CHANGES IN DEOXYRIBONUCLEOPROTEIN DURING SPERMIOGENESIS IN THE BULL: INCREASED [³H]ACTINOMYCIN D BINDING TO NUCLEAR CHROMATIN OF MORPHOLOGICALLY ABNORMAL SPERMATOZOA

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Summary. Previously reported work has shown that a reduction in the rate of tritium-labelled actinomycin D ([³H]AMD) binding to spermatids and spermatozoa reflects the extent of the change in nuclear chromatin which occurs during spermiogenesis. Therefore, the kinetics of binding to the deoxyribonucleoprotein (DNP) of live, morphologically abnormal, testicular and ejaculated spermatozoa from five infertile and three control bulls have been studied by the use of quantitative autoradiography in order to examine suspected defects in the nuclear chromatin and particularly in the binding of nuclear protein to DNA.

Abnormally shaped heads bound up to sixteen times more [³H]AMD than did neighbouring normally shaped heads. There was no difference between the infertile and the control groups in the binding capacity of all types of spermatozoa. There was no single abnormal form which constantly bound more [³H]AMD than other forms although those malformed heads which tended to be round seemed generally to be the most heavily labelled. Testicular spermatozoa usually bound more [³H]AMD than did ejaculated spermatozoa. Ageing of ejaculated spermatozoa *in vitro* had no apparent effect on the binding of the isotope to nuclear chromatin. A pattern of labelling over the nuclei of elongated spermatids was noted which seemed to mimic the pattern of chromatin condensation of spermateliosis.

The greater availability of [³H]AMD binding sites in morphologically abnormal sperm heads suggests a defect in the condensation of nuclear chromatin. This defect has not been elucidated.

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INTRODUCTION

Clinical cases of male infertility are frequently found to be associated with percentages of morphologically abnormal spermatozoa in the ejaculate which are considerably higher than those found in semen from fertile individuals (Lagerlöf, 1934). Although many forms of insult to the testis will result in the production of spermatozoa with visible defects, the morphogenesis of these abnormalities is still unknown. Attention has been focused mainly on the processes transpiring during spermateliosis, although a defective distribution of chromosomes during meiosis has been suggested as being responsible for the increased numbers of abnormal head forms seen in cases of testicular degeneration in the bull (Knudsen, 1954, 1958). Results from more recent studies tend to disagree with this suggestion while not presenting definitive evidence (Gledhill, 1966; Henricson & Bäckström, 1964). Improper packing of chromatin may be involved in the formation of misshapen heads although increased numbers of abnormal forms were not seen in ejaculates from an infertile bull that had a defect in the transformation of deoxyribonucleoprotein (DNP) during spermiogenesis (Gledhill, Gledhill, Rigler & Ringertz, 1966a).

Actinomycin D (AMD) can be used as a tool to study some of the properties of nuclear chromatin (Brachet & Hulin, 1969; Ringertz & Bolund, 1969): AMD will bind specifically to DNA (Hurvitz, Furth, Malamy & Alexander, 1962) and complexes formed between AMD and DNA are stable (Rauen, Kersten & Kersten, 1960). These characteristics make possible the autoradiographic detection of the DNA-nuclear protein complex in cells when isotopically labelled AMD is used (Ringertz, Daryźnkiewicz & Bolund, 1969). A markedly increased binding of [^3H]AMD to morphologically abnormal spermatozoa (Plate 1) was observed in preparations made for other studies (Darżynkiewicz, Gledhill & Ringertz, 1969; Gledhill, Ringertz & Darżynkiewicz, unpublished data). It was recognized that this finding might be pointing to a physicochemical difference between normal and misshapen head forms, since the availability of AMD-reactive sites reflects both the structural and functional states of the DNP within the living cell. Previous cytochemical studies (Gledhill, 1966) have shown that, while there is no difference in mean DNA content, similar abnormally shaped heads do have a lower mean total dry mass than do their normal counterparts. The reason for this difference is not known. While the present report still does not fully answer this question, the data presented is interpreted as demonstrating a physicochemical difference between morphologically normal and abnormal spermatozoa.

