The Biosynthesis of Protamine in Trout Testis

II. POLYSOME PATTERNS AND PROTEIN SYNTHETIC ACTIVITIES DURING TESTIS MATURATION*

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SUMMARY

Protamines are synthesized in the cytoplasm of rainbow trout (Salmo gairdnerii) spermatid cells. Following a pulse of 14C-arginine, the majority of the labeled nascent protamine is found on diribosomes (disomes) sedimenting at 120 S (LING, V., TREYTHICK, J. R., AND DIXON, G. H., Can. J. Biochem., 47, 51 (1969)). Polysonme profiles obtained at various times during testis maturation, which, in this species, takes place from September to December, show the appearance of high concentrations of such disomes during the period of rapid protamine synthesis. This effect is not observed in trout liver polysomes isolated under the same conditions, the polysomes being independent of the sexual maturation of the fish. Spermatid cell disomes are not disaggregated by lowering Mg++ concentration during isolation or by the addition of pancreatic ribonuclease. Pulse-chase experiments with 14C-arginine show that disomes are labeled more rapidly than heavier polysomes. The distribution of incorporated 14C-arginine and 14C-lysine in the polysomes suggests an ordered synthesis of protamine only during the early protamine stage of development, suggesting that messenger RNA for protamine is synthesized before protamine synthesis takes place and is metabolically stable.

We have previously presented several lines of evidence that at a late stage of spermatogenesis protamine is synthesized in the cytoplasm of rainbow trout testis cells and is quickly transported into the nucleus where it eventually replaces the histones on DNA (1). A special class of cytoplasmic polysomes, the diribosomes (disomes), sedimenting at 120 S, appears highly active in incorporating 14C-arginine and has been implicated as the species of polysome synthesizing protamine (1). In this present report, we substantiate further our previous observation of cytoplasmic disomes as the site of protamine synthesis and present evidence of specific developmental changes in the testis polysome population as the testis cell matures. Concomitant with the changes in polysome profiles, there are major alterations in the relative incorporation and distribution of labeled lysine and arginine in the polysomes which suggest an ordered synthesis of different proteins as development proceeds.

MATERIALS AND METHODS

Trout Testis—Testes at various stages of sexual maturation were obtained at the middle of each month during August to December 1968 from naturally maturing rainbow trout (Salmo gairdnerii) (13 to 18 months old) raised in the Sun Valley Trout Farm, Coquitlam, British Columbia. Excised testes were placed on ice, transported to the laboratory, and washed with cold running tap water. A number of testes (usually about 20) were pooled and minced with scissors, and a portion of the mince was removed for incubation with radioactive amino acids. The excised testis could be kept on ice for up to 4 hours without appreciable loss in ability to incorporate labeled amino acids into protamine. The testes not required for incubation were quickly frozen on Dry Ice and stored at −80° until use.

Cell Incubations—Cell suspensions were prepared with 10 to 20 g of fresh testes in Buffer TMKS (Tris-HCl, 0.05 M; magnesium acetate, 0.005 M; potassium chloride, 0.025 M; and sucrose, 0.25 M), pH 7.6, as previously described (1). Identical aliquots of the cell suspension were incubated separately with 14C-arginine and 14C-lysine (0.5 μCi per ml of labeled amino acid per incubation mixture, specific activities of 226 to 241 mCi per mg, New England Nuclear) at 20° for 30 min in a gyratory water bath (New Brunswick). Incubations were terminated by the addition of cycloheximide (Calbiochem) to a final concentration of 0.4 μM.

