and Immunochemistry, New York, N. Y., Holt, Rinehart and Winston, pp 105–108.
Kabat, E. A., Baer, H., Day, R. L., and Knaub, V. (1950), *J. Exp. Med. 91*, 433.
Karush, F. (1956), *J. Amer. Chem. Soc. 78*, 5519.
Karush, F. (1957), *J. Amer. Chem. Soc. 79*, 3380.
Kleczkowski, A. (1965), *Immunology 8*, 170.
Kleinschmidt, W. J., and Boyer, P. D. (1952), *J. Immunol. 69*, 247.
Kolecki, B. J., and Springer, G. F. (1965), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 24*, 631.
Kuhn, R., and Osman, H. G. (1956), *Hoppe-Seyler's Z. Physiol. Chem. 303*, 1.
Landsteiner, K. (1945), Specificity of Serological Reactions, New York, N. Y., Dover, p 254.
Landsteiner, K., and Van der Scheer, J. (1932), *J. Exp. Med. 56*, 399.
Litman, G. W., Frommel, D., Finstad, J., Howell, J., Pollara, B. W., and Good, R. A. (1970), *J. Immunol. 105*, 1278.
Marrack, J. R. (1934), The Chemistry of Antigens and Antibodies, London, H. M. Stationary Office.
Ouchterlony, Ö. (1962), *Progr. Allergy 6*, 61.
Pauling, L., Pressman, D., Campbell, D. H., Ikeda, C. E., and Ikawa, M. (1942), *J. Amer. Chem. Soc. 64*, 2994.
Reiner, L., and Fischer, O. (1929), *Z. Immforschg. 61*, 317.
Sanger, F. (1945), *Biochem. J. 39*, 507.
Schiffman, G., Kabat, E. A., and Thompson, W. (1964), *Biochemistry 3*, 113.
Somogyi, M. (1945), *J. Biol. Chem. 160*, 69.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Springer, G. F., Ansell, N., and Ruelius, H. W. (1956), *Naturwissenschaften 43*, 256.
Springer, G. F., Desai, P. R., and Kolecki, B. (1964), *Biochemistry 3*, 1076.
Springer, G. F., and Horton, R. E. (1964), *J. Gen. Physiol. 47*, 1229.
Springer, G. F., and Horton, R. E. (1969), *J. Clin. Invest. 48*, 1280.
Springer, G. F., Horton, R. E., and Forbes, M. (1959), *J. Exp. Med. 110*, 221.
Springer, G. F., Nagai, Y., and Tegtmeyer, H. (1966), *Biochemistry 5*, 3254.
Springer, G. F., Takahashi, T., Desai, P. R., and Kolecki, B. J. (1965), *Biochemistry 4*, 2099.
Springer, G. F., and Williamson, P. (1962), *Biochem. J. 85*, 282.
Springer, G. F., Williamson, P., and Brandes, W. C. (1961), *J. Exp. Med. 113*, 1077.
Warner, C., Schumaker, V., and Karush, F. (1970), *Biochem. Biophys. Res. Commun. 38*, 125.
Warren, L. (1959), *J. Biol. Chem. 234*, 1971.
Watkins, W. M. (1966), *Science 152*, 172.
Watkins, W. M., and Morgan, W. T. J. (1952), *Nature (London) 169*, 825.
Wiener, A. S., Moor-Jankowski, J., and Gordon, E. B. (1966), *Int. Arch. Allergy 29*, 82.
Winkler, S. (1934), *Hoppe-Seyler's Z. Physiol. Chem. 228*, 50.

# Physicochemical Properties of the Eel Anti-Human Blood-Group H(O) Antibody*

Anatoly Bezkorovainy, Georg F. Springer,† and Parimal R. Desai

ABSTRACT: The eel anti-human blood-group H(O) antibody isolated as described in the preceding paper was homogeneous by ultracentrifugal and electrophoretic criteria. It is a FS globulin, has a molecular weight of 123,000, and is of nearly spherical shape. It consists of three physically bonded subunits of apparently identical molecular weight of 40,000. Each subunit consists of four polypeptide chains of identical size; the chains, whose molecular weight is near 10,000, are joined by disulfide linkages.

