and Ou. Another subgroup is based on similarity to the variable sequence of the γ1 heavy chain Eu. This second kind Cunningham *et al.* (*8*) refer to as subgroup I and the kind first discovered as subgroup II. We have extensive sequence data on μ chains that show that both of these subgroups are found in γM immunoglobulins, and we have discovered a third subgroup that differs from the first two in having a free NH_2-terminal residue of glutamic acid and its own characteristic variable region sequence (*20, 21*). This new subgroup, which we have designated subgroup III (*22*), includes two μ chains and an α_1 chain sequenced in our laboratory and a γ4 chain sequenced by Pink *et al.* (*10*). The light chains in each of these four immunoglobulins are all of different subgroups.

Our data for the constant region of the μ chain (*21*) indicate that the latter has less than 40 percent identity in amino acid sequence with the constant region of γ chains. Figure 1 gives evidence that the μ chain constant region may be somewhat more related in structure to that of subclass γ4 than to γ1. The disulfide bridge to the light chain involves a half-cystine that is homologous in μ and γ4 chains but which is replaced by serine in γ1 chains (*4–7*). As a result, the bridge between the light and heavy chains in γ1 is located at Cys-220 instead of at Cys-131 (*23*). In human γG1 globulin two disulfide bridges link the pair of μ chains.

This demonstration of a unique sequence for the variable regions of the light and heavy chains of the same γM molecule without any evidence for sequence ambiguity excludes a duality in the primary structure of macroglobulins. Such a duality has been suggested by the finding of two categories of binding sites, five weak and five strong, in the γM pentamer (*24*). This heterogeneity of binding sites is thus probably attributable to stereochemical masking of sites in such multivalent molecules.

The results illustrated in Fig. 2 show that the same subgroup of light chain (the κI type) can combine with heavy chains of different class and subgroup. Although antibody activity has not been demonstrated in these two molecules, this finding raises three questions: (i) Is antigen-combining specificity dependent on the subgroup of either the light or heavy chain? If so, does the light chain have an equal role in specificity for antigen or only a stabilizing function for heavy chain? (ii) Is the subgroup and even the variable sequence identical on the μ and γ chains, respectively, of the γM and γG antibodies formed successively in the primary and secondary immune responses to the same antigen? (iii) In the latter case is the light chain identical on the γM and γG antibodies against the same antigen?

HEINZ KÖHLER, AKIRA SHIMIZU
CLAUDINE PAUL, FRANK W. PUTNAM
Department of Zoology, Indiana University, Bloomington 47401

