BIOSYNTHESIS OF PROTAMINE DURING SPERMATOGENESIS IN SALMONOID FISH*

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The protamines are small (M.W. 4-5,000), highly basic proteins which occur associated with DNA in the nuclei of certain spermatozoa (reviewed by Felix, 1960). Their content of arginine is very high, for example in clupeine (from herring, Clupea pallasii), of 31 residues, 21 are arginine (Ando et al, 1962). Miescher (1874) first observed that protamines appeared late in the maturation of the Rhine salmon (Salmo salar) testes and this has been confirmed in several other species (Ando and Hashimoto, 1957; Felix et al, 1958). Alfert (1956) concluded on the basis of histochemical studies that during spermatogenesis in Chinook salmon (Oncorhynchus tshawytscha) protamines replaced histones at the spermatid stage.

In the experiments described here, the biosynthesis of protamine has been examined in developing salmonoid testes. Sperma-

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togenesis was induced in immature Steelhead trout (Salmo gairdnerii) by injection of salmon (Oncorhynchus) pituitary extracts (Schmidt et al, 1965) to provide a continuing source of experimental material. Using cell suspensions from these testes and also from those in naturally maturing Pacific salmon, active incorporation of arginine-H^3 into protamine has been observed. The effects of the inhibitors cycloheximide, puromycin and chloramphenicol indicate that protamine, despite its small size and unusual composition is synthesized by the familiar route involving messenger RNA, ribosomes and sRNA.

MATERIAL AND METHODS

Steelhead trout aged 10-20 months were kept at 12°C and given twice-weekly intraperitoneal injections (0.05 ml) of a standard Chinook salmon pituitary gland extract containing gonadotrophic activity (Schmidt et al, 1965). Fish were killed at appropriate intervals and acid extracts of the testes were made by homogenizing the tissue briefly in 0.2N HCl^- and centrifuging at 12,000 g for 30 minutes. The proteins in the lyophilized acid extracts were examined by vertical electrophoresis (200 volts, 75 ma., 4 hours) in polyacrylamide gels (0.1 M acetate, pH 4.5) (Cruft, 1962) cast in standard gel trays. The gels were stained for one hour with a solution of Amido Schwarz 10B in 2% acetic acid, and destained electrophoretically.

Suspensions of intact cells were prepared from the testes of Steelhead trout receiving the pituitary extract injections, or from the maturing testes of Chinook or Sockeye (O. nerka) salmon caught during their spawning migrations upriver. One ml aliquots of scissor-minced testes in 5 ml Hanks medium (Baltimore Biological Laboratory) were gently homogenized at 0°C by hand in Potter-

Elvehjem tubes with teflon pestles. The resulting suspension was twice centrifuged (5 min. at 900 g) and resuspended in Hanks medium. The cells at a final dilution of 1 ml packed cells in 8 ml Hanks medium were preincubated in 1.0 ml aliquots at 0°C for 90 minutes to equilibrate with the appropriate inhibitor. The incubations were begun by the addition of 5 μC L-arginine-H^3 (final concentration of arginine, 1 x 10^{-5} M) and transfer of the tubes to gyratory shaking in a 20°C water bath. The incubation was stopped by addition of 2.0 ml Hanks medium, 1 x 10^{-3} M in unlabelled arginine and cooling to 0°C. After centrifugation (5 min. at 900 g) the packed cells were suspended in 0.5 ml water and frozen.

Protamine was purified by electrophoresis (as above) of lyophilized 0.2N HCl extracts of the cells. The position of the protamine band in the gel was determined by surface staining the gel briefly with 1% (aqueous) Wool Fast Blue BL which penetrates polyacrylamide extremely slowly. The stained protamine area was then cut out, minced by passing through a disc of stainless steel screening (150 mesh) in a syringe, and the gel particles packed in a small column for elution with 0.1 M acetic acid. Lyophilized eluates were hydrolyzed for 16 hours with 5.7N HCl at 105°C, dried, and dissolved in H_2O. Aliquots were removed for counting in the scintillation fluid of Bray (1960) and arginine was determined by a Sakaguchi reaction (Satake and Luck, 1958) modified to include a preacetylation step (Izumi, 1965).

