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Lipid peroxidation, low-level chemiluminescence and regulation of secretion in the mammary gland

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Abstract. In mammary explants of lactating mice, changes in the intensity of chemiluminescence (CL) were observed after the addition to the incubation medium of hormones and mediators that are involved in the regulation of secretion: oxytocin, acetylcholine, epinephrine and norepinephrine. A 15-min period of treatment with oxytocin, epinephrine or norepinephrine changed the level of thiobarbituric acid-reactive substances (TBARS). Two mammary explants, one of which was treated with oxytocin, acetylcholine, epinephrine or norepinephrine, were found to interact even when separated by a quartz glass wall. Analysis of the level of TBARS formation in these two explants showed that the observed interactions might be connected with light emission resulting from lipid peroxidation (LP) processes. The possible role of LP and low-level CL in the regulation of mammary gland secretion is discussed.

Key words. Mammary gland; regulation of protein secretion; chemiluminescence; lipid peroxidation.

Changes in lipid peroxidation (LP) processes are often responsible for rapid changes in the fatty acid composition of cell membranes influencing their permeability, the activity of membrane enzymes, etc. LP reactions are involved in the synthesis of many biologically active derivatives of polyunsaturated fatty acids[1–7]. In addition to this, it is well known that the processes which accompany radical chain LP reactions induce low-level chemiluminescence (CL)[8–10]. Biological photoemission has been under discussion since the 1920s[11–14], and there is still considerable controversy about it. A number of questions remain unanswered. The main objection to the hypothesis that there may be optical interaction between cells was that light emission from biological tissues could not be reliably detected by conventional physical methods. However, this difficulty was removed when photomultipliers became available[15–18]. Lipid peroxidation has been studied mainly in connection with the development of free radical-mediated diseases, tissue injuries, etc. Hence, though there is little doubt that LP processes play a physiological role, there is often no evidence for their participation in the neurohormonal control of a particular function. First, for the majority of tissues there are no data about the triggering of the LP mechanism by regulatory factors. If we consider secretion by the mammary gland, for example, there are only a few indirect indications in the literature. It was shown that prooxidant factors of leucocytes (whose migration into tissues is known to be under neuro-hormonal control) affect the functional activity of the secretory epithelium[19]. LP was observed to increase within the period of inhibition of secretion when an intensive leucocyte infiltration of mammary tissue occurred[20]. In the present work we studied whether hormones and mediators which participate in the regulation of mammary epithelial cell exocytosis could directly influence the LP and CL processes in mammary explants. In addition to this, bearing in mind that the functional significance of biological CL is not widely recognized, we tried to examine whether the photoemission which accompanies LP processes could affect the physiological activity of secretory cells.

Methods

The experiments were carried out on 2–3 h mammary tissue cultures (explants) of lactating albino mice (10–16 d of lactation). All explants weighed 10 mg (wet weight). The incubation medium was Hank's physiological solution (37 °C). In earlier electrophysiological and histological experiments it was shown that the mammary gland of lactating mice contains at least two populations of secretory cells which function in different phases[20,21]. In order to get reliable results which would permit us to characterize the activity of a single secretory cell we used the following approach. The last suckling of pups before they were weaned occurred 4–5 h prior to the mammary gland excision. This caused inhibition of the secretory processes in most of the cells. In tissue culture, these cells appeared to be synchronized by the phase of the secretory cycle, as shown by electronmicroscopic analysis and other methods. The rate of the secretory process was judged by the protein-excreting activity of the secretory epithelium, which was assayed by measuring the protein content of the incubation medium at the end of the experiment by the method of Lowry (ref. 22). Methods of tissue

culture preparation, as well as a detailed discussion of the validity of this physiological model for the investigation of the control mechanisms of the secretory process, are described elsewhere[20,21].

