Stimulation of Ornithine Decarboxylase Activity by Luteinizing Hormone in Immature and Adult Rat Ovaries†

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ABSTRACT: Intraperitoneal injection of 30 μg of luteinizing hormone (LH) into 20- to 22-day-old rats resulted in an increase in the specific activity of ornithine decarboxylase in the 38,000g_{max} supernatant fraction of ovarian extracts. A significant increase was apparent 1 hr after injection; the peak value (a 10–16-fold increase) occurred at 4 hr, and thereafter activity declined but at 8 hr was still significantly above the basal level. Adult rats also responded to exogenous LH by an increase in specific activity of ornithine decarboxylase. This rise was seen at every stage of the estrous cycle, even between 1400 and 1800 hr on the day of proestrus when there is a physiological rise in ornithine decarboxylase activity following the endogenous secretion of LH. The spontaneous cyclic rise in ornithine decarboxylase activity was prevented by injection of pentobarbital at 1400 hr on the day of proestrus. The ability of the ovary to respond to LH injection with increased ornithine decarboxylase activity first appears in the second week after birth and continues to increase at least until day 20. In prepuberal rats (20-day old) the effect of LH on ornithine decarboxylase activity was inhibited by injection of puromycin (180 μg/g), cycloheximide (6 μg/g), or actinomycin D (8 μg/g). These results indicate that the action of LH results in *de novo* synthesis of ornithine decarboxylase, and that enhanced RNA synthesis may be required for this response.

Ornithine decarboxylase (EC 4.1.1.17) catalyzes the conversion of ornithine to putrescine, which in turn is used in the biosynthesis of the polyamines spermidine and spermine. Present knowledge of polyamine biosynthesis and suggestions for a role of polyamines in the stabilization of nucleic acids and the biosynthesis of nucleic acids and proteins have been reviewed in a recent monograph (Cohen, 1971) and in the proceedings of a recent meeting (Herbst and Bachrach, 1970).

Several hormones capable of stimulating growth and cell division cause a rise in the activity of ornithine decarboxylase at an early stage in their action. Thus, growth hormone stimulates putrescine and spermidine synthesis in rat liver (Jänne *et al.*, 1968); epidermal growth factor produces an increase in ornithine decarboxylase activity when added to epidermal cells grown in cell culture (Stastny and Cohen, 1970); testosterone injection into castrated rats increases the specific activity of ornithine decarboxylase in the ventral prostate (Pegg *et al.*, 1970); and injection of 17β-estradiol into ovariectomized (Cohen *et al.*, 1970) or immature (Kaye *et al.*, 1971) rats stimulates ornithine decarboxylase activity in the uterus.

Kobayashi *et al.* (1971) reported that there was an increase in ornithine decarboxylase activity in the ovary associated with the release of luteinizing hormone (LH)[1] from the pituitary during the estrous cycle. These authors as well as Jänne and Williams-Ashman (1971) and Williams-Ashman *et al.* (1972) also found an increase in ornithine decarboxylase activity in the ovaries of LH or gonadotropin treated adult rats. In the present paper, we describe some characteristics of ovarian ornithine decarboxylase and the kinetics of LH stimulation of ornithine decarboxylase activity in immature rat ovaries. In addition, we define the time course of the development of this response during postnatal development and report the responsiveness of the ovaries to LH throughout the estrous cycle as well as evidence that the effect of LH represents induction of the synthesis of enzyme protein.

Materials and Methods

Animals. Rats of the Biodynamics Department colony, derived from Wistar stock, housed in air-conditioned quarters with light from 500 to 1900 hr, had free access to pelleted food (Purina Laboratory Chow, Ralston Purina Co., St. Louis, Mo.,) and water. Immature rats were used at the age of 20–22 days, unless otherwise stated. Their mean weight at 20 days was 33 g. Adult rats were used at different stages of their estrous cycle, when their mean weight was 200 g. Vaginal smears were made daily and only those rats were used which showed at least two regular 4-day cycles immediately preceding the experiment. Ovaries were removed and trimmed while on a cooled Teflon watchglass or in ice-cold saline, under a magnifying glass or binocular microscope.

