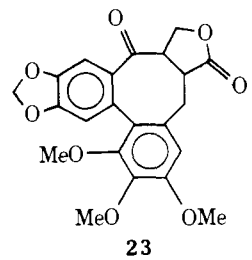
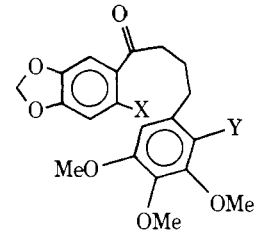
produces **21**, which is converted in three steps[18] to the enone **17**. Then reaction with tetrakis(triphenylphosphine)nickel(0) in DMF at 50° for 40 hr produces the dimethyl ether (**22**) of alnusone (**16**), which is isolated as a colorless solid in high purity (52% yield).[19] The product is identical in comparison of ir, mass, and ^{1}H NMR spectral data, and TLC behavior with a sample of **22** prepared by O,O-dimethylation of alnusone (**16**) from natural sources.[24]

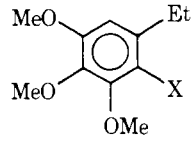
Hindered aryl halides provide the most important general limitation on the method. Direct approaches to the bisbenzocyclooctadienone structures as in steganone (**23**)[20] have not succeeded. For example, the related system **24** reacts with zerovalent nickel reagents at 40–50° to give rapid insertion into the aryl–bromide bond followed by slower insertion into the aryl–iodide bond. However, no aryl–aryl coupling is observed either inter- or intramolecularly; the products from hydrogen substitution for halogen (i.e., **25** and **26**) are obtained in yields of 39 and 24%, respectively. Tetrahydrofuran as solvent produces **25** in 93% yield; addition of D_2SO_4–D_2O during isolation leads to unlabeled **25**.[21] The failure to achieve aryl–aryl coupling is general for compounds with serious steric hindrance around the aryl iodide substituent. For example, aryl iodide **27** gave only the reduction product **28** when treated with zerovalent nickel (70–100% yield, depending on solvent and phosphine ligands). Further definition of the ring size and steric limitations on biaryl coupling with nickel(0) reagents will be presented in the full paper describing this work.



23



24, X = Br; Y = I
25, X = H; Y = I
26, X = H; Y = H



27, X = I
28, X = H

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(15) The yield of **19** is 86%; bp 82° (0.02 Torr); cf. E. J. Corey and M. Chaykovsky, *J. Am. Chem. Soc.*, **87**, 1353 (1965).
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(18) Acetylation of **21** followed by base-promoted elimination of acetic acid (diazabicyclononene–chloroform, 25°, 0.5 hr) and then silver(I) promoted iodination[12] gives **17** in 62% yield overall from **19** and **20**.
(19) The major by-products appear to be high molecular weight products, presumably from intermolecular aryl–aryl coupling.
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(21) Efficient reduction of an aryl iodide with a bulky ortho substituent was shown to occur by hydrogen atom transfer from tetrahydrofuran.[2b] We assume a similar process is operating with **24** and **27**.
(22) Fellow of the Alfred P. Sloan Foundation (1973–1975) and recipient of a Camille and Henry Dreyfus Teacher–Scholar Grant (1973–1978).
(23) National Science Foundation Predoctoral Trainee (1970–1974).
(24) We are grateful to Professor T. Tokoroyama (Osaka City University, Oaska, Japan) for providing generous samples of natural alnusone.

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Specific Enrichment with ^{13}C of the Methionine Methyl Groups of Sperm Whale Myoglobin[1]

Sir:

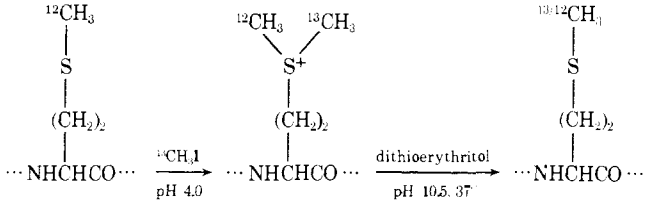
The value of ^{13}C NMR spectroscopy to the study of protein structure and mobility is substantially increased when single carbon resonances can be observed; under favorable conditions such resonances from proteins have been studied at natural abundance.[2] Efforts to enrich protein samples with respect to ^{13}C have facilitated the study of a number of proteins.[3,4] We report here a selective method to enrich in ^{13}C the methyl group of the two methionyl residues, 55 and 131, in covalently intact sperm whale myoglobin. In the ^{13}C NMR spectrum of this protein sample, which possessed in

