DEOXYRIBONUCLEOPROTEIN CHANGES IN RAM AND BULL SPERMATIDS

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The ability of nuclear chromatin to bind actinomycin-D appears to be related to the synthesis of RNA (Berlowitz, Pallotta & Sibley, 1968, 1969; Brachet & Hulin, 1970). It has also been suggested that binding of actinomycin-D is associated with physicochemical changes in the deoxyribonucleoproteins (Bolund, 1969; Darzynkiewicz, Gledhill & Ringertz, 1969; Pederson & Robbins, 1970). During spermatogenesis, RNA synthesis ceases before the structure and composition of the nucleoproteins change (Monesi, 1964; Utakoji, 1966; Darzynkiewicz et al., 1969; Gledhill, 1971; Gledhill, Darzynkiewicz & Ringertz, 1971).

Ram germinal cell squashes were fixed in 40% acetic acid and then incubated at 37°C for 15 min in Ringer–phosphate solution containing 0.166 µCi [3H]-actinomycin-D/ml (190 mCi/mmoll. Unfixed smears of bull germinal cells were incubated in the same solution for 1 hr at 30°C and were then fixed for 20 min in formol vapour.

Autoradiographs were prepared with Ilford K5 emulsion and exposed for 1 to 3½ months. For each step of spermiogenesis, the intensity of labelling in at least fifty spermatids was measured photometrically using a Leitz microspectrophotometer or by visual grain counting. The mean intensities of labelling have been corrected for background.

Small pieces of ram testis were fixed in acetic acid/alcohol and 4-µm paraffin wax sections were stained by the following methods:

1. toluidine blue at pH 4.1 (Prescott & Bender, 1962) and azure B at pH 4 (Flax & Himes, 1952). Both these stains are taken up by the free phosphoryl groups of nucleic acids.
2. Sakaguchi’s test, as modified by Notenboom, Van de Veerdonk & Van de Kamer (1967), which is specific for protein-bound arginine.
3. ninhydrin–Schiff reaction (Yasuma & Ichikawa, 1958) and dinitrofluorobenzene reaction, as modified by Danielli (1949), which demonstrate protein-bound lysine.

The intensity of nuclear staining was estimated by a single observer using an arbitrarily chosen subjective range from 0 to 5. Estimation was facilitated by comparing spermatid nuclei at different stages of development in adjacent tubules. Variations in the intensity of staining reflected variations in the quantity of stain bound by the whole nuclei because the shape and the volume of the nuclei (step 9 to step 15 spermatids) varied little and only nuclei judged to

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be lying entirely within the section were used. The validity of such semi-quantitative determinations has been confirmed by spectrophotometric measurements of squashes after Sakaguchi's staining (C. Ésnault, unpublished observations).

In both species, spermatids fixed before or after incubation with $^{3}$H]actinomycin-D were labelled. The intensity of labelling was low in the round spermatids and at the start of the phase of nuclear lengthening (steps 1 to 11 of Clermont & Leblond, 1955). The intensity doubled during steps 12 and 13, diminished during steps 14 and 15, and differed little from the background in testicular spermatozoa (Text-fig. 1).

![Graph showing variation in intensity of labelling (corrected for background) of bull and ram spermatid nuclei after incubation with $^{3}$H]actinomycin-D. Hatched columns: ram; stippled columns: bull; mean number of silver grains per nucleus ± 2 S.E.M. Spz: testicular spermatozoa.

**Text-fig. 1.** Variation in intensity of labelling (corrected for background) of bull and ram spermatid nuclei after incubation with $^{3}$H]actinomycin-D. Hatched columns: ram; stippled columns: bull; mean number of silver grains per nucleus ± 2 S.E.M. Spz: testicular spermatozoa.

![Graph showing variation in staining intensity of step 9 to 15 spermatid nuclei in the ram with the following stains: ▼, azure B at pH 4; ●, toluidine blue at pH 4.1; ○, DNFB-2-4 dichloro α-naphthol; △, Sakaguchi's test. Spz: testicular spermatozoa.

**Text-fig. 2.** Variation in staining intensity of step 9 to 15 spermatid nuclei in the ram with the following stains: ▼, azure B at pH 4; ●, toluidine blue at pH 4.1; ○, DNFB-2-4 dichloro α-naphthol; △, Sakaguchi's test. Spz: testicular spermatozoa.

The intensity of staining of the free phosphoryl groups by toluidine blue and azure B increased in step 9 to step 11 spermatid nuclei, reached a maximum at step 12, and then declined (Text-fig. 2). By contrast, there was a rapid reduction in the intensity of staining of lysine during steps 10, 11 and 12 and the
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testicular spermatozoa did not take up the stain. The intensity of the Sakaguchi reaction for arginine increased in step 12 and step 13 spermatids.

The uptake of $[^{3}\text{H}]$actinomycin-D by step 14 and 15 spermatids has been studied in the bull by Gledhill et al. (1971), who found that the intensity of labelling diminished at the end of spermiogenesis. Brachet & Hulin (1970) did not state which types of spermatids were studied in the mouse. Neither group reported any increase in tritiated actinomycin uptake during spermiogenesis. Brachet & Hulin (1970) found that the intensity of labelling was much greater in primary spermatocytes than in spermatids. Similarly, in the bull, the intensity of labelling is greater in pachytene primary spermatocytes than in round spermatids (steps 1 to 9).

The parallel changes in the degree of actinomycin binding and in the intensity of staining with toluidine blue and azure B suggest an increase of the number of active sites in the nuclear chromatin of step 12 and 13 spermatids, due to unmasking of DNA during this phase of spermiogenesis. Similar changes in the intensity of staining of spermatids by toluidine blue have also been found in the rat (Lison, 1955).

Vaughn (1966) reported that during spermiogenesis in the rat, lysine disappears from the spermatid nuclei and then the amount of arginine increases. Similar changes have been demonstrated by autoradiographic studies of lysine and arginine incorporation in the ram (Loir, 1972). Vaughn suggested that the alteration of spermatid amino-acid composition was due to the replacement of somatic-type histone by new, arginine-rich histone. Thus, during spermiogenesis, depletion of nucleoprotein appears to cause unmasking of active sites in nuclear DNA because practically all histone of the somatic type disappears from the nuclei before being replaced by arginine-rich histone.

Incorporation of $[^{3}\text{H}]$uridine occurs in round spermatids but ceases at step 9 in both the ram (Loir, 1972) and the bull (M. T. Hochereau-de Reviers, unpublished data). It is also absent in steps 12 and 13 spermatid nuclei in spite of the apparent unmasking of DNA. This may be due to the absence of RNA polymerase in these nuclei which was reported by Moore (1971). In both species, therefore, the binding of actinomycin in the elongated spermatids is not related to the synthesis of RNA. It is of interest that studies in the nucleated erythrocytes of the chicken and in the cells of Microtus agrestis have led Bolund (1969) and Sieger, Garweg & Schwarzacher (1971) to the same conclusion.

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