Biochemicals. DL-[*carboxy*-^{14}C]Ornithine monohydrochloride (4.62 mCi/mmol) was purchased from New England Nuclear Corporation, Boston, Mass. [*methyl*-^{3}H]Methionine (5.4 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, England, and [5-^{3}H]uridine (15 Ci/mmol) from the Nuclear Research Center—Negev, Beer Sheva, Israel. 17β-Estradiol was obtained from Ikapharm, Ramat-Gan, Israel. Luteinizing hormone (NIH LH S-16 ovine and NIH LH-S-17 ovine) was a gift of the Endocrine Section, U. S. National Institutes of Health. Pyridoxal phosphate and Tris were purchased from Sigma Chemical Co., St. Louis, Mo., and putrescine dihydrochloride, spermidine trihydrochloride spermine tetrahydrochloride, and dithiothreitol were from Calbiochem, Lucerne, Switzerland. Actinomycin D, cycloheximide, and puromycin were bought from Nutritional Biochemicals Corporation, Cleveland, Ohio. Pentobarbital

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[1] Abbreviations used are: LH, luteinizing hormone; FSH, follicle stimulating hormone; PGE_2, prostaglandin E_2.

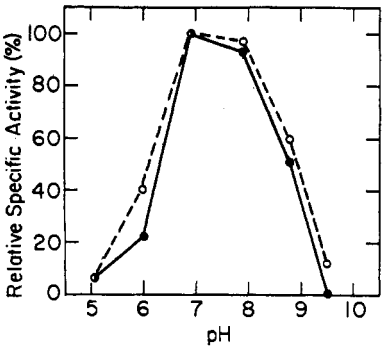

FIGURE 1: Specific activity of ornithine decarboxylase from ovary and uterus as a function of pH. Ovaries were collected from 20 prepuberal rats (24 days old) injected 4 hr previously with LH (30 μg/rat). Uteri were obtained from five prepuberal rats (21 days old) injected 4 hr previously with 17β-estradiol (0.5 μg/rat). Organs were homogenized and the 150,000g_{max} supernatant fraction was used as the enzyme source. The incubation mixture (100 μl total volume) consisted of 100 mM buffer (sodium acetate buffer for pH 5.1; potassium phosphate buffer for pH 6.0; Tris-HCl buffer for pH 6.9, 7.9, and 8.8; sodium borate buffer for pH 9.5), 0.05 mM pyridoxal phosphate, 5 mM dithiothreitol, 0.5 mM (1–3 Ci/mmol) DL-[*carboxy*-^{14}C]ornithine, and 96 μg (ovarian) or 125 μg (uterine) of protein. All pH measurements were done at 37° using a Radiometer Model 27 pH meter. Tubes were incubated at 37° for 1 hr and processed as described under Materials and Methods. Incubations with acid-inactivated enzyme extracts yielded a mean count of 160 cpm (range 80–360). The maximal activity of ovarian enzyme (100% point) was 6800 cpm and of uterine enzyme was 1600 cpm per tube. The data presented are from one of three experiments, all of which showed the same pH optimum: (●–●) uterine extract; (○---○) ovarian extract.