References and Notes

1. K. Titani, E. J. Whitley, Jr., L. Avogardo, F. W. Putnam, *Science* **149**, 1090 (1965).
2. N. Hilschmann and L. C. Craig, *Proc. Nat. Acad. Sci. U.S.* **53**, 1403 (1965).
3. F. W. Putnam, *Science* **163**, 633 (1969).
4. E. M. Press and N. M. Hogg, *Nature* **223**, 807 (1969).
5. G. M. Edelman, B. A. Cunningham, W. E. Gall, P. D. Gottlieb, V. Rutishauser, M. J. Waxdal, *Proc. Nat. Acad. Sci. U.S.* **53**, 78 (1969).
6. E. M. Press and P. J. Piggott, *Cold Spring Harbor Symp. Quant. Biol.* **32**, 45 (1967).
7. M. Wikler, H. Köhler, T. Shinoda, F. W. Putnam, *Science* **163**, 75 (1969).
8. B. A. Cunningham, M. N. Pflumm, U. Rutishauser, G. M. Edelman, *Proc. Nat. Acad. Sci. U.S.* **64**, 997 (1969).
9. P. D. Gottlieb, B. A. Cunningham, M. J. Waxdal, W. H. Konigsberg, G. M. Edelman, *ibid.* **61**, 168 (1968).
10. J. R. L. Pink, S. H. Buttery, G. M. De Vries, C. Milstein, *Biochem. J.* **117**, 33 (1970).
11. C. Milstein, in *Gamma Globulins, Structure and Biosynthesis*, F. Franêk and D. Shugar, Eds. (Academic Press, New York, 1969), p. 43 (this is vol. 15, Fed. Eur. Biochem. Soc. Proc., 5th Mtg., Prague, July 1968); J. R. L. Pink and C. Milstein, *Nature* **214**, 94 (1967).
12. F. W. Putnam, M. Kozuru, C. W. Easley, *J. Biol. Chem.* **242**, 2435 (1967).
13. We thank Dr. E. Appella of the National Cancer Institute, Bethesda, Md., for performing the aminoethylation with [^{14}C]ethylenimine and for advice in this experiment.
14. The specificity of thermolysin for the NH_2-terminus of hydrophobic residues, as reported for other proteins (*15*), also holds for the immunoglobulins. Thermolysin has a high specificity for cleaving bonds that yield peptides with NH_2-terminal leucine, isoleucine, phenylalanine, and tyrosine. Besides these, cleavage occurred at only 5 percent of the sites involving alanine or threonine and at no others.
15. H. Matsubara, A. Singer, R. Sasaki, T. H. Jukes, *Biochem. Biophys. Res. Commun.* **21**, 24 (1965); T. C. Vanaman, J. Salih, A. Wakil, R. L. Hill, *J. Biol. Chem.* **243**, 6409 and 6420 (1968).
16. W. R. Gray and B. S. Hartley, *Biochem. J.* **89**, 379 (1963).
17. F. W. Putnam, K. Titani, E. J. Whitley, Jr., *Proc. Roy. Soc. London Ser. B* **166**, 124 (1966).
18. F. W. Putnam and H. Köhler, *Naturwissenschaften* **56**, 439 (1969).
19. H. D. Niall and P. Edman, *Nature* **216**, 262 (1967); C. Milstein, *ibid.*, p. 330.
20. H. Köhler, A. Shimizu, C. Paul, V. Moore, F. W. Putnam, *ibid.*, in press.
21. A. Shimizu, H. Köhler, C. Paul, F. W. Putnam, unpublished results.
22. The term subgroup has been approved by a nomenclature discussion group of the World Health Organization to signify a set of similar variable-region sequences in heavy chains analogous to those in light chains. However, in heavy chains the subgroups are independent of the class of the chain, which is determined by the sequence of the constant region, whereas in light chains the kappa subgroups are restricted to kappa chains and lambda subgroups to lambda chains. The nomenclature for heavy chain subgroups is still undecided [*Bull. World Health Org.* **41**, 975 (1969)].
23. Abbreviations for amino acid residues: Ae-Cys, aminoethylcysteine; Lys, lysine; His, histidine; Arg, arginine; Asp, aspartic acid; Asn, asparagine; Asx, aspartic acid or asparagine, identity not established; Thr, threonine; Ser, serine; Glu, glutamic acid; Gln, glutamine; Glx, glutamic acid or glutamine, identity not established; Pro, proline; Gly, glycine; Ala, alanine; Val, valine; Ile, isoleucine; Leu, leucine; Tyr, tyrosine; Phe, phenylalanine; Trp, tryptophan; Cys, half-cystine; and PCA, pyrrolidonecarboxylic acid. The first amino acid of the chain is designated Asp-1 and so forth.
24. K. Onoue, A. L. Grossberg, Y. Yagi, D. Pressman, *Science* **162**, 574 (1968).
25. We thank Jeanne Madison and Marleen Kawahara for technical assistance and Dr. John L. Fahey, NIH Clinical Center, Bethesda, Md., for serum from patient Ou. Supported by grant CA-08497-04 from NIH.

9 February 1970; revised 1 May 1970

Sulfate-Binding Protein from Salmonella typhimurium: Physical Properties

Abstract. *This protein binds sulfate strongly and is implicated in sulfate transport in* Salmonella typhimurium. *It has a molecular weight of 32,000 and an axial ratio of 4 : 1. Crystals are elongate prisms up to 0.5 millimeter. X-ray diffraction photographs give discrete crystalline reflections to a spacing of at least 2 angstroms. The unit cell is orthorhombic* $P2_12_12_1$, *with four molecules per unit cell of 40.8 by 47.5 by 136 angstroms. This is consistent with a highly asymmetric molecule such as the prolate ellipsoid suggested by the other physical measurements. Addition of sulfate had minimum effects on the physical properties as measured by light absorption, optical rotary dispersion, circular dichroism, fluorescence and its depolarization, nuclear magnetic resonance, and sedimentation velocity.*

A protein that binds sulfate strongly and specifically has been isolated and crystallized (*1*) from *Salmonella typhimurium*. It appears to be characteristic of a class of similar proteins that may function in transport of the bound substrate (*2*). Preliminary physical and chemical characterization of the sulfate-binding protein has been reported (*3*). We report here detailed studies, par-

ticularly with x-ray diffraction, with the purpose of obtaining some insight into function from structure.

The molecular weight was determined by chromatography on Sephadex G-100 (*4*), with bovine serum albumin, α-chymotrypsin, and horse heart cytochrome c as standards. A curve of the ratio of elution volume to void volume (V_e/V_o) plotted against molecular weight on a logarithmic scale (Fig. 1) is linear and indicates a molecular weight for the binding protein of 32,000. This is in agreement with the 32,000 determined by sedimentation velocity and 31,000 determined by sedimentation equilibrium (*3*).

