BIREFRINGENCE OF SPERMATOZOA

I. Birefringence Melting of Squid, Bull, and Human Sperm Nucleoprotein

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ABSTRACT

In experiments designed to determine the thermal stability and bonding strength of a natural nucleoprotein structure, the loss of birefringence as a function of time and temperature was investigated for both mammalian and nonmammalian sperm nuclei. At a constant temperature, this reaction was found to be first order for both types over a range of temperatures. The methods of chemical kinetics applied to results of these reactions, called birefringence melting reactions, produced values for the enthalpy and entropy of activation in the reactions, which gave some indication of the strength of binding in the nucleoprotein structure; and these results, plus those on the influence of chemicals on the structure, were consistent with the molecular structures which have been proposed by others for the nucleoprotein complex of sperm nuclei. For both bull and human sperm in ethylene glycol, the rate-limiting step in the melting reactions appeared to be the breakage of disulfide bonds. For squid sperm in ethylene glycol, and bull or squid sperm in ethylene glycol plus β-mercaptoethanol, the identity of this step was more ambiguous, but a possibility consistent with these and other results would be a cooperative breakage of ionic bonds.

INTRODUCTION

The sperm cells of many different species are birefringent, or optically anisotropic, as first reported by Valentin (1861) and Engelmann (1875). The studies of Schmidt (1928, 1937) and Pattri (1932) on the negative birefringence of sperm heads showed that it was caused by the orientation of the chromatin in the nucleus, and specifically, by the alignment of the long axes of the DNA molecules primarily parallel to the long axis of the sperm head. More recent studies have shown that quantitative variations in birefringence, and in the loss of birefringence caused by polarized ultraviolet light, can be used to develop very detailed models of the orientation of the DNA in the sperm head (Inoué and Sato, 1962, 1966; Sato and Muller, 1966); the interpretation of such measurements has been challenged by others using different experimental methods (MacInnes and Uretz, 1968; Zirwer et al., 1970), but they still illustrate the potential value of quantitative polarization microscopy as a method for studying the structure of the sperm nucleus.

The chromatin of the sperm nucleus of most species consists almost entirely of a complex of negatively charged DNA and positively charged proteins, which are related to the histones of somatic cells but are usually simpler in composition and smaller in molecular weight (Busch,

1965). In cephalopod molluscs, such as squid (studied in this paper), and in fish, these basic sperm proteins are called protamines (Miescher, 1897; Kossel, 1884, 1921); they usually contain large amounts of arginine—as much as 70%–80% of the amino acid residues in some fish sperm (Felix et al., 1956) and almost this much in squid sperm (Bloch, 1962). In mammalian sperm cells the basic proteins have been more resistant to extraction and analysis, but recent work on bull sperm (Coelingh et al., 1969) shows that the major protein fraction of the nucleus contains a large amount of arginine, as do fish protamines, and differs from them primarily in its large content of half cystine. The components of mammalian sperm nuclei, therefore, may be as simple, and as small in number, as those of cephalopod and fish sperm.

The structure of complexes between DNA and basic proteins is a subject of considerable interest today, because of the increasing evidence during the past decade that the histones of somatic nuclei are capable of repressing DNA-dependent synthesis of RNA, and are, therefore, important components of the system of genetic control in eucaryotic cells (this subject has been reviewed by Stellwagen and Cole, 1969; Bonner et al., 1968; Hnilica, 1967; de Reuck and Knight, 1966; Busch, 1965; and Bonner and Ts'o, 1964). These results have prompted interest in a number of physical studies on DNA-protein complexes to determine the structural basis for the biological effects of basic proteins. Such studies have used two basic approaches: (a) to "reconstitute," or assemble, the nucleoprotein complex from a few well-defined components (Leng and Felsenfeld, 1966; Olins et al., 1967, 1968; Inouę and Ando, 1966, 1968), which raises the question of the biological relevance of the assembled complexes (Stellwagen and Cole, 1969); or (b) to attempt physical studies on undissociated or partially dissociated whole chromatin (Shih and Bonner, 1970; Ansevin and Brown, 1971; Li and Bonner, 1971; Spelsberg et al., 1971), which presents a formidable problem of interpretation because of the large and variable number of chromosomal components. The components of the sperm nucleus, in contrast to this, are much simpler and smaller in number, as discussed above, which means that among all natural nucleoprotein complexes in eucaryotic nuclei, spermatozoa are probably the most amenable to physical studies.

Two studies on the thermal denaturation of

sperm nuclei have appeared previously (Chamberlain and Walker, 1965; Ringertz et al., 1970). These papers showed that the bonds in the sperm nucleus were both qualitatively different from, and quantitatively stronger than, the bonds in free DNA, but neither of these studies attempted to explain their results in terms of molecular structure or bond energies. It was hoped that the more quantitative methods of chemical kinetics used in this study would yield more information of this kind.

The studies of Inners and Bendet (1969) on the thermal denaturation of the DNA of T2 bacteriophage showed the possibility of using the loss of birefringence of a biological structure as a means of measuring its denaturation. Several difficulties in the interpretation of these results, caused by the necessity of measuring the average birefringence of a large number of imperfectly oriented virus particles, would not be true of sperm cells, which are large enough that the birefringence of individual particles can be measured. If the loss of birefringence as a function of temperature could be shown to be a general property of sperm cells, as Schmidt (1928) showed it was for *Sepia officinalis* (cuttlefish) sperm, this might prove to be as useful a physical property for following the progress of thermal denaturation as the measurement of UV absorption.

