and succinate esters.[9,11] A possible explanation for this difference could reside in the preferential conformation of the ground states for the dimethylamines and the carboxylic acids. Possibly owing to the solvation of the carboxyl anion the glutarate and succinate monoesters exist preferentially in an extended conformation, whereas the relatively non-polar amines exist in a coiled conformation due to formation of hydrophobic bonds (Chart II). In this connection it should be noted that rate studies conducted in 50% dioxane–water and also in 8 *M* aqueous urea solution failed to alter this ratio.[37]

(37) These experiments are probably not critical since the uncoiling of hydrocarbon chains due to clathrate formation requires at least a 7-membered chain (see L. F. Fieser and M. Fieser, "Advanced Organic Chemistry," Reinhold Publishing Corp., New York, N. Y., 1961, pp. 131–133). Also, the water content of 50% dioxane–water would probably not be low enough to bring about uncoiling.

A second explanation would implicate steric hindrance imposed by the dimethyl substitution of the amino group on the rate of closure to form the five-membered ring (though the magnitude of the rate constants would not appear to suggest any steric hindrance; see Table VII). It should be noted that the ratio of the rate constants for the formation of five- and six-membered rings in the cyclization of ω-aminoalkyl bromides in water is 800.[38] Our investigations in this area are continuing.

Acknowledgments.—This work was supported by grants from the National Science Foundation and the National Institutes of Health. S. J. Benkovic particularly acknowledges support in the nature of a predoctoral fellowship from the National Institutes of Health.

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[CONTRIBUTION FROM THE CHEMISTRY DIVISION, ARGONNE NATIONAL LABORATORY, ARGONNE, ILL.]

Isolation, Amino Acid Composition and Some Physico-chemical Properties of the Protein Deuterio-phycocyanin[1]

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The isolation and purification of a fully deuteriated protein, deuterio-phycocyanin, from blue-green algae grown autotrophically in 99.8% D_2O is described. Sedimentation behavior in the ultracentrifuge shows the protein to be a system of reversibly interacting components. The amino acid compositions of ordinary and deuterio-phycocyanin isolated from *Plectonema calothricoides* have been established and it appears that the amino acid compositions are identical within experimental error. Ordinary and deuterio-phycocyanin therefore probably differ only in isotopic composition. Thermal denaturation of ordinary and deuterio-phycocyanin, both dissolved in H_2O, has been studied by measuring the quenching of fluorescence. Deuterio-phycocyanin undergoes thermal denaturation at a temperature 5° lower than is the case for ordinary phycocyanin. Since the two proteins appear to differ primarily in the isotopic composition of the non-polar side chains, differences in denaturation behavior are probably to be ascribed to differences in hydrophobic bonding.

Introduction

The successful cultivation of fully deuteriated organisms on the large scale by Katz, Crespi and coworkers[3] has made a great variety of fully deuteriated substances accessible for the first time. Proteins in which hydrogen has been entirely replaced by deuterium may be expected to make a useful contribution to problems of protein structure and function, and efforts have therefore been directed to the preparation of such substances. In the present communication the isolation, purification, amino acid composition and behavior on thermal denaturation of the fully deuteriated protein phycocyanin, extracted from the fully deuteriated blue-green alga *Plectonema calothricoides*, are described; a preliminary description of some aspects of this work has already appeared.[4]

Phycocyanin is a blue photosynthetic pigment widely distributed in blue-green algae. A member of the class of biliproteins, for which molecular weights of the order of 200,000 to 300,000 have been quoted, phycocyanin appears in fact to be a system of reversibly interacting components. The chemical properties of phycocyanin are reviewed by O'hEocha.[5] For purposes of the present discussion the ordinary, hydrogen-containing phycocyanin extracted from algae grown in H_2O will be referred to as ordinary or protio-phycocyanin; the protein extracted from algae grown in 99.8% D_2O will be designated deuterio-phycocyanin. The prefix deuterio- will also be used when the deuterio-phycocyanin is dissolved in H_2O and the exchangeable positions of the protein are occupied by hydrogen. The sharp distinction between the protein described here, in which the non-exchangeable positions in the molecule are occupied by deuterium, and ordinary proteins into which deuterium is introduced into exchangeable positions by treatment with D_2O[6] can be readily inferred from the discussion of Scheraga[7] on hydrogen–deuterium exchanges in proteins.

Experimental

Isolation and Purification.—Deuterio-phycocyanin and protio-phycocyanin were isolated from the blue-green alga *Plectonema calothricoides* by either of two procedures. In the first, approximately 25 g. of algae (wet weight) were frozen and thawed twice to rupture the cells. About 200 ml. of aqueous acetate buffer (*p*H 4.7, μ = 0.1) was added and the solution allowed to stand, first for several hours at room temperature, and then in the refrigerator at 5°. After several days, the supernatant solution was quite blue and intensely fluorescent. The supernatant solution was removed by centrifugation, fresh buffer was added, and the extraction continued until most of the phycocyanin was removed from the algae. An olive-green appearance of the residue indicated that most of the blue pigment had been extracted. The aqueous extract was then centrifuged for 10 minutes at 4° at 12,000 r.p.m. to remove debris. Phycocyanin was then precipitated from the clarified extract by adding ammonium sulfate to 50% saturation. The second procedure for the extraction of phycocyanin from algae omitted freezing and thawing. Instead, cell lysis was achieved by the action of lysozyme (Worthington 2× crystallized, 10 μg. per ml.) in phosphate buffer (*p*H 7, μ =

(1) Based on work performed under the auspices of the U. S. Atomic Energy Commission. Presented in part at the 141st National Meeting of the American Chemical Society in Washington, D. C., April, 1962.

(2) Resident research associate, 1961–1962.

(3) H. DaBoll, H. L. Crespi and J. J. Katz, *Biotechnology and Bioengineering*, to be published.

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(5) C. O'hEocha, in "Comparative Biochemistry of Photoreactive Systems," M. B. Allen, ed., Academic Press, Inc., New York, N. Y., 1960, pp. 181–205.

(6) L. G. Augenstine, C. A. Ghiron, K. L. Grist and R. Mason, *Proc. Natl. Acad. Sci.*, **47**, 1733 (1961); J. Bello and D. Harker, *Nature*, **192**, 756 (1961).

(7) H. A. Scheraga, "Protein Structure," Academic Press, Inc., New York, N. Y., 1961, pp. 192–208.