Table I. Characterization of ^{13}C Enriched Ferrimyoglobin[c]

	Enriched Mb	Virgin Mb
$\epsilon_{409}/\epsilon_{280}$	5.32	5.36 ± 0.15
$[\theta]_{208}$, deg/(g cm^2)	-2.36×10^4	-2.36×10^4
pI	8.21[a]	8.27[a]
$p_{1/2}$ (Torr), 20°, O_2	0.44[b]	0.42[b]

[a] Obtained by the method of E. T. Nakhleh, Ph.D. dissertation, American University of Beirut, 1972. [b] Ferrimyoglobin converted to ferro-form with dithionite.[10] [c] All measurements were made at 20°.

Scheme I



other respects the distinctive characteristics of native myoglobin, the two enriched nuclei each produced a narrow resonance, which stood out prominently from the natural abundance spectrum of the protein. The behavior of these resonances has the potential of providing information concerning the environment and rotational mobility of the nonpolar interior of the protein.[5]

Scheme I shows the sequence of reactions for the conversion of the methionine methyl groups in apomyoglobin to a form enriched with respect to ^{13}C. The pH of the apoprotein solution (0.5–0.6 m*M*) was lowered to 4.0 to minimize the reactivity of other nucleophiles in the protein and to render the normally buried methionyl residues[6] more accessible to reaction by disruption of the native structure.[7] The solvent-exposed methionyl residue of ribonuclease was methylated under conditions[8] similar to those reported here. Methyl iodide enriched in ^{13}C was added in a molar proportion to the apoprotein of 100-fold and the two-phase solution stirred at room temperature in the dark for 18 hr. The half-life for the methylation reaction under these conditions is approximately 2.7 hr. At the end of the 18-hr reaction period, the clear homogeneous solution was dialyzed for 24 hr against numerous changes of NaN_3 solution (0.2 g/l.). Amino acid analysis of *p*-toluenesulfonic acid[9] hydrolysates of the methylated protein showed that 95% of methionyl residues had been converted into those of *S*-methylmethionine; no unusual components were observed in the chromatograms apart from the *S*-methylmethionine. Furthermore, a preparation of the methylated protein was concentrated to 7–10 m*M* and showed a single, narrow, intense resonance at 167.8 ppm upfield from CS_2. The corresponding methyl adduct resonance in a sample of free *S*-methylmethionine was also at 167.8 ppm. See paragraph at end of paper regarding supplementary material.

The conversion to the original covalent structure capable of regaining native conformation was best effected by exposure to mercaptans. A solution, 0.8 m*M*, of methylated protein was brought to pH 10.5 and treated at this pH with batches of dithioerythritol until the final concentration of dithiol was 0.5 *M*. The slightly turbid solution was left at 37° for 18 hr. Under these conditions the half-life for the demethylation reaction is approximately 3.2 hr. Heme was reintroduced into the apoprotein[4] after extensive dialysis against water of the demethylation reaction mixture. After removal of protein insoluble in 0.1 μ phosphate buffer at pH 6.5, the reconstituted ferrimyoglobin was purified on carboxymethyl-Sephadex using this same phosphate buffer.

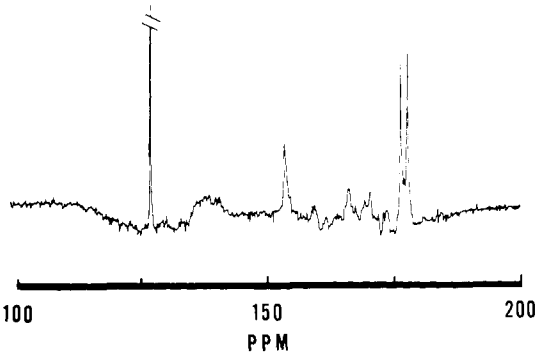


Figure 1. Proton decoupled Fourier transform ^{13}C NMR of the CO myoglobin prepared with enrichment of the methyl groups of the two methionine residues. Chemical shifts are referenced to external CS_2, with internal dioxane at 126.30 shown as the most prominent resonance. The instrument was the Varian XL-100-15 operating at 23.5 kG at 30.0 ± 0.5°. The data were accumulated from 32K transients with 16K data points.

