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Polyphasic Increase of [^{3}H-uridine Incorporation into RNA of 17 β-0estradiol Stimulated Mammary Gland

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SEZIONE III

(Botanica, zoologia, fisiologia e patologia)

Patologia. — *Polyphasic Increase of* [^{3}H]-*uridine Incorporation into* RNA *of* 17 β-*Oestradiol Stimulated Mammary Gland.* Nota di FERDINANDO AURICCHIO, ADA QUIRINO e FRANCESCO BRESCIANI, presentata (*) dal Socio L. CALIFANO.

RIASSUNTO. — 3 settimane dopo ovariectomia le ghiandole mammarie di topine mostrano (i) nessuna modificazione del peso fresco, (ii) una velocità residua di incorporazione di uridina-[^{3}H] nello RNA del 20% e (iii) una velocità residua di incorporazione di leucina-[^{3}H] nelle proteine del 90%.

Un singolo inoculo di 17-β-estradiolo in questi topi ovariectomizzati produce, durante le successive 17 ore, una serie di rapidi aumenti di incorporazione di uridina-[^{3}H] nello RNA della ghiandola mammaria; gli aumenti che sono di entità variabile e possono raggiungere fino a 5 volte i livelli di sintesi della ghiandola prima dell'inoculo, presentano « picchi » ad 1, 9, 12 e 16 ore dopo la somministrazione di ormone: un aumento significativo è già svelabile 15 minuti dopo l'inoculo.

DNA synthesis and cell proliferation in the mammary gland of adult mice slow down and come to virtual arrest after ovariectomy; the cells go into a " standby " or G_0 state, ready to re-enter the cell replication cycle upon administration of oestrogen to the animals [1, 2]. When 17 β-oestradiol is injected, DNA synthesis and mitosis in the gland resume in a synchronous fashion after a 48-72 h lag, with the number of responsive cells doubling in the next 72-96 h [3]. Cell proliferation resulting in duct elongation appears to be the main response of the gland to oestrogen [4].

The mammary gland is unique among oestrogen-target organs in that hormone-induced ductal growth is a purely hyperplastic response, and this offers a specific advantage for the study of oestrogen-induced cell proliferation. However, exceedingly little is known about the mechanism of the oestrogen effect on the mammary gland. We know that the gland contains specific oestrogen receptor proteins [5, 6, 7] and that the oestrogen-receptor complexes, once formed in the cytoplasm, rapidly move to the nucleus [8]. However, there is a gap in our knowledge of biochemical events following the initial interaction of the hormone with the gland and preceding the later surge of DNA synthesis and cell division.

In this paper we report that following injection of 17 β-oestradiol into ovariectomized mice, changes occur in the rate of incorporation of [^{3}H]-uridine into RNA of mammary gland. Several discrete bursts of increased uridine uptake into RNA were found to occur after a single administration of hormone. The results suggest a complex pattern of activation of transcription in the oestrogen stimulated gland and open interesting perspectives for future work.

(*) Nella seduta del 9 marzo 1974.

Materials and Methods

Materials.

Oestradiol-17 β was purchased from Mann Research Laboratory, N.Y., U.S.A., [^{3}H]-uridine (29 Ci/mmole) and [^{3}H]-L-leucine (39 Ci/mmole) were purchased from Amersham, Buckinghamshire, England. L–leucine, yeast RNA (type XI) and bovine plasma albumin were from Sigma, St. Louis, Missouri, U.S.A. Uridine was from Boehringer, Milan, Italy; Insta gel from Packard, Downers Grove, Illinois, U.S.A. Other materials were analytical grade reagents (from C. Erba, Milan, Italy).

Tritium measurement.

A three-channel, Nuclear Chicago spectrometer was used. The samples were prepared for radioactivity measurement by mixing with Insta gel. The efficiency (about 25 %) was determined by the " channel ratio " method and radioactivity expressed as d.p.m.

Animals, surgical procedure and treatments.

