# Polarographic Characteristics of Human Albumin, Gamma Globulin, Ceruloplasmin and Transferrin.* (31045)

ANANDA S. PRASAD, MIROSLAV D. POULIK AND SIGMUNDUR GUDBJARNASON
*Departments of Medicine and Paedriatrics, Wayne State University, School of Medicine, Detroit, Mich., and Department of Medicine, U.S. Veterans Administration Hospital, Dearborn, Mich.*

Brdička described a catalytic reaction at the dropping mercury electrode (DME) produced by certain amino acids, polypeptides and proteins dissolved in a cobalt-containing buffer of suitable pH(1). This reaction has gained considerable significance and has found application not only in clinical diagnosis of malignancies and other diseases but also in biochemical studies of native and denatured proteins(2).

Most workers have reported currents in millimeters of deflection of a galvanometer with unspecified sensitivity and characteristics of their DME were not defined so that an accurate evaluation of their data and their comparison with those obtained by others becomes impossible. According to Müller(2) the only satisfactory method for reporting results is in terms of current density, expressed in microamperes per square millimeter ($\mu a/mm^2$) of electrode surface. This obviously requires accurate knowledge of current and of surface area of DME, but it is applicable to any single measurement.

The purpose of this paper is to report results of our studies in pure normal human plasma protein fractions such as albumin, gamma globulin, ceruloplasmin and transferrin. Master curves relating current density to concentration for various proteins giving rise to Brdička reaction are proposed in this paper. Effects of temperature and alkaline digestion on wave heights for each protein fraction were also studied.

*Methods.* Ultracentrifugation studies were carried out in a Spinco model E analytic ultracentrifuge.† Initial concentrations for 4 protein fractions, namely, albumin, gamma globulin, transferrin and ceruloplasmin were 4.9, 3.0, 2.2 and 3.5 g% respectively. These samples were run in 3 different dilutions in normal saline and S rate was calculated for infinite dilution. Protein concentrations were determined by the biuret method(3).

*Serum protein fractions.* Human albumin was prepared by starch block electrophoresis (4). The front half of the albumin was removed, eluted, dialyzed, and lyophilized prior to use. Crystalline transferrin was prepared by Dr. K. K. Inman, Department of Public Health, Lansing, Mich., according to a method previously described(5). Ceruloplasmin and gamma globulin were obtained by large scale methods of preparation (Cohn fractionation) by American National Red Cross (courtesy of Dr. J. H. Pert). Purity of materials was determined by ultracentrifugation, immunoelectrophoresis, and starch gel electrophoresis. Albumin and transferrin sedimented as single peaks and on immunoelectrophoresis (Fig. 1 b, d), these two materials gave single precipitin arcs with a horse antiserum directed against whole human serum (Pasteur Institute). On starch gel electrophoresis, each material contained a main component and a minor slower migrating component as shown in Fig. 1 e, f. Minor component of albumin represents a small amount of a polymer (possibly dimer) as previously shown(6,7). Minor component present in transferrin preparation represents most probably that fraction of transferrin from which sialic acid was cleaved by natural sialidases (8).

Both ceruloplasmin and gamma globulin afforded a major and a minor peak by ultracentrifugal analysis. The materials were found homogeneous by immunoelectrophoresis (Fig. 1 a, c), but heterogeneous by starch gel electrophoresis (Fig. 1 g, h). All pure preparations of ceruloplasmin prepared by the method described above contain a number

* Supported in part by U.S.P.H.S. Grant AM-08013-02, a grant from American Cancer Society T-353, U.S.P.H.S. Grant No. HE-07995 and the Children's Leukemia Foundation of Michigan.

† Beckman Model E Analytical Ultracentrifuge, Spinco Division, Palo Alto, Calif.