0.1). The enzymatic disintegration of the algal cell walls can be carried out at 5°, but the process proceeds more rapidly at room temperature. The slurry so obtained was then stored in the refrigerator at 5°. Phosphate buffer is employed because lysozyme appeared to be inactive in acetate buffer at *p*H 4.7$_0$. Since lysozyme is soluble in 50% saturated ammonium sulfate solution, phycocyanin can be precipitated free of lysozyme. The use of lysozyme considerably facilitates the extraction of phycocyanin; details of the action of lysozyme on a number of blue-green algae are described elsewhere.[8] The phycocyanin isolated by freezing and thawing appeared identical with that obtained from lysozyme-lyzed algae; no effects were likewise discernible whether acetate or phosphate buffers were used.

Deuterio-algae are much more easily lyzed than ordinary algae either by freezing and thawing or by lysozyme. The greater ease of cell-wall rupture in deuteriated blue-green algae appears consistent with previously reported differences in the cell walls of ordinary and deuteriated green algae.[9]

Crude phycocyanin precipitated in 50% saturated ammonium sulfate was then recovered by centrifugation at 12,000 r.p.m. for 10 minutes; the colorless, clear supernatant solution was decanted away and discarded, and the blue precipitate taken into solution in a small volume of acetate buffer. This solution was then centrifuged at 10,000 r.p.m. for 10 minutes at 4°. The blue supernatant solution was collected and any precipitate was discarded. (The precipitate was generally green in color and obviously consisted of algal cell debris.) Precipitation in 50% saturated ammonium sulfate and dissolution in buffer were alternated until little or no residue was present on centrifugation of the protein in buffer solution. To achieve this usually required four or five reprecipitations.

A sample of the protein in buffer solution was then dialyzed against fresh phosphate buffer (*p*H 7.0, $\mu = 0.1$) and the visible and ultraviolet spectra measured on a Cary 14 recording spectrophotometer. The buffered protein preparation was then made 25% in saturated ammonium sulfate and allowed to stand in the refrigerator at 5° overnight. Crystallization normally occurred. The precipitate was then collected by centrifugation at 12,000 r.p.m. for 10 minutes. The supernatant solution was decanted, and the precipitate dissolved in a small volume of acetate buffer. A small sample of supernatant solution and precipitate were dialyzed against phosphate buffer (*p*H 7.0, $\mu = 0.1$), and the spectra examined on the Cary 14. When the ratio of the optical density at 620 mμ to that at 280 mμ was 4.00 or greater,[10] the protein was considered sufficiently pure. The spectra were also checked to make sure that the absorption in the 400 to 500 mμ region was at a minimum. Absorption in this region is indicative of cytochromes and other chromoproteins and carotenoids that are possible contaminants. The procedure outlined above is most suitable for purifying phycocyanin derived from algae in which a single type of phycocyanin is present and which are devoid of phycoerythrin. The presence of other phycobilins necessitates the use of Sephadex chromatography or chromatography on hydroxyapatite.[11,12]

Ordinary water, H_2O, was used for all the isolation and purification procedures for both the deuterio- and protio-phycocyanin preparations.

Amino Acid Analyses and Hydrolysis of Proteins.—All amino acid analyses were carried out by the Moore–Stein method with a Spinco 120 amino acid analyzer. The hydrolysis procedure used was that of Kimmel and Smith.[13,14] The protein samples were hydrolyzed in vacuum with 6 *N* HCl (Fisher Reagent Grade) at 110°. The time of hydrolysis varied from 20 hours to 100 hours in order to assure complete hydrolysis of the proteins, and in order to evaluate the destruction of particular amino acids by the acid hydrolysis. Analyses were at the least in duplicate for each reported experiment. In addition to the normal 50° amino acid analysis for the eighteen common amino acids, 30–50° separations were carried out to detect unhydrolyzed peptides and for the less common amino acids.

The protein content of each sample was determined by micro-Kjeldahl analyses for nitrogen. Samples of deuterio-phycocyanin were dialyzed against distilled ordinary water for 3 days with daily changes of water. This procedure resulted in the presence of hydrogen in all exchangeable positions of the protein. The sample was then thermally denatured at 80°, carefully dried on a vacuum line, and the nitrogen content determined by a micro-Kjeldahl method. Several aliquots of each amino acid sample were used to determine the nitrogen content of each sample. The techniques used yielded results of adequate reproducibility. Hydrolyses were performed on protein samples of approximately 10-mg. weight dissolved in from 1 to 2 ml. of solution (approximate concentration determined by optical density at 620 mμ). An equal volume of stock hydrochloric acid was added and the reaction mixture freed from air by freezing with liquid nitrogen, evacuating the reaction vessel on a vacuum line, and sealing the evacuated tube off. The tube was then placed in an oven at 110° for the prescribed hydrolysis period. Upon completion of hydrolysis the tube was opened, the acid evaporated on the vacuum line, and the residue taken up in distilled water and filtered. The filtered solution was again evaporated to dryness and the residue dissolved in 10 ml. of *p*H 2.2 buffer used for the amino acid analysis.

Physical Chemical Measurements.—Cellulose acetate electrophoresis measurements were made with a Shandon electrophoresis apparatus (Chicago Apparatus Co.). All visible and ultraviolet spectra were measured on a Cary 14 spectrophotometer. Infrared spectra were measured on a Perkin–Elmer 221 infrared spectrophotometer. Fluorescence spectra were observed with an Aminco–Kiers spectrofluorimeter adapted for fluorescence.[15] Sedimentation constants of the proteins were determined at a rotor speed of 59,780 r.p.m. in the analytical ultracentrifuge (Spinco model E) using Schlieren optics. The partial molar volume used in the calculation of S_{20} values was an arbitrary value, and the same value was used for the deuterio- and protio-proteins.

Thermal Denaturation.—The thermal denaturation of the deuterio- and protio-proteins was investigated by examining the fluorescence intensity. A Brice–Phoenix light scattering photometer (series 1000) was adapted for this purpose. A Corning 4303 filter was placed on the incident beam, and a Corning 2403 filter in front of the photomultiplier. The fluorescence intensity at various angles to the incident beam could be easily measured and proved to be a very convenient procedure for following the course of the denaturation. Details of the method and other applications will be presented elsewhere.[16] All measurements were taken at an angle of 90° to the incident beam. The cuvette was placed in a metal block through which water could be circulated. The temperature response of the cuvette in the temperature block was checked with a copper–constantan thermocouple. Over the temperature range examined, the temperature could be measured to a precision of ±0.1°.

Criteria for Purity and Deuterium Content of Deuterio-phycocyanin.—The absorption spectra serve as an initial measure of the purity and homogeneity of phycocyanin, but additional criteria are desirable. Cellulose acetate strip electrophoresis was performed on both the deuterio- and protio-phycocyanins. In each case this procedure was carried out in acetate buffer (*p*H 4.7$_0$, $\mu = 0.02$) and a single migrating band was observed. A slight difference in mobility and diffuseness of the band may exist in the two proteins, and a mixed sample of deuterio- and protio-phycocyanin in fact shows some tendency to separate. The homogeneity of the preparations, however, as indicated by the cellulose acetate strip electrophoresis, is excellent.