MATERIALS AND METHODS

Specimen preparation

Data from eight bulls are reported here (Table 1). Five of the animals exhibited clinical infertility, the other three forming a control group (Table 2). Testicular spermatozoa, admixed with other cells, were obtained by scraping the freshly cut surfaces of macroscopically normal testes within a few minutes of castration. Doubtless spermatids in the final stages of the maturation phase of

TABLE 1
SEMINAL CHARACTERISTICS OF THE EXPERIMENTAL BULLS

<i>Bull and Exp. no.</i>	<i>Breed*</i>	<i>Age (years)</i>	<i>No. of ejacs.</i>	<i>Sperm conc. (10⁶/mm³)</i>	<i>Abnormal heads (%)</i>	<i>Prox. cytopl. drops. (%)</i>
532R (1)	SRB	2	13	1.7 ± 0.1	9.5 ± 0.4	1.3 ± 0.3
35L (2)	SRB	1½	15	0.9 ± 0.1	9.9 ± 0.2	1.9 ± 0.1
139C (3)	SRB	10	8	0.8 ± 0.1	14.7 ± 0.3	1.5 ± 0.2
956B (4)	SRB	7½	26	1.4 ± 0.1	23.2 ± 0.2	3.1 ± 0.4
181T (5)	SRB	5½	9	1.5 ± 0.4	8.3 ± 1.3	1.3 ± 0.3
106W (6)	SRB	3½	35	1.1 ± 0.1	14.3 ± 1.0	6.9 ± 1.0
8SD (7)	SRB	3½	36	1.1 ± 0.1	21.7 ± 0.8	7.6 ± 0.9
21P (8)	SAB	1½	4	1.0	21.5	17.4

Values for sperm concentration, % abnormal sperm heads and % proximal cytoplasmic droplets expressed as Mean ± S.E.

* SRB: Swedish Red and White breed; SAB: Swedish Ayrshire breed.

spermateliosis, as well as newly released spermatozoa, were morphologically grouped together as testicular spermatozoa. Ejaculated spermatozoa were obtained with an artificial vagina shortly before castration. In order to obtain ejaculates which contained spermatozoa of a relatively uniform age, all the bulls but 139C and 21P were kept on a collection frequency of two ejaculations twice a week for at least 1 month before initiation of the experiment.

The cells were washed twice in a buffered balanced salt solution (Mann, 1964) and, with the exception described below, transferred within 30 min of collection to a tube containing medium TC 199 (Morgan, Morton & Parker, 1950). The temperature of all the cell suspensions was kept between 30° and 35° C.

[³H]actinomycin D incubations

The [³H]AMD labelling was performed separately on testicular and ejaculate spermatozoa as follows: cell suspensions were incubated at concentrations of

TABLE 2
LIFETIME FERTILITY RATES OF THE EXPERIMENTAL BULLS

<i>Bull</i>	<i>Years recorded (inclusive)</i>	<i>Total no. of AIs</i>	<i>Average 56-day non-return rate above or below that of AI station (%)</i>
<i>Control group</i>			
532R	Young bull with approved semen but not used for artificial breeding		
35L	Young bull with approved semen but not used for artificial breeding		
139C	1961 to first half 1969	16445	+2.3
<i>Infertile group</i>			
956B	1963 to 1967	1952	-10.3
181T	1965 to first half 1969	2558	-11.3
106W	1966 to 1967	1160	-18
8SD	1967	1176	-15
21P	Young bull not approved for use in artificial breeding		

about 10^6 testicular cells/ml or about 10^7 ejaculate cells/ml in 1-ml aliquots of medium TC 199 in a 5% CO_2 -in-air gassed incubator. The final concentration of $[^3\text{H}]\text{AMD}$ was $1\ \mu\text{g}/\text{ml}$. The labelled AMD was purchased from Schwartz BioResearch Inc., Orangeburg, N.Y. (original specific activity $3.38\ \text{Ci}/\text{mmol}$) and was diluted with unlabelled AMD to a final specific activity of $524\ \text{mCi}/\text{mmol}$. The cells were incubated in the presence of $[^3\text{H}]\text{AMD}$ for periods varying from 10 to 90 min. Following incubation, the cells were washed twice with an excess of medium TC 199. Thereafter, a drop of cell suspension was smeared on to a gelatinized slide. For each experiment, the smears for each time of incubation, and each type of cell suspension (testicular or ejaculate) were made on one slide to ensure identical conditions of autoradiography (Text-fig. 1)