Isolation of Ribosomes—Ribosomes were prepared from trout testis and liver by a method similar to that described by Wettstein, Stachelin, and Noll (2). All operations were performed at 2–4°. Frozen or fresh tissue was minced with scissors in 1 to 2 volumes of Buffer TMKS and homogenized in a Tri-R tissue homogenizer (Tri-R Instruments, New York) at 5000 rpm for 20 sec. The homogenate was centrifuged at 15,000 × g for 15 min to obtain a postmitochondrial supernatant. The post-
TABLE I

Synthesis of protamine as function of testis development in naturally maturing rainbow trout

Testes were collected at the middle of each month, from August to December, and cell suspensions were prepared from a number of testes. The cells were incubated with 0.05 µCi of 14C-arginine per ml of incubation mixture of 14C-arginine at 20°C for 30 min and were fractionated into a nuclear fraction and cytoplasmic fraction by differential centrifugation after homogenization (1). Ribosomes were prepared from the cytoplasmic fraction as described under "Materials and Methods" and the amount of ribosomal RNA obtained per g of testis was determined by the orcinol method (5) with yeast RNA as standard. The nuclei were extracted with 0.2 M H2SO4 and protamine was isolated by chromatography of the acid-soluble proteins on Bio-Gel P-10 (1). The amount of radioactivity incorporated into protamine was determined.

| Month   | Stage       | Average weight per testis (g) | Ribosomal RNA | 14C-Arginine incorporated into protamine |
|---------|-------------|------------------------------|---------------|------------------------------------------|
| August  | Preprotamine| 1.6                          | 5.9%          | mg/g testis                             |
| September | Early protamine | 7.1                      | 1.54         | mg/g testis/30 min                       |
| October | Protamine   | 10.0                        | 0.71         | <10                                      |
| November | Protamine    | 9.3                        | 0.29         | 2,420                                    |
| December | Late protamine | 4.0                      | 0.08         | 9,380                                    |
| Liver   | All         | 5.6                         |              | Total radioactivity                      |

a Average weight of six testes.
b Liver weight is constant.

mitochondrial supernatant was treated with the nonionic detergent Triton X-100 (Rohm and Haas) at a final concentration of 2% for 10 min and centrifuged again at 15,000 x g for 15 min. The detergent-treated postmitochondrial supernatant was layered over 3 ml of dense sucrose (40% w/v) made up in Buffer TMK (Tris-HCl, 0.05 M; magnesium acetate, 0.005 M; and potassium chloride, 0.025 M), pH 7.6, in a 13-ml polylamellar high speed centrifuge tube (Beckman-Spinco). Centrifugation was performed at 60,000 rpm for 2 hours in a No. 65 rotor (Spinco), the supernatant above the dense sucrose layer was aspirated off, and then the dense sucrose layer itself was similarly removed, leaving a clear ribosomal pellet. The inside wall of the centrifuge tube was wiped with absorbent paper tissue and the ribosomal pellet was briefly rinsed with a small volume of cold Buffer TMK. The pellet was then dissolved in Buffer TMK, yielding an opalescent solution.

Sucrose Density Gradient Analysis of Ribosomes—Sucrose solutions of appropriate concentrations were prepared in Buffer TMK with ribonuclease-free density gradient grade sucrose (Mann). Linear sucrose gradients (35 ml) of 10 to 34% w/v were generated by a Beckman density gradient former (DGF-IM-3) in cellulose nitrate tubes at room temperature. The gradients were then cooled at 4°C for 2 to 3 hours. Appropriate concentrations of ribosome solutions were made up in Buffer TMK, layered over the gradients, and centrifuged at 4°C in a SW-27 swinging bucket rotor (Spinco) at 26,000 rpm for 2.5 hours. The rotor was allowed to stop without braking. The centrifuge tube was punctured at the bottom with a needle and equal fractions were collected for analysis of absorbance at 260 nm and radioactivity. For the assay of radioactivity, an aliquot was removed from each fraction, carrier proteins (bovine serum albumin and protamine) were added, and total protein was precipitated with cold pH 2 trichloroacetic acid-tungstate solution (3). The precipitate was collected by centrifugation, washed once with trichloroacetic acid-tungstate, resuspended in 0.5 ml of distilled water, and transferred to a glass scintillation vial. Radioactivity was assayed in 10 ml of Bray's fluid (4) with a Unilux liquid scintillation counter.

