The Polarographic Behavior and Determination of Orotic Acid (Vitamin B_{13}) in Milk

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The polarographic behavior of orotic acid at the dropping mercury electrode was studied in the Britton-Robinson buffer solution; it was found to be a complex function of the pH. Three polarographic waves were observed over the pH range from 2 to 12. In the 1M $HClO_4$ electrolyte, orotic acid showed a very well-defined, diffusion-controlled, two-electron polarographic reduction wave which enabled us to determine orotic acid polarographically. A polarographic method for the determination of orotic acid in milk was proposed, and the effect of the presence of surface-active substances like proteins was investigated. The proposed method is very simple and rapid, and permits the accurate determination of 4—1000 μg/g of orotic acid in milk.

In recent years, the electrochemical reduction and oxidation of biologically-important compounds have been investigated with much interest. An examination of the oxidation-reduction behavior of compounds of biological significance is often of considerable value. Although actual biological redox systems may be of extreme complexity as a result of enzyme interactions, much information can be obtained from the study of these compounds in aqueous solutions with regard to factors affecting the electron-transfer processes, such as the pH.

Orotic acid (2,6-dihydroxypyrimidine-4-carboxylic acid), one of the uracil derivatives, is also known as vitamin B_{13}. Icha[1] has described orotic acid as polarographically-active. However, no detailed study of the electrode process and no application of polarography to the analysis of orotic acid in biological materials have been carried out. Recently, Archer[2] determined the orotic acid content in milk bread by the colorimetric method[3] as modified by Brieskorn and Wallrauch.[4] This method, however, is time-consuming and complicated.

The present work was carried out in order to study the polarographic behavior of orotic acid in detail and to investigate the applicability of polarography to the rapid analysis of orotic acid in cow's milk.

Experimental

Chemicals. The orotic acid (reagent of the Wako Pure Chemical Co.) was recrystallized three times from distilled water before drying at 60 °C *in vacuo*. A 5 mM stock solution was prepared by dissolving an appropriate amount of orotic acid in distilled water. The solutions used for the analysis were prepared by the dilution of the stock solution with aqueous buffer solutions and acid solutions to give the desired concentrations. The other chemicals were of a reagent grade and were used without further purification.

Apparatus. The polarograms were recorded using a Yanagimoto P-8 type Polarograph. Cyclic voltammetry was performed with a versatile solid-state instrument constructed in this laboratory following the design of Goolsby and Sawyer,[5] while the voltammograms were recorded using a Hewlett Packard Model 7045A X-Y recorder.

The dropping mercury electrode had the following characteristics: mercury flow rate, $m=2.25$ mg/s and drop time, $t=3.38$ s at open circuit and at a height of the mercury reservoir of 70 cm in 1 M $HClO_4$. A hanging mercury-drop electrode was used as a working electrode for the cyclic voltammetric experiments, while a mercury pool was employed for the controlled potential coulometric measurements. The reference electrode was SCE, and a platinum coil was used as an auxiliary electrode. All the experiments were carried out at 25.0±0.1 °C.

Results and Discussion

Polarographic Behavior of Orotic Acid. Effect of the pH: The effect of the pH on the polarographic curves was investigated by recording the polarograms of 0.5 mM orotic acid in the Britton-Robinson buffer solution. The half-wave potential and the reduction current of orotic acid with respect to the pH values are shown in Fig. 1.

