[CONTRIBUTION FROM THE DEPARTMENT OF PHYSICS, UNIVERSITY OF MICHIGAN]

Macromolecular Weights Determined by Direct Particle Counting. II. The Weight of the Tobacco Mosaic Virus Particle[1]

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A method is described for the determination of the particle weight of the tobacco mosaic virus in which the number of virus particles in a known dry weight of virus material is determined by counting particles in a known volume with the aid of the electron microscope. Use is made of indicator particles of polystyrene latex whose concentration in numbers of particles per unit volume has been calibrated. The monomeric unit of length of the tobacco mosaic virus particle, determined in the preceding paper, is taken as the elementary particle whose weight is determined. The dry weight of the monomer of centrifugally purified tobacco mosaic virus is found to be 49×10^6 g./mole. Sources of error are discussed, and it is concluded that the primary source of error resides in the calibration of the polystyrene indicator particles.

Introduction

The weight of the particles of tobacco mosaic virus has been the subject of much experimental work and discussion in the fifteen years that have elapsed since the practical isolation and purification of the virus were announced by Stanley.[3] The investigations of particle weight published up to 1943 are reviewed in detail by Bawden,[4] and by Lauffer and Stanley.[5] Subsequent to the dates of these reviews determinations of the weight of the tobacco mosaic virus (TMV) particle have been reported by Lauffer,[6] and by Schramm and Bergold,[7] using the method of sedimentation velocity combined with determinations of viscosity and diffusion rate. Some results which bear on the problem of the particle weight of TMV have also been published by Schachman,[8] who studied the viscosity of very dilute TMV solutions, and by Schachman and Lauffer,[9] who investigated the sedimentation characteristics of the virus particles in media of differing densities.

The particle weight of TMV has been determined in the past by two quite distinct methods: sedimentation velocity as corrected for the kinetic effects of the high dissymmetry of the rod-like particles, and by direct calculation of mass based upon the size and shape of the TMV particle as determined from electron micrographs and from X-ray analysis. The results obtained from the first method contain great uncertainties in the evaluation of the frictional effects of the elongated shape of the particles, and although there has been fair numerical agreement for the particle weight from experiment to experiment (from about 40 $\times 10^6$ to 60 $\times 10^6$ g./mole), there is reason to suspect the presence of serious systematic errors. The frictional ratio of the particles, f/f_0, can be obtained either by viscosity measurements or by a determination of the diffusion constant. Without entering into a detailed discussion of the sources of error, one can make the general statement that the primary source of uncertainty lies in the likelihood of some departure from random orientation of the elongated particles in the suspensions of virus in the concentrations used. Such orientation would affect all kinetic phenomena, such as the sedimentation velocities, the diffusion rates, and the viscosity values, and would therefore affect the values calculated for the particle weight. As mentioned above, the weight of the TMV particle has also been calculated from measurements of length and diameter, combined with measurements of the dry density. A value of 280 to 300 mμ has been suggested[10] as the appropriate length, while a lateral lattice spacing of 15.2 mμ found for the dry gel,[11] has been taken as the equivalent "diameter" of the particle. It should be pointed out, however, that there is no good evidence for believing that the individual TMV particles are smoothly cylindrical in shape, and that the shape of the particles when packed in a dry gel is an open question. It is unquestionably an oversimplification to identify the X-ray spacing of 15.2 mμ with the diameter of what are only presumed to be smooth, cylindrical rods.

The most important question, of course, in connection with ascertaining the particle, or molecular, weight of the tobacco mosaic virus is whether or not there is any uniform particle to weigh. There can no longer be any question about the uniformity of the short dimension of the particle, but there exists debate about uniformity in the long dimension. Unfortunately, the experimental criteria involved in resolving this uncertainty about uniformity are themselves subject to question. The degree of monodispersity of a suspension of virus particles is commonly estimated by analytical centrifugation, by experiments on free diffusion rates and by electron microscopy. The degree of monodispersity of a TMV suspension can be analyzed by observing the change with time of the contour of a sedimenting boundary, and comparing the result with theoretical curves based on the observed diffusion constant for the same suspension. Lauffer[12] has done this for the bushy-stunt virus, and has obtained evidence of a high degree of monodispersity. Lauffer,[6] and Schramm and Bergold,[7] have performed similar analyses for TMV, and have exhibited curves indicating a considerable deviation of the sedimentation-boundary

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(3) Stanley, *Science*, **81**, 644 (1935).