With this in mind, the kinetics of the loss of birefringence caused by heat was investigated for two major types of sperm cells. Mammalian sperm, characterized by their high content of disulfide bonds, are obviously of great interest because of the inclusion of humans in this class. Human sperm, however, are not very large and manifest very small retardations, so only a few experiments were performed on them. The majority of the experiments were on bull sperm, which are much larger and more birefringent. The squid (*Loligo pealii*) was selected as a nonmammalian species whose sperm cells are relatively large, highly birefringent, and fairly well-studied structurally. It was hoped that this would permit comparisons to be made between the two types, and conclusions to be drawn as to the nature of the relatively unknown structure of mammalian sperm.

MATERIALS AND METHODS

Sperm Samples and Chemicals

The squid sperm were obtained from spermatophores of *Loligo pealii* specimens collected by Marine

Biological Laboratory, Woods Hole, Mass., and frozen for transport to Pittsburgh. Bull sperm were furnished by Dr. Charles Kiddy, United States Department of Agriculture, Beltsville, Md. Human sperm were obtained from donors of normal health and fertility.

Samples were washed twice with 0.14 M NaCl, and washed once and resuspended in the solvent in which experiments were to be performed. All washings were removed by pelleting the cells at $5000\,g$ for 10 min (6500 rpm in the SS-34 rotor of the Sorvall RC-2 refrigerated centrifuge). In most cases the final solvent was ethylene glycol, in which the cells could be kept for several months at 4°C with no problem of bacterial contamination.

The use of ethylene glycol was suggested by preliminary attempts to melt bull sperm in distilled water: they showed no loss of birefringence, even when heated at 100°C for 30 min, by which time all the water had evaporated. The same cells, in distilled water, did lose birefringence when heated in an autoclave to 120°–125°C for 20 min, but these conditions could not be used for melting experiments requiring rapid removal and cooling of the cells at intermediate stages of the reaction. Other solvents with boiling points higher than 100°C were tried, such as glycerol and dimethyl sulfoxide, but it was decided to use ethylene glycol because it is a hydrophilic solvent, forms hydrogen bonds similar to those of water, has a fairly low viscosity and a boiling point of 198°C, and has been used previously as a solvent for melting experiments on DNA (Duggan, 1961; Eliasson et al., 1963; Luzzati et al., 1964; Nelson and Johnson, 1970; Green and Mahler, 1971).

Since equilibration of some solvents through the cell membrane occurred very slowly, taking 24 hr or more in some cases, the samples were allowed to equilibrate for at least 1 wk at 4°C after final resuspension in the intended solvent.

Diagrams of the sperm cells of the three species used for these experiments are shown in Fig. 1. This figure also shows the reference axes for each type of cell, which will be used to describe the various orientations of the cells, and the dimensions of the sperm heads along each axis.

All chemicals used were reagent grade, obtained from either Fisher Scientific Company, Pittsburgh, Pa., or J. T. Baker Chemical Co., Phillipsburg, N. J., and were used without further purification, unless otherwise stated.

Birefringence Melting Experiments

All melting experiments were performed in a Haake temperature bath, Model F, which was filled with ethylene glycol. 1 ml of a sperm sample was placed in a small test tube suspended in the bath, and a zero-time sample of about 0.05 ml was withdrawn and placed on a microscope slide which had
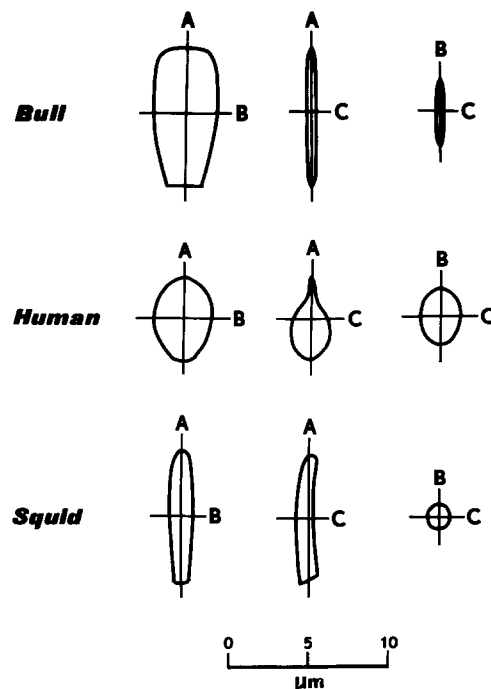

FIGURE 1 Diagrams of sperm heads of the species studied in this work, showing the reference axes used. "Flat" orientation, or top view, for each species, is on left; "on-edge" orientation (side view), in center; and "head-on" orientation (end view), on right. Dimensions, in micrometers, for bull sperm are $A = 8.5$, $B = 4.0$, $C = 0.3$–0.4; human, $A = 5.0$, $B = 3.5$, $C = 2.5$ (both from Altman and Dittmer, 1962); squid, $A = 7.8$, $B = C = 1.4$ (from Inoué and Sato, 1966).

been kept refrigerated. Subsequent samples of about 0.05 ml each were withdrawn at time intervals appropriate for the temperature of the experiment, and placed on cold microscope slides to stop the thermal reaction as quickly as possible. Since the loss of birefringence is irreversible, the retardations of all the samples were measured after the reaction was complete. Control experiments showed that the maximum error in temperature measurements was ±0.5°C, and that the times of heating were accurate within 5–10 sec.

