RADIATION DAMAGE TO BULL SPERM MOTILITY

III. FURTHER X-RAY STUDIES

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ABSTRACT The results of previous radiation experiments, which indicated that the centriole serves as a control center for bull sperm motility, appear to be in conflict with experiments showing that the bull sperm flagellum is an autonomous oscillator. To resolve this conflict experiments were conducted to calibrate absolutely the dose-response curves for the radiation damage, and to measure the force production and the mechanochemical energy conversion after irradiation in bull sperm. The results indicate that the centriole acts as a mechanical anchor for the contractile fibers.

INTRODUCTION

In recent papers (van Herpen and Rikmenspoel, 1969; Rikmenspoel and van Herpen, 1969) we have described the effects of X-rays and high energy protons on the motility of bull spermatozoa. It was found that even at low doses of irradiation a fraction of the sperm were "killed" (ceased motion), and that the forward velocity of the surviving sperm was reduced. This is contrary to the effects of chemical inhibitions, which show a threshold below which the number of moving sperm is unaffected as the forward velocity of the sperm is lowered (Rikmenspoel et al., 1973).

Dose response curves as mentioned above for radiation effects are considered to be indicative of a small target which is sensitive to the radiation (Zimmer, 1961). Application of target theory, adapted specially for the case of sperm motility (van Herpen and Rikmenspoel, 1969) indicated that in bull sperm the sensitive target was approximately spherical, with a diameter of $\approx$ 1,500 Å. The centriole is the only organelle in a bull sperm having approximately the correct size and shape required for this target.

From estimates of the efficiency of conversion of respiratory energy into mechanical work by proton irradiated sperm, Rikmenspoel and van Herpen (1969) concluded that the contractile system of the flagella was largely intact after irradiation. Since the dose response curves for the motility of the sperm in the case of proton irradiation showed a rather large spread, and since the effects of fructolysis were neglected in the energy calculations, the reported values for the efficiency as a function of the dose should be considered as rough estimates, however.

In the studies related above, it was proposed that the radiation damage was to a con-

trol system for the motility located in the centriole. Very recently new evidence has been reported, however, that the centriole is not a control center.

Lindemann and Rikmenspoel (1972) have shown that dissected fragments of bull sperm flagella which do not contain the centriole are capable of coordinated wave motion, albeit at reduced intensity. Furthermore, from experiments in which the motility of bull sperm was inhibited by high viscosity and by potassium cyanide (KCN), it was found that the frequency of the flagellar wave is reduced upon inhibition whereas the amplitude remains largely constant (Rikmenspoel et al., 1973). This indicates that the flagellar oscillation is a relaxation oscillation (Weber, 1965). Relaxation oscillators can in general not be synchronized well to an external signal (Klotter, 1960). All of these observations suggest that the contractile system of a bull sperm flagellum is an autonomous oscillator, and that it is not under control of an extra-flagellar oscillator.

This paper describes experiments done to clarify the above apparent conflict between the results of the experiments with radiation and those of chemical inhibition and dissection. The experiments were aimed at showing more clearly which function is damaged by irradiation. An absolute calibration has been done of the dose response curves for X-ray damage to motility and respiration. A measurement was made of the change in wave characteristics of individual sperm after irradiation to allow a more precise evaluation of the efficiency of energy conversion and of the contractile forces in the sperm.

EXPERIMENTAL METHODS

Bull spermatozoa were generously provided by the Eastern Artificial Insemination Cooperative at Ithaca, New York. After collection the semen was diluted five times in a medium described below, cooled to 4°C, and transported to the laboratory for experimentation.

The medium for the original and all subsequent dilutions was a modified Krebs-Ringer solution containing 140 mM NaCl, 4 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, and 2% of 0.1 N phosphate buffer. 10% (vol/vol) of egg-yolk was added to the medium to protect the sperm from cold shock. Na lactate at 2 mM concentration served as substrate. This medium was made optically clear and free from unwanted minerals by centrifugation and dialysis as described before (Rikmenspoel et al., 1969).