(4) Bawden, "Plant Viruses and Virus Diseases," 2nd ed., Chronica Botanica Co., Waltham, Mass., 1943, pp. 216-229.

(5) In "Colloid Chemistry," Vol. V, Reinhold Publ. Corp., New York, N. Y., 1944, pp. 791-798.

(6) Lauffer, THIS JOURNAL, **66**, 1188 (1944).

(7) Schramm and Bergold, *Z. Naturforsch.*, **26**, 108 (1947).

(8) Schachman, THIS JOURNAL, **69**, 1841 (1947).

(9) Schachman and Lauffer, *ibid.*, **71**, 536 (1949).

(10) Stanley and Anderson, *J. Biol. Chem.*, **139**, 325 (1941).

(11) Bernal and Fankuchen, *J. Gen. Physiol.*, **25**, 111, 147 (1941).

(12) Lauffer, *J. Biol. Chem.*, **143**, 99 (1942).

contour from that expected as a consequence of undisturbed diffusion. The latter authors have attempted no quantitative interpretation of this deviation, but Lauffer has interpreted his curves as indicating that the weights of the sedimenting particles have a spread in values amounting to a standard deviation of 14%. According to recent, unpublished studies of Schachman[13] this figure for the standard deviation is almost certainly far too low, since there is clear evidence for the existence of a marked sharpening of the sedimenting boundaries in the case of particles (like TMV) whose sedimenting rates are strongly dependent upon concentration. Although Schachman finds that the analytical centrifuge is capable of detecting extremely small fractions of monodisperse admixtures on the order of 1% by weight, his results indicate that at this time it is impossible to interpret quantitatively the shapes of sedimentation contours in terms of relative heterodispersity of the TMV particles.

The rather abundant electron microscopic evidence bearing on the monodispersity of TMV suspensions has clearly indicated that there is a most common length of particle; some of the uncertainties associated with this evidence are discussed in the immediately preceding paper (to be here called Paper A). It is shown in Paper A that, at least under certain conditions of preparation and observation, it is possible to obtain unequivocal electron micrographic evidence that the particles in a TMV suspension can exist almost entirely in precisely monomeric lengths, or in multiples of the monomeric length. This result is of considerable importance in its bearing on the problem of the particle weight of the virus, since it implies that one can think of a unit TMV particle in suspension as a unique entity with a definite and determinable weight. This weight can be measured by means of a slight variation of the method previously described.[14,15] In this method one obtains the average weight of macromolecular particles by first counting on electron micrographs of droplet patterns the number of particles in a known volume of a known dilution of suspension, and dividing this into the dry weight of the same number of particles. The advantage of the method is its directness and simplicity; one needs no information about the absolute size of the particles, nor about their shape or density, and the statistical reliability of the method can be internally checked.

Experimental[16]

The TMV Preparations.—White Burley tobacco plants were inoculated with the same virus inoculum as was used in connection with Paper A. Two batches of infected plants were harvested and pooled; one batch had been infected for 5 days and one for 15 days. The virus was extracted and purified by differential centrifugation in a manner similar to that described by Stanley.[17] The leaves were frozen overnight, chopped while frozen, and the cold juice pressed out through cheesecloth. The juice was then clarified by an acceleration to 7,700 g. followed by immediate deceleration. The clarified suspension was spun at 50,000 g. in a 10° Grebemeir rotor for one hour, the supernatant liquid discarded, and the pellet resuspended in fresh, double distilled water. The resuspension was clarified at 7,700 g. for 5 minutes. This clarified suspension was then spun at 50,000 g. for one hour and the supernatant liquid discarded. The pellet obtained was resuspended and clarified at 7,700 g. for 5 minutes. This resuspended, clarified pellet was called P_1 and a sufficient aliquot was set aside for analysis. The remainder of P_1 was carried through another cycle of high-speed sedimentation, resuspension, and low speed clarification to yield a P_2 suspension. A sufficient sample of P_2 was removed, and the remainder carried through another cycle of high-speed sedimentation. This time, and for all succeeding pellets, the clarification procedure was slightly changed. The surface of each pellet was lightly washed to remove possible non-virus material which might have formed on the upper layer of the pellet. Then the main portion of the pellet was carefully suspended in freshly double distilled water, leaving undisturbed the portion of the pellet against the wall of the tube. The suspended virus was then decanted. This cycle was repeated until aliquots of P_1, $P_2, \ldots, P_6$ were obtained. The entire purification was completed in 48 hours, with the temperature of all materials kept near 4°.