The retardation, R, of an object is related to its thickness, d, and its birefringence, $(n_e - n_o)$, as follows:

$$R = d(n_e - n_o), \tag{1}$$

where the retardation and thickness are both measured in nanometers (nm). Very small retardations, such as those of the sperm cells in this study, are most easily measured by using a Brace-Köhler, or elliptic, compensator (Köhler, 1921; Hartshorne and Stuart, 1970). This is a very thin plate of a birefringent

material, usually mica, which is introduced between the two polarizing filters so that the light passes through it and the object whose retardation is to be measured. When the slow direction of this plate is oriented at an angle, θ, of 45° from the polarizer direction, it has a calibrated maximum retardation, R_0, of between $\lambda/30$ and $\lambda/10$. The retardation at any other orientation of the compensator is determined by measuring the angle θ through which it is rotated, and using the following equation:

$$\tan \delta = 2 \tan (\delta_0/2) (\sin 2\theta), \qquad (2)$$

where the phase angles (measured in degrees), $\delta = (360°/\lambda) R$, and $\delta_0 = (360°/\lambda) R_0$, are related to the values of the retardations R and R_0 as shown. For values of R and R_0 less than $\lambda/10$, which is true of all cases considered here, the following approximation can be substituted (Hartshorne and Stuart, 1970, p. 311):

$$R = R_0 \cdot \sin 2\theta \qquad (3)$$

Operationally, then, the retardation of the birefringent object of interest is measured by cancelling its retardation with an equal, measurable retardation of opposite sign introduced by the compensator. The birefringence can then be determined according to Equation (1).

This procedure was used to measure the average retardation per cell for each sample taken in the melting experiments. Early experiments used an American Optical "Microstar" (Series 2) polarization microscope (American Optical Corporation, Southbridge, Mass.) equipped with the AO Ortho-Illuminator, 1.25 NA Abbé condenser, 43 ×/0.66 NA dry objective, and 15 × eyepieces. A Brace-Köhler compensator was constructed on which angles of rotation could be measured to 1°.

Later experiments used a Zeiss "RP-48" polarization microscope equipped with the 12 v, 60 w illuminator, 1.30 NA achromatic-aplanatic polarization condenser, 63 ×/0.90 NA Neofluar dry objective, and 12.5 × Kpl eyepieces. Two Brace-Köhler compensators (made by Carl Zeiss Inc., New York) were interchanged for different specimen retardations: one had a maximum retardation of 56.40 nm (approximately $\lambda/10$); the other, 22.02 nm ($\lambda/30$). Angles of rotation on each could be measured to 0.1°.

In theory the Brace-Köhler compensator is most accurate when measurements are made in monochromatic light; however, it was found, by comparing measurements made with a yellow-green filter ($\lambda = 557$ nm) to those made with white light, that for the small retardations of these specimens the dimmer monochromatic light produced no actual increase in accuracy, so all subsequent measurements were made using white light.

For each melting experiment at a constant temperature, the average retardation of each sample taken at a different time was determined by measuring the retardations of 10 different cells in the sample with the Brace-Köhler compensator. Cells measured were selected so as to be typical of the entire sample; in particular, it was found necessary to avoid measuring any cells which were aggregated, since they were affected by the heat of the solvent more slowly than individual cells. Measurements on bull and human sperm cells were made only on cells whose "B" axes (in Fig. 1) were parallel to the optic axis of the microscope. For all three species, the rotating stage of the microscope was oriented for each cell measured so that the "A" axis of the cell was 45° from the crossed polaroids. In this way, an average retardation was obtained for each timed sample which showed how much of the original birefringence had been lost in that time.

Since many denaturation reactions have been shown to follow first-order reaction kinetics (Lauffer, 1959), it was postulated that the loss of birefringence might follow such kinetics, and the points would, therefore, fit the following equation:

$$\ln \left[\frac{R_t - R_f}{R_0 - R_f} \right] = -k_1 t, \qquad (4)$$

where R_0 is the retardation at time 0, R_t is the retardation at time t, R_f is the final retardation, and k_1 is the first-order reaction-rate constant. This treatment of the data assumed that there would be one step among those leading to the loss of birefringence which could be considered rate limiting.

For squid and human spermatozoa, the final retardation was found to be zero, but it was found to be positive for bull sperm. This latter value was used for R_f in the equation, but it was also found that the assumption of first-order kinetics for the bull sperm was only good for the negative retardations measured in the first part of the reaction, so only these points were included in the calculations. For each experiment at a particular temperature, the best straight line passing through the origin and the timed points was calculated and the reaction-rate constant was determined from the slope of this line.

In order to determine the temperature dependence of a particular birefringence melting reaction, rate constants were calculated at several different temperatures for each set of conditions. These data were then graphed on an Arrhenius plot to find the thermodynamic parameters characteristic of a particular reaction. Five different sets of melting experiments were performed for the different sperm samples, as follows:

(a) Bull sperm in ethylene glycol.
(b) Human sperm in ethylene glycol.

(c) Squid sperm in ethylene glycol.

