G. D., Ed., New York, N. Y., Marcel Dekker, Chapter 6.

Yang, J. T. (1967b), *in* Manual of the Analytical Methods in Protein Chemistry, Vol. 5, Alexander, P., and Lundgren, H. P., Ed., Oxford, Pergamon (in press).

Yang, J. T., and Doty, P. (1957), *J. Am. Chem. Soc. 79*, 761.

Functional Properties of a Hemoglobin Carrying Heme Only on α Chains*

Kaspar H. Winterhalter, Gino Amiconi, and Eraldo Antonini

ABSTRACT: The functional properties of a hemoglobin carrying heme only on the α chains ($\alpha^h\beta$) are described. The protein shows nearly hyperbolic oxygen equilibrium curves with three to ten times higher oxygen affinity than normal hemoglobin, depending on pH. The Bohr effect is present, but is about one-half that of normal hemoglobin. The velocity constant of the reaction between $\alpha^h\beta$ and CO is much higher than in hemoglobin (initial second-order rate constant, $l' \simeq 2 \times 10^6$ M^{-1} sec^{-1}); the same kinetic pattern is obtained in rapid mixing and flash photolysis experiments. Addition of the full heme complement to the $\alpha^h\beta$ compound gives a product with all the properties of natural hemoglobin both in respect to the O_2 equilibrium and to the kinetics of reaction with CO. The functional properties of the $\alpha^h\beta$ compound are different from those of isolated α chains, which indicates that the reactivity of the heme in a given chain is modified by interaction with the partner chain, even if the latter is devoid of prosthetic group.

The existence of compounds resulting from incomplete reconstitution of hemoglobin from globin and heme has been suspected for several years (Winterhalter and Huehns, 1964; Gibson and Antonini, 1966). A compound of this type has been isolated by two groups of investigators (Winterhalter, 1966; Banerjee and Cassoly, 1967). The compounds obtained by the two groups are similar to each other (Banerjee and Cassoly, 1967; Winterhalter and Deranleau, 1967) in many respects. Thus both groups found that the partially saturated compound had, at protein concentrations above 10 mg/ml, a molecular weight corresponding to the hemoglobin tetramer, that it has only two hemes per four polypeptide chains, both attached to the α chains, with the two specific binding sites for heme on the β chains unoccupied. The present paper reports observations on the equilibria and kinetics of the reaction of the partially reconstituted compound obtained by Winterhalter with O_2 and CO, before and after its conversion into hemoglobin by the addition of 2 moles of heme/mole of protein. It may be anticipated that the partially reconstituted compound shows marked changes in functional behavior as compared with hemoglobin. The significance of these results is strengthened by the finding that the addition of heme converts it into functionally normal hemoglobin.

Material and Methods

All chemicals used were of the highest purity available. The compound carrying the heme only on the α chains was obtained by the method previously described (Winterhalter, 1966). The compound referred to before as ICII will be designated here as $\alpha^h\beta$;[1] this does not imply any statement in regard to the actual particle size of the compound (which at protein concentrations above 1% has a molecular weight similar to that of hemoglobin; Winterhalter and Deranleau, 1967). $\alpha^h\beta$ was converted into the compound fully saturated with heme (hemoglobin) by the addition of excess hemin dissolved in potassium phosphate buffer containing 100 mg of KCN/l. (pH 7.5). Cyanide ferric heme in excess over the stoichiometric amount of one per chain was removed by chromatography on a DEAE column (15 × 100 mm) equilibrated with a 0.1 M phosphate buffer (pH 7.0). Elution was carried out with the same buffer. In one experiment $\alpha^h\beta$ was converted into hemoglobin by adding stoichiometric amounts (in re-

---

* From the Laboratorium für Molekularbiologie der Eidenössisch Technischen Hochschule, Zürich, Switzerland, and the Consiglio Nazionale delle Ricerche, Centro di Biologia Molecolare, Università di Rome, Rome, Italy. *Received February 1, 1968.* This work was supported by the Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung, Grants No. 4329 and 3426, and in part by Research Grant AM 09807 from the U. S. Public Health Service and Grant No. E00AR-68-0004 from the Office of Scientific Research through the European Office of Aerospace Research, Office of Aerospace Research, U. S. Air Force.

