Synthesis of Nuclear Proteins in Ram Spermatids

The Staput method by velocity sedimentation at unit gravity allows the useful separation of spermatids[1]. Using this technique we have investigated the synthesis of the nuclear proteins in ram spermatids. Ram germ cells were pulse-labelled with 5 amino-acids and processed concurrently by quantitative autoradiography and sedimentation at 1 *g* to determine the patterns of incorporation into the nuclear proteins as a function of spermatid maturation.

Materials and methods. Immediately after castration, small fragments of ram testis were incubated at 30–32 °C in 10 ml of phosphate buffered saline containing 0.1% glucose (PBSG) for 27 or 45 min with one of the following amino-acids: L-arginine-^{3}H, L-lysine-^{3}H, L-leucine-^{3}H, DL-tryptophan-^{3}H : 10 μCi/ml; L-cysteine-^{35}S and DL-cysteine-^{14}C : 1 μCi/ml. Two incubations were carried out simultaneously, one with 100 μg/ml cycloheximide added 10 or 15 min before adding the labelled amino-acid, the other without the inhibitor. Suspensions of testis cells were prepared by the trypsin method[1] in the presence of 100 μg/ml cycloheximide. An alternative procedure, trypsinization, was performed during the last 21 min of the labelling. Testis cells were separated by velocity sedimentation at unit gravity[1]. When entire cells were under consideration A_{280} was determined for every fraction[1]. When nuclei were studied, they were prepared in every fraction with cetrimide[2] and counted for total number.

To measure the incorporation of the amino-acids, the cells or nuclei of every fraction were washed with cold PBS and resuspended in cold 10% TCA (20 min at 4 °C). The precipitates were washed with 5% TCA, dissolved in 1 *M* NaOH by heating at 37 °C for 20 min, neutralized with 1 *M* HCl, re-precipitated with 10% TCA and washed with 5% TCA before being dissolved in 0.3 ml 1 *M* NaOH for counting of radioactivity. The TCA solutions contained the unlabelled amino acid at a concentration 1000 times that of the labelled amino acid in the incubation medium. When ^{35}S or ^{14}C-cysteine was used, the precipitates were extracted with chloroform and methanol and with TCA at 90 °C. After similar incubations with ^{3}H-arginine or ^{35}S-cysteine, but at 0 °C, the acid-insoluble radioactivity was 2 and 2.5% of that measured at 30–32 °C. In some experiments (Figure 2), the cells or nuclei of every fraction were collected on Whatman GF/A filters and treated according to LEE and DIXON[3].

Aliquots of labelled cell suspensions were washed twice at 4 °C with PBS (containing 1000 times the unlabelled amino-acid), smeared onto gelatin-coated slides, then after 15 min fixation by formol vapor, they were washed for 20 min at 2 °C with 5% TCA plus the unlabelled amino-acid (×1000) and processed for quantitative autoradiography as described before[4].

Results and discussion. Under our conditions of pulse-labelling, uptake and incorporation of ^{14}C-leucine by ram germ cells proceeded at a linear rate and the cell concentration remained constant for at least 2.5 h (Figure 1). When ram germ cells were labelled in vivo (Figure 2), the patterns of incorporation into the proteins of testis cells were similar to those observed after pulse-labelling except in magnitude. These facts enable us to consider the data obtained after pulse-labelling as indicative of the

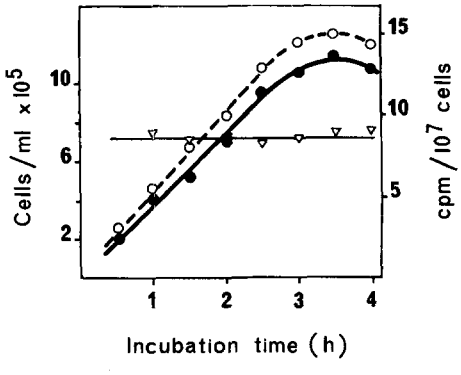

Fig. 1. Kinetics of ^{14}C-leucine uptake (○) and incorporation into total proteins (●) in ram germ cells. Variation of the concentration of cells in the labelling medium (△). 2×10^7 germ cells mechanically isolated. Incubation in 20 ml of PBSG plus 0.5 μCi/ml, at 31 °C. At various times, 1 ml samples were trypsinized in the presence of 100 μg/ml cycloheximide. Cells were collected free of cellular fragments by low speed centrifugation on 0.5% Ficoll in PBS. The total radioactivity and the TCA-insoluble radioactivity were determined (see methods) (cpm × 10^4). Cell counts were performed with a Coulter Counter. 3 rams; average curves.