The previous paper (Springer and Desai, 1971) described the immunochemical properties, isolation, and composition of the eel anti-human blood-group H(O) antibody. The present communication is concerned with some physical properties of this protein, and its apparent tertiary and quaternary structures. These studies were deemed especially worthwhile in view of the novel phenomenon of specific precipitation of the eel protein with blood-group active monosaccharides, and because of the profound difference in the conformation of the eel and mammalian antibodies (Jirgensons *et al.*, 1970). The quantity of the eel anti-human blood-group H(O) antibody available for this study was limited, and extensive repetition of the operations performed was impossible. Some experiments done with the eel protein were therefore accompanied by identical procedures on a previously characterized protein, the bovine colostral IgG, in order to test the reliability of our methodology. In contrast to the eel antibody, the colostral IgG (Kickhöfen *et al.*, 1968) was shown not to be split into subunits either in urea or by succinylation, but was dissociable into two heavy and two light chains by reduction–alkylation (Bezkorovainy *et al.*, 1970).

* From the Department of Biochemistry, Rush-Presbyterian-St. Luke's Medical Center, Chicago, Illinois 60612, and from the Department of Immunochemistry Research, Evanston Hospital, Northwestern University, Evanston, Illinois 60201. *Received June 2, 1971.* This work was supported by U. S. Public Health Service Grant GM-11985, National Science Foundation Grant GB-8378, and National Institutes of Health General Grant 5-S01-RR05629-04. The Immunochemistry Department is maintained by the Susan Rebecca Stone Fund.

† To whom correspondence should be addressed.

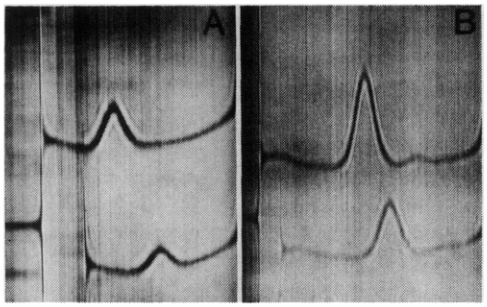


FIGURE 1: Ultracentrifugal analyses of native eel antibody and bovine colostral IgG. (A) Native eel antibody at concentrations of 6 and 3 mg per ml for the upper and lower patterns, respectively. Picture was taken 60 min after reaching speed at a bar angle of 50°; (B) native colostral IgG at concentrations of 10 and 7 mg per ml for the upper and lower patterns, respectively. Picture was taken 59 min after reaching speed at a bar angle of 60°.

## Materials and Methods

*Proteins.* The antibody was isolated from pooled eel sera as described in the previous paper (Springer and Desai, 1971). Bovine colostral IgG was prepared by the method of Smith (1946). Pepsin, twice crystallized from dilute alcohol, was obtained from Mann Research Laboratories (New York, N. Y.). Chymotrypsinogen A and ribonuclease A were purchased from Pharmacia Fine Chemicals, Inc. (Piscataway, N. J.).

*Reagents.* Succinic anhydride and 2-mercaptoethanol of reagent grade quality were purchased from Eastman Organic Chemical Corp. (Rochester, N. Y.). Iodoacetic acid came from K & K Laboratories (Plainview, N. Y.). Acrylamide (for electrophoresis), methylenebisacrylamide, and *N,N,N′,N′*-tetramethylethylenediamine were purchased from Eastman Organic Chemical Corp. Ammonium persulfate (Analytical Reagent) and coomassie blue used for disc electrophoresis were from Mallinckrodt Chemical Works (St. Louis, Mo.) and Canalco (Rockville, Md.), respectively; SDS,[1] 95% pure (Matheson Coleman & Bell, East Rutherford, N. J.) was recrystallized from ethanol before use. Urea and all other common chemicals were of reagent grade quality and were purchased from Fisher Scientific Co. (Pittsburgh, Pa.).

*Physical Measurements.* Ultracentrifugation was done in a Spinco Model E apparatus at 20° and 59,780 rpm. Proteins were analyzed at concentrations of 3–10 mg/ml and the results extrapolated to zero concentration. Diffusion studies were carried out in a Spinco Model H apparatus at 4° at protein concentrations of 2–6 mg/ml. Patterns were evaluated in a Kodak contour projector. Viscosity was determined in an Ostwald viscometer at 27°. Intrinsic viscosity was calculated from plots of reduced viscosity against concentration. Unless otherwise stated, the solvent used in all the above studies was 0.05 M cacodylate–0.1 M NaCl buffer (pH 7.0). Partial specific volumes were calculated from composition data (amino acids and carbohydrates); molecular weights were computed from diffusion and sedimentation data using the Svedberg–Pedersen equation (Schachman, 1957). $\beta$ values were calculated from the molecular weight, sedimentation constant, and intrinsic viscosity data by the method of Scheraga and Mandelkern (1953).