Swiss female mice, 3-4 months old were used. Ovariectomy was carried out *via* the dorsal approach, under light ether anesthesia. Chromatographically pure 17 β-oestradiol was dissolved in 0.15 M NaCl : ethanol (2 : 1) to the final concentration of 100 mg/l. Ovariectomized mice were injected intraperitoneally with 0.1 ml of the above 17 β-oestradiol solution (10 μg of hormone per animal).

Mice were injected intraperitoneally with 0.2 ml of 0.15 M NaCl containing 100 μCi of [^{3}H]-uridine of 10 Ci/mmole specific activity; or, with 0.2 ml of 0.15 M NaCl containing 50 μCi of [^{3}H]-L-leucine of 0.8 Ci/mmole specific activity. The mice were killed by cervical dislocation 30 min after [^{3}H]-uridine injection or 15 min after [^{3}H]-L-leucine injection. Uptake of precursors by mammary gland was assessed as described below.

Uptake of [^{3}H]*-uridine by mammary gland.*

After injection of [^{3}H]-uridine and sacrifice after 30 min, the 5th pair of mammary glands (inguinal) was rapidly dissected. After removing lymph nodes, the glands were frozen (— 20° C) before further treatment. Glands from mice which had undergone identical treatments were pooled together. After thawing, the gland pool was minced with scissors and then homogenized in an Ultraturrax homogenizer at 90 V in 4 bursts lasting 20 s each, with 60 s intervals between bursts. Homogenization was carried out in 3 ml of 1 mM uridine in 0.01 M NaCl, with the homogenate container immersed in crushed ice.

To measure total radioactivity, 0.1 ml of the homogenate was added to 2.9 ml of water, mixed with 6 ml of Insta gel and the radioactivity assessed as described above.

Incorporation of [^{3}H]-*uridine into mammary gland* RNA.

The mammary gland homogenate prepared as described above was submitted to the following procedure. Ice-cold acetone, 16 ml, was added and, after vigorous shaking, the mixture was centrifuged; the pellet was resuspended in acetone and the procedure repeated once more. The pellet after acetone extraction was resuspended in 16 ml of ice-cold 5 % (*w/v*) trichloroacetic acid containing NaCl (0.01 M) and uridine (1 mM); after 30 min in the cold, the suspended material was sedimented by centrifugation; the procedure was repeated twice more. Thereafter the pellet was resuspended in 16 ml of ether : ethanol (1 : 3), centrifuged and the procedure repeated once more. The final pellet was resuspended in 2 ml of 1 M KOH and the mixture incubated at 37° C for 20 h in a stoppered vial to hydrolyze RNA. Thereafter 0.4 ml of 6 M HCl and 2 ml of 5 % (*w/v*) trichloroacetic acid were added to the KOH hydrolysate and the precipitate sedimented by centrifugation. The clear supernatant was neutralized with KOH. A portion of the supernatant was mixed with Insta gel and used for radioactivity measurement; another portion was used to determine RNA content [9] using yeast RNA for standard curves. Specific radioactivity of RNA could thus be computed.

Incorporation of [^{3}H]-L-*leucine into mammary gland protein.*

After injection of [^{3}H]-L-leucine and sacrifice of the animal after 15 min as described in the section on Animals, etc., mammary glands were subjected to the same procedure as used for measurement of [^{3}H]-uridine incorporation into RNA, except for the following changes: (*a*) 1 mM L-leucine substituted uridine in the various solutions used; (*b*) KOH hydrolysis was carried out at 40° C for 1 h only; (*c*) after KOH hydrolysis and subsequent HCl and trichloroacetic acid addition followed by centrifugation, the *pellet*, and not the supernatant, was used for radioactivity and protein determination. The pellet was dissolved in 1.3 ml of 10^{-2} M NaOH. A portion of the dissolved material was mixed with Insta gel and used for radioactivity measurement; another portion was used to determine protein content according to a modified Lowry procedure [10] using crystallized bovine plasma albumin as standard.

RESULTS

As shown in fig. 1, the ability of mammary gland of adult mice to incorporate either [^{3}H]-uridine into RNA or [^{3}H]-L-leucine into protein decreases as a function of time after ovariectomy. At 3 weeks after ablation of the

ovaries, the decrease of rate of precursor uptake into RNA is very substantial (residual activity is about 18 % of control) while incorporation into protein is only slightly affected (residual activity is about 90 % of control). The weight of the gland is not significantly changed.