(d) Bull sperm in ethylene glycol plus 0.25 M β-mercaptoethanol, in order to determine the effect of the elimination of the disulfide bonds.

(e) Squid sperm (which have no disulfide bonds) in ethylene glycol plus 0.25 M β-mercaptoethanol, to determine whether the β-mercaptoethanol in (d) was affecting any sperm structures other than disulfide bonds.

Chemical Experiments

To provide more information on the type of bonds which maintain the integrity of the sperm nucleus, the effect of various chemicals upon the birefringence and morphology of the cells was investigated. The chemicals used on both bull and squid sperm were 0.25 M β-mercaptoethanol in ethylene glycol, 3 M sodium chloride in distilled water, and 6 M guanidine hydrochloride (dissolved in distilled water and filtered through a Millipore filter of pore size 0.45 μm). Chemicals that were used only on bull sperm also included 0.25 M β-mercaptoethanol plus either 3 M sodium chloride or 6 M guanidine hydrochloride (both in water), pure n-propanol, and 2% formaldehyde in ethylene glycol. Since the effect of any of the chemicals (those which had an effect) was fairly rapid and irreversible, and it was not possible to stop these reactions at any intermediate stages as it had been with the thermal reactions, no attempts were made to obtain quantitative kinetic data. The qualitative effects of each treatment on cell birefringence and morphology, however, were observed in the polarization and phase-contrast microscopes.

RESULTS

Birefringence Melting Experiments

The effect of heat upon the sperm cells used in this study is shown in a series of photomicrographs (Figs. 2–7). In Figs. 2–3, it can be seen that the squid sperm lose their characteristic cylindrical morphology when melted, and that the final birefringence is zero. The losses of birefringence and morphology did not always occur simultaneously. In general, the loss of birefringence appeared to occur more rapidly than the changes in morphology at higher temperatures, while the situation was reversed at lower temperatures or in β-mercaptoethanol. The squid sperm which were melted in β-mercaptoethanol appeared identical to those shown in these figures (which are in ethylene glycol) both before and after melting.

Bull sperm, shown in Figs. 4–5, and human sperm, shown in Figs. 6–7, can be seen to react to

heating somewhat differently from squid sperm. Neither one of these showed any visible change in morphology when melted in ethylene glycol, and both species showed some positively birefringent areas after heating. While these areas seemed to be the same as those which showed negative retardations before melting in bull sperm, which prompted an investigation of form birefringence described in the accompanying paper, they seemed to be at different places in the cell in human sperm. The appearance of most bull sperm cells which were melted in β-mercaptoethanol was the same, both before and after the reaction, as that of the cells in Figs. 4–5 (in ethylene glycol); a few cells showed slight changes of shape after the reaction, such as a rounded or hooked appearance at the anterior end.

The quantitative results for the first-order reaction-rate constant for each melting experiment are presented in Table I. Examples of the first-order reaction plots used to obtain these rate constants are also shown in Fig. 8; the loss of birefringence followed first-order kinetics over the temperature ranges investigated for all three species. An Arrhenius plot of all of the birefringence melting data from Table I is shown as Fig. 9. It can be seen from this that the different sperm samples seem to fall into two distinct classes, on the basis of several features, as shown in Table II, which presents the final thermodynamic results for each type of reaction.

Chemical Experiments

One of the original intentions of the chemical experiments was to try to duplicate the effect of melting by the use of reagents acting primarily on certain types of bonds. This attempt was successful for the squid sperm, but less so for the bull sperm.

Both sodium chloride and guanidine hydrochloride, reagents which would presumably have their greatest effects on ionic bonds (and also, in the case of guanidine hydrochloride, on hydrogen bonds), caused squid sperm to lose both their birefringence and morphology, just as in the melting experiments. In addition, in some cases the cell membrane seemed to break or dissolve, and the cells broke into smaller particles, as previously reported by Schmidt (1928). The squid sperm in β-mercaptoethanol showed no significant differences in birefringence or morphology, although, as shown in Table I and Fig. 9, they melted at slightly lower temperatures; Table II shows that β-mercaptoethanol did not significantly alter the thermo-

dynamic parameters for the squid sperm melting reaction.

