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The Secondary Structures of Nucleic Acids in Organic Solvents[1]

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Calf thymus DNA and pea microsomal RNA lose all of their formal secondary structure when dissolved in formamide or dimethylsulfoxide. The following observations on the nucleic acid solutions are used as evidence for this conclusion: low specific rotation; independence of rotation on temperature; similarity of the optical and hydrodynamic properties of nucleic acids once dissolved in organic solvents at room temperature to those of nucleic acids denatured by heat; and finally, the opposite signs of simple rotatory dispersion curves of nucleic acids, but not of the nucleotides, in aqueous and organic systems.

The secondary molecular structure of proteins and nucleic acids has been shown to be dependent on factors such as ionic strength, *p*H, nature of solvent and temperature.[2-5] These macromolecules may exist in a helical conformation which has a high degree of intramolecular hydrogen bonding to maintain a formal secondary structure or in a random-coil conformation which has little ordered arrangement. We have found that calf thymus deoxyribonucleic acid (DNA) and pea microsomal ribonucleic acid (RNA), when dissolved in formamide or dimethylsulfoxide, exhibit properties which have been interpreted in terms of loss of intra- and inter-molecular hydrogen bonding with consequent disruption of formal secondary structure.

Several lines of evidence have been developed which indicate that DNA and RNA exist in random-coil conformation when dissolved in the organic solvents just mentioned. In contrast to the behavior of aqueous solutions, these organic solutions of nucleic acids have a very low, or even negative, optical rotation which is not sensitive to temperature. Furthermore, some of the high, positive rotatory power associated with a helical conformation can be restored to a formamide solution by the addition of a salt such as potassium chloride. After having had a history of solution in either solvent, the nucleic acids in buffer solution show an ultracentrifuge pattern, or a temperature *vs.* optical density or rotation curve, characteristic of that of a nucleic acid solution which had been denatured by heat at 100°. Such a denaturation process was observed in the sharp and instantaneous transition of optical rotation during titration of an aqueous solution of DNA with organic solvents. Additional support for the interpretation of solvent effects was gained through the observation that the optical rotatory dispersion of either nucleic acid in organic solution differed widely from that in an aqueous system, whereas such an effect was small for the monomeric units of nucleotides and nucleosides.

In the case of DNA, a change in conformation from helix to random-coil, induced for example by raising the temperature of its aqueous solution, can be followed by measurements of the change in optical density (hyperchromic effect) or specific optical rotation. Two characteristics of this system, which may be used as criteria for denaturation, were the presence of a sharp transition temperature at about 85° for the native material and the absence of this phenomenon for denatured material[5] (Fig. 1). After a history of heat treatment above the "melting temperature" of the helix, the DNA showed a reversible temperature dependence of optical rotation or optical density.

The temperature dependence of optical rotation of DNA in organic solvents was compared with that in aqueous buffer at *p*H 5.5 (Fig. 1). Both the low or negative specific rotation and the absence of major temperature effects suggested that the DNA had lost much or all of the secondary structure which provided the high, positive contribution to specific rotation. On the other hand, the specific rotations of the nucleotides (Table I) were only 20–30% lower (or more negative) in the organic solvents as compared with those of the aqueous systems.

TABLE I

SPECIFIC ROTATIONS OF NUCLEOTIDES IN VARIOUS SOLVENTS AT 436 mμ AND 25°

Nucleotide	Solvent	$[\alpha]_{436}^{25}$
Adenylic acid (2′ and 3′)	Buffer, *p*H 5.5	−100°
	Formamide	−120°
	Dimethylsulfoxide	−122°
Cytidylic acid (2′ and 3′)	Buffer, *p*H 5.5	+ 48°
	Formamide	+ 30°
	Dimethylsulfoxide	+ 28°

To further verify the denaturation of DNA having a history of solution in formamide or dimethylsulfoxide at room temperature, the nucleic acid was precipitated from the organic solvents and redissolved in buffer solution. As in the case of heat-denatured DNA, which gave cyclic reproducibility of temperature *vs.* specific rotation or optical density curves, this recovered DNA showed no sharp transition zone on heating in buffer, and indeed, its behavior was quite comparable to that of once-heated DNA.