The ultracentrifuge patterns of the deuterio and protio preparations were also investigated, and it will be seen below that these observations also indicate good homogeneity and purity for both the protio and deuterio preparations. The amino acid analysis and elemental analysis of the deuterio- and protio-protein preparations are further evidence of the purity of both preparations, since there is very close agreement between these analyses.

To establish unambiguously the fully deuteriated nature of the deuterio-phycocyanin, the deuterium content has been ascertained by direct analysis. The phycocyanin isolated from completely deuteriated algae was dialyzed against D_2O for 3 days at 5° with daily changes of D_2O to re-exchange hydrogen introduced during the isolation and purification procedure. The protein was then thermally denatured by heating at 80° for 10 minutes. The denatured protein was recovered by centrifugation, washed with D_2O, centrifuged again and carefully dried on a vacuum line overnight. The dried protein was burned, the water of combustion collected, and analyzed for D_2O content spectrophotometrically.[17] The analysis showed 98.4% D_2O in the water of combustion. The infrared spectrum of the solid protein was also measured. A small amount of the fully deuteriated phycocyanin was mixed in a mull with Perkin–Elmer fluorocarbon oil and a search was made for the characteristic C–H absorption at 2900 to 3000 cm.$^{-1}$. No detectable C–H or N–H absorption was observed, but absorption in the 2220–2240 and 2450 cm.$^{-1}$ regions characteristic of C–D and N–D bonds, respectively, were present. As a control a sample of ordinary phycocyanin extracted from *P. calothricoides* was examined in the infrared by the fluorocarbon oil technique.

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(12) A. Tiselius, S. Hjerten and D. Levin, *Arch. Biochem. Biophys.*, **65**, 132 (1956).

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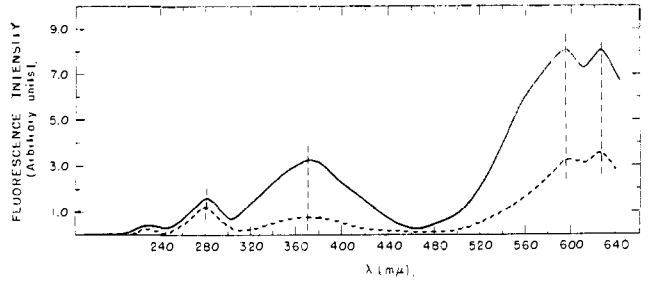


Fig. 1.—Fluorescence excitation spectra of phycocyanins in phosphate buffer (pH 7.15, μ = 0.02); the fluorescence at 648 mμ is measured in arbitrary units as a function of exciting wave length; the concentrations are not equal: ———, protio-phycocyanin; – – – –, deuterio-phycocyanin.

The characteristic C–H absorption in the 2900 to 3000 cm.$^{-1}$ region and the characteristic N–H absorptions were easily observed to be present.

An elemental analysis of ordinary and deuterio-phycocyanin extracted from *P. calothricoides* was made with results as summarized in Table I. The elemental composition of deuterio-phycocyanin was calculated from the hydrogen content of protio-phycocyanin, assuming each hydrogen atom was replaced by a deuterium atom. The calculated and experimental carbon and nitrogen analyses agree quite well, but the experimental hydrogen analysis is somewhat lower than the calculated value. Since the deuterium analysis is very sensitive to any hydrogen exchange occurring during any stage of the analysis, it is reasonable to suppose that a small amount of exchange during the several manipulations involved in the preparation and collection of the water of combustion occurs and that this accounts for the discrepancy. Small amounts of exchange at any stage will have a significant effect on the deuterium analysis, whereas the carbon and nitrogen analyses will only be affected by hydrogen exchange only if it occurs before the sample is weighed.

TABLE I

ELEMENTARY COMPOSITION OF DEUTERIO-PHYCOCYANIN

Element	Protio-phycocyanin, found, %	Deuterio-phycocyanin, calcd., %	Deuterio-phycocyanin, found, %
Nitrogen	15.50	14.40	14.29
Hydrogen	7.63	14.15	12.15
Carbon	51.14	47.60	47.76

Absorption and Fluorescence Spectra.—The visible and ultraviolet absorption spectra of both the deuterio- and protio-phycocyanin were examined in acetate (pH 4.70, μ = 0.02) and phosphate (pH 7.15, μ = 0.02) buffers in both H_2O and D_2O solutions. At both pH values, the absorption maximum is at 620 mμ, and appears to be the same within 1 mμ for both deuterio- and protio-phycocyanin. A broad maximum occurs in the 345–360 mμ region, as well as the usual absorption peak at 278 mμ. Differences in absorption spectra attendent on the replacement of hydrogen by deuterium thus appear to be less striking than is the case for chlorophyll and carotenoids.[18]

An attempt was made to compare the specific extinction coefficient of the deuterio- and protio-phycocyanin at 620, 350 and 280 mμ. However, it appears that phycocyanin does not obey Beer's law at these wave lengths, and a meaningful comparison of the specific extinction coefficients is not possible. Apparently, complex equilibria exist, and since these may well be quite different for the two phycocyanins, the question of extinction coefficient has been deferred.

The 648 mμ fluorescence excitation spectra of deuterio- and protio-phycocyanin were investigated in phosphate buffer at pH 7.15, μ = 0.02. No attempt to compare quantum yield was made but rather the wave lengths of maximum fluorescence excitation was ascertained. Figure 1 shows the fluorescence excitation spectra. The fluorescence excitation spectra of the two phycocyanins are essentially identical, and within experimental error no differences in the position of the fluorescence excitation maxima were found.