TEXT-FIG. 1. Schematic illustration of the placement of multiple specimens on a single $77 \times 25\ \text{mm}$ gelatinized object glass. At the conclusion of preselected durations of incubation with $[^3\text{H}]\text{AMD}$ (10, 20, 40, 80 min), a drop of ejaculate-suspension (e) or testis-suspension (t) is allowed to air-dry in a square. The preparations are identified by the number of the experiment (1 to 8).

and make the results of the grain counts comparable within the experiment. The smears were fixed overnight in absolute ethanol, washed for 2 hr both in 70% ethanol and in distilled water and coated for autoradiography with Kodak AR-10 stripping film. After air drying, the preparations were exposed in a dry CO_2 atmosphere, at 4°C , for 4 days. The slides were processed in Kodak D-19 developer (4 min, 18°C), fixed in Kodak X-ray fixer and counterstained with haematoxylin and eosin.

The grains over 100 morphologically normal cells of both testicular and ejaculate spermatozoa were counted. Grains were also counted over all the morphologically abnormal cells found within the same autoradiographs during

routine scanning procedures. This generally resulted in the scoring of fifteen to twenty-five abnormally shaped heads of both testicular and ejaculate cells for each incubation period. Only heads that had tails attached were counted because of the possibility that torn cellular membranes would make penetration of the [³H]AMD easier. Duplicate autoradiographs were analysed for each experiment.

Effects of ageing in vitro

In order to determine if cellular death accounted for a significant portion of the differences in the mean numbers of grains, fresh and aged spermatozoa from bull 21P were compared as follows: an ejaculate was subdivided into three portions and each portion was put into a double volume of one of the following diluents: egg yolk-citrate (Swanson, 1949), Krebs-Hanseleit-Ringer (Mann, 1964, p. 347), or boiled milk (Thacker & Almquist, 1953). After a few minutes in these diluents, 60 to 70% of the spermatozoa were still motile in a progressive manner. These samples were placed in a light-shielded area for 48 hr at 22° C. Another ejaculate of similar concentration was obtained from the same bull (21P) 48 hr after the first and the spermatozoa were treated in an identical manner except that they were transferred from diluent to medium TC 199 at 30 min after collection. After centrifugation and resuspension in medium TC 199, spermatozoa from the three aged and three fresh sub-samples were incubated in the presence of [³H]AMD as outlined above.

Less than 10% of the spermatozoa in the aged samples showed progressive motility 24 hr after collection and only occasional cells moved in a progressive manner after 48 hr. Between 60 and 70% of the spermatozoa in the second ejaculate showed progressive motility in each of the diluents after 30 min. Although we are sure that not all of the spermatozoa were dead in the aged samples, we are certain that many were dead and that most were moribund. Therefore, if differences in grain counts were to be influenced by the 'viability' (ageing and/or diluent) of the spermatozoa, these differences should become apparent when the counts for aged cells are compared to those for fresh spermatozoa. We assume the same reasoning holds true for testicular spermatozoa.

RESULTS

Only limited numbers of abnormal shapes were found in each preparation at each time of incubation. All misshapen head forms were considered in the single category of morphologically abnormal. The classification scheme proposed by Lagerlöf (1934) was followed and due consideration was given to possible differences because of the use of different stains and optical systems.

