Treatment of Immature Mice with Gonadotrophins

EFFECTS ON SOME ENZYMIC ACTIVITIES OF UNFRACTIONATED OVARIAN HOMOGENATES

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Treatment of immature mice with both follicle-stimulating hormone and human chorionic gonadotrophin *in vivo* resulted in large increases in the specific activities of ovarian alkaline phosphatase and alkaline nucleotidase. The specific activities of other ovarian enzymes studied were not altered by gonadotrophin treatment. A simultaneous change in the Michaelis constant of ovarian alkaline phosphatase accompanied the increase in specific activity. These changes commenced 6-8 h after injection of human chorionic gonadotrophin plus follicle-stimulating hormone. Injection of human chorionic gonadotrophin induced the change in Michaelis constant and increased ovarian alkaline phosphatase activity. Treatment with follicle-stimulating hormone had no effect on ovarian alkaline phosphatase. However, follicle-stimulating hormone synergistically augmented the response to human chorionic gonadotrophin. A latent period of about 24 h elapsed before this augmentation was expressed. Augmentation of ovarian alkaline phosphatase was directly related to the dose of follicle-stimulating hormone at a fixed dose of chorionic gonadotrophin. No response of ovarian alkaline phosphatase was observed after treatment of immature mice *in vivo* with oestrogens, progesterone, growth hormone or prolactin. Unlike chorionic gonadotrophin, sheep luteinizing hormone over a wide dose range induced no response within 24 h. However, a response in ovarian alkaline phosphatase was observed when sheep luteinizing hormone was administered in combination with follicle-stimulating hormone. The specific activity and K_m of ovarian alkaline phosphatase increased during normal maturation. The Michaelis constant ceased to increase as sexual maturity was reached. The changes in alkaline phosphatase activity were of a similar magnitude to those induced by gonadotrophin treatment. It is concluded that the changes induced acutely by treatment *in vivo* with unphysiological doses of gonadotrophins occur in the maturing mouse under the influence of endogenous, homologous gonadotrophins at physiological concentrations.

Probably the first step in the action of luteinizing hormone and chorionic gonadotrophin is the binding of the hormone to specific receptor sites on the target cell plasma membrane (Lee & Ryan, 1972; Rajaniemi & Vanha-Pertulla, 1972, 1973; Gospodarowicz, 1973). This results in the activation of ovarian adenylate cyclase (Fontaine *et al.*, 1971) and the intracellular production of adenosine 3':5'-cyclic monophosphate (Mason *et al.*, 1973) which then initiates or alters metabolic events within the cell. However, adenylate cyclase activation would seem to be part of a more general alteration of the structure and function of the cell surface membrane, and changes in the transport of small molecules and in enzyme activities characteristic of plasma membranes have been observed after activation of surface receptors.

Gonadotrophins have been shown to affect the transport of small molecules by the ovary. Low doses of luteinizing hormone or chorionic gonadotrophin

(and higher doses of follicle-stimulating hormone) *in vivo* rapidly increase the subsequent uptake of glucose *in vitro* by prepubertal rat ovaries (Armstrong *et al.*, 1963; Hamburger & Ahren, 1967; Ahren *et al.*, 1968). Follicle-stimulating hormone *in vivo* rapidly stimulates the uptake of amino acids *in vitro* by prepubertal rat ovaries (Ahren & Kostyo, 1963; Ahren & Rubinstein, 1965; Ahren *et al.*, 1967), mainly in the theca (Rubinstein, 1967).

Hormones have also been shown to affect certain surface-membrane enzyme activities. $\text{Na}^+ + \text{K}^+$ -stimulated ATPase* is concerned with active transport of Na^+ and K^+ and is located at the plasma membrane. This enzyme activity has been shown to be inhibited by luteinizing hormone in toad seminiferous tubules (Burgos *et al.*, 1968) and by insulin in rat uterus (Lostroh & Krahil, 1973), whereas the $\text{Na}^+ + \text{K}^+$ -stimulated ATPase of rat and human

* Abbreviation: ATPase adenosine triphosphatase.

prostate is activated by low concentrations of androgens (Farnsworth, 1970, 1972). Finally, Rubin *et al.* (1973) have demonstrated changes in the activity of three liver surface-membrane enzymes (Mg^{2+} stimulated ATPase, $Na^+ + K^+$ -stimulated ATPase and 5'-nucleotidase) as well as alterations in phospholipid distribution and protein conformation after treatment of hypophysectomized rats with growth hormone.