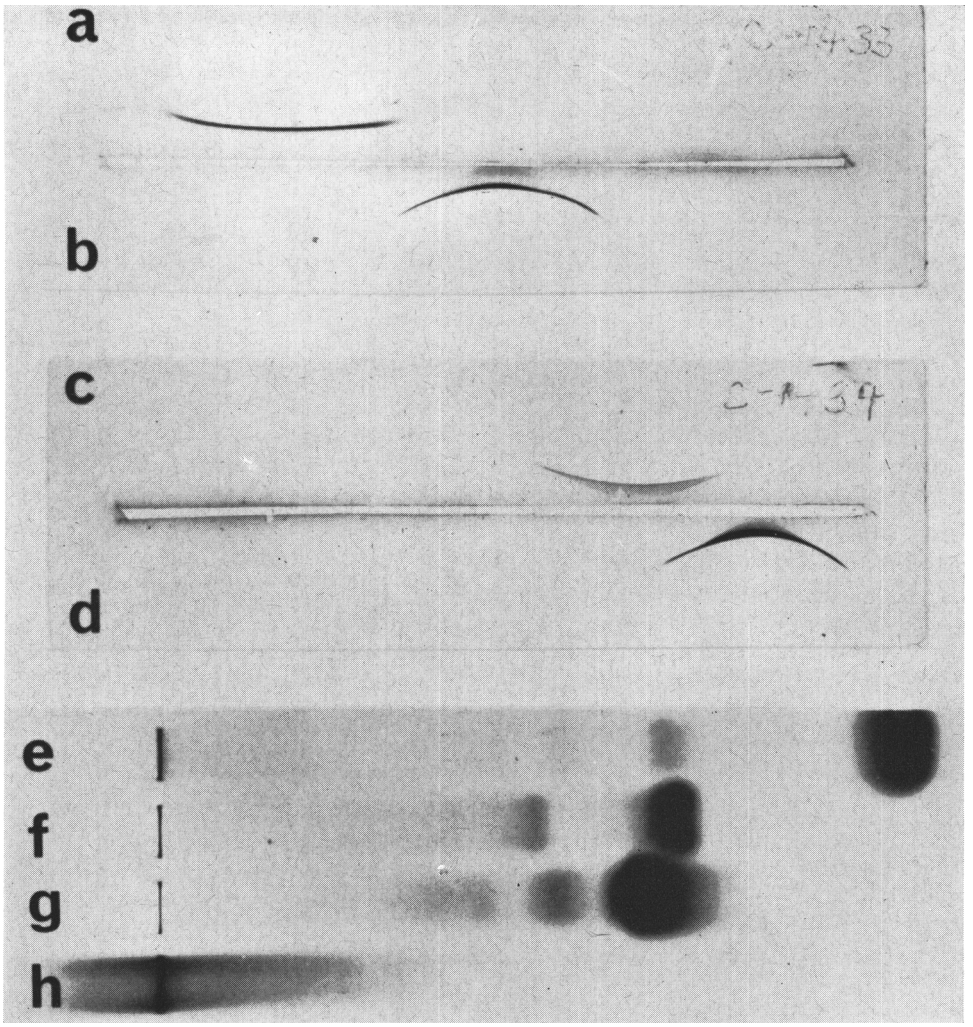



FIG. 1. Immunoelectrophoretic and starch gel electrophoretic patterns of albumin, gamma globulin, transferrin and ceruloplasmin: a & g — ceruloplasmin, b & e — albumin, c & h — gamma globulin, d & f — transferrin.

of polymers as previously shown(6,9). Gamma globulin preparations obtained by the Cohn method usually show the presence of a small amount of aggregated material.

*Polarographic methods.* Surface area of mercury drop was determined by the method of Müller(2). The height of the mercury column was kept at 80 cm and the size of the capillary tube‡ was such that the mercury drop time averaged 2 to 5 seconds.

For each 9 ml of DiCo buffer ($1.6 \times 10^{-3}$ M $CoCl_2$, 0.1 N $NH_4Cl$ and 0.1 N $NH_4OH$) used, 1 ml of 1 N ammonium hydroxide was added just before use to assure minimal loss of ammonia. Nitrogen was bubbled through the solution to expel atmospheric oxygen, before adding the protein. The protein was then added and nitrogen bubbled for a few seconds. The pH of the protein solution in buffer was 9.5.

