neutralized with solid sodium bicarbonate. The product was extracted with ether, the ethereal solution dried and the solvent removed. The residue was chromatographed on 18 g. of Woelm neutral alumina (Act. III). Elution with pentane gave a crude ester fraction which was saponified with 5% potassium hydroxide in methanol. The resulting alcohol was chromatographed on 5 g. of Woelm neutral alumina and elution with petroleum ether followed by crystallization from acetone yielded 40 mg. (8%) of XII, m.p. 102–103°, $[\alpha]^{27}D$ +2°.

Anal. Calcd. for $C_{26}H_{44}O$ (372.61): C, 83.31; H, 11.90. Found: C, 83.53; H, 11.78.

(b) **From B-Norcoprostane-3α,6α-diol 3-Monotosylate (XX).**—A solution of 150 mg. (0.275 mmole) of XX[11] in 50 ml. of dry *tert*-butyl alcohol containing 2.3 g. of potassium *tert*-butoxide was heated at 50° for 4 hours. The solution was allowed to stand at room temperature overnight, neutralized with glacial acetic acid, and concentrated under reduced pressure to a small volume. The remaining solution was poured into water and extracted with ether. After removal of the ether the solid residue was recrystallized from acetone containing a few drops of water; yield 67 mg. (65%), m.p. 102–103°, no depression on admixture with material prepared above.

Reaction of B-Norcoprostane-3α,6α-diol 3-Tosylate (XX) with Pyridine.—A solution of 85 mg. (0.16 mmole) of XX in 4 ml. of pyridine was heated at 90° for 40 minutes. The product was isolated in the usual manner and chromatographed on Woelm neutral alumina (Act. I). Elution with petroleum ether yielded 36 mg. (58%) of B-norcoprostane-3α,6α-oxide (XIX), m.p. 82.0–85.0°.

[CONTRIBUTION FROM THE DIVISION OF LABORATORIES AND RESEARCH, NEW YORK STATE DEPARTMENT OF HEALTH, ALBANY, AND THE DEPARTMENT OF BIOCHEMISTRY, ALBANY MEDICAL COLLEGE, ALBANY, N. Y.]

Studies of Completely Deuteriated Proteins. I. The Immunochemistry of the Deuteriated Protein and its Hydrogen Analog

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Qualitative and quantitative immunochemical evidence, double diffusion studies and precipitin curves are presented to establish the identity of a deuterio protein and its hydrogen analog in primary, secondary and tertiary protein structure.

The isolation and characterization of a completely deuteriated algal protein from blue-green algae have been reported by Berns, *et al.*[1] The physical and chemical properties described indicate that deuterio and hydrogen proteins are quite likely chemically identical and physically similar although isotopically different. The question of whether there are significantly different primary, secondary, tertiary and quaternary structural characteristics in the deuterio protein and its hydrogen analog may be answered in part but not entirely by the extremely sensitive techniques of immunochemistry. Certain positive conclusions concerning structural features of the protein can be reached and these are of considerable importance in determining the validity of additional investigations of the physical chemistry of deuterio[2] and protio proteins and the applicability of this system to elucidating the role of hydrogen bonding and possibly internal rotation in protein structure. It must be established first that the proteins are in fact the same protein except for isotopic differences and that the isotopic substitution has not altered the over-all protein structure so that comparison of the physical and chemical behavior of the systems is meaningless, since the so-called deuterio and hydrogen proteins are in actual fact different proteins.

Experimental

Materials.—All samples of phycocyanin were isolated and purified from algal cultures as described by Berns, *et al.*[1] In all experiments the protein samples were dialyzed into 0.15 *M* saline previous to use.