In all of the organic solutions just described there were no inorganic salts present. The aqueous solution, however, had an ionic strength of about 0.2. Since it is known that the helix to random-coil transition occurs more readily as the ionic strength is lowered,[6,7] it was of interest to investigate the

(1) This work was supported in part by grants RG-3977, RG-5143 and A-3102 from the National Institutes of Health, United States Public Health Service.

(2) P. Doty, H. Boedtker, J. R. Fresco, B. D. Hall and R. Haselkorn, *Ann. N. Y. Acad. Sci.*, **81**, 693 (1959).

(3) P. Doty, H. Boedtker, J. R. Fresco, R. Haselkorn and M. Litt, *Proc. Natl. Acad. Sci. U. S.*, **45**, 482 (1959).

(4) J. R. Fresco, *Trans. N. Y. Acad. Sci.*, **21**, 653 (1959).

(5) J. R. Fresco, *Federation Proc.*, **18**, 904 (1959).

(6) H. S. Rosenkranz and A. Bendich, THIS JOURNAL, **81**, 6255 (1959).

(7) J. R. Fresco and E. Klemperer, *Ann. N. Y. Acad. Sci.*, **81**, 730 (1959).

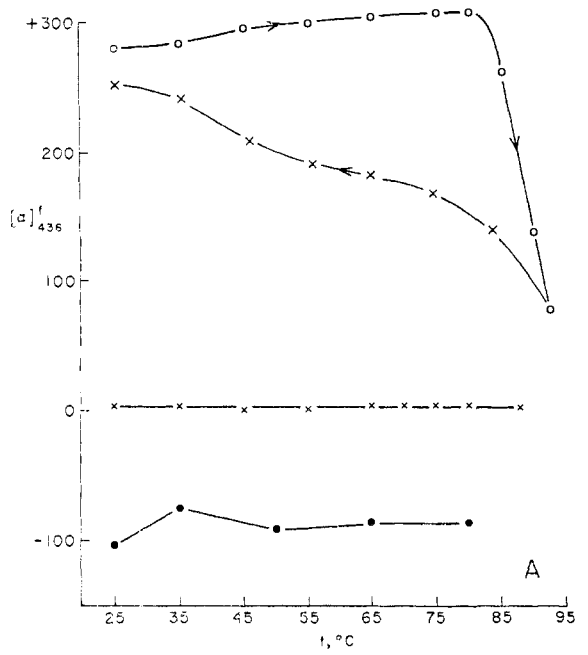

Fig. 1.—Temperature dependence of specific rotation of DNA in: —O—O—, buffer; —X—X—, formamide; —●—●—, dimethylsulfoxide.

effect of salts in the organic system. Formamide, with a very high dielectric constant and the ability to cause ionization of dissolved salts, was chosen as the solvent. When DNA was dissolved in formamide containing 0.1 *M* potassium chloride, the specific optical rotation was dependent on temperature below 45°, and at 10° a moderately high, positive rotation was observed (Fig. 2). The behavior was interpreted in terms of a denatured material which reached a maximum random-coil conformation at 45°.

In contrast to the behavior of DNA, the specific rotation *vs.* temperature curve (or a superimposable optical density *vs.* temperature curve) of pea microsomal RNA (Fig. 3) indicated no narrow zone of transition temperature. Furthermore, the system exhibited essentially reversible characteristics with only slight changes in magnitude and general shape of the melting curves after one heating cycle. Although the RNA at 25° had a higher degree of intramolecular hydrogen bonding and helical conformation than at 90°, the heated and unheated materials could not be distinguished from one another by the dependence of their optical rotation or optical density on temperature; another measurement was required to identify the material with a history of heat treatment which had caused the transformation of helix to random-coil.

Upon examination in the analytical ultracentrifuge, the pea microsomal RNA was found to consist of two components with sedimentation coefficients of 27–28 *S* and 17–18 *S*[8] (Fig. 4). After heat treatment at 85–95° for about 15 minutes, it consisted only of a single component with a very broad boundary of about 8–11 *S* (Fig. 4). Therefore, by analysis of ultracentrifuge, it was possible to detect RNA which had been once heated.

(8) P. O. P. Ts'o and R. Squires, *Federation Proc.*, **18**, 341 (1959).