Samples of the testes were fixed in Bouin's or buffered formalin solutions and sections stained with hematoxylin and eosin for light microscopy.

RESULTS AND DISCUSSION

As reported by Robertson and Rinfret (1957) and Schmidt et al

(1965) the weights of testes in immature trout receiving salmon pituitary extracts increased remarkably. This increase in weight (from 10-20 mgm to 1-5 gm over two months) was accompanied by marked histological and biochemical changes in the testes. The development of the immature testes, containing only cysts of spermatogonia at the start, paralleled that of the adult fish testes during the spawning migration. Within two months complete differentiation had occurred and mature spermatozoa were produced. This system, therefore, offers considerable potential for studying biochemical aspects, both of hormonally controlled cellular differentiation and the process of meiosis and gamete formation.

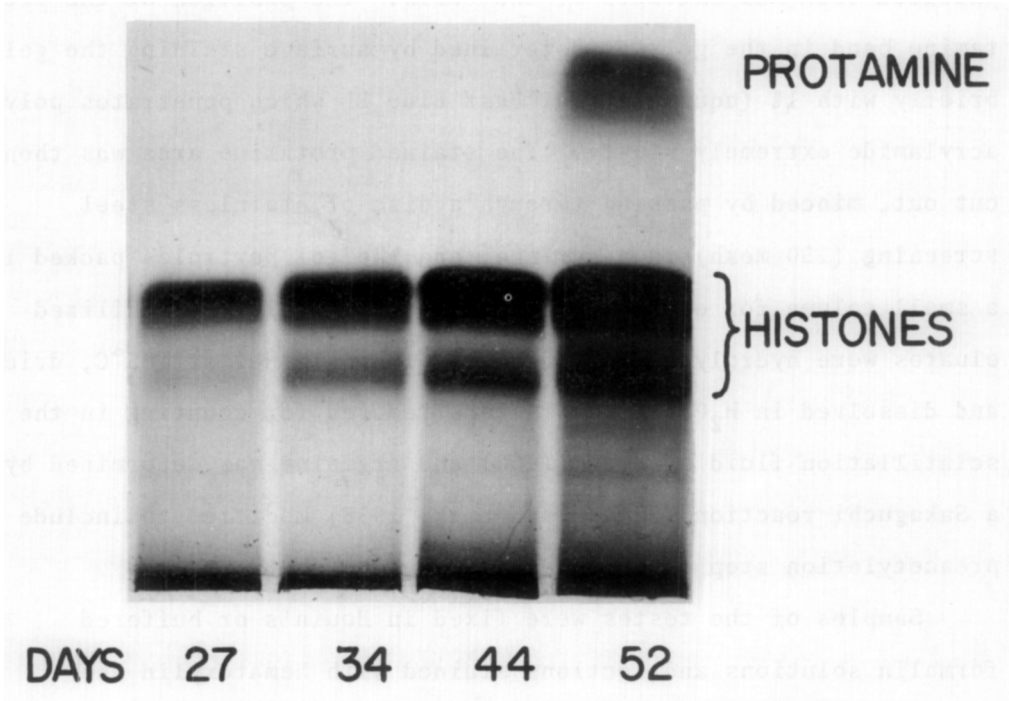

Fig. 1. Polyacrylamide electrophoresis of the proteins extracted by 0.2N HCl from Steelhead trout testes excised at the indicated intervals from the start of pituitary extract injections.