Methods.—Ouchterlony plates and Oudin tubes were set up as described by Kabat and Mayer,[3] and the precipitin procedure used was similar to that of these authors.[3] The amount of nitrogen in the precipitates was determined with a micro-Kjeldahl procedure similar to the Markham modification.[3] Rabbits were inoculated with a suspension of phycocyanin in complete Freund's adjuvant. The suspension was equal volumes of adjuvant[4] and protein solution. Each rabbit was injected with about 3 mg. of protein. Approximately 0.3 ml. of suspension was injected into each toe pad and 0.4 ml. subcutaneously in the back of the neck. Five rabbits were injected with deuterio phycocyanin isolated from *Plectonema calothricoides* and five with the protio protein. One month later the injections were repeated, the animals rested for one week, and then approximately 50 ml. of blood was collected from each rabbit by cardiac puncture. The animals were rested for a month, reinoculated and one week later bled again.

First course antisera to the deuterio phycocyanin from *P. calothricoides* and also to the corresponding protio protein were diluted 1:5 and 1 ml. of this dilution was added to 1 ml. of serially diluted antigen. Both antisera and antigen preparations were clarified previous to their use. All precipitin procedures were done at constant volume and at the antiserum dilution. The precipitins were then allowed to stand for 3 days at 1°. Appropriate antigen and antiserum blanks were done simultaneously. The precipitates were spun down and washed[3] and transferred to Kjeldahl flasks for nitrogen determination. The supernatants were saved and checked for the presence of antibody and antigen. In all the experiments reported in this study the results of the tests of supernatant for excess antibody and antigen were in general agreement with the information derived from the determination of the amount of nitrogen in the precipitates. Precipitin experiments were also carried out with crude algal extracts. An antigen concentration of about 0.2% was used in the agar diffusion experiments.

The precipitin lines on Ouchterlony plates were examined for fluorescence with a long wave length, ultraviolet mineral light. Sedimentation values for the deuterio phycocyanin from *P. calothricoides* and for the protio phycocyanin from the same source, both in 1% saline solution, were determined by a Spinco model E analytical ultracentrifuge. Both proteins were sedimented in the same run in which one cell was used with a quartz 1° positive wedge window.

Results

I. Diffusion Studies.—First course antisera in agar, in Oudin tubes against which the protio and deuterio antigen diffused, exhibited definite precipitin lines. Sharp single lines were observed in the antisera prepared against protio protein. The antideuterio antisera in Oudin tubes exhibited multiple lines. Double diffusion Ouchterlony plates were then set up in which antideuterio *P. calothricoides* antisera were allowed to diffuse simultaneously against deuterio and protio phycocyanin from *P. calothricoides*, deuterio phycocyanin from *Phormidium luridum* and protio phycocyanin from *Synechococcus lividus*.[5] Antisera to protio phycocyanin were allowed to diffuse against the same antigens. The results of this double diffusion and other

(1) D. S. Berns, H. Crespi and J. J. Katz, *J. Am. Chem. Soc.*, **85**, 8 (1963).

(2) The term "deuterio protein" refers to the protein with deuterium substituted for hydrogen in all normally non-exchangeable positions. "Protio protein" is the normal hydrogen-containing protein.

(3) E. Kabat and M. Mayer, "Experimental Immunochemistry," C. C. Thomas, Springfield, Ill., second edition, 1961, pp. 22–96. Note that antigens used in the Ouchterlony plates were of less purity than those used in immunizing.

(4) Prepared by C. Brown of this Laboratory.

(5) A culture of this alga was kindly supplied by D. L. Dyer, Martin Co., Denver, Colo.

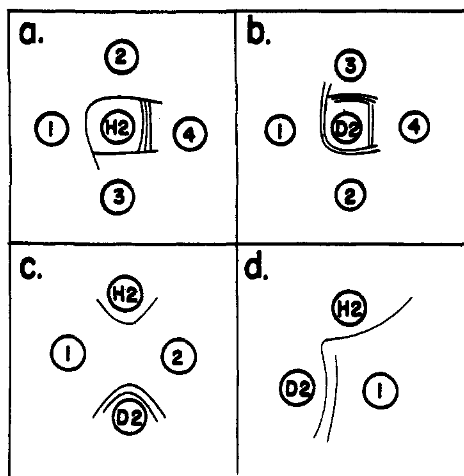

Fig. 1.—Line representations of agar diffusion plates. (a) First course antiserum to protio *P. calothricoides* phycocyanin from rabbit H2 is in the reservoir marked H2 and has been allowed to diffuse against antigens 1, 2, 3 and 4; all antigens are in 0.15 molar saline: 1, protio *P. calothricoides* phycocyanin; 2, deuterio *P. calothricoides* phycocyanin; 3, deuterio *P. luridum* phycocyanin; 4, protio *S. lividus* phycocyanin.

