[CONTRIBUTION FROM THE LABORATORIES OF THE SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH AND THE SLOAN-KETTERING DIVISION OF CORNELL UNIVERSITY MEDICAL COLLEGE]

Studies on the Effect of Heat on Deoxyribonucleic Acid[1]

BY HERBERT S. ROSENKRANZ[2] AND AARON BENDICH

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The effect of heat on solutions of DNA, with or without salt present, was investigated. Chromatographic and sedimentation behaviors of these macromolecules are presented which indicate that all molecules do not react to thermal denaturation n a uniform manner. Salt was found to exert a protective effect.

Introduction

The heating of DNA in water or in solutions of very low ionic strength leads to a considerable decrease in sedimentation coefficient, viscosity and molecular weight.[3-10] When heated in salt-free water solution, the sedimentation coefficient, for example of T2 phage DNA, drops from a value of 20–40 to about 4–6.5 *S*.[9]

The interpretation of the results of the heating of DNA in salt solution is complicated by apparently conflicting results in the literature. Some investigators have reported a decrease in sedimentation coefficient after such treatment, whereas others have noticed an increase (presumably due to aggregation).[7] Shooter and his colleagues[9,11] have found a limited decrease in sedimentation constant together with a decrease in viscosity. They interpreted these results as possibly due to a decrease in molecular weight (to $^1/_4$ or $^1/_6$ of the original). On the other hand, Schachman[5] reported that heating of DNA in salt solution results in a decrease in viscosity with hardly any change in the sedimentation behavior. Such were also the findings of Rice and his colleagues who in addition found no change in the molecular weight of the DNA so treated.[12,13] The effects of the heating of DNA in salt solutions appear to depend upon the concentration of DNA[11] as well as on the ionic strength.[8,10] It must be borne in mind, however, that a variety of different approaches including the ion-exchange chromatographic technique[14,15] have clearly shown that many preparations of DNA are mixtures of different polynucleotides. It therefore is pertinent to ask whether conclusions drawn from changes in the physico-chemical properties of unfractionated DNA should not be tempered by this fact. It may be that all of the component polynucleotides in a preparation of DNA are not affected equally by a treatment such as heating. In the hope of shedding more light on this question, the experiments reported below were performed.

Experimental

Nucleic Acid.—The DNA was prepared from fresh calf thymus glands by the Schwander and Signer method.[16] Several of the properties of this preparation (designated as S-II in a previous publication[17]) have been reported.[14,17,18]

Chromatography.—Solutions of the DNA were applied to 0.8 cm. columns containing 0.5 g. of the anion-exchange cellulose derivative ECTEOLA-SF-1 and chromatographed at a rate of about 5 ml. per hour using the two-mixing chamber technique previously described.[14] Every chromatographic analysis was carried out in duplicate. Four distinct elution schedules as shown on the abscissa (Fig. 1) were employed: I (a continuous gradient increase from 0.0 to 0.5 *M* NaCl); II (0.1 *M* NH_3 in 2.0 *M* NaCl); III (a gradient increase from 0.1 to 1.0 *M* NH_3 in 2.0 *M* NaCl), and IV (0.5 *M* NaOH). For convenience, these regions will be referred to by number in this study.

Dialysis of Fractions.—Selected chromatographic fractions were equilibrated by dialysis against 0.20 *M* NaCl in the cold prior to analysis in the ultracentrifuge and prior to alkalinization.

Heating.—Solutions of DNA (1 mg. per ml.) either in 0.01 *M* phosphate buffer, *p*H 7, or in distilled water were heated in glass-stoppered tubes for 1 hour in a boiling water-bath (100°). They were then cooled in an ice-bath.

Hyperchromic Shifts.—It has been found that DNA exhibits an increase in absorbancy at 260 mμ upon treatment with alkali (for references and discussion see ref. 18). This is referred to as the "hyperchromic shift." The magnitude of this effect is taken to be a measure of the intactness of the proposed twin-helical structure[19] of DNA.