The Stokes radius (*a*) was calculated by Sephadex chromatography (*4*), with the equation of Laurent and Killander (*5*): $K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_t is the total bed volume. The value of K_{av} was 0.427. The correlation with Stokes radius is given by the equation $(-\log K_{av})^{\frac{1}{2}} = \alpha(\beta + a)$. The constants α and β were calculated from the known Stokes radii of cytochrome c (17 Å) and bovine serum albumin (35 Å) and from the measured K_{av} for these proteins. The Stokes radius for the binding protein was 24.9 Å.

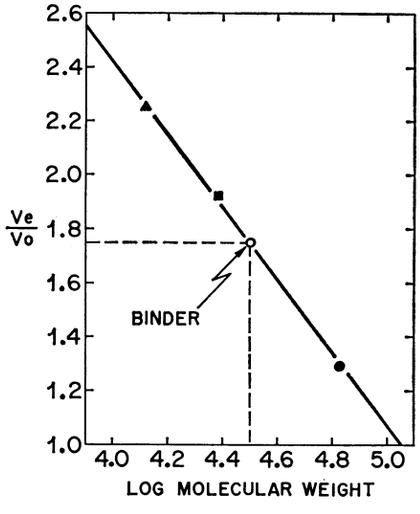

Fig. 1. Molecular weight of sulfate-binding protein determined by Sephadex G-100 column chromatography. The column (K 15/90) was packed to the height of 76.5 cm; the bed volume was 120 ml. A sample (0.5 ml) containing 0.1 percent blue dextran and about 4 mg each of bovine serum albumin, horse heart cytochrome c, α-chymotrypsin, and binder was added to the column. Elution was performed with 50 m*M* potassium phosphate buffer at *p*H 7.0, and 1.8 ml fractions were collected. The blue dextran was measured by absorption at 450 nm, serum albumin (●) at 280 nm and cytochrome c (▲) at 415 nm; α-chymotrypsin (■) was assayed by the method of Schwert and Takenaka (*11*). The binder (○) was measured by the resin assay. The ratio of elution volume (V_e) to void volume (V_o) was plotted against the log of the molecular weight for each known protein, and the molecular weight of the binder was determined by interpolation.

The Stokes radius was also calculated from the diffusion coefficient (*6*). The average diffusion coefficient D_{20} was 8.7×10^{-7} cm^2 sec^{-1}, as compared to values obtained by sedimentation velocity of 7.4 and 8.1×10^{-7}. The Stokes radius corresponding to the new value is 24.7 Å, in good agreement with the radius obtained with Sephadex. The average Stokes radius from the two methods is 24.8 Å and with this value the hydrated volume is 64×10^3 Å^3. The anhydrous volume is 35×10^3 Å^3, if the partial specific volume $\bar{v} = 0.72$ cm^3 g^{-1}.

The frictional ratio calculated from the Stokes radius [$f/f_o = (3\bar{v}M/4\pi N)^{\frac{1}{3}}/a$] was 1.19, where M is the molecular weight and N is Avogadro's number. This value gives an axial ratio for either an oblate or a prolate ellipsoid of about 4. Earlier viscosity data (*2*) indicated an axial ratio of about 6. An axial ratio of 4, if one approximates the prolate ellipsoid with a cylinder, gives the binder protein dimensions of 112 Å long and 27 Å diameter. For a flat cylinder it is 17 Å thick and 72 Å in diameter.

Crystals for x-ray diffraction were obtained from 2-methyl-2,4-pentanediol and 8 m*M* citrate buffer *p*H 5.1 (*1*) at room temperature and with very slow growth of crystals (several months).

The crystals were prismatic. The best developed crystals had 222 symmetry. The longest dimension in the largest crystal was about 0.5 mm; the other two dimensions were of the order of 0.2 mm. The crystals were mounted with a small drop of mother liquor in thin-walled quartz capillaries with an external diameter of approximately 1 mm. The crystal was in equilibrium with columns of mother liquor on either side. Precession cameras with 0.2 mm collimators were used.

With a fresh crystal a "still" diffraction photograph gives spots out to a spacing of at least 2 Å; thus the data are potentially available for a full, single-crystal, structure analysis. Figures 2 and 3 are 12° precession photographs with the crystal rotated 90° about the long crystal axis between exposures. The specimen to film distance is 10 cm. The cell is orthorhombic—$a = 40.8$, $b = 47.5$, and $c = 136$ Å. There are no general *hkl* absences. Only even-order reflections appear on *h*00, 0*k*0, and 00*l*. Thus the space group is $P2_12_12_1$. The density of the crystal measured in a gradient column is 1.20 g/cm^3. In conjunction with the unit cell volume of 264,000 Å^3, this implies one molecule per asymmetric unit of the unit cell. The long axes of the molecules must be oriented almost parallel to the long axis of the unit cell.