Irradiation with 180 KeV X-rays of samples of the five times diluted semen was carried out in an arrangement identical to that described before (van Herpen and Rikmenspoel, 1969). The temperature of the sperm was maintained at 4°C during the irradiation.

Dosimetry was performed as described before (van Herpen and Rikmenspoel, 1969), except that a model 7767 Roentgen Rate Meter (Victoreen Instrument Div., VLN Corp., Cleveland, Ohio) was used. The dosimeter had been calibrated by the factory just prior to the series of experiments.

Respiration and Motility Measurements

Respiration was measured with the Clark oxygen electrode as described before (Rikmenspoel, 1969). 0.5 cm^3 of the irradiated sperm samples was suspended into 1.5 cm^3 of diluent in the measuring chamber. 2 mM of 2-deoxy glucose was added to suppress fructolysis by the

sperm. It has been shown that this concentration suffices to inhibit fructolysis completely (Rikmenspoel et al., 1969).

After 4 min for warm up to 37°C the respiration was measured during approximately 5 min. The experiment was then terminated by the addition of 50 μl of 0.01 M KCN. Any remaining slope of the oxygen electrode tracing was considered to be instrumental drift and the measured respiration values were corrected accordingly.

For motility and cinemicrographic measurements 0.1 cm^3 of the irradiated sperm was suspended into 0.9 cm^3 or 1.9 cm^3 (dependent on the concentration) of diluent medium to which 2 mM 2-deoxy glucose had been added. The diluted sperm was placed in a microscopic slide chamber 40 μm deep and 15 mm in diameter (Rikmenspoel, 1957). Data were taken after a 4 min warm-up period to 37°C.

Motility measurements were made with the "dark field track" method introduced by Rothschild (1953). The sperm samples are illuminated in this method in dark field, and photographs are made with an exposure time of 1 s. The head of a motile sperm produces a track on the photographs, with the length of the track corresponding to the forward velocity of the sperm. The number of tracks on a photograph gives the number of moving sperm. This method, even though rather laborious, yields absolute values for velocity and concentration of the motile sperm. The details of the method have been discussed before (Rikmenspoel, 1957).

To reduce the influence of sampling error all motility measurements reported were averaged over three slides drawn from the same sample.

Cinemicrographs of sperm samples at 200 frames per second were made using a Milliken DBM-5C camera (D. B. Milliken, Arcadia, Calif.), as described before (Eykhout and Rikmenspoel, 1960). The procedure for frame by frame analysis of the wave motion of the sperm flagella has been described previously in detail (Rikmenspoel, 1965).

RESULTS

Sperm samples of a number of ejaculates were irradiated with doses of 6, 12, and/or 24 krad. Nonirradiated sperm served as control. The dose-response curves obtained for respiration, average velocity, and number of surviving sperm are shown in Fig. 1. The lines drawn in the graphs for the average velocity and the number of surviving cells are the dose-response curves reported before, using the indirect photoelectric motility measurement (Rikmenspoel and van Herpen, 1969). It can be seen that the present absolute motility data confirm these earlier dose-response curves.

Sperm samples of a single ejaculate were subjected to doses of 0, 6, 12, and 24 krad and filmed. The average velocities of the sperm in these samples as measured from the cinemicrographs are inserted in Fig. 1.

For each of the irradiation doses a number of sperm were selected (four or five) on the films for detailed measurements. The sperm were chosen to be free of interference from neighboring sperm. Care was taken that the average of their velocities was reasonably close to the average velocity for the particular dose. The very large amount of work involved in the frame by frame analysis set the limit to the number of sperm studied.

Viewing of the cinemicrographs showed that at all radiation doses the wave in motile flagella was a traveling wave. At the higher X-ray doses (12 and 24 krad) the motion appeared slightly irregular, but the frequency of the flagellar motion of each cell analyzed could still be determined with an accuracy of approximately $\pm 20\%$.