To 2.0 ml. of 0.003% DNA solution in 0.20 *M* NaCl in a quartz cuvette, 0.04 ml. of 19 *N* NaOH (final *p*H *ca.* 13.5) was added. The solution was thoroughly mixed. The hyperchromic effect (per cent. increase in optical density at 260 mμ, read against the appropriate blank) was corrected for the dilution (2%) caused by the addition of the alkali. The addition of extra alkali did not cause a further increase in the hyperchromic shift.

Ultracentrifugal Analysis.—Solutions containing 0.003% DNA in 0.20 *M* NaCl were analyzed at 59780 r.p.m. (259,700 × *g*) in 12 mm. cells in a Spinco Model E ultracentri-

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(2) Taken from the thesis submitted by H. S. R. in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of Medical Sciences of Cornell University, New York, February, 1959.

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(19) J. D. Watson and F. H. C. Crick, *Nature*, **171**, 737 (1953).

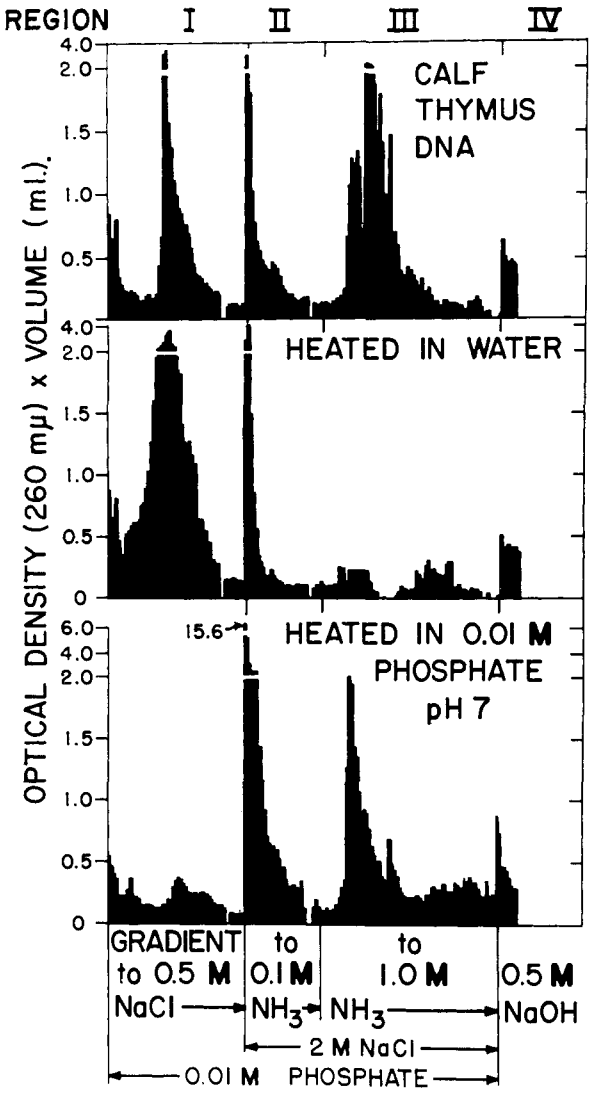

Fig. 1.—Chromatography of calf thymus DNA after heating at 100° for 1 hour in distilled water (center profile) and 0.01 *M* phosphate buffer, *p*H 7 (bottom profile). Upper profile is a chromatogram of the unheated calf thymus DNA. Each column (0.8 × *ca.* 5.3 cm.) contained 0.5 g. of ECTEOLA-SF-1. Recovery in each case was 100 ± 5%.

fuge equipped with ultraviolet optics.[20,21] The integral distributions of sedimentation coefficients, corrected to 20° and water, were calculated by an adaptation[18] of the method of Schumaker and Schachman.[21]

The distributions are reproduced in Fig. 2 and 3, in which values of $S_{20,w}$ on the abscissa are plotted against the fraction of total DNA concentration on the ordinates. The average sedimentation coefficients $s_{50\%}$ were estimated from the 0.5 intercepts.