Optical properties are similar to those described (*3*) and are typical of a protein. No change of optical density between 210 and 310 nm was observed when 1 m*M* sulfate was added. Fluorescence excited at 283 nm and emitted at 330 nm (Aminco Bowman)

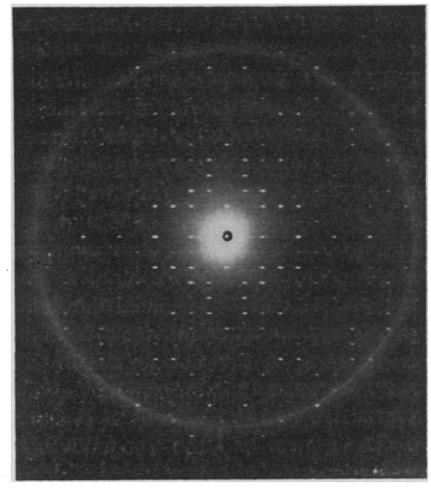

Fig. 2. Twelve-degree precession photograph [001] axis.

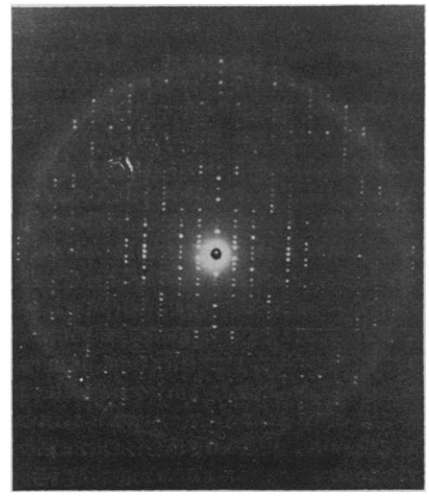

Fig. 3. Twelve-degree precession photograph [010] axis, crystal rotated 90° about [100] axis between Figs. 2 and 3.

increased about 12 percent when 1 m*M* sulfate was added (*3*). This finding suggests a small change in the environment of one or more tryptophan residues. Changes in the polarization of fluorescence spectra were at the limit of significance, an indication that the molecule undergoes no serious rearrangement involving tryptophan residues.

Nuclear magnetic resonance measurements were made with binding protein whose exchangeable groups were deuterated (*7*). No discernible separate peak was detected, and there was no change when sulfate was added.

Optical rotary dispersion (ORD) measurements (Cary Model 60) were made on protein (1 mg/ml) in 10 m*M* phosphate buffer, *p*H 6.9. The spectrum between 220 and 310 nm was characteristic of protein with the α-helix structure, having a trough at 233 nm, $[\alpha] = -5800$ or $[m'] = -5900$, and with a crossover point at 225 nm. The α-helix content calculated from the trough, assuming $[m']$ at 233 nm $= -13{,}000$ for a complete helix and -2000 for a random coil, gives an α-helix content of 35 percent (*8*). From the equation of Shechter and Blout (*9*) the percentage of α-helix was calculated at 193 and 225 nm to be 37.0 and 33.5 percent, respectively. A minimum decrease of 6 percent, at the 233 nm minimum was observed when 1 m*M* sulfate was added.

Circular dichroism was measured on the samples that were used for ORD. A minimum reading of -36×10^5 deg cm^2/dmole was found at 223 nm. It was reduced by 3 percent when 1 m*M* sulfate was added. There was a smaller minimum, -4.8×10^4 deg cm^2/dmole at 273 nm. It was reduced about 6 percent when sulfate was added. This must be caused by minor changes in the aromatic residues. The α-helix content calculated (*10*) from the trough at 233 nm was 38 percent.