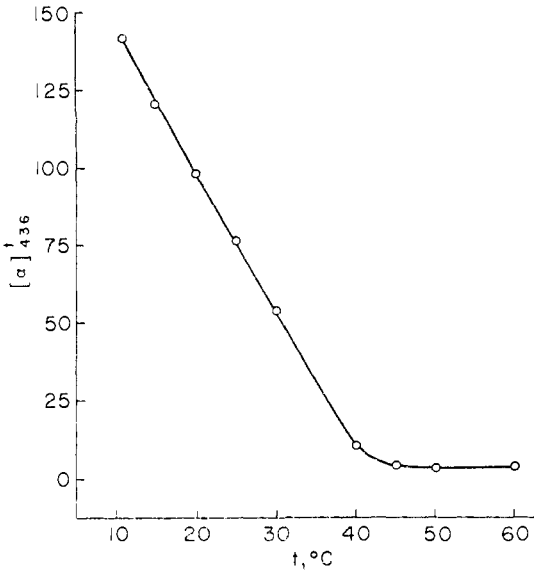

Fig. 2.—Temperature dependence of specific rotation of calf thymus DNA in formamide containing 0.1 *M* potassium chloride.

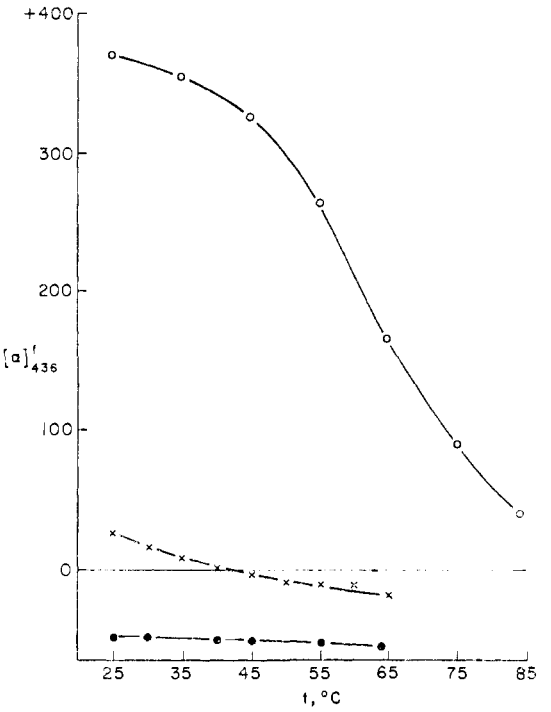

Fig. 3.—Pea microsomal RNA. Specific rotation *vs.* temperature for solutions in: —O—O—, buffer, *p*H 5.5; —X—X—, formamide; —●—●—, dimethylsulfoxide.

The variation of specific rotation with changes in temperature for RNA in formamide and dimethylsulfoxide is shown in Fig. 3. Again, these values were quite low, and a slight temperature dependence of rotation was shown only by the formamide solution. Also, as in the case of heat-denatured RNA, the material recovered from organic solution and redissolved in buffer could not be differentiated from native RNA by deviations in the optical rotation. The ultracentrifuge pattern (Fig. 4), however, was similar to that of the heated RNA and markedly

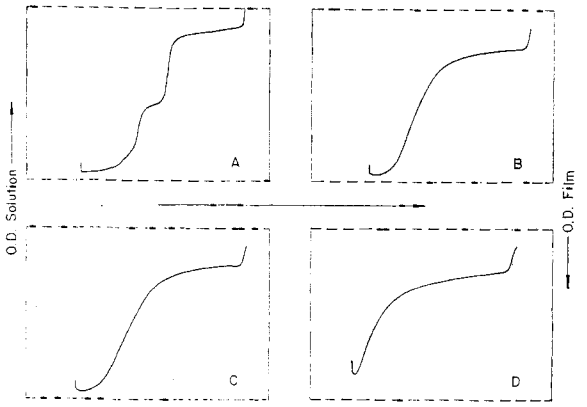

Fig. 4.—Ultracentrifuge pattern for pea microsomal RNA. (A) Native; (B) heat denatured; (C) formamide denatured; (D) dimethylsulfoxide denatured.

