

## Adenylyl Cyclase Activities in Ovarian Tissues. I. Homogenization and Conditions of Assay in Graafian Follicles and Corpora Lutea of Rabbits, Rats, and Pigs: Regulation by ATP, and Some Comparative Properties

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**ABSTRACT.** Responsiveness of ovarian adenylyl cyclases to luteinizing hormone (LH), found to be 5 to 10-fold in cell-free preparations under optimal conditions, required gentle homogenizations and storage in sucrose-containing media. Assay conditions required the use of an ATP-regenerating system consisting of creatine kinase, creatine phosphate, and myokinase for the preservation of ATP levels.

LH-stimulated adenylyl cyclase (AC) in rabbit CL showed the following properties: 1) The pH optimum of basal activity was about 8.0; that of LH-stimulated activity was about 7.5. 2) The relative response to LH was low (1.5 to 2-fold) at 0.1 mM ATP and increased with increasing ATP, but not with increasing GTP. At low (0.1 mM) ATP, GTP increased catalytic efficacy of the system, both in the absence and in the presence of LH (no effect on relative stimulation). 3) The optimal relative stimulation by LH was obtained at about 1.0 mM MgCl<sub>2</sub> in excess of added magnesium-binding ingredients. 4) The sensitivity to stimulation by LH (about 0.2 µg/ml NIH-LH-B8) was unaffected by either pH, nucleotides (ATP and GTP), or MgCl<sub>2</sub> concentration. 5) Under the assay conditions used, activity was stimulated by prostaglandin E<sub>1</sub> (PGE<sub>1</sub>) about 1.5

to 2-fold, and by epinephrine about 3 to 4-fold.

In all aspects tested, LH-stimulated AC in rat CL resembled that in rabbit CL, except that about 5-fold higher concentrations of NIH-LH-B8 were needed for half-maximal stimulation. The AC activity in pig Graafian follicles, however, differed from that in rabbit CL in that 1) the ATP concentration needed for optimal stimulation by LH was lower (in the micromolar rather than the millimolar range); 2) catecholamines elicited only a 1.3 to 1.4-fold stimulation; and 3) NIH-LH-B8 elicited half-maximal stimulation at 0.008 to 0.020 µg/ml.

We were unable to detect LH-responsive AC activity in either homogenates or washed particles of CL from either cycling or pregnant pigs.

LH fractions of three origins (human, bovine, and ovine) and of varying specific activities (from 0.041 to 2.0 NIH-LH-S18 units/mg) were tested and the relative potencies by OAAD assay were found to correlate well with the relative potencies in the adenylyl cyclase assays (rat CL, rabbit CL, and pig follicles), consistent with the possibility that AC receptors are responsible for biologic actions of LH. (*Endocrinology* 99: 163, 1976)

**I**T IS currently assumed that luteinizing hormone (LH), like many other hormones, exerts its actions on ovarian tissues by stimulating the membrane-bound enzyme adenylyl cyclase (AC), thereby increasing the second messenger, cAMP (1-7). It seems, therefore, that useful information will be gained about the regulation of mammalian reproduction processes if the molecular and biochemical bases of the interaction(s) of LH with its AC system are explored in cell-free preparations, and if, at the same time, the quantity and state of responsiveness of this system under various physiological and pharmacological conditions are described in more than one species. It is the purpose of this article, the first in a series

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dealing with molecular aspects of LH action at the ovarian level, to report on adequate experimental conditions for the determination of LH-sensitive AC in the follicles and corpora lutea (CL) of various species, and to present the basic properties of LH-responsiveness that confer a special character upon these adenyl cyclase systems, when compared with AC systems from other organs responsive to other hormones.

Subsequent articles report on *in vivo* regulation of LH-stimulated AC in rabbit (8) and rat (9) ovarian structures and on hCG- and LH-induced desensitization of AC to LH stimulation as seen *in vivo* in corpora lutea and follicles of rabbits and rats (10), and *in vitro* in isolated Graafian follicle membranes of pigs (11).

## Materials and Methods

### *Animals and sources of ovaries, CL, and follicles*

Rabbits (3.5–4.5 kg), primarily New Zealand Whites which had littered at least once, were used throughout. Females which were neither pregnant nor pseudopregnant were considered in estrus. Pregnancy was induced by successive mating to two different experienced bucks. Pseudopregnancy was induced by the injection (iv) of 100 IU hCG (Ayerst) dissolved in 0.5 ml of 0.9% saline. The rabbits were sacrificed by cervical dislocation between 6 and 9 days after the induction of pregnancy and pseudopregnancy. Their ovaries were then removed and placed until dissection of the CL or follicles in iced Krebs-Ringer bicarbonate buffer (KRB) prepared with  $\frac{1}{2}$  the recommended amount of  $\text{CaCl}_2$  (12).

Prepubertal rats (Charles River, CD-outbred) were received at 20 days of age and “superovulated” by injection at 22 or 23 days of age with PMSG (50 IU, sc) and 56 h later with hCG (50 IU, sc). Superovulated rats were sacrificed by decapitation on the 7th day after PMSG injection. Their ovaries were removed, freed from the ovarian bursa, and kept until homogenization in iced KRB.

Pig ovaries were obtained at a slaughterhouse, placed in iced KRB as soon as possible (usually within 30 min of sacrifice), and transported to

the laboratory where they arrived between 1 and 3 h after sacrifice. Rabbit CL of pregnancy and pseudopregnancy, pig CL of the cycle and of early and late pregnancy, rabbit estrous follicles (diameter larger than 2 mm), and pig estrous follicles (diameter larger than 6 mm) were dissected with angular fine-point forceps (Clay Adams) and kept in iced KRB until homogenization. Rat CL of superovulation were not dissected from their ovaries; rather, the whole ovaries (90% + CL) were homogenized.

### *Preparation of homogenates*

Unless indicated otherwise, dissected rabbit ovarian structures and rat ovaries were homogenized by either of two methods:

*Method A (used with CL and superovulated ovaries only).* One part of tissue was homogenized with 5 parts of 27% (wt/wt) sucrose in 1 mM EDTA and 10 mM Tris-HCl, pH 7.5, using a Polytron equipped with PT-10ST generator. Homogenization, final volume between 1.5 and 4 (usually 2.5) ml, was carried out in 15-ml Corex tubes and consisted of 2 treatments of 20 seconds each at a setting of 40% of maximum, separated by 20 seconds. The resulting homogenate was diluted 4-fold and filtered through no. 12 Japanese silk screen (13).

*Method B (used with rat ovaries and rabbit CL and follicles).* Dissected ovaries, CL, or follicles (the latter popped in KRB immediately before homogenization) were homogenized in 7-ml Dounce homogenizers (Kontes) in 20 (CL) or 10 (follicles) volumes of homogenization medium (the same as used in Method A) with 10 strokes of the loose pestle followed by 10 strokes with the tight pestle. The resulting homogenate was filtered through silk screen as in Method A.

### *Preparation of membrane particles from CL and follicles from rats and rabbits*

For the preparation of membrane particles from either rat ovaries (membrane particles of CL of superovulation), rabbit CL of pregnancy or pseudopregnancy, or rabbit estrous follicles, the filtered homogenates were subjected to 2 consecutive centrifugations. The first centrifugation (5 min,  $160 \times g$ ) removed tissue fragments, nuclei, and major cell debris. The second cen-

trifugation (30 to 45 min at  $10,000 \times g$ ) carried out in a SS-34 rotor of the Sorval RC-2B centrifuge, concentrated membrane particles contained in the supernatant fluid after the first centrifugation. The resulting pellet was resuspended in homogenization medium (5 volumes with respect to original tissue weight) with the aid of a small Dounce homogenizer (10 strokes with the loose pestle).

Membrane particles from pig Graafian follicles were prepared as described in detail elsewhere (11). Briefly, the dissected material (*ca.* 12 g from 30 to 40 ovaries) was homogenized in 10 volumes of the Sucrose-Tris-EDTA medium described in Method A, but using a 30-ml Dounce homogenizer (Blaessig Glass Co.) and 20 strokes with the loose pestle only. After filtration, first through gauze and then through silk screen, a first pellet was obtained by centrifugation at 4 C for 12 min at  $12,000 \times g$ . This pellet was resuspended with the aid of the Dounce homogenizer in wash medium (10 mM Tris-HCl, 1.0 mM EDTA, pH 7.5). After 5 min at room temperature, the membranes were repelleted at 4 C for 12 min at  $12,000 \times g$ . This wash procedure was repeated twice more. The final pellet was then resuspended in 5 volumes (with respect to initial tissue weight) of the initial homogenization medium. After aliquoting, membranes were stored at  $-70$  C.

Descending thin-layer chromatography was performed on plastic-backed polyethyleneimine (PEI)-cellulose plates (Brinkmann) using 1M LiCl as the developing solvent.

#### Adenylyl cyclase assay

Unless described otherwise, 20- $\mu$ l aliquots of homogenates or membrane particles suitably diluted in homogenization medium were tested for AC activity in a final volume of 50  $\mu$ l containing 3.0 mM ATP (with 2 to  $5 \times 10^6$  cpm of [ $\alpha$ - $^{32}$ P]ATP), 5.0 mM  $MgCl_2$ , 1.0 mM EDTA, 1.0 mM cAMP (with *ca.* 10,000 cpm [ $^3$ H]cAMP), 20 mM creatine phosphate, 0.2 mg/ml creatine kinase, 0.1 mg/ml myokinase, and 25 mM bis-tris-propane (BTP)-HCl buffer, pH 7.5. Incubations were at 37 C for 10 min. The reaction was stopped with 0.1 ml of a solution containing 10 mM cAMP, 40 mM ATP, and 1% sodium dodecylsulfate, followed by immediate boiling for 3.5 min. The boiling step was omitted when ATP levels were measured. [ $^{32}$ P]cAMP formed

and [ $^3$ H]cAMP added to monitor recovery were then isolated essentially according to the method of Salomon *et al.* (14) with the following modifications: a), as described recently (15), the Dowex 50-X4 column had a 1.5 ml bed volume and was subjected to a more rigorous regenerating procedure and to a slightly different elution scheme, resulting in 80% recovery of cAMP; and b), the aluminum oxide column was made with 1.2 g of alumina, rather than 0.6 g, and eluted with 4 ml of imidazole buffer, rather than 3 ml. This resulted in an overall recovery of cAMP of 65 to 75%, and reaction blanks ranging from 3 to 6 cpm per  $1 \times 10^6$  cpm of ATP added. The imidazole eluates from the alumina columns were collected in scintillation vials containing 3a-70B, a premixed scintillation cocktail with a high capacity for water, and counted in a Packard TriCarb liquid scintillation counter. With this methodology, duplicate determinations agreed within 5% of the mean. Unless otherwise indicated, the points on the graphs represent results from single incubations. Each of the reported experiments was repeated at least once with a different batch of membrane particles to assure reproducibility.

Protein was determined by the method of Lowry *et al.* (16), using bovine serum albumin (Fraction V) as standard.