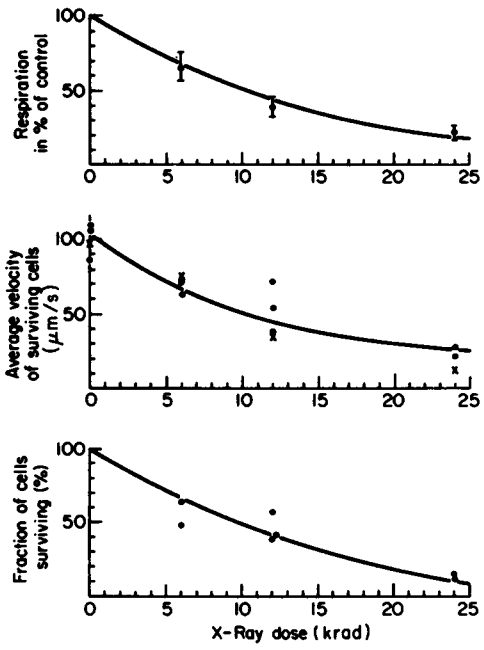

FIGURE 1 Dose response curves for X-ray effects on bull spermatozoa. The curves drawn in the graphs for the average velocity and the fraction of cells surviving are the curves reported before by van Herpen and Rikmenspoel, 1969. The crosses indicate average velocities measured cinematographically. The line in the graph for the respiration was drawn by eye for heuristic purpose.

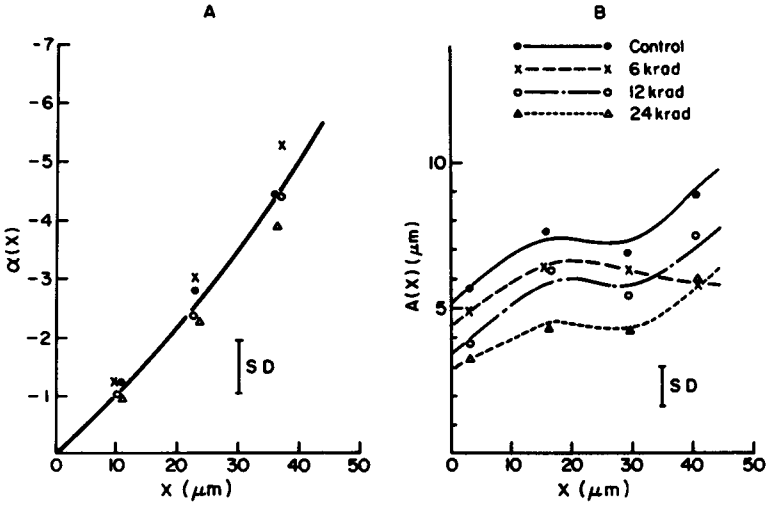

FIGURE 2 Wave function of the flagellar motion at different X-ray doses. (*A*) The function $\alpha(x)$ defines the progression of the wave (see text). The line was fitted by eye. (*B*) Amplitude of the flagellar wave as a function of x averaged at each dose over the cells analyzed. The vertical bars marked SD indicate typical standard deviation between cells at one dose.

TABLE I

AVERAGE FREQUENCY, f, AMPLITUDE, b, OF THE TAIL WAVE, AND FORWARD VELOCITY OF BULL SPERM ANALYZED AT THE VARIOUS X-RAY DOSES

Dose	No. of sperm analyzed	Average frequency	Average amplitude	Average forward velocity
krad		*cps*	*μm*	*μm/s*
0	4	21.4	7.2	102
6	5	20.6	6.4	82
12	5	14.6	5.9	32
24	5	13.6	4.5	22

