	Pyruvic acid content, %	
	Polaro. OPD method	Direct polaro.*
Lactic acid (95%)	0.0112	0.0113
Lactic acid (75%)	0.0084	0.0105
Acidophilus milk (liquid)	0.0119	0.0153
Acidophilus milk (powder)	0.0064	0.504

* According to the procedure of Ref. 7, p. 269.

With regard to the oxidation wave, a linear relationship was also observed between the concentration of each α-keto acid and the wave height under strictly controlled conditions. However, the wave height changes with the concentration of OPD since the equilibrium constant for the condensation reaction seems to be relatively small, much like that for monoaldehydes,[16,17] and it decreases with time since the condensation product is easily oxidizable with the dissolving oxygen. For analytical purposes, therefore, the concentration of OPD in the reaction mixture should be kept in a large excess and should be kept constant, and the dissolving oxygen should be removed by a stream of nitrogen immediately after the preparation of the electrolytic solution.

The authors wish to express their thanks to Dr. Masanosuke Takagi for his helpful discussions and suggestions during this work and to Mr. Iwao Abe for his assistance in the experimental work.