Counting, Measuring and Weighing the TMV Particles.—Spray-drop patterns containing TMV particles of P_5 and P_6, and containing known concentrations of polystyrene latex particles, were formed for subsequent electron microscopy and counting as described in Paper I. The aliquots of purified virus suspensions to be sprayed were diluted with uninfected plant juice at a concentration of $^1/_{160}$ that of the freshly extracted juice. This was done in order to reduce side-to-side aggregation of the particles upon drying of the droplets, since this interferes with precise counting and measurement of lengths. P_5 was sprayed at 3000-fold dilution, and P_6 at 2000-fold. The length of each virus particle in each droplet pattern was measured and corrected for electron microscopic field distortion, as described in Paper A. From the counts of virus and of polystyrene particles, and from the corrected lengths, the number of TMV particles per ml. of suspension, as sprayed, and the total length of the particles of TMV per ml. of suspension, could be obtained. Small quantities (about 20 mg.) of the purified suspensions of P_5 and P_6 were weighed after having been dried to constant weight over phosphorus pentoxide *in vacuo* at room temperature.

Tests for Homogeneity of the TMV Suspensions.—Spectrophotometric absorption measurements were made on all six TMV suspensions ($P_1, \ldots, P_6$) in order to obtain an indirect indication of relative purity of the suspensions. The suspensions were also examined directly for evidence of particulate impurities by spraying them in a sufficiently concentrated form (about 0.6 mg./ml.) to cause each droplet pattern to contain about 30,000 TMV particles. Several of these patterns were photographed in order to detect small-sized impurities. About 100 of the concentrated patterns were examined directly on the fluorescent screen of the microscope in a search for the presence of bacteria and other large sized material.

Results

The Standard Polystyrene Latex Suspensions.—The number of polystyrene latex particles per ml. in the standard latex suspension was taken to be 1.78×10^{12}; the same value as adopted in Paper I. There has appeared to be no good reason to change this value and, in fact, its continuation is indicated strongly in a recent summary by Gerould.[18]

The Counts, Lengths and Weights of the TMV Particles.—In Table I are shown the results of the counts and measurements of length of the TMV particles in preparations P_5 and P_6.

(13) Schachman, paper presented at the Virus Symposium, California Institute of Technology, March, 1950.

(14) Williams and Backus, THIS JOURNAL, **71**, 4052 (1949), to be here called Paper I.

(15) Backus and Williams, *J. Appl. Physics*, **21**, 11 (1950).

(16) The reader is referred to the two previous articles[10,11] for additional details of the method of obtaining particle weights by direct particle counts.

(17) Stanley, *J. Biol. Chem.*, **135**, 437 (1940).

(18) Gerould, *J. Appl. Physics*, **21**, 183 (1950).

TABLE I

DATA FOR THE DETERMINATION OF THE WEIGHT OF THE TMV PARTICLE

	P_5	P_6
No. of drop patterns analyzed	24	22
No. of PSL[a] counted	743	783
Ratio[b] of TMV/PSL	1.33 ± 0.053[c]	1.27 ± 0.067
Mean length[d] of TMV per PSL	11.6 ± 0.043	12.7 ± 0.068
No. of PSL/ml. as sprayed ($\times 10^{-10}$)	3.57	3.57
Dilution[e] of TMV as sprayed	3000	2000
Length[d] of TMV/ml.[f] ($\times 10^{-15}$)	1.24	0.907
Dry wt. of TMV susp. (mg./ml.)	13.30	9.65
Wt. of unit length[d] of TMV ($g. \times 10^{17}$)	1.07	1.06
Length[d] of TMV monomer	7.68	7.68
Wt. of one TMV monomer ($g. \times 10^{17}$)	8.23	8.14
Particle wt. of TMV (g./mole $\times 10^{-6}$)	49.6	49.1

[a] PSL = polystyrene latex particles. [b] Ratio of number of TMV particles to PSL particles as sprayed. [c] Standard deviation of the mean. [d] Unit of length is arbitrary, but same as in Paper A. [e] Dilution compared to suspension as weighed. [f] In the concentration of virus as weighed.

