# THE CHEMISTRY OF GONADOTROPINS

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Following a brief historical review of the chemistry of gonadotropins leading up to the recent exciting discovery of the subunit nature of these hormones, it is pointed out that despite these impressive advances, the true identity of the physiologically active gonadotropins is yet to be established. Studies carried out by the present authors on PI-2 — an ovine pituitary glycoprotein with dual gonadotropic activity — are described. On the basis of extensive data, it is concluded that this glycoprotein is a functional complex of follicle-stimulating and luteinizing hormones. The results are viewed in the perspective of other observations and discussions in the literature bearing on the possibility that the physiologically active hormonal entities are indeed complexes of the two gonadotropins.

## INTRODUCTION

Gonadotropins have been the subject of investigation for well over four decades. While a relationship between the pituitary gland and the gonads had been discussed as early as 1909 by Crowe *et al.*, definite evidence for the existence of hypophyseal gonadotropic principles was presented by Smith (1930) and Smith and Engle (1927), eighteen years later. On the basis of their studies, Aschheim and Zondek (1927) postulated that the pituitary elaborates had two gonadotropic hormones, which later came to be referred to as the follicle-stimulating hormone (FSH) and the luteinizing or interstitial cell-stimulating hormone (LH/ICSH) (Li and Evans 1948). Evidence supporting such a concept was furnished by Fevold, Hisaw and Leonard (1931), who demonstrated that these two activities could be fractionated from crude extracts of ovine pituitaries by relatively simple means. On the other hand, scepticism as to the existence of two distinct gonadotropins was expressed even at this early date by Smith and Engle (1927). They raised the question whether a single hormone could not elicit different responses depending on the dosage used.

It is not intended here to recount in detail the growth of knowledge of the chemistry of pituitary as well as urinary gonadotropins. Several reviews have assessed the 'state of the art' at intervals (Li and Evans 1948; Evans and Simpson 1950; Hays and Steelman 1955; Segaloff and Steelman 1959; Steelman and Segaloff 1959; Albert 1961; Dixon 1964; Geschwind, 1964, 1969; Wolstenholme and Knight 1965; Hellema 1971; Canfield *et al.* 1971). In the present survey, only the major advances will be delineated, primarily with the object of providing a perspective for the evaluation of the investigations of the present authors.

## HISTORICAL REVIEW

In the decade between 1940 and 1950, the major concern of investigators was to work out procedures for the efficient extraction and fractionation of gonadotropins.

Amongst the significant contributions were : (i) the observation of Li and co-workers that FSH is the only pituitary hormone soluble in 50 per cent saturated ammonium sulphate (Li and Evans 1946), (ii) the use by McShan, Kagawa and Meyer (1954) of saturated NaCl and heating to 70°C to dissociate and preferentially solubilize gonadotropic activity from acetone-dried pituitary powder, and (iii) the introduction by Koenig and King (1950) of an acetone-ethanol solvent system for a highly selective extraction of ovine gonadotropins that was to prove popular in the extraction of gonadotropins from the pituitaries of other species as well.

These developments, together with the introduction of ion-exchange celluloses (Peterson and Sober 1956) as well as dextran gels (Porath and Flodin 1959) for the chromatographic separation of proteins, resulted in another spurt of activity. By the early 1960s, homogeneous preparations of pituitary gonadotropins from several species, including man, as well as of urinary gonadotropins, had been isolated and subjected to evaluation by physico-chemical techniques. Many of these were adjudged sufficiently homogeneous to warrant analysis for amino acid and carbohydrate composition and for the initiation of structural studies. At the time of the 'Fifth International Symposium on Comparative Endocrinology', Geschwind (1969) was able to give details of molecular weight, iso-electric point and chemical composition for some gonadotropins of pituitary origin. Further work in several laboratories (Rosemberg 1968; Butt 1969; Butt, Crooke and Ryle 1970) added more definitive details to the physico-chemical and compositonal characteristics of pituitary as well as urinary gonadotropins.