A single intraperitoneal injection of 17 β-oestradiol into these ovariectomized mice (10 μg per animal) results in a complex time-course change in rate of precursor uptake into total RNA of mammary gland. The data are

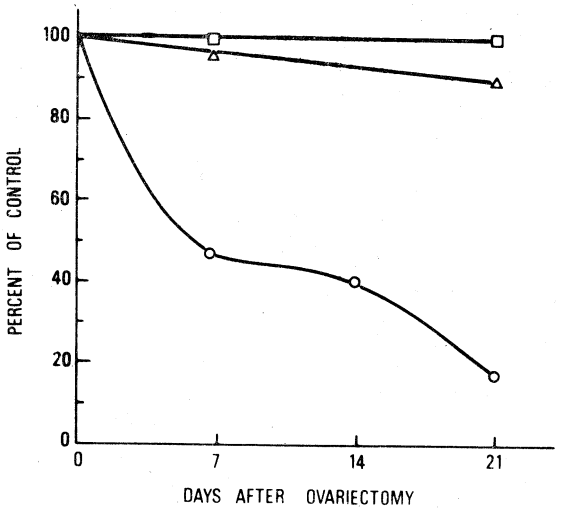

Fig. 1. – Effect of ovariectomy on wet weight (□) and radioactive precursor incorporation into mammary gland RNA (○) and protein (△).

Adult ovariectomized mice were injected with either 100 μCi of [^{3}H]-uridine 30 min before sacrifice, or 50 μCi of [^{3}H]-L-leucine 15 min before sacrifice. At the times indicated, animals were killed in groups of 3 and their inguinal mammary glands (5th pair) excised, weighed and pooled for homogenization and further treatment. Specific radioactivity of RNA and protein was determined in the gland homogenate. Data are expressed as percentage of control value i.e., values for mammary glands of mice killed immediately after ovariectomy (time 0). The following control values were obtained: wet weight of 6 glands, 846 mg; specific activity of RNA, 57,000 d.p.m. per mg; specific activity of protein, 6,700 d.p.m. per mg.

presented in graph form in fig. 2 and a statistical evaluation of significance of changes is summarized in Table I (each observation in Table I is a measure on homogenate of a pool of 6 glands from 3 mice). A first burst of increased incorporation into RNA occurs during the initial 2 h after injection of the hormone. The increase is significant already at 15 min after hormone administration, the earliest measurement in this study, as well as at 30, 60, 90 and 120 min. There is variability among animals with regard to both the time at which the increased activity peaks (30 to 120 min.) and the height of the peak (2- to 5-fold the control value). After the first burst, incorporation rate returns to pre-stimulation level, with no significant difference from basal level detected at 3, 4-6, 7 and 8 h after hormone injection. A 2nd burst of increased activity shows a statistically significant peak at the 9th h after

17 β-oestradiol injection; a 3rd at the 12th and a 4th at the 16th h. The peaks are intercalated by periods in which precursor uptake decreases up to about pre-stimulation values, with no significant difference from basal level detected at 10, 11, 13-15 and 17 h after hormone injection. Also in the case of these later peaks, there is variability among animals with regard to height of the peak. Qualitative occurrence of these peaks is, however, remarkably reproducible.