Reagents acting primarily on any single type of bond produced no visible effect on either the birefringence or morphology of bull sperm. This included either sodium chloride, guanidine hydrochloride, or β-mercaptoethanol, each used individually, and it was also true of formaldehyde, which would be expected to produce less specific effects. The use of combinations of reagents, however, had much greater effect. Sodium chloride plus β-mercaptoethanol caused the sperm heads
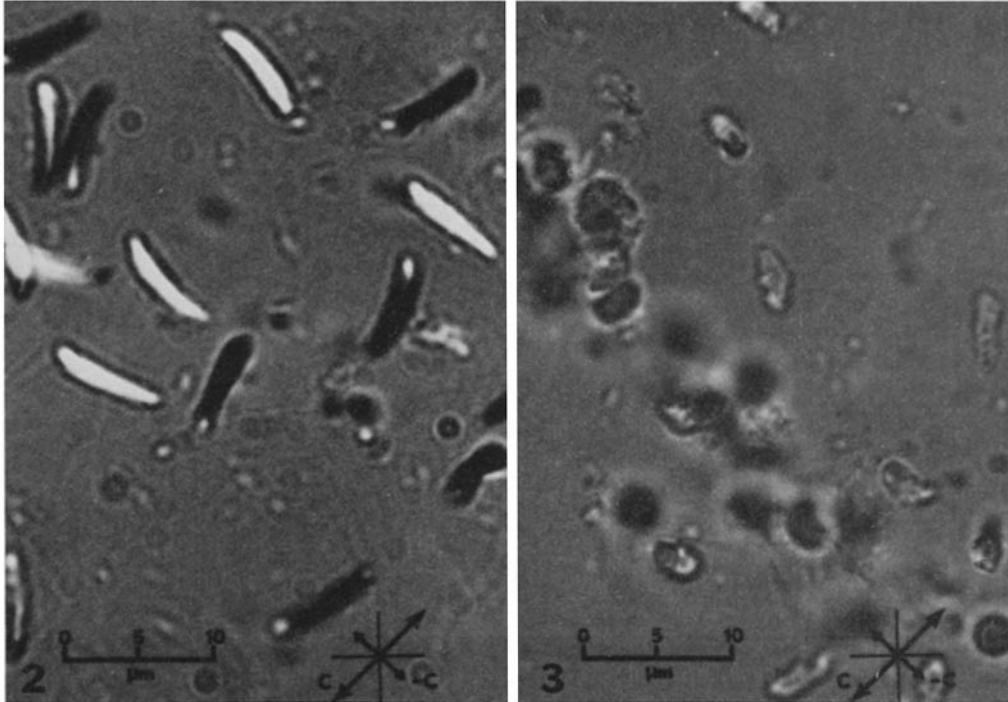

FIGURE 2   Squid sperm, unmelted, in ethylene glycol; polarized light with the Brace Köhler compensator. The diagram to the right of the magnification scale shows the directions for the crossed polaroids (light vertical and horizontal lines) and the compensator directions C and $-C$ (heavier diagonal arrows). Cells which are oriented so that their retardations have the same algebraic sign as that of the compensator appear lighter than the background; those with retardations of opposite sign appear darker. The retardations, in nanometers, of the compensator for the C direction (longer arrow) will be given in this figure, and similar figures following, as $R(C)$ = ⸺; the compensator retardation for the direction perpendicular to this $(-C)$ is of the same magnitude, but of opposite sign. For this figure, $R(C)$ = + 56.4 nm; all cell retardations, therefore, are negative. × 2000.

FIGURE 3   Squid sperm in ethylene glycol, melted. Changes in morphology from Fig. 2 are apparent. $R(C)$ = + 20 nm; cell birefringence is zero, although scattered, depolarized light causes some cells to appear lighter or darker than background. × 2000.

FIGURE 4   Bull sperm in ethylene glycol, unmelted. Different orientations (as in Fig. 1) can be seen. $R(C)$ = − 20 nm; cell retardations are negative (more easily seen for cells "on edge"). × 2000.

FIGURE 5   Bull sperm in ethylene glycol, melted. $R(C)$ = − 20 nm; cell retardations now appear to be positive, but no change in cell morphology is apparent. × 2000.

FIGURE 6   Human sperm in ethylene glycol, unmelted. $R(C)$ = − 10 nm; negatively birefringent areas can be seen in some cells. × 2000.

FIGURE 7   Human sperm in ethylene glycol, melted. No change in cell morphology. $R(C)$ = + 10 nm; positively birefringent areas can be seen in some cells. × 2000.

TABLE I
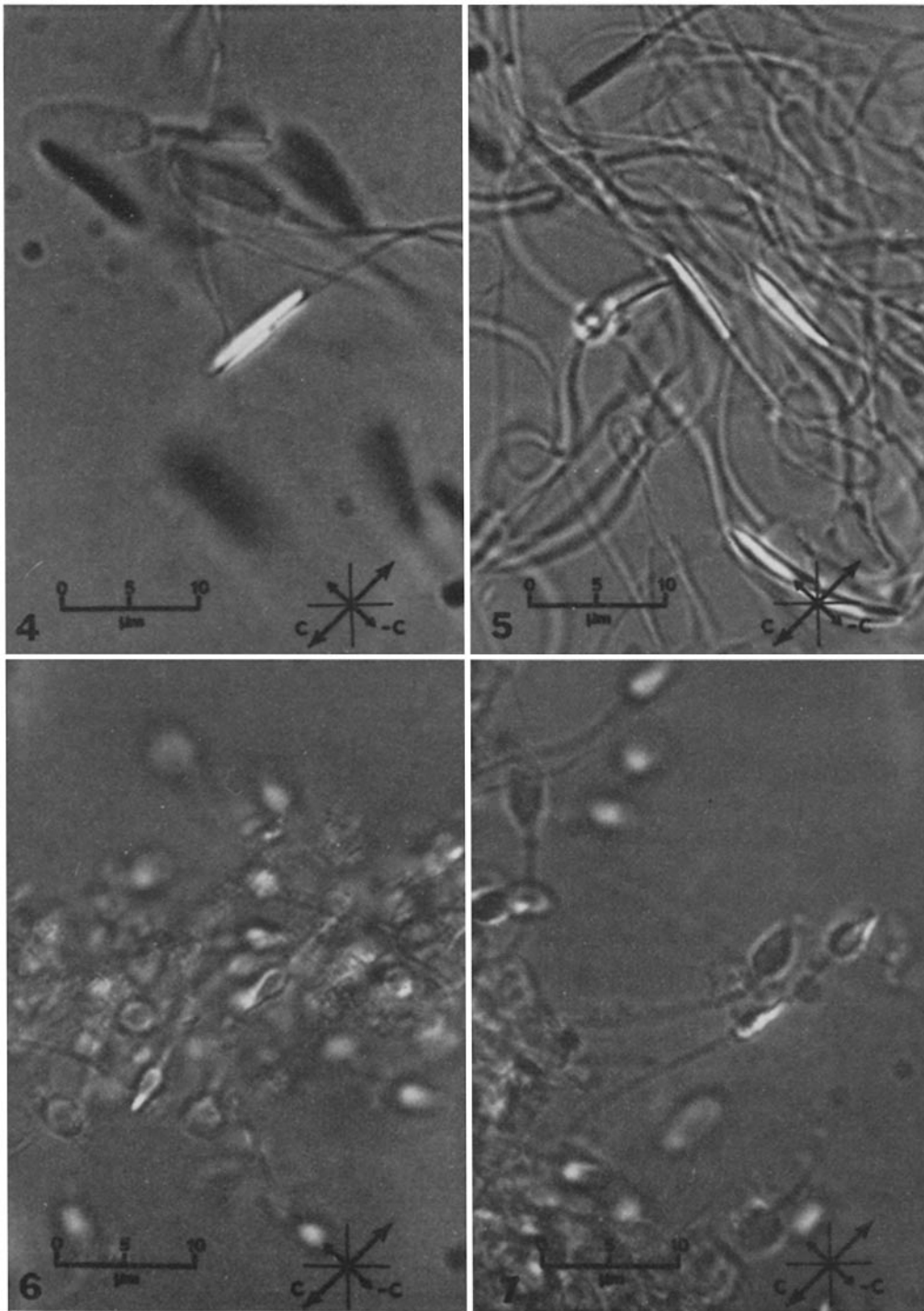
*Results of Birefringence Melting Experiments**