Moving-boundary electrophoresis was done in Veronal buffer of pH 8.6, $\mu$ 0.1, at 60 V (0.75 mA) in an Antweiler microelectrophoresis apparatus. The cells were enclosed in a water-cooled jacket at 10–15°. Paper electrophoresis was performed as previously described (Springer *et al.*, 1965) on Whatman No. 1 paper at 3–10 V/cm and 0.15–1.0 mA/cm for 5–16 hr at 22–25° (room temperature). The following electrolytes were used: sodium borate 0.118 M with respect to borate at pH 10.0; 0.06 M sodium barbital, pH 8.6; 0.02 M sodium phosphate, pH 7.0; 0.025 M potassium phthalate, pH 5.0; and 0.2 M sodium acetate, pH 4.0. Human serum and starch served as controls. Cellulose acetate electrophoresis was carried out in a Gelman chamber (Gelman Instrument Co., Ann Arbor, Mich.) using Sepraphore III strips and the Gelman high-resolution buffer (Tris–barbital–sodium barbital, pH 8.8, $\mu$ 0.05) at 11.8 V/cm and 0.8 mA/cm for 80–90 min at 22–25°. Human serum served as control. Paper electropherograms were stained with Amido-Schwarz 10B, bromophenol blue, or iodine, whereas cellulose acetate strips were stained with 0.2% ponceau S in 5% trichloroacetic acid.

Disc electrophoresis in 10% polyacrylamide gel (0.5 × 6.0 cm) containing 0.1% SDS and 0.1 M phosphate buffer (all final concentrations) at pH 7.0 was performed according to Weber and Osborn (1969). Canalco disc electrophoresis equipment Model 1200 was used at a constant current of 8 mA/gel for 4 hr.

Optical activity was determined in a Perkin–Elmer Model 141 digital readout polarimeter at 29° using a 1-ml cell, and ultraviolet absorption was measured in a Beckman DU spectrophotometer equipped with a Gilford reading device.

*Chemical Procedures.* Succinylation and reduction–alkylation of proteins were performed by methods previously described (Bezkorovainy *et al.*, 1969). Succinylation with 1 mg of succinic anhydride for each 2 mg of protein in 1% solution was at pH 8.0 and 0°. The succinylated protein was characterized physically (see Results) and then dissolved in aqueous 8 M urea containing 0.1 M 2-mercaptoethanol. The reaction mixture was kept under $N_2$ for 16 hr at room temperature, thereafter a 1.5-fold molar excess of iodoacetate over the amount of 2-mercaptoethanol present was added. The reaction mixture was incubated for 4 hr at room temperature and was then dialyzed against distilled water and freeze-dried.

Reduced eel antibody was prepared for electrophoresis by incubation of a 1% protein solution in 1% 2-mercaptoethanol and 1% SDS buffered with 0.01 M phosphate (all final concentrations) at pH 7.0, at 37° for 6 hr with mixing every 20 min. The mixture was stored in a refrigerator, and incubated at 37° for 2 hr just before use. Standards and native eel antibody used were prepared in the same manner except that the 2-mercaptoethanol was omitted.

## Results

*Homogeneity and Physical Properties of Native Eel Antibody.* Moving-boundary electrophoresis at pH 8.6 showed that this protein migrated as a single component. Zone electrophoresis on paper and cellulose acetate membranes showed single zones at all pH values tested. Below pH 5.25 the protein had a positive charge and above this pH a negative one. At pH 8.6 the eel antibody migrated like an $\alpha$-2 globulin as determined by moving-boundary and zone electrophoresis.

Ultracentrifugal analysis of the eel protein revealed a single symmetrical boundary (Figure 1A). However, a small amount of a faster sedimenting component was observed in some preparations. The colostral IgG showed a single major boundary with traces of a more rapidly migrating component (Figure 1B). The sedimentation coefficients obtained at different protein concentrations are plotted and extrapolated to zero con-

[1] Abbreviation used is: SDS, sodium dodecyl sulfate.