Polyacrylamide gel electrophoresis of the proteins in the 0.2N HCl extract of testes excised at intervals (fig. 1) shows the appearance at a late stage in spermatogenesis of a new protein band corresponding in mobility to the protamine extractable from mature testes. In the testes at this stage large numbers of spermatids were present. These results confirm the studies of Alfert (1956) mentioned above and those of Bloch (1962) who, with snail (Helix aspersa) and squid (Loligo opalescens), and grasshopper (Chortophagia viridifasciata) (Bloch and Brack, 1964) has observed both cytochemically and by autoradiography the appearance of arginine-rich histones and protamines in spermatid cells.

The biosynthesis of this newly appearing protamine was studied in the cell suspension sytem. Using cells from naturally maturing Chinook salmon testes, the time course of incorporation of

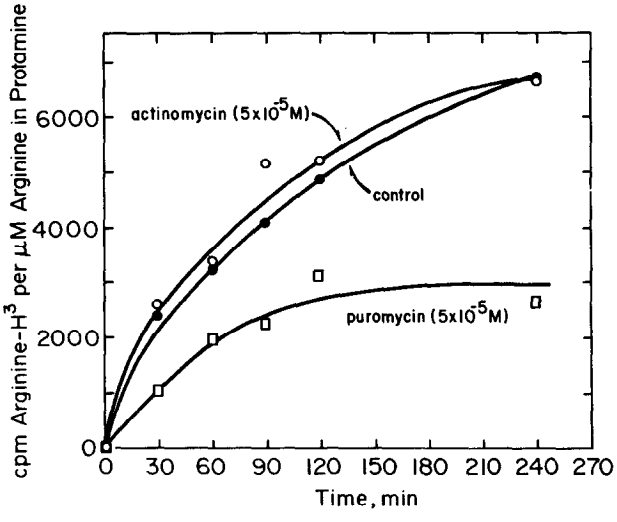

Fig. 2. Time course of incorporation of L-arginine-H^3 into protamine by a suspension of Chinook salmon testes cells.

arginine-H^3 into protamine was examined (fig. 2). Similar results were obtained in other experiments using testes cells from

both naturally maturing Sockeye salmon and immature Steelhead trout in which spermatogenesis had been induced by the pituitary extracts. There was strong inhibition of this incorporation by puromycin but not by actinomycin D. The effects of these inhibitors, puromycin and actinomycin D, as well as chloramphenicol and cycloheximide at different concentrations on cells of induced immature trout testes is shown in figure 3. Cycloheximide

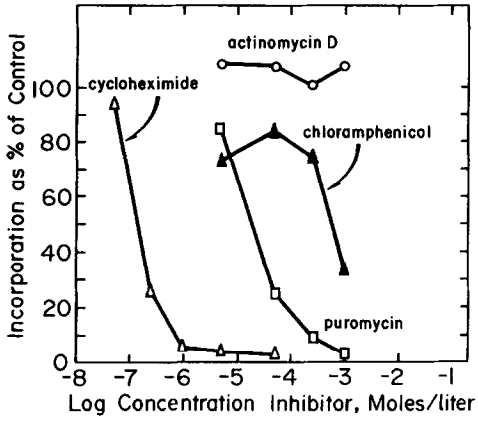

Fig. 3. Incorporation of L-arginine-H^3 into protamine by Steelhead trout testes cells in the presence of varying concentrations of inhibitors of protein synthesis. The incubations were for 90 minutes under conditions described in text.

is the most potent inhibitor producing 50% inhibition at 1.4×10^{-7} M. This extent of inhibition was only produced by puromycin at 1.9×10^{-5} M. Bennett et al (1964) found that in mammalian cells also, cycloheximide was more effective than puromycin, but the inhibition in the present system occurs at much lower levels of cycloheximide than in the mammalian system. Chloramphenicol, the potent inhibitor of bacterial protein synthesis was only effective at concentrations greater than that for puromycin, while actinomycin D did not inhibit protamine synthesis at all over a four hour period.