Results and Discussion

Effect of Heating on Unfractionated DNA.—Per unit weight, the DNA dissolved in water had an optical density at 260 mμ which was 17.2% higher than that of DNA dissolved in phosphate buffer. This hyperchromic effect confirms the previous findings of Thomas,[22] of Beaven, *et al.*,[23] and of Cavalieri, *et al.*,[17] that exposure of DNA to water results in denaturation.

When these solutions were heated at 100°, each showed an increase in their 260 mμ absorbancies

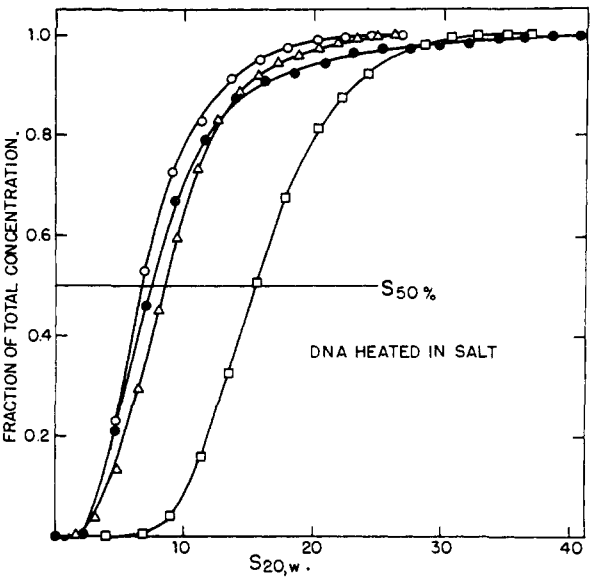

Fig. 2.—Effect on the sedimentation behavior of DNA of heating in the presence of salt: □, DNA dissolved in salt ($s_{50\%}$ = 15.8 *S*); △, as above, after heating ($s_{50\%}$ = 8.4 *S*); ○, first fraction of heated DNA eluted with 0.1 *M* NH_3, 2 *M* NaCl (Region II) ($s_{50\%}$ = 6.7 *S*); ●, second fraction of heated DNA eluted in Region II ($s_{50\%}$ = 7.4 *S*).

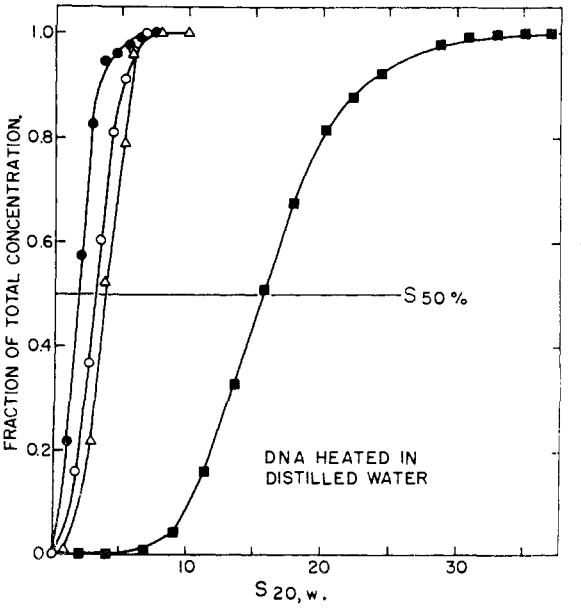

Fig. 3.—Effect on the sedimentation behavior of DNA of heating in water: ■, DNA dissolved in salt ($s_{50\%}$ = 15.8 *S*); ○, DNA dissolved in water, after heating ($s_{50\%}$ = 3.0 *S*); ●, fraction eluted with 0.18 *M* NaCl ($s_{50\%}$ = 1.8 *S*); △, fraction eluted with 0.26 *M* NaCl ($s_{50\%}$ = 3.8 *S*).