Each protein was dissolved in normal saline and diluted to make a 5 g% solution. Protein solutions were polarographed repeatedly at time intervals to check the difference in result, if any, due to the lapse in time between the start and finish of a series of determinations. The effect of temperature on

‡ E. H. Sargent, Chicago, Ill.

the polarographic tracings of the buffer blank and each protein solution were recorded at 2°, 23°, 37° and 60°C.

The polarogram was recorded immediately after addition of each protein solution by means of a Sargent‡ model XX polarograph between applied voltages of —0.8 to —1.9 v. In each phase the sensitivity of the polarograph was adjusted to produce maximum height of the curve for each protein. For each protein concentration tracings were obtained at 2 sensitivities. Current density was calculated from wave heights and sensitivity of the galvanometer and expressed as $\mu a/mm^2$ surface area of mercury drop. The tracings were obtained at room temperature (23 ± 1°C) except when the effect of temperature was studied. A blank buffer wave was always recorded at 2 different sensitivities and proper correction for protein wave height made each time.

The polarographic curves for L-cysteine in DiCo buffer, between the concentrations of $1 \times 10^{-6}$ to $1 \times 10^{-4}$ M, was obtained in a manner similar to that described above.

The alkali digestion was performed as described by Müller(2). The protein digest was polarographed in 10 ml of the standard DiCo buffer open to air. An undigested protein solution to which water had been added in exactly the same proportion was used as control. The catalytic waves were expressed in $\mu a/mm^2$ surface area of the mercury drop and plotted against time on a semilogarithmic graph paper.

*Results.* Polarographic tracings for each protein fraction were reproducible under the conditions of our experiments. In some preliminary experiments, polarographic tracings were obtained initially and one hour after addition of protein solution to the buffer, at room temperature and at 2°C. No change in the tracings was observed.

Fig. 2 shows the effect of temperature on the polarograms. The first wave was higher at room temperature but at 2°C the second wave was greater, thus exhibiting the "crossing effect." Suppressive effects of ceruloplasmin and transferrin on cobalt peak at 2°C were markedly decreased. Both waves showed a slight shift towards positive potential, as

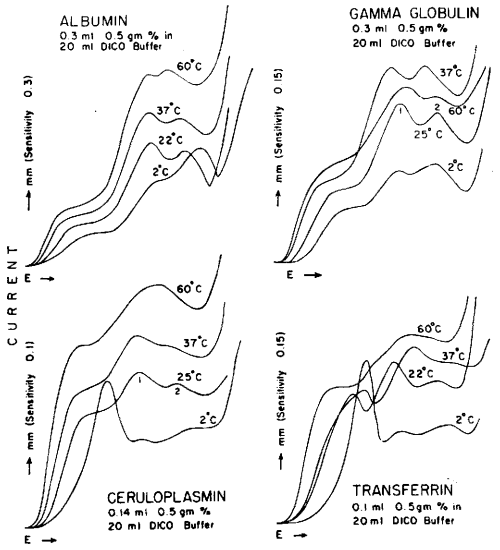


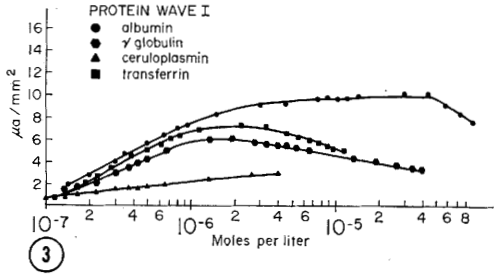

FIG. 2. Effects of temperature change on polarographic tracings of differnt protein fractions. The x-axis (E→) represents the change in applied voltages of —0.8 to —1.9 v during the polarographic tracing and the y – axis represents the wave height (current). The protein double waves have been marked 1 and 2 in the Figure.