Sedimentation Behavior.—Sedimentation experiments of an exploratory nature were carried out in acetate buffer (pH 4.70, μ = 0.02) and in phosphate buffer (pH 7.20, μ = 0.02) with both deuterio- and protio-phycocyanin. In acetate buffer both the deuterio- and protio-protein sedimentation patterns showed four peaks. The S_{20} values for this experiment are listed in Table II. The 14.1_6 S component in the deuterio- and the 11.1_5 S in the protio-protein are present in the largest amount and are responsible for the largest portion of the light absorption. Further sedimentation experiments with deuterio-phycocyanin in acetate buffer indicated that the sedimentation constants were highly concentration dependent not only in magnitude but in number as well. For example, in acetate buffer at a protein concentration

TABLE II

SEDIMENTATION CONSTANTS IN ACETATE BUFFER

$S_{20} \times 10^{13}$, deuterio-phycocyanin		$S_{20} \times 10^{13}$, protio-phycocyanin	
4.80	14.16	3.73	11.15
8.74	22.67	7.17	15.84

TABLE III

AMINO ACID COMPOSITION OF DEUTERIO-PHYCOCYANIN EXTRACTED FROM *P. calothricoides*

Amino acid[a]	—Grams of amino acid residue per 100 g. of protein— 20-hr. hydrolysate	45-hr. hydrolysate	70-hr. hydrolysate	90-hr. hydrolysate	Average	N as % of total N
Lysine	4.07	3.96	3.96	4.14	4.02 ± 0.07	5.65
Histidine	0.69	0.65	0.57	0.62	0.63 ± .04	1.29
Ammonia (NH_3)	1.80	1.68	1.87	1.81	1.71 ± .11[b]	10.28
Arginine	6.30	6.06	6.25	6.25	6.20 ± .08	14.09
Aspartic acid	9.14	9.02	8.92	9.35	9.11 ± .13	7.50
Threonine	3.68	3.12	3.02	2.99	4.30[c]	3.93
Serine	3.67	2.78	2.02	1.90	4.35[c]	4.65
Glutamic acid	6.75	6.80	7.05	7.00	6.89 ± 0.09	4.99
Proline	2.35	2.15	2.27	2.18	2.22 ± .07	2.05
Glycine	3.48	3.40	3.40	3.68	3.48 ± .10	5.70
Alanine	8.01	8.28	8.06	8.38	8.18 ± .14	10.48
Half cystine	..	..	..	0.79	0.79[d]	0.91
Valine	4.66	4.78	4.98	4.85	4.82 ± 0.09	4.35
Methionine	0.46	0.26	0.73	3.29	3.29[d]	2.28
Isoleucine	4.60	4.41	4.50	4.62	4.60 ± 0.07	3.60
Leucine	7.55	7.45	7.40	7.42	7.44 ± .05	5.82
Tyrosine	4.91	4.97	4.29	4.85	4.75 ± .24	2.71
Phenylalanine	3.26	3.24	3.46	3.60	3.40 ± .12	2.12
					80.18 ± 1.29	92.40

[a] Trace amounts of hydroxylysine, ornithine and 1-methylhistidine are present, but in amounts too small to make quantitative estimation possible. [b] Value not included in total. [c] Value arrived at by extrapolation. [d] Maximum value used.

TABLE IV

AMINO ACID COMPOSITION OF PROTIO-PHYCOCYANIN EXTRACTED FROM *P. calothricoides*

Amino acid[a]	Grams of amino acid residue per 100 g. of protein 20-hr. hydrolysate	40-hr. hydrolysate	60-hr. hydrolysate	100-hr. hydrolysate	Average	N as % of total N
Lysine	3.77	3.96	3.77	4.14	3.91 ± 0.14	5.52
Histidine	0.59_8	0.63_8	0.56_5	0.68_5	0.62_2 ± 0.04	1.23
Ammonia (NH_3)	1.33	1.54	1.60	2.36	1.71 ± 0.32[b]	9.65
Arginine	5.95	6.02	5.94	5.74	5.92 ± .08	13.71
Aspartic acid	9.05	9.11	9.13	9.04	9.09 ± .04	7.15
Threonine	3.68	3.46	3.09	1.83	4.25[c]	3.80
Serine	3.53	3.03	2.20	0.87	4.20[c]	4.35
Glutamic acid	6.66	6.65	6.27[d]	6.63	6.65 ± .01	4.59
Proline	1.68	2.41	1.96	2.19	2.06 ± .24	1.92
Glycine	3.36	3.41	3.32	3.56	3.41 ± .08	5.40
Alanine	7.79	7.75	7.76	8.00	7.82 ± .09	9.95
Half cystine	0.54_5	0.73_5	0.53_5	0.66_2	0.62 ± .09	0.54
Valine	4.40	4.52	4.71	4.73	4.58 ± .13	4.17
Methionine	3.17	2.84	3.00	2.99	3.00 ± .09	2.07
Isoleucine	4.25	4.36	4.16	4.56	4.44 ± .14	3.54
Leucine	7.67	6.93	7.60	6.97	7.29 ± .34	5.80
Tyrosine	4.56	4.78	4.72	4.30	4.63 ± .16	2.58
Phenylalanine	3.02	2.91	2.90	3.35	3.05 ± .15	1.87
					75.54 ± 1.82	87.84

[a] Trace amounts of hydroxylysine, ornithine and 1-methylhistidine are present, but in amounts too small to make quantitative estimation possible. [b] Value not included in total. [c] Value arrived at by extrapolation. [d] Value not included in average.

(18) H. H. Strain, M. R. Thomas, H. L. Crespi and J. J. Katz, *Biochim. Biophys. Acta*, **52**, 517 (1961).

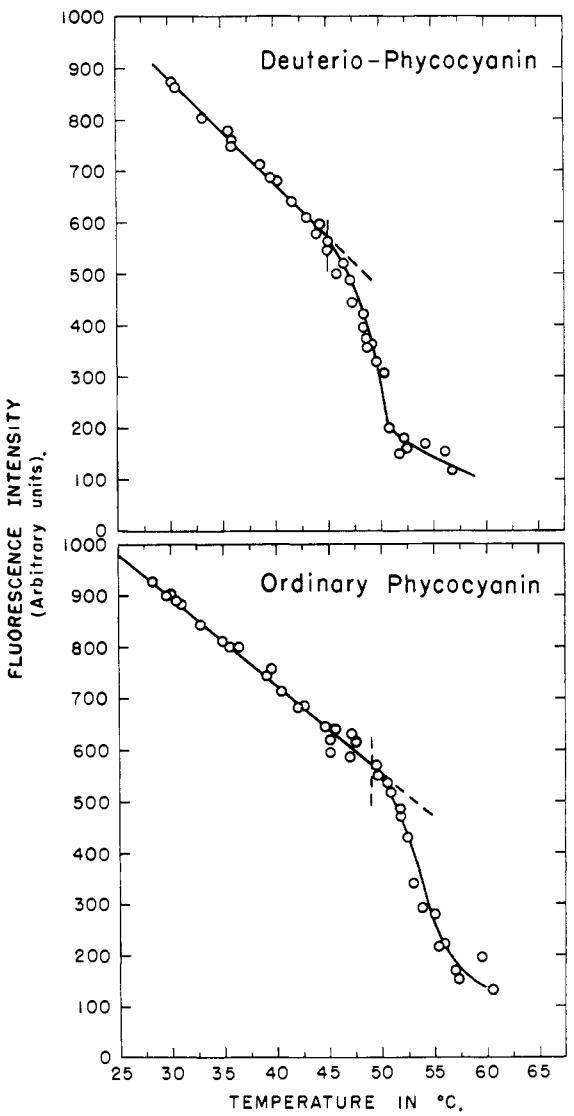


Fig. 2.—Thermal denaturation of phycocyanins in acetate buffer, pH 4.70, μ = 0.1.