TABLE I: Physical Parameters of Native and Modified Eel Antibody and Bovine Colostral IgG.

| Sample | $s^0_{20,w}$ (S Units) | $D^0_{20,w}$ ($\times 10^7$ cm²/sec) | Mol Wt | $\bar{v}$ |
|---|---|---|---|---|
| Eel antibody | | | | |
| Native | 7.2 | 5.0 | 123,000 | 0.705 |
| Succinylated | 2.9 | 6.1 | 40,000 | 0.705[a] |
| Succinylated–reduced–alkylated | 1.4 | 11.5 | 10,000 | 0.705[a] |
| Colostral IgG | | | | |
| Native | 6.8 | 3.7 | 170,000 | 0.725[b] |
| Succinylated | 5.9 | 3.1 | 168,000 | 0.725[b] |

[a] Assumed to be identical with the calculated $\bar{v}$ of the native protein. [b] Assumed from Bezkorovainy *et al.* (1970).

centration in Figure 3, and the sedimentation constants ($s^0_{20,w}$ values) thus obtained are given in Table I; they were very similar for the eel antibody and bovine IgG.

The partial specific volume ($\bar{v}$) of the eel antibody was 0.705 ml/g. The diffusion constants for the eel protein were 4.96, 5.17, 4.76, and 5.09 $\times 10^{-7}$ cm² per sec for protein concentrations of 6.0, 3.0, 2.5, and 2.2 mg per ml, respectively. This indicated little if any variation of the diffusion constant with protein concentration; the constants were therefore averaged to give a $D^0_{20,w}$ value of 5.0 $\times 10^{-7}$ cm²/sec. The reduced viscosity values for the eel antibody protein were 4.45, 3.93, 3.68, and 3.86 ml per g at protein concentrations of 5.9, 4.4, 2.7, and 1.8 mg per ml. An intrinsic viscosity value of 3.4 ml/g was calculated from these data.

The molecular weight of the eel antibody, calculated from the sedimentation and diffusion data, was 123,000. The molecular weight of the colostral IgG was 170,000, in good agreement with the previously published data (Bezkorovainy *et al.*, 1970). The $\beta$ value of the eel antibody was 2.0 $\times 10^{-6}$, indicating an axial ratio of near 1:1. The frictional ratio was 1.3.

The absorbance ($A^{1\%}_{278\ nm}$) of the eel antibody was 12.696 in water, and its $[\alpha]^{29}_D$ was $-77°$ (*c* 0.5, water, 1 dm).

*Quaternary Structure of the Eel Anti-H(O) Protein.* A rapid and simple procedure for testing the quaternary structures of proteins is that of Greene and Feeney (1968), in which the unknown protein in its reduced–alkylated form is compared with similarly treated nondissociable standard proteins in urea solution. This procedure was employed in the present study. First, both the eel antibody and bovine IgG were dissolved in 8 M urea containing 0.1 M $NaHCO_3$ and ultracentrifuged. At a concentration of 7 mg/ml, the eel antibody had a sedimentation coefficient of 1.49 S and the colostral IgG had a sedimentation coefficient of 2.58 S. When 0.1 M 2-mercaptoethanol (final concentration) was added to the solutions of these proteins in 8 M urea, the sedimentation coefficients were lowered to 0.6 and 0.9 S for the eel antibody and the bovine globulin, respectively. This suggested that the eel antibody protein consisted of subunits that were dissociable by urea with further dissociation by the 2-mercaptoethanol. It has been shown previously that urea alone does not dissociate the colostral IgG although its sedimentation constant is lowered (Bezkorovainy *et al.*, 1970); the colostral protein, however, was dissociable by 2-mercaptoethanol.