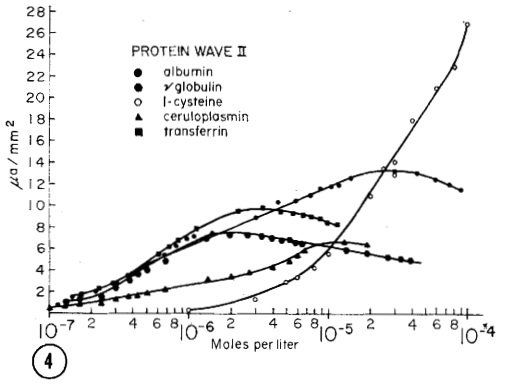
temperature increased. In cases of ceruloplasmin and transferrin the first protein wave could not be distinguished at 60°C.

Fig. 3 & 4 present the master curves for the two waves in the 4 protein fractions. In most dilute solutions of albumin, gamma globulin and transferrin, both protein waves, seemed to overlap each other; however, as the concentration was increased beyond $10^{-6}$ M/liter, the curves were distinct and characteristic for each fraction. Ceruloplasmin showed differences from others even in more dilute solutions. The first wave in ceruloplasmin became gradually indistinct as the concentration was increased until it disappeared at $4 \times 10^{-6}$ M concentration. The ceruloplasmin second wave showed similar phenomena and disappeared at $2 \times 10^{-5}$ M concentration. Generally speaking, the current density of each wave increased with increase in protein concentration up to a certain point beyond which an increase in protein concentration was accompanied by a decrease in the wave heights. Fig. 4 also shows the curve for L-cysteine in DiCo buffer. No limiting height for the current density could be reached even

with an increase in hundred-fold concentration of L-cysteine.

The results of alkali digestion test are presented in Fig. 5. Within the first few minutes following digestion, wave heights of albumin increased and subsequently showed decrease with increase in time of digestion. Initial rise in wave height following digestion was present in other fractions also but not as prominent as in albumin and in case of gamma globulin the change was very minimal. However, all fractions showed a decrease in wave heights as digestion was prolonged, such that a straight line could be drawn through the points when the wave heights in $\mu a/mm^2$ surface area of mercury were plotted against the time of digestion in minutes on a semilogarithmic scale. From this one could obtain T ½ for "molecular decay reaction" as described by Müller(2) for each fraction. Each protein fraction gave a typical value for T ½ as indicated in the Figure. These half lives were the same for the first and second protein waves of each fraction.



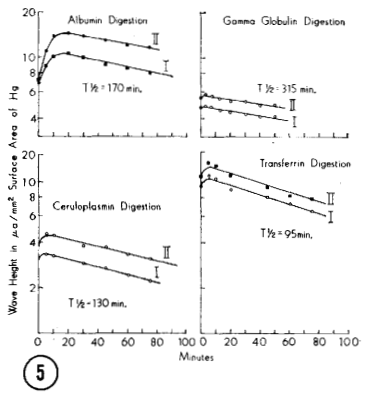




FIG. 3. Master curves relating current density to protein concentrations for protein wave I.

FIG. 4. Master curves relating current density to protein concentrations for protein wave II. The master curve for l-cysteine is also presented in this Figure.

FIG. 5. Change in current density *vs* time following alkaline digestion.

*Discussion.* Until recently separation and purification of plasma protein fractions have been very difficult. Previous workers studying the Brdička reaction used human sera and only a few reports describe studies on proteins obtained by Cohn's fractionation techniques(2). Human sera represent mixtures of various proteins; therefore, results of such studies cannot be regarded as specific for any proteins. The techniques of our protein fractionation yielded pure material; therefore, studies on these gave characteristic results of Brdička reaction.