#### Materials

**Chemicals and enzymes.** Free acids of cAMP, EDTA and EGTA, ATP (Tris-salt), BTP (1,3 bis[Tris[hydroxymethyl-methylamino] propane (or bis-tris-propane), free base), Tris (free base), GTP, and myokinase (crystalline in saturated ammonium sulfate) were purchased from Sigma. Creatine kinase and creatine phosphate were purchased from Cal Biochem. Dowex 50-X4 (AG, 200 to 400 mesh,  $H^+$  form) was purchased from Bio Rad. A solution of 2 mg/ml myokinase was prepared by centrifuging the commercial suspension at  $10,000 \times g$  for 15 min at 4 C, and then resuspending the pellet in 0.9% saline. This suspension was used within one week of preparation. 3a-70B cocktail was purchased from RPI Corporation.

**Hormones.** LH (NIH-LH-S18, NIH-LH-B8, NIH-LH-B9, human LER 960, bovine LER 1716-2, and ovine LER 1374-A), and FSH (NIH-FSH-P1) were a gift from the Endo-

crinology Study Section, National Institutes of Health. PMSG (NIAMDD-PMSG-1) was a gift of the Rat Pituitary Hormone Distribution Program of the National Institute of Arthritis, Metabolism, and Digestive Diseases. Dr. Leo E. Reichert kindly supplied us with the following LH-containing fractions: LER-1056-C (immunochemical grade ovine LH), LER-1056-A1 (a large molecular aggregate obtained during purification of ovine LH), LER-1056-A3 (a small molecular weight component obtained during purification of ovine LH), LER-1733-2 (a biologically active but low specific-activity fraction that has different chromatographic behavior than highly purified LH), and LER-1733-3 (highly purified ovine LH). hCG was a gift from Ayerst Laboratories (Dr. J. B. Jewell). Prostaglandin E<sub>1</sub> (PGE<sub>1</sub>) was a gift from the Upjohn Company (Dr. J. E. Pike). Stock solutions of gonadotrophins were prepared at a final concentration of 1 mg/ml in 0.9% saline with 50 mM potassium phosphate buffer pH 7.5. Stock solutions of PGE<sub>1</sub> (1 mg/ml) were prepared as recommended by the Upjohn Company.

## Results

### I. Preliminary

Contrary to findings with other AC systems, whose responses to hormones are not much affected by homogenization conditions and are stable to prolonged storage at -70 C, initial studies with LH-sensitive AC showed the strict dependence of absolute activities and relative stimulation upon the type of homogenization and the composition of homogenization and storage media. We therefore searched for satisfactory conditions that would yield reproducible results. As model systems we used homogenates and membrane particles from six to nine-day-old CL from either pregnant or pseudo-pregnant rabbits. Some features were subsequently tested using pig Graafian follicles of more than 6 mm in diameter and CL of pregnant rats and of PMSG-primed, hCG-induced, superovulated rats (CL of superovulation). All these systems were found to be highly responsive to LH (5 to 10-fold stimulation) when tested under appropriate conditions.

Experiments carried out at ATP concentrations lower than 1 mM revealed the presence of a potent ATP-pyrophosphohydrolase activity in CL membrane particles. We therefore added myokinase (adenylate kinase) to our standard assay medium and thereby assured the adequate maintenance of ATP levels throughout the incubations (see below for further details).

### II. Tissue homogenization

*a. Mechanical devices.* Extensive homogenization in hypotonic or hypertonic media, such as those obtained with conical motor-driven ground glass homogenizers or Polytron-type homogenizers at high settings for 10 seconds or more, resulted in membrane particles with low adenylyl cyclase activities and low (sometimes undetectable) responses to LH.

On the other hand, homogenates and particles prepared using more gentle homogenization techniques that include the use of Dounce homogenizers, Polytron-type homogenizers at setting of less than 40% of maximum for not longer than 20 seconds, and glass-teflon homogenizers used at low speeds for short times, were found to have much higher activities (basal or fluoride-stimulated) and to respond well to LH. We used primarily the Polytron PT-10ST homogenizer and the 7 ml Dounce homogenizers (Methods A and B described under *Methods*). Essentially identical results were obtained with either method, and we used them interchangeably depending mainly on the quantity of tissue available for homogenization: Method A (Polytron) was used with tissue samples of more than 500 mg and Method B (Dounce) was used with tissue samples of less than 500 mg.

*b. Homogenization medium.* As shown in Fig. 1, for membrane particles from rabbit corpus luteum, the composition of the homogenization medium has a profound influence on the stability of the LH-stimulated AC system. Thus, membrane particles prepared in hypotonic media (1 mM KHCO<sub>3</sub>

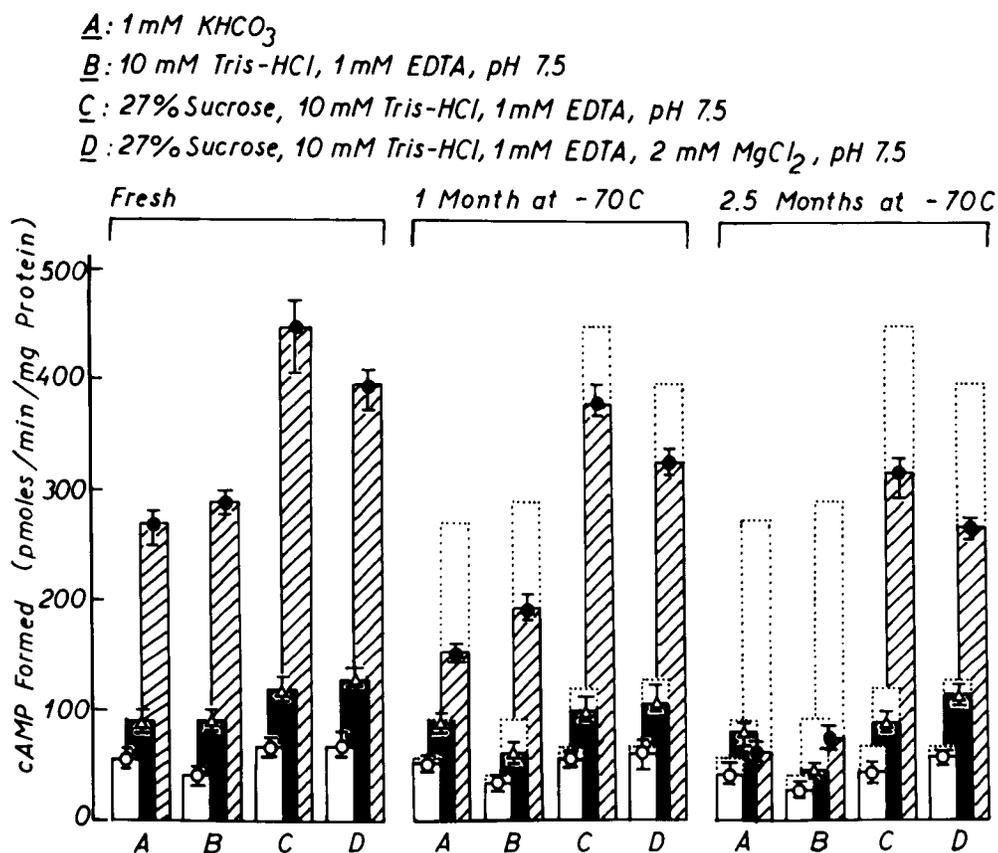


FIG. 1. Effect of composition of homogenization medium on stability to storage and responsiveness to LH of rabbit corpus luteum (CL) adenylyl cyclase (AC). Eight rabbits were sacrificed on the eighth day of pregnancy. Sixty-four CL (between 20 and 30 mg each) were dissected and distributed in four groups, each containing one CL from each of the 16 ovaries. The dissected CL were kept in ice-cold KRB saturated with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$  until homogenization by Method B (Dounce). After removing an aliquot for the assay of activities (results not shown), the 5% homogenate was centrifuged in the SS-34 rotor of the Sorval RC-2B refrigerated centrifuge at 4 C, first for 5 min at  $160 \times g$ , and then, after removal of the pellet containing poorly homogenized cell debris and nuclear material, for 30 min at  $10,000 \times g$ . The  $10,000 \times g$  pellets were resuspended in 5 volumes (with respect to original tissue weight) of the corresponding initial homogenization medium with the aid of a 7ml Dounce homogenizer (10 strokes with the loose pestle) and separated into 0.4 ml aliquots which were used for the determination of AC activity either on the same day or after 1 and 2.5 months of storage at  $-70 \text{ C}$ . Incubation conditions: standard assay conditions described under *Methods* (3.0 mM ATP, 5.0 mM  $\text{MgCl}_2$ , 0.1 mg/ml myokinase, 25 mM BTP-HCl buffer, pH 7.5) were used throughout. When present, LH and  $\text{PGE}_1$  were 10  $\mu\text{g}/\text{ml}$  each. Twenty- $\mu\text{g}$  aliquots either of homogenate or of 1:8 dilutions of the resuspended pellets in the corresponding resuspension media were tested. No corrections were made for the varying ionic composition of the aliquots tested. Results are expressed as specific activities. Membrane particles prepared under conditions A, B, C, and D contained, before storage, 50%, 53%, 60%, and 65% of the LH-stimulated activity and 58%, 55%, 47%, and 49% of the protein present in the respective homogenates. Open bars represent basal adenylyl cyclase activities; hatched bars represent LH-stimulated activities; black bars represent  $\text{PGE}_1$ -stimulated activities; and broken lines represent activities present in fresh membrane particles.

or 10 mM Tris-HCl plus 1 mM EDTA) lost, upon storage for 2.5 month at  $-70 \text{ C}$ , 30% of their basal activity and between 75 and 80% of their LH-stimulated activity. The selective loss of LH-stimulated activity

could be prevented by the addition of 20 to 27% (wt/wt) sucrose. When this was done (condition C on Fig. 1) the 2.5-month storage still resulted in a loss of activity (30%), but it was the same for both basal and

LH-stimulated activity. In other, less controlled experiments, but where the whole procedure for the preparation of membrane particles was carried out in about ½ the time, a 4-month storage period resulted in only 20% loss of tested activities, with no further change for up to 7.5 months, the longest time tested. We were never able to preserve LH stimulation when the initial homogenization had been carried out in hypotonic media. Under any of the conditions used, prostaglandin-stimulated activity, although less than that produced by LH, was consistently more stable to storage. The homogenization of CL-containing ovaries from superovulated rats in hypotonic media also resulted in preparations that were unstable to storage and showed low stimulation by LH (1.5 to 2-fold stimulation in "hypotonic" homogenates *vs.* 4 to 6-fold stimulation in "sucrose" homogenates). On the other hand, the LH-stimulated activity in Graafian follicles from pig ovaries appeared to be less sensitive to hypotonic media. As reported in detail elsewhere (11), the best results (with regards to stability) were obtained when the membrane particles derived from "sucrose" homogenates of Graafian follicles were subjected to 3 successive washings with a solution containing 10 mM Tris-HCl (pH 7.5) and 1.0 mM EDTA, prior to resuspension in the original sucrose-containing homogenization medium for aliquoting and freezing for storage.

The experiment shown in Fig. 1 was also carried out including 1 mM dithiothreitol in the homogenization and storage media, but its addition appeared to be without effect on any of the variables tested.

