Stimulation of Fatty Acid Synthesis *in vitro* by Gonadotrophin-Induced Testicular Ribonucleic Acid

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RNA from testes of hypophysectomized rats treated with follicle-stimulating hormone and luteinizing hormone markedly stimulates *in vitro* the incorporation of acetate and malonate (as CoA derivatives) into polyunsaturated fatty acids. The system *in vitro* contains the components necessary for both protein and fatty acid synthesis. That the RNA is a hormone-induced messenger type that causes enzyme synthesis that then causes fatty acid synthesis is supported by the following observations: (1) the stimulation of RNA synthesis by follicle-stimulating hormone and luteinizing hormone is decreased by injection of the animals with actinomycin D; (2) puromycin in the system *in vitro* decreases the synthesis of polyunsaturated fatty acids; (3) the activity of the RNA preparation is destroyed by digestion with ribonuclease; in fact, the digest is inhibitory, which is a characteristic of messenger-RNA-mediated protein synthesis; (4) protein that might be denatured enzyme is virtually absent from the effective RNA preparations.

The early stimulation of nuclear RNA synthesis by various hormonal agents has been described (Hamilton, Widnell & Tata, 1965; Notides & Gorski, 1966) and the implications for the mechanism of hormone action have been discussed (Korner, 1965). Nuclear RNA preparations can induce synthesis of specific enzymes by cell-free ribosomal preparations (Lang, 1965a) and increase chromatin activity (Breuer & Florini, 1966). Specific changes in the physical characteristics of DNA in relation to the synthesis of specific nuclear RNA under hormonal influences have been demonstrated (Goldberg & Atchley, 1966). Sekeris (1965) reported that a nuclear RNA synthesized under the influence of the insect hormone, ecdysone, would stimulate cell-free synthesis of the enzyme 3,4-dihydroxy-phenylalanine decarboxylase by a liver microsome system.

It has been reported that the gonadotrophic hormones, FSH* and LH, either individually or, better, in combination, would stimulate biosynthesis of polyunsaturated fatty acids in testicular tissue (Goswami & Williams, 1967). Since capacity for synthesis of polyunsaturated fatty acids was demonstrated to reside in the microsomal fraction, we attempted to produce the hormonal effects on these structures. Direct additions of FSH, LH and testosterone had, however, no effect on fatty acid biosynthesis and we investigated whether a nuclear

RNA preparation from hormone-treated animals would have any effect on the capacity to synthesize polyunsaturated fatty acids by microsomal preparations from untreated hypophysectomized animals. The present paper is a report of the results of such experiments.

MATERIALS AND METHODS

Fifty hypophysectomized immature male Sprague-Dawley rats were obtained from Endocrine Laboratories, Madison, Wis., U.S.A., and were maintained after arrival for at least 2 weeks. Half the rats were then treated with 1 mg. of FSH and 50 i.u. of LH each at 0, 24, 48 and 72 hr., and the other half were treated with the same volume of 0.9% NaCl. The animals were killed by decapitation at 74 hr.

Extraction of nuclear RNA. Basically, the procedure of DiGirolamo, Henshaw & Hiatt (1964) was used for this purpose. The testicular tissues of all the hormone-treated animals were pooled in the cold after removal of the cuticles, minced for 30 sec., and suspended in 3 vol. of cold 0.25 M-sucrose in 3 mM-CaCl₂-50 mM-tris-HCl buffer, pH 7.5 (medium A). The tissue was then homogenized with five strokes of a Teflon-glass Potter-Elvehjem homogenizer. The nuclei were isolated from the homogenate by the method of Chauveau, Moule & Rouiller (1956) as modified by Blobel & Potter (1966). Essentially this consisted of suspending the 600 g pellet in 1 vol. of medium A in a polyallomer tube, mixing it thoroughly with 2 vol. of 2.3 M-sucrose in 1.5 mM-CaCl₂-10 mM-tris-HCl buffer, pH 7.5, underlaying the mixture with 2.3 M-sucrose-CaCl₂-tris, and centrifuging it at 44 000 g for 1 hr. in a Spinco SW 39 rotor at 0-4°. The nuclear pellet was then suspended in a volume of medium A

* Abbreviations: FSH, follicle-stimulating hormone; LH, luteinizing hormone.

twice the weight of the tissue. The rest of the extraction was done according to the method described by DiGirolamo *et al.* (1964) and the final precipitate was dissolved in 1 mM-MgCl₂.