The sample of enriched ferrimyoglobin, which was obtained in 60% yield based on the quantity of original apoprotein, showed, in addition to the characteristics presented in Table I, an intense band of protein of the same electrophoretic mobility as virgin ferrimyoglobin; a trace of protein of higher pI was also present. The data from amino acid analysis were in excellent agreement with expected values. The enriched ferrimyoglobin was converted to the ferro- form with dithionite[10] and equilibrated with carbon monoxide.

In Figure 1 is presented the proton noise decoupled ^{13}C NMR spectrum of a 6.5 m*M* solution of the CO myoglobin at pH 7.83. The two prominent resonances due to the enriched carbons have chemical shifts at 176.77 and 178.15 ppm upfield from CS_2. These chemical shift differences likely arise from differences in the microenvironment of the two internally located methyl groups. For comparison, a value of 178.7 ppm upfield from CS_2 was found for the methyl carbon resonance in methionine[11] and in the pentapeptide GlyGlyMetGlyGly.[11]

Measurements of spin-lattice relaxation time, T_1, by the inversion-recovery method[12] yield values in the range of 140–160 msec at 15.1 MHz for both methionine methyl resonances. Such values support the evidence from the observed line widths that indicates appreciable internal rotational motion sensed by the enriched nuclei within the protein.[3,4,11,13]

Studies of the effect of solution pH, oxidation state of the iron ion, the nature of the heme ligand, and the assignment of these prominent resonances are in progress.

Supplementary Material Available. Complete amino acid composition data, the ^{13}C NMR spectrum of the methylated apoprotein, and the absorbance (uv and visible) spectrum of the reconstituted CO-myoglobin shown in Figure 1 will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 24× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journals Department, American Chemical Society, 1155 16th St., N.W., Washington, D.C. 20036. Remit check or money order for $4.00 for photocopy or $2.50 for microfiche, referring to code number JACS-75-3875.

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The Two-Step Mechanism of Noncatalytic Aromatic Bromination. Controlled Variation of the Rate-Controlling Step

Sir:

By a systematic variation of the initial conditions, the rate-controlling step in an aromatic bromination has been changed from that of arenium ion formation to that of proton removal from arenium ion. The results support the simple two-step mechanism, eq 1 and 2, with no significant contribution by mechanisms involving more than one bromine molecule.

The well-known kinetic complications that often arise in noncatalytic aromatic bromination in the absence of added excess Br^- have been expressed in the form of eq 5.[1,2] Such behavior has been interpreted literally; that is, as meaning that the first term is due to an activated complex containing the elements of one Br_2, the second term due to an activated complex containing the elements of two Br_2, and so on. Various mechanisms have been assigned to each literally interpreted term. However, the *same* kinetic behavior will be shown by the simple two-step mechanism of eq 1 and 2 *alone*, provided only that $v_{-1}/v_2 = k_{-1}[Br^-]/k_2$ grows to significance during a kinetic run as the result of Br^- production. Equation 4, the general rate expression for the simple two-step mechanism, can be shown to be the *equivalent* of the power series equation, 5. Starting with $[Br_2]_{stoich} + [Br^-]_{stoich} = [Br_2]_0$, one expresses the variable $[Br^-]$ in terms of $[Br_2]$. The denominator of eq 4 then takes the form $C_1(1 + C_2[Br_2])^{-1}$ which then is expandable as a power series in $C_2[Br_2]$. This leads to eq 5, in which the constants k_I, k_{II}, and k_{III} have the complex form shown in eq 6.

$$ArH + Br_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Ar^+(H)Br + Br^- \quad (1)$$

$$Ar^+(H)Br + B_i \longrightarrow ArBr + HB_i^+;\ k_2 = \Sigma k_{2_i}[B_i] \quad (2)$$

$$Br^- + Br_2 \rightleftharpoons Br_3^-;\ K_3 = [Br_3^-]/[Br^-][Br_2] \quad (3)$$

$$-d[ArH]/dt = \frac{k_1k_2[ArH][Br_2]}{k_{-1}[Br^-] + k_2} \quad (4)$$

Table I. Rate Constants and Kinetic Isotope Effects in the Para-Bromination of *N*-Methylacetanilide in 50% HOAc at 25°