It appears therefore that several membrane functions are altered by the activation of cell-surface receptors. This suggests that a general membrane response may occur in stimulated tissues and that binding to surface receptors may result in a rapid alteration of the activities of cell surface-membrane enzymes.

The effects of gonadotrophin treatment on some enzymic activities of mouse ovarian homogenates are reported in the present paper. The activities that were chosen for study (alkaline phosphatase, nucleotidase and naphthylamidase) were selected since these activities have been shown to be associated with cell surface membranes in a variety of other tissues. Alkaline phosphatase (EC 3.1.3.1) has been shown to be associated with cell surface-membrane preparations isolated from liver (Emmelot *et al.*, 1964), kidney (Coleman & Finean, 1965) and intestinal mucosa (Clark & Porteous, 1965; Hübscher *et al.*, 1965). 5'-Nucleotidase (EC 3.1.3.5) has been demonstrated in surface-membrane preparations isolated from liver (Emmelot *et al.*, 1964), and kidney and intestinal mucosa (Coleman & Finean, 1965). Naphthylamidase (EC 3.4.11.1) has been shown to be a constituent of membranes from kidney, intestinal mucosa and liver (Coleman & Finean, 1965). Acid naphthylamidase and acid phosphatase are marker enzymes for lysosomes.

Materials and Methods

Materials

Substrates, fine chemicals and oestrogens (oestradiol-17 β , oestrone and oestriol) were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. All reagents were of the highest grade obtainable.

Human chorionic gonadotrophin (Pregnyl) was obtained from Organon Laboratories Ltd., Morden, Surrey, U.K. Dr. W. R. Butt (Women's Hospital, Birmingham, U.K.) kindly provided two preparations of human pituitary follicle-stimulating hormone, CM₁ and CP₁. Preparation CM₁ has a potency of 100 i.u. of follicle-stimulating hormone/mg (in terms of the Second International Reference Preparation of human menopausal gonadotrophin), and a follicle-stimulating hormone/luteinizing hormone ratio of 1. Preparation CP₁ has a potency of 560 i.u. of follicle-stimulating hormone/mg, and a follicle-stimulating

hormone/luteinizing-hormone ratio of 4 (Butt *et al.*, 1961). Dr. S. S. Lynch (Women's Hospital, Birmingham, U.K.) kindly provided preparations of ovine luteinizing hormone (NIH-LH S₁₂), sheep prolactin (Armour, no. 759-39BB), and human pituitary growth hormone obtained from the Medical Research Council.

Mice

Mice used in these experiments were of two strains: an outbred LACA strain purchased from Olac Laboratories, King's Norton, Birmingham 30, U.K. (varying in age from 23 to 25 days on arrival and used the following day), and an outbred CFW sub-strain bred in the laboratory and reared from birth in standard foster litters of ten females (Shirley, 1972). The age of these mice was known precisely. The strain used for each experiment is indicated in the appropriate legend. No differences were observed in the behaviour of their enzymes.

Gonadotrophin treatment

Gonadotrophins were dissolved in 0.9% NaCl containing 1% (w/v) bovine serum albumin in 0.010M-Tris-HCl, pH 7.4, and were administered by subcutaneous injection according to the doses and schedules described in the legends to the appropriate experiments. Mice were killed by chloroform anaesthesia. The ovaries were removed, blotted, freed of fat and connective tissue, pooled and stored in cold 0.3M-sucrose-0.01M-Tris-HCl, pH 7.4. The volume was adjusted to give approx. 5ml of sucrose-Tris medium/0.1g of tissue, and the ovaries were homogenized by hand in an all-glass Potter homogeniser (clearance, 2mm), filtered through glass wool, and the homogenates were stored at -20°C until required.

Enzyme assays

All enzyme assays were performed at least in duplicate with the appropriate controls.