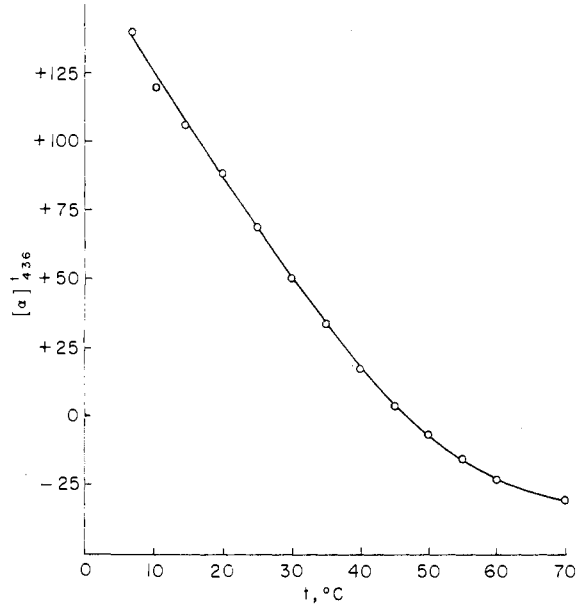

Fig. 5.—Temperature dependence of specific rotation of pea microsomal RNA in formamide containing 0.1 *M* potassium chloride.

different from that of the native, unheated material. Pea microsomal RNA, then, like calf thymus DNA, lost most of its ordered secondary structure when dissolved in formamide or dimethylsulfoxide at room temperature, and an irreversible structural transformation occurred.

When RNA was dissolved in formamide containing 0.1 *M* potassium chloride, it exhibited a reasonably high specific rotation which was temperature dependent (Fig. 5), but there was neither the slight transition zone shown by aqueous solutions around 65° nor the sharp minimum shown by DNA in salt-containing formamide. Again it appeared that some of the charge-repulsion effect had been diminished by the presence of salt and a small amount of ordered secondary structure had reformed.

Titration of buffer solutions of nucleic acids with formamide or dimethylsulfoxide at a given temperature was followed by means of specific rotation measurements. DNA gave a sharp transition zone at 58 volume per cent (28 mole per cent) dimethyl-

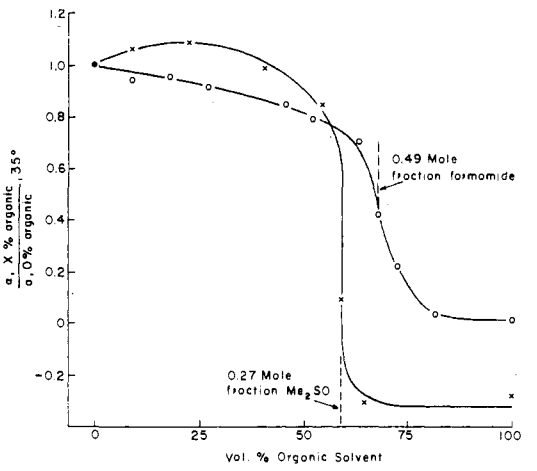

Fig. 6.—Titration of buffer solution of DNA with: -O-O-, formamide; -X-X-, dimethylsulfoxide.

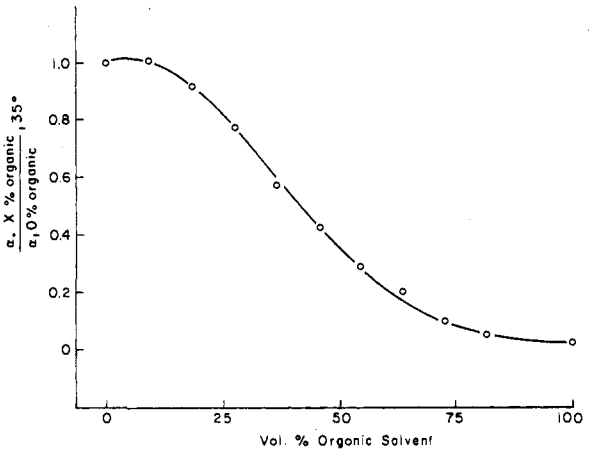

Fig. 7.—Titration of buffer solution of RNA with formamide.

sulfoxide or at 70 volume per cent. (51 mole per cent.) formamide (Fig. 6). RNA, on the other hand, gave a curve (Fig. 7) which, with appropriate coördinate scale, could be superimposed on that of the typical rotation *vs.* temperature curve.

