The stimulation of follicular growth in cultured mouse ovaries by lactate salts

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When intact ovaries of infantile mice are cultured in the presence of gonadotrophins, steroids or dibutyl cyclic adenosine 3':5' monophosphate follicular growth is stimulated. If [³H]thymidine is supplied in the medium, its accumulation in the tissue is enhanced as a result of this growth (Ryle, 1969, 1972, 1974; Kent, 1973). During other experiments it was noted that lithium lactate also increased the uptake of [³H]thymidine by cultured ovaries, and the following studies were designed to extend this observation.

A single foster litter of ten 15-day-old mice, of the CFW strain, provided the ovaries for each experiment. The medium and culture procedure, the dissolution of the tissue and the counting routine for radioactivity were as described previously for the assay of FSH (Ryle, 1971, as modified by Boggis & Ryle, 1972). The medium containing 5% (v/v) serum which, in most experiments, was from a hypophysectomized sheep. However, owing to limited supplies, normal calf serum was occasionally used. [Me-³H]Thymidine was supplied by the Radiochemical Centre, Amersham. Other chemicals were obtained from Sigma (London) Chemical Company Ltd. The cultures were maintained for 3, 4 or 5 days. Those intended for definitive morphological study were gassed with air at 0.2 kg/cm² above atmospheric pressure, since this minimizes tissue damage.

For morphological studies 2 µg Colcemid (CIBA Laboratories, Horsham, Sussex) were added to each dish 4 hr before the tissue was transferred to Bouin's aqueous fixative. Serial sections (10 µm) were cut and stained with Harris's haematoxylin and eosin. Code numbers were assigned to the slides before examination. Every section was inspected and each recorded follicle was examined in that section which showed its oocyte nucleolus. The number of granulosa cell layers and the numbers of mitotic figures within the granulosa cells were noted.

Experiments were carried out to examine: (a) the relationship between lithium lactate concentration and [³H]thymidine uptake; (b) the importance of the anion and cation for the ovarian response; (c) the follicle population which grows in response to lithium lactate; (d) possible steps involved in the stimulation of follicular growth. Unless otherwise stated four ovaries were assigned to each treatment group in each experiment. When any procedure enhanced the uptake of [³H]thymidine its effect on follicular growth was subsequently checked morphologically.

The effects of varying the concentration of lithium lactate. In three experiments ovaries were cultured for 4 days in concentrations of lithium lactate ranging from 1.25 to 60 mM. [³H]Thymidine was added on the 2nd day. The quantity which accumulated in the tissue increased linearly with log concentration of lithium lactate up to 10-15 mM, then declined (Table 1).

Table 1. The uptake of [³H]thymidine (ct/min⁻¹ per ovary) by mouse ovaries (4/group) in response to different concentrations of lithium lactate

		Lithium lactate (mM)								
Exp.		0	1.25	2.5	5	10	15	30	45	60
1	Mean	442	569	751	940	1137	—	—	—	—
	S.E.M.	21	36	112	87	64				
2	Mean	—	—	—	—	—	1226	805	234	27
	S.E.M.						42	71	34	1

The role of the cation and anion. Groups of ovaries were cultured with 0, 5, 10, 20 and 40 mM-lithium hydroxide, the pH of the medium being adjusted to 7.6 with 0.1 N-HCl. Significantly more ($P < 0.05$) [^3H]thymidine was taken up at 5 mM than by the control ovaries, but apparently in a non-specific manner for a subsequent histological study showed that the tissue was badly damaged and there was no indication of follicular growth. In two experiments in which groups of ovaries were cultured with 5 and 10 mM-sodium lactate [^3H]thymidine uptake was enhanced, although not as much as by lithium lactate. The lactate ion was therefore stimulatory but the extent of its effect was modified by the associated cation.

The responsive follicle population. In a preliminary experiment groups of 4 ovaries were cultured in medium alone or with 5-mM-lithium lactate for 3 days. The mean ($\pm$ S.E.M.) number of follicles with three layers of granulosa cells per ovary was 11.0 ± 0.1 in the control group and 22.3 ± 4.6 in the treated group ($P < 0.05$). Neither the number of follicles with four or more layers of granulosa cells nor the mean number of mitotic figures per three-layered follicle were significantly affected. Smaller follicles were not examined.

In a second experiment groups of ovaries were cultured for 4 days with no additive, with 5 mM-lithium lactate or with 5 mM-sodium lactate. The results in Table 2 show that lithium lactate enhanced mitotic activity in follicles with 2 layers of granulosa cells but not in larger ones. The numbers of follicles with 2 and 3 layers of granulosa cells were increased, the former presumably as a result of enhanced growth in follicles with 1 layer, although these were not examined. Associated with the lack of increased mitotic activity in follicles with 3 layers, the number with 4 layers was unchanged. Sodium lactate was again less effective than lithium lactate.