For the preparation of the ribonuclease digest, a sample of the hormone-treated RNA preparation from 25 hormone-treated rats was digested with 1 ml. of ribonuclease T₁ (Schwarz BioResearch Inc., Orangeburg, N.J., U.S.A.) (5 µg./ml.) at 37° for 30 min., and the digest was processed as outlined for nuclear RNA.

Determination of the sedimentation profile of nuclear RNA. A sample of the RNA preparation was layered over a gradient of 5–20% (w/v) sucrose in 0.01 M-sodium acetate, pH 5, and centrifuged in a Spinco SW 39 rotor at 38 000 rev./min. for 12 hr. at 4°. Extinctions at 260 and 280 mµ were then determined on 10-drop fractions obtained by piercing the bottom of the tube with a 24-gauge needle.

Determination of base ratios of nuclear RNA preparations. This was done according to the method outlined by MacGee (1966). For gas-liquid chromatography an F & M (model 5750) dual flame ionization detector was used. Separation was done on a 6 ft. × 1/8 in. stainless-steel column packed with 0.5% Carbowax 20M on 80–120-mesh glass beads (Applied Science Laboratories Inc., State College, Pa., U.S.A.). The operating temperatures were: detector, 285°; column, 150–225° (programmed at 4°/min.); injection port, 300°. Helium was used as a carrier gas at a flow rate of 80 ml./min. The bases were identified by comparing their retention volumes with those of standards, and the base ratios were obtained by the comparison of the areas under the peak of each base.

Preparation of cell fractions from testicular tissue of control rats. The tissues were homogenized in 3 vol. of medium A as outlined above. The 600g pellet was discarded and the supernatant was centrifuged at 17 000g for 15 min. The mitochondrial pellet was discarded and the supernatant obtained was centrifuged at 104 000g for 1.5 hr. The microsomal pellet was suspended in and washed three times with medium A and the final pellet was suspended in 1 mM-MgCl₂–10 mM-tris-HCl buffer, pH 7.5.

Incorporation of radioactivity from [¹⁴C]acetyl-CoA or [¹⁴C]malonyl-CoA into fatty acids in vitro. The incubation mixture contained in a final volume of 0.8 ml.: 4.2 µmoles of MgCl₂; 3 µmoles of KCl; 14 µmoles of tris-HCl buffer, pH 7.5; 0.09 µmole of each of the 20 amino acids (see below); 6.0 µmoles of GSH; 2.5 µmoles of phosphoenolpyruvate; 0.0625 µmole of GTP; 25 µg. of pyruvate kinase; 0.25 µmole of NADH (disodium salt); 0.25 µmole of NADPH (tetrasodium salt); 0.5 µmole of ATP. The amino acid mixture was made up of L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-glutamine, L-glutamic acid,

glycine, L-histidine, L-hydroxyproline, L-isoleucine, DL-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine and L-valine. [¹⁴C]-Acetyl-CoA, [¹⁴C]-malonyl-CoA, [¹⁴C]-methionine, supernatant fraction from hormone-treated testicular tissue and nuclear RNA preparations were added as indicated.