Alkaline phosphatase was measured by the hydrolysis of *p*-nitrophenyl phosphate at 30°C in a 1.0ml incubation system containing 150 μ mol of piperazine-HCl buffer, pH 10.4, 0.5 μ mol of EDTA adjusted to pH 10.4 with NaOH, 3 μ mol of magnesium acetate, 3 μ mol of *p*-nitrophenyl phosphate and ovarian homogenate containing 10-100 μ g of protein. Free *p*-nitrophenol was measured at 420nm after the addition of 3ml of 0.5M-NaOH-0.5% (w/v) EDTA to stop the reaction. Nucleotidase activity was measured by the hydrolysis of adenosine 5'-monophosphate (5'-AMP) at 30°C in a 1.0ml incubation system containing 150 μ mol of piperazine-HCl, pH 9.4, 0.5 μ mol of EDTA, 3 μ mol of magnesium acetate, 3 μ mol of

5'-AMP and ovarian homogenate containing 0.1–0.5mg of protein. The reaction was stopped by the addition of 1ml of 10% (w/v) trichloroacetic acid, and the mixture was centrifuged to remove denatured protein. P_i was estimated on a 1.0ml sample of the deproteinized supernatant by the method of Fiske & SubbaRow (1925). Acid phosphatase activity was measured by the hydrolysis of *p*-nitrophenyl phosphate at 30°C in a 1.0ml incubation system containing 50 μ mol of sodium acetate–acetic acid buffer, pH 6.2, 1 μ mol of EDTA (disodium salt), 10 μ mol of magnesium acetate, 3 μ mol of *p*-nitrophenyl phosphate, pH 6.2, and an amount of ovarian homogenate containing 0.1–0.5mg of protein. The reaction was stopped and the colour developed by the addition of 3.0ml of 0.5M-NaOH–1% (w/v) EDTA, and the absorption at 420nm due to free *p*-nitrophenol was measured. Alkaline naphthylamidase activity was measured by the hydrolysis of L-leucyl- β -naphthylamide at 37°C in a 1.0ml incubation system containing 50 μ mol of glycine–NaOH buffer, pH 10.0, 10 μ mol of magnesium acetate, 5 μ mol of cobalt nitrate, 1.3 μ mol of L-leucyl- β -naphthylamide, adjusted to pH 10.0 with NaOH, and an amount of ovarian homogenate containing 0.1–0.5mg of protein. The reaction was stopped by the addition of 1ml of 10% (w/v) trichloroacetic acid, and the mixture centrifuged to remove denatured protein. A 1.0ml sample of the deproteinized supernatant was taken and free β -naphthylamide was measured by a slight modification of the method of Hübscher *et al.* (1965). Where comparison of reaction velocity between homogenates was necessary, reaction rates were adjusted for the protein contents of the homogenates. Protein was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as a standard [Sigma (London) Ltd., A4378, no. 436–8120].

Michaelis constants were calculated from Lineweaver–Burk plots by using measurements of rates of hydrolysis at several different substrate concentrations. At least six different substrate concentrations were used for each estimate of V and K_m . Substrate concentrations were chosen to cover a fifteenfold range of substrate concentration which included points above and below the value of K_m . The standard error of the estimation of V and K_m was computed for single experiments, and means $\pm$ S.D. of values of V and K_m were calculated where sufficient similar experiments had been performed.

Ovarian alkaline phosphatases

Ovarian homogenates contain two distinct phosphomonoesterase activities which are optimal at alkaline pH. The two phosphatase activities have different pH optima, different substrate specificities, different inhibitor sensitivities and the kinetic behaviour of the two activities differs with pH. One

phosphatase activity is optimal above pH 10.2, is inhibited by Ca^{2+} and is active against *p*-nitrophenyl phosphate, but much less active against other organic phosphates (T. A. Bramley, unpublished work). This activity is therefore referred to as alkaline phosphatase (EC 3.1.3.1). The second phosphatase activity is optimal at pH 9.5, is insensitive to Ca^{2+} and is active against a wide range of organic phosphates, including 5'-nucleotides (T. A. Bramley, unpublished work). The activity was measured by using adenosine 5'-phosphate as substrate and therefore has been termed 'alkaline 5'-nucleotidase (EC 3.1.3.5)'. Other organic phosphates are hydrolysed more rapidly than 5'-nucleotides, however, and this nomenclature is adopted solely to differentiate the two phosphatase activities.