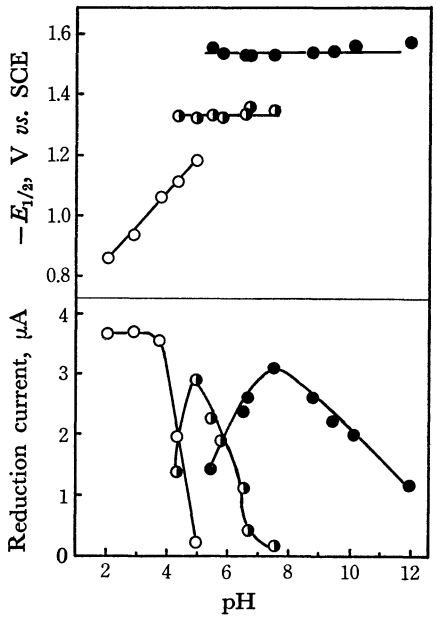

Fig. 1. The effect of pH on the reduction current and the half-wave potential of 0.5 mM orotic acid (Britton-Robinson buffer).
○: 1st wave, ◑: 2nd wave, ●: 3rd wave

Between pH 2 and 12, three polarographic reduction waves were observed. The first wave appeared in the pH range of 2—5. The half-wave potential shifted linearly towards a negative potential with an increase in the pH; this implies that hydrogen ions are consumed in the first reduction step. At pH values below 5, the half-wave potential of the first wave is given by the equation:

$$E_{1/2} = -0.635 - 0.108\ \mathrm{pH} \quad (1)$$

The height of the wave remained almost constant up to about pH 3.8. Above pH 3.8 it decreased sharply, and it vanished at about pH 5.1. The diffusion-current constant ($I=i_d/Cm^{2/3}t^{1/6}$) of the first wave at pH 3.1 was 3.92, corresponding to a two-electron Faradaic process.

The half-wave potentials of the second wave and the third wave were independent of the pH of the electrolyte. The second wave appeared in the pH range of 4—8, and the third wave, above pH 5. The reduction current of both waves was dependent on the pH. The sum of the heights of these three waves was almost constant up to about pH 7.5.

Calculation of αn_a *and* P: The values of αn_a and of P(the number of hydrogen ions involved per molecule of the reactant in the rate-controlling step) were calculated on the basis of Eqs. (2) and (3);[6,7] they are summarized in Table 1.

$$E_{1/4} - E_{3/4} = 0.0517/\alpha n_a \quad (2)$$

$$\mathrm{d}(E_{1/2})/\mathrm{d}(\mathrm{pH}) = -0.05915\,P/\alpha n_a \quad (3)$$

For the first wave, an average αn_a value of 1.32 and an average P value of 2.41 were determined. The average αn_a values of the second and third waves were 1.48 and 0.99 respectively.

Effects of the Drop-Time and the Temperature: The effect of the drop-time on the limiting current was investigated by recording the polarograms of 0.5 mM orotic acid at various heights of the mercury column. The $I/\sqrt{h}$ values, where h is the height of the column after correction for the back pressure, for all three polarographic waves were constant. The temperature coefficient of the limiting current of the three polarographic waves of orotic acid was 0.6—1.4% per degree in the temperature range from 15 to 45 °C. These results indicate that each of the three polarographic waves is diffusion-controlled.

Controlled Potential Coulometry. Coulometric reductions at the controlled potential of orotic acid in the Britton-Robinson buffer were performed in an attempt to determine the number of electrons involved in the overall electron transfer reactions. At pH 2.57 the potential of the mercury pool was first controlled at −1.15 V *vs.* SCE In this experiment, 5.23 coulombs were consumed in the reduction of 2.5×10^{-5} mol of orotic acid, which yielded $n=2.17$ for the first reduction wave. The experiments were repeated at pH 5.00 and 7.90 with new solutions containing the same amount of orotic acid and with the potentials of the working electrde controlled at−1.45 and−1.75 V respectively. The n values of the second and third reduction waves were 1.83 and 1.86 respectively. These experiments imply that two electrons are involved in each of the three polarographic steps.

Cyclic Voltammetry. Cyclic voltammetric experiments were performed at a hanging mercury drop electrode. The mercury drop was renewed each time. If the potential sweep was repeated at the same mercury drop, the peak height decreased and the peak potential shifted towards a more negative value, indicating the adsorption of a reduction product at the electrode. Orotic acid gave a cathodic peak corresponding to each of the three polarographic waves observed at various pH values, the heights of which were proportional to the square root of the scan rate in the range from 1.0 to 15 V/min. However, no anodic peak resulting from the reoxidation of the reduction product was observed at any scan rate or any switching potential.