Abnormally shaped heads bound [³H]AMD at a greater rate than did normally shaped heads. No single abnormal form was solely responsible for the increased number of grains per nucleus reported for the abnormally shaped group, although those forms which tended to be 'roundish' (e.g., round, pear-shaped) gave the impression of being the most heavily labelled. This impression cannot be evaluated by statistical testing since there were great numbers of different head abnormalities but only small numbers of cells of any one

TABLE 3
UPTAKE OF TRITIATED ACTINOMYCIN D BY UNFIXED TESTICULAR SPERMATOCYTES AT VARIOUS TIMES OF INCUBATION

Incubation time (min)	Experiment 1		Experiment 2		Experiment 3	
	N*	A*	N	A	N	A
Control	0.8 ± 0.1	12.8 ± 4.5	1.6 ± 0.3	19.0 ± 5.8	—	—
10	1.6 ± 0.2	23.5 ± 2.3	3.5 ± 0.3	38.0 ± 4.7	5.6 ± 0.6	8.9 ± 2.1
20	3.5 ± 0.2	11.3 ± 2.6	8.9 ± 0.4	—	7.5 ± 0.6	10.7 ± 3.4
40	7.6 ± 0.4	25.3 ± 4.3	14.0 ± 0.7	—	14.4 ± 0.9	8.5 ± 0.9
80						

Incubation time (min)	Experiment 4		Experiment 5		Experiment 6		Experiment 7	
	N	A	N	A	N	A	N	A
Infertile	1.6 ± 0.2	1.5 ± 0.5	1.7 ± 0.1	16.0 ± 0.3	2.9 ± 0.2	7.3 ± 2.7	3.1 ± 0.2	3.3 ± 0.9
10	3.7 ± 0.3	8.0 ± 0.3	3.5 ± 0.2	—	4.0 ± 0.4	—	5.5 ± 0.5	6.1 ± 2.1
20	5.6 ± 0.6	5.7 ± 0.4	5.8 ± 0.3	22.0 ± 0.7	6.2 ± 0.4	8.2 ± 0.3	9.3 ± 0.6	8.5 ± 0.3
40	9.8 ± 0.7	15.0 ± 0.4	9.8 ± 0.5	29.0 ± 0.8	†14.4 ± 0.7	†22.6 ± 0.7	12.0 ± 0.7	12.3 ± 0.9
80								

Values expressed as mean number of silver grains per head ± S.E.

* N = morphologically normal spermatozoa; A = morphologically abnormal spermatozoa.

† 90-min incubation.

TABLE 4
UPTAKE OF TRITIATED ACTINOMYCIN D BY UNFIXED EJACULATED SPERMATOZOA AT VARIOUS TIMES OF INCUBATION

Incubation time (min)	Experiment 1		Experiment 2		Experiment 3	
	N*	A*	N	A	N	A
Control	0.8±0.1	1.0±0.1	2.0±0.1	6.3±1.2	—	—
10	1.1±0.1	8.7±0.4	3.1±0.1	8.7±0.3	2.4±0.2	3.6±0.3
20	2.1±0.1	25.0±2.0	6.1±0.2	18.5±4.6	4.2±0.2	5.0±0.3
40	3.3±0.1	11.0±0.8	9.8±0.3	19.5±5.4	4.5±0.4	4.4±0.2
80						

Incubation time (min)	Experiment 4		Experiment 5		Experiment 6		Experiment 7	
	N	A	N	A	N	A	N	A
Infertile	1.1±0.1	2.9±0.8	1.6±0.1	—	2.2±0.1	1.6±0.2	1.5±0.1	1.8±0.5
10	1.6±0.1	5.8±0.8	1.8±0.1	—	3.0±0.1	3.4±0.2	2.2±0.1	2.4±0.3
20	2.6±0.2	6.7±2.1	3.0±0.2	6.0±0.3	4.0±0.2	6.5±1.3	4.1±0.2	5.0±0.9
40	4.0±0.2	7.2±1.5	3.6±0.2	4.8±0.8	†5.6±0.2	†7.0±0.8	4.9±0.2	6.7±0.9
80								

Values expressed as mean number of silver grains per head ± S.E.

* N = Morphologically normal spermatozoa; A = morphologically abnormal spermatozoa.
† 90-min incubation.