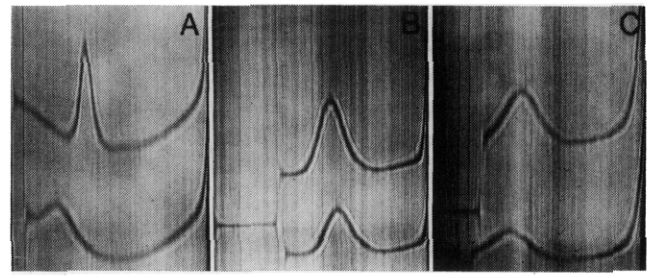


FIGURE 2: Ultracentrifugal analyses of modified eel antibody and bovine colostral IgG. (A) Colostral and eel proteins (upper and lower patterns, respectively) at concentrations of 7 mg/ml. Picture was taken 203 min after reaching speed at a bar angle of 50°; (B) succinylated eel antibody at concentrations of 9 and 6 mg per ml for upper and lower patterns, respectively. Picture was taken 83 min after reaching speed at a bar angle of 60°; (C) succinylated–reduced–alkylated eel antibody at concentrations of 8 and 4 mg per ml for upper and lower patterns, respectively. Picture was taken 183 min after reaching speed at a bar angle of 50°.

The quaternary structure of the eel antibody was then studied in greater detail by succinylation followed by reduction–alkylation. After succinylation both proteins showed single boundaries in the ultracentrifuge (Figure 2B), and the relationships of their sedimentation coefficients to concentration are shown in Figure 3. Other physical parameters are summarized in Table I; whereas the colostral IgG had retained its molecular weight of near 170,000 following succinylation, the molecular weight of the eel antibody was reduced to 40,000. This finding was in agreement with the urea experiment described above.

The succinylated proteins were further fragmented by reduction–alkylation in the presence of 8 M urea. Ultracentrifugal analysis of the reduced–alkylated proteins in 0.1 M glycine buffer at pH 11.5 showed that the eel material migrated as a

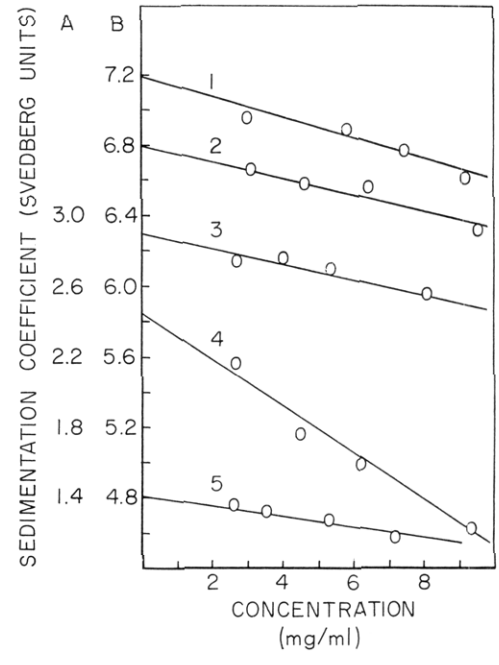


FIGURE 3: Dependence of sedimentation coefficients on concentration. Scale A to be used with curves 3 and 5, scale B with curves 1, 2, and 4. Curve 1, native eel antibody; curve 2, native colostral IgG; curve 3, succinylated eel antibody; curve 4, succinylated colostral IgG; curve 5, succinylated–reduced–alkylated eel antibody.

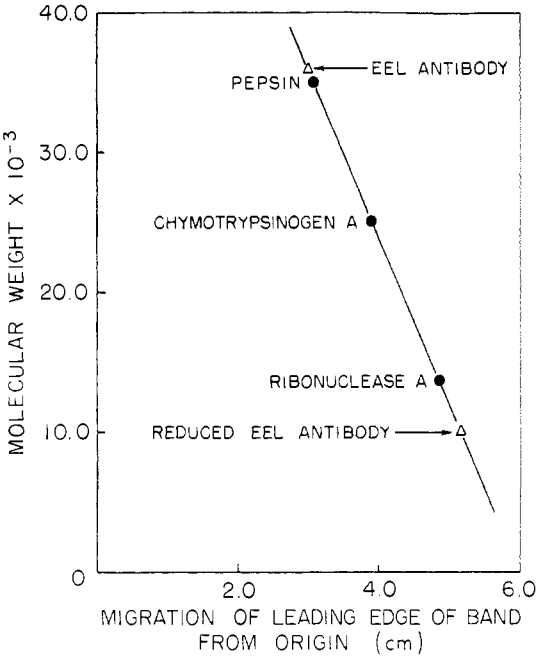


FIGURE 4: Relationship between molecular weights of proteins and their migration in the SDS–polyacrylamide gel electrophoresis system. Pepsin, chymotrypsinogen A, and ribonuclease A served as standards.