[1] The superscript h denoting one molecule of heme

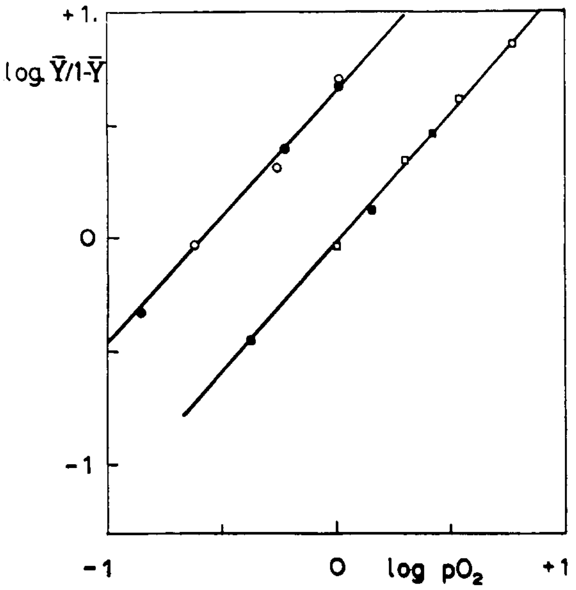

FIGURE 1: Hill plot of oxygen equilibrium of $\alpha^h\beta$. Conditions: 21°; potassium phosphate buffer, 0.2 M, pH 7.0 (●○); 2% borax, pH 9.1 (■□). Different symbols indicate different experiments on the same preparation (n = 1.1). Protein concentration 2–2.5 × 10^{-4} M in heme.

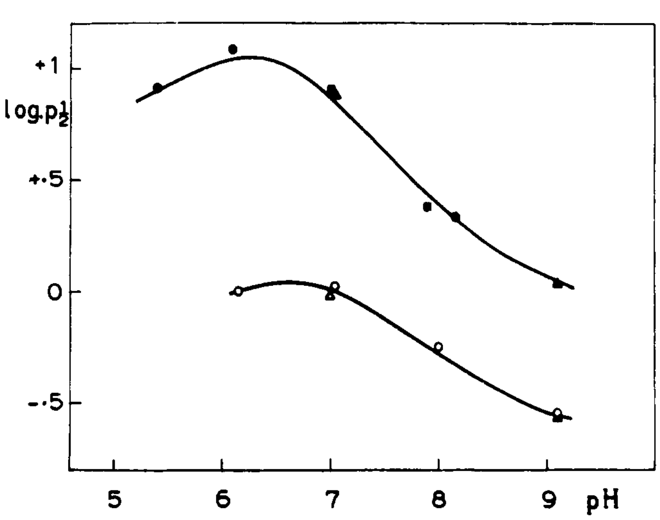

FIGURE 2: pH dependence of log $p^{1/2}$ for: △○ = $\alpha^h\beta$; ● = natural hemoglobin; ■ = hemoglobin obtained from direct recombination of globin and heme; ▲ = $\alpha^h\beta$ + hemin leading to the formation of hemoglobin. Conditions: 21°; 0.2 *M* phosphate buffer (pH 6–8); 0.4 M acetate (pH 5.4); 2% borax (pH 9.1).

spect to the unoccupied β chains) of ferric heme 5 min before the kinetic experiments. The other compounds were stored, up to about 2 weeks, at 4° in 0.01 M sodium phosphate buffer (pH 6.8).

Conversion of the cyanide ferric heme proteins into the oxygenated derivatives was carried out by the addition of minute amounts of sodium dithionite to the protein solutions and subsequent dialysis against several changes of potassium phosphate buffer, 0.1 M (pH 7.0), containing 10^{-3} M EDTA.