Based on all of these findings, we chose condition C (27% (wt/wt) sucrose, 10 mM Tris-HCl and 1.0 mM EDTA, pH 7.5) as our standard homogenization condition for all subsequent studies.

### III. Assay conditions

*a. Requirement for myokinase (adenylate kinase).* In experiments using relatively high

concentrations of membrane particles (more than 50  $\mu$ g of protein per assay) and ATP concentrations lower than 1.0 mM, we were initially unable to obtain linear time courses, regardless of whether we added GTP or varied the temperature of incubation (30 or 37 C), the MgCl<sub>2</sub> concentration (2 or 5 mM), or the pH of the assay (6.5 or 8.5). Since this lack of linearity was partially relieved when the concentrations of ATP were raised to 3 mM or higher, we investigated the possibility that our ATP-regenerating system (0.2 mg/ml creatine kinase and 25 mM creatine phosphate) was defective and the lack of linearity was due to inadequate preservation of the ATP levels throughout the incubation period. Chromatography of the incubate on PEI-cellulose before and after 10 min at 37 C revealed that, indeed, the ATP levels were not preserved throughout the incubation and that ATP degradation was associated with an almost quantitative appearance of AMP in the incubation medium. The addition of as much as 1 mg/ml creatine kinase and 50 mM creatine phosphate did not result in improvement. Since AMP cannot be regenerated to ATP by the simple ATP-regenerating system used, we tested whether the addition of myokinase would improve the situation by establishing an equilibrium between ATP, ADP, and AMP and hoped that this new equilibrium would then be displaced towards ATP by excess creatine phosphate. We found (Fig. 2, A through F) that the addition of myokinase to the incubation medium was indeed necessary, and also that adequate preservation of the ATP levels required the concomitant use of lower concentrations of membrane particles (less than 25  $\mu$ g of CL membrane protein per assay). From these findings, it seems likely that the formation of AMP from ATP is due to direct hydrolysis by nucleoside triphosphate pyrophosphohydrolase activity catalyzing the reaction  $\text{ATP} \rightarrow \text{AMP} + \text{PP}_i$ .

The results shown in Fig. 2 also indicate that the adequate maintenance of ATP

levels does not require the addition of myokinase when high concentrations of ATP are used, as long as the concentration of membranes is low; and that it requires not only myokinase addition but also shortened incubation times if activity is to be determined at high membrane concentrations and with added myokinase, we were unable to maintain adequate ATP levels when the initial concentrations of ATP were less than 0.1 mM (Fig. 3, panel A, open symbols), possibly because under these conditions the levels of the nucleotide substrate for creatine kinase (ADP), are not enough to allow the added amount of creatine phosphate to drive the reaction towards ATP formation. Consistent with this idea is the finding that below 0.1 mM ATP, the addition of 0.04 mM GTP, and hence

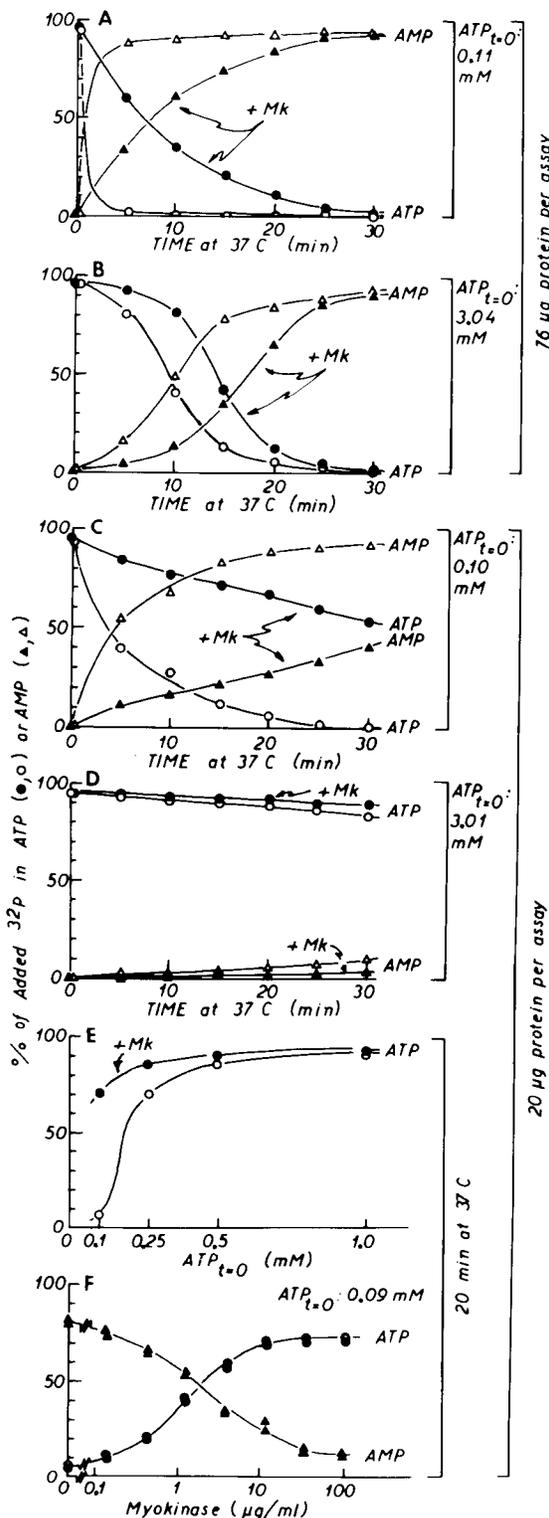
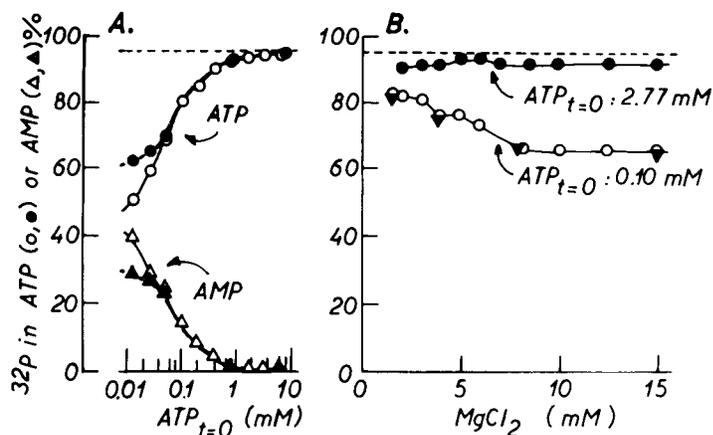


FIG. 2. Panels A-D. Effect of myokinase and membrane particle concentration at various initial ATP concentrations on the maintenance of ATP and the formation of AMP during incubation under adenylyl cyclase assay conditions. ATP<sub>t=0</sub> represents concentration of [ $\alpha$ -<sup>32</sup>P]ATP added to the incubations (ca.  $5 \times 10^5$  cpm/50  $\mu$ l); open symbols represent incubations in the absence of added myokinase; closed symbols (except in F) represent incubations to which 0.1 mg/ml myokinase (Mk) has been added; circles represent the percent of total added <sup>32</sup>P that chromatographed with an R<sub>f</sub> equal to that of ATP on PEI-cellulose thin-layer sheets; triangles represent the per cent of total added <sup>32</sup>P that chromatographed with an R<sub>f</sub> equal to that of AMP on PEI-cellulose thin-layer sheets. Membrane particles from 8-day-old CL of pregnant rabbits were used for these studies. Panel E: Effect of a fixed amount of myokinase on the maintenance of ATP levels at varying initial concentrations of ATP. Panel F: Effect of myokinase concentrations on maintenance of ATP levels at fixed ATP concentration.

The incubation media contained, in addition to the listed concentrations of ATP, membrane particles and myokinase, 5.0 mM MgCl<sub>2</sub>, 1.0 mM EDTA, 1.0 mM cAMP, 0.2 mg/ml creatine kinase, 25 mM creatine phosphate, and 25 mM BTP-HCl buffer, pH 7.5. Incubations were at 37 C. The reactions were terminated by removing at the indicated times between 1 and 2  $\mu$ l of the incubation media and applying them to the PEI-cellulose TLC sheets. The remaining conditions are described in the figure.



$4 \times 10^{-5}\text{M}$  GTP. Same experiment as shown in Fig. 7. Both panels: — — — represent the per cent of total added  $^{32}\text{P}$  in ATP at the beginning of the incubations. For the rest of the incubation conditions see *Methods* and legends to Figs. 5 and 7; for a description of the determination of distribution of  $^{32}\text{P}$  in ATP and AMP see the legend to Fig. 2 and *Methods*.

of GDP (resulting from hydrolysis by nucleotidase activity present in membrane fractions of the degree of impurity used here), acted as a "helper" in establishing an equilibrium more in favor of ATP (Fig. 3, panel A, closed symbols). Thus, studies on the dependency of AC activity in membranes from CL on ATP concentrations had to be limited at the lower end to concentrations above 0.05 mM, if "adequate" maintenance of the ATP levels is defined as that leading to preservation by the end of the incubation of more than 50% of the initially added ATP. On the other hand, when the concentration of  $\text{MgCl}_2$  in the incubation medium was varied, it did not appear to be associated with major deleterious effects on the maintenance of ATP levels. At 0.1 mM of initial ATP, the substrate level was maintained better when the concentration of total added  $\text{MgCl}_2$  was below 5 mM than when it was higher than 5 mM (Fig. 3, panel B, open circles and closed inverted triangles), but we did not investigate the reasons for this phenomenon. The addition of GTP with the total added ATP being either 2.77 mM (not shown) or 0.1 mM (Fig. 3, panel B, closed inverted triangles) was without effect on the maintenance of ATP levels at any of the concentrations of  $\text{MgCl}_2$  tested.

While potent pyrophosphohydrolase ac-

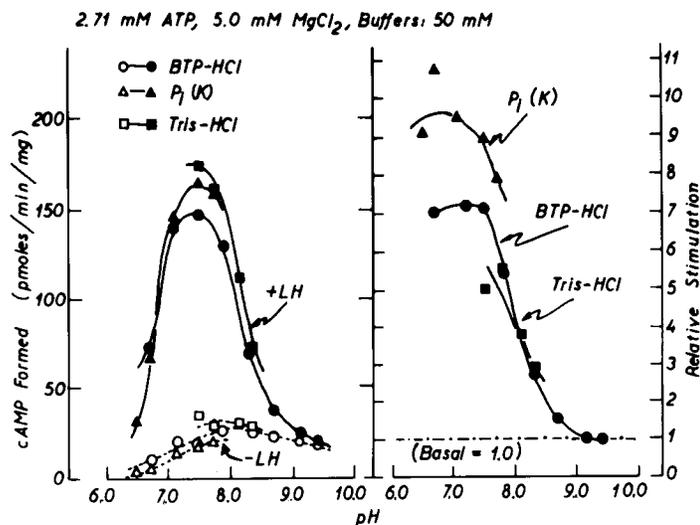
tivity was also present in membrane particles from CL of rats, (*i.e.*, myokinase was necessary to maintain ATP levels adequately), it appeared to be absent or negligible in membrane particles from the Graafian follicles of rabbits and pigs, where adequate levels of ATP could be maintained in the absence of myokinase. Thus, in one of several experiments, we found that the incubation of membrane particles from the Graafian follicles of pigs (14.2  $\mu\text{g}$  protein per assay, ATP at  $t = 0$ : 0.05 mM) and of rabbits (16  $\mu\text{g}$  protein per assay, ATP at  $t = 0$ : 0.10 mM) under AC assay conditions (5.0 mM  $\text{MgCl}_2$ , 1.0 mM EDTA, 1.0 mM EGTA, 1.0 mM cAMP, 0.2 mg/ml creatine kinase, 20 mM creatine phosphate, and 25 mM BTP-HCl buffer, pH 7.0), but in the absence of added myokinase, resulted in the preservation, respectively, of 65 and 77% of the ATP at the end of a 25 min incubation at 37 C.