Table 2. Effects of lithium lactate and sodium lactate on the mean number of follicles of different sizes and on mitotic activity in their granulosa cells (means $\pm$ S.E.M.) in mouse ovaries (5/group)

No. of granulosa cell layers	No. of follicles			No. of mitotic figures/follicle		
	Control	Lithium lactate	Sodium lactate	Control	Lithium lactate	Sodium lactate
2	11.2 ± 2.4	$27.4 \pm 5.5^*$	15.6 ± 2.7	0	$0.36 \pm 0.11^*$	0.25 ± 0.14
3	1.4 ± 0.49	$6.0 \pm 1.6^*$	$4.8 \pm 1.4^\dagger$	1.0 ± 0.55	0.46 ± 0.07	0.21 ± 0.13
4	0.2 ± 0.2	0.22 ± 0.22	0.4 ± 0.24	0	0	0.2 ± 0.2

Significantly greater than controls, $*P < 0.01$, $^\dagger P < 0.05$.

Table 3. Effects of DL- and L-lithium lactate on the mean ($\pm$ S.E.M.) uptake of [^3H]thymidine (ct/min $^{-1}$ per ovary) by mouse ovaries (3/group)

Exp.	Control	DL-Lactate		L-Lactate	
		2.5 mM	5 mM	2.5 mM	5 mM
1	789 ± 46	—	1389 ± 80	887 ± 20	1389 ± 46
2	631 ± 76	1046 ± 39	1491 ± 332	1097 ± 168	1333 ± 74

Possible metabolic steps involved in ovarian stimulation by lactate. It seemed likely that the conversion of lactate to pyruvate by ovarian lactic dehydrogenase would be the first step in the stimulatory process. However, concentrations of pyruvate between 0.025 and 160 mM did not stimulate [^3H]thymidine uptake, and the addition of excess pyruvate to inhibit the reduction of nicotinamide adenine dinucleotide (NAD) by lactate dehydrogenase did not prevent the stimulation of [^3H]thymidine uptake by lithium lactate. Moreover, the sodium salts of two analogues of lactic acid (hydroxycaproic acid and hydroxybutyric acid), which can serve as substrates for lactic dehydrogenase and so promote the reduction of NAD, failed to enhance [^3H]thymidine uptake at concentrations from 0.5 to 80 mM. Finally it was found that the effects of L-lactate were not significantly different from those of DL-lactate at the same concentrations (Table 3), showing that the non-metabolizable isomer was as effective as the metabolizable one.

It was hoped that the response to lithium lactate might throw some light on the mechanism of action of FSH, especially because in three tests in which the uptake of [^3H]thymidine was measured, the dose-response curves for the two substances were parallel (J. Boggis, unpublished observations). However, both lithium and sodium lactate stimulated growth only in follicles with less than 3 granulosa cell layers whereas, in similar prolonged cultures, FSH, stimulates growth in follicles of all sizes (Ryle, 1972). Since FSH rapidly enhances lactate production by rat ovaries *in vitro* (Ahrén, Hamberger & Rubinstein, 1968) its stimulation of growth in small follicles may to some extent result from the conditions of culture.

Although gonadotrophins supplied in the medium during prolonged cultures stimulate ovarian follicle growth, and presumably protein synthesis, they fail, during brief incubations, to enhance the incorporation of amino acid into ovarian protein. However if injected into the animal an hour or more before removal of the ovaries they are effective (Ahrén *et al.*, 1965). Nilsson & Selstam (1975) found that, after injecting LH, augmented lactate production preceded augmented amino acid incorporation. Perhaps lactate accumulates to a sufficient local concentration to enhance protein synthesis when the ovary has already been uniformly stimulated *in vivo*, but does not reach adequate levels during incubations with gonadotrophins in the medium which last only a few hours. Pregnenolone, testosterone, oestradiol-17 β and also dbcAMP—unlike gonadotrophins—all stimulate growth in the same limited mouse follicle population as does lithium lactate (Kent, 1973; Ryle, 1974). Since cyclic AMP rapidly enhances lactate production by incubated rat ovaries (Herlitz, Hamberger & Ahrén, 1974), it is possible that its growth-stimulating effects on cultured mouse ovaries, and also those of the three steroids, are mediated by this metabolic product.

Follicles with 2 (and presumably 1) layers of granulosa cells grew in response to lactate salts while those with 3 and 4 layers did not. This suggests that some change in membrane function of thecal cells, granulosa cells or the intervening basal lamina occurs at this stage of follicular development. The present results have shown that a small, non-hormonal molecule can stimulate mitotic activity and organized growth *in vitro* and have suggested that the initial step of this stimulation does not require the action of lactic dehydrogenase, but there is as yet no evidence to indicate by what mechanism mitosis is induced.

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