The O_2 equilibrium curves (Rossi-Fanelli and Antonini, 1958) were determined at 21° in 0.2 M potassium phosphate, 0.4 M sodium acetate, and 2% borax buffers. In these experiments the concentration of heme protein was between 2 and 2.5 × 10^{-4} M in terms of heme. At the end of each experiment the pH of the solutions was measured with a Radiometer Model 4 pH meter. The spectrophotometric data were obtained on a Beckman Model DK 1 recording spectrophotometer. Stopped-flow measurements were performed with a Gibson-Durrum stopped-flow apparatus (Gibson and Milnes, 1964). Flash photolysis experiments were made with the apparatus described before (Antonini *et al.*, 1967). The experimental procedures were the same as those described previously (Antonini *et al.*, 1965).

Results

O_2 Equilibria. The shape of the O_2 dissociation curve of $\alpha^h\beta$ is nearly hyperbolic, the value of n in the Hill equation $y/(1 - y) = kp^n$ being equal to 1.0–1.1 (Figure 1). The O_2 affinity is higher (three to ten times) than that of hemoglobin; the Bohr effect is present however, reduced to about one-half that of normal hemoglobin (Figures 1 and 2). The protein was stable during the determination of the O_2 equilibrium curve except at the lowest pH, near 6 (the value of log $p^{1/2}$ shown in Figure 2 is the mean of three experiments which gave values of log $p^{1/2}$ of 0.0, 0.15, and 0.87–1). The instability at the lower pH prevented measurements of the reverse Bohr effect.

The product obtained by the addition of heme to $\alpha^h\beta$, thus having the full heme complement, shows the same oxygen affinity and Bohr effect than normal hemoglobin. This is evident from Figure 2 showing the O_2 affinity of this product, of the hemoglobin reconstituted by adding initially the full heme complement to globin, and of normal hemoglobin, all stored, reduced, and studied under similar conditions. The values of n for the hemoglobin reconstituted from $\alpha^h\beta$ were near 2.1; the ones for the directly reconstituted hemoglobin were 2.3–2.5 and for the "natural" hemoglobin 2.6. These and the values of log $p^{1/2}$ shown in Figure 2 are somewhat lower than the values found for fresh preparations of oxyhemoglobin.

Kinetics of the Reaction with Carbon Monoxide. The essential results obtained in rapid mixing experiments are shown in Figure 3. The incompletely reconstituted compound $\alpha^h\beta$ reacts with CO with a much faster rate than hemoglobin. The progress curve, when the reaction is treated as a second-order reaction, shows a tendency of the rate to decrease as the reaction proceeds. The initial second-order rate constant, l', is about 2 × 10^6 M^{-1} sec^{-1}. On the other hand, the kinetic behavior becomes the same as that of hemoglobin when more heme, enough to saturate the heme-free β chains, is added. It is noteworthy that this may be achieved even if the lacking heme complement is added to the compound just before the kinetic run. This is evident both in respect to the shape of the progress curves and to the value of the apparent second-order rate constant.

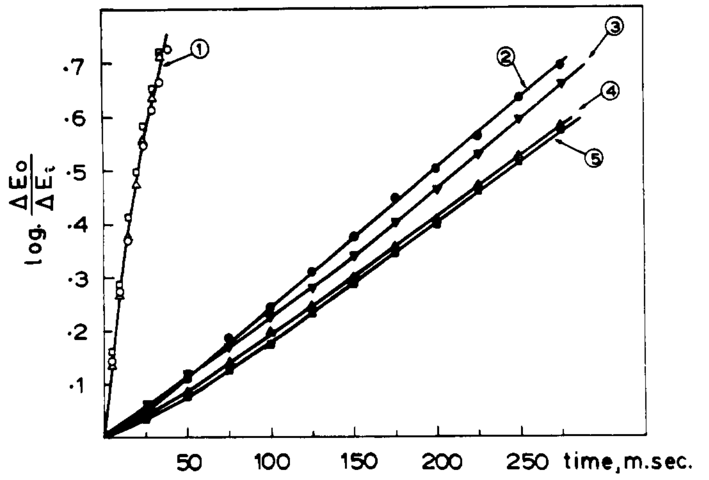

FIGURE 3: Combination of reduced heme-proteins with CO in rapid mixing experiments. Curve 1: $\alpha^h\beta$ (empty symbols ○, △, and □ representing three different preparations of $\alpha^h\beta$). Curve 2: natural hemoglobin (●). Curve 3: $\alpha^h\beta$ converted into hemoglobin by addition of stoichiometric amounts of hemin (▼), same sample as □. Curve 4: $\alpha^h\beta$ converted into hemoglobin several days before the experiment (▲). Curve 5: directly reconstituted hemoglobin (■). Conditions: 23°; CO 2.5 × 10^{-5} M; phosphate buffer, 0.1 M, pH 7.0; λ 430 mμ. Concentration $\sim$2.5 × 10^{-6} M in heme.