ROBERT LANGRIDGE
HIDEO SHINAGAWA*
ARTHUR B. PARDEE
Department of Biochemical Sciences,
Princeton University,
Princeton, New Jersey 08540

References and Notes

1. A. B. Pardee, *Science* **156**, 1629 (1967).
2. ———, *ibid.* **162**, 632 (1968).
3. ———, *J. Biol. Chem.* **241**, 5886 (1966).
4. P. Andrews, *Biochem. J.* **91**, 222 (1964).
5. T. C. Laurent and J. Killander, *J. Chromatogr.* **14**, 317 (1964).
6. T. Svedberg and K. O. Pedersen, *The Ultracentrifuge* (Clarendon Press, New York, 1940), p. 298.
7. D. H. Meadows, J. L. Markleg, J. S. Cohen, O. Jardetzky, *Proc. Nat. Acad. Sci. U.S.* **58**, 1307 (1967).
8. N. S. Simmons, C. Cohen, A. G. Szent-Gyorgi, D. B. Wetlaufer, E. R. Blout, *J. Amer. Chem. Soc.* **83**, 4766 (1961).
9. E. Shechter and E. R. Blout, *Proc. Nat. Acad. Sci. U.S.* **51**, 695 (1961).
10. J. T. Yang, in *Conformation of Biopolymers*, G. N. Ramachandran, Ed. (Academic Press, London, 1967), p. 147.
11. G. W. Schwert and Y. Takenaka, *Biochim. Biophys. Acta* **26**, 570 (1955).
12. Supported in part by NIH grants AI-04409 and GM-16539 and NSF grant GB-7857 and in part by funds provided by the Eugene Higgins Trust Fund. Facilities were made available by the Whitehall and John A. Hartford Foundation to the Department of Biology, Princeton University. We thank Dr. A. Rawitch for polarization measurements and Dr. O. Jardetsky at Merck, Sharp, and Dohme Laboratories for nuclear magnetic resonance measurements.

* Present address: Department of Biology, University of Osaka, Osaka, Japan.

22 December 1969; revised 6 March 1970

Biological Nitrogen Fixation in Lake Erie

Abstract. *Biological nitrogen fixation, as determined by acetylene reduction, occurs in Lake Erie. Fixation potential by blue-green algae in situ in water and by bacteria in collected sediments was demonstrated. Nitrogen-fixing activity occurred from June through November suggesting that it is significant over the extremes of seasonal variation in light, temperature, and nutrients.*

Biological nitrogen fixation by blue-green algae and bacteria occurs in freshwater lakes (*1–3*). We report here studies on the nitrogen-fixing activity in the water and sediments of Lake Erie during the period from 1 June through 22 November 1969. Samples were taken at various locations in the vicinity of the Bass Islands, which lie at the eastern edge of the shallow, rapidly eutrophying western basin of the lake. The sample sites included shorelines, bay areas, and open lake waters.

Nitrogen-fixing activity was determined by the method employed in the study of other freshwater environments (*1, 3*) which measures acetylene reduction to ethylene by nitrogen-fixing organisms. For measurements of algal fixation in situ, vaccine bottles (72 ml), capped with serum stoppers, were evacuated and filled to atmospheric pressure with a gas mixture containing acetylene, argon, and oxygen (5 : 75 : 20) (*4*). Either water containing algae or algae concentrated by plankton net and resuspended in water was added at the rate of 50 ml per sample to the gas-filled bottles by injection. Another needle was introduced through the serum stopper to relieve the gas pressure to atmospheric as the algal sample was injected. Immediately after the injection was completed, the bottles were placed back in the lake to permit incubation in situ. Samples from each site were incubated for 0, 30, 60, and 90 minutes in Lake Erie and for 5 days at 22° to 24°C under an incandescent light in the laboratory. At the end of the desired incubation time, fixation was stopped by the addition of 5 ml of 50 percent trichloroacetic acid (TCA) solution. Three controls were used for each sample. One control was the sample to which TCA had been added immediately (0 minutes); the second was a gas-filled bottle to which only TCA had been added; and the third was an untreated gas-filled bottle. Nitrogenase activity was estimated by determination of both acetylene loss and ethylene production. Acetylene and ethylene were quantitated by gas-liquid chromatography (GLC) (*5*).

Nitrogen-fixing activity in the bottom sediments was estimated in undisturbed sediments collected either by dredging or by manually collecting the samples. Sediment samples consisting of undisturbed cores (1 cm in diameter to a depth of about 6 cm) were placed in vaccine bottles (72 ml) immediately after they were collected. The bottles were capped, evacuated, and filled with a gas mixture of 6.56 percent acetylene and 93.44 percent argon (*4*). The filled sample bottles were incubated in the dark at 22° to 24°C for 5 days; the gas in the bottles was then analyzed by GLC (*5*). Nitrogenase activity was determined as described for algal samples.

Nitrogen-fixing activity of the blue-green algae was expressed as a rate based on total biuret detectable protein in all algae (Table 1). Therefore the total indicated protein is higher and, consequently, the apparent specific nitrogenase activity lower than would be expected if all activity were based on the protein from nitrogen-fixing algae only. All ethylene produced could be accounted for by a corresponding decrease of acetylene in the sample bottles.

Nitrogen-fixing activity in the water was not detected in samples taken in