We studied the action of oxytocin and other hormones or mediators which are known to be involved in the regulation of protein excretion by mammary secretory cells[20,21]. The following concentrations of physiologically active substances (PAS) were used: oxytocin ($1 \cdot 10^{-8}$ M), acetylcholine hydrochloride ($5 \cdot 10^{-5}$ M), epinephrine hydrochloride ($5 \cdot 10^{-6}$ M), norepinephrine hydrotartrate ($5 \cdot 10^{-6}$–$5 \cdot 10^{-5}$ M).

The intensity of spontaneous CL of the explants was recorded using a chemiluminometer HLM1C-01, which measured ultra weak light emission within the range of 400–600 nm[23]. The readings were made every 10 s. Dynamics of CL was determined for 2 min before and 5 min after the application of a PAS. In all other experiments the test duration was 15 min.

LP level was estimated by determining the TBARS content. In the assay used, only water soluble but not protein-bound products were examined[24]. While bearing in mind the possible selectivity of TBA-tests[3,25–27], we expressed the obtained values as nanomoles of TBARS per g wet weight, using the molar extinction coefficient for malondialdehyde ($1.56 \cdot 10^5$ $M^{-1} \cdot cm^{-1}$).

To test possible effects of spontaneous CL on the functional activity of cells, we looked for mutual optical effects of one mammary explant on another. An explant exposed to a PAS served as a source of low-level CL, or inductor (*I*), while another one, not treated with a PAS, was used as a detector (*D*). The explants were incubated in a special experimental chamber (fig. 1A). The *I* explant was incubated in a horizontal plane, at a fixed horizontal angle to control (*C*) and *D* explants. With the arrangement shown, the studied effect was observed even when the distance between two cultures was rather large (about 1 cm) and the exposure period was short (several hours)[28]. The explants were in separate chambers divided by transparent walls of quartz glass 0.1 mm thick. An additional non-transparent screen was fixed between the *I* and the *C* explants, so that the *C* tissue could serve as a control for optical influences of *I* on *D*. The chamber construction allowed for two *D* and two *C* explants (fig. 1B). To test the possibility of an optical influence from the *D* to the *I*, one of the controls (control treated, *CT*) was exposed to the action of the same PAS as the *I* (control for *I*). After incubation, the intensities of protein secretion and of LP were compared in the tissues of the *D* and *I* explants, and also compared with the respective controls. The experiments were carried out under normal ambient light conditions. All the results are presented as means ± standard errors. Values of the experimental observations varied considerably from one trial to another, owing mainly to variations in tissue status in the model object used.

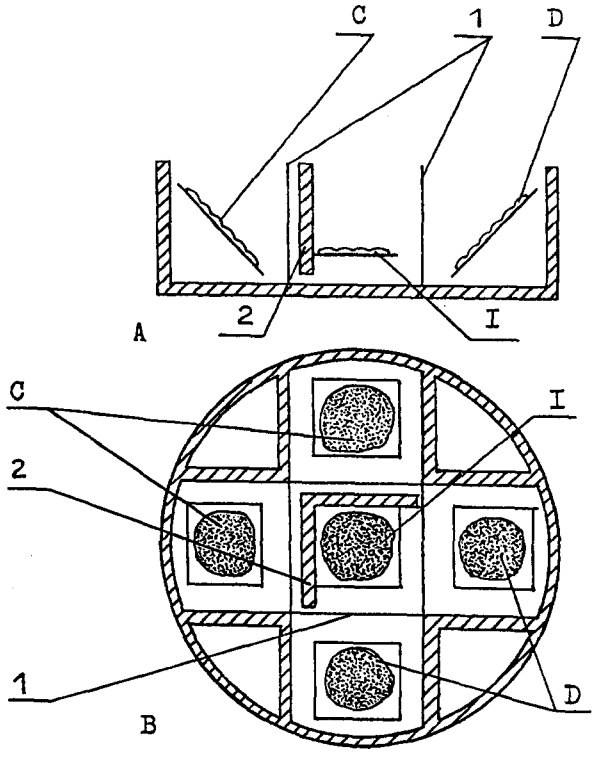

Figure 1. Diagram of the experimental set-up for the investigation of the possibility of optical influences being exerted by one explant on the other. *A*, vertical plane; *B*, horizontal plane; *I*, *D* and *C*, explants (*I*, inductor; *D*, detector; *C*, control); 1, quartz glass wall; 2, non-transparent screen. Refer to text for details.