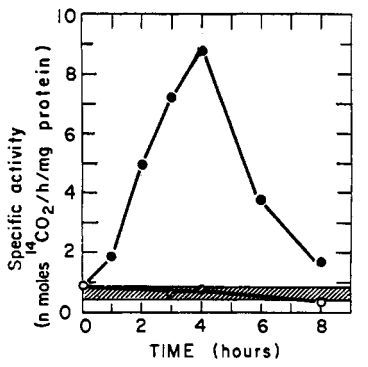

FIGURE 2: Time course of stimulation of ornithine decarboxylase activity by luteinizing hormone. Ovaries from 20- to 22-day old rats that had received a single intraperitoneal injection of LH (30 μg/rat) at the time indicated were homogenized and portions of the 38,000g_{max} supernatant fraction (0.06–0.3 mg of protein) were used for the assay described under Materials and Methods in a total volume of 125 μl. All points represent averages of at least two independent experiments, each done on a pool of ovaries from three rats: (●–●) after LH injction; (○–○) after water injection. The cross-hatched band represents the 95% confidence limits of the mean value for all control rats.

sodium (Nembutal) was obtained from Abbot Ltd., Queensborough, England.

LH was dissolved to appropriate concentrations in double distilled water and injected in a volume of 0.5 ml. 17β-Estradiol was dissolved in ethanol and diluted with water to give solutions containing 1% or less of ethanol. Control animals were given 0.5 ml of water or dilute ethanol solution. All injections were made by the intraperitoneal route.

Extraction and Assay of Ornithine Decarboxylase. Enzyme extracts were prepared essentially as described previously (Kaye *et al.*, 1971). Ovaries were homogenized in a Potter-Elvehjem homogenizer in 5 vol of isotonic sucrose (0.25 M) containing 10 mM Tris-HCl buffer, pH 7.5, 5 mM dithiothreitol, and 0.1 mM disodium EDTA. The homogenate was centrifuged at 38,000g_{max} for 10 min and the supernatant solution assayed for ornithine decarboxylase activity. Centrifugation at 150,000g_{max} for 45 min did not produce a supernatant solution with significantly higher specific activity.

For the standard ornithine decarboxylase assay, the reaction mixture consisted of 50 mM Tris-HCl buffer, pH 7.8, 0.05 mM pyridoxal phosphate, 5 mM dithiothreitol, 0.5 mM DL-ornithine (1–3 mCi/mmol), and an enzyme preparation containing 0.04–1.0 mg of protein. This standard reaction mixture was used in 100–500 μl total volume, depending on the concentration and amount of enzyme extract that was used.

Assays were made at two or more protein concentrations in the range giving linear $^{14}CO_2$ release as a function of both protein concentration and of time. When the assay was carried out under special conditions these are stated under the corresponding figure.

Incubations were carried out at 37° for 60 min in 13 × 57 mm glass centrifuge tubes containing a polypropylene center well suspended from the rubber stopper of a serum bottle (which provided a gas-tight seal). Trapping and counting of $^{14}CO_2$ released was as described previously (Kaye *et al.*, 1971).

Control values for incubations in the absence of enzyme or using acid-inactivated enzyme were subtracted from all experimental values.

For removal of low molecular weight substances, enzyme extracts were passed through a 8 × 1 cm column of Sephadex G-25 equilibrated with the homogenization medium at 4°.

Protein was determined according to Lowry *et al.* (1951) using crystalline lysozyme as a standard.

Calculations of 95% confidence intervals were based on a table of "*t*" values (Fisher and Yates, 1948).

Results

Characteristics of Ovarian Ornithine Decarboxylase. Ornithine decarboxylase activity in the 38,000g_{max} supernatant fraction of rat ovaries has an apparent pH optimum of 7.0 with a plateau extending to pH 8.0 (Figure 1). The corresponding uterine enzyme preparation showed an almost identical pH optimum. We routinely used pH 7.8 in assaying both uterine and ovarian ornithine decarboxylase activities.

After passage of the 38,000g_{max} supernatant fraction through Sephadex G-25, enzyme activity was completely dependent on the presence of pyridoxal phosphate with an apparent K_m of activation of 8×10^{-7} M. Such an enzyme preparation also showed a dependence on the concentration of L-ornithine, with an apparent K_m of 3×10^{-5} M. The presence of an equimolar concentration of D-ornithine had no effect on enzyme activity.