Results

Trout Testis Development and Time of Protamine Synthesis—The immature testis collected from 1.5- to 2-year-old rainbow trout during August weighs approximately 1.6 g and rapidly increases in weight to a maximum of 10 g by October (Table I). During this period of rapid weight increase, the spermatogonial stem cells in the testis are presumably undergoing repeated mitoses to form a large number of cells which, after the meiotic divisions, subsequently develop into sperm cells (6, 7). By December, however, there is a sharp decrease in the average weight of the testis, partly as a result of the release of mature spermatozoa. These fish normally spawn during January and February.

The rate of incorporation of 14C-arginine into protamine varies considerably as development progresses (Table I). As previously shown (1), at the stage when protamine synthesis is rapid there is little histone synthesis. Table I indicates that rapid protamine synthesis occurs at a late stage of spermatogenesis during October and November when there is no further increase in testis weight. Recent experiments involving fractionation of trout testis cells by the method of Lam, Furrer, and Bruce (8) have shown clearly that protamine synthesis occurs in spermatid cells and these cells are predominant in the trout testis during October and November. Although the differentiation of secondary spermatocytes first to spermatids and then to mature spermatozoa is occurring during this period, no cell divisions are involved and there is no increase in cell number. When the greater proportion of the cells in the testis is mature spermatids and sperm cells, protamine synthesis has largely ceased (Table I). From Table I it may also be seen that the number of ribosomes per g of testis progressively decreases with development until the

1 A. Louis, D. Lam, and G. H. Dixon, manuscript in preparation.
profiles of trout testis were examined at various stages of spermatogenesis, changes in the relative amounts of different classes of polysomes were observed (Fig. 1). The monosomes and the disomes sediment at 77 S and 120 S, respectively (1). It may be seen in Fig. 1 that large peaks of disomes are present in the poly-
some profiles obtained from October and November testes. In
September and December, these disomes are present in only relatively small numbers. It is significant that protamine syn-
thesis is most rapid during October and November when the relative proportion of disomes in the polysome population is greatest (Table I). This suggests that the rate of protamine
synthesis is a function of the number of disomes present and is consistent with previous observations (1) that protamine is syn-
thesized on disomes. During late November, the number of monosomes increases sharply, although at this stage the disome peak is still large. The source of these monosomes appears to be a degradation of the larger polysomes but in December, when there is a marked decrease in the total number of ribosomes (Table I), the disomes themselves largely disappear and the great majority of polysomes are converted to the monomeric state.

Effect of Mg++ on Polysome Profiles—Various investigators
(13–15) have noted that dimeric ribosomes sedimenting at 110 to
120 S are formed as the result of an artifactual aggregation of
ribosomes at high magnesium concentrations. Since these
dimeric ribosomes either dissociate at 1 mM Mg++ ion concentra-
tion or are absent when extracted at this lower magnesium
concentration (15), two parallel extractions of polysomes were
performed on testes obtained at the protamine stage. Two
TMK buffers, one containing the usual 5 mM magnesium acetate and the other 1 mM magnesium acetate, were used for preparation of polysomes. It may be seen in Fig. 2 that polysomes extracted with 5 mM Mg++ show few monosomes. Conversely, the ribo-
somes extracted with 1 mM Mg++ appear to have substantially fewer large polysomes than those extracted with 5 mM Mg++. However, the two profiles are similar in that both have prominent peaks of disomes sedimenting at 120 S. Since the disomes are

late protamine stage in December when there is an even sharper
decrease. This decrease in the number of ribosomes during spermatogenesis is consistent with the observations of various
investigators (9–11) who have shown that cytoplasm is sloughed
off from developing sperm cells in rat testis. Crecelius and
Tominson (12) have also observed a progressive decrease in RNA
content in the maturing salmon testis.

It is clear also that the total number of ribosomes present at a
particular stage does not reflect the rate of 14C-arginine incorpora-
tion into protamine since, at the preprotamine stage when little
protamine is synthesized, there are more ribosomes than during
the protamine stage when protamine is rapidly synthesized.