Differences in CL under the same conditions resulted from differences in the surface areas of explants. High values for standard errors were usual (fig. 2A, tables 1 and 2). However, the data obtained from the parallel samples, D_1 and D_2, were in some cases very similar (fig. 2B). The type of difference (increase/decrease) between the values of experimental and control explants (*C*/*D*, *C*/*I*, etc) was stable and reproducible. The only method to prove the significance of differences between such data is a non-parametric test. The Wilkoxon tests were used, preferably the Wilkoxon (Mann-Whitney) test for paired data where appropriate. During the studies of simultaneous processes, correlation analysis between the values obtained simultaneously was performed. The results of 151 experiments are presented in this work.

Results and discussion

The initial level of spontaneous photoemission of mammary explants averaged 190 ± 18 counts per min (the wet weight of the explants was 10 mg in all the trials). Changes in CL intensity in response to a PAS are shown in figure 3. Both oxytocin and acetylcholine application first resulted in a decrease of the intensity of luminescence. The difference from the initial level was significant ($p < 0.05$) in the 10–20 s and the 80–90 s time-periods, starting from the moment of application.

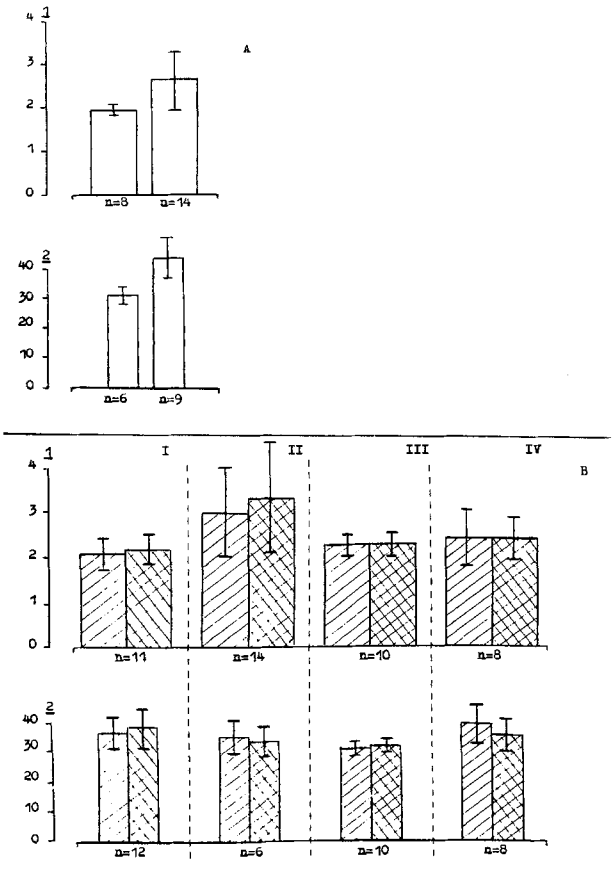

Figure 2. The 'biological variance' of mean values and standard errors observed under the same experimental conditions. *A* Average values from different series of experiments/untreated control (*CU*). The most different results are selected for demonstration. *B* Each pair represents the data of one series of experiments. All examples are parallel samples of detector/explants, incubated simultaneously in two detector sectors of experimental chamber. Experimental series: I, oxytocin ($1 \cdot 10^{-8}$ M)-treated inductor; II, acetylcholine ($5 \cdot 10^{-5}$ M)-treated inductor; III, norepinephrine ($5 \cdot 10^{-5}$ M)-treated inductor; IV, epinephrine ($5 \cdot 10^{-6}$ M)-treated inductor. *1*, protein content in the incubation medium following 15 min incubation (average values: mg/g wet weight $\pm$SE); *2*, TBARS content in explants following 15 min incubation (average values: nmol/g wet weight $\pm$ SE). Blank bars, control; hatched, 1st detector; cross-hatched, 2nd detector. Refer to figure 1 and text for details.