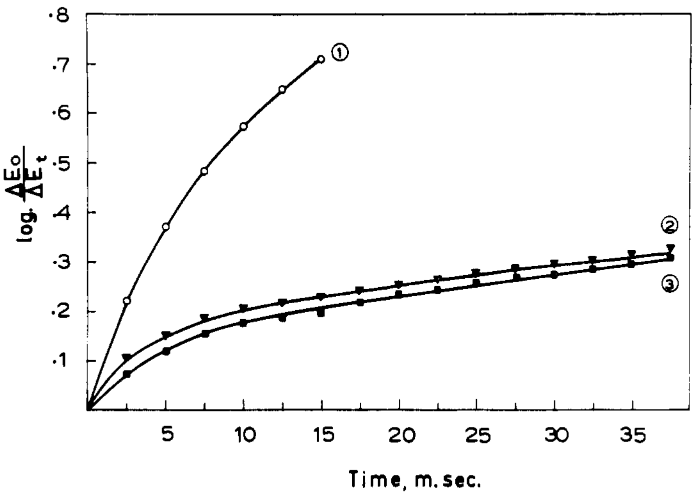

FIGURE 4: Recombination of heme proteins with CO after flash photolysis. Curve 1: $\alpha^h\beta$ (○). Curve 2: $\alpha^h\beta$ + stoichiometric amounts of hemin (▼), same preparation as curve 1. Curve 3: directly reconstituted hemoglobin (■). Conditions: 24°; CO concentrated 1 × 10^{-4} M phosphate buffer, 0.1 M, pH 7.0; λ 430 mμ. Flash energy 300 J. Flash duration (to 10% peak concentration) $\sim$300 μsec. Protein concentration 0.5 × 10^{-4} M in heme.

Flash Photolysis. The results of some of the flash photolysis experiments are shown in Figure 4. The $\alpha^h\beta$ compound shows, in flash photolysis, kinetics of the reaction with CO similar to that observed in rapid mixing experiments, both in respect to the initial value of the second-order velocity constant ($l' \simeq 2 \times 10^6$ M^{-1} sec^{-1}) and to the heterogeneity. The reconstituted hemoglobins behave, also in the flash photolysis experiments, similarly to natural hemoglobin under the same conditions (Antonini *et al.*, 1967).

Discussion

The results of the present work lead to interesting conclusions in respect to both the problem of reconstitution of hemoglobin from its components and of the equilibrium and kinetic aspects of the reaction of hemoglobin with ligands.

When heme is added to "native" globin the reconstituted hemoglobin exhibits the same properties in the reactions with ligands as native hemoglobin. This is a confirmation of previously reported observations (Rossi-Fanelli and Antonini, 1958; Antonini and Gibson, 1960). Moreover, the present experiments show that reconstitution of functionally normal hemoglobin from globin and heme can take place in steps, with first addition of heme to the α-chain globin and then binding of heme to the β chains. Taken together with recent studies on the reconstitution of hemoglobin from isolated α^h and β^h chains (Bucci and Fronticelli, 1965; Antonini *et al.*, 1966; De Renzo *et al.*, 1967) these results confirm the conclusion that no matter how the final stoichiometric mixture is achieved, the hemoglobin molecule, with all its attributes, is spontaneously reassembled by simply mixing its components (Antonini, 1967). The results reported here for $\alpha^h\beta$ are at variance with those obtained by Banerjee and Cassoly (1967) which indicated that the α-heme β-chain compound could not be readily converted into hemoglobin, since it maintained the abnormal functional properties even after saturation with heme. It should be pointed out, as mentioned in the introduction, that in several respects the compound studied by Cassoly *et al.* (1967) is similar to that described here. This is true to some extent also in respect to the reaction with ligands. We have ourselves observed a certain instability of the $\alpha^h\beta$ compound which is particularly evident on prolonged storage. Thus it appears that the possibility of reobtaining hemoglobin from these compounds carrying heme groups only on the α chains might depend critically on the preservation of the naked β chains from irreversible changes during manipulations and storage.