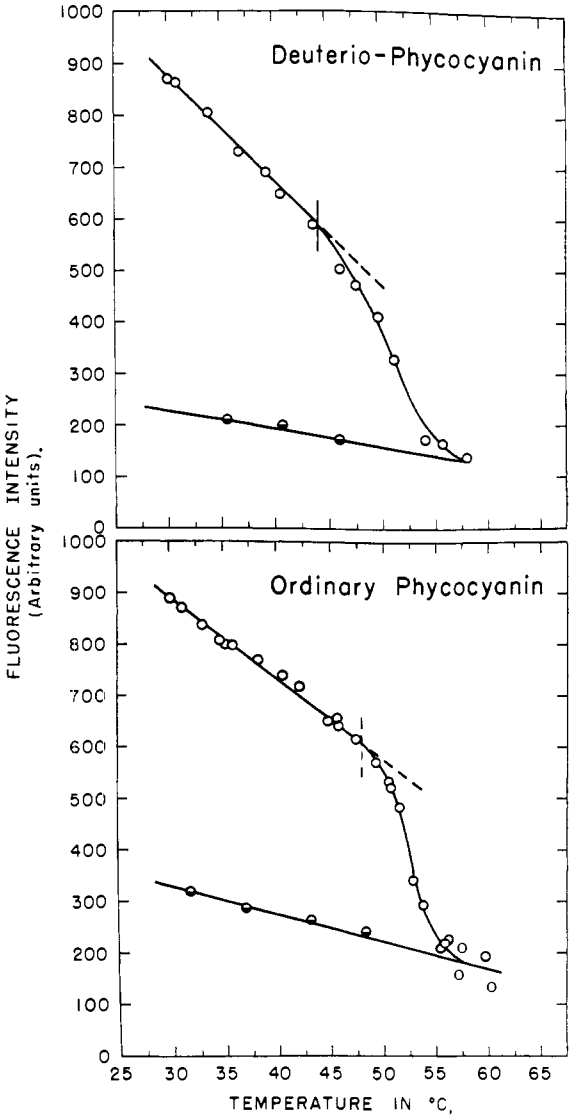


Fig. 3.—Thermal denaturation of phycocyanins in acetate buffer, pH 4.70, μ = 0.01: ◒, descending temperatures.

of 12 mg./ml. the deuterio-protein exhibited a sedimentation pattern with S_{20} values of 4.51×10^{-13}, $9.9_0 \times 10^{-13}$ and 11.8×10^{-13}. Simple dilution of the protein concentration to one-half of the value of the first experiment gave a sedimentation pattern with peaks at $S_{20} = 3.83 \times 10^{-13}$ and 13.65×10^{-13}. In phosphate buffer at a protein concentration of about ~15 mg./ml. a single peak is observed with $S_{20} = 5.84 \times 10^{-13}$; at one-half this concentration a single peak with $S_{20} = 7.72 \times 10^{-13}$ is found. Some experiments with relatively less pure samples of phycocyanin were carried out early in this work. Although only of a preliminary nature, these observations are reported here because of the light they may shed on the nature of phycocyanin. In phosphate buffer (pH 6.70, μ = 0.015), protio-phycocyanin shows a single peak with $S_{20} = 3.10 \times 10^{-13}$; upon dilution by a factor of 3/2, $S_{20} = 6.20 \times 10^{-13}$ was observed. In acetate buffer (pH 4.70, μ = 0.01) the same sample showed peaks at $S_{20} = 4.5$ and 14.1×10^{-13} (acetate buffer, pH 4.7, μ = 0.01) and $S_{20} = 1.00 \times 10^{-13}$ (phosphate buffer, pH 6.70, μ = 0.015). Deuterio-phycocyanin from phosphate buffer was transferred by dialysis to the original acetate buffer and now showed peaks at $S_{20} = 3.96$ and 14.8×10^{-13}. The sedimentation patterns are thus a function of pH, and appear to be reversible.

The sedimentation behavior of deuterio-phycocyanin was entirely analogous to that of the protio-phycocyanin. It is evident that an associating system is involved in both cases. The association of the protein apparently is quite different at a pH of 4.7, where two peaks are seen from that at a pH near 7.0 where only one peak is observable. The magnitude of the sedimentation constants at both pH values is affected by change in concentration, and in the acetate buffer even the number of peaks is changed. This is certainly the type of behavior generally encountered in associating systems. The fact that the phycocyanin solutions in acetate and phosphate buffers apparently do not obey Beer's law would seem also to indicate complex equilibria. That a change of buffer medium by dialysis and restoration of the original medium reproduces the original sedimentation patterns would seem to support the belief that the observed behavior is not an artifact. It is important to point out that the sedimentation patterns observed here are in agreement with the original work of Svedberg, *et al.*[19] The results reported here are difficult to reconcile with those recently reported by Brody and Brody,[20] but we are unable to account for the discrepancy. The pH dependence of fluorescence depolarization reported by Goedheer[21] is also consistent with the association equilibria that probably are responsible for the observed sedimentation properties of phycocyanin.

Amino Acid Composition.—For comparisons between deuterio- and protio-phycocyanin to be valid, it is important to establish the chemical identity of the two species. It is conceivable that culturing *P. calcothricoids* in D_2O may involve the selection of a mutant, and that the chemical composition of the proteins synthesized by hydrogen and the deuteriated organism may be significantly different. Should this be the case, variation in physical properties as between deuterio- and protio-proteins might not be particularly meaningful. Although sequential analysis of the amino acids in phycocyanin would in principle be desirable, we have contented ourselves with establishing the amino acid

(19) T. Svedberg and I. Erickson, *J. Am. Chem. Soc.*, **54**, 3998 (1932); T. Svedberg and T. Katsumai, *ibid.*, **50**, 525 (1928); I. G. Erickson-Quensel, *Biochem. J.*, **32**, 585 (1938).

(20) S. S. Brody and M. Brody, *Biochim. Biophys. Acta*, **50**, 348 (1961).

(21) S. C. Goedheer, Doctoral Thesis, Rijksuniversiteit, Utrecht, 1957.