Ultraviolet Absorption.—The results of the measurement of the optical density at 2600 Å. are shown in Table II.

TABLE II

ULTRAVIOLET ABSORPTION OF TMV PREPARATIONS

Pellet	Opt. dens.,[a] D	Dry wt., mg./ml.	(D) per a 0.1% susp.
P_1	1.01	0.292	3.46
P_2	0.75	.230	3.26
P_3	1.25	.366	3.42
P_4	1.10	.350	3.14
P_5	0.93	.266	3.50
P_6	1.12	.321	3.49

[a] For an absorption cell 1 cm. thick.

Discussion

Precision of the Determination of Particle Weight.—The major source of uncertainty in the determination of the particle weight of TMV by this method continues to reside in the determination of the number of polystyrene latex particles per unit volume, as discussed in Paper I. Since the appearance of that paper, however, the more recent paper by Gerould[18] indicates that our adopted diameter for the latex particles is in agreement with that determined in several other laboratories.

Another source of uncertainty is in the measurement of the lengths of the TMV particles as they appear in the electron micrographs of the droplet patterns. The origin of these uncertainties is discussed in Paper A. It should be pointed out, however, that a certain amount of compensation of systematic error may be anticipated in the present work. This is because the actual lengths (in cm.) of the virus particles are not measured in the course of determining their weights; what is measured is the ratio of their lengths to the monomeric length discussed in Paper A. A systematic error of measurement resulting in an incorrect value for the monomeric length in cm. might be expected to act in the same direction in this investigation, and cause the error in the *ratio* of the lengths to be largely compensated for.

The magnitude of the random errors of mixing and measuring are indicated by the standard deviations (s.d.) in Table I. Although the ratio of the number of TMV particles to PSL particles (row 3 in Table I) is not needed for the particle weight calculation, its value is listed for its bearing on the statistics of mixing. It is seen that the s.d. of the mean of the *counts* is between 4.0 and 5.3%. The figure to be expected from *random mixing* of particles, when the number counted is on the order of 700–800 for the PSL and 1000 for the TMV, is 5.8%. Hence, it would seem that the mixing of the rod-shaped TMV particles, in very high dilution, with the spherical PSL particles is as good as the mixing exhibited by the spherical bushy-stunt virus particles (Paper I). The s.d. of the measurements of particle length, in terms of mean length of the TMV particles per PSL particle in each drop pattern, are shown in row 4, Table I, and can be seen to be approximately the same as the s.d. of the counts. This result can only mean that the degree of mixing of the numbers of latex particles with the *total lengths* of the virus particles in each droplet is as good (random) as the mixing of the *numbers* of the two kinds of particles. This could hardly be expected to occur if there were great dispersity in the lengths of the particles in suspension, since some droplets would then contain a relative excess of long particles, and some an excess of short particles, thereby increasing the scatter of the measurements of mean length. The simplest way to interpret the approximate identity of the s.d. of the *counts* and of the mean *lengths* is to assume that the particles in suspension are largely monodisperse, in general agreement with the conclusions of Paper A.