If the traveling wave in a flagellum were purely sinusoidal, the wave could be described as $U(x,t) = b\sin[\omega t + \kappa x]$, where x is a running coordinate along the equilibrium position of the flagellum; $U(x,t)$ is the deviation of the flagellum from the equilibrium position at x at time t; b is the amplitude, ω is the angular frequency, and κ the wavenumber ($\kappa = 2\pi/\lambda$) of the wave. In an actual flagellum the wave is not purely sinusoidal, since the amplitude varies with x, and the wavelength decreases distally. It has been shown (Rikmenspoel, 1965) that this can be adequately expressed by writing:

$$U(x,t) = A(x)\sin[\omega t + \alpha(x)], \tag{1}$$

where $A(x)$ is the amplitude of the traveling wave as a function of x. The function $\alpha(x)$ defines the rate of traveling of the wave, with the derivative $\alpha^1(x)$ of $\alpha(x)$ having the meaning of a "local" wave number.

The shape of the wave motion of the sperm flagella at the different X-ray doses represented by $A(x)$ and $\alpha(x)$ is shown in Fig. 2. The data were averaged over the various sperm analyzed for each dose. Table I gives the average frequency, f, of the motion and the average amplitude b (taken as the amplitude midway along the flagellum) for the irradiated sperm. It can be seen that both the frequency and the mean amplitude of the flagellar waves decrease with higher X-ray dose.

ACTIVE MOMENTS AND ENERGY CONVERSION

The data on the shape of the flagellar wave in Fig. 2 make it possible to evaluate the active moments produced by the contractile structures in the flagella of irradiated sperm. The active moment $M_{act}(x,t)$ at location x on the flagellum at time t is composed of the moment due to the viscous resistance of the medium M_{visc} and the elastic bending resistance due to the stiffness of the flagellum M_{el}:

$$M_{act}(x,t) = M_{visc} + M_{el}. \tag{2}$$

When the expressions derived earlier for M_{visc} and M_{el} (Rikmenspoel, 1971) are inserted, Eq. 2 becomes:

$$M_{act}(x,t) = -\int_x^L k(\xi - x)(\partial U/\partial t)\,d\xi - IE(\partial^2 U/\partial x^2), \tag{3}$$

where L is the length of the flagellum, k is the viscous drag coefficient of the flagellum, IE is the stiffness of the flagellum, and U is the wave function defined in Eq. 1. At the base of the flagellum ($x = 0$) the values of $k_o = 2.1 \times 10^{-2}$ dyn · cm^{-1} · s and $IE_o = 1.8 \times 10^{-12}$ dyn · cm^2 have been determined (Rikmenspoel, 1965; Lindemann et al., 1973). The variation of k and IE along the flagellum due to its tapered shape were reported by the same authors.

With the wave functions described in Fig. 2 for the various radiation doses the evaluation of $M_{act}(x,t)$ is straightforward; a detailed explanation has been given before (Rikmenspoel, 1971). Since U in Eq. 3 is periodic with an angular frequency ω, M_{act} has the same periodicity, and one can write

$$M_{act}(x,t) = M(x)\cos(wt + \mu), \tag{4}$$

where $M(x)$ represents the magnitude of the active moment at x, and μ is an x-dependent phase factor.

The magnitude of the active moment $M(x)$ for the four radiation doses, derived as described above is presented in Fig. 3. Even though the calculated points in Fig. 3 show appreciable spread, the approximately linear decrease of the magnitude of the active moment towards the tip of the flagellum remains present for all radiation doses. The phase factor μ of Eq. 4 was found to be small (< 1 rad) for all values of x at all doses, indicating that in all cases the moment is developed in phase over the flagellum.

Apparently the coordination of the contractile activity is not affected by X-ray irradiation. In the cells which are still motile after irradiation the contractile system operates at a lower intensity.