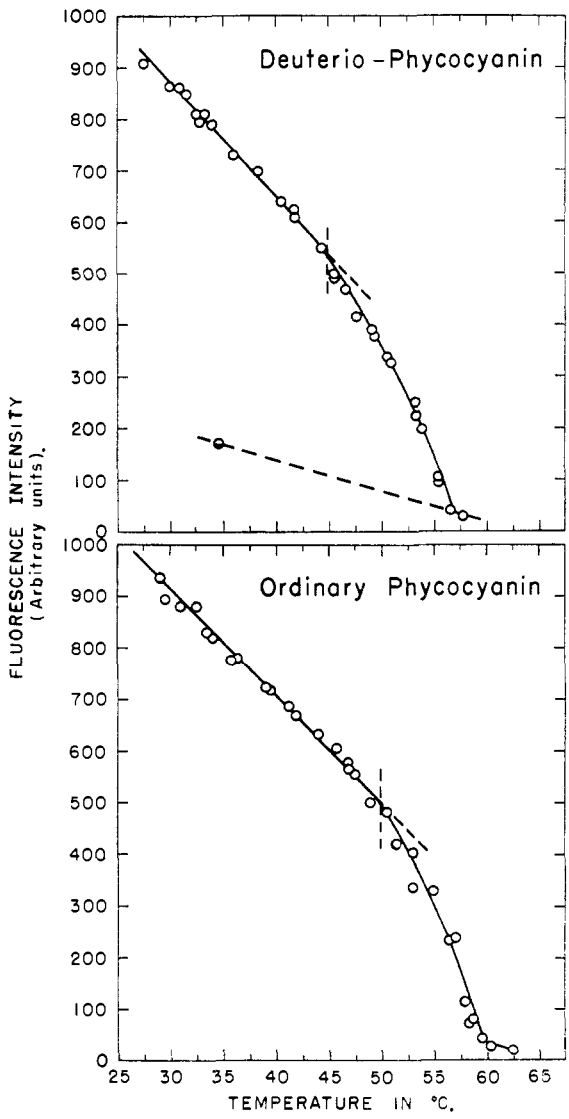


Fig. 4.—Thermal denaturation of phycocyanins in phosphate buffer, *p*H 7.00, $\mu = 0.1$: ◒, descending temperatures.

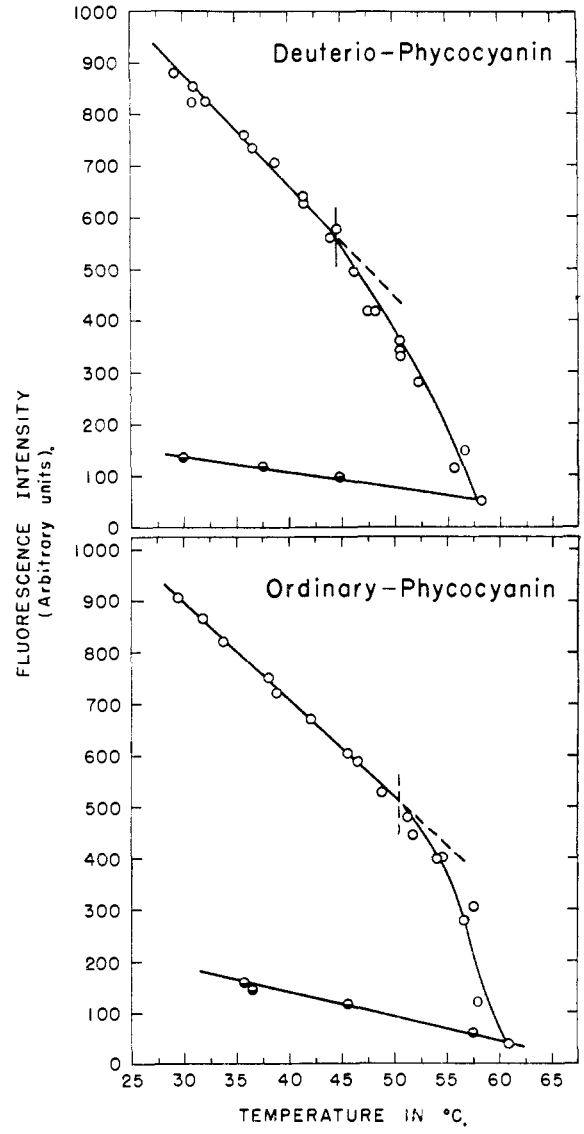


Fig. 5.—Thermal denaturation of phycocyanins in phosphate buffer, *p*H 7.15, $\mu = 0.01$: ◒, descending temperatures.

compositions of deuterio- and protio-phycocyanin. The analyses were performed by the Moore–Stein method with a Spinco amino acid analyzer. Varying periods of hydrolysis were used to assure complete hydrolysis and to evaluate the magnitude of decomposition for amino acids during the acid hydrolysis. The results of the amino acid determinations are given in Tables III and IV.

The total amino acids recovered in the protein hydrolysates is somewhat less than 100% of the protein sample weight. In the amino acid analysis of the closely related protein phycoerythrin, Kimmel and Smith[13] found that the amino analysis did not account for the entire weight of the sample, but that the nitrogen recovery in terms of amino acids was very nearly 100%. In the analyses reported here, the nitrogen recovery is only about 90%. Part of the nitrogen unaccounted for is probably to be attributed to tryptophan that has been lost during hydrolysis. The tetrapyrrole chromophores have been estimated to constitute 5% of the weight of the phycocyanin.[22] The nitrogen discrepancy may therefore be associated with nitrogen-containing non-amino acid chromophores. As the amino analyses improve, and particularly when the tryptophan content is determined, it may be possible to make more specific statements about the nitrogen content of the chromophoric groups.

From the data in Tables III and IV it can be concluded at this time that deuterio- and protio-phycocyanin extracted from *P. calothricoides* are essentially identical in amino acid composition. Differences in physical properties of the two proteins thus would appear to reflect the effects of isotopic substitution rather than differences in chemical composition.

Isotope Effects in the Hydrolysis of Deuterio-phycocyanin.—Some interesting kinetic isotope effects were observed incidental to the hydrolysis of the proteins for amino acid analysis. Both serine and threonine appear to be lost much more slowly in the hydrolysis of deuterio-phycocyanin than is the case for the protio compounds. Again, the protio-phycocyanin is completely hydrolyzed in 20 hours; further heating leads only to loss of some amino acids. In hydrolysates of deuterio-phycocyanin, however, no cystine and only small amounts of methionine are found after 20 hours. Cystine and methionine do not reach their maximum values until the protein is subjected to 90 hours of hydrolysis. Under conditions of incomplete hydrolysis, it appears from the chromatograms that peptides are present, and these appear to be hydrolyzed very much more slowly in the case of cystine and methionine when present in deuteriated form. The sudden jump in

(22) F. T. Haxo and C. O'hEocha, in *Encyclopedia of Plant Physiology*, **5**, pt. I, pg. 497. Berlin, 1960.

yield of methionine and cystine from 70 to 90 hours is puzzling and perhaps not completely interpretable in terms of a slow liberation from a peptide. In the absence of more detailed information, however, it would appear to be reasonable to attribute differences in the rates at which methionine and cystine appear in the hydrolysis of protio- and deuterio-phycocyanin to the operation of a kinetic isotope effect.