(20) K. V. Shooter and J. A. V. Butler, *Trans. Faraday Soc.*, **52**, 734 (1956).

(21) V. N. Schumaker and H. K. Schachman, *Biochim. et Biophys. Acta*, **23**, 628 (1957).

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(23) G. H. Beaven, E. R. Holiday and E. A. Johnson, in "The Nucleic Acids," Vol. 1, Chargaff and Davidson, eds., Academic Press, Inc., New York, N. Y., 1955, p. 493.

(Table I). This effect was small with the DNA which had already been denatured due to its exposure to distilled water. When the heated samples were made alkaline, both again exhibited a hyperchromic shift. The sum of the three hyperchromic shifts was very close to the alkali hyperchromic effect shown by the untreated DNA (DNA dissolved in phosphate).

The data of Table I suggest that not all denaturation procedures are equally effective in causing a hyperchromic shift (*i.e.*, denaturation). Thus heating seems to be less effective than alkalinization.

TABLE I

INCREASE IN 260 mμ ABSORBANCY OF DNA FOLLOWING VARIOUS TREATMENTS

Procedure		
Exposure to water	17.2%	
Heating of water soln.	2.9%	
Heating in salt soln.		15.2%
Alkali treatment of DNA which had been heated in		
(a) Water solution	8.0%	
(b) Salt solution		15.8%
Total effect	28.1%	31.0%
Alkali treatment of DNA dissolved in salt	27.8%	

Whereas both solutions (DNA in water and in phosphate) did not yield substances dialysable against 0.01 *M* phosphate and 0.20 *M* NaCl, they did so after heating at 100°. Of the ultraviolet absorbing material in the DNA solution heated in water, 5.1% was found to be dialysable against 0.01 *M* phosphate, and 21% was dialysable against 0.20 *M* NaCl. This indicates that about 16% of the ultraviolet absorbing material was composed of dialysable oligonucleotide material presumably larger than tetranucleotides. Of the DNA heated in salt solution, 4.7% was found to be dialysable against 0.20 *M* NaCl.

This dialysability could have resulted either from the production of an apurinic acid or from a rupture of phosphodiester bonds or both (see also ref. 5). Free guanine was identified in the solution of DNA heated in water as judged by paper chromatography in 1-butanol–water[24] and sodium monohydrogen phosphate–isoamyl alcohol[25] solvent systems. This indicates that the thermal denaturation of DNA dissolved in water involves more than a disturbance of the hydrogen-bonded secondary structure. This also has been observed by Greer and Zamenhof[26] who have found that about 1% of adenine and of guanine were liberated when calf thymus DNA was heated at 81° for 12 hours in phosphate buffer, *p*H 6.8.[26a]

The sedimentation distribution curves of the samples that had been subjected to heat treatment differed radically from the curve for the untreated DNA (DNA dissolved in salt) (see Fig. 2 and 3 and Table II). The ratios of number-to-weight-average molecular weights (M_w/M_n) estimated from S_w and S_n[18] suggest that the thermal breakdown was not a random process, for had that been the case it would have been expected that the M_w/M_n ratios would have remained the same. These results would indicate that some of the macromolecules in the original unfractionated DNA were more resistant to heat denaturation than others.

TABLE II

PHYSICAL PROPERTIES OF SOLUTIONS OF DNA BEFORE AND AFTER HEATING

	DNA in salt soln., untreated	DNA heated in salt soln.	DNA heated in water soln.
$s_{50\%}$, S	15.8	8.2	3.0
M_w/M_n[a]	2.03	2.75	2.83

[a] M_w/M_n was calculated from S_w/S_n by a procedure described previously.[18]

The sedimentation distribution for the DNA specimen that had been heated in salt indicates a high degree of heterogeneity with a small proportion of material with high sedimentation coefficient (see also ref. 27).