Analytical Application. Effect of Buffer and Acid Components: Polarographic experiments revealed that a well-defined wave appeared at low pH values; the height of this wave was almost constant up to about pH 3.8. Therefore, the polarographic behavior of the first wave at low pH values was investigated by using other buffers (acetate and chloride buffer) and acids (HCl, H_2SO_4, and $HClO_4$) as electrolytes. In these electrolytes, the half-wave potential of orotic acid shifted towards a positive potential with a decrease in the pH. The $E_{1/4}$–$E_{3/4}$ value decreased with a decrease in the pH of the electrolytes. The buffer and acid components, however, had no influence on the wave-height.

When HCl and H_2SO_4 were used as supporting electrolytes, a large maximum appeared in any acid concentration. However, in the case of a $HClO_4$ background, no maximum appeared and the polarographic

TABLE 1. EFFECT OF pH ON THE POLAROGRAPHIC REDUCTION WAVES OF 0.5 mM OROTIC ACID IN THE BRITTON-ROBINSON BUFFER

pH	1st wave			2nd wave		3rd wave	
	$E_{1/4}-E_{3/4}$(mV)	αn_a	P	$E_{1/4}-E_{3/4}$(mV)	αn_a	$E_{1/4}-E_{3/4}$(mV)	αn_a
2.93	40	1.29	2.36				
3.86	39	1.33	2.43	39	1.32		
4.36	39	1.33	2.43	38	1.36		
5.02				34	1.52		
5.54				33	1.57	68	0.76
5.83				32	1.61	50	1.03
6.60				35	1.47	51	1.01
6.72						54	0.95
7.56						57	0.90
8.80						54	0.95
9.53						49	1.05
10.20						51	1.01

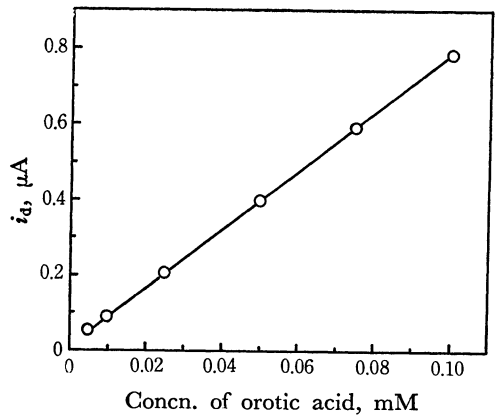

Fig. 2. Calibration curve for orotic acid in 1 M $HClO_4$.

wave was well-defined. The effect of the drop-time was investigated by recording polarograms of 0.1 mM orotic acid in 1 M $HClO_4$ at various heights of the mercury column. The limiting current of orotic acid was approximately proportional to $h^{1/2}$, indicating that the reduction current is controlled by diffusion. The diffusion current of orotic acid in 1M $HClO_4$ was proportional to the concentration in the 5×10^{-6}—10^{-4} M range as is shown in Fig. 2.

Consequently, 1M $HClO_4$ was used as the supporting electrolyte in the following experiments for the determination of orotic acid.

Effect of Protein Addition: A polarographic wave of orotic acid was observed even in the presence of 50% milk. However, the proteins present in milk are surface-active, and so the polarographic wave of orotic acid was partly distorted by the adsorbed layer of proteins on the electrode. The limiting current was depressed, and the half-wave potential shifted towards a more negative potential.