Assays of 5% (wt/vol) homogenates of either rat or rabbit CL, using between 2 and 3 mM ATP did not require the addition of exogenous myokinase, possibly because of the presence of sufficient endogenous myokinase activity (not shown).

*b. Ionic strength and pH.* Contrary to findings in other AC systems (liver, fat, renal

FIG. 3. Maintenance of ATP levels under adenyl cyclase assay conditions at varying concentrations of ATP (Panel A) and Mg (Panel B). Panel A: Levels of ATP (circles) and AMP (triangles) present at the end of 10 min incubations at pH 7.5 and 37 C in the absence (open symbols) and the presence (closed symbols) of  $4 \times 10^{-5}\text{M}$  GTP. Same experiment as shown in Fig. 5. Panel B: Level of ATP at the end of 10 min incubations at pH 7.5 and 37 C. Incubations were carried out in the absence (circles) and the presence (inverted triangles) of

FIG. 4. Effect of the pH of incubation on basal and LH-stimulated adenylyl cyclase activities (left panel) and on LH stimulation (right panel). Membrane particles from the CL of pseudo-pregnant rabbits were used. The pH values reported are those determined in mock incubations (substituting unlabeled ATP for labeled ATP) after 5 min of incubation at 37 C. The relative activities were calculated by dividing LH-stimulated activities by the corresponding basal activities.  $P_i(K)$  = potassium phosphate buffer; open symbols = basal adenylyl cyclase activities; closed symbols = LH-stimulated activities. The rest of the incubation conditions are described on the figure and in *Methods*.



membranes), the LH-sensitive system in the rabbit CL was found to be influenced by the ionic composition of the medium, and it was found convenient to assay activity at the lowest ionic strength possible. For example, increasing the concentration of buffer from 25 mM to 50 mM resulted in about a 30 to 40% reduction of LH-stimulated activity without effect on the basal activity. Similar results were obtained with 50 mM NaCl, indicating that the inhibition was due to ionic strength and was unrelated to the type of ion.

pH optima differed for basal and LH-stimulated activities. As shown in Fig. 4 the optimal pH for basal activity is around 8.0, while that for LH-stimulated activity is sharper and about one half a pH unit lower, *i.e.*, 7.5. In this and two other experiments carried out with separate batches of membranes, we found that optimal relative stimulation, using BTP-HCl as buffer, was obtained between pH 6.5 and 7.5. Similar results were obtained with Tris-HCl as the sole added buffer, and with potassium phosphate buffers ( $P_i(K)$ ). The latter buffer system, however, gave very low basal activities at pH's below 7.0, which resulted in large errors in the calculation of relative activities (see Fig. 4, right panel, closed tri-

angles). All experiments testing for activities in either follicles or CL reported elsewhere (9,10), were carried out at a final pH of 7.0, using 25 mM Tris-HCl as the buffer system. Since, however, Tris is not a good buffer at this pH, the ionic strength of the incubation media was kept to a minimum by separately adjusting, with the aid of a pH stat, the pH of all of the ingredients used (ATP, cAMP, EDTA, regenerating system, and other solutions) to pH 7.0 with minimal amounts of Tris-base. Under these conditions, and using 5% homogenates of rabbit CL prepared in 27% sucrose, 1.0 mM EDTA and 10 mM Tris-HCl, pH 7.5, and 3.0 mM ATP as substrate, we found the pH of the reaction when tested in 0.2-ml mock incubations after 5 min at 37 C, to vary between 7.0 and 7.1. In all experiments presented in this article, however, we used bis-tris-propane-HCl (BTP-HCl) as the buffer system. This buffer has two  $pK_a$  values ( $pK_{a1} = 9.0$  and  $pK_{a2} = 6.8$ ) which give it a useful range, spanning from 6.0 to 10.0.

#### IV. Properties

*a. Effects of ATP and GTP.* Previous experiments reported elsewhere (17), had indicated that the AC system of the rabbit CL of

pregnancy responds not only to LH but also to  $PGE_1$ , and that responsiveness to LH but not to  $PGE_1$  is enhanced markedly by ATP. This finding was somewhat surprising, since, in most other AC systems, this regulatory role is fulfilled by guanyl nucleotides. This is not to say that GTP had no effect on this system, since it was found to enhance to about the same degree both basal activity and LH-stimulated activity without alteration of the responsiveness to LH. Since, however, those (17) experiments were carried out in the absence of myokinase in the incubation medium, the possibility existed that one or the other of the effects of ATP and GTP was an artifact of the presence of nucleoside triphosphate pyrophosphohydrolase activity. We repeated and expanded

those studies under conditions in which we controlled ATP levels by the addition of creatine kinase, creatine phosphate, and myokinase (Fig. 3) and determined the effects of varying concentrations of ATP, on basal and LH-stimulated activity, in the presence and the absence of GTP. For comparative purposes, we also determined  $PGE_1$ -stimulated activity. The results of one of three such experiments are shown in Fig. 5. In agreement with our earlier report, ATP, but not GTP, enhances the responsiveness of the rabbit CL system to LH. GTP appears to enhance the general "efficiency" of the system by stimulating the formation of cAMP, both in the absence and the presence of LH, to about the same degree. Thus, GTP clearly interacts with the system.

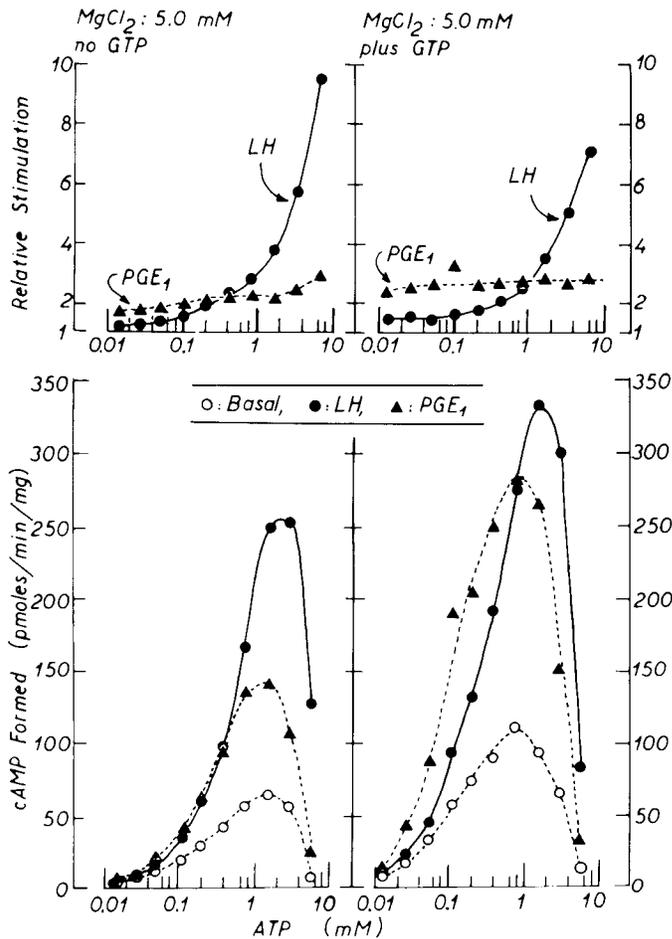


FIG. 5. Effect of ATP on activity (lower panels) and hormonal responsiveness (top panels) of rabbit CL adenylyl cyclase. Activities were determined in the absence (left panels) and the presence (right panels) of GTP.  $\circ$  = basal activities;  $\bullet$  = absolute or relative LH-stimulated activities;  $\blacktriangle$  = absolute or relative  $PGE_1$ -stimulated activities. Membrane particles ( $22 \mu\text{g}$  protein per assay) from CL of pregnant rabbits were used. Incubations were carried out at  $37^\circ\text{C}$ , for 10 min, under standard assay conditions described under *Methods*, in the presence of the indicated concentrations of ATP,  $5.0 \text{ mM MgCl}_2$ , and, when present,  $4 \times 10^{-5} \text{ M GTP}$ ,  $10 \mu\text{g/ml LH}$  and  $10 \mu\text{g/ml PGE}_1$ . The rest of the conditions are described under *Methods*.

GTP does not seem to interfere competitively with the LH-enhancing action of ATP, since a slight inhibitory action of GTP seen at low ATP, either increased (Fig. 5) or remained constant (Fig. 6), but did not decrease, with increasing concentrations of ATP. These findings are consistent with our previous suggestion that ATP and GTP interact with separate regulatory sites of the system, one (specific for GTP) regulating the catalytic efficiency of AC, and the other (specific for ATP) altering the degree to which occupied hormone receptor can enhance catalytic activity. As shown in Fig. 5, the addition of high ATP concentrations (1 to 2 mM) that result in limiting concentrations of free Mg ion, resulted in decreased absolute activities and increased ATP-dependent enhancement of LH responsiveness. While the decrease in adenylyl cyclase activities under these conditions may be due either to lack of  $Mg^{++}$  at an allosteric site (18,20) or to increased inhibition of the enzyme by the free form of the substrate  $ATP^{4-}$  or  $ATPH^{3-}$  (21), the finding that ATP-dependent enhancement of LH responsiveness was increased under these conditions is consistent with a recent model for AC regulation proposed by Rodbell's group (22), in which the regulatory effects of nucleotides are due to their interaction with the system in the free (in this case  $ATP^{4-}$  or  $ATPH^{3-}$ ) rather than the Mg-bound ( $MgATP^{2-}$ ) form.

The enhancement of LH responsiveness by ATP can be seen over a wide range of pH (Fig. 6), and appears to be more pronounced at more acid pH's, suggesting that the actual form interacting with the system might be  $ATPH^{3-}$  rather than  $ATP^{4-}$ . However there may be other explanations, such as the actual activating process depending on the pH and having a pH optimum that is in the acidic range.