In addition to demonstrating the relative effectiveness of the two solvents in a denaturation process, these titrations suggested that the optical phenomena previously described were not due to chemical reaction between solvent and solute, for it seemed unlikely that reaction rates would show the discontinuous and practically instantaneous effects observed. Further evidence for the absence of chemical modification was gained from spectra, for DNA with a history of organic solution had an ultraviolet spectrum with extinction coefficient and maximum/minimum ratio identical with those of heat-denatured material.

The optical rotatory dispersion measurements of the nucleic acids and the monomeric nucleotides in various solvents also support the notion that the nucleic acids in organic solution exist in a conformation of random-coil. The native, unheated DNA in buffer solution gave a strongly positive dispersion curve (Fig. 8) which fit a single term Drude equation fairly closely. When the same

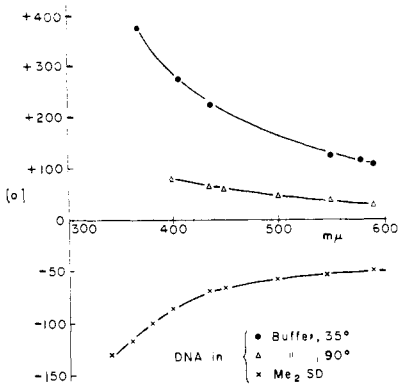

Fig. 8.—Rotatory dispersion of calf thymus DNA in various solvents.

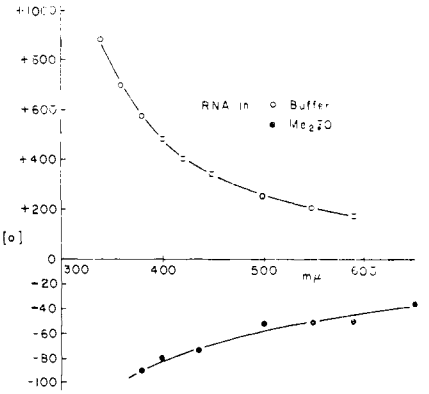

Fig. 9.—Rotatory dispersion of pea microsomal RNA in various solvents.

material was denatured by heating 20 minutes at 100°, dispersion measurements at 90° again showed increasing specific rotation at decreasing wave lengths but with much lower values of $[\alpha]$. In both situations, the calculated values for λ_0 were 237 ± 5 mμ, with indications of a positive Cotton effect. However, when the DNA was dissolved in dimethylsulfoxide the rotations were strongly negative (Fig. 8) and suggested a negative Cotton effect. The data did not fit a one term Drude equation with any precision. These same phenomena were observed for RNA (Fig. 9). The observed optical rotations of the nucleic acids in formamide solution were very low and the experimental values lacked enough precision to warrant further analyses. On the other hand, solvent changes alone had no major effect on specific rotations or rotatory dispersions of individual nucleotides. This is illustrated in the studies with adenylic acid and cytidylic acid (Fig. 10). Thus, the organic solvents had a profound effect on the rotatory dispersion measurement of nucleic acids and only a slight effect on that of nucleotides.

In a comparison of the solvent effect of formamide and dimethylsulfoxide some interesting anomalies appeared. The dispersion curves for mononucleotides in each of these solvents were virtually identical (Fig. 10), yet the nucleic acids showed a much larger negative rotation in the dimethylsulfoxide. This implied that a specific solvent effect was operative in the polymers but not in the monomers. Furthermore, in a formamide solution of DNA containing sodium chloride, an abrupt minimum in rotation was reached at about 45° which coincided with the value at any temperature in the absence of salt. Consequently, whatever changes had occurred during solvent denaturation in formamide had been carried to completion. These solvent effects may also have played an important role in the preparation of solutions of the nucleic acids. Native DNA was difficult to dissolve in dimethylsulfoxide, but solutions could be prepared by heating at 60° for two days or by initially starting with 10% water and 90% dimethylsulfoxide (see Experimental). However, heat-denatured DNA could be dissolved relatively easily in the anhydrous solvent. No difficulties were encountered in preparing solutions of RNA in dimethylsulfoxide or in preparing solutions of either nucleic acid in formamide.