Polysome Profiles during Development—When the polysome

FIG. 2. Effect of low Mg++ ion concentration on testis disomes.
Ribosomes were isolated from trout testis in Buffer TMKS (A) containing the usual 5 mM magnesium acetate and (B) 1 mM magnesium acetate. The ribosomes were analyzed in sucrose gradients containing the appropriate magnesium concentration on a SW-27 rotor. The profiles of the testis polysomes were monitored as in Fig. 1.
Effect of Pancreatic Ribonuclease on Polysome Profiles—The decrease in the proportion of larger polysomes observed in ribosomes extracted in 1 mM Mg\(^{++}\) (Fig. 2B) is likely to be the result of degradation of these polysomes by nucleases released during isolation in the presence of the lower magnesium ion concentration. The increase in the number of monosomes (Fig. 2B) supports this idea since nuclease usually degrades polysomes into monosomes (16–18). This, however, would suggest that the disomes observed in Fig. 2 are resistant to nuclease action since there appears to be no decrease in the number of disomes. The effect on testis polysomes of a mild treatment with pancreatic ribonuclease was therefore examined (Fig. 3). The ribosomes were purified from testis cells previously incubated with \(^{14}\)C-arginine and hence contained labeled nascent proteins associated with them. It may be seen in Fig. 3A that there are two major peaks of 260 nm absorbance (M and D) in the polysome profiles corresponding to monosomes and disomes, respectively. The major peak of radioactivity is associated with the disomes (D) and radioactivity is also found in the large polysomes. After ribonuclease treatment (Fig. 3C), there is a decrease in the amount of absorbance at 260 nm in the large polysome region and an increase in monosomes (M). Radioactivity associated with the larger polysomes is also removed by the ribonuclease treatment. When bentonite (18, 20) is added before the ribonuclease to moderate the severity of the treatment (Fig. 3B), there is partial protection of the larger polysomes and some \(^{14}\)C-arginine remains associated with them. These observations are consistent with those of other investigators (16, 18) who have studied the effect of mild ribonuclease treatment on bacterial and animal polysomes. As may be seen from Fig. 3C, the disomes (D), in contrast to the large polysomes, are relatively unaffected by mild ribonuclease treatment.

Time Dependence of Incorporation into Polysomes and Disomes—To determine whether the disomes might result from degradation of larger polysomes during the isolation procedure, ribosomes were prepared from two identical testis cell suspensions after a pulse of \(^{14}\)C-arginine at 20° for 2 min and 40 min, respectively. The polysome profile in cells from each incubation was examined by sucrose density gradient centrifugation (Fig. 4). After 2 min of incorporation, the major peak of radioactivity is associated with disomes sedimenting at 120 S while the larger polysomes and the monosomes (77 S) contain little radioactivity. After 40 min of incorporation, however, the amount of radioactivity associated with the disomes and monosomes remained essentially the same as that observed after 2 min of incorporation, but now more radioactivity is associated with the larger polysomes. Since the \(^{14}\)C-arginine appears first on the disomes and only after a longer incubation period on the larger polysomes, the disomes cannot be the breakdown products of larger polysomes. Further, nuclease usually degrades polysomes into monosomes (16–18) and since after a longer incubation period the increase in label on the larger polysomes is not reflected by an increase in radioactivity on the monosomes (Fig. 4), it appears that testis polysomes are degraded neither by nuclease during the incubation nor during the isolation procedure at the standard 5 mM Mg\(^{++}\) concentration.

