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7. Benzylpenicillinic acid potassium salt.
8. D(—)-Threo-2,2-dichloro-*N*-[β-hydroxy-α(hydroxymethyl)-*p*-nitrophenethyl] acetamide.
9. 4-Dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide.
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24 July 1968

Proton Magnetic Resonance of Proteins Fully Deuterated except for ^{1}H-Leucine Side Chains

Abstract. *The fully deuterated proteins C-phycocyanin, C-phycoerythrin, and cytochrome c have been obtained by biosynthesis with the leucine side chains, and only the leucine side chains, of normal (^1H) isotopic composition. In these (isotopic hybrid) proteins, proton magnetic resonance analysis shows that the ^1H-leucine side chains are in a variety of environments. During protein biosynthesis, the alpha hydrogen of leucine is exchanged with a hydrogen (^2H) from the aqueous medium.*

There are a number of ways in which fully deuterated proteins can be used for magnetic resonance studies (*1*). We now report results on the incorporation of exogenous (^{1}H)-L-leucine into proteins synthesized by organisms growing in 99.8 percent D_2O (*2*). Algae and photosynthetic bacteria, grown in D_2O with ^{1}H-leucine, synthesized protein in which the leucine amino acid residues that are present contain ordinary hydrogen, and all other nonexchangeable side-chain hydrogen is present as deuterium. We refer to such proteins of mixed isotopic composition as isotopic hybrids. Leucine was chosen for these experiments because it appeared likely that its hydrogen would not appear in other amino acid side chains of the deuterated protein, and this expectation has been confirmed.

The blue-green algae *Phormidium luridum, Fremyella diplosiphon,* and *Synechococcus lividus* (*2*) were cultured in a 99.8 percent D_2O medium to which 0.02 percent of (^{1}H)-L-leucine was added. The cells were harvested, and the proteins C-phycocyanin (and C-phycoerythrin from *Fremyella*) were extracted and purified (*3*). A 50-mg portion of purified protein was then hydrolyzed in 6*N* DCl; the hydrolyzate was taken to dryness, dissolved in D_2O, filtered, dried, and then taken up in 2*N* DCl for proton magnetic resonance analysis (Fig. 1). The (^{1}H)-L-leucine is incorporated into the protein of all three of these algal species, and there is no significant incorporation of protons into other amino acid side chains. The incorporation of leucine into protein occurs with exchange of hydrogen at the α-carbon of leucine with the water of the medium, as no protons attributable to this position are observed in the hydrolyzed protein. From a known concentration of acetate used as an internal standard, we calculate the incorporation of ^{1}H-leucine (as mole percentage of total leucine content of the protein) to be 16 ±5 percent in isotopic hybrid phycocyanin derived from *Phormidium,* 60 ± 15 percent in both phycocyanin and phycoerythrin from *Fremyella,* and 75 ± 15 percent in phycocyanin from *Synechococcus.*

Isotopic hybrid cytochrome c was isolated from *Rhodospirillum* (*4*) grown in D_2O with deuterated algal hydrolyzate (*5*) and added ^{1}H-leucine. In this protein, we found that 80 percent of the leucine was ^{1}H-leucine; this amount was close to the ratio of ^{1}H-leucine to deuterated leucine in the culture medium (*6*). In this case, too, leucine was the only amino acid containing protons which was present in the acid hydrolyzate, and the hydrogen at the α-carbon of leucine had likewise been exchanged by deuterium (*7*).

We have examined native isotopic hybrid deuterated phycoerythrin (molecular weight ~2.3×10^5) containing 60 percent of ^{1}H-leucine by proton magnetic resonance analysis with time-averaging techniques (Fig. 1). The main resonance absorption, arising from the two methyl groups of leucine, is centered at 1.23 ± 0.02 ppm [measured from external hexamethyldisiloxane (HMS)], as compared to 1.26± 0.01 ppm for the center of the methyl lines in monomeric leucine dissolved in D_2O. A considerable fraction of the leucine resonances is observed at higher field, centering at about 0.6 ppm. Thus, the proton magnetic resonance data