Results

Ovarian 5'-nucleotidase specific activity (V /mg of protein) was increased after injection of immature mice with follicle-stimulating hormone plus human chorionic gonadotrophin, but no change in Michaelis constant (K_m) was observed (Table 1). The specific activity of ovarian alkaline phosphatase was also increased by gonadotrophin treatment. In addition, its Michaelis constant was also significantly altered, suggesting that a different alkaline phosphatase had been induced. No changes were observed for other ovarian enzymes studied (acid phosphatase, alkaline naphthylamidase, or acid naphthylamidase, not shown).

Time course of responses

Treatment of immature mice with follicle-stimulating hormone alone failed to affect ovarian alkaline phosphatase activity (Fig. 1). Human chorionic gonadotrophin alone caused an initial increase in the specific activity of alkaline phosphatase at 24h, with alteration of K_m . Once this initial change had occurred, however, the specific activity and K_m of ovarian alkaline phosphatase were not affected by treatment for a further two days with chorionic gonadotrophin alone.

Treatment with follicle-stimulating hormone plus chorionic gonadotrophin increased ovarian alkaline phosphatase activities at 24h compared with those observed after treatment with chorionic gonadotrophin alone. At 48h and 72h, however, further marked increases in specific activity were observed. Changes in K_m were significant at 24h and thereafter did not change. At all times after 24h, the increment in alkaline phosphatase specific activity obtained with follicle-stimulating hormone plus chorionic gonadotrophin was significantly greater than the sum of the increments of the two hormones alone. Follicle-stimulating hormone synergistically augmented the

Table 1. *Effects of gonadotrophin treatment of immature mice on the specific activities and Michaelis constants of some ovarian enzymic activities*

Immature LACA mice were injected subcutaneously with a total dose of 60 i.u. of human chorionic gonadotrophin plus 5.6 i.u. of follicle-stimulating hormone (CP₁) in three equal daily doses. Mice were killed on the morning of the fourth day. Control mice received saline medium only. Ovarian homogenates were prepared and V and K_m for each enzyme were determined from Lineweaver-Burk plots. V was adjusted for the protein content of the homogenates. Six or more mice were used for each homogenate. Numbers in parentheses indicate the number of homogenates used in computing the statistical data. Results represent means $\pm$ s.d. Significance was calculated by using Student's t test on paired results for alkaline phosphatase and the Bessel correction was applied to the smaller groups of results.

	Immature untreated		Immature gonadotrophin-treated	
	V (nmol/min per mg of protein)	K_m (mM)	V (nmol/min per mg of protein)	K_m (mM)
Alkaline phosphatase (EC 3.1.3.1)	42.7 $\pm$ 8 (13)	0.55 $\pm$ 0.17 (13)	383.3 $\pm$ 166* (13)	1.02 $\pm$ 0.40*
5'-Nucleotidase (EC 3.1.3.5)	25.6 $\pm$ 8 (6)	1.13 $\pm$ 0.15 (6)	183.3 $\pm$ 22* (6)	1.18 $\pm$ 0.12†
Acid phosphatase (EC 3.1.3.2)	27.3 $\pm$ 5 (4)	0.51 $\pm$ 0.08 (4)	35.0 $\pm$ 12† (4)	0.50 $\pm$ 0.02†
Alkaline naphthylamidase (EC 3.4.1.1)	5.17 $\pm$ 0.3 (4)	0.09 $\pm$ 0.02 (4)	4.0 $\pm$ 0.7† (4)	0.11 $\pm$ 0.05†

* $P < 0.001$.

† $P > 0.1$.

‡ $P > 0.01$, < 0.05 .

response of alkaline phosphatase initiated by chorionic gonadotrophin, but this augmentation only became clearly expressed after 24 h after a single hormone injection.

Measurement of the specific activity and K_m of ovarian alkaline phosphatase activity at various times after a single injection of follicle-stimulating hormone plus chorionic gonadotrophin showed that no change took place in either parameter until 6 to 8 h after injection (Fig. 2). Thereafter both the specific activity and K_m rose sharply and continued to increase in parallel.

