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EXCHANGEABLE HYDROGEN IN PHYCOERYTHROBILIN*

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WE WISH to report here the presence of exchangeable hydrogen atoms in phycoerythrobilin (PEB) and to discuss the implications of this data for the structure of this bilin. A structure for PEB has recently been proposed by Chapman *et al.*[1]

C-phycoerythrin was extracted from freeze-dried *Fremyella diplosiphon*[2] cells and purified by ammonium sulfate fractionation to a purity index (A_{565}/A_{280}) between 4 and 5. The phycoerythrin was then refluxed for 16 hr in 90 per cent methanol and phycoerythrobilin isolated according to the procedure described by Crespi *et al.*[3] for phycocyanobilin (PCB). TLC revealed, however, that this isolation procedure yielded phycoerythrobilin contaminated with a considerable amount of orange pigment. Consequently, the bilin was further purified by adsorption from pyridine solution onto a column of crystalline sodium acetate trihydrate. The orange pigment accompanied by some brown passed through the column. After washing the column with 3–4 bed volumes of pyridine saturated with sodium acetate, the column was drained and the deep purple adsorbent was dissolved in water. This solution was then acidified with glacial acetic acid and the phycoerythrobilin again isolated by the methods used for PCB.[3] Chromatography on Adsorbosil 5 (Applied Science Labs., State College, Pa.) in butanol–pyridine–water (3:1:1) and in butanol–acetic acid–water (4:1:1) showed a single pigment with only traces of other colored components visible at high loads. Table 1 gives the results of PMR (Varian HA-100 spectrometer) analysis of purified phycoerythrobilin. Our data, obtained with solutions of the free acid in pyridine-d_5 and in trifluoroacetic acid (TFA), are in essentially complete agreement with the PMR results of Chapman *et al.*[1] that were obtained with the dimethyl ester in deuteriochloroform.

We have also examined PEB isolated by refluxing fully deuterated phycoerythrin with ordinary (^1H) methanol. PMR analysis of this material dissolved in pyridine-d_5 showed protons at 1·57 and 5·86 ppm. If we assume complete exchange at the methine position, then $1{\cdot}1 \pm 0{\cdot}3$ protons are observed in the ethylidene methyl group. These two positions, then, are presumably exchanged during the isolation procedure. If similarly isolated deuterated PEB is analyzed in TFA solution, lines are observed at 1·79 and 5·95 ppm, as expected from the pyridine data. In addition to these lines, proton resonances appear at 3·31 ppm, apparently because of exchange of the hydrogen atom at carbon 1. In pyridine-d_5 there are resonance lines in the region of the spectrum at which the proton at carbon 1 would be expected to appear (about 3·15 ppm) but interpretation is made uncertain by the possible presence of small methoxy resonances. We also have observed that there is no further exchange at

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[1] D. J. CHAPMAN, W. J. COLE and H. W. SIEGELMAN, *J. Am. Chem. Soc.* **89**, 5976 (1967).
[2] H. F. DABOLL, H. L. CRESPI and J. J. KATZ, *Biotechnol. Bioeng.* **4**, 281 (1962).
[3] H. L. CRESPI, U. SMITH and J. J. KATZ, *Biochemistry* 7, 2232 (1968).

1·79 ppm on standing in TFA and that the proton at 5·95 is easily exchanged upon solution of PEB in TFA-d_1.

In summary, then, we have observed that in PEB (1) the hydrogen at carbon 1, which is alpha to a carbonyl group may be exchangeable in boiling, neutral methanol, and is exchangeable in TFA; (2) the hydrogen of one of the methine bridges is easily exchanged both in boiling, neutral methanol, and in TFA; (3) the hydrogen of the ethylidene methyl group shows exchange or the introduction of a proton by chemical reaction, or both, in boiling, neutral methanol, but no exchange in TFA.

Since the mode of attachment of PEB to its apoprotein has not yet been elucidated, it is possible that a proton is introduced into the ethylidene methyl group by chemical reaction

TABLE 1. PHYCOERYTHROBILIN—CHEMICAL SHIFTS

Chemical shift			
Pyridine*	TFA†	Multiplicity‡ and |J|, Hz ± ·02	Assignment
1·25	1·31	*d*, 7·2	CH_3 at C_1
1·57	1·79	*d*, 7·0	CH_3—CH=
1·83	1·94	*s*	β-CH_3
1·96	1·97	*s*	β-CH_3
2·01	2·00	*s*	β-CH_3
2·70	2·62	*m*	CH_2
3·05	3·00	*m*	CH_2
4·50	4·59	*u*	Bridge-CH_2
5·25	—	*m*	CH=CH_2
5·86	5·95	*s*	Bridge-CH
6·17	—	*m*,§ 7·0, 2·2	CH_3—CH=
6·50	—	*m*	CH_2=CH
6·56	7·41	*s*	Bridge-CH

* ppm downfield from internal hexamethyldisiloxane (HMS).
† From external HMS.
‡ *d*=doublet; *m*=multiplet; *s*=singlet; *u*=unresolved.
§ A quartet of doublets.

during detachment. We have suggested a mechanism for the detachment of bilin from the apoprotein[3] according to which it might be expected that a proton from the medium would be introduced into this methyl group. Consequently, to provide evidence on this point, we have refluxed ordinary (all ^1H) PEB for 42 hr in neutral, 90 per cent methanol-d_1 (CH_3O^2H) and again isolated PEB on a sodium acetate column. From integration of the PMR spectrum of this PEB, we estimate 45 ± 15 per cent ^2H at the ethylidene methyl position introduced by exchange with refluxing methanol.

Based on assignments made in previous works[3] the exchangeable methine proton in PEB is at bridge position *a*. However, in phycocyanobilin the proton at bridge *a* is non-exchangeable, which suggests a different location for the pyrrolenine nitrogen in PEB as compared to PCB. We favour the structure shown in Fig. 1. This structure is the same as that proposed by Chapman *et al.*[1] However, we believe the exchange data presented here provides a more firm basis for the location of the pyrrolenine nitrogen. The structure of Fig. 1 requires the

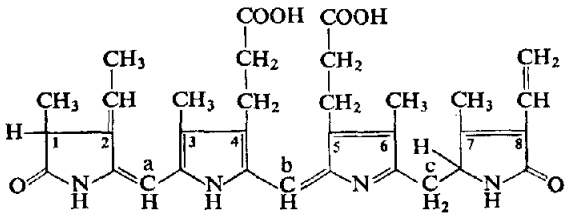

FIG. 1. STRUCTURE PROPOSED FOR PHYCOERYTHROBILIN.

activation of the ethylidene group for exchange to proceed through a prototropic form; consequently, the rate of exchange of the hydrogen in the ethylidene group in PEB should be much lower than in PCB. This may well be the case, as under similar conditions PCB showed about 50 per cent exchange at the ethylidene methyl position after 16 hr reflux, while 42 hr were required to show about 50 per cent exchange at this same function in PEB. These data leave open the possibility that a proton is introduced into the ethylidene methyl group by chemical reaction during detachment of PEB and a further small amount of hydrogen is incorporated into this methyl group by subsequent exchange.