with the experimental data. The tosylhydrazone I reacts with base reversibly to give the conjugate base of I and methanol. The anion loses *p*-toluenesulfinate, giving diazocamphane in the rate-determining step.[10,11] In the absence of excess strong base the intermediate diazocamphane attacks a proton either on the liberated methanol or on the tosylhydrazone which is present in abundance. The methoxide ion and tosylhydrazone again set up an equilibrium while the resulting diazonium decomposes directly to products,[9] or in a two-step process loses nitrogen and becomes a poorly solvated carbonium ion.[12] This carbonium ion can then collapse by two routes: (1) by a transannular 1,3-proton elimination to give tricyclene in low yield (*ca.* 35%), and (2) by a Wagner–Meerwein rearrangement and proton expulsion to give camphene in higher yield (*ca.* 65%).[3]

At higher base concentrations (*e.g.*, 4.00 equiv) the equilibrium between the tosylhydrazone and its conjugate base highly favors the latter, which decomposes to diazocamphane as previously mentioned. The diazocamphane can then abstract a proton from methanol to set up an equilibrium between methoxide ion and the resulting diazonium ion, the former being present in large excess. Therefore, in the presence of a large excess of methoxide the diazocamphane should be favored in the equilibrium, and this can then lose nitrogen, giving the carbene intermediate.

The reaction leading to camphene has its interesting aspects also. First the deuterium incorporation in the labeling experiments amounted to about 80% instead of the calculated 97.5% (99.7% D_2O in a 40-fold excess). This can be the result of two phenomena: (1) some of the camphene results from a carbene intermediate, or (2) the selectivity of the diazocamphane for hydrogen is about seven times that for deuterium. Because the amount of deuterium incorporation is independent of base concentration, the second explanation seems more reasonable and is being investigated further. Another interesting point is that the poorly solvated cation generated in our system, aside from giving tricyclene, undergoes rearrangement. This behavior is not consistent with that previously reported[12] where rearrangement of poorly solvated cations is apparently inhibited.

The results of this investigation have shown that "carbenic" products do not always result from carbene intermediates.[13] We are presently studying reactions such as the base-catalyzed dehydrohalogenation of isobornyl chloride in aprotic solvents in order to determine the generality of this phenomenon.

Acknowledgment. The authors wish to express their gratitude to Arapahoe Chemical Co., Division of Syntex, Inc., and to the National Science Foundation (Grant No. GP-5753) for support of this work.

(10) L. Friedman and H. Shechter, *J. Am. Chem. Soc.*, **81**, 5512 (1959).
(11) J. W. Powell and M. C. Whiting, *Tetrahedron*, 7, 305 (1959).
(12) A. T. Jurewicz, J. H. Bayless, and L. Friedman, *J. Am. Chem. Soc.*, **87**, 5788 (1965); J. H. Bayless, F. D. Mendicino, and L. Friedman, *ibid.*, **87**, 5790 (1965).
(13) See also J. W. Wilt, C. A. Schneider, H. F. Dabek, J. F. Kraemer, and W. J. Wagner, *J. Org. Chem.*, 31, 1543 (1966), for a similar conclusion using N-methylpyrrolidone as a solvent.

Robert H. Shapiro, J. H. Duncan, J. C. Clopton
Department of Chemistry, University of Colorado
Boulder, Colorado 80304
Received August 24, 1966

A Method for Obtaining Three-Dimensional Structural Information about Protein Molecules in Solution

Sir:

Despite the noteworthy success of X-ray crystallography in obtaining complete three-dimensional structures for several protein molecules,[1] the possibility remains that protein conformations in aqueous solution under physiological conditions differ from those in the crystal. The observation of what are felt to be conformational changes in protein molecules in solution produced by changes in temperature and pH, or by allosteric effects induced by small molecules, indicates a degree of flexibility which might easily result in structural change as a result of packing into the crystalline lattice. The structural questions raised by these changes in solution are, of course, of great interest in themselves. We wish to report results leading toward a structural method applicable to proteins in aqueous solution under a variety of conditions.