(b) First course antiserum to deuterio *P. calothricoides* phycocyanin from rabbit D2 is in the reservoir marked D2 and has been allowed to diffuse against antigens 1, 2, 3 and 4.

(c) First course antiserum to deuterio *P. calothricoides* phycocyanin from rabbit D2 and first course antiserum to protio *P. calothricoides* from rabbit H2 have been allowed to diffuse simultaneously against antigens 1 and 2.

(d) First course antiserum to deuterio *P. calothricoides* phycocyanin from rabbit D2 and first course antiserum to protio phycocyanin from rabbit H2 are allowed to diffuse simultaneously against antigen 1. Similar results were found using antigen 2 in place of 1.

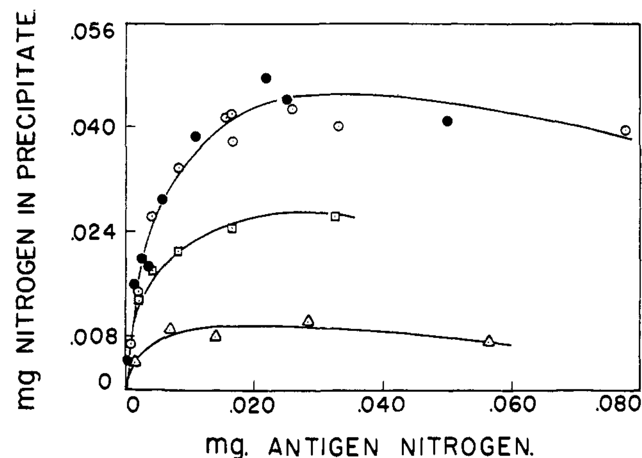

Fig. 2.—Quantitative precipitin curve for antiserum to protio *P. calothricoides* phycocyanin from rabbit H2 and several antigens; antigens: ●, deuterio *P. calothricoides* phycocyanin; ⊙, protio *P. calothricoides* phycocyanin; ⊡, deuterio *P. luridum* phycocyanin; ⚠, protio *S. lividus* phycocyanin.

agar diffusion studies are shown in Fig. 1. Ouchterlony plates were also set up with pooled normal rabbit γ-globulin diffusing against the above-mentioned array of antigens and no lines were detected. The antiprotio antisera in Ouchterlony plates exhibit single sharp lines between protio and deuterio *P. calothricoides* phycocyanin and antisera with no spurring. The deuterio *P. luridum* phycocyanin and protio *S. lividus* phycocyanin lines spur with relation to the *P. calothricoides* phycocyanin. The antideuterio *P. calothricoides* antisera exhibit behavior similar to that of the

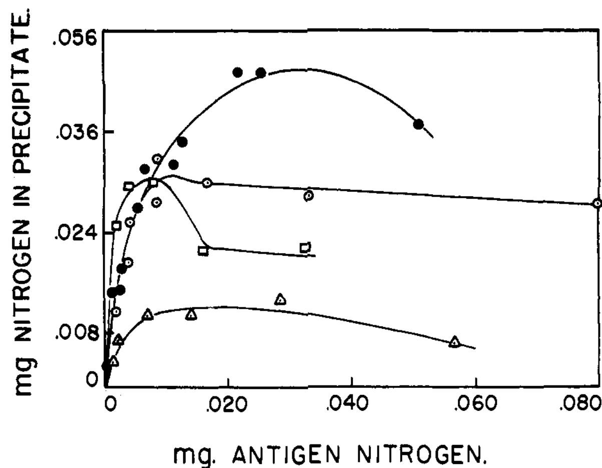

Fig. 3.—Quantitative precipitin curve for antiserum to deuterio *P. calothricoides* phycocyanin from rabbit D2 and several antigens: ●, deuterio *P. calothricoides* phycocyanin; ⊙, protio *P. calothricoides* phycocyanin; ⊡, deuterio *P. luridum* phycocyanin; ⚠, protio *S. lividus* phycocyanin.