TEXT-FIG. 2. Kinetics of $[^3\text{H}]\text{AMD}$ binding to morphologically normal testicular (O) and ejaculate (●) spermatozoa, and morphologically abnormal testicular (Δ) and ejaculate (▲) spermatozoa. The capacity of testicular spermatozoa to bind $[^3\text{H}]\text{AMD}$ is usually higher than that of ejaculate spermatozoa and abnormally shaped heads generally bind more of the isotope than do normally shaped heads. S.E.M. is represented by a vertical line.

abnormality. As found in previous experiments (Darzynkiewicz *et al.*, 1969), normally formed testicular spermatozoa usually bound [³H]AMD at a greater rate than did ejaculated spermatozoa. Likewise, the same pattern of silver grain distribution over the elongated spermatid nucleus was again observed with more grains located distal to the area of the equatorial segment than proximal to it. Grain counts of testis-cell suspensions revealed that two populations of elongated spermatids with different mean values were clearly noticeable. We were unable to classify these spermatids morphologically as normally or abnormally shaped.

TABLE 5

FACTORIAL DIFFERENCE IN UPTAKE OF TRITIATED ACTINOMYCIN D BETWEEN MORPHOLOGICALLY NORMAL AND ABNORMAL TESTICULAR AND EJACULATED SPERMATOZOA AT VARIOUS TIMES OF INCUBATION

Incubation time (min)	Experiment 1		Experiment 2		Experiment 3	
	T*	E*	T	E	T	E
Control						
10	16.0	1.3	8.4	3.2		
20	14.7	7.9	10.9	2.8	1.6	1.5
40	3.2	11.9		3.0	1.4	1.2
80	3.3	3.3		2.0	0.6	1.0

Incubation time (min)	Experiment 4		Experiment 5		Experiment 6		Experiment 7	
	T	E	T	E	T	E	T	E
Infertile								
10	0.9	2.6	9.4		2.5	1.4	1.1	1.2
20	2.2	3.6				1.1	1.1	1.1
40	1.0	1.2	3.7	2.0	1.3	1.6	0.9	1.2
80	1.5	0.9	3.4	1.3	1.6	1.3	1.0	1.4

Values expressed as mean silver grain count for morphologically abnormal sperm heads divided by mean value for normally shaped heads (cf. Tables 3 and 4).

* T = testicular spermatozoa; E = ejaculated spermatozoa.

Based on silver-grain counts, misshapen sperm heads in testicular preparations (Table 3) and in ejaculate preparations (Table 4) bound [³H]AMD at a higher rate than did spermatozoa with normally shaped heads (Plate 1). While there were sporadic exceptions for isolated incubations within an experiment, this was a general trend seen for each bull (Text-fig. 2). On one occasion, isotope binding by the malformed heads was sixteen-fold higher than that of normally shaped heads; usually the mean grain count for the abnormally shaped heads was increased by a factor of between 1.3 and 3.0 (Table 5). Of the forty-eight comparisons shown in Table 5, only seven have a value of 1 or less. Thus, the abnormally shaped sperm heads showed a higher rate of binding than did the normally shaped heads in forty-one out of forty-eight comparisons and in only one of the forty-eight comparisons did the normally shaped spermatozoa

bind substantially more [^3H]AMD than the misshapen ones (Exp. 3, 80 min, testicular spermatozoa).

These differences in mean grain count per nucleus are particularly noticeable in testicular spermatozoa from the control bulls, less pronounced for ejaculated spermatozoa of these bulls, and least apparent in both types of spermatozoa from the infertile bulls (Text-fig. 2). However, there is no clear-cut division between the infertile and control groups and only slight dissimilarity between testicular and ejaculate spermatozoa in regard to the ratio formed by comparison of grain counts for normally and abnormally shaped heads.