Sample	T, °C	k_1, min^{-1}
A. Bull sperm, in ethylene glycol	90	0.00514
	95	0.0156
	100	0.0285
	100	0.0323
	100	0.0346
	105	0.116
	105	0.119
	110	0.257
B. Human sperm, in ethylene glycol	80	0.0122
	85	0.0169
	90	0.0371
	95	0.0803
	100	0.356
C. Squid sperm, in ethylene glycol	80	0.00605
	80	0.00449
	82	0.00777
	85	0.0593
	87	0.164
	90	0.627
D. Bull sperm, in ethylene glycol + β-mercaptoeth-anol	75	0.00869
	80	0.0167
	82	0.233
	85	0.415
E. Squid sperm, in ethylene glycol + β-mercaptoeth-anol	70	0.00131
	72	0.00726
	75	0.0569
	77	0.155
	80	0.427
	85	0.936

* Experiments are grouped by sample type (species and solvent); all initial and final measurements of compensator angle for each sample were averaged together to give θ_0 and θ_f for that sample. Experimental points for bull sperm in ethylene glycol at 100°C (A: 3, 4, and 5) are graphed in an example of the first-order reaction plot in Fig. 8.

to begin swelling, eventually becoming spherical and losing all birefringence; the membranes of most of these cells finally ruptured at about the midline of the head, releasing the nuclear contents. The midpieces and tails did not appear to be affected. Guanidine hydrochloride plus β-mercaptoethanol caused similar changes to occur in the sperm head, although the dissolution of the head membrane and the nucleus appeared to occur more rapidly, and before as much swelling had
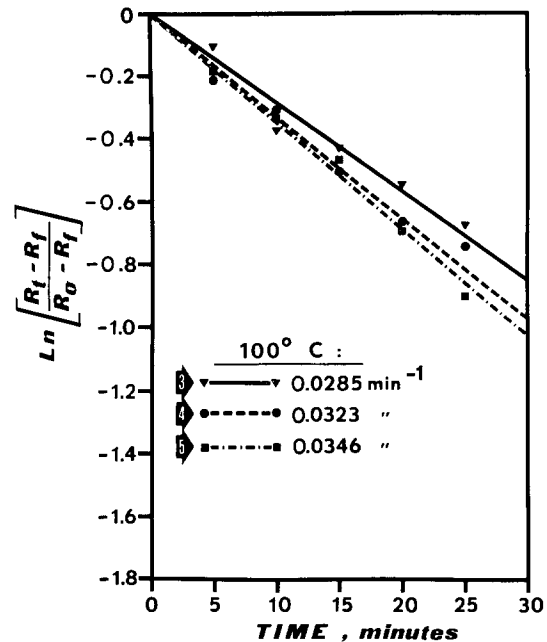

FIGURE 8 First-order reaction plots for bull sperm in ethylene glycol at 100°C. Values are from experiments 3, 4, and 5, in Section A of Table I. It was assumed that all lines drawn would pass through the origin, at $\ln(1) = 0$.

occurred. In addition, the midpieces and tails of these cells came apart (the midpiece initially appeared to become an open helix) and eventually dissolved completely. No cell structures of any type could be seen by microscopic examination of the final stage of these samples, and the solutions appeared completely clear and extremely viscous.