The functional properties of $\alpha^h\beta$ differ considerably from that of hemoglobin. The O_2 equilibrium shows a value of *n* close to 1, which indicates disappearance of heme–heme interactions; the O_2 affinity is increased, but a substantial part of the Bohr effect remains. These differences are abolished by adding the full heme complement, although the value of *n* in the hemoglobin reconstituted from $\alpha^h\beta$ is lower than normal. However, a lower value of *n* is invariably seen after almost any manipulation of hemoglobin, which is not surprising since the O_2 equilibrium of hemoglobin is very sensitive to slight modifications which might introduce functional heterogeneities in the system. Even the normal or reconstituted hemoglobins studied as controls showed a lower value of *n* and slightly higher O_2 affinity than freshly prepared, untreated HbO_2. The low value of *n* associated with a large Bohr effect observed with the $\alpha^h\beta$ represents another case of dissociation between changes in the heme–heme interaction and in the Bohr effect (see for instance Hb Chesapeake; Nagel *et al.*, 1967). The meaning of this in relation to the general problem of functional interactions in hemoglobin has been discussed elsewhere (Antonini, 1965).

The O_2 equilibrium of $\alpha^h\beta$ is different from that of isolated α^h chains. The O_2 affinity of isolated α^h chains is higher and the Bohr effect is absent (Antonini *et al.*, 1965; De Renzo *et al.*, 1967). A low value of n, associated with high oxygen affinity and the presence of a Bohr effect, has also been observed for hemoglobin Gun Hill (Bradley and Rieder, 1966), an abnormal hemoglobin in which, due to deletion of five amino acid residues, (Bradley and Wohl, 1967) heme-free abnormal β chains are associated with normal α^h chains.

The general features of the ligand equilibrium of $\alpha^h\beta$ are similar to those of compounds in which functioning α^h chains are associated with heme carrying but inactive β^h chains, such as nitric oxide intermediates of the type $\alpha\beta^{NO}$.[2] However, the O_2 affinity of these compounds is lower than that of $\alpha^h\beta$. They are also similar to those of some hemoglobins M (Murawski *et al.*, 1965; Hayashi *et al.*, 1966), although these hemoglobins exhibit some variability in their oxygen equilibria between one another.

In the kinetics of the reaction with CO the $\alpha^h\beta$ compound shows a value for the initial second-order rate constant about 20 times higher than that of hemoglobin. The progress curve shows a certain degree of kinetic heterogeneity since the rate tends to slow down as the reaction proceeds. Since the kinetic experiments are done at a low protein concentration, a possible explanation of the kinetic heterogeneity might be dissociation of the deoxy $\alpha^h\beta$ compound into single-chain molecules, *i.e.*, $\alpha^h\beta \rightleftharpoons \alpha^h + \beta$, assuming that the rate of combination with CO of $\alpha^h\beta$ is lower than that of α^h. This suggestion is supported by the tendency of the compound to dissociate as observed in ultracentrifuge experiments even at much higher protein concentrations (Winterhalter and Deranleau, 1967) and by the fact that the initial rates of reaction with CO of $\alpha^h\beta$ are similar to those of isolated chains (Brunori *et al.*, 1966).[3] In this connection it is important to emphasize that in the kinetics of the reaction with CO, the $\alpha^h\beta$ compound behaves very differently from the compounds quoted above in which the functioning α^h chains are associated to functionally inert, but heme-carrying, β^h chains, with the possible exception of hemoglobin M radon (Chicago) (Gibson *et al.*, 1966). In rapid mixing experiments these compounds react with CO at a rate similar to that of fully unliganded hemoglobin and vastly different therefore from that corresponding to the isolated α^h chains. In this context it might be interesting to report some preliminary experiments in which protoporphyrin, instead of heme, was added to the $\alpha^h\beta$ compound.[4] This new compound $\alpha^h\beta^p$ in which presumably the protoporphyrin occupies the free heme sites on the β chain, although the reaction was also kinetically heterogeneous, showed a much slower rate than $\alpha^h\beta$ in the reaction with CO. These results as outlined above might be at least in part due to the different tendency to dissociate into single chains of the deoxygenated derivatives of the various compounds.