Advances up to this stage may be summarized as follows : (i) A broad consensus with respect to the size of the hormone molecules was reached. While initial estimates of the molecular weight ranged from 100,000 for a porcine LH preparation to 26,000 for human LH, it is currently held that they have molecular weights of the order of 30,000, (ii) The carbohydrate moiety of all the glycoprotein hormones is made up of sialic acid, L-fucose, D-galactose, D-mannose, *n*-acetyl-D-glucosamine and *n*-acetyl-D-galactosamine. The proportion of an individual monosaccharide is variable not only between the different hormones but also in relation to the animal species. Compared to HCG, human FSH, LH and TSH have much less sialic acid and galactose. Bovine and ovine LH have even less of the monosaccharides than their human counterparts, (iii) These hormones have a high content of proline and half-cystine and none has any free sulphhydryl groups. In terms of amino acid composition, HCG is much closer to human LH than to FSH or TSH. While the amino acid compositon of FSH, LH, TSH and HCG show striking differences, their relative proportions follow a similar pattern.

## Recent Advances

An important development was the observation of Li and Starman (1964) that under acidic conditions, ovine LH behaves as a monomer with a molecular weight of 16,000. These workers suggested that the hormone may consist of two non-identical subunits. Papkoff and Samy (1967) working in Li's laboratory, furnished proof by effecting a separation of the subunits using a counter-current distribution procedure followed by gel filtration on Sephadex G-100. Other investigators quickly

followed up this lead and have documented the existence of two dissimilar subunits, α and β, in human and ovine FSH; human, ovine and bovine LH, HCG (Saxena, Beling and Gandy 1972); and, finally, human and bovine TSH (Pierce *et al.* 1971).

The consequences of this exciting breakthrough may well be imagined. Structural studies which had been initiated on the native, dimeric molecules were rapidly extended to include the subunits. Detailed comparisons of the amino acid and carbohydrate composition, as well as the primary structure of the subunits have already furnished a wealth of information. It is of interest that there is a considerable degree of similarity between the α–subunits of ovine, bovine and human LH, as well as HCG. Similar is the case for the β-subunits. On the other hand, the two subunits of anyone hormone are strikingly different. For instance, while α-HCG is rich in lysine, histidine, glutamic acid, half-cystine, methionine, tyrosine and phenylalanine, the β-subunit has higher amounts of arginine, proline and isoleucine. Similar differences extend also to the carbohydrate composition of the subunits. In the words of Sairam *et al.* (1972) : "... the elucidation of the primary structures of ovine ICSH, bovine TSH, and the realization of the subunit nature of other glycoprotein hormones (FSH, HCG) have opened new avenues for important investigations on the biosynthesis, mode of action, validity of immunoassay, and structure-function relationships of these glycoprotein hormones."

## The Problem in Perspective

Exciting as these new developments are, there are other aspects relating to the nature and identity of the gonadotropins that have remained largely unexplored. One of them was formulated several years ago by Meyer in the following terms : "I would like to ask....if the present day methods do not yield a *group of pure proteins** with gonadotropic activity. I think the implication is very important here in terms of what really constitutes the FSH or LH protein of the *pituitary gland.* In our laboratory, Dr. McShan specifically working on isolation, and more recently studying other fractions which are not satisfactory as pure FSH or LH, proteins, finds proteins having both activities, one or two with primarily LH and others with FSH activity, based on assays using follicular growth and ventral prostrate growth in hypophysectomized rats. So we are concerning ourselves with the isolation of one protein with certain characteristics, but what happens to the other fractions with their activities, and what is the FSH or the LH hormone of the *pituitary gland* in physico-chemical terms ?" (Cole 1964, p. 37)

The investigations in this laboratory, which were initiated some years ago, have been based on the hypothesis that more than one molecular species of FSH or LH may exist in a crude extract of the pituitary and that they may differ not only with respect to their chemical structure, but also in relation to their characteristic biological activities. We were aware of reports on microheterogeneity amongst glycoproteins (Gibbons 1966), the basis for which is presumed to be variations in the carbohydrate moiety in the absence of hererogeneity in the peptide part of the molecule. However, we anticipated a more fundamental correlation between structure and biological

*Italics in this and other quotations have been placed by the present authors.

activity, particularly with fractions showing marked overlap in FSH and LH activities.