Suggestions for the sites of action of these inhibitors in protein synthesis have been made. Three appear to act upon the transfer of aminoacyl groups from sRNA to peptide linkage; puromycin inhibits the transfer reaction by acting as an analog of amino-acyl-sRNA (Nathans, 1964), chloramphenicol competes with mRNA on the ribosomes (Weisberger and Wolfe, 1964), while cycloheximide interrupts completion of polypeptide chains and their release (Siegel and Sisler, 1964; Williamson and Schweet, 1965). The inhibition of arginine-H^3 incorporation into protamine by these three inhibitors is strong evidence for the involvement of a mRNA, sRNA, ribosome system in the synthesis of protamine.

The fact that actinomycin D did not inhibit the synthesis of protamine is not due to the impermeability of the cells to actinomycin D. In control experiments with similar cells, actinomycin D (5×10^{-5} M) inhibited the incorporation of Uridine-H^3 into RNA 77%. Since the site of action of actinomycin D has been clearly shown to be upon the transcription of the DNA sequence into RNA (Reich and Goldberg, 1964), the lack of inhibition indicates that a putative mRNA for protamine has a relatively long life in the testes cells.

A study of the synthesis of protamine in this system is being pursued because of the small size and unusual amino acid composition of this protein. The messenger RNA for protamine might consist of only 90-100 nucleotides and due to the overwhelming predominance of arginine in protamine, where it is known to occur in "blocks" of up to four residues (Ando et al, 1962), should possess sections of repeating nucleotide sequence. Sequence determination on this mRNA might allow the determination of the naturally occurring codon(s) for arginine out of the five possibilities suggested by in vitro experiments with synthetic poly-

nucleotides (Nirenberg et al, 1965; Khorana, 1965).

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REFERENCES

Alfert, M. (1956). J. Biophys. Biochem. Cytol. 2, 109.
Ando, T., and Hashimoto, Ch. (1957). Proc. Intern. Symp. on Enzyme Chem., Tokyo and Kyoto, p. 380.
Ando, T., Iwai, K., Ishii, S., Azegami, M., and Nakahara, C. (1962). Biochim. Biophys. Acta 56, 628.
Bennett, L.L., Smithers, D., and Ward, C.T. (1964). Biochim. Biophys. Acta 87, 60.
Bloch, D.P. (1962). J. Histochem. Cytochem. 10, 137.
Bray, G.A. (1960). Anal. Biochem. 1, 279.
Clark, J.M., and Chang, A.Y. (1965). J. Biol. Chem. 240, 4734.
Cruft, H.J. (1962). Biochem J. 84, 47P.
Felix, K. (1960). Advan. Protein Chem. 15, 1.
Felix, K., Goppold-Krekels, A., and Lehmann, H. (1958). Z. Physiol. Chem. Hoppe-Seyler's 312, 57.
Izumi, Y. (1965). Anal. Biochem. 10, 218.
Khorana, H.G. (1965). Fed. Proc. 24, 1473.
Miescher, F. (1874). Ber. deut. chem. Ges. 7, 376.
Nathans, D. (1964). Fed. Proc. 23, 984.
Nirenberg, M., Leder, P., Bernfield, M., Brimacombe, R., Trupin, J., Rottman, F., and O'Neal, C. (1965). Proc. Nat. Acad. Sci. 53, 1161.
Reich, E., and Goldberg, I.H. (1964). In Prog. in Nucleic Acid Res. and Mol. Biol., J.N. Davidson and W.E. Cohn, eds., Academic Press N.Y. Vol. 3, p. 183.
Robertson, O.H., and Rinfret, S.P. (1957). Endocrinology 60, 559.
Satake, K., and Luck, J.M. (1958). Bull. Soc. Chim. Biol. 40. 1743.
Schmidt, P.J., Mitchell, B.S., Smith, M., and Tsuyuki, H. (1965). Gen. Comp. Endocrin. 5, 197.
Siegel, M.R., and Sisler, H.D. (1964). Biochim. Biophys. Acta 87, 83.
Weisberger, A.S., and Wolfe, S. (1964). Fed. Proc. 23, 976.
Williamson, A., and Schweet, R. (1965). J. Mol. Biol. 11, 358.