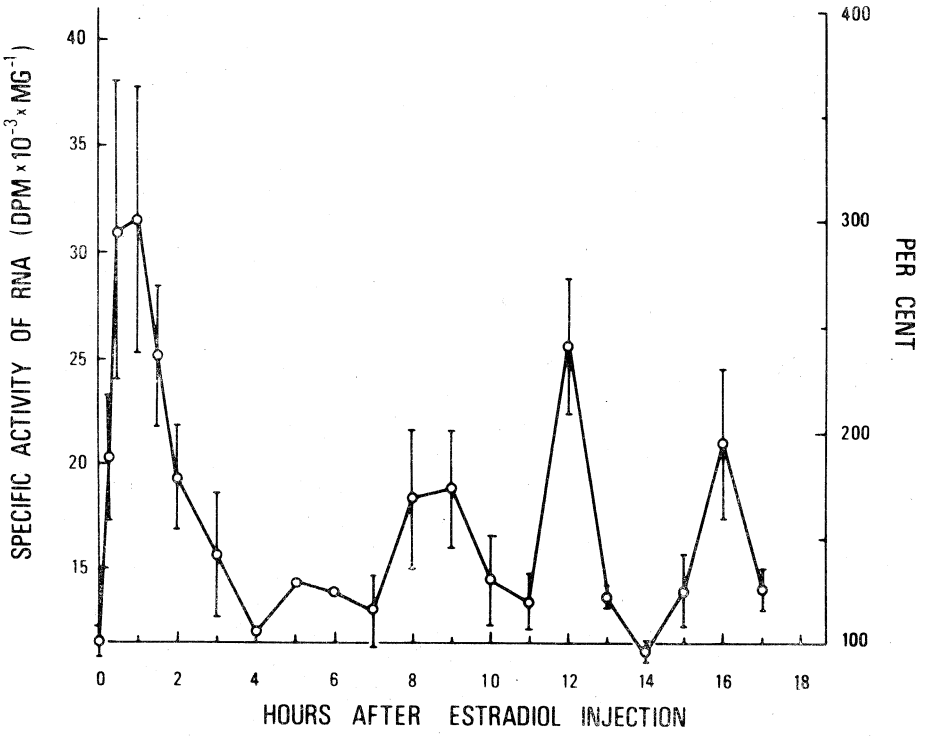

Fig. 2. – Effect of single injection of 17 β–oestradiol on [^{3}H]-uridine incorporation into RNA, in mammary glands of ovariectomized adult mice.

Adult mice 3 weeks after ovariectomy were used. They were given 10 ûg of 17 β–oestradiol intraperitoneally and injected with 100 μCi of [^{3}H]-uridine 30 min before sacrifice. At the times indicated, animals were killed in groups of 3 and their inguinal mammary glands (5th pair) excised and pooled for homogenization and further treatment. Specific radioactivity of RNA was determined in the gland homogenate. A total of 291 animals was used. Experimental points are average values. Standard error is shown only when average is the result of at least three different measures of specific radioactivity, each on a different pool of three mice (6 glands). For further specifications and significance of peaks see also Table I.

As shown in fig. 3 for the first burst of increased precursor incorporation into RNA, total uptake of [^{3}H]-uridine into the gland shows only minor increases during the oestrogen induced periods of highly augmented incorporation into RNA. Also, the weight of the gland is not affected. An increase in total uptake of at most 1.2 fold corresponds to an increase in specific activity of RNA of 5-fold. These results make it clear that the increased specific

activity of RNA cannot be accounted for by an increase of the specific activity of the precursor pool resulting from higher intracellular concentration of [^{3}H]-uridine.

TABLE I

Test of significance of time-dependent changes of [^{3}H]*-uridine uptake into mammary gland* RNA *after a single injection of* 17 β*-oestradiol into ovariectomized mice* (1).

Time after 17 β-oestradiol (h)	Observation Number (2)	Mean	$\Sigma x^2 \times 10^{-6}$	Standard Deviation (percent)	P
0	9	11581	—	16.8	—
0,25	4	20312	358	30.7	< 0.05
0,5	4	31053	1670	45.0	< 0.05
1	6	31633	2634	48.0	< 0.05
1,5	3	25233	519	23.3	< 0.05
2	5	19280	353	29.8	< 0.05
3	3	15800	122	32.2	> 0.05
4 + 5 + 6	5	13400	52	12.4	> 0.05
7	4	13150	72	26.0	> 0.05
8	6	18483	542	44.6	> 0.05
9	6	18941	467	36.7	< 0.05
10	6	14608	208	36.8	> 0.05
11	4	13561	64	20.5	> 0.05
12	4	25805	729	26.3	< 0.05
13	4	13700	45	6.7	> 0.05
14	4	11150	41	5.0	> 0.05
15	4	14025	85	25.5	> 0.05
16	4	21175	441	34.0	< 0.05
17	4	14100	62	15.4	> 0.05

(1) Groups of values at different time-intervals after hormone injection were compared with the group of values at time 0 (basal value) according to the " null hypothesis " method. The method for groups with different numbers of values was applied according to Snedecor [20]. When $P > 0.05$ the null hypothesis may not be rejected and the difference is not significant.