During assay of ovarian ornithine decarboxylase, release of CO_2 with time was linear for up to 90 min; linearity of activity with protein concentration was found up to at least 4.8 mg of protein/ml. The use of 5 mM dithiothreitol in the homogenizing buffer, rather than 0.5 mM as used previously for the uterine enzyme (Kaye *et al.*, 1971), enabled the enzyme preparation to be frozen and stored at −15° for at least 1 week, without loss of activity. A similar increase in the concentration of dithiothreitol in the incubation mixture also resulted in a slight enhancement in activity.

Stimulation of Ornithine Decarboxylase Activity by Luteinizing Hormone. A maximum increase (10- to 16-fold) in

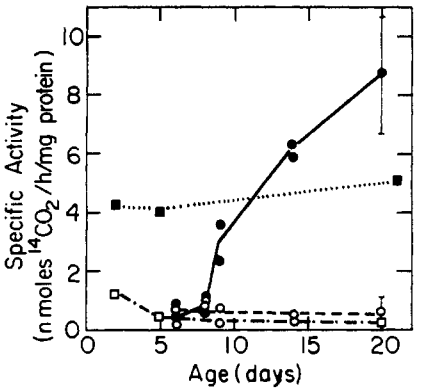

FIGURE 3: Hormonal induction of ornithine decarboxylase as a function of age. Ovaries (at least five pairs per point) collected from rats injected 4 hr previously with LH (30 μg/rat) were homogenized and portions of the 38,000g_{max} supernatant fraction (0.04–0.6 mg of protein) were used for the assay described under Materials and Methods in a total volume of 125 μl. Each point represents the result of a single experiment, except for the 20-day value which is the mean of 13 independent determinations. Uteri were collected from rats injected 4 hr previously with 17β-estradiol (~15 ng/g-wt, *i.e.*, 50–500 ng/rat depending on age) and assayed for ornithine decarboxylase as described under Materials and Methods, using pooled uteri from 15 rats (day 2) to 5 rats (day 21) per analysis: (●–●) enzyme from ovaries of LH-treated rats; (○---○) enzyme from ovaries of water-injected rats; (■···■) enzyme from uteri of 17β-estradiol-treated rats; (□---□) enzyme from uteri of dilute ethanol treated rats. The vertical bracket represents the 95% confidence limits of the mean value.

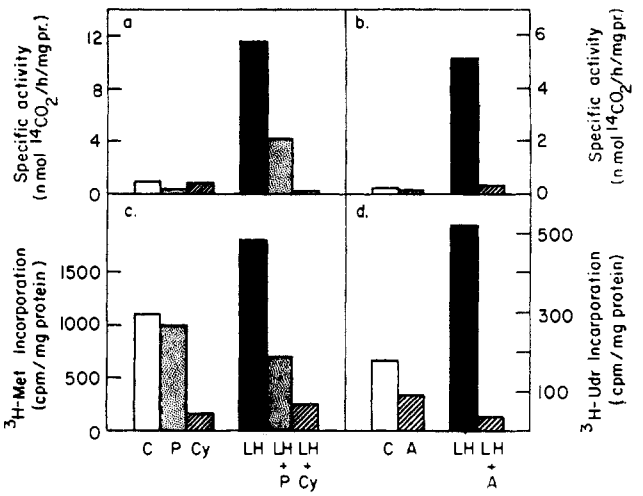

FIGURE 4: Suppression of the LH effect on ornithine decarboxylase by inhibitors of protein and RNA synthesis. Rats were injected 4 hr before killing with either LH or water (controls) and 30 min before killing with either [*methyl*-^{3}H]methionine (1 μCi/g, the cycloheximide experiment; 2 μCi/g, the puromycin experiment) or [^{3}H]uridine (3 μCi/g, actinomycin D experiment). Ovaries were homogenized and protions (0.04–0.2 mg of protein) of the 38,000-g_{max} supernatant fraction were used for the assay of ornithine decarboxylase activity as described under Materials and Methods. Portions of the 38,000g_{max} supernatant fraction (cycloheximide and puromycin experiments) or the total ovarian homogenate (actinomycin D experiment) were precipitated by 5% trichloroacetic acid. The precipitates were collected on Whatman GF/C glass fiber filters, washed, and counted as previously described (Kaye *et al.*, 1971). Values are derived from duplicate determinations using a pool of three–five rats for each point: C, control rats injected with 0.5 ml of water; LH, luteinizing hormone, 30 μg/rat; Cy, cycloheximide (190 μg/rat) injected 4 hr before killing; P, puromycin (6 mg/rat) injected 1 hr before killing; A, actinomycin D (260 μg/rat) injected 4 hr before killing.