Chloroform (85 mg) made from T_2O by the haloform reaction and containing 1 curie of tritium was distilled along with 2 ml of water into 200 mg of chymotrypsinogen A in the absence of air. The solution was then exposed to a ^{60}Co γ-ray source of 0.125 Mrad/hr for 30 min. After freeze drying and dialysis to remove exchangeable OH and NH protons, this material had an activity of 1,125,000 cpm/mg. A control run where the γ irradiation was omitted was then conducted. After freeze drying and dialysis 6000 cpm/mg was found. The tritiated chloroform was subsequently used successfully for several more labeling experiments.

The sample of chymotrypsinogen which had been labeled in the ^{60}Co source was chromatographed and rechromatographed on Amberlite GC-50 resin. Following the chromatography by ultraviolet spectroscopy, peaks of the same shape as those found using nonlabeled chymotrypsinogen were obtained. The radioactivity was found to follow the peak detected by ultraviolet spectroscopy. The activity of the rechromatographed labeled chymotrypsinogen was 611,000 cpm/mg. The decrease in specific activity is probably due to further exchange during dialysis of hydrogens such as those α to the amide carbonyls. Some radioactivity might have been present in chemically altered protein and been removed by chromatography, but this is less likely. Repetition of the chromatography on Sephadex G-50 gave similar results.

We feel that these results indicate that a considerable amount of labeling through exchange of C–H hydrogens for tritium has occurred, presumably through abstraction of hydrogen atoms from the protein by the OH radicals produced by irradiation of water, followed by donation of tritium atoms to the protein radicals by the chloroform. The chromatographic evidence indicates that it is likely that the exchange was accomplished without structural damage to the protein.

Because of the size of the $CTCl_3$ molecule, we feel that this labeling process could occur readily only on the *outside* of the protein molecule. Larger donors of tritium might be used, if necessary, to make a better distinction between inside and outside. With labeling

(1) J. C. Kendrew, *Science*, **139**, 1259 (1963); M. Perutz, *ibid.*, **140**, 863 (1963); D. C. Phillips, *Sci. Am.*, **215**, No. 5, 78 (1966).

on the outside, degradation of the chymotrypsinogen into peptides and separation and measurement of their radioactivity should provide information about which peptides and, through further degradation, about which amino acid residues occupy the surface of the intact protein molecule in its natural conformation in aqueous solution. We further expect that a similar experiment performed in the presence of an enzyme substrate or inhibitor will give labeled protein molecules in which the radioactivity incorporated at and around the active site will be substantially reduced or eliminated, thus providing a general technique for pinpointing enzyme active sites. Such experiments are now in progress.

Tritium has previously been incorporated into solid ribonuclease using the Wilzbach procedure[2] (exposure to gaseous tritium) and recently by the production of radicals on ribonuclease through γ-ray irradiation followed by treatment with tritiated hydrogen sulfide.[3] It is possible that procedures similar to that proposed here might yield information about the structures of solid proteins.

Acknowledgment. We should like to acknowledge support for this project by Grant GM 11675 from the National Institutes of Health. We should also like to thank R. Wolfgang for valuable advice concerning the mechanisms of incorporation of tritium and F. Richards for considerable help and advice on handling proteins and peptides.

(2) D. Steinberg, M. Vaughan, C. B. Anfinsen, and J. Gorry, *Science*, **126**, 447 (1957).
(3) P. Riesz, F. H. White, Jr., and H. Kon, *J. Am. Chem. Soc.*, **88**, 872 (1966).

Martin Saunders, Herbert A. Jung, William L. Hamilton
Sterling Chemistry Laboratory, Yale University
New Haven, Connecticut 06520
Received November 7, 1966

Electrochemical Generation of the Phenanthrene Triplet

Sir:

The luminescence properties of phenanthrene have been thoroughly studied by many investigators.[1-5] Normal,[1] delayed,[2] and excimer[1] fluorescence emission have been detected. In addition, phosphorescence from this molecule has been observed in the crystal,[3] in rigid glasses,[4] and in fluid solution even at room temperature,[5] although the last of these conditions is not generally suitable for such detection.

By consecutive electrochemical oxidation and reduction or reduction and oxidation in double-potential-step experiments, rubrene[6] and other fluorescent organic molecules[7] can be raised to electronically excited singlet states. The potential range employed in these double-step experiments need not be large enough to encompass both the anion and cation of the fluorescer in order for fluorescence emission to be seen.[6,7] We wish to report here that phenanthrene, when subjected to this pre-annihilative treatment, produces a green emission which is not characteristic of phenanthrene fluorescence.