Other differences are evident from the chromatograms. In both the 20-hour deuterio and protio hydrolysates, peaks are found in the interval between the normal elution time for ammonia and for arginine. In the case of the deuterio-phycocyanin, 20-hr. hydrolysates show elution peaks at 98 ml. and 103 ml., while in the protio-phycocyanin 20-hr. hydrolysates reveal only the 97-ml. peak in sizable amount; the 104-ml. peak here is barely detectable. These peaks, probably due to peptides, are found on 45-hr. and 70-hr. deuterio-phycocyanin hydrolysate chromatograms in decreasing amounts, and in the 90-hr. hydrolysate they are no longer detected. The 90-ml. peak is present in decreasing amounts in the 40- and 60-hr. hydrolysate. After 100 hr., the peak area is quite small and has an integrated absorbance of about 1% of that of the ammonia peak. It was also noted that in the neutral and acidic portion of the chromatogram a peak appeared between methionine and isoleucine in the 20-hr. protio-phycocyanin hydrolysate. This same peak became increasingly larger in area in the 40-, 60- and 100-hr. hydrolysates. While this peak did not appear in the 20-hr. deuterio-phycocyanin hydrolysate its appearance was noted in the 45-hr. hydrolysate and in all subsequent hydrolysates. This peak is undoubtedly due to alloisoleucine.[23] The combined amount of alloisoleucine and isoleucine is used as the amount of isoleucine found in the hydrolysates. The area under these curves in 20-, 40-, 60- and 100-hr. hydrolysates are approximately 1.8, 2, 4 and 11% of the area of the isoleucine peak, respectively. The alloisoleucine peak area in the deuterio-phycocyanin hydrolysates is approximately 3% of the area of the isoleucine peak in all the 40-, 60- and 90-hr. hydrolysates. The rate of isomerization of isoleucine is thus distinctly lower in deuterio-isoleucine than in the usual hydrogen-containing isoleucine.

Whether ionic equilibria are significantly different for deuterio- and protio-amino acids and peptides was studied by ion exchange chromatography of mixtures of deuterio- and protio-glycine and deuterium- and protio-phycocyanin hydrolysates. No separations were achieved, nor was there even any asymmetry present in the elution curves of these mixtures. Consequently it is concluded that the ionic equilibria are essentially the same for protio- and deuterio-amino acids insofar as ion exchange behavior is concerned.

Thermal Denaturation.—The thermal denaturation of deuterio- and protio-phycocyanin was studied in acetate buffer (pH 4.70, μ = 0.01 and 0.1) and in phosphate buffer (pH 7.00, μ = 0.1 and pH 7.15, μ = 0.01). The relative intensity of fluorescence of the protein is used to obtain a temperature profile in a fashion analogous to that recently reported by Steiner and Edelhoch.[24] The temperature profiles are shown in Figs. 2 to 5. In each case the profiles are a result of at least three separate experiments and are quite reproducible. Particular care was taken to determine the point at which the change in relative fluorescence intensity became irreversible. This was done by slowly increasing the temperature and then lowering it to the temperature region in which the fluorescence intensity had already been measured. It was observed that the change in fluorescence intensity was generally not dependent upon the rate of temperature change. In regions where irreversible changes occurred there may very well be some dependence on the rate of temperature change, and the exact shape of the curves in the transition zone therefore may be somewhat arbitrary. The curves obtained on cooling from the end of the transition was always linear and at a considerably attenuated relative fluorescence intensity as compared to the forward direction.

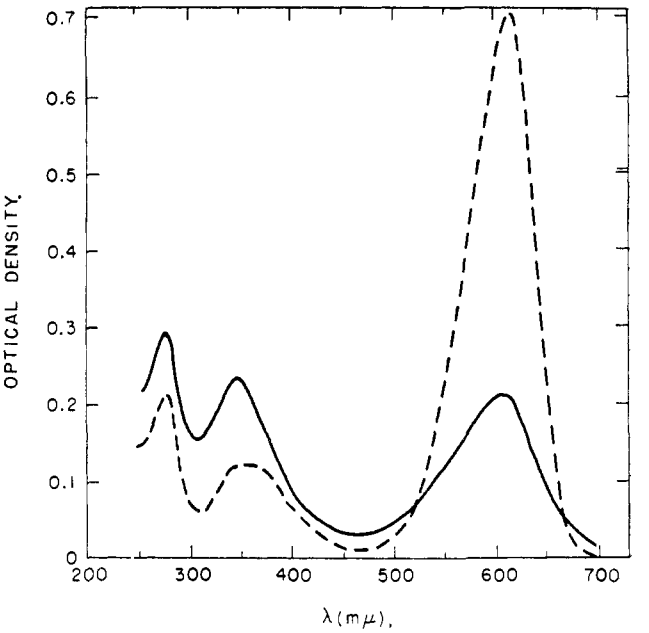


Fig. 6.—Absorption spectra of thermally denatured deuterio-phycocyanin; phosphate buffer, pH 7.0, μ = 0.1: ———, denatured; - - - -, native protein.

In phosphate buffer, the protein solutions remained clear and free of any colloidal material; no protein precipitated at any time during the thermal transition. There was, however, a marked decrease in intensity of the blue color of the solution corresponding to the quenching of the fluorescence. In Fig. 6 the absorption spectrum of a typical phosphate buffered protein solution is shown before and after thermal denaturation. The acetate buffered solutions always became colloidal within a degree or two after entering the transition region. The major portion of the protein eventually precipitated as higher temperatures were reached. The spectrum of the supernatant solution in these cases was quite similar to that of the phosphate solutions in that the 620 mμ peak was greatly attenuated and the 350 mμ peak was somewhat enhanced.

The thermal profiles clearly indicate a significant difference between the temperature at which the thermal transition begins in the deuterio- and protio-phycocyanins. In acetate buffer of 0.1 or 0.01 ionic strength the deuterio transition begins at about 44° and the protio at 48°; the mid-point of the transition region of the deuterio-protein is at about 50° and in the protio-protein at about 53°. In phosphate buffer the deuterio transition begins at 45° and the ordinary protein transition begins at 51°; the mid-point of the transition is at about 52° for the deuterio- and 57° for the protio-protein. The difference between deuterio- and protio-phycocyanin transitions is approximately 5° in both cases. There is little or no detectable difference in transition on change in ionic strength, but the thermal denaturation does occur at a slightly lower temperature in acetate buffer than in phosphate.

(23) K. A. Piez, *J. Biol. Chem.*, **207**, 77 (1954); S. Moore, D. H. Spackman and W. H. Stein, *Anal. Chem.*, **30**, 1190 (1958).