Michaelis constants were determined by using the ovarian homogenate obtained at 12 h after injection. By using a very much wider range of substrate concentration than normal, the presence of two alkaline phosphatase activities, one with a K_m of 0.28 mM and the second with a K_m of 0.82 mM, was clearly demonstrated.

Specificity of response

The response of ovarian alkaline phosphatase appeared to be quite specific. As demonstrated previously, follicle-stimulating hormone had little effect (Table 2), but chorionic gonadotrophin alone or in combination with follicle-stimulating hormone caused a change in K_m of the expected magnitude and an increase in specific activity. (It should be noted that the response in this experiment was measured 24 h after injection. This period was chosen as it is

sufficiently long to allow the changes induced by chorionic gonadotrophin in specific activity and K_m to be clearly shown, but insufficient to allow the augmenting effects of follicle-stimulating hormone on this response to be fully expressed.)

No response was observed with oestradiol, oestrone or oestriol (alone or in combination with progesterone) at concentrations that caused large changes in uterine weight. Similarly, no response was observed with progesterone, sheep prolactin, human growth hormone, or with sheep luteinizing hormone alone. The lack of response to ovine luteinizing hormone was in marked contrast with the effects of human chorionic gonadotrophin. However, ovine luteinizing hormone in combination with follicle-stimulating hormone induced a typical change in kinetics and an increase in specific activity.

Comparison of the effects of ovine luteinizing hormone and human chorionic gonadotrophin alone or in combination with follicle-stimulating hormone further emphasized their differences (Fig. 3). The response was measured 24 h after injection.

Low doses of chorionic gonadotrophin alone induced a response in specific activity and K_m of ovarian alkaline phosphatase. Higher doses were less effective. In combination with follicle-stimulating hormone the response was greater, particularly at higher doses of chorionic gonadotrophin, where the fall observed with chorionic gonadotrophin alone was prevented.

Ovine luteinizing hormone alone induced no response in ovarian alkaline phosphatase over a wide range of doses (although a slight change in K_m was

Fig. 1. Effect of time on gonadotrophin stimulation of ovarian alkaline phosphatase activity

Immature LACA mice were injected subcutaneously at 24h intervals. The total dose of hormone administered was ●, 5.6i.u. of follicle-stimulating hormone (CP₁); △, 30i.u. of human chorionic gonadotrophin; ▲, 30i.u. of human chorionic gonadotrophin plus 5.6i.u. of follicle-stimulating hormone. Other mice received saline only (○). Mice were killed at various times after injection, ovarian homogenates were prepared and V and K_m for alkaline phosphatase measured. Values of V were corrected for protein contents of the homogenates. Each homogenate was prepared from at least six animals. Points represent means $\pm$ S.D. and were calculated from values obtained on between three and six homogenates. * indicates a significant alteration in K_m from control values ($P < 0.001$). There was no significant difference in V or K_m between controls and follicle-stimulating hormone-treated mice at all times. Treatment with chorionic gonadotrophin significantly increased specific activity at all times ($P < 0.001$). In addition, values of K_m for all mice which received chorionic gonadotrophin ($\pm$ follicle-stimulating hormone) were significantly altered at all times (1.14 ± 0.3 mM) from control values (0.53 ± 0.2 mM). There was no significant difference in specific activity between chorionic gonadotrophin-treated and follicle-stimulating-hormone-plus chorionic-gonadotrophin-treated mice at 24h ($P > 0.1$), but a highly significant difference at 48 and 72h ($P < 0.001$).