The work, W, performed by a motile sperm flagellum can be written as (Machin,

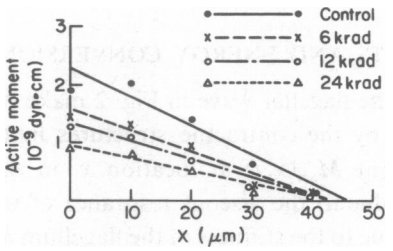

FIGURE 3 Magnitude of the active moment developed in bull sperm flagella at the various X-ray doses.

TABLE II

WORK PERFORMED BY BULL SPERM AS A FUNCTION OF X-RAY DOSE

The line "total work in sample" shows the work per sperm multiplied by the fraction of sperm surviving, normalized to the control sample = 100. The "absolute efficiency" is obtained by normalizing to the control sample = 0.2.

	Dose (krad)			
	0	6	12	24
Viscous work (10^{-7} erg/sperm/s)	5.9	4.2	1.8	0.9
Elastic work (10^{-7} erg/sperm/s)	1.6	1.2	0.7	0.4
Total work (10^{-7} erg/sperm/s)	7.5	5.4	2.5	1.3
Percent sperm surviving	100	64	41	10
Total work in sample (% of control)	100	46	14	1.7
Respiration (% of control)	100	67	39	20
Relative efficiency (% of control)	100	69	36	8[5]
Absolute efficiency (control = 0.2)	0.2	0.14	0.07	0.017
Degree of coupling, q	0.8	0.63	0.46	0.26

1958):

$$W = b^2L\{\tfrac{1}{2}k\eta\omega^2 + IE\kappa^4\omega/2\pi\}, \tag{5}$$

where η is the viscosity of the medium; all other symbols in Eq. 5 have been defined above. The first term in Eq. 5 represents the work against the viscous resistance of the medium, the second term the elastic work.

Gray and Hancock (1955) have shown that to evaluate the work of a flagellum, the use of the average amplitude b will not result in large errors. Miles and Holwill (1971) have shown that for bull sperm the value of $IE\kappa^4$ is constant along the flagellum, the decrease of IE with x being compensated by the increase of κ (κ being the derivative of $\alpha(x)$ in Fig. 2). The work by the sperm has therefore been calculated using the average value b for the amplitude and the value for $IE\kappa$ at the base $x = 0$.

In combination with the measurements of the respiration (Fig. 1), an evaluation of the work by the sperm yields the mechanochemical efficiency of conversion of energy. Table II presents the results obtained. The present more accurate measurement shows that the efficiency of energy conversion decreases with the dose much faster than was indicated in the earlier estimate after proton irradiation (Rikmenspoel and van Herpen, 1969).

A system such as a sperm flagellum in which mechanochemical conversion of energy takes place, can be characterized by a driving reaction and a driven one. In the present case the driving reaction is the respiration/oxidative phosphorylation, the driven reaction is the motility of the flagellum. Kedem and Caplan (1965) have introduced the concept of the degree of coupling, denoted by q, between the driving and the driven reaction. They have shown that the maximal efficiency obtainable in a system is dependent only on the degree of coupling q.

Machin (1958) has shown that optimal efficiency for flagellar propulsion is present

when the ratio of viscous to elastic work is as 3 to 1. From Table II it can be seen that at all radiation doses the ratio of viscous to elastic work is not far from 3. The mechanical performance of the flagella is therefore close to optimal even at the reduced rates after irradiation.

The absolute efficiency of mechanochemical conversion in bull sperm flagella under normal conditions has been reported as being 20–25% (Rikmenspoel et al., 1969). This leads to a degree of coupling of the respiration to the motility of $q = 0.8$ (Rikmenspoel et al., 1973).

At a dose of 24 krad the rate of the driving reaction (the respiration) is reduced fivefold. If the degree of coupling were not affected by the irradiation the efficiency would then have decreased threefold, according to Table I in Kedem and Caplan (1965). The present data show a 12-fold decrease in efficiency at 24 krad, indicating that q has decreased. The value of q corresponding to the decreased efficiency as a function of dose, as derived from Kedem and Caplan (1965) is inserted in Table II. At 24 krad the degree of coupling of the motility to respiration has decreased by a factor of 3.