single component (Figure 2C), whereas that from colostrum moved as a single component with a shoulder. The dependence of the sedimentation coefficients of the eel antibody on concentration is given in Figure 3, and other physical parameters are summarized in Table I. It will be seen that both the sedimentation constant and the molecular weight of the succinylated–reduced–alkylated eel antibody protein are considerably lower than those of the eel protein after succinylation alone. The succinylated–reduced–alkylated colostral protein was not subjected to procedures separating it into the light and heavy chains in the present study, since this has been described previously (Bezkorovainy *et al.*, 1970).

Confirmation of the eel antibody quaternary structure described above was provided by SDS–polyacrylamide gel electrophoresis. A linear relationship was obtained between the molecular weights of standard proteins and the distance of their leading edge of migration from the origin (Figure 4). Electrophoresis of the eel antibody gave a molecular weight of 36,000 before reduction and 10,000 following reduction. These values are in general agreement with those determined by the hydrodynamic measurements.

## Discussion

The eel antibody protein was homogeneous by the moving-boundary and the zone electrophoretic procedures as well as by ultracentrifugal analyses.

The $\beta$ value of $2.0 \times 10^{-6}$ calculated for the eel anti-blood-group H(O) protein was within experimental error of the $2.12 \times 10^{-6}$ value characteristic of ellipsoids of revolution of nearly spherical shapes (Scheraga and Mandelkern, 1953). It may be concluded that the shape of the eel anti-H(O) protein, as that of mammalian immunoglobulins, approaches a sphere. The molecular weight of the eel antibody, however, was considerably lower than that of mammalian immunoglobulins (123,000 *vs.* 160,000).

Ultracentrifugal analyses after either urea treatment or succinylation indicated that the eel antibody protein consisted of three subunits joined by physical interactions only. Similar disaggregation was also observed in presence of SDS. The colostral IgG did not dissociate into subunits upon treatment with urea or upon succinylation.

Reduction–alkylation of the succinylated eel antibody resulted in its further fragmentation. Ultracentrifugal analysis of this preparation, as well as polyacrylamide gel electrophoresis of the reduced protein in SDS, indicated that the subunits produced by this procedure were of nearly identical size.

It may thus be concluded that each eel antibody molecule consists of three identical, physically joined subunits with a molecular weight of near 40,000, and each subunit, in turn, consists of four polypeptide chains with a molecular weight of near 10,000 each. On the other hand, the ultracentrifugal analysis of the reduced–alkylated colostrum IgG showed that its subunits were not identical.

These findings do not prove that the polypeptide chains of the eel antibody are identical. In fact, this is suggested by the two $NH_2$-terminal and the two COOH-terminal amino acid residues detected in the eel antibody protein (Springer and Desai, 1971). Some phylogenetic implications of these findings are discussed in the preceding paper.

## Acknowledgments

The assistance of Messrs. D. Grohlich and R. Zschocke is gratefully acknowledged.

## References

Bezkorovainy, A., Zschocke, R., and Grohlich, D. (1969), *Biochim. Biophys. Acta 181*, 295.

Bezkorovainy, A., Zschocke, R., and Grohlich, D. (1970), *J. Immunol. 104*, 648.

Greene, F. C., and Feeney, R. E. (1968), *Biochemistry 7*, 1366.

Jirgensons, B., Springer, G. F., and Desai, P. R. (1970), *Comp. Biochem. Physiol. 34*, 721.

Kickhöfen, B., Hammer, D. K., and Scheel, D. (1968), *Hoppe-Seyler's Z. Physiol. Chem. 349*, 1755.

Schachman, H. K. (1957), *Methods Enzymol. 5*, 32.

Scheraga, H. A., and Mandelkern, L. (1953), *J. Amer. Chem. Soc. 75*, 179.

Smith, E. L. (1946), *J. Biol. Chem. 164*, 345.

Springer, G. F., and Desai, P. R. (1971), *Biochemistry 10*, 3749.

Springer, G. F., Takahashi, T., Desai, P. R., and Kolecki, B. (1965), *Biochemistry 4*, 2099.

Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.