both reactions are characterized by an early proliferative phase followed by the appearance of specifically cytotoxic lymphocytes (1, 3, 24, 25).

The graft-versus-host reaction, as assayed by splenomegaly and lethality, is mediated by a synergistic interaction between two subpopulations of thymus-derived cells (11, 26). One subpopulation (T1) is: (1) present in high concentration in spleen and thymus, (2) nonrecirculating, (3) insensitive to antilymphocyte serum, (4) rapidly depleted by adult thymectomy, and (5) possesses relatively large amounts of surface-associated θ antigen. The second population (T2) is: (1) present in high concentration in lymph nodes and peripheral blood, (2) recirculating, (3) sensitive to antilymphocyte serum, (4) slowly depleted by adult thymectomy, and (5) possesses relatively small amounts of surface-associated θ antigen.

Although the mechanism underlying the cooperative interaction between these two cell populations remains unknown, T1 cells have been shown to be the direct precursors of effector lymphocytes while the T2 cells somehow potentiate activation of the T1 population. The demonstration by the current studies of in vitro synergism between thymus and lymph node cells in the proliferative and effector phases of the mixed lymphocyte reaction closely coincides with the T1–T2 model. With respect to tissue distribution, the cell population responsible for the proliferative phase of the mixed lymphocyte reaction appears to correspond to the T2 population while that responsible for effector cell production resembles the T1 population (3, 27). In addition, we have found that pretreatment of spleen-cell suspensions with appropriate dilutions of anti-θ serum and complement more effectively inhibits effector cell generation than proliferation (27). These results suggest that the subset of cells responsible for effector cell production has more surface-associated θ antigen than that responsible for proliferation and provide further evidence that the cell populations defined by the current studies correspond to T1 and T2.

Although the current studies clearly demonstrate synergism between lymphoid cell populations participating in the mixed lymphocyte reaction, many questions concerning the nature of this cooperative interaction remain to be answered. For example, it will be of interest to determine the time necessary for the interacting cell populations to be cocultured in order to obtain optimal responsiveness. Another question is whether the increases in the proliferative and effector responses observed in the cell mixtures are mediated by the same or different cell populations. It will also be of interest to determine whether the cell populations responsible for synergism are of thymus or lymph node origin. Further, it is not known whether the observed cooperation requires cell-to-cell contact or is mediated by soluble cell products. Answers to these and other questions gained through study of the in vitro model described herein will undoubtedly provide insight into the mechanisms of thymus cell synergism normally occurring in vivo.

Portions of this investigation were made by L. C. in partial fulfillment of the requirements for the degree of Master of Science, Long Island University, Brooklyn, N.Y. This investigation was supported by United States Public Health Service Grant AI-10158-03.


Correction. In the article “Characteristics of Polargraphic Catalytic Waves Observed with Bovine-Serum Albumin: Kinetic or Diffusion Control,” by Kolthoff, I. M., Yamasita, K., Hie, T. B. & Kanbe, A., which appeared in the July 1973 issue of Proc. Nat. Acad. Sci. USA 70, 2020–2024, the legend to Fig. 3, p. 2022, fourth line should read: “... curves 8–12, with 18, 36, 54, 72, 90, 108, 126, 144, 162, and 180 μM BSA, respectively.”
Characteristics of Polarographic Catalytic Waves Observed with Bovine-Serum Albumin: Kinetic or Diffusion Control

I. M. KOLTHOFF, K. YAMASHITA, TAN BOEN HIE, AND A. KANBE

School of Chemistry, University of Minnesota, Minneapolis, Minn. 55455

Contributed by I. M. Kolthoff, April 26, 1973

ABSTRACT Conditions are described under which the catalytic Brdicka currents observed in ammoniacal buffer solutions containing cobalt(III) hexammine chloride or cobalt(II) and very little bovine-serum albumin (0.1-0.2 μM) are purely kinetically controlled. Under these conditions the kinetic current at pH 9.3-10.3 exhibits a sharp maximum followed by a decrease of the current to a well-defined minimum. The maximum and minimum are eliminated in the presence of 5 mM or more concentrated calcium chloride when a short plateau occurs at the potential of the maximum followed by a well-defined second wave. Both currents are kinetically controlled. When the albumin concentration is 2 μM or greater, the kinetic currents approach diffusion control, the rate of diffusion of cobalt(III) or (II) being close to rate determining. Under these conditions the currents become proportional to the cobalt(III) or (II) concentration in the concentration range between 25 and 200 μM.