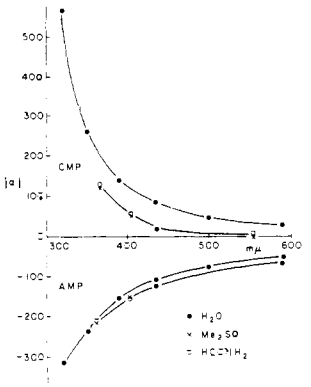

Fig. 10.—Rotatory dispersion of adenylic acid and cytidylic acid in various solvents: —●—●—, aqueous (*p*H 5.5); -O-O-, formamide; -×-×-, dimethylsulfoxide.

Although the basic difference in the mechanism of solvation and denaturation between formamide and dimethylsulfoxide is not immediately apparent, it is possible that formamide, like water, was effective because of its ability to act either as an electron acceptor or electron donor in hydrogen bond formation. In contrast, dimethylsulfoxide should have been an electron donor but not an acceptor in a bonding sense, and initiation of solvation at the highly anionic molecular surface of the nucleic acids would have been difficult.

Experimental

Materials and Reagents.—All of the DNA used for the described experiments was calf thymus DNA purchased from Nutritional Biochemical Corporation, and some of its properties have previously been described.[9] Aqueous solutions of about 0.1% were prepared by mixing solvent and DNA in a slowly rotating flask for two days at room temperature. Formamide solutions were prepared similarly, but the rate of solution was considerably higher. Dimethylsulfoxide failed to show any significant effect on DNA after several days, even when the temperature was raised to about 40°; solvation was effected by the addition of 10% by volume of water and rotating the mixture for 12 hr. Most of this water was removed by partial distillation at reduced pressure to about one-third original volume. DNA could be precipitated from formamide solution by the addition of two volumes of 100% ethanol and one-half volume of absolute ethyl ether at 0°; or the formamide could be replaced directly by buffer solution by three dialyses over a period of five days in 20-fold amounts of buffer at 0°. The

(9) W. F. Dore, F. A. Wallace and N. Davison, *Biochem. Biophys. Res. Com.*, 1, 312 (1959).

addition of four volumes of benzene precipitated DNA from dimethylsulfoxide. Measured physical constants in buffer solution were $[\alpha]^{25}D + 135°$ and $[\alpha]^{25}_{436} + 270°$.

Pea microsomal RNA was prepared according to the method described previously.[8] Immediately after preparation it was quick-frozen in buffer solution and stored at $-50°$ until needed. Solutions in the two organic solvents could be prepared directly from the precipitated RNA (isolated from buffer solution by the addition of three volumes of cold ethanol and washed with absolute ethanol). Isolation from formamide solution was carried out by precipitation with two volumes of buffer and six volumes of ethanol at ice temperature. Last traces of solvent could be removed by repeated washing with absolute ethanol or ethyl ether or by redissolving the precipitate in buffer and separating it a second time by alcohol dilution. The addition of two parts of benzene and two parts of ethanol (ethyl ether may be substituted for benzene) brought about precipitation of RNA from dimethylsulfoxide. Measured physical constants were $[\alpha]^{25}D + 170°$ and $[\alpha]^{25}_{436} + 370°$.

The organic solvents were 99% formamide (Eastman White Label) and practical grade dimethylsulfoxide (Eastman) with a melting point of about 18°. The aqueous solvent was an 0.1 *M* acetate buffer at *p*H 5.5 containing 0.1 *M* sodium chloride and 0.001 *M* magnesium chloride.

Instrumentation and Methods of Analysis.—Measurements of optical rotations were made on a Rudolph Model 200S polarimeter with oscillating polarizer and xenon and mercury arc lamps. The polarimeter tubes were of unitized glass construction with water jacket, center-fill device and quartz windows. The windows were sealed to the optically ground tube ends with an epoxy resin, Epocast 502. Although some problem of strain birefringence may have been inherent in the system, it was possible to reproduce zero readings at various temperatures with pure solvents in the polarimeter tubes. In most of the measurements it was possible to duplicate results on the instrument to better than ± 2%.

Optical density measurements were made with a Beckman DK-2 ultraviolet spectrophotometer fitted with a modified temperature control device. Quartz cells were fitted with 20 mm. immersion, standard taper thermometers for direct reading of solution temperatures. The cell compartment cover was adapted to pass the calibrated portion of the thermometer.