(2) Each observation is carried out on a pool of six glands from three different mice.

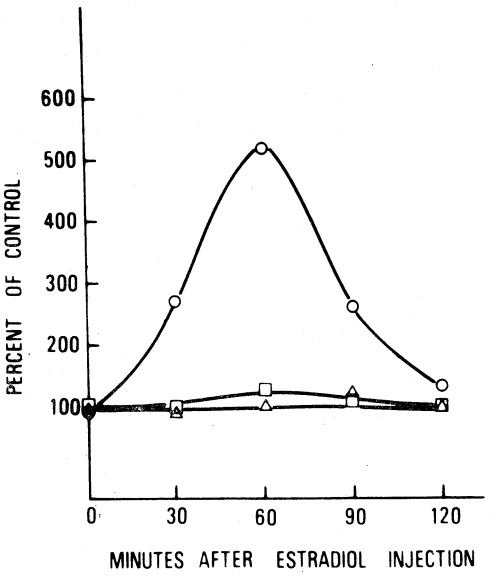

Fig. 3. – Effect of single injection of 17 β-oestradiol on wet weight (△), total [^{3}H]-uridine uptake (□) and specific radioactivity of RNA (○) of mammary glands in ovariectomized adult mice.

Experimental procedure are as described in legend to fig. 2 except that glands were carefully dissected and weighed before further processing. Total radioactivity was referred to gm of fresh tissue. Data in figure are expressed as percentage of control values. The control values are as follows: wet weight of 6 glands, 874 mg; total radioactivity, 785,300 d.p.m. per gm of wet weight; specific radioactivity of RNA, 11,600 d.p.m. per mg.

DISCUSSION

The finding of significant discrete bursts of increased rate of precursor uptake into mammary gland RNA following a single injection of 17 β-oestradiol into ovariectomized mice is undoubtedly the result of measurements having been made at short intervals. Had, for instance, the measurements been taken at 2 h intervals, instead of a maximum of 1 h as done in this study, most of the peaks would have been missed and the resulting apparent pattern would have been quite different from what was actually found. Such a difference in experimental approach may at least in part justify differences from findings with oestrogen-stimulated rat uterus [11-13]. In general, the studies with uterus cover in detail the initial 2 to 4 h after oestrogen injection, while later measurements are taken at long intervals. On the whole, increased nucleoside incorporation into uterus RNA is found to begin within a few minutes after oestrogen administration and to peak within the 1st h; thereafter, incorporation decreases to return to about twice the pre-stimulation level within 2 h from hormone administration; measurements at 12 and 24 h show about the same rate of incorporation as observed at 2 h. Thus, while the first burst of precursors uptake into RNA appears to be a common feature of mammary gland and uterus, there is insufficient data for a detailed comparison of later

behaviour of these two oestrogen target organs. It is not unreasonable, however, that organ- and species-specific differences exist.

Of course, an increased incorporation of injected radioactive precursors into cell RNA does not necessarily mean an increased rate of synthesis of the macromolecules. An increase in the specific activity of the precursor pool in the cell, brought about by either an increased influx of the labeled precursor or a decreased size of the pool of endogenous precursor, or both, could account for a higher specific activity of cellular macromolecules. The fact that despite the many studies with uterus bearing on this subject the answer is still not univocal [14, 15, 16] is symptomatic of the difficulty of resolving this problem. The most solid evidence suggesting that a least in part the early increases in precursor uptake into RNA of stimulated uterus represent true increase of synthesis are those showing that specific radioactivity of particular species of cellular RNA [17, 18] is increased; and, furthermore, that endogenous RNA polymerase activity of isolated nuclei increases after oestrogen stimulation [19].