During the 210–220 s period, photoemission increased ($p < 0.05$).

Epinephrine caused a steady increase in the intensity of CL, which started to develop 10 s after the application and continued during the entire period of recording (5 min). Under the influence of norepinephrine, significant differences compared to the initial level ($p < 0.05$) were observed in the values obtained from measurements at 20–30 s, 90–100 s (decrease) and 120–240 s (increase).

It should be noted that no changes in the CL-intensity were observed in control experiments in which instead of PAS-solutions similar volumes of physiological salt solution were added to the incubation medium.

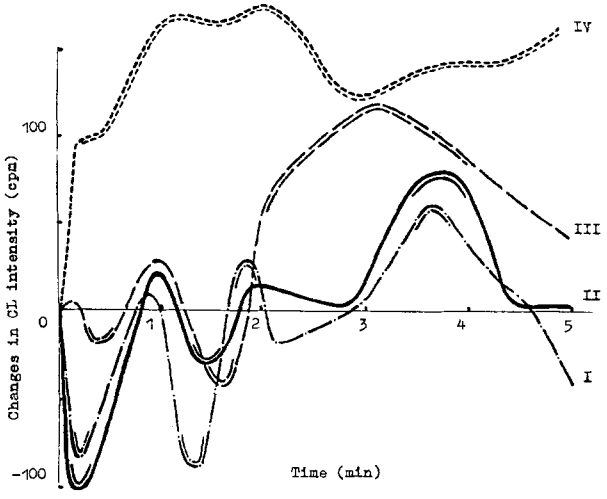

Figure 3. Changes in chemiluminescence (CL) intensity in mammary explants of lactating mice (average values) under the influence of physiologically active substances. I, oxytocin ($1 \cdot 10^{-8}$ M), n = 8; II, acetylcholine ($5 \cdot 10^{-5}$ M), n = 8; III, norepinephrine ($5 \cdot 10^{-6}$ M), n = 8; IV, epinephrine ($5 \cdot 10^{-6}$ M), n = 8. Zero axis, average initial level of CL before the application of physiologically active substances. CL intensity is expressed as a unit of counts per min (cpm), the wet weight of each explant being 10 mg in all the trials. Duplication of curves means that the deviations of CL intensity from the initial level are statistically significant ($p < 0.05$; Wilkoxon's W-test for paired data; comparison of discretely recorded data).

The CL dynamics registered, though they were of a very complex character, show some similarities between different PAS, for example oxytocin and acetylcholine. The correlation coefficient between the changes of CL intensity in tissues under the action of these substances had the value of 0.73 ± 0.17 ($p < 0.01$). This similarity could indicate a common mechanism in their action on the effector cell. Such a common mechanism was suggested previously[20,21].

According to the present concepts, visible-range spontaneous CL of cells and organs often, but not always, reflects the occurrence of radical-chain LP reactions[8,10]. Thus, the observation of CL activity on the application of a secretion-regulating PAS does not necessarily mean that the PAS influences the intensity of LP as well. To clarify this point, an analysis of the accumulation of LP products (TBARS) in the mammary explants was performed. Explants were subjected either to the independent action of one PAS, or to the action of several such substances simultaneously, which more closely resembled natural regulatory mechanisms.