Mitochondrial Ribosomes—In the treatment of the postmitochondrial cytoplasmic fraction with Triton X-100 during the preparation of ribosomes, there was the possibility that a small number of mitochondria still present after differential centrifugation would be lysed (21) and the mitochondrial ribosomes (22, 29)
would then be released into the cytoplasm. As a result, it was possible that the peak of radioactive protamine appearing in the disome region of the cytoplasmic testis polysomes (1) could be due to the presence of mitochondrial ribosomes. To check the possibility of a mitochondrial site of synthesis for protamine, the ribosomes of the postnuclear supernatant (cytoplasm plus mitochondria) and those of the postmitochondrial supernatant were analyzed after testis cells were incubated with 14C-arginine for 2 min at 20°. The results are shown in Fig. 5, A, B, and C. The "mitochondrial ribosomes" of Fig. 5C were prepared from the sediment obtained by centrifugation of the postnuclear supernatant of Fig. 5A. It is observed that the absorbance profiles of the ribosomes prepared from the postnuclear and postmitochondrial supernatants (Fig. 5, A and B) are essentially the same; the radioactivity profiles are also similar with the major peak of radioactivity in the disome region. While the total amount of radioactivity incorporated into the disome region is similar for the postnuclear and postmitochondrial fractions, the specific activity, i.e. counts per min of 14C-arginine per absorbance at 260 nm, in the disome region is higher in the postmitochondrial fraction than in the postnuclear ribosomes. This suggests that additional mitochondrial ribosomes deliberately released by detergent treatment of the postnuclear supernatant are not contributing to the synthesis of protamine; otherwise, the specific activity as well as the total incorporation into the disome region of the postnuclear supernatant ribosomes would be higher than with the postmitochondrial supernatant ribosomes. Further, it is seen that the ribosomes prepared directly from a crude mitochondrial fraction incorporate little radioactivity with low specific activity in the disome region (Fig. 5C).

Variation in Pattern of Incorporation of 14C-Arginine during Testis Maturation—The qualitative protein-synthesizing ability of testis ribosomes during testis development was approached by examining the distribution of 14C-arginine and 14C-lysine incorporated into nascent proteins on polysomes prepared from whole testis cells pulsed for 30 min with the two amino acids. In Fig. 6, I to IV, it may be seen that incorporation of each basic amino acid follows a definite pattern from September to December which varies at each stage of development. In particular, with October and November testis (Fig. 6, II and III), the disome fraction, sedimenting at 120 S, incorporates arginine but very little lysine. This observation is consistent with the previous location of protamine synthesis in this fraction (1) since two-thirds of the total residues of protamine are arginines and its lysine content is negligible.2 This lack of lysine label in the disome region suggested that the ribosomes prepared directly from a crude mitochondrial fraction incorporate little radioactivity with low specific activity in the disome region (Fig. 5C).

Footnote:
2 No lysine residue was observed when the amino acid composition of total unfractionated trout protamine was analyzed. However, a very small lysine content was seen in a minor component of protamine (V. Ling, B. Jergil, and G. H. Dixon, manuscript in preparation).
FRACTION NO. FRACTION NO.

FIG. 6. Developmental changes in protein synthetic activity of testis polysomes. Cell suspensions were prepared from testes of naturally maturing trout obtained at the middle of each month. The cell suspensions were incubated with $^{14}$C-arginine (specific activity, 226 mCi per mm) and $^{14}$C-lysine (specific activity, 214 mCi per mm) (0.5 μCi per ml of labeled amino acid per incubation mixture) at 20°C for 30 min and ribosomes were purified from these cell suspensions as before. The polysome profile and radioactivity associated with the polysomes were analyzed after sucrose density gradient centrifugation on a SW-27 rotor. I, September; II, October; III, November; IV, December. In each case, Frame A, the continuous recording of $A_{420}$ in the effluent from the sucrose gradients is included since the resolution of polysomes is much more clearly seen by this method than by the absorbance of collected fractions shown by the closed circles in Frames B and C.

TABLE II
Incorporation of $^{14}$C-arginine into protamine in presence of actinomycin D

Two testes (3 g each) were obtained from a naturally maturing trout at the early protamine stage of spermatogenesis. A cell suspension prepared from the testes was divided into two equal portions, one as control and the other containing 0.01 mM actinomycin D. After a preliminary incubation at 20°C for 30 min, 1 μCi of $^{14}$C-arginine was added to each suspension and the incubation was continued for 60 min, when it was terminated by the addition of cycloheximide to 0.4 mM. Basic proteins were extracted from testis nuclei and the histones and protamine were separated on a Bio-Gel P-10 column as described previously (1). Aliquots of the tubes comprising the protamine peak were counted in Bray's solution and the total counts per min under the peak in each case are given in the table.

| Incubation            | $^{14}$C-Arginine incorporated into protamine | Inhibition |
|-----------------------|---------------------------------------------|------------|
| Control               | 1.36 ($\times 10^{4}$)                       | 0%         |
| Actinomycin D (0.01 mM)| 1.05 ($\times 10^{4}$)                       | 23%        |

suggests that testis disomes are not making any other basic protein and may be exclusively engaged in protamine synthesis.