The underlying reaction of the catalytic current is complicated and not well understood. It is believed that this involves a catalytic reduction of hydrogen ion from the cobalt containing buffer to which protein is added(2). Cystine and cysteine give single waves in DiCo buffer with peaks at the same potential at which the second peak occurs in the protein-containing solutions. Most investigators believe that this wave is due to the presence of SH and SS groups present in the protein(1,2). If one compares the rise in the curves of the human protein fractions and of cysteine in Fig. 4, one finds a close parallelism in dilute solutions. One may therefore speculate that perhaps a fixed multiple of SH groups in each molecule of protein is activated. However, when the concentration of proteins increases beyond $3 \times 10^{-5}$ M this parallelism no longer remains valid. These

curves suggest protein-protein interaction at higher concentrations of the proteins.

The result of the alkali denaturation test is interesting. Each protein fraction gave a specific T ½ for "molecular decay reaction." Both protein waves were affected in similar fashion, indicating that decay reaction involved molecule as a whole. Activation reaction for albumin, however, revealed differences between two waves indicating that this probably concerns different places on same protein molecule. Similar observations in various Cohn fractions and plasma albumin obtained from different species have been reported in the literature(2).

*Summary.* The polarographic characteristics of pure protein fractions appear to be specific in nature. These techniques therefore could be utilized as useful tools for studying normal and abnormal serum proteins.

We gratefully acknowledge the technical assistance of Mrs. Ann Petrowsky and Mr. Virgil Schwandt in this study.

1. Brdička, R., Collection Czechoslov. Chem. Commun., 1933, v5, 112.

2. Müller, O. H., Methods of Biochemical Analysis, D. Glick, ed., Interscience Publishers, New York, 1963, v11, 329.

3. Weichselbaum, T. E., Am. J. Clin. Path. Tech. Sec., 1946, v10, 40.

4. Kunkel, H. G., Methods of Biochemical Analysis, D. Glick, ed., Interscience Publishers, New York, 1954, v1, 141.

5. Inman, J. K., Doctoral Thesis, Harvard University, 1956.

6. Poulik, M. D., Protides in Biological Fluids, H. Peeters, ed., Elsevier Publishing Co., 1963, v10, 170.

7. ———, Ann. N. Y. Acad. Sci., 1964, v121, 470.

8. ———, Clinica Chim. Acta, 1961, v6, 493.

9. Poulik, M. D., Bearn, A. G., Clin. Chim. Acta, 1962, v7, 374.

Received November 15, 1965. P.S.E.B.M., 1966, v122.

## Differentiation of Muramidase and β-Lysin. (31046)

DAVID M. DONALDSON* AND JOHN G. TEW
*Department of Bacteriology, Brigham Young University, Provo, Utah*

β-lysin and muramidase (lysozyme) are 2 bactericidal substances found in the sera from a variety of mammalian species. These two substances have numerous similarities which make them difficult to differentiate but which separate them from bactericidal antibody-complement systems. Both β-lysin and muramidase are lethal for Gram-positive species in contrast to the antibody-complement systems which are primarily bactericidal for Gram-negative organisms. They are also less specific than antibody in their bactericidal spectra. β-lysin and muramidase resist inactivation at boiling temperatures for short periods of time; whereas, complement is readily destroyed by heat. Furthermore, it has been demonstrated that β-lysin can be separated from both antibody and complement by Seitz filtration of serum(1). Data will be presented indicating that this is also the case with serum muramidase. Early evidence indicating that β-lysin and muramidase were probably different substances was obtained by Myrvik and Weiser(2) who demonstrated cytologic differences in the appearance of *Bacillus subtilis* exposed to egg white muramidase and to rabbit serum. Their observation that the destruction of the bacterial cell was more complete with rabbit serum than with egg white muramidase could be due to the combined effects of serum β-lysin and serum muramidase. Much of the evidence to be reported demonstrating that these substances are different was dependent on the discovery of purification techniques for β-lysin(1) and the subsequent production of neutralizing anti-β-lysin serum(3).

*Materials and methods.* Rabbit β-lysin and anti-rabbit-β-lysin were prepared and as-

* Supported by USPHS Grant AI 01171 and career program award 5-K3-AI-22, 162, Nat. Inst. Allergy and Infect. Dis.