The data summarized in table 1 show that under the experimental conditions used both stimulation and inhibition of secretory processes occurred. Following the application of acetylcholine, the amount of protein in the incubation medium was on the average 20% higher than in the control, the difference being significant ($p < 0.01$). When both acetylcholine and oxytocin were used the increase was more pronounced, 53% ($p < 0.01$). The protein content increased by 25% under the

Table 1. Influence of physiologically active substances (PAS) on protein excretion and lipid peroxidation in mammary explants of lactating mice

PAS	Process examined	Number of trials	Controls	
			no treatment (CU)	with Pas (CT)
oxytocin ($1 \cdot 10^{-8}$ M)	1	20	2.1 ± 0.2	2.3 ± 0.2
	2	14	42.9 ± 6.5	50.7 ± 8.4*
acetylcholine ($5 \cdot 10^{-5}$ M)	1	38	2.5 ± 0.5	3.0 ± 0.5*
	2	24	34.2 ± 4.0	35.5 ± 4.0
epinephrine ($5 \cdot 10^{-6}$ M)	1	23	2.5 ± 0.5	2.3 ± 0.5
	2	23	40.4 ± 6.2	51.6 ± 6.8*
oxytocin ($1 \cdot 10^{-8}$ M) + acetylcholine ($5 \cdot 10^{-5}$ M)	1	8	2.1 ± 0.2	3.3 ± 0.2*
	2	7	34.2 ± 3.4	37.8 ± 3.7
oxytocin ($1 \cdot 10^{-8}$ M) + acethycholine ($5 \cdot 10^{-5}$ M) + epinephrine ($5 \cdot 10^{-6}$ M)	1	8	2.1 ± 0.2	2.7 ± 0.2*
	2	7	35.0 ± 3.5	32.9 ± 3.3
norepinephrine ($5 \cdot 10^{-6}$ M)	1	14	2.3 ± 0.2	2.9 ± 0.2
	2	9	44.0 ± 7.0	36.6 ± 5.0
norepinephrine ($5 \cdot 10^{-5}$ M)	1	8	1.9 ± 0.1	1.6 ± 0.1*
	2	8	33.5 ± 2.8	29.2 ± 2.6*

1, protein content in the incubation medium after 15 min of incubation (average values: mg/g wet weight ± SE); 2, TBARS content in explants following 15 min incubation (average values: nmol/g wet weight ± SE); ** – differences are significant compared to CU (Mann-Whitney U-test; *$p < 0.05$; *$p < 0.01$)

influence of acetylcholine, oxytocin and epinephrine, $p < 0.05$. However, with norepinephrine alone, at $5 \cdot 10^{-5}$ M, protein content decreased by 16% on the average ($p < 0.05$). In some cases the differences in relation to the norm were not significant (norepinephrine at $5 \cdot 10^{-6}$; oxytocin; epinephrine).

LP was shown to be intensified under the action of oxytocin or epinephrine. The amount of TBARS increased in explants treated with these PAS by 12% ($p < 0.01$) and by 11% ($p < 0.05$) respectively, compared to the norm. TBARS content decreased by 22% in explants subjected to the action of norepinephrine ($5 \cdot 10^{-5}$ M, $p < 0.01$), which means that the LP intensity lessened. In other cases no statistically significant changes in the LP intensity were observed (table 1).

Of particular interest is the following observation: hormones and mediators that exert a unidirectional effect on secretion influence the LP differently. Correlation analysis did not reveal a linear dependence between the indices of changes in LP and secretion intensities ($r = -0.03 \pm 0.11$; $p > 0.05$). The curvilinear correlation coefficient, however, was significant: $r_\eta = 0.50 \pm 0.10$; $p < 0.01$, which means that there is an interrelation between the observed processes, but it is of a complex character.