The incorporation of label into the larger polysome areas also changes at different stages of development. In September (Fig. 6, I) there is little incorporation into the larger polysomes but by November (Fig. 6, III) large amounts of label, particularly $^{14}$C-arginine, are found in this region. At the present time, the label incorporated into the larger polysome region has not been characterized but it is not likely to be associated with nascent histones since there is very little histone synthesis at the protamine stage of development (1). In December (Fig. 6, IV), when the testis consists largely of late spermatids and mature spermatozoa, few polysomes remain and protein synthetic activity has almost ceased.

Effect of Actinomycin D on Protamine Synthesis—Actinomycin D, at low concentrations, specifically inhibits the synthesis of RNA in animal cells (24). Preliminary observations (25) indicated that the incorporation of $^{14}$C-arginine into protamine was not inhibited by actinomycin D at a time when protamine synthesis was rapid (cf. protamine stage, Table I). These results were interpreted to suggest that protamine is synthesized on a stable mRNA template formed at an earlier stage of development. In a reexamination of these findings, testis cell suspensions were prepared from trout at a stage of spermatogenesis at which protamine synthesis had just begun (early protamine stage, Table I) and the effect of actinomycin D on the incorporation of $^{14}$C-arginine into protamine was determined (Table II).

At this stage of maturation (September) actinomycin D (0.01 mM) inhibits incorporation by 23%, suggesting that the synthesis of some protamine mRNA is still taking place although at later times (October and November) there was no inhibition, indicating that mRNA synthesis had ceased. In September, the number of disomes (Fig. 1) is small but increasing rapidly to the peak observed in October and November when protamine synthesis is maximal. A reasonable interpretation of this data is that protamine mRNA synthesis occurs just before the onset of protamine synthesis in September and that, once formed, it binds ribosomes to form a stable disome complex on which protamine synthesis can take place.
result of enzymatic degradation of messenger RNA connecting to monosomes following a mild treatment with ribonuclease is the ribosomes in the polysome complex (16, 18, 26), the resistance of are resistant to a mild treatment with pancreatic ribonuclease the result of Mg++-induced aggregation of monosomes (Fig. 2) Characterization of these disomes indicates that they are neither communicating (1) that disomes in the cytoplasm of rainbow Though there is no direct evidence of which set is present in codons have been found to code for arginine, one completely must contain predominantly codons for arginine. Two sets of codons for lysine are AA$^+$ or AGX, where X can be any of the four bases, and the other partially degenerate, AGY, where Y is either A or G. Although there is no direct evidence of which set is present in protamine messenger RNA, one piece of indirect evidence suggests that the CGX set may be more probable. Salmonid and the closely related clupeiform fishes (29) produce protamines which are extremely arginine-rich and contain either no lysine or the disome complex, might well be highly resistant to ribonuclease attack. In support of this hypothesis it has been shown, for example, that ribosomes bound to synthetic mRNA (poly U) or viral messenger (f2) are able to protect certain portions of the polynucleotide from degradation by pancreatic ribonuclease (27, 28).

Second, since protamine is so arginine-rich, its messenger RNA must contain predominantly codons for arginine. Two sets of codons have been found to code for arginine, one completely degenerate, CGX, where X can be any of the four bases, and the other partially degenerate, AGY, where Y is either A or G. Although there is no direct evidence of which set is present in protamine messenger RNA, one piece of indirect evidence suggests that the CGX set may be more probable. Salmonid and the closely related clupeiform fishes (29) produce protamines which are extremely arginine-rich and contain either no lysine or at most minute traces of it. The codons for lysine are AAA or AAG, which are related by single transition (purine $\rightarrow$ purine) base changes to the AGA and AGG codons for arginine. If AGA or AGG were to code for arginine in protamine, then it would seem likely that single step mutations (30) would have occurred during evolution to give rise to mutant lysine-containing protamines.