Fig. 2. Effects of gonadotrophin on ovarian alkaline phosphatase activity at various times after injection

Immature LACA mice were given a single subcutaneous injection of 60i.u. of human chorionic gonadotrophin and 11.2i.u. of follicle-stimulating hormone (CP₁). At various times after injection, the mice were killed and ovarian homogenates prepared. Values of V and K_m for the alkaline phosphatase activity of each homogenate were calculated from Lineweaver-Burk plots and values of V were adjusted for the protein contents of the homogenates. Ten mice were used for each point. Points represent V , columns represent K_m . Bars represent standard error of estimations.

observed at the highest dose). However, in the presence of follicle-stimulating hormone, a clear increase in both parameters was observed. This response was maximal at the lowest dose of luteinizing hormone tested ($1 \mu\text{g}$). The difference in behaviour of the two gonadotrophins was not due to contamination of chorionic gonadotrophin with follicle-stimulating hormone. The follicle-stimulating hormone content of the chorionic gonadotrophin preparation used was extremely low (0.01i.u. of follicle-stimulating hormone per 1000i.u. of chorionic gonadotrophin by radioimmunoassay; S. S. Lynch, personal communication).

Effects of follicle-stimulating hormone

The effects of the response of ovarian alkaline phosphatase to follicle-stimulating hormone were investigated in the presence and absence of a constant dose of chorionic gonadotrophin. Mice were injected

Table 2. *Specificity of response of ovarian alkaline phosphatase activity*

Immature LACA mice were given a single subcutaneous injection of 0.9% NaCl-1% bovine serum albumin containing the appropriate amount of hormone. Steroid hormones were dissolved in ethanol before dilution in saline medium. Ethanol controls were included. Mice were killed 24 h after injection, ovarian homogenates were prepared and V and K_m for alkaline phosphatase were determined from Lineweaver-Burk plots. Values of V were adjusted for the protein content of the homogenate. Eight mice were used for each treatment. Oestrogens all greatly increased uterine weight at the doses used.

Hormone	Dose	Hormones	Dose	V (nmol/min per mg of protein)	K_m (mM)
No hormones				38.3	0.38
Human pituitary follicle-stimulating hormone (CP ₁)	5.6 i.u.			40.0	0.38
	11.6 i.u.			40.0	0.38
Human chorionic gonadotrophin	5.0 i.u.			78.5	0.55
	60.0 i.u.			77.5	0.54
Human pituitary follicle-stimulating hormone	5.6 i.u.	Human chorionic gonadotrophin	5.0 i.u.	91.6	0.58
	11.6 i.u.		60.0 i.u.	77.5	0.55
Ovine luteinizing hormone	10.0 μ g			43.3	0.40
Human pituitary follicle-stimulating hormone	5.60 i.u.	Ovine luteinizing hormone	10 μ g	66.7	0.53
Ovine prolactin	30 μ g			36.7	0.33
Human growth hormone	50 μ g			42.6	0.36
Oestradiol-17 β	0.5 μ g			36.0	0.40
	5 μ g			36.7	0.38
Oestriol	5 μ g			39.0	0.38
Oestrone	5 μ g			43.6	0.42
Progesterone	2.5 μ g			43.6	0.39

daily on 3 consecutive days. This injection regime allowed the augmenting effects of follicle-stimulating hormone to be clearly expressed. Injection of immature mice with different doses of follicle-stimulating hormone alone induced little response in ovarian alkaline phosphatase activity (Fig. 4). However, injection of mice with follicle-stimulating hormone in the presence of a constant dose of chorionic gonadotrophin caused a dose-related increase in the specific activity of ovarian alkaline phosphatase.

Effects of endogenous mouse gonadotrophins

The gonadotrophins used in this study were, in all cases, foreign to the mouse. Similarly, the doses of gonadotrophins used to elicit these acute responses were large compared with physiological concentrations of gonadotrophins. Therefore the effects of physiological concentrations of homologous mouse gonadotrophins were investigated by studying alkaline phosphatase activities in the maturing animal. Fig. 5 shows that the specific activity and K_m of ovarian alkaline phosphatase increased with increasing age. The value of K_m appeared to reach a plateau

at approx. 40 days of age. This corresponded to the age at which corpora lutea were first observed (i.e. sexual maturity). The magnitude of the changes in K_m and specific activity were similar to the changes observed after gonadotrophin treatment. Thus treatment with exogenous gonadotrophins induced changes in mouse ovarian alkaline phosphatase activity similar to those observed during normal maturation under the influence of physiological concentrations of endogenous gonadotrophins. The specific activities of some other ovarian enzymes were not increased as the animal reached maturity.