Testicular spermatozoa clearly bind more [^3H]AMD than do ejaculate spermatozoa in terms of absolute amounts of the isotope. Although binding of

TEXT-FIG. 3. Frequency distribution of the mean [^3H]AMD silver-grain count of morphologically normal (open) and abnormal (stippled) ejaculate spermatozoa: 48 hr of ageing *in vitro* (1 to 3); fresh semen (4 to 6). Diluents: egg yolk-citrate (1 and 4), Krebs-Hanseleit-Ringer (2 and 5) and boiled milk (3 and 6). S.E.M. is represented by a vertical line. Length of incubation was 80 min; Bull 21P, Exp. no. 8.

[^3H]AMD increases with increasing time of incubation, there seems to be a leveling off of the rate between 40 and 80 min for both normal and abnormal forms. However, the difference in rate of binding between normally and abnormally shaped heads is most apparent for the short incubation time periods (Text-fig. 2).

The difference in average grain count between normally and abnormally shaped heads of spermatozoa from bull 21P was also observed in the ageing experiment (Text-fig. 3). Ageing *in vitro* had an effect on the viability of the cells, but had no effect on the overall [^3H]AMD binding capacity of ejaculated spermatozoa and particularly did not alter the increased binding of the isotope characteristically seen for abnormally shaped heads. Although this experiment is not as conclusive as it might be with several animals, it suggests that penetration of the isotope into the nucleus is not significantly affected by cellular death.

DISCUSSION

The kinetics of [^3H]AMD binding to the DNP of morphologically abnormal testicular and ejaculated spermatozoa have been studied by means of quantita-

PLATE 1

Photomicrographs of typical patterns of silver-grain distribution over morphologically abnormal (Fig. 1) and normal (Fig. 2) testicular spermatozoa and ejaculate spermatozoa (Fig. 3). Ethanol. Haematoxylin and eosin. $\times 1220$.

(Facing p. 34)

tive autoradiography. Previous work in this laboratory (Darżynkiewicz *et al.*, 1969) and by Brachet & Hulin (1969) has shown that the maturation of the male germ cell nucleus is paralleled by a reduction in the rate of binding of [³H]AMD to the DNP. Although difficult to evaluate at present, this decrease in the rate of binding is probably a consequence of the physicochemical changes undergone by the DNP complex during the nuclear condensation typical of mammalian spermateliosis.

The time curves (Text-fig. 2) obtained for the binding of [³H]AMD to normally and abnormally shaped spermatozoa of both testicular and ejaculate origin differ from curves obtained from phytohaemagglutinin stimulated and unstimulated human lymphocytes in that they are more or less linear. The early phase of rapid [³H]AMD binding sometimes observed in other cell-types (Ringertz *et al.*, 1969; Bolund, 1970) is missing in the spermatozoa. The quantity of [³H]AMD bound to the sperm nuclei is much smaller than the amount bound to lymphocytes (Darżynkiewicz, Bolund & Ringertz, 1969a, b; Ringertz *et al.*, 1969). The rate at which spermatozoa bind [³H]AMD probably reflects their ability to penetrate both the cellular membranes and condensed chromatin, as well as the availability of sites in the DNP which are capable of binding [³H]AMD.

It may be argued that the known difference in membrane permeability between living and dead cells would explain the increased mean grain counts of the abnormally shaped heads. Although it is generally accepted that, under most conditions, the percentage of dead cells is greater in a population of malformed spermatozoa than it is in a population of morphologically normal spermatozoa, it is highly unlikely that all the misshapen spermatozoa were dead. The results of the experiment on fresh and aged spermatozoa further suggests that differences in permeability of the cellular membranes have no effect on binding. Moreover, the pattern of labelling showing preferential binding to the incompletely condensed nuclear areas of elongating spermatids (Darżynkiewicz *et al.*, 1969) indicates that most, if not all, of the limitation in distribution of the isotope is related to the extent of chromatin condensation and not to membrane permeability. Results obtained by Bolund (1970), using the technique of dry autoradiography so that unbound [³H]AMD is not eliminated from the cell, suggest that, at least in lymphocytes and HeLa cells, membrane permeability is of relatively little importance in determining whether or not the nuclei bind the drug. In these experimental systems, therefore, the rate of binding is most likely to reflect the properties of the chromatin and DNP rather than the state of the cellular membrane.