40 Years ago Brdicka (1) observed typical catalytic polarographic currents (waves) with sulphydryl- and/or disulfide-containing proteins in ammoniacal buffers containing cobalt(III) hexammine chloride [denoted in this paper as Co(III)] or cobalt(II) added as chloride. These catalytic currents are usually referred to as Brdicka currents. Since their discovery, a very large number of papers has been published, and several summaries are found in the literature (2-7). Most papers dealing with Brdicka currents present factual information, the kinetics of these currents and the reaction mechanism at the electrode still being incompletely understood. During the last several years, we have made extensive studies of Brdicka currents observed with bovine-serum albumin (BSA), a protein that contains close to 1 sulphydryl and 17 sulfide groups. In the present paper, we deal mainly with the kinetics of these currents and the reactions at the electrode surface under specified conditions, in ammoniacal buffers; in subsequent papers, characteristics will be described in other buffers and different conditions.

In some ammoniacal buffers in the presence of Co(III) or Co(II) BSA in very small concentrations yields three catalytic waves, the third one occurring also in the absence of cobalt(III) or (II) and generally being referred to as the "presodium wave," an unfortunate notation that might better be replaced by "protein hydrogen wave." We have observed that, at small BSA concentration, calcium ions in small concentrations greatly affect the shape of the Brdicka currents (see Experimental) and make the kinetics sharply defined.

Studies of the kinetics of Brdicka currents observed with human serum albumin, gamma globulin, and ovalbumin in a buffer that was 0.1 M in both ammonia and ammonium chloride have been made by Kútová and Březina (8). Their results with their interpretation are discussed in a later section.

EXPERIMENTAL

Chemicals. All chemicals used were of reagent-grade quality. Over the years, three high-quality commercial bovine-serum albumin products were used, Armour product, lot A69050, Cyclochemical albumin, lot H-3691-E, and Calbiochem., lot 46225. They behaved in an identical way. Their water content was determined by drying for 2 h in a vacuum oven at 90°. Stock solutions, 1-4% in BSA, were stored in a refrigerator at 5°. The concentration of the various dilutions was often checked spectrophotometrically.

Polarography. Prior treatment before polarography: In general, the solution containing BSA, buffer, and Co(III) or Co(II) was made air-free in a cylindrical polarographic cell of the same design as that described (9). Nitrogen was passed in a brisk stream through three washbottles partly filled with the same buffer as used in the polarographic cell, then through a fritted-glass filter in the solution in the cell. Especially at relatively low pH much foam is formed. In order to find whether protein was concentrated in the foam, the latter was passed into an inverted funnel that was connected with a U tube, the foam being collected in a graduate cylinder. After proper dilution, the protein in the cylinder was determined polarographically by measurement of the Brdicka currents and comparison of the magnitude of these currents with those of BSA solutions of known concentration. In most of these experiments the initial BSA concentration was 1 μM. Foaming was most pronounced close to the isoelectric point; e.g., in an acetate buffer of pH 5.5. After nitrogen was passed through for 15-30 min, the solution was protein free, all BSA being recovered in the foam. Foaming became progressively less with increasing pH. In experiments in Tris buffers of pH values 7.8 and 9, all the BSA was transferred with the foam after 1 hr of passage of nitrogen. Foaming was also considerable in ammonia buffers, but when nitrogen was passed through in a slow stream, the foam dissolved when stirring was discontinued, and no protein was lost. Foaming was prevented completely by addition of a fraction of a drop of octanol to 50 or 100 ml of the solutions at any pH above 5. The octanol had no effect on the Brdicka currents, but displaced the first reduction current of Co(III) to more negative potential, the
alcohol being desorbed at potentials where Brdička currents are observed. Oxygen has no effect on these currents and many workers in the literature did not make solutions air-free in studies of Brdička currents. When working with Co(II) in Tris buffers, we find that the cobalt is fairly easily air-oxidized to Co(III) and that it is advisable to make solutions air-free before adding the cobalt(II).

In the present determinations of the effect of the height of mercury (hHE) in the reservoir and of current-time curves, solutions were always made air-free. In deriving the effect of hHE on the first Brdička current, i1, the diffusion current of Co(III) or (II) plus the residual current of the protein in the cobalt-free buffer was subtracted from the current measured at the proper potential. Values of the second wave, i2, were taken equal to i1 - i1, i3 being taken at the potential of i1.