The Tests of Purity of the TMV Suspensions.—Subsequent to the appearance of Paper I, some doubts have been raised as to the adequacy of electron microscopic observations in general in detecting and assessing impurities and, as a result of these doubts, a fuller discussion is offered here. Let us set 200 droplet patterns as the upper number which can be conveniently and quickly examined on the fluorescent screen of the microscope. Each droplet pattern has a volume of about 5×10^{-9} ml., so the total volume of the small sample to be surveyed is 10^{-6} ml. The impurities which can be detected will vary in mass from about 4×10^{-20} g. (a protein sphere of 40 A. diameter) to 10^{-11} g. (a large sized bacterium). When impurities of the smallest size are sought, it is necessary to spray the suspension under investigation in quite dilute form, on the order of 0.02 mg./ml. This concentration will cause an average sized droplet pattern to be covered with dried suspension over roughly 2–5% of its area, and leave ample area for the detection of small impurity particles. A contamination of this dilute suspension by the smallest size impurities in the amount of 1% by weight will result in some 4×10^7 impurity particles being present in the 200 droplet patterns. These would obviously be detectable, and countable in even greater dilutions. For the detection of 1% of impurity particles of intermediate size, in the range of mass from 10^{-17} to 10^{-15} g., one would spray at a concentration of about 0.1 mg./ml.

This would place about 10^4 such impurity particles in the 200 observed droplet patterns. If the impurity particles were very similar in shape and size to the primary particles whose weight is being determined, they might be difficult to distinguish. Their presence would cause little error, however, since they would be counted and weighed along with the primary particles.

When the presence of 1% contamination by large particles, such as bacteria of masses as great as 10^{-11} g., is being sought, the suspension should be sprayed in concentrated form as great as 1 mg./ml. This will produce a drop pattern in which the area is well covered with the particles of the primary suspension (such as tobacco mosaic virus or bushy-stunt virus), but objects as large as bacteria are still easily seen. The 200 drop patterns would then contain, as the most likely number, about 10 particles of mass 10^{-12} g. and only 1 particle of mass 10^{-11} g. In the latter case, the statistics of counting would be very poor, and the method of assaying would have to be supplemented by other observation. The light microscope can be used to assay the number of bacteria and other particles if their mass is as great as 10^{-12} g. Turbidity can also be used as a guide, since say a 2% primary suspension of protein material, with a 1% contamination of bacteria of average size (5×10^{-13} g.), will contain 4×10^8 bacteria/ml. This will create a very evident turbidity which can be evaluated by well known bacteriological methods.

It is evident that in the search for impurities in the present work the droplet patterns containing TMV were sprayed at a somewhat incorrect dilution. They were about tenfold too concentrated for optimum detection of small-sized impurities, and were somewhat too dilute, and too few of them scanned, for adequate information about the presence of bacteria from counts alone. The TMV suspensions which were examined after spraying in concentrated form showed an observable increase in particulate purity from P_1 to P_3, with no observable change from P_3 to P_6. The three most highly purified pellets exhibited small amounts of roughly spherical particles of about 100 Å. diameter, but it is estimated that the total volume of these impurity particles is far less than 1% of that of the TMV in the droplet pattern. No bacteria, nor particles of comparable size, were found in the survey made of 100 droplet patterns containing about 3,000,000 TMV particles. If we assume that the possible contaminating bacteria had masses on the order of 10^{-12} g. (large size *E. coli* organisms), then the odds are about 20 to 1 that the suspension was contaminated less than 1% by such bacteria.

The foregoing conclusion concerning the presence of bacteria is based on counts alone. Visual inspection of the TMV in a 2% suspension showed it to be clear, with the characteristic bluish sheen due to scattered light, but with no sign of turbidity compatible with the presence of as much as 4×10^8 bacteria/ml. The procedures used in sedimenting, suspending, and clarifying the TMV material would be calculated to reduce the bacterial count certainly below 10^6/ml.

The measurements on ultraviolet absorption are rather inconclusive. We had anticipated an increased specific optical density at 2600 A. for the later pellets, as was reported for the bushy-stunt virus in Paper I, but the data indicate no regular trend for TMV. The interpretation of this result is that either (1) P_1 was already sufficiently pure that a negligible fraction of it was non-virus, or that (2) there are impurities in the earlier pellets, and perhaps in all the pellets, but they consist of material with virus-like content of nucleic acid.

Comparison with Other Determinations of the Particle Weight of the Tobacco Mosaic Virus.—It is difficult to attempt a comparison with the previously obtained particle weights of TMV for the reason that, in the early work, the origins of the data necessary for the calculations are highly heterogeneous. Only Lauffer,[6] and Schramm and Bergold,[7] have experimentally determined particle weights from homogeneous data obtained wholly in their own laboratories.