[1] M. LOIR and M. LANNEAU, Expl Cell Res. *83*, 319 (1974).
[2] M. LOIR and A. WYROBEK, Expl Cell Res. *75*, 261 (1972).
[3] I. P. LEE and P. L. DIXON, Toxic. appl. Pharmac. *23*, 20 (1972).
[4] M. LOIR, Annls. Biol. anim. Biochim. Biophys. *12*, 411 (1972).

Distribution of spermatids at different stages of maturation[6], in percentage of nuclei (average ± SD), as a function of sedimentation velocity after 4 h of sedimentation in a non-linear Ficoll gradient

Sedimentation velocity (mm/h)	Classes of spermatids Stages of spermatid maturation			
	L + M 1–8a + 8b	N 9–12a	O 12b–13	P 14–S
3.5–4.8	88 ± 4.5	7 ± 2	1 ± 0.9	1 ± 0.6
2.35–3.5	55 ± 11	25 ± 6	12 ± 3	5 ± 1.5
1.2 –2.35	4 ± 2	43 ± 4	34 ± 3	15 ± 4
0.45–1.2		13 ± 2	30 ± 5.5	56 ± 4

Mean of 6 experiments.

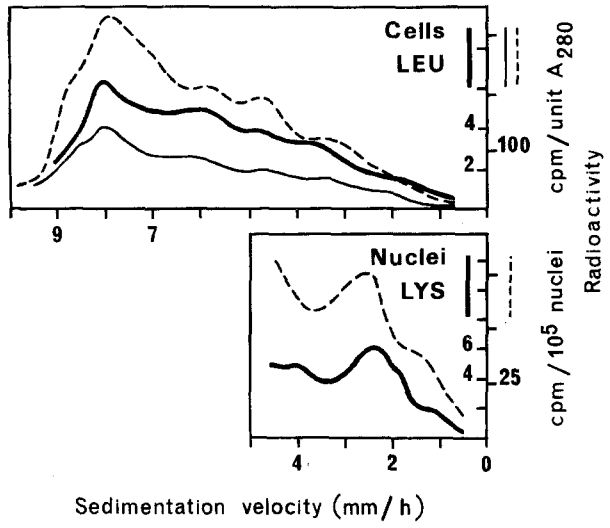

Fig. 2. Incorporation of ^{3}H-leucine and ^{3}H-Lysine in cellular and nuclear proteins of ram testis cells as a function of sedimentation velocity. Cells were labelled by 3 methods. 1 h in vivo labelling[6] (12 µCi/g testis, ——); 27 min in vitro labelling before trypsinization (- - - -), and simultaneously with trypsinization (——).