That lysine does not occur in Salmonid and clupeiform protamines suggests that, in fact, the other codon set, CGX, may be predominant and, in this case, mutation of arginine to lysine would require at least two steps and would, therefore, be much less probable. It is also interesting to note that codons for several of the limited range of neutral amino acids in protamine, namely proline (CCX), serine (AGcU), and glycine (GGX), are also related by single base changes to the CGX codons for arginine.

When the sequence of nucleotides for the putative mRNA for protamine is written with CGX as the arginine codon, the resulting mRNA is extremely CG-rich and its sequence allows a high degree of secondary structure if written in a "hairpin" structure. Such an mRNA, particularly when in complex with ribosomes in the disome complex, might well be highly resistant to ribonuclease.

The stability of the disomes and the general lack of RNA (29, 31) during the later stages of spermatogenesis raise the question of when the messenger RNA for protamine is synthesized. The appearance of a large number of disomes in trout testis simultaneously with rapid protamine synthesis (Fig. 1, B and C, and Table I) suggests that protamine messenger RNA and ribosomes combine immediately to form active disomes and that the rate-limiting step for the onset of protamine synthesis is the appearance of protamine messenger and formation of active disomes. The resistance of disomes both to low Mg++-induced degradation (Fig. 2) and mild ribonuclease treatment (Fig. 3) suggests that, once synthesized and in complex in disomes, protamine mRNA is very stable. This stability is also reflected by the insensitivity of protamine synthesis to inhibition by actinomycin D once the disome population has become appreciable as in October and November testis (Fig. 1).

A striking feature of spermatogenesis is the marked reduction in the amount of cytoplasm in the differentiating spermatid cell first noted by Caspersson (9), who measured the progressive disappearance of ribonuclease acid from the developing sperm cell by cytochemical and spectrophotometric means. Daoust and Clermont (10) have also shown that, in the rat testis, the RNA of the spermatid is collected into larger and larger granules until they are set free as "residual bodies" shortly before the mature spermatid is released into the lumen of the seminiferous tubules. Lacy (11), studying these residual bodies in greater detail at various stages of their formation, has noted that, in addition to the mass containing numerous RNA particles, these bodies include mitochondria, Golgi membranes, and vesicles, and, also, a concentration of cytoplasmic membranes. Clearly, therefore, a mechanism exists for the removal of cytoplasm from the maturing sperm cell. Consistent with these observations, the data summarized in Table I also indicate that there is a progressive loss of ribosomes from the cytoplasm of developing rainbow trout sperm cells. It may also be seen from the table that the total number of ribosomes present in the testis does not reflect the rate of protamine synthesis in that during October and November the rate of protamine synthesis increases while the ribosome content decreased. Further, in Fig. 1 it may be seen from the changing profile of testis polysomes during development that the proportion of disomes active in the synthesis of protamine increases at the stage of rapid protamine synthesis. This suggests that the decrease in numbers of ribosomes in the testis cells is not strictly the result of a general decrease in the cell cytoplasm but may be the result of specific degradation of certain ribosomes or polysomes not required for further protein synthesis after a particular stage of testis maturation. In this respect, the removal of cytoplasm from the developing sperm cell is probably a specific and well regulated process. In accord with this idea of selectivity in removal of cytoplasm, it has been noted in the developing salmon testis (Oncorhynchus nerka), for example, that the decrease in total RNA was accompanied by a change in its base composition, the mature testis RNA being richer in guanosine (12, 31).

It is generally thought that specific functional polysomes are formed for the synthesis of specific proteins at different stages of cell development. In synchronized HeLa cells, for example, distinct peaks of small cytoplasmic polysomes are observed when ribosomes obtained during the S phase of the cell cycle are fractionated on a sucrose gradient (32). These polysomes are not present at other stages of cell development and have been shown to be associated specifically with the synthesis of histones which is, in turn, coupled with DNA synthesis. In the trout testis
also, striking changes in the polysome profiles are observed (Figs. 1 and 6, I to IV) and reflect the functional state of the tissue in that large numbers of disomes are present in testis cells at a stage of development when the synthesis of protamine is at its peak.

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