(24) R. F. Steiner and H. Edelhoch, *Nature*, **193**, 376 (1962).

All of the experiments on thermal denaturation reported here were carried out in H_2O solution. The deuterio-phycocyanin was isolated and stored in H_2O. The deuterio-phycocyanin therefore probably has hydrogen at all the exchangeable positions. A sample of deuterio-phycocyanin dialyzed against H_2O for several days was denatured, dried in vacuum, and burned. The water of combustion showed about 83% D_2O. This result is in agreement with the observation that about 20% of the hydrogen in many proteins is exchangeable. Even though it is recognized that some exchangeable hydrogen atoms may exchange only slowly, the great majority of the hydrogen bonding positions in H_2O solutions of deuterio-phycocyanin can be expected to be occupied by hydrogen in view of the protracted exposure of the protein to H_2O. It would appear, therefore, that the observed differences in transition temperature are not primarily a consequence of a change in hydrogen bonding or of ionic bonds as well. Rather, the differences must be associated with differences in side chain interactions.[25]

Phycocyanin appears to possess an about average number of non-polar amino acids (Table III). It is hard to avoid the conclusion that it is the replacement of hydrogen by deuterium in the non-polar amino acids and the consequent differences in apolar or hydrophobic bond interactions that are primarily responsible for the present observations. Until the configurational stability of deuterio-phycocyanin is studied by other means, and until these studies are extended to other deuteriated proteins, it is premature to attempt an assessment of the relation between hydrogen and deuterium apolar bonds. It would appear, however, that fully deuteriated proteins present a unique experimental situation in which attention can be focused directly on the role of non-polar amino acids in protein configuration.

Acknowledgments.—We wish to express our thanks to Messrs. Jon Palmer and Norman Solliday who provided very capable technical assistance in securing the amino acid analyses.

(25) W. Kauzmann, "Advances in Protein Chemistry," Vol. XIV, Academic Press, Inc., New York, N. Y., 1959, p. 33 et seq.; I. Klotz, *Brookhaven Symp. Biol.*, **13**, 25 (1960).

[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT, BROOKHAVEN NATIONAL LABORATORY, UPTON, N. Y.]

The Secondary α-Deuterium Isotope Effect in the Thermal Decomposition of α-Phenylethylazo-2-propane[1,2]

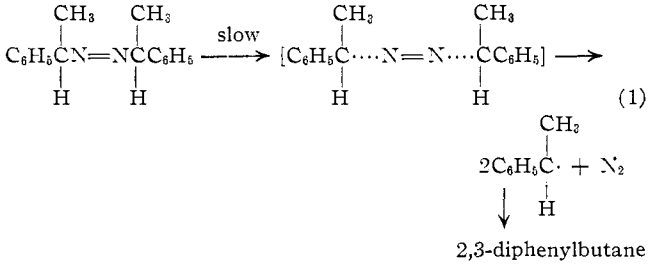
BY STANLEY SELTZER

RECEIVED JUNE 6, 1962

α-Phenylethyl-α-*d*-azo-2-propane (II) and α-phenylethylazo-2-propane-2-*d* (III) were prepared. The rates of decomposition of these compounds, in addition to the natural compound I, were measured and compared. The isotope effects are k_I/k_{II} = 1.15 and k_I/k_{III} = 1.04. The conclusions drawn from these isotope effects are: (1) both carbon–nitrogen bonds break simultaneously, and (2) the α-phenylethyl carbon–nitrogen bond is stretched considerably more than the 2-propyl carbon–nitrogen bond in going from the starting state to the transition state. An apparent contradiction of two rules predicting the structure of the transition state is discussed.

Introduction

Recently, it has been reported from this Laboratory, that the secondary α-deuterium isotope effect in the radical decomposition of azo-bis-α-phenylethane-α-α'-d_2 in ethylbenzene at 105° is k_H/k_D = 1.27.[3] Since this effect is approximately twice as large as those encountered in other "unimolecular" reactions, it was taken as evidence for both carbon–nitrogen bonds breaking simultaneously in the transition state of the rate-controlling step (eq. 1).



(1)

The interpretation of this isotope effect rests upon the comparison of its magnitude (observed in a radical reaction) with those encountered in reactions approaching SN1 character. The Arrhenius activation energies, measured in the solvolytic reactions, are a little more than half the 32.6 kcal./mole activation energy observed[4] for the decomposition of azo-bis-α-phenylethane. If a stepwise mechanism of decomposition is assumed, with the first step being rate controlling, then the high activation energy for this endothermic step suggests a transition state closely resembling products[5] (*i.e.*, α-phenylethyl and α-phenylethylazo radicals). A less likely interpretation of the large isotope effect follows from this assumption: that it represents a greater reorganization of atoms (hence a greater change in the α-hydrogen vibrational frequencies) in going from the reactant state to the transition state in the case of the azo compound decomposition than in the solvolytic reactions. In other words, the observed effect, k_H/k_D = 1.27, would be due to only one of the two D atoms in azo-bis-α-phenylethane-α,α'-d_2. (This interpretation is referred to in the Discussion as case A, while the previous interpretation is designated as case B.) This alternate possibility was investigated by measuring the two secondary α-deuterium isotope effects in the unsymmetrical azo compound α-phenylethylazo-2-propane.

Experimental

α-Phenylethylhydrazine (I).—Acetophenone azine, m.p. 120.4–122.1°, 10.3 g., in 200 ml. of ethyl acetate in the presence of 1.7 g. of 5% Pd–C (Baker Catalyst Co.) was hydrogenated at room temperature at a pressure of approximately 14 p.s.i. The reduction was stopped after one mole equivalent of H_2 was added. After filtering and evaporating the solvent, the yellow oil was added to a solution of 9 g. of oxalic acid dihydrate in 30 ml. of absolute alcohol plus 30 ml. of ether. This solution was stirred for 20 hours at room temperature. The white solid, α-phenylethylhydrazinium oxalate, was filtered off, washed, and dried; m.p. 170.2–170.8° dec., lit. 172°[6]; yield 6.1 g., 62%.

(1) Presented in part at the American Chemical Society Meeting, Chicago, Ill., September, 1961.

(2) Research performed under the auspices of the U. S. Atomic Energy Commission.

(3) S. Seltzer, *J. Am. Chem. Soc.*, **83**, 2625 (1961).

(4) S. G. Cohen, S. J. Groszos and D. B. Sparrow, *ibid.*, **72**, 3947 (1950).

(5) G. S. Hammond, *ibid.*, **77**, 334 (1955).

(6) C. G. Overberger and A. V. DiGiulio, *ibid.*, **80**, 6562 (1958).