In all instances, the pH of the solution was measured before and after the polarographic measurement and found unchanged.

**Instrument.** Most polarograms were determined with a Sargent polarograph S-29311, model XVI. Capillaries of similar characteristics have been used over the years by the various authors of this paper; under usual working conditions the drop time was 5–7 sec, and the amounts of mercury flowing per second were of the order of 1.2 mg. Whenever desirable, exact figures are given. Potentials refer to the saturated calomel electrode.

All experiments were performed at 25.0°C.

**RESULTS**

**Kinetically controlled currents**

The shape and pattern of Brdička waves vary with the kind of buffer, concentration and ratio of concentrations of buffer constituents, ionic strength, presence of divalent cations, (calcium, see below), and particularly with concentrations and ratio of concentrations of BSA and cobalt(III) or (II). In ammonia buffers patterns of Brdička currents are similar in the presence of cobalt(III) or (II), in general these currents being somewhat larger with Co(III) than with Co(II) at BSA concentrations less than 0.3 μM. (This was not found to be true in Tris buffers.)

In Fig. 1 an example is presented of a polarogram with Co(III) in a buffer of pH 9.65, 0.1 M in ammonium chloride, and 0.26 M in ammonia (t = 5.15 sec at E = 0 V against a saturated calomel electrode (S.C.E.) and m = 1.472 mg/sec). A sharp maximum, referred to as i1, is observed at −1.32 V, followed by a poorly defined minimum plateau. The striking effect of calcium chloride is also illustrated in Fig. 1. It appears that i1 is only slightly affected and that a second wave is observed with a limiting value close to −1.6 V before the “presodium current” starts. The sharp maximum in the cobalt reduction at −1.26 V is greatly suppressed, but not completely eliminated, by BSA in a concentration of 0.28 μM. At a BSA concentration of 0.15 μM the maximum is suppressed to a much smaller extent and is still acute. In buffers like that in Fig. 1, but at ammonia concentrations of 0.1 and 1 M, the polarograms have slightly different shape but the calcium effect remains similar to that in Fig. 1. When the height of the mercury hHE (corrected for back pressure) is changed from 92.5 to 42.5 cm under conditions as in Fig. 1, the current decreased only from 13.65 to 12.70 μA.

In Fig. 2 is given an example of plots of log i against log t under conditions as in Fig. 1, but at a cobalt(III) concentration of 50 μM. All plots yield straight lines with slopes reported in Table 1. This table also presents data obtained under different conditions.

**Diffusion control of Brdička currents**

The pattern of the Brdička current–voltage curves at relatively large BSA concentrations varies greatly with the BSA and cobalt concentrations and also with the kind of buffer used. In 0.1 M NH4Cl–1 M NH3 at a Co(III) concentration of 25 μM there occurs a sharp maximum at −1.48 V at a BSA concentration of 1.8 μM. This maximum becomes less pronounced with increasing BSA concentration and no longer occurs when [BSA] = 7 μM.

As is illustrated in Fig. 3 under the experimental conditions a limiting value of the Brdička current is obtained at [BSA] = 1.8 μM. With further increase of BSA concentration the Brdička current does not change. The limiting Brdička current is attained at larger BSA concentrations with increasing cobalt concentrations. However, when the latter is equal to about 100 μM or greater, the Brdička current starts to coalesce.

---

**Fig. 1.** Brdička currents at small BSA concentration and effect of calcium chloride. Buffer is 0.1 M in ammonium chloride, 0.26 M in ammonia (pH 9.65). Curve 1, 400 μM [Co(NH4)2]Cl2; Curve 2, as I + 0.28 μM BSA; Curve 3, as 2 + 0.01 M CaCl2. Currents are maximum currents, i.e., current when drop falls.