$[Br_2]_0$	$[NaBr]_0$	10^2k_H[a]	$10^2k_H(corr)$[b]	k_H/k_D[c]
$\sim 2 \times 10^{-4}$	0	8.59	8.59	0.93 ± 0.02[d]
$\sim 2 \times 10^{-3}$	0	(7.90)[e]		
$(2-20) \times 10^{-4}$	0.050[f]	2.23	5.31	1.41 ± 0.05
$(2-20) \times 10^{-4}$	0.150[g]	0.654	3.36	1.85 ± 0.04
$(2-20) \times 10^{-4}$	0.300	0.248	2.30	2.14 ± 0.02
$(2-20) \times 10^{-4}$	0.500	0.151	2.23	2.27 ± 0.02
$(2-20) \times 10^{-4}$	0.050[h]	1.96	4.66	1.18 ± 0.01

[a] $k_H = k_{obsd}(sec^{-1})/[ArH]$, average of several runs. [b] $k_H(corr) = k_H[Br_2]_{stoich}/[Br_2] = k_H(1 + K_3[Br^-])$; $K_3 = 27.6$.[6] [c] k_D is the rate constant for *N*-methylacetanilide-*2,4,6-d*$_3$. [d] Standard deviation. [e] Based on initial slope, 20% reaction, of a curved rate plot. [f] Contained 0.250 *M* $NaClO_4$. [g] Contained 0.150 *M* $NaClO_4$. [h] Contained 0.250 *M* NaOAc.

$$-d[ArH]/dt = [ArH](k_I[Br_2] + k_{II}[Br_2]^2 + k_{III}[Br_2]^3) \quad (5)$$

$$k_I = \frac{k_1k_2}{k_{-1}[Br_2]_0 + k_2}$$

$$k_{II} = \frac{k_I(k_{-1} + 2k_{-1}K_3[Br_2]_0)}{k_{-1}[Br_2]_0 + k_2} \quad (6)$$

$$k_{III} = \frac{k_{II}(k_{-1} - 2k_2K_3)}{k_{-1}[Br_2]_0 + k_2}$$

Consider the effect of added excess Br^-, which is experimentally that of simplifying the kinetics to straight first order in Br_2. The literal interpretation of eq 5 requires that this be due to enough of a reduction in $[Br_2]$, as the result of equilibrium 3, to make negligible the probability of forming activated complexes containing the elements of more than one Br_2. On the basis of just the simple two-step mechanism, the kinetic simplification is due to the constancy of $k_{-1}[Br^-]$ and does not require a reduction in $[Br_2]$. Indeed, in the bromination of benzene in 78% of CF_3CO_2H, kinetically complex in the absence of added Br^- ($[Br_2]_0 \sim 3 \times 10^{-3}$ *M*), the addition of excess NaBr reduced the kinetics to cleanly first order in Br_2 under conditions that did *not* significantly decrease $[Br_2]$.[3] This result is contrary to the multimechanistic interpretation, but consistent with the simple two-step mechanism.

Given a suitable value of k_{-1}/k_2 it should be possible to change at will the rate-controlling step of the two-step mechanism by controlling $[Br^-]$. This has been realized in the para bromination of *N*-methylacetanilide in 50.1% acetic acid at 25°.[4] Rates were determined by following Br_2 absorption at 410 nm, an excess of the aromatic being employed ($[ArH] = 0.01-0.1$ *M*).

Without added excess Br^-, kinetic complications arose when a 1-cm Beckman cell was employed as the reaction vessel ($[Br_2]_0 \sim 2 \times 10^{-3}$ M). First-order plots were noticeably curved (decreasing apparent rate constant), especially beyond about 20% reaction. The curvature was substantially greater than could be attributed to an increasing tie-up of Br_2 in the form of Br_3^-,[5] and is consistent with $v_{-1}/v_2 = k_{-1}[Br^-]/k_2$ growing to significance during the production of Br^-. However, by the use of 10-cm cells, the amount of Br^- produced could be reduced tenfold ($[Br_2]_0 = 2 \times 10^{-4}$ *M*). Under these conditions, the reaction was cleanly first order for three half-lives of observation. Thus v_{-1} was throughout negligible relative to v_2, making the first step essentially completely rate controlling, with $k_{obsd} = k_1[ArH]$. Also consistent with rate control by the first step was the observation of a slightly smaller rate constant for 2,4,6-trideuterio-*N*-methylacetanilide under the same conditions (Table I).

The addition of excess NaBr to 1-cm cells runs also pro-