The results obtained in the present investigation show that all the factors which cause a multiphase luminescent reaction, with the weakening of emission during the first 2 min and its intensification by the third minute of exposure (oxytocin, acetylcholine, or norepinephrine,

Table 2. Mutual optical influence of mammary explants of lactating mice, one of the explants being under the action of a physiologically active substance (PAS)

PAS	Process examined	Number of trials	Controls		Experiment	
			no treatment (CU)	with PAS (CT)	untreated (detector)	with PAS (inductor)
oxytocin ($1 \cdot 10^{-8}$ M)	1	11	2.3 ± 0.4	2.4 ± 0.4	2.1 ± 0.3	2.7 ± 0.4**,***
	2	12	37.8 ± 5.9	47.4 ± 6.5	37.2 ± 5.9	27.9 ± 4.0**,***
acetylcholine ($5 \cdot 10^{-5}$ M)	1	14	2.6 ± 0.7	3.0 ± 0.8*	3.2 ± 1.1*	2.4 ± 0.6**,***
	2	6	31.6 ± 2.8	33.5 ± 5.3	34.2 ± 5.5	31.3 ± 4.3
epinephrine ($5 \cdot 10^{-6}$ M)	1	10	2.6 ± 0.7	2.4 ± 0.6	2.4 ± 0.5	2.1 ± 0.5*
	2	10	34.0 ± 5.6	34.7 ± 5.7	37.8 ± 5.9	38.1 ± 5.9
norepinephrine ($5 \cdot 10^{-5}$ M)	1	8	1.9 ± 0.1	1.6 ± 0.1*	2.3 ± 0.1*	1.5 ± 0.1*,***
	2	8	33.5 ± 2.8	29.2 ± 2.6*	31.0 ± 2.5	44.0 ± 6.5**

1 and 2, see table 1; * and *, signicantly different compared to CU; ** and **, significantly different compared to CT; *** and ***, significantly different compared to detector (Wilkoxon W-test for paired data; *,** and ***, $p < 0.05$; *, ** and ***, $p < 0.01$)

$5 \cdot 10^{-6}$ M) appear to stimulate protein secretion in secretory cells (fig. 3, table 1). It is known that exocytosis in secretory cells of the mouse mammary gland occurs mainly within the first two minutes of the application of a stimulus[20,21], just at the time when a decrease of CL is observed. We believe that exocytosis, with a simultaneous decrease of tissue CL, reflects an interrelation between the processes of secretion and generation of free radicals, under the action of regulatory factors. By direct study of the mutual influence of one of the processes on the other it might be possible to elucidate these mechanisms. We therefore attempted to determine whether the CL which accompanies LP changes resulting from the action of regulatory factors can influence the secretory process. We observed that protein excretion and LP intensities in an untreated mammary explant could be influenced by a treated explant if the barrier between them was transparent. When the barrier was non-transparent (i.e. control conditions) then the activities (table 2) were similar to those presented in table 1. But they changed significantly with a transparent wall. In the untreated explant, *D* (photoemission detector), changes occurred when a second experimental explant (the inductor, *I*) was subjected to the action of acetylcholine or norepinephrine. An increase of protein secretion was observed in the *D* explant compared to a control (*CU*) which was not treated and was separated from the treated explant by an opaque barrier. The increase was 20% ($p < 0.01$) with acetylcholine and 18% ($p < 0.01$) with norepinephrine (table 2). When explant *I* was treated with oxytocin or epinephrine no statistically significant changes were observed in *D*. In all cases there were no changes in the LP intensity in *D* compared to *CU*.

Differences were also observed in the *I* explants compared to controls (*CT*) treated with the same PAS. These were: extra protein secretion in the *I* treated with oxytocin (16%, $p < 0.05$), and inhibition of the exocytosis stimulated by acetylcholine – protein content in the incubation medium decreased by 20% compared to *CT* ($p < 0.01$). In the *I* explant treated with catecholamines no statistically significant changes were revealed. Differences in the LP intensity in the *I* explants were observed when these were treated with oxytocin or norepinephrine. In the first case TBARS content decreased by 27%, and in the second it increased by 42% ($p < 0.01$).