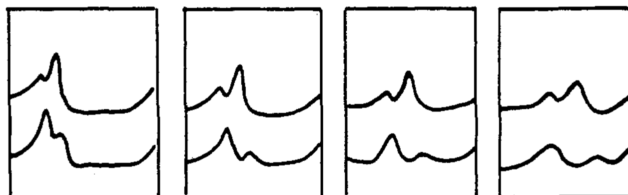

Fig. 4.—Line representation of sedimentation patterns of deuterio *P. calothricoides* phycocyanin, upper pattern, and protio *P. calothricoides* phycocyanin, lower pattern; speed 59,780 r.p.m.; exposures taken at 8.00-minute intervals after reaching speed; concentration of both deuterio and protio *P. calothricoides* phycocyanin solution ~1%; sedimentation is from left to right.

antiprotio *P. calothricoides* antisera except that double bands are present. All the lines found on the Ouchterlony plates fluoresced when exposed to a long wave length mineral light. The fluorescence was in the red region coinciding with the observed fluorescence[1] of the phycocyanin.

II. Precipitin Studies.—The antibody–antigen reaction qualitatively characterized by the Oudin and Ouchterlony diffusion techniques was quantitatively characterized by precipitin studies. These results are presented in Fig. 2 and 3. Precipitin studies carried out with crude algal extracts did not exhibit an increase in total nitrogen found in the precipitates.

III. Ultracentrifuge Studies.—To characterize to some extent the immunizing system, ultracentrifuge runs were made on the saline solution of the protio and deuterio *P. calothricoides* proteins. Typical sedimentation patterns are represented in Fig. 4 and the S_{20} values are presented in Table I. Viscosity studies were not performed at this time for these systems and the same partial specific volume corrections were used for deuterio and protio proteins.[6]

TABLE I

SEDIMENTATION CONSTANTS OF PHYCOCYANINS IN SALINE

Deuterio- *P. calothricoides* phycocyanin S_{20}	Protio *P. calothricoides* phycocyanin S_{20}
14.80×10^{-13}	9.60×10^{-13}
6.55×10^{-13}	5.77×10^{-13}

Discussion

The most obvious and most important consequences of the results of the immunochemical investigation are

(6) Extensive studies of $\overline{V}$ and other physical–chemical studies of the protio and deuterio protein are in progress by E. Scott of this Laboratory.

that the deuterio *P. calothricoides* and protio *P. calothricoides* phycocyanin can now be established as structurally identical, as far as the extremely sensitive immunochemical technique can be used as an indication of structural differences. Previous amino acid analysis of these two proteins[1] established that there is quite probably identity of primary protein structure. The immunochemical evidence indicates the identity of secondary and tertiary structure. It is not proposed that all bond lengths and helix dimensions are identical but that, aside from the small differences in interatomic parameters induced by the substitution of D for H, the secondary and tertiary structures are quite likely identical. The sensitivity of the immunochemical technique to small differences in protein structure is well documented.[7-10] While it cannot be unequivocally stated that the primary, secondary and tertiary structures of the protio and deuterio protein are identical, the amino acid analysis and the immunochemical studies favor this conclusion. In fact, no evidence uncovered to date would seem to support a proposed structural difference.