In view of this, it was felt that a complete 'accounting' of the gonadotropic activity of a pituitary extract, in terms of the constituent glycoprotein components that could be isolated from it, would enable a better understanding of the nature of gonadotropins. In what follows, the results, to date, of such a study using extracts of ovine pituitaries obtained by a modified Koenig–King (1950) procedure will be described. An important early decision was the use of disc gel electrophoresis to monitor the course of fractionation.

*Isolation of an ovine pituitary glycoprotein with dual gonadotropic activity*

It was demonstrated earlier that gel chromatography on Sephadex G–100 could be used as a convenient means of analysing lyophilized Koenig-King extracts of ovine pituitaries (Bullemma and Duraiswami 1969). Following gel filtration, biological activity was recovered in the major protein peak (Pl–2, $K_d$=0.25) and in another fraction (Pl–1, $K_d$=0.07) that preceded Pl–2 either as a shoulder or as a distinct peak. Disc electrophoresis at *p*H 8.6 indicated that while the original extract as well as Pl–1 are polydisperse, Pl–2 is homogeneous (Sreemathi *et al.* 1971).

The following observations strongly suggest that Pl–2 is indeed monodisperse: (i) When this material was repeatedly chromatographed on Sephadex G–100, using 0.2M KCl, 0.05M NaCl or 0.05M phosphate buffer (*p*H 7.4), a symmetrical peak with a $K_d$ value of 0.25 was consistently obtained (Sreemathi *et al.* 1971); (ii) Attempts to resolve Pl–2 further by chromatography on Biogel P–60, in the expectation that the aromatic-retarding characteristics of the polyacrylamide might be more effective than the hydrophilic Sephadex, led to no fractionation. Here again, a single, symmetrical peak with a characteristic elution volume was obtained (Sreemathi *et al.* 1971); (iii) Whether examined before or after rechromatography on G–100, Pl–2 yielded but a single stained band following disc-gel electrophoresis. Moreover, the picture was not altered by overloading the gel with the sample (Plate XXV, Figs. A 4, A 5); (iv) When Pl–2 was subjected to disc electrophoresis using gels of different porosities—$R_{0.5}$=1.64, 1.45 and 1.28 respectively (Gordon 1969)—it failed to be resolved into more than one component (Plate XXVI, Fig. A); (v) Similarly, only one stained band was obtained in all cases when samples representing different fractions of Pl–2, following rechromatography on G–100, were examined by disc- and starch-gel electro phoresis (Plate XXVI, Figs. B, C); (vi) The material exhibited typical monodisperse behaviour in the analytical ultracentrifuge (Sreemathi *et al.* 1971 and plate XXV, Fig, C); (vii) Gel electro-focusing in the *p*H range of 4–6 yielded a single, sharply stained band (Plate I Fig. B), (viii) In an immuno-chemical study, hyper-immune rabbit antiserum raised against the original Koenig–King extract was tested against Pl–2, Pl–1 and the crude extract, using both the Ouchteriony double diffusion as well as the immuno-electrophoretic techniques. Pl–2 alone gave a single precipitin line (Sreemathiand Duraiswami 1972).

The physico-chemical parameters of Pl–2 are summarized in Table I. The relation between protein content as determined by several methods and the total solids is presented in Table II. Information on the carbohydrate composition is included in Table VII.

TABLE I

*Physico-chemical parameters of Pl-2*

| M | $S^{\circ}_{20W}$ | $D^{\circ}$ (XI0$^{7}$ cm) | Stokes radius (Å) | |
|---|---|---|---|---|
| | | | A | B |
| 63,000 | 4.13 | 6.414 | 34 | 30±1 |

A, from sedimentation data
B, estimated from $K_d$ value on G–100 (Sreemathi 1971)

TABLE II

*Determination of protein content of Pl-2 by different methods* (Sreemathi 1971)

| Dry weight mg/ml | Protein content (mg/ml) | | | | |
|---|---|---|---|---|---|
| | Biuret method | Lowry | Kalckar | Warburg | Roos |
| 4.0±0.2 | 4.0±0.1 | 4.8±0.1 | 2.3±0.1 | 2.1±0.1 | 2.3±0.1 |