ovarian ornithine decarboxylase specific activity was seen 4 hr after a single injection of 30 μg of LH into 20- to 22-day old rats (Figure 2). The activity then rapidly declined from its peak value (see half-life estimates below). At 8 hr the value was indistinguishable from that at 1 hr; both levels were significantly higher than the control mean.

When rats of different ages were tested for their capacity to respond to LH (Figure 3), no response was found before day 9. Thereafter, responsiveness increased with age, with the steepest rise in ornithine decarboxylase activity occurring between days 8 and 10 and a continued rise to day 20.

By contrast, 17β-estradiol stimulated ornithine decarboxylase activity in the uterus severalfold even in rats as young as 2 days old (Figure 3).

Mature rats responded to injection of LH by an increase in ornithine decarboxylase activity at all stages of the estrous cycle. Although control values in immature and cycling rats were comparable, the peak specific activity (nanomoles of CO_2 per hour per milligram of protein) attained after LH injection in the mature animals (4.2) was lower than that in immature rats (8.8).

Ornithine decarboxylase activity in ovaries of untreated rats increased during the afternoon of proestrus from a specific activity of 0.3 at 14 hr to 1.3 at 19 hr. A similar rise was observed by Kobayashi *et al.* (1971) and attributed to the endogenous release of LH. Since pentobarbital given early in the afternoon of proestrus is known to suppress this physiological release of LH (Barraclough *et al.*, 1971; Ayalon *et al.*, 1972), it was used to confirm the conclusion that it is indeed LH which is responsible for the rise of ornithine decarboxylase during the estrous cycle. When pentobarbital was injected at 14 hr, the specific activity of ornithine decarboxylase at 19 hr remained at the control level of 0.3 nmol of CO_2/hr per mg of protein; however, if pentobarbital was injected together with exogenous LH, ornithine decarboxylase increased to a specific activity of 2.3. It is therefore clear that pentobarbital is not interfering with the stimulation of ornithine decarboxylase by LH, but only with its release from the pituitary.

Effects of Inhibitors on the Stimulation of Ornithine Decarboxylase Activity by Luteinizing Hormone. Cycloheximide, which was capable of almost complete suppression of incorporation of [^{3}H]methionine into protein at a dose of 190 μg/rat, blocked the rise in ornithine decarboxylase activity when injected together with LH to immature rats (Figure 4a). In preliminary experiments, when cycloheximide was given at 3, 4, or 5 hr after LH injection, the apparent half-life of ornithine decarboxylase activity was calculated to be from 12 to 21 min (unpublished results).

Puromycin (6 mg/rat) inhibited the rise in [^{3}H]methionine incorporation into ovarian protein caused by LH (Figure 4c). When this dose of puromycin was given 3 hr after administration of LH, *i.e.*, at a time of rapid ornithine decarboxylase synthesis (Figure 2), the activity of this enzyme measured 4 hr after the injection was one-third of the level induced by LH without puromycin treatment (Figure 4a).

Actinomycin D injected at a dose (260 μg/rat) shown previously to inhibit stimulation of uterine ornithine decarboxylase activity (Kaye *et al.*, 1971) inhibited the incorporation of [^{3}H]uridine into ovarian RNA and prevented the stimulation of uridine incorporation by LH (Figure 4d). When this dose of actinomycin D was injected into rats together with LH, there was no increase in ovarian ornithine decarboxylase activity (Figure 4b).