In conclusion, all the studies on hemoglobin-like compounds in which only the one type of chain is active in respect to ligand binding show that the reactivity of the heme on a given chain is affected by the interaction with a partner chain, quite apart from the presence or absence of the ligand, or even of the heme group on the interacting chain. This conclusion, especially in view of the presence of a considerable Bohr effect in the $\alpha^h\beta$ compound, has obvious implications in respect to the regulation of the activity in proteins as envisaged by "allosteric" mechanisms.

References

Antonini, E. (1965), *Physiol. Rev. 45*, 123.

Antonini, E. (1967), *Science 158*, 1417.

Antonini, E., Bucci, E., Fronticelli, C., Chiancone, E., Wyman, J., and Rossi-Fanelli, A., (1966), *J. Mol. Biol. 17*, 29.

Antonini, E., Bucci, E., Fronticelli, C., Wyman, J., and Rossi-Fanelli, A. (1965), *J. Mol. Biol. 12*, 375.

Antonini, E., Chiancone, E., and Brunori, M. (1967), *J. Biol. Chem. 242*, 4360.

Antonini, E., and Gibson, Q. H. (1960), *Biochem. J. 76*, 534.

Banerjee, R., and Cassoly, R. (1967), *Biochim. Biophys. Acta 133*, 545.

Bradley, T. B., Jr., and Rieder, R. F. (1966), *Blood 28*, 976.

Bradley, T. B., Jr., and Wohl, R. C. (1967), *Science 157*, 1581.

Brunori, M., Noble, R. W., Antonini, E., and Wyman, J. (1966), *J. Biol. Chem. 241*, 5238.

Bucci, E., and Fronticelli, C. (1965), *J. Biol. Chem. 240*, PC551.

Cassoly, R., Bucci, E., Iwatsuko, M., and Banerjee, R. (1967), *Biochim. Biophys. Acta 133*, 557.

De Renzo, E., Ioppolo, E., Amiconi, G., Antonini, E., and Wyman, J. (1967), *J. Biol. Chem. 242*, 4850.

Gibson, Q. H., and Antonini, E. (1966), Hemes and Hemoproteins, Chance, B., Estabrook, R. W., and Yonetani, T., Ed., New York, N. Y., Academic.

Gibson, Q. H., Heller, P., and Yakulis, V. (1966), *J. Biol. Chem. 241*, 1650.

Gibson, Q. H., and Milnes, L. (1964), *Biochem. J. 91*, 161.

Hayashi, N., Motokawa, Y., and Kikuchi, A. (1966), *J. Biol. Chem. 241*, 79.

Murawski, K., Carta, S., Sorcini, M., Tentori, L., Vivaldi, G., Antonini, E., Brunori, M., Wyman, J., Bucci, E., and Rossi-Fanelli, A. (1965), *Arch. Biochem. Biophys. 111*, 197.

Nagel, R. L., Gibson, Q. H., and Charaches, S. (1967),

---

[2] M. Brunori, R. W. Noble, and E. Antonini, unpublished data.

[3] It may be pointed out that the O_2 equilibrium experiments are done at a protein concentration (few milligrams per milliliter) where ultracentrifuge and gel filtration experiments indicate no dissociation into single chains.

[4] Results similar to those reported here for the kinetics of reaction with CO of $\alpha^h\beta$ and $\alpha^h\beta^p$ have been obtained recently by Gibson (Q. H. Gibson, personal communication).

Biochemistry 6, 2395.

Rossi-Fanelli, A., and Antonini, E. (1958), *Arch. Biochem. Biophys. 80*, 229.