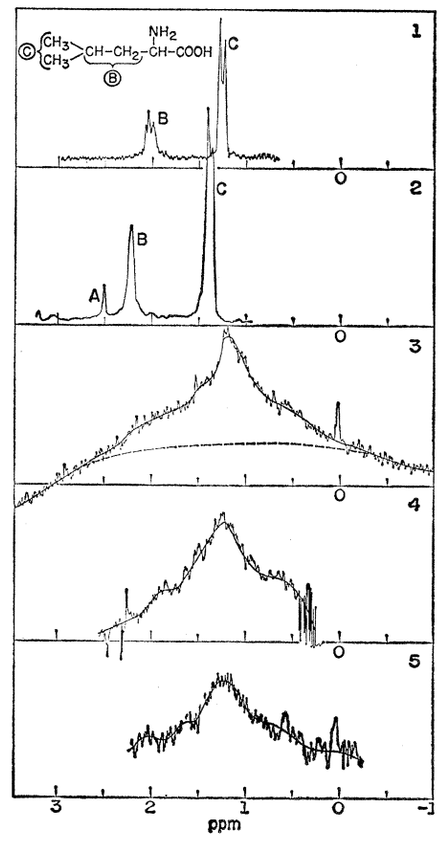

Fig. 1. *Spectrum 1.* The upfield portion of the proton magnetic resonance (PMR) spectrum of ^{1}H-leucine, 1 percent in D_2O-sodium phosphate buffer, 0.01*M*, *p*D 7.5. *Spectrum 2.* The upfield portion of the PMR spectrum of acid-hydrolyzed phycoerythrin substituted with ^{1}H-leucine. The sample (7 percent amino acids) is dissolved in 2*N* DCl, so the leucine lines are shifted downfield. Peak A is internal acetate, 0.0042*M*; accumulated scans, 67. *Spectrum 3.* A 1.7 percent solution of leucine-substituted deuterated phycoerythrin in D_2O-phosphate buffer; accumulated scans, 882; sweep rate, 2 cycle/sec^2. The dashed line is our estimate of the position of the base line. *Spectrum 4.* Solution as in spectrum 3; accumulated scans, 489; sweep rate, 5 cycle/sec^2. *Spectrum 5.* An 0.8 percent solution of leucine-substituted deuterated phycoerythrin in D_2O-phosphate buffer; accumulated scans, 969; sweep rate, 5 cycle/sec^2. The relatively sharp line at 0.6 ppm is probably real and associated with the protein. The line near 0 ppm in both spectra 3 and 5 is an artifact, perhaps due to a trace of silicone. All chemical shifts are given from external hexamethyldisiloxane. The protein spectra were recorded with external benzene to provide the field-frequency lock. Probe temperature, 32°C.