Sedimentation data were not obtained for the unheated DNA sample dissolved in water since Shooter and Butler[20] have shown such samples to be unstable in solution.

Chromatography of Heated DNA Samples.—The chromatograms (Fig. 1) of the heated samples are clearly different from that of the untreated DNA since the bulk of the DNA heated in water was recovered in Region I, whereas the heating in 0.01 *M* neutral phosphate solution gave rise to a product which on chromatography showed hardly any of the first large peak characteristic of the unheated DNA. Since the amount of DNA is directly proportional to the black areas shown in this plot (Fig. 1), the heating of the DNA in the 0.01 *M* salt solution resulted in almost a quantitative loss of the first peak and in a considerable loss of the DNA from elution Region III (*i.e.*, in the 0.1 to 1.0 *M* NH_3/2 *M* NaCl range). These fractions were obtained in Region II, presumably together with that material ordinarily eluted in this region.

Since the chromatogram of the unheated DNA (Fig. 1, upper profile) shows the largest amount of DNA to be in Region III, it can be expected that this region contains much of the larger molecular weight DNA which is almost quantitatively lost upon heating in water, and which is recovered in the first peak (Region I). Since DNA heated in water contains polynucleotide material of rather small size, it can be inferred that fractions eluted in Region I are also small. The position of a particular DNA peak in a chromatographic region can be used[28,29] as an indicator of molecular size.

This interpretation of the chromatographic profile of DNA heated in water is consistent with the physico-chemical changes which are known to occur when such a treatment is used (see above) and with the changes in the distribution of sedimenta-

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(26a) NOTE ADDED IN PROOF.—The liberation of purines following the heating of calf thymus DNA solutions also has been observed by V. N. Schumaker, *J. Franklin Inst.*, **266**, 233 (1958).

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(29) M. Rosoff, G. di Mayorca and A. Bendich, *Nature*, **180**, 1335 (1957).

tion coefficients (Table II). If fractions of DNA obtained in Region I in an ECTEOLA chromatogram are smaller than the average, whereas those in Region III are much larger (some evidence for these suppositions has been presented (see ref. 28, 18)), it then could be said, as one possibility, that the heating in salt solution had apparently resulted in an aggregation of most of the smaller DNA particles and a degradation of some of the larger polynucleotides present in the original DNA. Another possibility is that the shift in the position of fractions in the chromatogram, upon heating in salt, is due to denaturation.

The chromatographic profiles of the heated DNA samples are consistent with the sedimentation data. A less drastic alteration accompanies the heating of DNA in salt than in water solution. This supports the conclusion of Sadron[8] (see also ref. 17, 22, 23, 30, 31) that salt exerts a limited protective effect against the thermal breakdown of DNA. The presence of salt also has been reported to protect against the loss of biological activity which otherwise occurs when transforming DNA is stored in solutions of very low ionic strength either at ordinary or ice-box temperatures.[32,33]

Properties of Column Fractions.—In the case of the DNA sample heated in salt, the first two fractions collected in Region II exhibited a hyperchromic shift of 1.2 and 11.3%, respectively, upon alkalinization. This indicates a dissimilarity in the extent of denaturation brought about by heating. Some DNA molecules seem to be more resistant to thermal denaturation than others.

In Fig. 2 and 3 are shown the distributions of sedimentation coefficients of selected fractions obtained from the heated specimen. These curves should be compared with those of Fig. 2 of ref. 28. A relationship between the position of the fraction on the chromatogram and molecular size is seen, with later fractions having the higher average sedimentation coefficients (see also ref. 29). It is to be expected that fractions from Region III of the DNA heated in salt will have higher average sedimentation coefficients than the unfractionated heated specimen (see Fig. 2).