While the LH responsiveness of CL AC increases with ATP concentrations ranging from 0.1 mM to as high as was tested (3 to 4 mM), the responsiveness of the Graafian follicle system to LH was affected by much

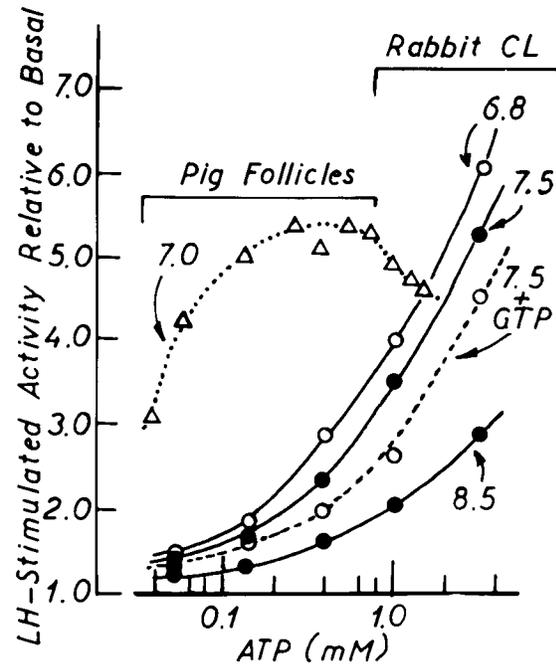


FIG. 6. Effect of ATP at various pH's on the responsiveness to LH of rabbit CL adenylyl cyclase and of pig Graafian follicle adenylyl cyclase. Assay conditions for rabbit CL adenylyl cyclase (membrane particles from CL of pregnant rabbits were used) were the same as described in the legend to Fig. 5, except that, when present, GTP was  $1 \times 10^{-5}M$ . Assay conditions for pig Graafian follicle adenylyl cyclase differed in that 1 mM EGTA was added, myokinase was absent, and the temperature and time of incubation were 30 C and 5 min, respectively.

lower concentrations of ATP. As shown in Fig. 6, an apparent half-maximal stimulation of LH responsiveness in the follicle cyclase occurred between 0.04 and 0.05 mM ATP and reached its maximum at about 0.1 mM. The responsiveness remained relatively constant between 0.1 and 1 mM ATP and decreased thereafter, possibly due to the activation of an ATP and Mg-dependent desensitization mechanism (for details on this decrease, see ref. 11). Experiments were carried out to determine whether the effects of GTP and ATP seen in the rabbit CL and pig follicles were characteristic of CL and follicles, or whether these differences might have had their origin in the fact that the tissues assayed were derived from different

TABLE 1. Effect of addition of GTP and ATP on responsiveness to LH of adenylyl cyclase from rat CL and rabbit follicles

Assay conditions			Adenylyl cyclase activity <sup>1</sup>		Relative stimulation
ATP	MgCl <sub>2</sub>	GTP	Basal	LH	
(mM)			(pmoles/mg/min)		
Rat CL of superovulation (homogenate) <sup>2</sup>					
0.09	2.0	—	2.0 ± 0.36	3.5 ± 0.13	1.78
0.09	2.0	0.01	4.1 ± 0.41	6.1 ± 0.5	1.49
2.7	5.0	—	38.6 ± 2.4	243.3 ± 10.9	6.30
Rabbit estrous follicles (membrane particles) <sup>3</sup>					
0.12	2.0	—	3.5 ± 0.4	20.1 ± 1.2	5.74
0.12	2.0	0.01	5.2 ± 0.9	32.3 ± 3.4	6.25
1.5	5.0	—	17.2 ± 2.0	79.3 ± 8.3	4.66

<sup>1</sup> Activities are mean ± SD of triplicate determinations. LH, when present, was 10 μg/ml.

<sup>2</sup> Homogenates were prepared as described under *Methods*. Twenty-μl aliquots containing approximately 100 μg of homogenate protein were assayed under standard assay conditions (myokinase present, 25 mM BTP-HCl buffer, pH 7.5) except for the changes indicated on the table. Incubations were for 12 min at 37°C. <sup>32</sup>P in ATP at the beginning and at the end of the 12 min incubation at 0.09 mM ATP, 2.0 mM MgCl<sub>2</sub>, in the absence of GTP, was 98.5 and 93%, respectively.

<sup>3</sup> Membrane particles of Graafian follicles from 6 rabbits were prepared in the same way as CL membrane particles (see *Methods*). Assays were performed on 7.5 μg membrane protein in media containing the concentrations of ATP (15 × 10<sup>6</sup> cpm of [<sup>32</sup>P]ATP), MgCl<sub>2</sub>, and GTP indicated on the Table, and 1.0 mM cAMP, 1.0 mM EDTA, 20 mM creatine phosphate, 0.2 mM creatine kinase and 25 mM Tris-HCl, pH 7.5. Incubations were for 5 min at 30°C.

species. Table 1 indicates that the pattern of ATP and GTP responsiveness seen in rabbit CL and pig follicles can also be observed in rat CL and rabbit follicles.

Thus, ATP was found to enhance LH responsiveness in both CL and follicles, with the follicular AC system being much more sensitive to ATP than the CL system. As in the CL system, GTP was also unable to substitute for ATP in the follicle system (not shown).

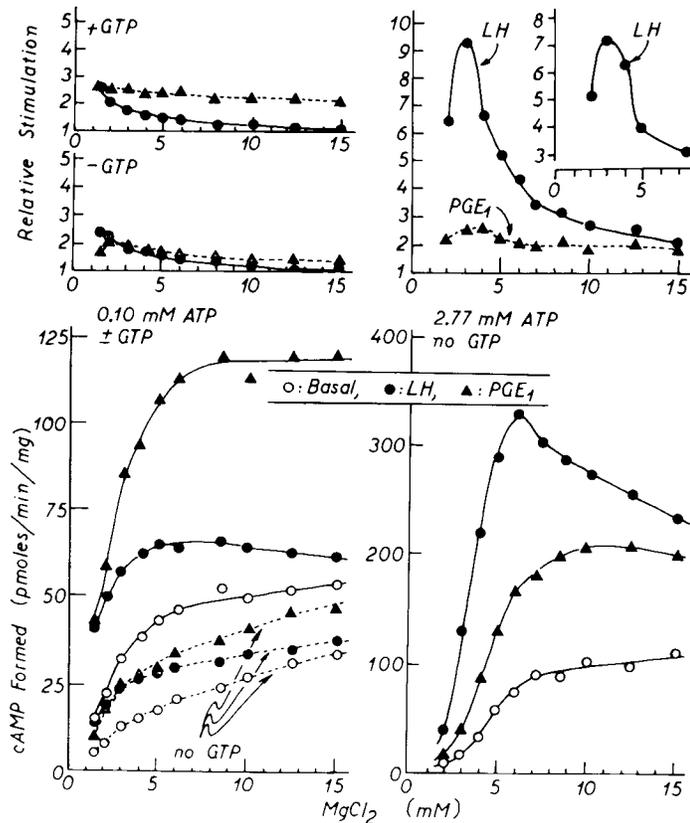
*b. Effects of Mg.* The dependence of AC systems and their stimulation by hormones on Mg ion is complex, and the LH-sensitive system is no exception. Figure 7 depicts the effects of varying concentrations of MgCl<sub>2</sub> on basal, LH- and PGE<sub>1</sub>-stimulated AC activities of rabbit CL of pregnancy. Determinations were carried out under

three nucleotide conditions: at 0.1 mM ATP in the absence of GTP, at 0.1 mM ATP in the presence of 4 × 10<sup>-5</sup>M GTP and at 2.77 mM ATP. As indicated indirectly by the ATP study commented on above, LH stimulations were best seen at high ATP and at rather low (limiting) concentrations of Mg ion. Clearly, excess MgCl<sub>2</sub> tends to inhibit LH stimulation, be it at low or high ATP in the absence or presence of GTP. One unexplained, but reproducible, finding (see inset of Fig. 7) was that, if the Mg concentrations were reduced below the concentration of added ATP, the relative stimulation by LH also diminished.

*c. Response to prostaglandin.* The response of the rabbit CL AC system to PGE<sub>1</sub> was little affected by either Mg ion (Fig. 7), ATP concentration (Fig. 5), or GTP (Figs. 5 and 7). The lack of effect of GTP and ATP is consistent with our previous report (17) that the action of PGE<sub>1</sub> on CL adenylyl cyclase was enhanced by GDP, the concentrations of which was kept to a minimum under our assay conditions, and was unaffected by purine nucleoside triphosphates. We have not investigated further this effect of GDP, or whether it is also present in the CL of the rat.

*d. Sensitivity of LH-responsive adenylyl cyclase to LH: Lack of effect of incubation conditions.* Rodbell *et al.* (23) reported that the concentration of glucagon required for half-maximal activation of liver AC is strongly dependent on the concentration of GTP in the medium and, therefore, on assay conditions. In fact, the addition of 10<sup>-5</sup>M GTP in their experiments, (0.1 mM substrate, 5.0 mM MgCl<sub>2</sub>, pH 7.6) resulted in a 10-fold increase in the apparent affinity of the system for glucagon. Since this type of result provides insight into the possible mechanisms involved in hormonal stimulation, we explored the possibility that a similar phenomenon might also be observable for LH stimulation of the CL membrane AC. We tested for the possible effects of

FIG. 7. Effect of Mg concentration on activity (lower panels) and hormonal responsiveness (top panels) of the rabbit CL adenylyl cyclase. Activities were determined in the absence and presence of GTP at 0.1 mM ATP (left panels) and at 2.77 mM ATP (right panels).  $\circ$  = basal activities;  $\bullet$  = absolute or relative LH-stimulated activities;  $\blacktriangle$  = absolute or relative PGE<sub>1</sub>-stimulated activities. Membrane particles (22  $\mu$ g protein per assay) from CL of pregnant rabbits were used. Incubations were carried out at 37 C for 10 min under standard assay conditions described under *Methods*, in the presence of the indicated concentrations of MgCl<sub>2</sub>, ATP, and, when present,  $4 \times 10^{-5}$ M GTP, 10  $\mu$ g/ml LH, and 10  $\mu$ g/ml PGE<sub>1</sub>. For the rest of conditions see *Methods*. Upper right hand inset: Relative stimulation by LH at varying concentrations of MgCl<sub>2</sub>. These data were obtained in a similar experiment carried out at 2.9 mM ATP with a different batch of membrane particles (19  $\mu$ g protein per assay) from CL of pregnant rabbits.



assay conditions (pH, ATP, Mg ion, and GTP) on the position of the LH dose-response curve. As shown in Figs. 8 and 9, none of the conditions tested resulted in significant differences in the concentrations of LH needed to activate AC to 50% of maximum, even though the change in conditions resulted in large changes in both absolute and relative activities. Thus, the absolute activity with 10  $\mu$ g/ml LH, at pH 6.8, 0.09 mM ATP, and 2.0 mM MgCl<sub>2</sub>, in the presence of GTP, was only 6.9% of that obtained at pH 7.5, 2.4 mM ATP, and 5.0 mM MgCl<sub>2</sub> in the absence of GTP (Fig. 8, closed circles in third panel *vs* closed circles in second panel), and the relative stimulation by LH changed from a 96% increase at pH 7.5, 0.23 mM ATP, and 5.0 mM MgCl<sub>2</sub> in the presence of GTP to a 454% increase at

pH 6.8, 2.4 mM ATP, and 5.0 mM MgCl<sub>2</sub> in the absence of GTP (Fig. 8, closed circles last panel *vs* open circles, second panel). Figure 9 shows not only that GTP did not influence the position of the LH dose-response curve, but also that LH did not influence the concentration dependency of the system on GTP. The possibility that the membrane particles used contain high amounts of endogenous nucleotides, thereby masking the effects ATP or GTP might have on the apparent affinity of the system for LH, seems unlikely. If this were so, we should have observed neither the effect of GTP on the catalytic activity of the system (Fig. 7, lower left panel; Fig. 8, first panel; and Fig. 9) nor the enhancement of LH responsiveness with increasing ATP concentrations (Figs. 5, 6, and 8).