Although these experiments were successful in causing a loss of the original birefringence, they did not duplicate the results of the melting experiments, since heating bull sperm cells had caused no apparent loss of cell morphology and resulted in a final positive birefringence. No combination of reagents was discovered which had exactly these effects, although some interesting results were discovered for bull sperm in n-propanol, since such cells appeared to have both positive and negative values of birefringence within the same cell, with no change in cell morphology. Results of all of the chemical experiments are briefly summarized in Table III.

DISCUSSION

The results obtained in this work are rather remarkable in view of the very large size and the
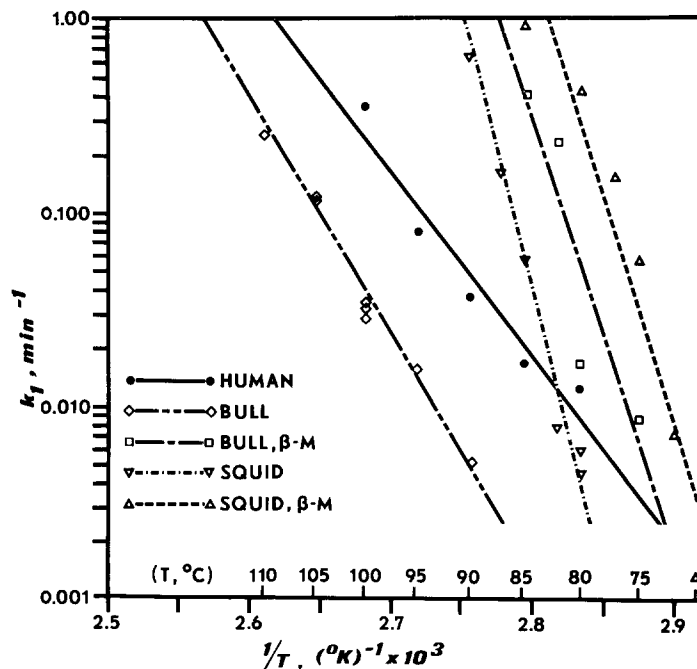
FIGURE 9 Arrhenius plot of reaction-rate constants from melting experiments in Table I. Lines shown were fitted to points by simple linear regression, rather than orthogonal regression, since experimental errors in the x direction are much smaller than those in the y direction. All samples shown were in ethylene glycol; those marked "β-M" were in 0.25 M β-mercaptoethanol, in addition.

complexity of the sperm cell nucleus, and the number of interrelated steps which are probably involved in the thermal denaturation process studied here. The observations that the birefringence melting reaction follows simple first-order kinetics, and that the temperature dependence of this reaction follows exactly the predictions of the transition-state theory developed for very simple molecules, both indicate that a single step, relatively simple in molecular terms, may be rate limiting for each type of melting reaction. The evidence from this and other experimental work, together with the molecular models proposed by others, although purely circumstantial, favors a particular type of molecular process as the rate-limiting step in each case. The possible identity of this step will be discussed briefly for each of the classes of sperm cells in Table II.

Squid Sperm

The structure of squid sperm nucleoprotein, in intact cells and after extraction, was studied during the early 1950s (Wilkins and Randall, 1953; Wilkins et al., 1953; Wilkins and Battaglia, 1953),

and a detailed molecular model for the complex was presented (Feughelman et al., 1955; Wilkins, 1956, 1959). This model placed the polypeptide backbone of the protein in the narrow groove of the DNA helix, with the charged arginine residues extended alternately to each side so that each one could combine with one DNA phosphate. The model was modified in later publications (Zubay and Doty, 1959; Luzzati and Nicolaïeff, 1959, 1963; Wilkins and Zubay, 1963; Luzzati, 1963) by the proposal that each protamine molecule was extended between, and attached to, two or more DNA molecules, resulting in the rigid cross-linked structure apparently characteristic of the squid sperm nucleus, which shows no osmotic response even in distilled water (Hilden and Sato, 1963).

The results of the chemical experiments of this paper, and of similar experiments by Schmidt (1928), are easily explained by such a model, since the DNA molecules would be released from their orientation, relative to each other, by any reagent which weakened the ionic bonding between arginine and phosphate groups. Since dissolution of these ionic bonds is probably the major effect of

TABLE II

Summary of Melting Experiment Data*

Sample	Melting range, °C	$(\Delta H^\circ)\ddagger$, $\left(\frac{kcal}{mole}\right)$	$(\Delta S^\circ)\ddagger$, $\left(\frac{cal}{mole - °K}\right)$
Bull sperm, in ethylene glycol	90–110	55.3 ± 5.3	83 ± 14
Human sperm, in ethylene glycol	80–100	43.2 ± 11.9	54 ± 33
Squid sperm, in ethylene glycol	80–90	126.2 ± 10.6	288 ± 30
Bull sperm, in ethylene glycol + β-mercaptoethanol	75–85	101.4 ± 66.5	222 ± 118
Squid sperm, in ethylene glycol + β-mercaptoethanol	70–85	106.4 ± 27.0	240 ± 96