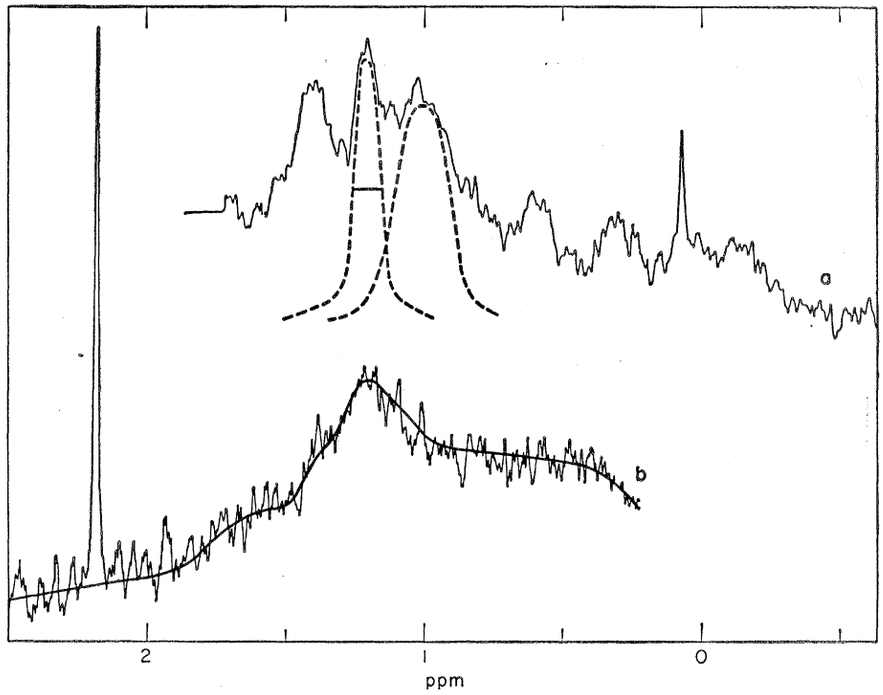

Fig. 2. *Spectrum a*: 2 percent solution of bacterial cytochrome c in D_2O-phosphate buffer; accumulated scans, 296; sweep rate, 2 cycle/sec^2. Other conditions as in Fig. 1. *Spectrum b*: 3.4 percent solution of leucine-substituted deuterated phycocyanin in D_2O-phosphate buffer; accumulated scans, 510; sweep rate, 2.5 cycle/sec^2. The sharp line at 2.23 ppm is internal acetate.

indicate that in phycoerythrin the (observable) leucine side chains occur in two different environments. From integration of the spectra, we estimate that at least 75 percent of the incorporated ^{1}H-leucine is observed in the protein. This calculation represents a very conservative estimate, in that the placement of the base line is difficult, and essentially all of the ^{1}H-leucine may in fact be detectable.

Proton magnetic resonance analysis of ^{1}H-leucine-substituted C-phycocyanin (under our conditions mainly a trimer with molecular weight of 1.4×10^5) gives a result similar to that obtained with phycoerythrin (Fig. 2,*b*). Here the main resonance peak of leucine centers at 1.23 ± 0.02 ppm. With deuterated-cytochrome c containing ^{1}H-leucine extracted from *Rhodospirillum* (molecular weight 1.2×10^4) we observe much sharper lines (Fig. 2,*a*). There are major resonance peaks at 1.24 ± 0.02 ppm and 1.06 ± 0.02 ppm; the line widths at half height are 11 and 22 hz, respectively. Smaller peaks are observed at 0.63, 0.32, and -0.15 ppm. These data indicate that, in these proteins, a sizable portion of the leucine side chains (those at about 1.24 ppm) are in a very mobile, aqueous environment. That portion of the leucine immediately upfield from the line at 1.24 ppm could represent leucine in a less mobile hydrophobic environment, for this line is broader than the one at 1.24 ppm and an upfield shift is to be expected if the surrounding environment is more hydrocarbon-like. In cytochrome c, the lines observed at very high field are most likely from leucine residues adjacent to aromatic residues of the protein.

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31 May 1968

Partial Hydroxylation of Certain Lysines in Collagen

Abstract. *Peptides derived from the $\alpha 1$ chain of collagen have been isolated in small amounts and have been shown to differ from ones found in expected amounts only by substitution of hydroxylysine for lysine. This observation indicates that hydroxylation of these lysines by protocollagen hydroxylase has been effected to a very minor extent.*

The biological formation of hydroxyproline and hydroxylysine in collagen involves hydroxylation of proline and lysine after incorporation of the latter amino acids into polypeptides (*1*) about the size of α-chains (*2*). The hydroxylation of both proline and lysine is probably catalyzed by one enzyme (*3*), namely protocollagen hydroxylase (*4*). Only proline located in position 3 of the collagen triplet (the repeating amino acid sequence—gly X.Y.—with glycine occupying position 1) appears to be hydroxylated (*5*).

The hydroxylysine contents of collagens derived from different tissues of the same animal are quite variable, although the total amounts of lysine and hydroxylysine, as well as the overall amino acid composition of these collagens, are constant (*6*). In the collagen of chick bone, the hydroxylysine content progressively decreases during maturation of the chicks (*7*). Since certain prolyl residues in rat skin and tendon collagens are incompletely hydroxylated (*8*), it was suggested that the variations observed in the amounts of hydroxylysine of chick bone collagen reflect an alteration in the ability of lysine (protocollagen) hydroxylase to act upon lysine residues that are candidates for hydroxylation.

I now present evidence that in rat skin collagen certain lysyl residues are hydroxylated to a very minor extent. These observations were made during investigations on the primary structure of collagen. Initial experiments involved splitting α-chains at methionyl residues with cyanogen bromide (*9*). Eight resultant peptides have been isolated and characterized from the $\alpha 1$ chain of rat skin collagen. One of these peptides, $\alpha 1$-CB5, contained equimolar quantities of glucose and galactose presumably attached glycosidically to the hydroxyl group of hydroxylysine (*10*).

Initial degradation experiments aimed at determining the amino acid sequence of $\alpha 1$-CB5 involved digestion of the