Another type of comparison also indicated a difference of tissue activities in experimental and control conditions. When comparing the treated explant *I* with the non-treated explant *D*, the observed effect of the PAS was different to that seen under control conditions. In the *I* treated with oxytocin we observed an increase of the protein secretion (29%, $p < 0.05$) and a decrease in the TBARS content (26%, $p < 0.05$) compared to the *D*. The exocytosis-stimulating action of acetylcholine in the *C* changes into an inhibitory one; the protein content decrease amounted to 35% ($p < 0.01$). The norepinephrine inhibitory action was stronger under experimental conditions than in *C* conditions, the protein discharge in the *I* decreased by 45% compared to *D* ($p < 0.01$).

It should also be mentioned that the tendency towards an inhibition of the protein excretion observed in explants under epinephrine in *C*-conditions becomes statistically significant in the experimental conditions: the protein content in the incubation medium declined by 19% ($p < 0.05$) compared to *C* without epinephrine. As the only difference in the experimental conditions in this series compared to *C*-conditions was the transparency of the wall between the *D* and the *I* explants, we believe that all the differences observed between the *D* and the *I* explants and the controls can be explained by the existence of interactions of an optical nature between these two explants.

To evaluate the contribution of LP-associated CL to the optical influences, we compared optically-induced changes in the secretory processes in one of the experimental (interacting) tissue explants with changes in the LP intensity in the other. The correlation analysis showed that these processes were interrelated. The correlation ratio of the dependency of changes in protein discharge in one of the experimental explants on the changes in TBARS production in the other is statistically significant ($p < 0.05$) and equals $\eta = 0.50 \pm 0.16$. The correlation ratio of the reverse dependence is also significant ($p < 0.05$), but its value is lower: $\eta = 0.40 \pm 0.17$. The existence of this dependence and its predominant direction allows us to suggest that LP reactions (or processes that accompany them) comprise one of the sources of the light emission which causes the observed effects.

The experimental results show that the type of influence of bio-CL on the mammary explant tissue depends on the original functional state of its epithelium. Thus the optical effect on the secretion was different in explants subjected to the action of various PAS. The correlation between the changes in protein discharge in these explants (*I*, according to the terminology used) and the LP changes in *D* explants (considered now as photoemission sources) also varied: correlation coefficient was 0.04 ± 0.30 ($p > 0.05$) in the experiments with oxytocin; 0.03 ± 0.50 ($p > 0.05$) with acetylcholine; 0.53 ± 0.33 ($p > 0.05$) with epinephrine and 0.69 ± 0.29 ($p < 0.05$) with norepinephrine ($5 \cdot 10^{-5}$ M).

A certain regularity was seen in the type of CL influence on explants that were incubated in hormone/mediator-free medium (*D*). Protein secretion, as a rule, was inhibited under the influence of CL which followed the intensification of fatty acid peroxidation. Conversely it was stimulated when CL originated from explants in which LP was weakened. The reverse correlation co-

efficient for these processes is 0.35 ± 0.17 ($p < 0.05$). It should be noted that in contrast to the tissues subjected to the action of norepinephrine or oxytocin, in these explants no significant optical influence on the intensity of peroxidation was revealed (table 2). It seems possible that these data characterize the peculiarities of the influence of bio-CL on intracellular self-regulatory mechanisms, or directly on the mechanisms of exocytosis in the secretory cells of mammary explants.

Of particular interest is the type of mutual optical influences observed in experimental explants (table 2): they exerted a contrary action on one another's excretory activity in all the trials except those when one of the explants was subjected to the action of epinephrine. These data might be important when considering the problem of coordination mechanisms in the mammary secretory epithelium. As has been noted, mammary epithelial cells are supposed to function 'asynchronously' [20,21]. Interaction of cells via CL might contribute to the development of a mechanism that controls this asynchronous functioning. The observation of unidirectional changes in the experiments where epinephrine was used allows us to suggest that optical cellular interactions might also induce a more complete inhibition of secretion in the mammary tissue under high concentrations of epinephrine.

In conclusion, the results obtained showed that LP processes may be involved in the regulation of secretion in the mammary gland, and that this involvement could be partially due to the accompanying CL.

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