* Data were derived from application of the transition-state theory (Barrow, 1966, p. 486–500) to the birefringence-melting data of Fig. 9 and Table I. The theory predicts that the reaction-rate constant varies according to the following equation:

$$\log k_1 = -\left[\frac{(\Delta H^\circ\ddagger)}{2.303\,R}\right]\left(\frac{1}{T}\right) + \left[\frac{(\Delta S^\circ)\ddagger}{2.303\,R}\right] + \log\left(\frac{kT}{h}\right),$$

where k_1 is the first-order reaction rate constant; R, the gas constant, 1.9869 cal/ mole-°K; k, the Boltzmann constant, 1.358×10^{-16} erg/°K; h, the Planck constant, 6.6256×10^{-27} erg-sec; T, the absolute temperature; and $(\Delta H^\circ)\ddagger$ and $(\Delta S^\circ)\ddagger$ are the enthalpy and entropy of activation per mole, respectively, for the transition to the activated complex. (The "mole" in this definition is 6.02×10^{23} of the individual molecular transitions which characterize the rate-limiting step). For these calculations, the average value of $\log (kT/h) = 12.878$ was used, since the variation in this quantity is much smaller than the experimental error. Error figures after the "$\pm$" signs above are 95% confidence limits, calculated from the corresponding limits for the slope of the regression line (Program 2.51 for the Olivetti-Underwood calculator).

TABLE III

Summary of Chemical Experiments*

Treatment	Effect on bull sperm	Effect on squid sperm
β-mercaptoethanol, in ethylene glycol	0	0
Sodium chloride, in water	0	1
Guanidine hydrochloride, in water	0	1
Sodium chloride + β-mercaptoethanol, in water	1	—
Guanidine hydrochloride + β-mercaptoethanol, in water	1	—
Formaldehyde, in ethylene glycol	0	—
n-Propanol	2	—

* Result of a particular treatment is indicated by a number corresponding to the following effects:

0 = No effect on either cell birefringence or morphology.

1 = Loss of both birefringence and morphology.

2 = Appearance of positive birefringence; no further changes.

– = Treatment not applied to that sample.

salts, this is apparently all that is necessary to cause the losses of birefringence and morphology in the chemical experiments, and the same process may be occurring in the thermal denaturations. Melting experiments on DNA in ethylene glycol have always shown melting temperatures to be less than 40°C (Duggan, 1961; Eliasson et al., 1963; Luzzati et al., 1964; Nelson and Johnson, 1970; Green and Mahler, 1971), so the destruction of the hydrogen bonding and base stacking of DNA at the temperatures of above 70°C used in these experiments is probably so rapid that these processes are not part of the rate-limiting step. This step is more likely to be the separation of proteins from the DNA, and the activated complex would then involve the same separation of oppositely charged arginine and phosphate groups as the chemical reactions.

The energy of a single arginine-phosphate ionic bond is not known, but it was determined from molecular model building that the bond energy for a single arginine-phosphate bond is unlikely to be more than 100 kcal/mole, and a more likely value is about 55 kcal/mole. This means that in order to account for the observed values of $(\Delta H^\circ)\ddagger$ in

Table II in terms of the separation of arginine-phosphate bonds, it is necessary to postulate a unit of several such bonds which display at least partial cooperativity in their attachment to, or release from, DNA.

Cooperative binding of the arginine groups would be predicted from the model of Feughelman et al. (1955), and has been observed in experimental binding studies using protamines, polylysine, or polyarginine, and DNA (Akinrimisi et al., 1965; Leng and Felsenfeld, 1966; Olins et al., 1967, 1968). Since, in spite of this possible cooperative binding, the melting of squid sperm still follows first-order kinetics, this could be an indication that some larger units, such as the tetrapeptides of arginine shown to occur frequently in protamines (Ando and Suzuki, 1966, 1967), may display cooperativity within themselves, but some independence of the melting of adjacent units. Whatever the true explanation, it can be seen from these considerations that no contradiction necessarily exists between the nucleoprotamine model proposed for squid sperm (Feughelman et al., 1955) and the thermodynamic results of these experiments.

Mammalian Sperm

The birefringence melting data of Fig. 9 and Table II, and the chemical experiments summarized in Table III, indicate that there is a remarkable similarity between the structure of bull sperm in β-mercaptoethanol, which presumably have lost their disulfide bonds, and that of squid sperm, either with or without β-mercaptoethanol. Apparently, from the chemical experiments, only ionic bonds between arginine and phosphate groups remain to maintain the structure in bull sperm without disulfide bonds; and from the similarities to squid sperm shown in the melting reactions, a similar cooperative breakage of ionic bonds may be involved in these reactions. At the molecular level, then, the nucleoprotein structure in mammalian sperm appears to differ from that in squid sperm, i.e. basically the model of Feughelman et al. (1955), primarily in the addition of disulfide bonds between protein molecules, which results in a further stabilization of the structure.

For both bull and human sperm in ethylene glycol (without β-mercaptoethanol), the rate-limiting step in the melting reactions appears to be the breakage of these disulfide bonds, on the basis of two types of evidence. In the model proposed

by Bril-Petersen and Westenbrink (1963) and extended by Coelingh et al. (1969), the disulfide bonds between protein molecules are distributed throughout the sperm nucleus and maintain a rigid, cross-linked structure which would enclose each DNA molecule and maintain its orientation; breakage of such bonds would be necessary for loss of birefringence to occur. Quantitatively, the values of $(\Delta H°)\ddagger$ for bull and human sperm in ethylene glycol (Table II) are both very close to, and include within their respective error limits, the value of 51 kcal/mole given by Barrow (1966, p. 176) as the bond energy of a disulfide bond. This is an average value for many disulfide compounds, so the bond energy for cystine in a sperm nucleus could be a few kilocalories different, but the agreement of these figures for the bond energy is still quite good.

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