### V. Responsiveness of LH-stimulated adenylyl cyclases to LH fractions of various specific activities

One of the goals of the studies presented in this series of articles was to investigate the role of AC in the mechanism of action of LH on ovarian tissues. Indirect evidence on the mediation of LH effects by cAMP and the actual fact that AC in ovarian tissues is stimulated by LH, strongly suggested that the cyclase system is a primary target of LH action. Further acceptance of this assumption required the existence of an acceptable relationship between the effects of LH fractions of varying degrees of purity on AC and the activity of LH fractions, as determined in *in vivo* bioassays. Thus, the possibility existed that, although LH is capable of stimulating AC and cAMP is capable of mimicking actions of LH, the actual primary mechanism by which LH affects target tissues is not AC activation, but rather by causing other, as yet undefined, changes, possibly through separate receptors with

higher affinities, as suggested for the LH-Leydig cell interaction (24-26). We determined the effect of a variety of LH fractions (kindly made available to us by Dr. L. E. Reichert) on three LH-sensitive AC systems derived from rabbit CL of pregnancy, rat CL of superovulation, and pig Graafian follicles. Ten LH fractions were tested in each of the three systems by obtaining the corresponding dose-response curves, linearizing the proportional range of these curves (Hill plots), and calculating by least squares regression analysis their potency and 95% confidence limits in both  $\mu\text{g/ml}$  and units relative to NIH-LH-S18. We used standard equations for these calculations (27). Figure 10 presents the actual adenylyl cyclase activation data and their Hill plots for five of the 10 LH fractions tested; Table 2 presents the calculated intercepts of the linearized dose-response curves (potencies) with their 95% confidence limits; and Fig. 11 presents in graphical form the relation of the biological potencies of the tested LH fractions, as seen in the *in vivo* ovarian ascorbic acid

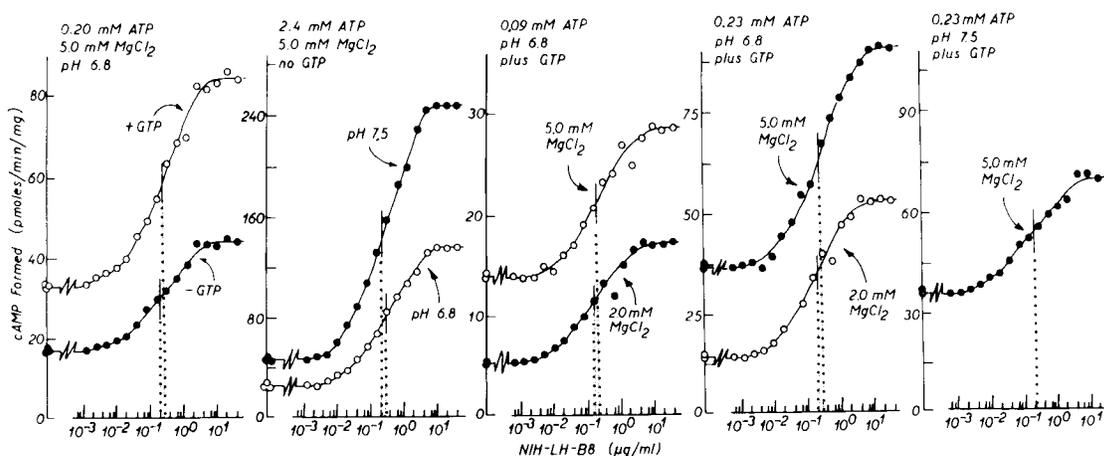
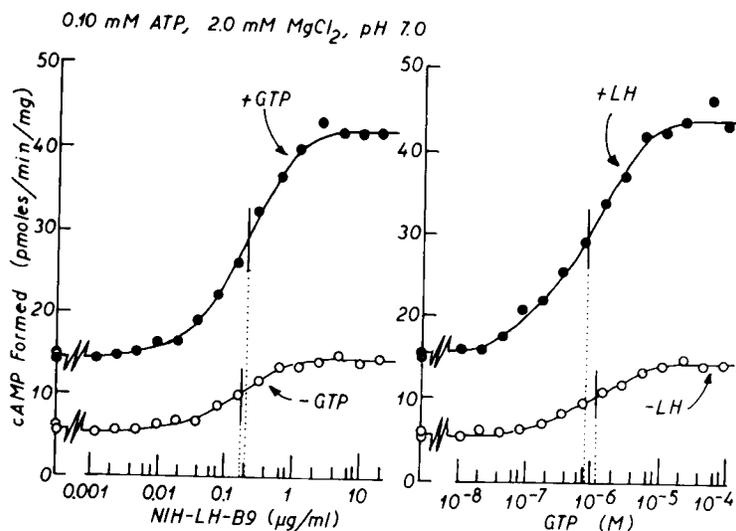


FIG. 8. Sensitivity of rabbit CL adenylyl cyclase to LH stimulation and lack of effect of some assay conditions. Experiments were carried out with CL of pregnant rabbits. Incubations were for 15 min at 37 C under standard assay conditions (myokinase present, 25 mM BTP-HCl buffer) described under *Methods* with the changes indicated on the figure. Two experiments are presented, each carried out with a different batch of membrane particles. Experiment 1 (18  $\mu\text{g}$  membrane protein per assay) is shown in the first and second panels; experiment 2 (14  $\mu\text{g}$  membrane protein per assay) is shown in the other panels. GTP when present, was  $1 \times 10^{-5}\text{M}$ . Concentrations of LH (NIH-LH-B8, in  $\mu\text{g/ml}$ ) giving half-maximal stimulation were, respectively: Panel 1: 0.20 in the absence and 0.25 in the presence of GTP. Panel 2: 0.27 at pH 6.8 and 0.20 at pH 7.5. Panel 3: 0.16 at 2.0 mM and 0.21 at 5.0 mM  $\text{MgCl}_2$ . Panel 4: 0.20 at 2.0 mM and 0.31 at 5.0 mM  $\text{MgCl}_2$ . Panel 5: 0.22 at 5.0 mM  $\text{MgCl}_2$ .

FIG. 9. Lack of evidence for interdependent interaction of LH and GTP with rabbit CL adenylyl cyclase. Membrane particles (17  $\mu\text{g}$  protein per assay) from CL of pregnant rabbits were used. Incubations were for 15 min at 37 C under standard assay conditions described under *Methods* (myokinase present, 25 mM BTP-HCl buffer) with the changes indicated in the figure. Concentrations of LH (NIH-LH-B9), giving half-maximal stimulation were 0.18 and 0.21  $\mu\text{g}/\text{ml}$  in the absence and the presence of GTP  $1 \times 10^{-5}\text{M}$ , respectively. Concentrations of added GTP giving half-maximal increases of activity were 1.05 and  $0.85 \times 10^{-6}\text{M}$  in the absence and the presence of 10  $\mu\text{g}/\text{ml}$  LH, respectively.



depletion test (performed in rats), to the relative potencies of these same fractions in each of the three AC systems. Several findings emerged from these studies.

First, it is apparent that the relative affinity characteristics of the rat CL cyclase system for the various LH fractions (that vary not only in purity but also in species origin) correlates relatively well with the characteristics expected if the receptors coupled to AC and those responsible for the initiation of ascorbic acid depletion in rat ovaries are similar or the same. The correlation curve, however, does not have a slope of 1.00, the theoretical value expected if the only factor that comes into play is receptor affinity, rather it has a slope of  $0.74 \pm 0.12$  (mean  $\pm$  SD) with a coefficient of determination of 0.79. One explanation for this deviation from ideal is that the bioassay data not only depend on receptor affinity, but also on hormone half-life, for which no correction has been introduced. In general, however, the correlation of rat cyclase data with rat bioassay data is consistent with the assumption that LH may initiate its actions on target tissues *via* AC activation.

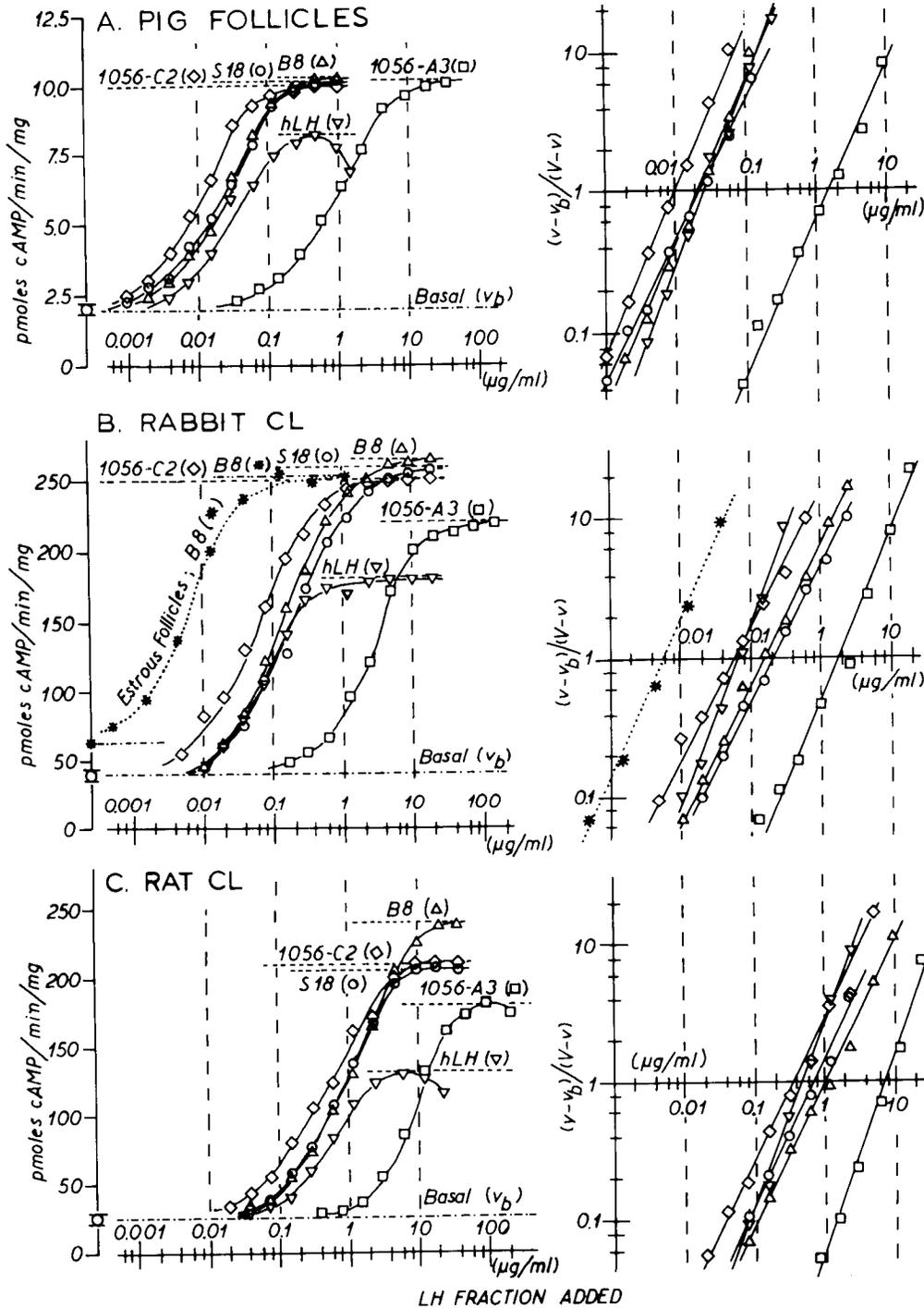
Second, the apparent affinities of the analyzed AC systems vary both with species

and tissue origin. Both CL preparations tested were considerably less sensitive to low LH concentrations than either the pig or the rabbit follicle preparation. About 20 times higher concentrations of bovine (B8 or, not shown, B9) LH were needed to stimulate half-maximally rabbit CL cyclase than rabbit follicle cyclase (160 ng/ml *vs* 7.5 ng/ml). While rabbit follicles appear to be 3 times more sensitive than pig follicles, this difference may not be significant. In either case, the sensitivity of the AC system to LH stimulation decreased by at least one order of magnitude upon luteinization of the follicle.