Incorporation in vivo of radioactivity from [⁵⁻³H]orotic acid into nuclear RNA. For this purpose hypophysectomized rats were divided into five groups of six animals each receiving the following injections: (1) 0.9% NaCl; (2) FSH (obtained from Endocrinology Study Section, National Institutes of Health, Bethesda, Md., U.S.A.); (3) LH (PLH, obtained from Armour-Baldwin Laboratories, Omaha, Nebr., U.S.A.); (4) FSH plus LH; (5) FSH plus LH and actinomycin D (Cosmegen, obtained from Merck, Sharp and Dohme, Rahway, N.J., U.S.A.). The hormones were dissolved in sterile 0.9% NaCl, each injection of 0.25 ml. containing 2 mg. of FSH or 40 i.u. of LH as appropriate. Actinomycin D was dissolved in the minimum quantity of 95% (v/v) ethanol and the dose (250 µg. in 0.15 ml./rat) was adjusted with 0.9% NaCl. The hormones were injected at 0, 24, 48 and 72 hr.

Each animal was injected at 72.5 hr. with a dose of 1 mc of [³H]orotic acid (obtained from New England Nuclear Corp., Boston, Mass., U.S.A.) and with actinomycin D for group 5 rats, and the animals were decapitated at 73 hr. The nuclei from the testicular tissue were isolated, the nuclear RNA was extracted by procedures described above and made up to suitable volumes, samples were dried on small-diameter (2.4 cm.) Whatman 3MM filter-paper disks and radioactivity was determined by scintillation counting.

Fatty acid analyses of microsomal preparations after incubation. The incubation was stopped in each flask by the addition of 1 ml. of ethanolic 10% (w/v) KOH, and the mixture was saponified on a steam bath in an atmosphere of N₂ for 60 min. After removal of the non-saponifiable material with light petroleum (b.p. 40–60°), the soap solution was acidified with conc. HCl and the free fatty acids were extracted with light petroleum. A sample was used for scintillation counting and the rest was methylated by the method of Metcalfe & Schmitz (1961).

Gas-liquid radiochromatographic analyses of the fatty acids were done by the methods reported by Goswami & Coniglio (1966a).

RESULTS

Pulse-labelling experiments. Table 1 shows the radioactivity incorporation from [⁵⁻³H]orotic acid into the nuclear RNA of the testes from control and hormone-treated animals. There was essentially

Table 1. *Incorporation in vivo of radioactivity from [⁵⁻³H]orotic acid (1 mc) into the nuclear RNA fraction of testicular tissue of hypophysectomized rats*

Group no.	Treatment	Radioactivity incorporated (counts/min./E ₂₆₀ unit)
1	0.9% NaCl (0.25 ml.) (control)	—
2	FSH (2 mg./day)	85
3	LH (40 i.u./day)	61
4	FSH + LH	110
5	FSH + LH + actinomycin D	48

no radioactivity incorporation into the testicular nuclear RNA fraction from hypophysectomized rats. This occurred in spite of the fact that the number of E_{260} units extractable/mg. of tissue from control animals was comparable with that obtained from hormone-treated groups, although the control animals had a much smaller testicular size than the hormone-treated animals, as reported by Goswami & Williams (1967). FSH stimulated this incorporation to a higher degree than LH, but the highest incorporation was obtained with animals treated with both FSH and LH. Treatment with actinomycin D simultaneously with the orotic acid inhibited the incorporation by about 50%.

Characteristics of the nuclear RNA preparation. Fig. 1 shows the sedimentation profiles of the nuclear RNA preparations extracted by methods outlined above. The E_{260}/E_{280} ratios for all the fractions varied between 1.85 and 2.00, showing them to be essentially free of protein. The preparation was essentially free of glycogen, but was slightly contaminated with DNA, as was expected (Blackburn & Klemperer, 1967). The main component was in the 25–30s region, although there was considerable molecular heterogeneity. This was expected, since no attempt was made to separate the nuclear chromosomal DNA-like RNA from the ribosomal RNA, which would contaminate the RNA preparations under the extraction conditions outlined above (Georgiev, 1967). The short peaks at the top of the gradient were probably indications of some degradation of the preparation. For the study of the distribution of radioactivity, several samples were pooled and centrifuged on a

sucrose gradient in the same manner. The radioactivity peaks corresponded in general with the extinction peaks. The actinomycin-induced inhibition of radioactivity distribution was also found to be general and not restricted to any particular molecular species.