Qualitative Aspects.—The antigenicity due to species differences in phycocyanins derived from *P. calothricoides*, *P. luridum* and *S. lividus* is well pronounced in the diffusion studies represented in Fig. 1a and b. The presence of two lines in the antideuterio diffusion plate does indicate an apparent difference in immunogenicity of deuterio and hydrogen protein; however, the antigenicity of both proteins is still seen to be identical as indicated by the lack of spurring of bands in Fig. 1c. Both these bands fluoresce red when exposed to long wave length ultraviolet light and it is, therefore, maintained that both lines represent an antibody–phycocyanin aggregate. Since all bands in the double diffusion studies fluoresced, it is quite likely that all the antibody produced is specific to phycocyanin. Ultracentrifuge data and other physical studies in progress in this Laboratory definitely indicate that the phycocyanins are an associating system. In the present study the ultracentrifuge data (Fig. 4 and Table I) demonstrate that there is definitely more of the higher polymeric form of the phycocyanin in the deuterio than in the protio preparation. The agar double diffusion study represented in Fig. 1d indicates that the second line in the antideuterio antisera studies is quite likely associated with antibody to the higher polymeric form, the faster diffusing lower molecular weight phycocyanin being closer to the antisera reservoir and present in both antideuterio and antiprotio antisera.

The curvature of the lines in the agar double diffusion experiments shows that the antigen diffuses more rapidly than the antibody, a fact that certainly favors the existence of phycocyanin in a form with a molecular weight less than that of antibody. This would place the values at considerably less than 150,000. Previous molecular weights reported were in the 200,000–300,000 region.[11,12] This, of course, does not preclude the existence of higher order aggregates of molecular weight 200,000 to 300,000.

Another interesting fact is that the phycocyanin derived from *S. lividus* crossreacts with antibody to *P. calothricoides* phycocyanin. *Synochococcus lividus* is a thermophilic alga and grows at temperatures up to 55° and phycocyanin from *P. calothricoides* has been reported to denature at about 50–55° depending on *p*H.[1] Preliminary investigations of denaturation of phycocyanin from *S. lividus* indicate that it denatures in the 80° region. Undoubtedly, there are sizable differences in the two proteins; however, a certain portion of the antigenicity of the phycocyanins is quite likely associated with the chromophore which is undoubtedly quite similar if not identical in all C-phycocyanins.

Quantitative Aspects.—The precipitin curves in Fig. 2 demonstrate that quantitatively there is little discernible difference between the antigenicity toward antiprotio antisera of the deuterio and protio phycocyanin from *P. calothricoides*. The deuterio *P. luridum* phycocyanin and protio *S. lividus* phycocyanin did not precipitate as much first course antiprotio antiserum as did the deuterio and protio *P. calothricoides* and this is as expected from the earlier observed difference on the agar double diffusion plates and the often observed difference in precipitability of antigens from different species.[8] The precipitin curves for the antideuterio antisera (Fig. 3) do not exhibit the same quantitative identity of protio and deuterio phycocyanin antigenicity. This difference can be explained to some extent by the fact that the antideuterio antisera have a large amount of antibodies present that are directed toward the higher molecular weight phycocyanin aggregate, and the deuterio phycocyanin, it is evident from the ultracentrifuge data, has a considerably greater amount of the higher molecular weight aggregates. Precipitin experiments carried out with crude algal extracts did not exhibit an increase in total nitrogen in the precipitates, which would indicate that all the precipitating antibody is quite likely directed against phycocyanin, and antibody directed against impurities is not an important factor. The position of the *P. luridum* and *S. lividus* antigens on the antideuterio antisera precipitin curves is quite similar to that found in the antiprotio antisera precipitins and this adds to the overall consistency of the experimental results.

Conclusions

The net result of the present investigation is to lay a firm basis for future studies of the deuterio and protio phycocyanins and their intercomparison. It is hoped that additional information can be gleaned from these comparisons about the relative importance of various contributions to protein structure in solution and protein interaction in solution. It is believed that the evidence presented favors strongly the conclusion that the unassociated deuterio and protio phycocyanins from *P. calothricoides* are identical in the structural aspects to which the techniques are sensitive. It, therefore, justifies the examination of observed differences in properties of these two proteins on the basis of a difference in only isotopic substitution.

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