When the stable phenanthrene anion ($E_{1/2} = -2.47$ v *vs.* sce) is generated electrochemically in a 4 m*M* solution of purified phenanthrene[8] and 0.1 *M* tetra-*n*-butylammonium perchlorate in deaerated N,N-dimethylformamide at a 3 × 10 mm platinum sheet electrode, and then oxidized at sufficient overvoltage, a green emission is seen at the electrode. By going to more positive voltages, short of phenanthrene oxidation or of the dimethylformamide decomposition potential ($\sim+1.5$ v), the green emission becomes brighter.

If phenanthrene anion is generated for a sufficient time so that it diffuses through the entire solution, thus destroying possible traces of oxidants, this "purged" solution gives the same green emission on the oxidation cycle. The emission from this solution is first detectable on oxidation at +0.15 v, and the intensity also increases up to background. The minimum thermodynamic driving force in the purged solution for the emission measured from the half-wave reduction potential is 2.6 v (or $\sim$60 kcal/mole).

The emission spectrum was photographed by focusing a 1:1 image of the electrode on the slit of a fast grating Raman spectrograph,[9] using Eastman Kodak 103aF spectrographic plates. The plates were microphotometered on a Jarrell Ash Model 23-100 recording microphotometer and were corrected for the spectral sensitivity of the emulsion. The emission was found to be broad and structureless with a maximum between 4900 and 5000 A. This corresponds very closely with the room-temperature phenanthrene phosphorescence in ethanol reported by Parker and Hatchard.[5]

As shown in Table I, addition of various substances which are electroinactive between −2.5 and +1.5 *vs.* sce in DMF to the green-emitting phenanthrene system affected the emission in a manner which can be related to the triplet energies of the substances. The presence of 1,3,5-hexatriene does decrease the stability of the phenanthrene anion but not sufficiently to account for the total loss of emission. The other added substances have no detectable effect on the phenanthrene anion-radical stability.

Table I. Effect of Electroinactive Triplet Quenchers on Phenanthrene Electrochemiluminescence

	E_t, kcal/mole	% quenched
1,3,5-*trans*-Hexatriene	47[a]	100
2,3-Dimethylbutadiene	59[b]	87
Phenanthrene	62[c]	
Biphenyl	65[d]	0

[a] D. F. Evans, *J. Chem. Soc.*, 1735 (1960). [b] R. E. Kellogg and W. T. Simpson *J. Am. Chem. Soc.*, **87**, 4230 (1965). [c] Reference 1. [d] V. L. Ermolaev, *Usp. Fiz. Nauk*, **80**, 3 (1963).

These results, together with the observation that triplet quenchers had no effect on the *fluorescence* electrochemiluminescence of 1,3,4,7-tetraphenylisobenzofuran,[7] indicate that in the acceptor–phenanthrene systems irreversible triplet energy transfer is responsible

(1) T. Azumi and S. P. McGlynn, *J. Chem. Phys.*, **41**, 3131 (1964).
(2) C. A. Parker and C. G. Hatchard, *Proc. Roy. Soc.* (London), **A269**, 574 (1962).
(3) R. M. Hochstrasser and G. J. Small, *J. Chem. Phys.*, **45**, 2270 (1966).
(4) E. C. Lim and J. D. Laposa, *ibid.*, **41**, 3257 (1964).
(5) C. A. Parker and C. G. Hatchard, *J. Phys. Chem.*, **66**, 2506 (1962).
(6) D. L. Maricle and A. H. Maurer, *J. Am. Chem. Soc.*, **89**, 188 (1967).
(7) A. Zweig, A. K. Hoffmann, D. L. Maricle, and A. H. Maurer, in press.
(8) The method of E. C. Kooyman and E. Farenhorst, *Trans. Faraday Soc.*, **49**, 58 (1943), was used. We thank Dr. W. A. Henderson, Jr., for making this material available.
(9) R. F. Stamm, *Ind. Eng. Chem., Anal. Ed.*, **17**, 318 (1945).