Gas-liquid chromatographic determination of base ratios. The minor peaks given by cytosine and adenine, reported by MacGee (1966), were minimized by the use of pure standards, and the major peaks yielded very satisfactory quantitative results. The standard curves for all components were essentially linear and, with careful matching of the flow rates in the two columns during programming, the sensitivity could be brought down to 0.5–1 μ g. A typical analysis yielded: guanine, 26.4%; cytosine 26.1%; uracil, 27.3%, adenine, 20.2%; with a $(G+C)/(A+U)$ ratio of 1.11. The $(G+C)/(A+U)$ value of 1.11 probably represented a heterogeneous mixture of both DNA-like RNA and ribosomal RNA (Notides & Gorski, 1966; Georgiev, 1967). The RNA preparations used for the subsequent incubations were all samples of this mixture.

Incorporation of radioactivity from $[^{14}C]$ acetyl-CoA into total fatty acids by microsomal preparations. Table 2 shows the results of some typical experiments on the incorporation of radioactivity from $[^{14}C]$ acetyl-CoA into total fatty acids by microsomal preparations from testes of hypophysectomized rats.

Though microsomal preparations alone did not incorporate any radioactivity from $[^{14}C]$ acetyl-CoA into total fatty acids, addition of nuclear RNA

Fig. 1. Sucrose-density-gradient analyses of bulk RNA from FSH+LH-treated rat testicular nuclei. Linear gradients of 5–20% (w/v) sucrose were centrifuged for 12 hr. at 38 000 rev./min. in the Spinco SW 39 rotor. Fractions (10 drops) were collected from the bottom of the tube. ●, E_{260} ; ○, radioactivity after pulse-labelling with $[5-^3H]$ -orotic acid for 30 min.; ▲, as for ○, after actinomycin treatment.

Table 2. *Incorporation in vitro of radioactivity from [1-¹⁴C]acetyl-CoA into fatty acids by microsomal preparations from testes of hypophysectomized rats*

Basic incubation medium and microsomes were present in all experiments, and 60 mμmoles of acetyl-CoA (25000 counts/min.) were added. Incubation was at 37° for 1 hr. The reaction was terminated by the addition of 1 ml. of 10% (w/v) KOH.

Additions	Radioactivity incorporated (counts/min./mg. of protein)
RNA (control) + supernatant (control)	32
RNA (FSH + LH) + supernatant (FSH + LH)	195
RNA (control) + supernatant (FSH + LH)	38
RNA (FSH + LH) + supernatant (control)	79
RNA (FSH + LH) + supernatant (FSH + LH) + puromycin	93

Table 3. *Incorporation in vitro of radioactivity from [1,3-¹⁴C₂]malonyl-CoA into fatty acids by microsomal preparations from testes of hypophysectomized rats*

Each incubation flask contained 90 μg. of [1,3-¹⁴C₂]malonyl-CoA (125000 counts/min.) and 0.1 ml. of supernatant fractions from FSH + LH-treated rat testes. Other additions are described in the Materials and Methods section. Incubation was at 37° for 1 hr. The reaction was terminated by the addition of 1 ml. of 10% (w/v) KOH.

Additions	Radioactivity incorporated (counts/min./mg. of protein)
0.1 M-Tris	6650
RNA (FSH + LH)	11700
RNA (digested with ribonuclease)	3330

preparations combined with supernatant fractions stimulated the radioactivity incorporation to various extents, depending on the source of these preparations. The nuclear RNA preparation from the hormone-treated animals appeared to be the most important component participating in the synthetic process, since it had a greater stimulatory activity when combined with the supernatant fraction from the control animals than that of the supernatant fraction from hormone-treated animals combined with the RNA preparation from control animals. The highest incorporation was obtained with a combination of nuclear RNA and supernatant fraction both from the hormone-treated animals. Addition of puromycin at a dose of 50 μg./incubation mixture suppressed the incorporation, but not to the level of the residual activity obtained with RNA preparations and supernatant fraction from untreated control animals.