Discussion

Ornithine decarboxylase is a particularly useful protein for the study of the mechanisms of hormone action. It is induced by both protein and steroid hormones. Protein hormones such as LH probably have their primary site of action at the cell surface (Schimmer *et al.*, 1968; Cuatrecasas, 1969; Selinger and Civen, 1971) and interact with adenylyl cyclase to affect cyclic AMP production (Sutherland, 1972). On the other hand, steroid hormones such as estradiol (Cohen *et al.*, 1970; Kaye *et al.*, 1971) and testosterone (Pegg *et al.*, 1970), which also induce ornithine decarboxylase, interact with soluble cytoplasmic and nuclear protein receptors; the receptor steroid complex then interacts with an "acceptor" molecule in the chromatin (see articles in Raspé, 1971). Despite such diverse pathways, both types of hormones stimulate ornithine decarboxylase activity. Moreover, the half-life of rat liver ornithine decarboxylase, approximately 12–20 min (Russell and Snyder, 1969), is the shortest enzyme half-life yet found, and hence makes it likely that this enzyme has a regulatory function.

To date, there is no indication of any significant organ specific differences in ornithine decarboxylase. The comparison of ovarian and uterine enzymes in this study (Figure 1) together with the description of the properties of ornithine decarboxylase in crude (Raina and Jänne, 1968) and purified rat liver preparations (Beck *et al.*, 1972; Ono *et al.*, 1972) and rat ventral prostate preparations (Williams-Ashman *et al.*, 1972) are compatible with the view that various hormones acting on different organs by diverse mechanisms elicit the increased synthesis of the same enzyme protein. Even the time course of induction of ovarian ornithine decarboxylase (Figure 2) is reminiscent of that for uterine (Kaye *et al.*, 1972), testicular (Stastny and Cohen, 1972), and hepatic ornithine decarboxylase (Panko and Kenney, 1971).

There is a close correlation between the acquisition of competence of the ovary to respond to exogenous LH with a rise in ornithine decarboxylase activity (Figure 3) and the age at which the ovary first becomes responsive with respect to cyclic AMP formation and stimulation of protein kinase by exogenous cyclic AMP (Lamprecht *et al.*, 1971, 1973): all three responses are first demonstrable during the second week after birth. During this week the ovary also shows other physiologic responses to gonadotropins (Falck, 1953; Hertz, 1963; Ben-Or, 1970) and at 8–9 days after birth, specific uptake of labeled human chorionic gonadotropin is first observed (Presl *et al.*, 1972).

The induction of ovarian ornithine decarboxylase appears to be a specific response to LH, since administration of FSH or steroid hormones to mature rats was reported to be ineffective (Kobayashi *et al.*, 1971). Furthermore, administration of pentobarbital to the rats in the early afternoon of proestrus, known to block the release of LH from the pituitary gland, prevented the normal cyclic rise in ovarian ornithine decarboxylase activity on the evening of the same day, whereas exogenous LH stimulated this enzyme activity even when given together with pentobarbital.

The studies using cycloheximide and puromycin (Figure 4) provided evidence for the *de novo* synthesis of ornithine decarboxylase following LH administration; the experiments with actinomycin D suggests that RNA synthesis may also be necessary. An increased incorporation of uridine and methionine into ovarian RNA and protein was noted (Figure 4c,d) 4 hr after LH administration (*cf.* Reel and Gorski, 1968a,b). A role for polyamines in the stimulation of RNA and protein synthesis has been suggested (Raina and Jänne, 1971; Takeda, 1969; Takeda and Igarashi, 1970a,b).