We plan similar studies with the mammary gland. However, we can already point out that available information bearing on this problem and presented in this paper does not suggest a primary effect of oestrogen on influx of radioactive precursors into the gland. Indeed, total weight of the mammary glands (unlike that of the uterus) does not change following ovariectomy and 17 β-oestradiol injection, and total uptake of [^{3}H]-uridine per unit weight of gland remains either unchanged or is at most 20 % higher when precursor incorporation into RNA is up 5-fold (fig. 3); such a slight increase of total precursor uptake cannot justify the much higher specific radioactivity of RNA and, in all likelihood, is the result and not the cause of the enhanced precursor incorporation into RNA. Also, the appearance of several discrete bursts of increased precursor incorporation into RNA is in itself an argument against explanation of these increases as a result of changes in the specific radioactivity of precursor pools. Indeed, it would require a rather unlikely series of discontinous and abrupt changes in specific activity of the precursor pools to explain the sequence of bursts of increased incorporation.

The observed changes in precursor uptake into RNA are thus likely to represent unfolding of an orderly pattern of sequential activation of RNA synthesis in response to the hormone and in preparation of further events leading to the full-fledged oestrogen effect on the gland. One must consider the following possible causes for each burst of synthetic activity: (i) the activity is induced by oestrogen itself; (ii) the activity is induced by the product of a previous synthetic burst; (iii) the activity is the result of an indirect effect of oestrogen via another oestrogen target tissue; for instance, oestrogen induced secretion of prolactin; (iv) different bursts represent responses of different tissues in the gland. Eventually, we plan to analyze these various possibilities in an effort to unravel the mechanism of oestrogen effect on the mammary gland.

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REFERENCES

[1] BRESCIANI F., « Scienze », *146*, 653 (1964).
[2] BRESCIANI F., « Exptl. Cell Res. », *38*, 13 (1965).
[3] BRESCIANI F., *in* Hubinot P. O., F. Leroy and P. Galand (eds.), *Basic Actions of Sex Steroids on Target Organs*, S. Karger, Basel and New York, 1971, p. 130.
[4] BRESCIANI F., « Cell Tissue Kinet. », *1*, 51 (1968).
[5] BRESCIANI F. and PUCA G. A., « Atti Soc. Ital. Patol. », *9*, 661 (1965).
[6] PUCA G. A. and BRESCIANI F., « Endocrinology », *85*, 1 (1969).
[7] SHYAMALA G. and NANDI S., « Endocrinology », *91*, 861 (1972).
[8] PUCA G. A., ORII H. and FRAGOMELE F., « Atti Soc. Ital. Patol. », *10*, 979 (1967).
[9] CERIOTTI G., « J. Biol. Chem. », *214*, 59 (1955).
[10] EGGSTEIN M. and KREUTZ F. H., « Klinische Wochenschr. », *33*, 879 (1955).
[11] TATA J. R., « Progr. Nucl. Acid Res. Mol. Biol. », *5*, 191 (1966).
[12] HAMILTON T. H., « Science », *161*, 649 (1968).
[13] HAMILTON T. H., *in* HUBINOT P. O., F. LEROY and P. GALAND (eds.), *Basic Actions of Sex Steroids on Target Organs*, S. Karger, Basel and New York, 1971, p. 56.
[14] BILLING R. J., BARBIROLI B. and SMELLIE R. M. S., « Biochim. Biophys. Acta », *190*, 52 (1969).
[15] OLIVER J. M. and KELLIE A. E., « Biochem. J. », *119*, 187 (1970).
[16] OLIVER J. M., « Biochem. J. », *121*, 83 (1971).
[17] KNOWLER J. T. and SMELLIE R. M. S., « Biochem. J. », *125*, 605 (1971).
[18] KNOWLER J. T. and SMELLIE R. M. S., « Biochem. J. », *131*, 689 (1973).
[19] GLASSER S. R., CHYTIL F. and SPELSBERG T. C., « Biochem. J. », *130*, 947 (1972).
[20] SNEDECOR G. W., *Statistical Methods*, ed. 5, Iowa State University Press, Ames, Iowa, 1956, p. 85.