Third, the degree of stimulation that can be obtained varied with the origin of LH. Thus, in rat CL, bovine LH fractions (B8 and, not shown, LER no. 1716-2) stimulated AC to the highest extent, giving LH-stimulated activities at saturation that ranged from 235 to 240 pmoles/min/mg. Then followed ovine LH fractions, which stimulated the enzyme to activities between 195 and 210 pmoles/min/mg (with the much less pure LER fractions nos. 1056-A1 and 1056-A3 giving somewhat less activation [190 and 175 pmoles/min/mg, respectively]), and human LH, which was even less effective (125

pmoles/min/mg). These differences, although present, are less pronounced in rabbit CL membranes and, except for the lower stimulating activity of human LH, are not

detected in membrane particles of estrous pig follicles (Fig. 10). The finding that human LH has about half the efficacy of bovine LH in stimulating the AC system



from the CL of superovulated rats is of interest because, if coupled to specific binding studies, it may afford a tool to investigate quantitative relationships between binding and AC activation, and because eventual knowledge of the tertiary and quaternary structure of bovine and human LH may be expected to shed some light on which molecular characteristics of LH are involved in enzyme activation. The very low apparent affinity of the rat system for LH is intriguing. We are currently investigating whether it is, at least in part, due to the presence of interfering enzymes (proteases) in our particulate preparations, and whether equally high concentrations of LH are required in membrane particles of CL obtained from "true" pseudopregnant (cervix-stimulated) or pregnant rats.

#### VI. Responsiveness to Catecholamines

In addition to LH and prostaglandins we also tested the effect of catecholamines (epinephrine and isoproterenol) on the AC activity of pig Graafian follicles, rat CL of superovulation, and rabbit CL of pregnancy. As shown in Table 3, the cyclase from follicles is barely responsive to catecholamines, but AC from CL (be they from rabbit

or rat) are highly responsive to the biogenic amines. From the data of Table 3, it is also apparent that catecholamines and LH stimulate the same catalytic unit, for their effects are not additive. In this respect, the CL adenylyl cyclase appears to resemble the AC in rat fat cells, which is also activated by more than one hormone, each thought to have its own specific receptor (28).

#### Discussion

In this article we have attempted to present some guidelines for the assay in ovarian tissues of LH-responsive adenylyl cyclase activity, especially with respect to homogenization and assay conditions. Using the type of strategy reported on, we were successful in finding adenylyl cyclase activity that responds to LH at least 5-fold (and often more) in corpora lutea of rabbits and rats, and in large Graafian follicles of rabbits, rats, and pigs. However, we were unable to determine LH-stimulated AC activity in pig CL of the cycle or of pregnancy, indicating that the above described strategies can only be taken as guidelines. One reason for the failure to detect a response of AC to LH (both basal and fluoride-stimulated activities were easily detectable) may be related to the re-

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FIG. 10. Effect of LH fractions of varying potencies on adenylyl cyclase activities in rabbit and rat CL and rabbit and pig follicles. A. Membrane particles of pig Graafian follicles of diameter larger than 6 mm were prepared as described in detail elsewhere (see *Methods* and 11) and assayed (14  $\mu$ g membrane protein) for adenylyl cyclase activity in medium containing 0.1 mM ATP, 3.0 mM MgCl<sub>2</sub>, 1.0 mM cAMP, 1.0 mM EDTA, 1.0 mM EGTA, 20 mM creatine phosphate, 0.2 mg/ml creatine kinase, and 25 mM BTP-HCl buffer, pH 7.0. Incubations were for 15 min at 30 C. B. Membrane particles of rabbit CL of pregnancy were prepared as described under *Methods* and assayed (21  $\mu$ g protein per assay) under standard conditions (myokinase present, 25 mM BTP-HCl buffer, pH 7.5) for 15 min at 37 C. Note: one experiment on the effect of NIH-LH-B8 on membrane particles from rabbit follicles (\*), is also shown. The preparation of rabbit membrane follicles (essentially the same as that for rabbit CL membranes) is described under *Methods*. These membrane particles (8  $\mu$ g protein per assay) were incubated for 5 min at 30 C in medium containing 1.5 mM ATP, 5.0 mM MgCl<sub>2</sub>, 1.0 mM cAMP, 1.0 mM EDTA, 1.0 mM EGTA, 20 mM creatine kinase, 0.2 mg/ml creatine kinase, 25 mM BTP-HCl buffer, pH 7.0, and the indicated concentrations of LH. C. Membrane particles from CL of superovulation were prepared as described under *Methods* and assayed under standard conditions (myokinase present, 25 mM BTP-HCl buffer, pH 7.5) for 15 min at 37 C. Left panels: Absolute activities obtained in the presence of the indicated concentrations of LH-containing fractions. Values in the absence of LH are means  $\pm$  range of triplicate determinations. Rest of values are the results of individual determinations. Lower and upper dashed lines represent basal ( $v_n$ ) and maximally stimulated ( $V$ ) activities. Right panels: Hill plots ( $\log[(v-v_n)/(V-v)] = f(\log[H])$ ) of the proportional sections of the dose-response curves shown on the left panels. Lines were drawn "by eye." Estimates of the intercepts of regressions with the abscissa calculated by the method of least squares are presented in Table 2.

TABLE 2. Activity of various LH-containing fractions in pig, rabbit, and rat ovarian adenylyl cyclase assays<sup>1</sup>

LH fractions <sup>2</sup>	Hill plots		Potencies relative to NIH-LH-S18 <sup>4</sup>		Potencies relative to NIH-LH-S18 by OAAD <sup>5</sup> Units
	Slope n ± SD	A <sub>0.5</sub> <sup>3</sup> μg/ml	Units	(95% conf. lim.)	
A. Pig Graafian follicles					
NIH-LH-S18	0.99 ± 0.04	0.023 (0.015) <sup>6</sup>	1.00 (1.00)	(0.85–1.16)	1.0
NIH-LH-B8	1.20 ± 0.05	0.021 (0.013)	1.05 (1.11)	(0.91–1.21)	1.03
hLH (LER #960)	1.29 ± 0.05	0.027 (0.017)	0.84 (0.88)	(0.73–0.98)	1.6
LER #1374-A	1.06 ± 0.04	0.007	3.14	(2.77–3.58)	2.0
LER #1056-C2	1.18 ± 0.03	0.009 (0.006)	2.49 (2.61)	(2.27–2.73)	1.67
LER #1716-2	1.03 ± 0.05	0.012	1.79	(1.46–2.18)	1.30
LER #1733-3	1.00 ± 0.03	0.015	1.54	(1.37–1.73)	1.5
LER #1733-2	1.10 ± 0.03	0.11	0.21	(0.19–0.23)	0.13
LER #1056-A1	1.01 ± 0.05	0.73	0.031	(0.026–0.037)	0.069
LER #1056-A3	1.02 ± 0.04	0.81 (0.60)	0.028 (0.025)	(0.024–0.016)	0.041
B. Rabbit corpora lutea of pregnancy					
NIH-LH-S18	0.96 ± 0.03	0.21 (0.19)	1.00 (1.00)	(0.90–1.11)	1.0
NIH-LH-B8	1.05 ± 0.05	0.16 (0.15)	1.31 (1.27)	(1.06–1.61)	1.03
hLH (LER #960)	1.32 ± 0.08	0.071 (0.066)	2.46 (2.90)	(2.42–3.64)	1.6
LER #1374-A	1.00 ± 0.01	0.050	4.18	(3.97–4.40)	2.0
LER #1056-C2	0.92 ± 0.03	0.055 (0.048)	3.80 (3.98)	(3.32–4.35)	1.67
LER #1716-2	1.05 ± 0.03	0.090	2.33	(2.06–2.62)	1.30
LER #1733-3	1.06 ± 0.05	0.069	3.04	(2.59–3.57)	1.5
LER #1733-2	1.01 ± 0.03	0.46	0.45	(0.40–0.51)	0.13
LER #1056-A1	0.97 ± 0.04	1.97	0.107	(0.091–0.125)	0.069
LER #1056-A3	1.21 ± 0.07	2.04 (1.94)	0.103 (0.098)	(0.083–0.128)	0.041
C. Rat corpora lutea of superovulation					
NIH-LH-S18	1.01 ± 0.03	0.77 (0.83)	1.00 (1.00)	(0.88–1.13)	1.0
NIH-LH-B8	1.01 ± 0.04	1.06 (1.20)	0.73 (0.69)	(0.63–0.84)	1.03
hLH (LER #960)	1.38 ± 0.06	0.49 (0.51)	1.59 (1.63)	(1.37–1.83)	1.6
LER #1374-A	1.12 ± 0.02	0.23	3.37	(3.22–3.53)	2.0

TABLE 2. (Continued)

LH fractions <sup>2</sup>	Hill plots		Potencies relative to NIH-LH-S18 <sup>4</sup>		Potencies relative to NIH-LH-S18 by OAAD <sup>5</sup>
	Slope n ± SD	A <sub>0.5</sub> <sup>3</sup> μg/ml	Units	(95% conf. lim.)	Units
LER #1056-C2	0.97 ± 0.04	0.40 (0.44)	1.92 (1.88)	(1.59-2.32)	1.67
LER #1716-2	0.94 ± 0.03	0.54	1.43	(1.25-1.64)	1.30
LER #1733-3	1.08 ± 0.04	0.42	1.83	(1.58-2.12)	1.5
LER #1733-2	1.22 ± 0.02	0.93	0.83	(0.77-0.90)	0.13
LER #1056-A1	1.52 ± 0.04	8.07	0.096	(0.087-0.105)	0.069
LER #1056-A3	1.41 ± 0.09	7.54 (8.10)	0.103 (0.102)	(0.082-0.129)	0.041

<sup>1</sup> For details of assays see legend to Fig. 10.

<sup>2</sup> For description of some of the properties of these fractions see *Materials*. These fractions were kindly supplied by Dr. L. E. Reichert (Emory University, Atlanta, Georgia) and the NIH.