Administration of prostaglandin E_2(PGE_2) (Lamprecht *et al.*, 1973) to prepuberal or adult rats also results in enhancement in the activity of ornithine decarboxylase in the ovary. Both LH and PGE_2 (Tsafriri *et al.*, 1972) stimulate ovarian cyclic AMP production. It is possible that cyclic AMP may be involved in the induction of ornithine decarboxylase in the ovary as it is in enzyme induction in bacteria (Pastan and Perlman, 1971). Recently, dibutyryl cyclic AMP was shown to stimulate ornithine decarboxylase activity in rat liver (Beck *et al.*, 1972). However, while 17β-estradiol induces ornithine decarboxylase in the rat uterus (Kaye *et al.*, 1971), this effect is unattended by increased formation of cyclic AMP (Zor *et al.*, 1973).

A first approximation of the half-life of ovarian ornithine decarboxylase was calculated in this study as 10–21 min. A very rapid turnover of the enzyme has previously been observed in rat liver (Russel and Snyder, 1969). The possibility of regulation of ornithine decarboxylase levels by changes in the rate of inactivation or degradation of ornithine decarboxylase caused by hormonal stimuli is thus particularly intriguing in view of the extremely short half-life of the enzyme.

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Human Luteinizing Hormone and Its Subunits. Physical and Chemical Characterization†

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ABSTRACT: Human luteinizing hormone, isolated and purified from human pituitary glands, has been separated into its constituent α and β subunits. The molecular weights of the native hormone and the subunits have been determined by the combined methods of density gradient centrifugation and analytical gel chromatography and compared to molecular weights derived from sedimentation equilibrium experiments. The agreement between the methods is good and furthermore the molecular weights obtained are consistent with the present knowledge of primary structure, amino acid composition, and carbohydrate content of the parent hormone and its subunits. The experimentally determined intrinsic viscosity, sedimentation coefficient, and partial specific volume of the native hormone are used to compute a molecular weight which agrees with those obtained by the other methods. The measured $\bar{V}$ agrees reasonably well with a $\bar{V}$ calculated from chemical composition. The viscometry indicates that the native hormone behaves as a relatively compact equivalent hydrodynamic particle of moderate asymmetry. The ultraviolet absorption spectra of native hormone and subunits have been determined and a difference spectrum derived from them. The difference spectrum is interpreted as indicating possible changes in environment of aromatic side chains when the α and β subunits combine to form the native hormone.

In the past few years, rapid progress has been made in elucidating the properties and primary structure of ovine and bovine LH[1] (Liu *et al.*, 1970, 1971; Papkoff *et al.*, 1971; Pierce *et al.*, 1971; Maghuin-Rogister *et al.*, 1971) and the related hormone, human chorionic gonadotropin (HCG) (Bahl *et al.*, 1972; Morgan and Canfield, 1972). All of these preparations consist of two nonidentical subunits, which are associated by noncovalent bonds.

Human LH has been shown to consist of noncovalently linked subunits (Ryan, 1968, 1969) and more recently these subunits have been separated and shown to differ in their amino acid and carbohydrate compositions (Hartree *et al.*, 1971; Closset *et al.*, 1972; Saxena and Rathman, 1971). The amino acid sequence of the α subunit has now been reported by Sairam *et al.* (1972) and the β subunit sequence by Closset *et al.* (1973) and by Shome and Parlow (1973). There has not been, however, a comprehensive study of the hydrodynamic properties of human LH and its subunits and an effort to compare them with chemical composition. This report is intended to provide some of these data.

At the present time, nothing is known concerning the mutual interactions responsible for the crucial fact that the biological activities of the intact native pituitary glycoprotein hormones are completely obliterated when the subunits are separated. With the further finding that one subunit (approximately one-half the mass of the native molecule) is constant or nearly so in primary structure through the series TSH, FSH, and LH, it appears that interesting information of a general sort might be gained concerning the various modes of noncovalent subunit–

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[1] Abbreviations used are: LH, luteinizing hormone; HCG, human chorionic gonadotropin; TSH, thyroid-stimulating hormone; FSH, follicle-stimulating hormone; CMC, carboxymethylcellulose.