Rossi Fanelli, A., Antonini, E., and Caputo, A. (1958), *Biochem. Biophys. Acta 30*, 608.

Winterhalter, K. H. (1966), *Nature 211*, 932.

Winterhalter, K. H., and Deranleau, D. A. (1967), *Biochemistry 6*, 3136.

Winterhalter, K. H., and Huehns, E. R. (1964), *J. Biol. Chem. 239*, 3699.

Phycocyanobilin. Structure and Exchange Studies by Nuclear Magnetic Resonance and Its Mode of Attachment in Phycocyanin. A Model for Phytochrome*

Henry L. Crespi, Ursula Smith, and Joseph J. Katz

ABSTRACT: Phycocyanobilin, the prosthetic group of phycocyanin, has been isolated in high yield by methanol extraction. Its structure has been determined by nuclear magnetic resonance and mass spectral studies to be mesobiliverdinoid, but with an ethylidene group at position 2. The methyl hydrogen atoms of this ethylidene group, as well as of methine bridge *c* and the hydrogen at position 1, α to a carbonyl group, have been found readily exchangeable. Present evidence indicates at least two possible ester modes of attachment of bilin to apoprotein, and one of these offers a model for the photochromic behavior of phytochrome.

Phycocyanin, a photosynthetic protein present in blue-green algae, is one of an important group of plant proteins that contain a bile pigment prosthetic group. The structure of the prosthetic group of phycocyanin, phycocyanobilin (PCB),[1] was first described by Lemberg (Lemberg and Bader, 1933; Lemberg and Legge, 1949) as a linear tetrapyrrole compound. A more definitive characterization of phycocyanobilin has been subsequently severely hampered by the lack of a good preparative procedure and the apparent lability of the compound (*cf.*, for example, Ó hEocha, 1963, 1966). It was found recently, however (Fujita and Hattori, 1962, 1963), that refluxing whole algae with methanol would liberate phycobilins. Ó Carra and Ó hEocha (1966) and Siegelman *et al.* (1966) have used this observation as the basis of their methods for the liberation of phycobilins from purified protein. The preparative procedure reported here is a considerable modification of those previously reported and allows very high yields of phycocyanobilin.

In our work, we have isolated phycocyanobilin from C-phycocyanin in 40–50% yield. Our product is essentially chromatographically pure and shows only traces of other pigments. In view of the possible lability of this bilin, we felt it of overriding importance to avoid any extensive chemical or chromatographic processing. To this end, we have confined ourselves to mild procedures involving solvent extraction and have worked with the free acid so obtained rather than resorting to esterification. From data obtained with material isolated by our procedure, we propose a structure for phycocyanobilin (Figure 7A). This structure is very similar to, but not the same as, that (Figure 7C) reported by other workers (Cole *et al.*, 1967; Rüdiger *et al.*, 1967) and provides a basis for the chemistry of the ethylidene functional group. Also, the placement of the ethylidene group at position 2 in the PCB molecule is established with some rigor. Further, with respect to the mode of attachment of phycocyanobilin to the apoprotein, we present data that suggests two possibilities: the first, an ester linkage to serine; the second, an attachment involving a C–C bond directly to the peptide backbone that is labilized by rupture of an ester bond. The second possibility appears to offer an interesting model for the photoresponses of phytochrome. A preliminary report of some aspects of this work has already appeared (Crespi *et al.*, 1967).

Experimental Section

Isolation of Phycocyanin. C-phycocyanin was isolated from *Phormidium luridum* or *Synechococcus lividus* grown in H_2O or 99.8% D_2O (DaBoll *et al.*, 1962). The protein was purified by ammonium sulfate fractionation to a purity index (ratio A_{620}/A_{280}) of 3.3–4.0 (Hattori *et al.*, 1965a). For experiments involving proteolytic digestion, further purification was effected by

---

* From the Chemistry Division, Argonne National Laboratory, Argonne, Illinois 60439. *Received January 10, 1968.* Based on work performed under the auspices of the U. S. Atomic Energy Commission.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: PCB, phycocyanobilin; HMS, hexamethyldisiloxane; TFA, trifluoroacetic acid; DPCB, deuterated PCB.