TABLE III

*Assay of LH potency by Parlow method*

| Assay Number | Relative potency in terms of NIH–LH–$S_{14}$ | 95% Limits | Lambda | Design |
|---|---|---|---|---|
| 1 | 1.338* | 1.032–1.685 | 0.220 | 2×3 |
| 2 | 1.289** | 0.950–1.689 | 0.142 | 2×2 |
| 3 | 1.971* | 1.476–2.493 | 0.169 | 2×2 |
| 4 | 1.597* | 1.178–2.116 | 0.271 | 3×2 |
| 5 | 1.744** | 1.322–2.136 | 0.264 | 2×2 |
| 6 | 1.666* | 1.316–1.110 | 0.197 | 2×2 |
| 7 | 1.000** | 0.782–1.319 | 0.194 | 2×2 |
| 8 | 1.103*** | 0.801–1.514 | 0.149 | 2×2 |
| 9 | 1.618† | 1.352–1.894 | 0.170 | 2×2 |

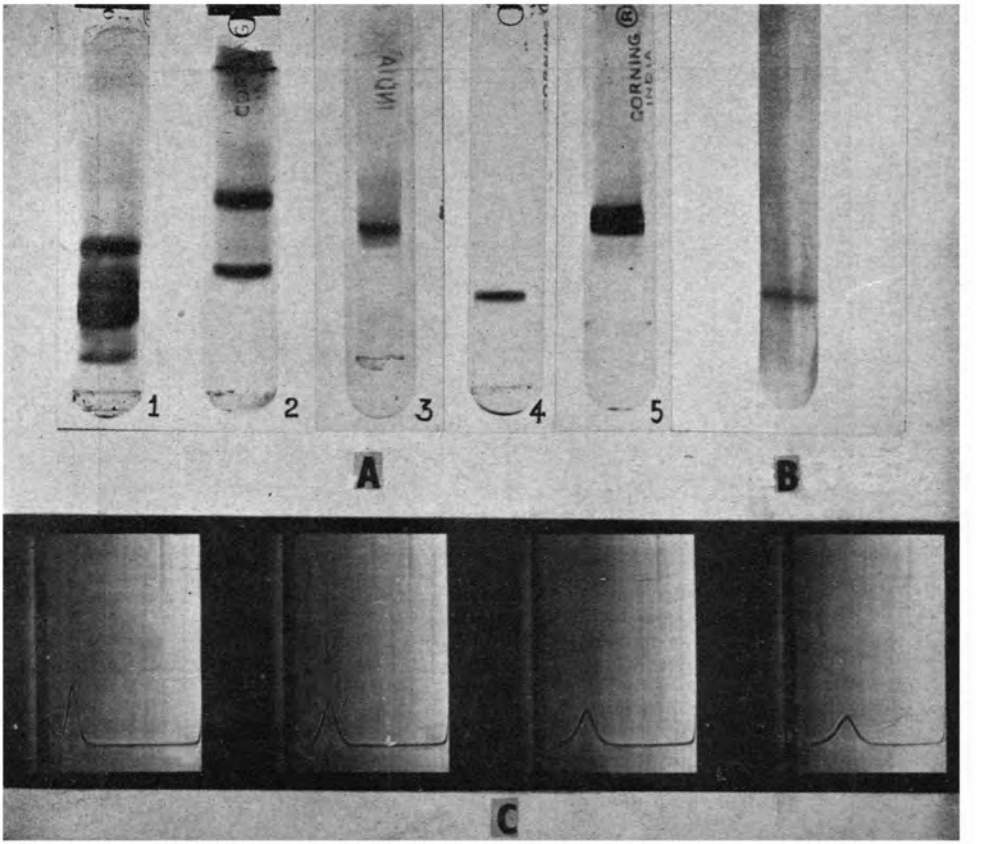
Mean Relative Potency=1.482 (in terms of NIH–LH–$S_{14}$. U/mg dry weight)

*The sample for assay was obtained by pooling individual fractions corresponding to Pl-2 following gel filtration of the crude extract.

**The sample for assay was obtained by pooling fractions of Pl–2 following rechromatography on G-100.

***Pl–2 sample assayed was lyophilized following rechromatography.

†Sample assayed was lyophilized and dissolved in phosphate buffer (*p*H 7.4) and stored in deep freeze for two months.