The effect of proteins on polarograms of 0.1 mM orotic acid in 1M $HClO_4$ was investigated by adding proteins refined by centrifuging milk several times. The results are shown in Table 2. On the addition of more than 0.3% of proteins, the half-wave potential became more negative and the $E_{1/4}$—$E_{3/4}$ value increased. However, the wave height of orotic acid was not affected by the presence of less than 0.5% of proteins. These results indicate that the presence of a large amount of proteins produces a drawn-out wave at a more negative potential with an increase in the protein concentration, and that the removal of most proteins is necessary for the determination of orotic acid in milk.

Table 2. Effect of protein on the polarograms of 0.1 mM orotic acid in 1 M $HClO_4$

Protein, wt%	$-E_{1/2}$, V *vs.* SCE	$E_{1/4}-E_{3/4}$, mV	i_d, μA
0	0.678	30	0.771
0.02	0.675	30	0.780
0.05	0.680	30	0.759
0.08	0.680	30	0.783
0.15	0.680	30	0.790
0.30	0.693	38	0.776
0.45	0.710	50	0.773
0.60	0.732	72	0.742
0.90	0.750	85	0.720
1.2	0.770	80	0.721
1.5	0.780	135	0.674

Determination of Orotic Acid in Milk: The method for the determination of orotic acid in milk is as follows: Weigh accurately a quantity of a sample expected to contain from 0.1 to 0.6 mg of orotic acid into a 100-ml beaker. Add 10—20 ml of 1M $HClO_4$, mix, and place the mixture in a water-bath at 65 °C for 30 min. Then remove the beaker from the water-bath, cool to room temperature, and filter the coagulated proteins through fluted filter-paper. Wash the precipitate with 20 ml of 1M $HClO_4$, transfer the solution into a 50-ml calibrated flask, and dilute to the mark with 1M $HClO_4$. Transfer this solution into a polarographic cell. Remove the dissolved air with nitrogen and record a polarogram.

Various types of artificial milk containing known amounts of orotic acid and known amounts of refined proteins were prepared, and the proteins present in these milk were removed by the proposed procedure. The recovery experiments were carried out by analyz-

Table 3. Recovery of orotic acid

Taken orotic acid (μg)	Added protein (wt%)	Found (μg)	Recovery (%)
99.8	1.02	98.2	98.4
99.8	1.57	97.5	97.7
171.6	0.44	170.0	99.1
171.6	0.63	170.0	99.1
171.6	1.04	161.5	94.1
240.2	0.59	234.0	97.4
255.1	0.35	243.7	95.5

Table 4. Determination of orotic acid in cow's milk

	Taken cow's milk (g)	Found (μg/g) This work (A)[a]	This work (B)[b]	Colorimery
A	1.001	109	102	106
	2.205	118	115	
	3.197	113	117	108
	4.763	105	117	
	Average	111	113	
Deviation coefficient (%)		4.7	6.1	
B	1.129	82	78	74
	2.100	78	80	
	3.331	79	72	71
	4.605	75	78	
	Average	79	77	
Deviation coefficient (%)		3.7	4.5	
C	1.022	118	122	120
	2.536	126	126	
	3.198	115	116	102
	4.501	122	117	
	Average	120	120	
Deviation coefficient (%)		4.0	3.9	

a) Determined by using the calibration graph.
b) Determined by the standard addition method.

ing orotic acid in these filtrates. The $E_{1/4}$—$E_{3/4}$ values of the polarograms recorded were about 30 mV, indicating that most proteins were removed by this procedure(see Table 2). The results, shown in Table 3, indicate that a tolerable agreement between the amounts of orotic acid added and the amounts recovered is obtained by this procedure.

The precision of this analytical method for orotic acid was determined by analyzing three samples(A, B, and C) of cow's milk four times each. The determination was carried out by both the calibration graph method and the standard addition method. The results, shown in Table 4, were compared with those obtained by the colorimetric method.[2)]

Compared with the colorimetric method, which involves very time-consuming and complicated procedures, the proposed polarographic method is very simple and rapid and permits the accurate determination of 4—1000 μg/g of orotic acid in milk.

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