Incorporation with [1,3-¹⁴C₂]malonyl-CoA. Table 3 shows the results of substituting [1,3-¹⁴C₂]malonyl-CoA for [1-¹⁴C]acetyl-CoA in the incubation medium. In comparison with acetyl-CoA, incorporation of radioactivity from [1-¹⁴C]malonyl-CoA into total fatty acids was much greater,

Table 4. *Percentage distribution of radioactivity from [1,3-¹⁴C₂]malonyl-CoA into long-chain fatty acids of testicular microsomes from hypophysectomized rats after incubation with nuclear RNA preparations from hormone-treated rats*

The results are expressed as percentages of total radioactivity in microsomal fatty acids. The first number of the fatty acids designation represents the number of carbon atoms and the second number the number of double bonds.

Fatty acid	RNA added	Control (no RNA)
< 16:0	7.15	6.48
16:0	47.50	66.10
16:1	4.34	3.42
18:0	6.55	8.06
18:1	3.75	2.73
18:2	2.57	1.71
18:3	3.63	1.59
20:3 (ω-9)	3.39	1.48
20:3 (ω-6)	3.39	1.25
20:4	2.41	1.03
20:5	3.16	0.68
22:4	3.16	1.36
22:5	5.61	3.07
22:6	3.28	1.03
> 18:2	28.03	11.49

amounting to 3-5% of the total dose added. In all these experiments, the supernatant fraction from FSH + LH-treated groups only was used. There was an almost twofold increase in incorporation of radioactivity with the addition of the nuclear RNA fraction from the testicular tissue of the hormone-treated animals. Digestion of the nuclear RNA preparation with ribonuclease T₁ and addition of an ethanol precipitate of the phenol extract of the digest to the incubation medium resulted in a marked inhibition of incorporation of radioactivity from [1-¹⁴C]malonyl-CoA into total fatty acids. This inhibition was probably due to contamination with oligonucleotides, which, according to DiGirolamo *et al.* (1964), would markedly inhibit protein synthesis in cell-free preparations. Apparently the contamination was still there even though the

precipitates were taken up in 2M-potassium acetate and reprecipitated with 25% ethanol before being dissolved in 1mM-magnesium chloride, in which form it was added to the incubation medium.

Distribution of radioactivity in the total microsomal fatty acids incorporated from [1,3-¹⁴C₂]malonyl-CoA. Table 4 shows the results of gas-liquid radiochromatographic analyses of the fatty acids extracted from the microsomal preparations after various incubations. A total of 5000 counts/min. was introduced into the injection port for each determination. The difference between the groups in the incorporation of radioactivity into various fatty acids is apparent. The radioactivity in the C_{18:2} fatty acid is presumably in an isomer of linoleic acid, probably the octadeca-6,9-dienoic acid reported in liver mitochondria and microsomes (Goswami & Coniglio, 1966b). This increase in incorporation is manifested in each individual polyenoic fatty acid, irrespective of whether it belongs to the $\omega-6$ or the $\omega-9$ family. The highest stimulation was, however, obtained with the C_{20:5} and C_{22:6} fatty acids, which, according to Mead (1963), belong to the $\omega-3$ family.

DISCUSSION

Though there have been extensive speculations on gene activation as a probable mechanism of hormonal action on target tissues, and a number of reports (Hamilton *et al.* 1965; Notides & Gorski, 1966; Korner, 1965) do indicate the existence of such a mechanism, direct demonstrations of this effect are meagre. Though the present study does not demonstrate unequivocally the existence of such a mechanism, the data are best explained by postulating a hormone-DNA-messenger RNA-enzyme-product sequence of events.