The possibility that the distinct alkaline phosphatase observed after gonadotrophin treatment was luteal in origin may be discounted, since no luteal tissue was present in these mice until after 40 days of age, by which time maximal changes in alkaline phosphatase had already occurred.

Discussion

Treatment of immature mice with gonadotrophins induced a large increase in both an ovarian alkaline nucleotidase activity and a kinetically distinct ovarian

Fig. 3. Effects of human chorionic gonadotrophin or ovine luteinizing hormone alone or in combination with follicle-stimulating hormone on ovarian alkaline phosphatase

Immature LACA mice were given a single subcutaneous injection containing the various gonadotrophins at the dose indicated and 24 h later the mice were killed and ovarian homogenates prepared. Values of V (a) and K_m (b) for the alkaline phosphatase activity of each homogenate were calculated from Lineweaver-Burk plots and values of V were adjusted for the protein contents of the homogenates. Six mice were used for each point. $\circ$, human chorionic gonadotrophin alone; $\bullet$, human chorionic gonadotrophin plus 5.6 i.u. of follicle-stimulating hormone (CP₁); $\triangle$, ovine luteinizing hormone alone; $\blacktriangle$, ovine luteinizing hormone plus 5.6 i.u. of follicle-stimulating hormone (CP₁); $\blacksquare$, 5.6 i.u. of follicle-stimulating hormone (CP₁) alone. Bars represent standard error of estimation.

alkaline phosphatase. Other ovarian enzymes tested were not affected by gonadotrophin treatment. Increased activities of the kinetically distinct alkaline phosphatase were detectable 6-8 h after treatment with human chorionic gonadotrophin alone; these activities continued to increase up to 24 h after injection, and thereafter remained constant for a further 48 h. In combination with follicle-stimulating hormone, the response to chorionic gonadotrophin was potentiated, resulting in greatly augmented activities of the enzyme. The augmentation of the chorionic gonadotrophin-induced response in ovarian alkaline phosphatase activity by follicle-stimulating hormone was not expressed until later than 24 h after an injection of both hormones. After 24 h, however, the increase in activity after the injection of follicle-

stimulating hormone plus chorionic gonadotrophin was significantly greater than the increase with either hormone alone. Evidence of synergism at the enzyme level between follicle-stimulating hormone and luteinizing hormone has been shown previously only for ovarian adenylate cyclase (Fontaine *et al.*, 1971), although this has not been confirmed (Mason *et al.*, 1973). This is despite the fact that follicle-stimulating hormone and luteinizing hormone are known to affect synergistically a variety of ovarian functions. Both gonadotrophins are required for correct ovarian development (Greep *et al.*, 1942; Simpson *et al.*, 1950; Eshkol & Lunenfeld, 1971), whereas the widely used bioassay for follicle-stimulating hormone (Steelman & Pohley, 1953) depends on the synergistic augmentation of follicle growth with human chorionic

Fig. 4. Effects of follicle-stimulating hormone alone or in the presence of chorionic gonadotrophin on the specific activity of ovarian alkaline phosphatase

Immature LACA mice were given the total dose of follicle-stimulating hormone indicated in three equal daily subcutaneous injections. Mice received either follicle-stimulating hormone alone (○) or the dose indicated, together with a total dose of 60 i.u. of chorionic gonadotrophin (●). On the morning of the fourth day mice were killed, and ovarian homogenates were prepared. Alkaline phosphatase activity was measured and adjusted for the protein contents of the homogenates. Six mice were used for each point. Points represent the means of four separate experiments, the bars represent standard deviation.

gonadotrophin. Synergism of follicle-stimulating hormone and luteinizing hormone has also been shown for ovarian oestrogen production (Greep *et al.*, 1942; Rosenberg & Joshi, 1968) and the luteinization *in vitro* of small monkey follicles (Channing, 1970). In addition, follicle-stimulating hormone and luteinizing hormone are both required for ovulation and show synergism in that ovulation occurs on injection of both hormones at doses which singly fail to induce

ovulation. (Greenwald, 1970; Labhsetwar, 1970; Jones & Nalbandov, 1972.)