Analysis for the concentrations of nucleic acids was made indirectly by the colorimetric phosphate determination described by Allen,[10] employing a Beckman DU spectrophotometer. Direct perchloric acid digestions were carried out only on the aqueous and formamide solutions. The thymus DNA was calculated to contain 9.30% phosphorus[11] and the microsomal RNA 9.09%, determined from the base-ratio analysis in our Laboratory.

Analytical ultracentrifugation was performed in the Model E ultracentrifuge, Spinco Division, Beckman, Inc., with phase-plate schlieren optics and within ultraviolet absorption optics. The instrument was provided with a temperature control system. The absorption patterns were taken on commercial film with a 20 second exposure time on solutions of O.D.^{260} of 1.0 in 12 mm. Kel-F cells. The photometric records were within the linear range of the characteristic curve of the film as shown by the trace of exponential aperture[12] in the counter balance cell in each run. The films were traced with a Double-Beam Recording Microdensitometer, Joyce Lobel Co., Newcastle upon Tyne, England. The direction of sedimentation of all the patterns is from left to right.

(10) R. J. L. Allen, *Biochem. J.*, **34**, 858 (1940).

(11) E. Chargaff in "Nucleic Acids," Vol. II, Chargaff and Davidson, Academic Press, Inc., New York, N. Y., 1955, p. 335.

(12) E. Robkin, M. Meselson and J. Vinograd, THIS JOURNAL, **81**, 1305 (1959).

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY MEDICAL COLLEGE, NEW YORK, N. Y.]

Synthesis of a Lysine-vasopressin Derivative with a Methyl Substituent on the Imino Nitrogen of the Peptide Bond between Lysine and Glycinamide (9-Sarcosine Lysine-vasopressin)[1]

BY JOHANNES MEIENHOFER AND VINCENT DU VIGNEAUD

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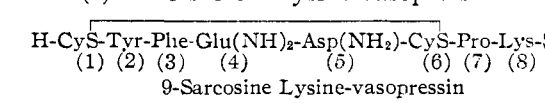
An analog of lysine-vasopressin has been synthesized in which the glycinamide residue in the side-chain is replaced by a sarcosinamide residue. The protected nonapeptide intermediate, which was reduced with sodium in liquid ammonia and then aerated to obtain the 9-sarcosine lysine-vasopressin, was itself prepared by the coupling of the appropriately protected pentapeptide and tetrapeptide derivatives with the use of dicyclohexylcarbodiimide. The nitrophenyl ester method was employed for the synthesis of the protected tetrapeptide used in the preparation of the nonapeptide intermediate. The 9-sarcosine lysine-vasopressin possessed 0.4–0.5 unit of pressor activity per mg. in contrast to the approximately 290 units per mg. of pressor activity exhibited by lysine-vasopressin. Moreover, the 9-sarcosine analog showed less than 0.01 unit per mg. of avian depressor activity and no detectable rat uterine-contracting activity. Thus it would appear that these pharmacological effects of lysine-vasopressin are either markedly decreased or eliminated entirely by the substitution of a methyl group for the hydrogen of the imino group of the terminal peptide bond in the side-chain of this hormone.

In the course of studies on the relation between structure and the biological activities of oxytocin and the vasopressins, an analog of lysine-vasopressin has been synthesized in which the glycinamide residue in position 9 is replaced by a sarcosinamide residue. The 9-sarcosine lysine-vasopressin (I) differs from lysine-vasopressin in the replacement of the hydrogen on the nitrogen of the terminal peptide bond of the side-chain by a methyl group.



9-Sarcosine Lysine-vasopressin

I

The synthesis of the protected nonapeptide intermediate for 9-sarcosine lysine-vasopressin, namely S-benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-N$^{\epsilon}$-tosyl-L-lysylsarcosinamide, was carried out by coupling S-benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine[2] with S-benzyl-L-cysteinyl-L-prolyl-N$^{\epsilon}$-tosyl-L-lysylsarcosinamide by the use of dicyclohexylcarbodiimide[3] in 90% tetrahydrofuran–water.

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(2) V. du Vigneaud, M. F. Bartlett and A. Jöhl, THIS JOURNAL, **79**, 5572 (1957).

(3) J. C. Sheehan and G. P. Hess, *ibid.*, **77**, 1067 (1955).