A comparison between these sedimentation distribution curves and those obtained for DNA fractions isolated from unheated DNA (see ref. 28) reveals that although there is a gradation of average sedimentation coefficients in all cases, Regions I and II of the chromatograms of heated specimen yield fractions of DNA with average sedimentation coefficients which are lower than the chromatographic fractions obtained from unheated DNA with the corresponding eluting fluid. The appearance of fractions of DNA with different sedimentation coefficients in the same chromatographic regions may be due to the different structures of denatured and undenatured DNA. Let us suppose that the position of the molecule of DNA (or a degradation product) on a chromatogram is determined by the number of its phosphate groups bound to the ECTEOLA. In the case of undenatured DNA, the relatively rigid structure imposed by the double helix will make only a certain number of phosphate groups available for binding with ECTEOLA. Short molecules will have fewer such groups along their length, while longer molecules will have more of them. The longer molecules would be bound to the surface of the exchanger with greater tenacity and would therefore require a higher ionic strength or *p*H for elution.

In the case of denatured or partially denatured DNA, the structure has lost all or part of its rigidity, and it is probable that for a given length more phosphate groups will be accessible for binding with ECTEOLA than for the corresponding length of undenatured DNA. For completely denatured or single-stranded DNA, the salt concentration required for dissociation from combination with the ECTEOLA might be identical with the one required to remove an undenatured molecule of twice its molecular weight (but of the same length).

Evidence in support of this hypothesis has been obtained from an analysis of the chromatographic profile of the single-stranded DNA of phage ϕX-174[34] (unpublished results with R. L. Sinsheimer).

The results obtained in this study suggest that the thermal denaturation of DNA yields a product which consists of molecules of different sizes and of different extents of denaturation. An electron microscopic examination also has revealed this to be the situation.[35] Recently, Marmur and Doty[36] found a relationship to exist between the guanine–cytosine content and the temperature of DNA denaturation. They found, with several specimens of DNA of differing base composition, that the higher the guanine–cytosine content, the higher the temperature of denaturation and hence the greater the resistance to heat denaturation. Since the DNA of calf thymus has been found to be a mixture of polynucleotides of varying base composition,[14] the finding of Marmur and Doty might serve as a basis to explain the heterogeneity of behavior of the DNA fractions toward heat denaturation reported here, and this is under investigation. These findings might have their applicability in the interpretation of the results obtained with inactivated transforming DNA. Even though a preparation of transforming DNA which has been inactivated, for example by heating, no longer exhibits the ability to transform other cells, it is quite possible that not all of the molecules have been inactivated. As is the case with calf thymus DNA, some molecules of transforming DNA may be more resistant to denaturation than others but may not be able to express their transforming activity due to inhibition by the inactivated DNA present. A fractionation of such an "inactive" DNA might reveal the existence of such mole-

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cules, since the properties of active molecules are obscured when present in a mixture.[37]

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NEW YORK 21, N. Y.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOPHYSICS, UNIVERSITY OF PITTSBURGH]

Specific Reactions of Hydrogen Peroxide with the Active Site of Hemocyanin. The Formation of "Methemocyanin"[1]

BY GARY FELSENFELD AND MORTON P. PRINTZ

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The effect of hydrogen peroxide on the hemocyanin of *Limulus polyphemus* and *Busycon canaliculatum* has been studied. In the case of *Limulus* it is found that the deoxygenated hemocyanin is much more sensitive to attack than is the oxygenated hemocyanin, that one equivalent of peroxide per mole of copper is sufficient to destroy most of the oxygen-carrying capacity of the deoxygenated hemocyanin and that peroxide acts by oxidizing the cuprous ion of deoxygenated hemocyanin to cupric ion. It has so far not been possible to regenerate hemocyanin from the attacked material by the use of reducing agents. *Busycon* hemocyanin behaves similarly except that it is possible to regenerate hemocyanin by the use of reducing agents, notably by use of an excess of peroxide. In both species the product of attack has a sufficiently strong affinity for the oxidized copper ions to prevent their removal by an IR-120 cationic exchange resin. It is proposed that the properties of the product of peroxide oxidation on hemocyanin for the first time justify the use of the name "methemocyanin." The remarkable effectiveness of peroxide in the oxidation of hemocyanin and in the reduction of *Busycon* methemocyanin, and the correlation between cuprous ion oxidation and active site destruction, provide important information about the active site structure of hemocyanin.