Changes in both the specific activity and K_m of ovarian alkaline phosphatase occurred simultaneously commencing 6–8 h after subcutaneous injection of gonadotrophin, indicating the appearance of a kinetically distinct enzyme activity. The time-lag observed before the onset of these changes is consistent with the synthesis of proteins *de novo*.

The Michaelis constant of the enzyme was altered by treatment with small doses of human chorionic gonadotrophin or ovine luteinizing hormone plus follicle-stimulating hormone *in vivo*. This change was complete at 24 h and was not affected by treatment for a longer period. Treatment with chorionic gonadotrophin plus follicle-stimulating hormone failed to increase K_m once the observed change had taken place, although this treatment resulted in the production of much more enzyme. This could be consistent with the 'switching-on' of protein synthesis to produce a new kinetically distinct phosphatase, or the activation of a mechanism which kinetically modifies an inactive or less-active phosphatase precursor. The contributions of these alternative mechanisms may be resolved by the use of antibiotics which specifically inhibit protein synthesis.

The lack of response of ovarian phosphatase to ovine luteinizing hormone, in contrast with the marked response to human chorionic gonadotrophin, was not in itself surprising in view of the differences in structure between the two molecules. The lack of response of the immature rat to ovine luteinizing hormone (Christiansen, 1967; Parlow *et al.*, 1972) has been attributed to the very short circulation half-life of ovine luteinizing hormone compared with human chorionic gonadotrophin (Parlow, 1968). This factor may explain the lack of response of sheep luteinizing hormone compared with chorionic gonadotrophin, but does not explain why a response was observed (at the lowest concentrations of luteinizing hormone tested) when sheep luteinizing hormone was given in the presence of follicle-stimulating hormone (a very clear synergism, since no response occurred with either hormone alone).

One possibility is that the prepubertal mouse ovary contains some co-operative binding sites which bind sheep luteinizing hormone only in the presence of follicle-stimulating hormone. There is some evidence for such a speculation. Figarova *et al.* (1973) showed that pre-treatment of hypophysectomized immature rats with follicle-stimulating hormone increased the ovarian binding of human chorionic gonadotrophin. Eshkol (1971) claimed that binding of follicle-stimulating hormone and chorionic gonadotrophin by immature mouse ovaries was increased very shortly after pre-treatment with preparations containing follicle-stimulating hormone and luteinizing hormone, and Goldenberg *et al.* (1972b) demonstrated

Fig. 5. Ovarian alkaline phosphatase in mice of different ages

Ovarian homogenates were prepared from CFW mice of different ages. Values of V (a) and K_m (b) for alkaline phosphatase were obtained from Lineweaver-Burk plots and values of V were corrected for the protein contents of the homogenates.

increased uptake of follicle-stimulating hormone after treatment of immature hypophysectomized rats with preparations containing follicle-stimulating hormone and chorionic gonadotrophin, an effect probably mediated by oestrogens (Goldenberg *et al.*, 1972a).

A puzzling aspect of this study was the complete and consistent lack of response to follicle-stimulating hormone alone, even though the follicle-stimulating hormone preparation was appreciably contaminated with luteinizing hormone as measured by the ovarian ascorbic acid-depletion method. A clear response was observed at very low doses of sheep luteinizing hormone in the presence of follicle-stimulating hormone, but even high doses of follicle-stimulating hormone containing appreciable contaminating luteinizing hormone failed to induce a response. The reasons for the failure of follicle-stimulating hormone contaminated with luteinizing hormone to induce a response are not clearly understood at this time.

Alkaline phosphatase has been demonstrated histochemically in mouse and rat ovaries, and shown to be localized predominantly in the theca, with some activity in interstitial tissue (Gomori, 1941; Deane,

1952; Moog & Wengler, 1952; Ford & Hirschman, 1955). However, mouse ovaries possess two distinct alkaline phosphatases, an active quite-specific *p*-nitrophenyl phosphatase and a less-active less-specific phosphatase (T. A. Bramley, unpublished work). Unfortunately, ovarian alkaline *p*-nitrophenyl phosphatase is almost completely inhibited under the conditions used by these workers for the histochemical demonstration of alkaline phosphatase and consequently only the less-specific activity is demonstrated. The histochemical localization of ovarian alkaline *p*-nitrophenyl phosphatase is not known at this time.

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