<sup>3</sup> Estimates of the intercepts of linear regressions of Hill plots with the abscissa. They represent concentration of hormone necessary to activate adenylyl cyclase to 50% of maximum, and are assumed to be a measure of the potency of the fractions tested.

<sup>4</sup> Calculated by dividing A<sub>0.5</sub> for NIH-LH-S18 A<sub>0.5</sub> for the hormone in consideration. No correction has been made to account for possible inaccuracies due to differences in the slopes of Hill plots.

<sup>5</sup> As assayed by Dr. L. E. Reichert.

<sup>6</sup> The numbers in italics represent the results obtained in a repeat experiment, in which only NIH-LH-S18, NIH-LH-B8, hLH (LER #960), LER #1056-C2, and LER #1056-A3 were tested. Different batches of membranes were used.

ported lower capacity to respond to LH by synthesizing progesterone, of pig CL slices than of slices of CL from other species (29). Another reason may be that the CL of pigs are not responsive to LH in terms of cAMP synthesis. We have not yet made use of imidodiphosphate analogues to explore whether the pig CL system requires a nucleoside diphosphate (ADP or GDP) for stimulation by LH, as was found for the response to prostaglandins in rabbit CL of pregnancy (17) and for arginine vasopressin stimulation in renal membranes (17,30), or whether the system can be tricked into showing LH responsiveness by adding the synthetic nucleotide 5'-guanylyl imidodiphosphate (GMP-P(NH)P), whose rate of stimulation of basal AC has recently been shown in several systems to be accelerated by the addition of hormone (31-33). The use of GMP-P(NH)P allowed Cooper *et al.* (34) to detect catecholamine stimulation in human fat cell AC that was hitherto unrecognized. At any rate, since both the rabbit and the rat are widely used models in

reproductive biology, and since the methodologies described allow for the determination of LH responsiveness in ovarian tissues (follicles and CL) of these animals, we hope that they will be of use in the study of the regulation of LH action at the ovarian level.

The finding reported here that ATP and GTP interact in a regulatory fashion with ovarian adenylyl cyclases, seemingly with distinct allosteric sites, both confirms and expands our previous findings on this point (17). The enhancement of LH responsiveness of the follicle AC by low (micromolar) concentrations of ATP (seen for pig follicles in Fig. 6) is reminiscent of a similar phenomenon observed in the kidney vasopressin-sensitive system, in which responsiveness to vasopressin is enhanced either by low ATP or by equally low concentrations of adenosine (35,30). We did not explore whether, likewise, adenosine can substitute for ATP in enhancing LH responsiveness. The question now arises of whether the multiplicity of regulatory sites observed

TABLE 3. Effect of catecholamines on adenylyl cyclase systems of various ovarian tissues

Additions <sup>1</sup>	Adenylyl cyclase activities	
	Absolute <sup>2</sup>	Relative <sup>3</sup>
A. Rat CL of superovulation (membrane particles) <sup>4</sup>		
None	30.9 ± 3.1	—
LH	300.3 ± 9.4	9.72
Iso	255.2 ± 15.2	8.26
PGE <sub>1</sub>	44.5 ± 5.5	1.44
B. Rat CL of superovulation (homogenates) <sup>5</sup>		
None	38.0 ± 0.7	—
LH	187.2 ± 6.4	5.20
Iso	329.6 ± 13.8	9.16
LH + Iso	354.2 ± 11.4	9.84
C. Rabbit CL of pregnancy (membrane particles) <sup>6</sup>		
None	33.7 ± 2.5	—
LH	207.7 ± 10.1	6.25
Epi	124.8 ± 5.0	3.70
PGE <sub>1</sub>	62.2 ± 1.2	1.85
D. Pig Graafian follicles (membrane particles) <sup>7</sup>		
1. "Low" ATP		
None	0.81 ± 0.05	—
LH	5.17 ± 0.20	6.54
Iso	1.07 ± 0.04	1.31
PGE <sub>1</sub>	1.52 ± 0.08	1.88
2. "High" ATP		
None	7.25 ± 1.12	—
LH	39.44 ± 1.76	5.44
Iso	10.73 ± 0.58	1.47
PGE <sub>1</sub>	11.76 ± 0.59	1.63

<sup>1</sup> When present, LH and PGE<sub>1</sub> were 10 μg/ml, isoproterenol (Iso) was 18.5 μg/ml, and epinephrine (Epi) was 21 μg/ml.

<sup>2</sup> Values are mean ± SD of triplicate determinations expressed as pmoles/min/mg protein.

<sup>3</sup> Calculated by dividing the stimulated activities by their corresponding basal activity.

<sup>4</sup> For the preparation of membrane particles (22 μg protein per assay) see *Methods*. Enzyme activities were determined under standard assay conditions (myokinase present, 25 mM BTP-HCl buffer, pH 7.5, 2.7 mM ATP, 5.0 mM MgCl<sub>2</sub>). Incubations were for 10 min at 37 C.

<sup>5</sup> Homogenates were prepared as described under *Methods* and assayed (20 μl, 93 μg protein per assay) in medium containing no myokinase, 2.92 mM ATP, 5.0 mM MgCl<sub>2</sub>, 1.0 mM cAMP, 1.0 mM EDTA, 20 mM creatine phosphate, 0.2 mg/ml creatine kinase, and 25 mM Tris-HCl. Final pH of the assay was 7.0. Incubations were for 10 min at 37 C.

<sup>6</sup> Membrane particles were prepared as described under *Methods* and assayed (19 μg protein per assay) under standard conditions (myokinase present, 25 mM BTP-HCl buffer, pH 7.5, 3.0 mM ATP, 5.0 mM MgCl<sub>2</sub>). Incubations were for 15 min at 37 C.

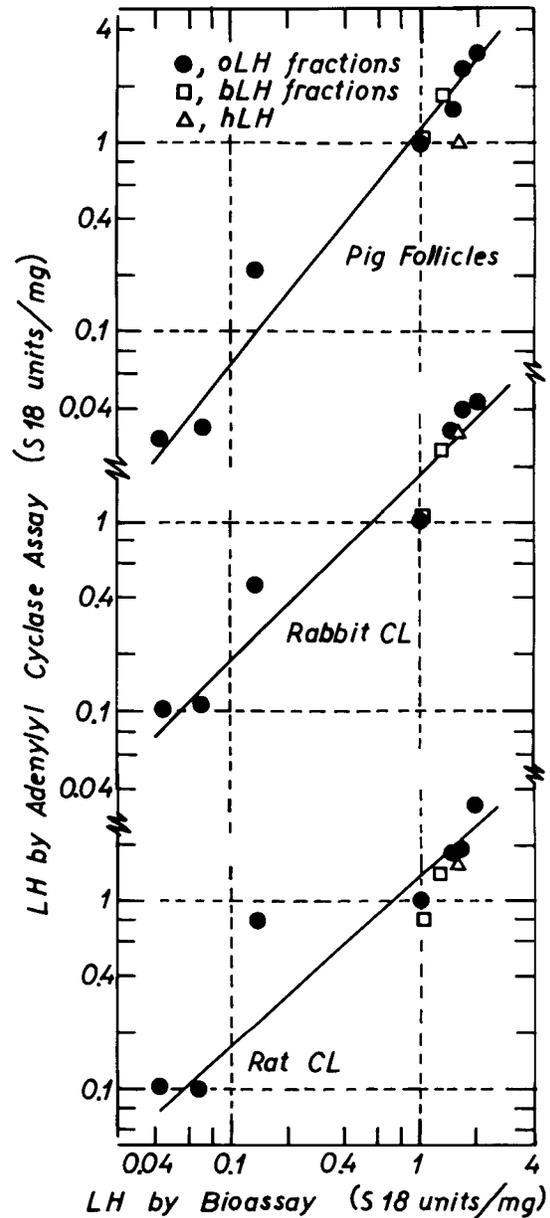


FIG. 11. Relation between potency estimates by adenylyl cyclase activity determination and potency estimates obtained by bioassay (ovarian ascorbic acid depletion test) in rats.

<sup>7</sup> Pig Graafian follicle membranes were prepared as described elsewhere and assayed (14 μg protein per assay) in medium containing 0.11 mM ("low") or 1.36 mM ("high") ATP, 5.0 mM MgCl<sub>2</sub>, 1.0 mM cAMP, 1.0 mM EDTA, 1.0 EGTA, 20 mM creatinine phosphate, 0.2 mg/ml creatine kinase, and 25 mM BTP-HCl buffer, pH 7.0. Incubations were for 5 min at 30 C.

here, in ovarian adenylyl cyclases and, earlier, in the renal AC system, is a general characteristic. Rendell *et al.* (36) recently used mathematical modeling to explore the interplay between catalytic site, regulatory site, and hormone receptor in the glucagon-sensitive AC system of the rat liver. In doing so, they assumed the existence of a single allosteric site for nucleotide interaction, for, in this system, ATP, which would clearly identify a secondary regulatory site, does not appear to play a role distinct from that of GTP. It is conceivable, however that the liver system also has multiple regulatory sites, all specific for GTP, and that each regulates a distinct aspect of the hormonally responsive system. This possibility may be particularly worth entertaining in view of the fact that GTP, and the recently more often employed GMP-P(NH)P analogue, have been found to affect various behavioral aspects of the system, including catalytic activity in the absence of hormone, rate of hormonal activation, and binding of hormone to its putative receptor sites. Clearly, further experimentation in this field will be needed to clarify the complexities involved in hormonal activation of adenylyl cyclases, before a unified model for the regulation can be proposed.

The AC systems of Graafian follicles and of CL, although both responsive to LH, were found to differ from each other in several respects, summarized in Table 4. The differentiation of follicle AC into CL cyclase (triggered by LH or hCG) is a phenomenon with at least two steps, in which the system first becomes desensitized to LH stimulation (see refs. 37 and 8,11), and then "re-acquires" LH responsiveness, but with altered characteristics: the requirement of higher ATP, lowered sensitivity, and a slowed-down desensitization response (10, 11). Since, at the same time, catecholamine responsiveness develops and other membrane functions are either increased or newly induced (such as nucleoside pyrophosphohydrolase), it seems reasonable to assume that one or more of the molecular

TABLE 4. Comparative properties of follicle and CL adenylyl cyclase systems

Variable studied	Follicles	CL
Stimulation by LH (responsiveness)	Large	Large
Sensitivity for LH (affinity)	High	Small
Nucleotide dependence of LH response <sup>1</sup>	Low ATP ( $\mu$ M)	High ATP (mM)
Nucleotide effect mimicked by GTP?	No	No
Stimulation by catecholamines	Small or absent	Large
Nucleoside pyrophosphohydrolase activity in membranes	Low or absent	High
Desensitization by LH (or hCG) <sup>2</sup>	Yes	Yes
Rate of desensitization by LH <sup>2</sup>	Fast	Slow

<sup>1</sup> See also ref. 17.

<sup>2</sup> References 10 and 11.

elements that comprise the CL adenylyl cyclase system are newly induced rather than being carryovers from the follicular stage. Studies of the appearance of these new elements should help our understanding of both corpus luteum formation and plasma membrane differentiation.

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