**Fig. 2.** Plots of log i against log t. Curve 1, first wave at −1.40 V, no CaCl2; curve 2, as I at −1.45 V + 0.01 M CaCl2; curve 3, total wave at −1.60 V + 0.01 M CaCl2. Conditions are as in Fig. 1.
with the presodium current before the limiting value is attained. When the BSA concentration is increased above 20 μM, the limiting current starts to decrease. The effect of the cobalt concentration on the pattern of the Brdička currents in a buffer 2 μM in BSA is illustrated in Fig. 4. At a given BSA concentration the pattern of the Brdička currents changes with cobalt(III) or (II) concentration. Under conditions given in Fig. 4 there is a plateau at cobalt(III) concentrations smaller than 100 μM, and a second wave with a well-defined plateau is observed at cobalt concentrations between 200 and 300 μM. At larger cobalt concentrations the second wave exhibits a well-defined maximum. A plot of the height of the first wave [corrected for \( i_2 \) of Co(III) and the residual current of BSA in the buffer] against cobalt concentration yields a straight line (Fig. 5). At BSA and cobalt concentrations where the limiting value of the Brdička current is attained, but where a sharp maximum occurs (Fig. 3, curves 3 and 4) calcium chloride has a large effect on the shape of the polarogram. With 5 mM calcium chloride the maximum is eliminated and a second wave is observed that coalesces with the "presodium" wave. When the BSA concentration is such (e.g., 5 μM or greater) that no maximum occurs, the calcium effect is no longer distinct. The polarograms have the same shape and the Brdička currents are of the same order of magnitude with Co(II) as with Co(III), while the effect of calcium chloride is also the same.

Values of Brdička currents at a concentration of 5 μM BSA, various cobalt(III) concentrations, and a concentration of 50 μM cobalt(II) are presented in Table 1. Also are presented the values of the slopes \( x \) of \( \log i \) against \( \log t \) and \( y \) of \( \log i \) against \( \log h_{Hg} \) plots under various conditions. Maximum currents \( (i_{\text{max}}) \) refer to currents at the moment the drop falls. In the last three columns of Table 1 are reported values of \( i_{\text{max}}/i_{\text{average}} \).

**DISCUSSION**

It is well known \((3, 7)\) that under conditions of kinetic control the instantaneous current \( i_t \) varies with time according to the expression:

\[
i_t = k t^{1/3}
\]

(I)

From data given in Table 1 it appears that at BSA concentration less than 0.28 μM, \( x \) in the plot of \( \log i_t \) against \( \log t \) is 0.60 in the absence of calcium chloride, which is very close to the value of 0.60 for kinetically controlled currents. In the presence of calcium chloride the values of \( x \) for \( i_t \) and \( i_2 \) correspond exactly to the theoretical value for kinetic control. Also, the value of \( y \):

\[
\log i = k h_{Hg}^3
\]

(II)

is close to or equal to zero, a value of zero being indicative of kinetic control. Under these conditions \( i_{\text{max}}/i_{\text{average}} \) should be equal to \( 1/3 = 1.7 \), a value practically equal to that observed (Table 1) in solutions with a BSA concentration equal to or smaller than 0.28 μM. Kútová and Březina \((8)\) obtained...
in ammoniacal buffers of pH 9.2, which were 0.1 M in ammonium chloride and 0.29 mM in human-serum albumin at various cobalt(III) concentrations, values of $y$ equal to zero (Table 1, ref. 8) but values of $x$ (with cobalt(II)) of 0.95–0.8 (Table 2, ref. 8), the latter values approaching that for surface kinetic currents, when $x$ should be equal to $1/4$ (10). At concentrations of 0.5 mM BSA we find values of $x$ and $y$ of 0.53 and 0.25 for the first wave at $-1.46$ V against S.C.E. and 0.58 and 0.24, respectively, in the presence of 0.01 M calcium chloride. For a catalyzed reaction at the surface of the electrode with incomplete coverage of the surface with active material (BSA) Weber et al. (11) derived values of $x$ and $y$ 1.12 and 0.5, respectively. Apparently at a concentration of 0.5 mM the Brdička current is partly kinetically and partly diffusion controlled. At a concentration of 5 mM BSA we find values of $x$ varying between 0.22 and 0.29, the average value being 0.25 (first wave). For diffusion control the Ilković value of $x = 1/4$ must be corrected for spherical diffusion (12) and thus becomes equal to 0.19, a value close to our average value. On the other hand, the average value of $y$ in the expression $i = k \gamma^{-1} t$ is 0.38 ± 0.3 (Table 1) as compared to 0.5 for diffusion control.