FIGS. A–C. A, disc electrophoretic patterns of crude extract and fractions. 7.5 per cent gels, 5.5 cm in length were used (*p*H 8.6). In all cases, the direction of migration of proteins is from top to bottom (towards anode). 1, crude extract ; 2, Pl–1 ; 3, Pl–2 ; 4 and 5, Pl–2 following rechromatography (4 was loaded with 15 μg, and 5 with 80 μg); B, iso-electric focusing of Pl–2 on polyacrylamide gel. material : 150 μg of Pl–2 following rechromatography on G–100. 7.5 per cent gels, 18 cm in length were used. Ampholine range : *p*H 4-6. Electrofocusing was for 12 hr at 4°C at 240 volts. C, sedimentation pattern of Pl–2 at a concentration of 0.8 per cent (w/v) in 0.05 μ, *p*H 7.4 phosphate buffer at 20°C and 56,000 rpm. Photographs (left to right) taken at a bar angle of 70° at 16 min intervals.

In terms of biological acitivity, Pl–2 behaved both as FSH and LH, as shown earlier (Sreemathi *et al.* 1971). Additional data on its LH potency are given in Table III. Table IV summarizes a comparison of Pl–2 and NIH–LH–$S_{14}$ as '*ovulating hormone*'. By this last criterion, the material appears to be twice as active as the standard LH preparation. In terms of the OAAD assay, it has 1.5 times the activity of the standard LH. Its FSH potency, however, is only 40 per cent of the ovine FSH standard.