Introduction

In 1933, Conant and his collaborators[2] reported that they had been able to prepare a material which they termed "methemocyanin" by the action of potassium permanganate or potassium molybdicyanide upon the hemocyanin of the horseshoe crab. The fact that this "methemocyanin" had the same oxygen-carrying properties as native hemocyanin and that there was no other evidence of oxidation of copper led Rawlinson[3] to conclude that Conant had not succeeded in attacking the active site of the molecule and that his product was not methemocyanin, but merely hemocyanin in which certain groups unrelated to physiological activity had been oxidized.

Our more recent knowledge of the nature of the active site in the hemocyanins permits us to understand why oxidizing agents strong enough to oxidize cuprous ion to cupric ion might not be able to attack the active site copper of hemocyanin, even though in deoxygenated hemocyanin this copper is entirely in the cuprous state.[4] The cuprous ions of hemocyanin, which are probably bound to the sulfur atoms of cysteine side chains,[4] have an equilibrium constant of about 10^{18} for associat on with the protein.[5] This equilibrium constant (which is nearly the same as that of the cuprous–cysteine complex)[6] is much larger than any of the equilibrium constants for association of cupric ion with the usual amino acid side chains of proteins; any difference of this kind would alter the oxidation potential of hemocyanin-bound cuprous ion in such a way as to reduce its susceptibility to oxidation.[7] It is also possible that the low dielectric constant of the protein may result in decreased oxidation rates if charged reactants or products are involved despite an equilibrium position favorable to oxidation.[9] Finally, steric factors may decrease the rate of oxidation sufficiently so that reagents which are capable of oxidizing free cuprous ion to cupric ion do not affect the active site.

In an attempt to find an effective oxidant, we have studied the action of hydrogen peroxide upon the cuprous ion of hemocyanin. We will show that the hemocyanin obtained from two different species, the arthropod *Limulus polyphemus* and the mollusc *Busycon canaliculatum*, is attacked by hydrogen peroxide. The attack is specifically upon the active site; it involves stoichiometric amounts of peroxide, and it results in specific oxidation of the cuprous ion with attendant loss of oxygen-carrying properties. In the case of *Busycon*, the oxidized material can be reduced with a suitable reducing agent, with a full restoration of ability to carry oxygen. We will propose that these properties justify the designation of the product of peroxide attack on hemocyanin as "methemocyanin."

(1) Contribution No. 63 from the Department of Biophysics. This research was supported in part by a grant (G5728) from the National Science Foundation. Presented in part at the 136th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept. 13–18, 1959.

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(6) W. Stricks and I. M. Kolthoff, THIS JOURNAL, **73**, 1723 (1951).

(7) This is contrary to the situation in hemoglobin and in the more highly coördinated complexes of copper, in which the higher oxidation state of the metal is usually stabilized (Ref. 8). In the case of copper ion which has only one coördination site occupied by a ligand other than water, the lower oxidation state is stabilized, since the first association constant of a copper complex with a given ligand is generally greater for cuprous ion than for cupric ion. (See, for example, F. R. N. Gurd and P. E. Wilcox, in "Advances in Protein Chemistry," Vol. XI, ed. M. L. Anson, K. Bailey and J. T. Edsall, Academic Press, Inc., New York, N. Y., 1956, p. 351.)

(8) A. E. Martell and M. Calvin, "Chemistry of the Metal Chelate Compounds," Prentice–Hall, Inc., New York, N. Y., 1952, pp. 57, 373.

(9) J. H. Wang, THIS JOURNAL, **80**, 3168 (1958).