Kůtová and Březina (8), working with 29 mM solutions of human-serum albumin and varying the cobalt(III) concentration between 20 and 2000 mM, report values of $y$ varying between 0.51 and 0.15 (Table 1, ref. 8) for the total Brdička current, and 0.2 for the first wave at the same concentration of human-serum albumin. At a concentration of 1 mM cobalt(II) they report (2 experiments) values of $x$ equal to 0.51 (Table 2, ref. 8). On the basis of our results we conclude that at BSA concentrations of 2–5 mM the electrode surface becomes rapidly covered with a layer of adsorbed protein and that the Brdička currents are close to being controlled by diffusion of BSA.

These conclusions are substantiated by the facts that the Brdička currents then become independent of BSA concentration and proportional to the cobalt(III) concentration in the range between 25 and 200 mM (but less than proportional at larger cobalt concentrations). Also, values of $i_{\text{max}}/i^2$ between 1.2 and 1.3 (Table 1) are close to the theoretical value of 1.19 for diffusion control.

Under conditions under which the Brdička current is determined by the rate of diffusion of cobalt(III) or (II), we account for the above results by the following sequence of reactions at the electrode surface in which BH denotes the acid form of the buffer used. BSA contains about 0.7 SH and 17 $-S-S-$ bonds per molecule. According to Cecil et al. (13) only about five disulfide groups are reduced at the dropping mercury electrode. For the sake of simplicity we use the notation of $\psi^{-x}$ for the electro-reduced form of BSA, the concentration of $\psi^{-x}$ at a given potential at the surface of the electrode being constant at BSA concentrations greater than 2 mM. With Co(III) as the catalyst, this ion is present in the form of Co(II) at potentials slightly less negative than at those where the Brdička currents are observed. The adsorbed protein is then present as $\psi^{-x}$, which reacts with Co(II) to yield $\psi S_x Co(II)_y$, the latter reacting with 2 electrons according to Eq. 2:

$$\psi S^{-x} + y Co(II) \rightarrow \psi S_x Co(II)_y$$

(1) Initiation, diffusion control

$$\psi S_x Co(II)_y + 2 ye^- \rightarrow \psi S_x Co(O)_y$$

(2) Propagation

$$\psi S_x Co(O)_y + x BH \rightarrow \psi(SH)_x Co(O)_y + xB$$

(3) $\theta(SH)_x Co(O)_y + xe^- \rightarrow \psi S_x Co(O)_y + x/2 H_2$ (4)

$$\psi S_x Co(O)_y \rightarrow \psi S^{-x} + Co(O)Hg$$

(5) Termination

$$\psi(SH)_x Co(O)_y \rightarrow \psi S^{-x} + xH^+ + yCo(O)Hg$$ (5a)

At the large BSA concentration the rate of diffusion of Co(III) or (II) from the bulk of the solution to the electrode becomes rate determining. Reactions (2), (3), and (4) constitute a chain mechanism. The rates of reactions (1), (2), (3), and possibly (4) and (5) affect the magnitude of the Brdička currents as they determine the length of the chain. The number of ionized sulfhydryl groups ($-SH \rightarrow -S^- + H^+$) depends on $[H^+]$, while the rate of reaction (3) varies with kind and concentration of the acid buffer constituent $BH^+$.

Evidence for formation of Co(O) complexes has been given in a study of Brdička currents observed with cysteine and cysteine-like molecules (9). It is not probable that cobalt(III) diffuses to the electrode in the form of a protein complex, as proportionality between the current and cobalt(III) concentration (Fig. 5) would then not have been found.

![Fig. 4. Effect of Co(III) concentration on Brdička currents in ammonia buffer pH 10.3, 5 mM BSA, $t = 5.15$ sec, $m = 1.472$ mg/sec; $i_{\text{max}}$. Curve 1, residual of buffer; curve 2, 5 mM BSA (no Co, note "presumed wave"), curves 3–16, with 25, 50, 75, 100, 125, 150, 175, 200, 250, 300, 400, 500, and 600 mM Co(III).](image-url)

![Fig. 5. Plot of $i_{\text{max}}$, first protein wave against concentration of Co(III) from data in Fig. 4.](image-url)
At very small concentrations when the currents are kinetically controlled the rate of adsorption of BSA seems to be rate determining, and hence the rate of \( \delta S_2^0Co(III)_p \) formation becomes the slowest reaction.

This investigation was supported by Public Health Service Grant Ca-89723-04 from the National Cancer Institute.