Some of the earlier values calculated for the particle weight of TMV are given by Lauffer and Stanley[5]: A value of 43×10^6 g./mole is found from the data on viscosity[19,20] combined with sedimentation velocity,[21] and a figure of 60×10^6 has resulted from data on diffusion rates[22] combined with sedimentation velocity.[21] Oster, Doty and Zimm[23] have reported a value of 40×10^6 from observations of light scattering by the TMV particles. More recently Lauffer[6] has reported values from 31.6 to 36.0×10^6 from data obtained by sedimentation, diffusion and viscosity. Schramm and Bergold[7] gave 41×10^6 as the value obtained from sedimentation and diffusion.

As is readily apparent, our method of determining the particle weight of TMV has nothing in common with the kinetic methods. It is closely related, however, to the static method in which the particle weight is calculated from measurements of length, thickness and density. This method has yielded a value of about 40×10^6 g./mole for the particle weight, if a length of 280 mμ, a rod-like diameter of 15.0 mμ and a dry density of 1.37 g./cc. are used.[24] This contrasts somewhat with the value of 49×10^6 g./mole obtained in the present study, by use of a method which requires no knowledge of the absolute dimensions or density of the particles.

If one takes the value of 300 mμ for the length of the monomeric TMV particle (Paper A), and the value of the particle weight (8.18×10^{-17} g.) determined by us, one can obtain directly the cross-sectional mass of the particle per unit of length: 2.73×10^{-20} g./Å. One can now calculate either the density, assuming the cross-sectional dimensions to be known, or *vice versa*. We believe that the value of 1.37 g./cc. for the density is a better determined quantity than the cross-sectional area, and so proceed to calculate a figure of 1.99×10^4 Å.^{2} for the latter. This corresponds to the

(19) Lauffer, *Science*, **87**, 469 (1938); *J. Biol. Chem.*, **126**, 443 (1938).
(20) Frampton and Neurath, *Science*, **87**, 468 (1938).
(21) Wyckoff, *J. Biol. Chem.*, **121**, 219 (1937).
(22) Neurath and Saum, *ibid.*, **126**, 435 (1938).
(23) Oster, Doty and Zimm, THIS JOURNAL, **69**, 1193 (1947).
(24) Stanley and Anderson, *J. Biol. Chem.*, **139**, 325 (1941).

area of a hexagon whose sides are of length 88 Å. A set of such hexagons packed tightly together would have their centers 152 Å. apart, the value obtained for the lattice spacing of TMV by Bernal and Fankuchen.[11] Thus, if we assume that the particles of dried TMV gel are packed in the cross-sectional form of hexagons, we find that a particle weight of 49×10^6 g./mole is consistent with the data for lattice spacing, density and particle length.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, STATE UNIVERSITY OF IOWA]

The Effect of Serum Albumin on the Polarographic Diffusion Current of Cadmium[1]

BY CHARLES TANFORD

A study has been made of the depression of the polarographic diffusion current of cadmium by bovine serum albumin. It is shown that the effect is due to complex formation between cadmium and the protein, and that adsorption of protein on the mercury drop and other non-specific factors do not play a significant role. The possibility that this type of diffusion current depression might be used to evaluate the thermodynamic constants of protein–metal interaction is discussed.

It is a well-known fact that very small quantities of protein added to a solution of a reducible substance will (provided the sign of the protein charge is correct) eliminate the polarographic maximum which may be exhibited by that substance. If somewhat greater quantities of protein are added however, it is usually found that a reduction in the diffusion current also takes place.[2,3] Kolthoff and Lingane have ascribed this phenomenon to complex formation between the protein and the reducible substance. Alternative explanations, however, have been offered, *e.g.*, that the phenomenon is due to reduction of the effective surface of the drop by protein adsorption.[4,5] It is also possible that viscosity changes may play a role in the phenomenon.[2] The objects of this paper are (a) to bring forward evidence to show that, at least in the example chosen for study, complex formation is indeed the predominant, and probably the only factor in the decrease of the diffusion current, and (b) to investigate the possibility of using this effect to evaluate the thermodynamic constants for complex formation between proteins and reducible molecules or ions.