DISCUSSION

The present measurement of the dose response curves, in which the fraction of surviving cells and their average velocity were measured absolutely and independently of one another, confirms the earlier observation that at low X-ray dose both quantities are decreased. This precludes the possibility that the damage is affected through radiation-produced chemical poisons which inhibit the oxidative metabolism or the contractile structure, because experiments with KCN and with thiourea have shown that in that case the number of motile cells is reduced only when the average velocity has been decreased to less than 50% (Rikmenspoel et al., 1973). It appears therefore that the effects of X-rays are due to direct radiation damage, and that the application of target theory to the earlier experimental data was justified. The quantitative results of the target theory of an approximately spherical target of 1,000–1,500 Å diameter reported before (Rikmenspoel and van Herpen, 1969) need not to be corrected since the present data (Fig. 1) agree with the earlier ones.

It has been shown (Rikmenspoel et al., 1973) that when the energy supply to bull sperm flagella is reduced by respiratory inhibition, all sperm remain motile, but the forward velocity of all cells is reduced. In that case the amplitude of the flagellar wave of the cells is decreased by approximately 30%, but the frequency of the wave is lowered by a factor of 6, when the inhibition has reduced the forward velocity to approximately 25 μm/s. These effects are very different from the results of irradiation as presented in Table I. This confirms that the reduction of motility after irradiation is not caused by a limitation of the energy supply to the sperm.

It is of interest to compare the effects of irradiation on bull sperm motility with the effects of thiourea. Thiourea is known to reduce the contractile activity of skeletal muscle (Ruegg et al., 1963), and flagellar activity (Brokaw, 1966; Rikmenspoel et al., 1973). It has been shown that the mode of action of thiourea in bull sperm flagella is to uncouple the contractile activity from the ATP-ase activity of the contractile ele-

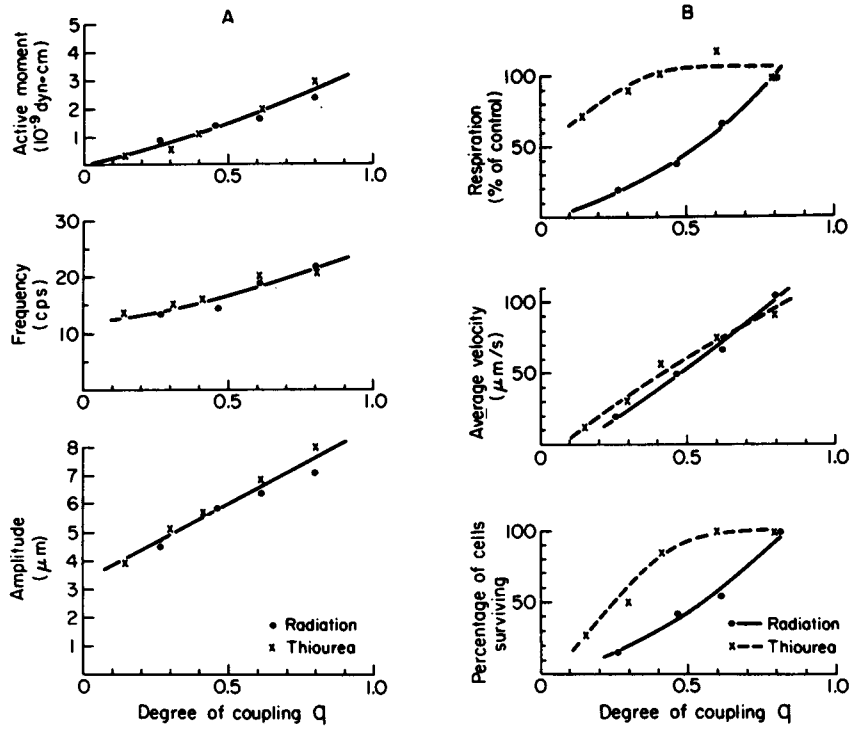

FIGURE 4 Properties of bull sperm motility, plotted as a function of the degree of coupling between respiration and motility, for irradiation and for thiourea inhibition. (*A*) Mean amplitude and frequency of the tail wave and magnitude of the active moment at the base of the flagellum. (*B*) Respiration and average velocity and percentage of surviving cells.