Differences in nuclear surface area and other factors of cellular configuration seem to create more favourable conditions for the efficient detection of tritium grains labelling morphologically normal spermatozoa in autoradiograms. Most forms of morphologically abnormal sperm heads have a smaller surface area and are probably thicker, at least at the base of the head, than are normally shaped heads (Plate 1) (Lagerlöf, 1934; Gledhill, 1966). The greater surface area and thinness of normally shaped heads favour increased efficiency of [³H]detection in these forms. Since the abnormally shaped heads generally had a higher mean grain count number than did the normally shaped heads

(even with their greater efficiency of grain formation), subtracting the average grain count for the normally shaped heads from that for the abnormally shaped heads results in estimates of difference which are probably conservative in extent.

Although the present results may be influenced in a slight manner by differences in the rate at which the drug penetrates the normally and abnormally shaped sperm heads, it appears most likely that the increased number of silver grains per abnormal nucleus is due to a defect in the condensation of the chromatin. This defect would not be gross enough to be visible with bright field or phase-contrast microscopy (Plate 1), but still might affect the rate at which [^3H]AMD penetrates the chromatin. It is also possible that the defect is expressed as an increased capacity of the DNP complex to bind the drug, i.e. there are more binding sites available for the drug in the chromatin. We have demonstrated that pronounced changes in the chemical and physical properties of the DNP normally occur during spermiogenesis in the bull (Gledhill, Gledhill, Rigler & Ringertz, 1966b; Ringertz, Gledhill & Darzynkiewicz, 1970). These changes, the unusually low value for the specific optical path difference of sperm heads (Carlson & Gledhill, 1966), and the essentially total resistance of spermatozoa to the action of DNase (Daoust & Clermont, 1955) probably reflect alterations in the chemical state and composition of DNP which, in turn, may be the molecular basis for the extremely dense packing of the chromatin in the normal sperm nucleus.

The present results become more intriguing when combined with the results of other investigators (Amann & Almquist, 1962; Gillette, 1965; Paüfler & Foote, 1968; Bane & Rao, personal communication, 1969) which show higher frequencies of occurrence of head abnormalities in spermatozoa from the testis and caput epididymidis than in spermatozoa from the cauda epididymidis and in ejaculated spermatozoa. If, in a physicochemical sense, the DNP complex of deformed heads is less stable because of weaker than normal binding of nuclear proteins to the DNA, these malformed heads would probably be more susceptible to the actions of lytic substances and macrophages. Nucleases (Waldschmidt, Karg & Kinzler, 1964; Quinn, 1968) and proteolytic enzymes (Waldschmidt, Hoffmann & Karg, 1966) in the luminal contents would be particularly important if a preferential removal of spermatozoa with abnormally shaped heads does occur in the excurrent male duct system (Waldschmidt & Karg, 1964; Gillette, 1965).

At present, we do not have a definitive explanation for the genesis of the difference in binding of [^3H]AMD by normally and abnormally shaped sperm heads. Preliminary study (Gledhill, unpublished) of the 'melting profiles' of abnormally shaped heads determined by acridine orange microfluorimetry (Ringertz *et al.*, 1970) has elicited some differences from morphologically normal heads in the stability of nuclear DNA to heat denaturation. Earlier work (Gledhill, 1966) has shown that morphologically abnormal ejaculated cells of the same type as investigated herein had the same amount of DNA but a lower total dry mass than did normally shaped heads when measured with ultraviolet microspectrophotometry and microinterferometry.

Further study will have to determine if the morphogenesis of abnormally

shaped sperm heads is related to the apparent preferential elimination of these spermatozoa in the male genital tract. Further study will also have to determine if morphologically normal sperm heads from the semen of certain types of infertile males might be defective in molecular structure but not defective enough to be misshapen. Although a specific defect has not been elucidated by the present studies, one point is clear: the general tendency seen in each bull indicates that an abnormally or otherwise incompletely condensed nuclear chromatin is involved in the formation of misshapen bull sperm heads.

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