The importance of polyunsaturated fatty acids in testicular development and maturation, probably in the formation of structural elements for the sperm plasma membrane, is well recognized (Davis, Bridges & Coniglio, 1966). The stimulation of spermatogenesis by pituitary gonadotrophins is accompanied by a marked increase in biosynthesis of polyunsaturated fatty acids in this tissue. The site of this biosynthesis is not definitely known, but since testes of immature hypophysectomized rats contain a significant amount of polyunsaturated fatty acids (particularly C_{22:5}, $\omega-6$) (Goswami & Williams, 1967) and since spermatogonia and Sertoli cells are the only structures known to remain intact after hypophysectomy (Smith, 1939), this biosynthetic activity probably resides in the microsomal structures in these cellular elements. The hormonal stimulation of spermatogenesis is associated with increased synthesis of polyunsaturated fatty acids, and this appears to be one of the most

important expressions of hormonal action in relation to stimulation of spermatogenesis by FSH and LH.

The demonstration of enzymic synthesis in cell-free preparations is fraught with many difficulties, inasmuch as the measurement of an end product of enzymic activity is of necessity the criterion of increased synthesis. For this the enzyme concerned must have a very high turnover rate so that differences in activities after short incubation periods can be measurable. Secondly, the reconstituted system for protein synthesis must also be favourable for optimum expression of enzymic activity. In our system, since we used microsomal preparations instead of ribosomal preparations, and since the microsomal membranes are known to be the most active sites for the elongation and desaturation mechanism for the biosynthesis of polyunsaturated fatty acids, our reconstituted system was probably more efficient with respect to the optimum expression of these enzymic activities. The membranous structures might provide a structure for more efficient organization of the enzyme units. Interestingly, Lang (1965b) reported that he could obtain much better results with microsomes than with ribosomes in his reconstituted system for the induction of the enzyme 3,4-dihydroxyphenylalanine decarboxylase.

Though we do not intend to interpret our present findings as providing unequivocal proof that enzymic synthesis is being initiated in cell-free preparations, which must await further experiments of a more rigorous nature, the results do present favourable indications. At least some stimulating factor is being carried over from hormone-treated testicular nuclei that results in an increased biosynthesis of polyunsaturated fatty acids in cell-free testicular preparations from hypophysectomized animals.

Several significant observations point towards the conclusion that one or more enzymes are probably being synthesized: (1) the freedom of the nuclear RNA preparations from protein contamination; (2) inhibition of the nuclear-RNA-induced stimulation of fatty acid biosynthesis by puromycin; (3) inhibition of the fatty acid biosynthesis by the ribonuclease-digest preparation. This digest probably contains a significant amount of oligonucleotides, which would inhibit aminoacylation of transfer RNA (Hayashi & Miura, 1966) and hence protein (enzyme) synthesis. Though the possibility exists that the oligonucleotides might have a direct effect on the fatty acid-synthesizing enzyme, this seems unlikely since oligonucleotides do not have any significant effect on microsomal radioactivity incorporation when very low amounts of microsomes are incubated, i.e. under conditions when the enzyme concentration is rate-limiting. The fact

that a supernatant fraction from the hormone-treated animals is necessary for the optimum incorporation of activity into polyunsaturated fatty acids is indicative of a participation of the transfer RNA fraction, but since addition of this fraction along with an RNA fraction from non-treated animals does not significantly alter radioactivity incorporation (Table 2), hormonal control is probably not at this level. The stimulation of radioactivity incorporation by the testicular nuclear RNA preparation from hormone-treated animals, inhibition of incorporation by the addition of a ribonuclease digest and the relative freedom of the preparation from protein contamination would all point towards a control at the level of transcription.

Very little is known about the rate-limiting step in polyunsaturated fatty acid biosynthesis, and therefore the exact mechanism of stimulation by the hormone-treated supernatant fraction cannot be fully explained. The possible existence of a rate-limiting enzyme or a cofactor in the hormone-treated supernatant fraction cannot be excluded, but, in view of the presently proposed models of enzyme synthesis, this stimulatory effect can also be explained in terms of a participation of the transfer RNA fraction from the supernatants of hormone-treated animals. If this is the case, a coupling of translational and transcriptional processes is probably operative in the regulatory process.

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