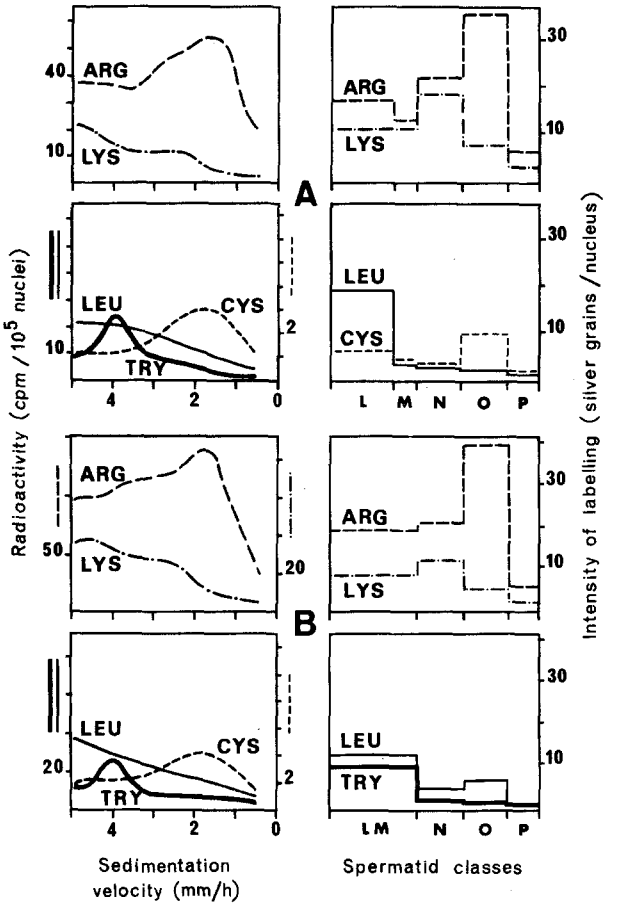

Fig. 3. Incorporation of 5 labelled amino-acids into the nuclear proteins of ram spermatids as a function of the sedimentation velocity (left) and as a function of steps of spermatid maturation as seen by autoradiography (right). Cells were pulse-labelled for 27 min (A) and for 45 min (B) before trypsinization. For spermatid classes L-P, see Table. Each curve (left and right) is the average for 2 or 4 rams.

in situ metabolism of the germ cells. Whether pulse-labelling proceeded before or concomitantly with trypsinization (Figure 2), the patterns of incorporation into the proteins of spermatids did not differ significantly, except in magnitude, this being in agreement with the observations of TSAN et al.[5].

Patterns of incorporation of the 5 amino acids into the nuclear proteins of spermatids pulse-labelled for 27 and 45 min are shown in Figure 3. Cycloheximide was found to inhibit these incorporations by 90–98%. The distribution of various classes of spermatids[6] as a function of the sedimentation velocity (SV) is reported in the Table. The variations in amino acid incorporation between 4.8 and 0.45 mm/h are similar to those during spermiogenesis as seen by autoradiography. However, small variations which concern spermatids 8b–12a, are less apparent between 1.2 and 3.5 mm/h, where most of these cells sediment, than on autoradiographs. This is due to the broad distribution of spermatids 12b–13. Our results confirm and complete, for short labelling times, our previous observations[4,7]. It appears that round spermatids synthesize mainly non-histone proteins since they are tryptophan-rich and cystein-moderately-rich. Appearance in spermatids 12b–13 of the basic sperm-specific nucleoprotein, arginine, cysteine-rich and lysine, tryptophan-poor is preceded by the synthesis in spermatids 9–12a of protein(s) which are acid-soluble[4], arginine and lysine-rich, tryptophan and cysteine-poor. 3 proteins with similar characteristics have been isolated from trout spermatids[8] and 1 from rat spermatids[9]. In ram[10], several basic proteins are present in nuclei of spermatids 9–12. Studies are in progress to elucidate which of these proteins result from the synthesis that our data demonstrate.

Résumé. La synthèse des protéines nucléaires dans les spermatides de bélier a été étudiée après marquage avec 5 acides aminés tritiés, soit par comptage à scintillation liquide après séparation des spermatides par sédimentation à 1 g, soit par autoradiographie. Les spermatides rondes synthétisent essentiellement des protéines non-histones. La synthèse de la nucléoprotéine spécifique du spermatozoïde dans les spermatides allongées est précédée par celle de protéine(s) de type histone.

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[5] M. F. TSAN, R. A. TAUBE and R. D. BERLIN, J. Cell Physiol. *81*, 251 (1973).
[6] M. LOIR, Annls. Biol. anim. Biochim. Biophys. *12*, 203 (1972).
[7] M. LOIR, Annls. Biol. anim. Biochim. Biophys. *12*, 531 (1972).
[8] K. MARUSHIGE and G. H. DIXON, J. biol. Chem. *246*, 5799 (1971).
[9] W. S. KISTLER, M. E. GEROCH and H. G. WILLIAMS-ASHMAN, J. biol. Chem. *248*, 4522 (1973).
[10] M. LOIR and M. LANNEAU, Expl. Cell Res., accepted for publication.
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