Experimental

The polarograph used was a modified Sargent Polarograph, Model XX. An H-type cell, designed to hold 2 to 3 ml. of solution, and containing a satd. calomel electrode, was used. Diffusion currents were measured by the extrapolation method, in a constant temperature bath at 25.1°, using a capillary with an m value of approx. 2.65 mg./sec. The drop time at the point of measurement was 3.8 sec. Oxygen was removed from the solutions by a special technique described elsewhere.[6] (The ordinary method of oxygen removal is not possible here, because solutions containing proteins foam if gases are bubbled through them.) The supporting electrolyte for all experiments, except that illustrated by Fig. 1, consisted of 0.15 M sodium chloride.

All ordinary reagents were commercial C.P. reagents. Armour crystalline bovine plasma albumin was used. It was found to contain 5% moisture, which was corrected for in all weighings. Stock solutions of the protein were adjusted to desired pH values by the careful addition of dilute sodium hydroxide.

Theoretical

If there is combination taking place between reducible substance and protein, we can envisage two limiting types. The first is one in which there is a very strong interaction, *i.e.*, in which there is a large negative free energy of combination. This would result in a large displacement of the half-wave potential of the reducible substance, and there would therefore be no difficulty in recognizing and interpreting the effect.[7] On the other hand, the interaction between protein and reducible ion may be relatively weak. To see what would occur then it is convenient to discuss a hypothetical case with appropriate values of the thermodynamic constants. Let us assume, for example, that we have a protein with 40 positions available for combination with a reducible molecule, and that the free energy of combination, per mole of reducible substance, is −4000 calories (average value).[8] If the reduction involves two electrons, the difference in half-wave potential between combined and uncombined reducible substance would then be only $4.186 \times 4000/2 \times 96{,}500$ or 0.186 volt. If a given solution were to contain both combined and uncombined reducible substance, the reduction waves of the two would therefore overlap, and would, in fact, appear as one, somewhat flattened wave. However, since reducible molecules bound to a protein molecule will have a much smaller diffusion coefficient than free reducible molecules, as well as, possibly, a slow reduction rate due, for example, to the necessity of correct orientation of the protein molecule at the drop, the diffusion current will be markedly decreased over what it would be in the absence of protein.

It remains to show that a weak interaction of this sort will cause an appreciable fraction of a reducible substance in a solution containing protein to be bound to the protein so as to cause the sizable diffusion current reduction which is actually observed. To do this we write the equilibrium constant for the combination of a reducible substance with a protein[9] neglecting for the moment the term for electrostatic interaction

$$\bar{\nu}/(n - \bar{\nu})c = K \qquad (1)$$

where c is the concentration of free reducible substance, n is the total number of sites on the protein available for combination and $\bar{\nu}$ is the average number of such sites covered. In our example n is equal to 40, and K has the value exp.

(1) Presented at the 118th National Meeting of the American Chemical Society, Chicago, Ill., Sept. 3–8, 1950.

(2) I. M. Kolthoff and J. J. Lingane, "Polarography," Interscience Publishers, Inc., New York, N. Y., 1941.

(3) J. K. Taylor and R. E. Smith, *Anal. Chem.*, **22**, 495 (1950).

(4) B. Keilin, THIS JOURNAL, **70**, 1984 (1948).

(5) K. Wiesner, *Coll. Czech. Chem. Comm.*, **12**, 594 (1947), has shown that the reduction of the diffusion current of certain organic molecules by eosin is due to this effect. The differences between eosin and bovine serum albumin in this respect will be pointed out below.

(6) C. Tanford and J. Epstein, *Anal. Chem.*, in press.

(7) Strong combination of this sort occurs in some specific cases, and some examples of it will be studied in this laboratory in the near future.

(8) It should be mentioned that the polarographic wave obtained will not at any time obey the fundamental equation of Heyrovsky and Ilkovic, $E_{d.e.} = E_{1/2} - 0.0591/n \log i/(i_d - i)$, even if all the reducible molecules are bound to the protein, since the free energy of combination will be different for each successive molecule or ion bound to the protein, *i.e.*, the half-wave potential will differ slightly for each successive molecule or ion reduced. For metals the result is always a curve less steep than that predicted by the Heyrovsky-Ilkovic equation.

(9) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).