ments. As a result the respiratory turnover is still 70% of the control value, when thiourea inhibition has reduced the amount of work performed by the contractile elements 50-fold (Rikmenspoel et al., 1973).

The degree of coupling between the respiration as the driving reaction and the motility of the flagella as the driven reaction was calculated by Rikmenspoel et al. (1973) for the case of thiourea inhibition, quite analogously to the calculation done in this paper. This makes a direct comparison possible between the effects of X-rays and thiourea.

In Fig. 4 A have been plotted the active moment $M(o)$ produced at the base of the flagellum, the amplitude, and the frequency of the tail wave, as a function of the degree of coupling, for the cases of irradiation and of thiourea inhibition. Fig. 4 B shows the variation of respiration, forward velocity, and surviving fraction of cells with the degree of coupling for irradiation and thiourea. Figs. 4 A and B show clearly the similarity of the effects of irradiation and thiourea on the moments produced, and the frequency and amplitude of the mechanochemical oscillation. The forward velocity of the surviving cells, which is the resultant manifestation of the contractile activity in the sperm flagella is from Fig. 4 B affected similarly by irradiation and thiourea.

Our present data indicate therefore strongly that X-ray irradiation affects the contractile system by uncoupling. The mechanochemical system of a flagellum can be schematized as:

$$\text{respiration} \xrightarrow{1} \text{ATP} \xrightarrow{2} \text{active moment} \xrightarrow{3} \text{motility.}$$

Step 1 in this scheme is apparently intact after irradiation as well as after thiourea, since the respiration remains oligomycin sensitive in both these cases (Rikmenspoel and van Herpen, 1969; Rikmenspoel et al., 1973). The uncoupling by thiourea is at step 2, as indicated by the fact that the respiration is largely intact when active moment and motility are strongly reduced. The uncoupling by X-rays is apparently at step 3 since the respiration is reduced as the contractile system becomes unable to produce active moment after irradiation, and the sperm appears not to be ATP limited.

The results of the present experiments seem to give rise to a conflict between the size of the target of the irradiation (approximately 10^{-15} cm^3, Rikmenspoel and van Herpen, 1969) and the size of the contractile fibers in bull sperm flagella (4.5×10^{-13} cm^3). It is possible to reconcile a small sized target with damage due to an uncoupling of the contractile apparatus, however. Summers and Gibbons (1971) have observed that Triton demembranated sea urchin flagella, when broken, only show wave motion (in the presence of ATP) in the fragments containing the centriole. In flagella and in cilia it has been found (Rikmenspoel, 1971; with Rudd, 1973) that the contractile forces are developed such that a mechanical attachment of the contractile elements at the base is required. This points to the centriole (or the basal body of cilia) having the function of a mechanical anchor point for the contractile fibers. Fawcett and Phillips (1970) have found that during spermatogenesis in mammalian organisms, the centriole is developed independently from the coarse contractile fibers. After the formation of centriole and the fibers is completed a lamellar structure develops which binds the fibers to the centriole. This can be taken to support the notion that the centriole has a mechanical function.

If the effect of irradiation is to modify the centriole area such that it no longer fulfills its role as a necessary tie point for the contractile fibers, the small target size and the effect on the contractile structure by mechanical uncoupling are reconciled. It will be desirable, however, to have an independent confirmation, for example from electron microscopy of irradiated sperm, of this interpretation.

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