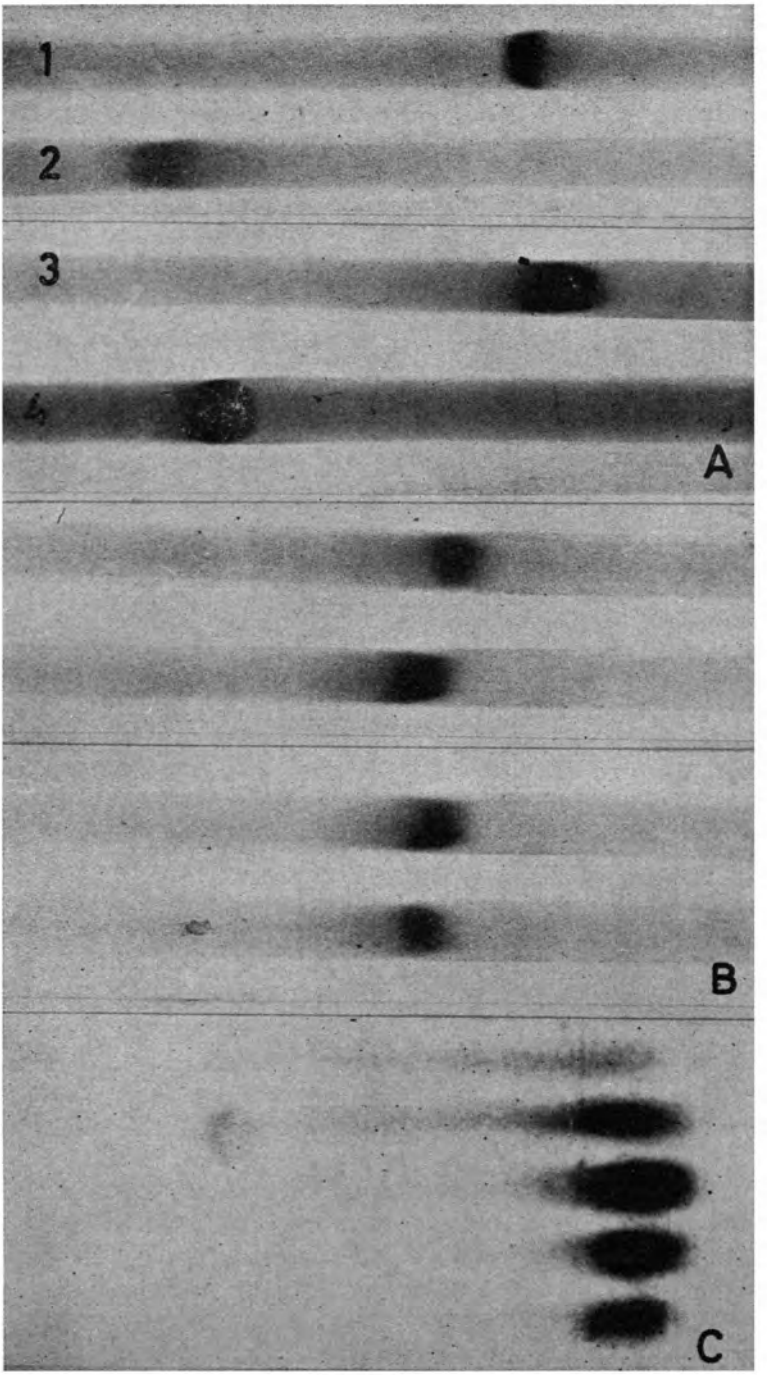

1
2
3
4
A
B
C

TABLE IV

*Ovulation-inducing ability of PI–2 preparation*

| Preparation | Dose ($\mu$g) | Average No. of ova per rat ± S.E. | % of rats ovulating |
|---|---|---|---|
| NIH–LH–$S_{14}$ | 1.0 | 0.0 | 0.0 |
| | 2.0 | 0.2 ± 0.1 | 25.0 |
| | 4.0 | 21.2 ± 6.6 | 87.5 |
| PI–2 | 1.0 | 2.2 ± 1.1 | 50.0 |
| | 2.0 | 26.6 ± 15.3 | 87.5 |
| | 4.0 | 63.6 ± 18.5 | 87.5 |
| Saline | - | 0.0 | 0.0 |

*Note* : 24-day-old female rats (Holtzman strain) were injected, subcutaneously with 15 IU of PMS at 7.30 AM. They were given 7.5 mg phenobarbital each (subcutaneous) on day 26 at 1.00 PM NIH–LH or PI-2 was injected at 3.00 PM on the same day (i.e., day 26) intraperitoneally. Autopsy was done on day 27, and the ova contained in the oviducts were counted.

(15 IU of PMS alone does not cause ovulation).

## SIGNIFICANCE OF THE DATA

Here, then, is an unusual pituitary gonadotropin molecule. It exhibits biological activities that would characterize it both as FSH and LH. Nevertheless, it is monodisperse when examined by several criteria. In terms of its molecular weight, and carbohydrate composition also, it represents a departure from what has been reported so far in the literature. There have been very few reports bearing on the possibility that a monodisperse pituitary glycoprotein may yet possess both FSH and LH activities. Duraiswami, McShan and Meyer (1964) interpreted their findings on a purified preparation of ovine FSH on the basis that the same protein which was active as FSH also had 'intrinsic' LH activity. A similar conclusion was drawn by Papkoff (1965) with respect to one of his FSH preparations. Unfortunately, these intriguing findings were not pursued further. Reichert (1971) recently reported that human FSH prepared by iso-electric focusing in a *p*H gradient of 3–6 had significant LH activity. Since human pituitary LH was found not to be iso-electric at *p*H values lower than 6, the possibility of contamination appears to be ruled out. As one of the

FIGS. A–C. A, effect of varying acrylamide concentration on the disc electrophoretic pattern of PI–2. Material : PI–2f ollowing rechromatography on G–100 (150 $\mu$g). Length of gel : 21 cm (Buchler apparatus). Electrophoresis performed at a *p*H of 8.6 for 18 hr at 180 volts at 4°C. Migration from left to right (towards anode). Percentage of acrylamide in the gels : 1—7.5; 3—11.25; 2 and 4—15.0; B, disc electrophoresis of fractions of PI–2. Material (top to bottom) : samples from tubes 48, 52, 58 and 62 (*cf.* Fig. 2). Percentage of acrylamide used — 11.25 ; other conditions as in Fig. A. C, starch-gel electrop horesis of fractions of PI–2. Material (top to bottom) : as in Fig. B. Migration from left to right (towards anode). Run at *p*H 8.6 for 18 hr at 200 volts at 4°C.

several alternative explanations, he has considered it possible that this molecular species may represent a complex of FSH and LH.

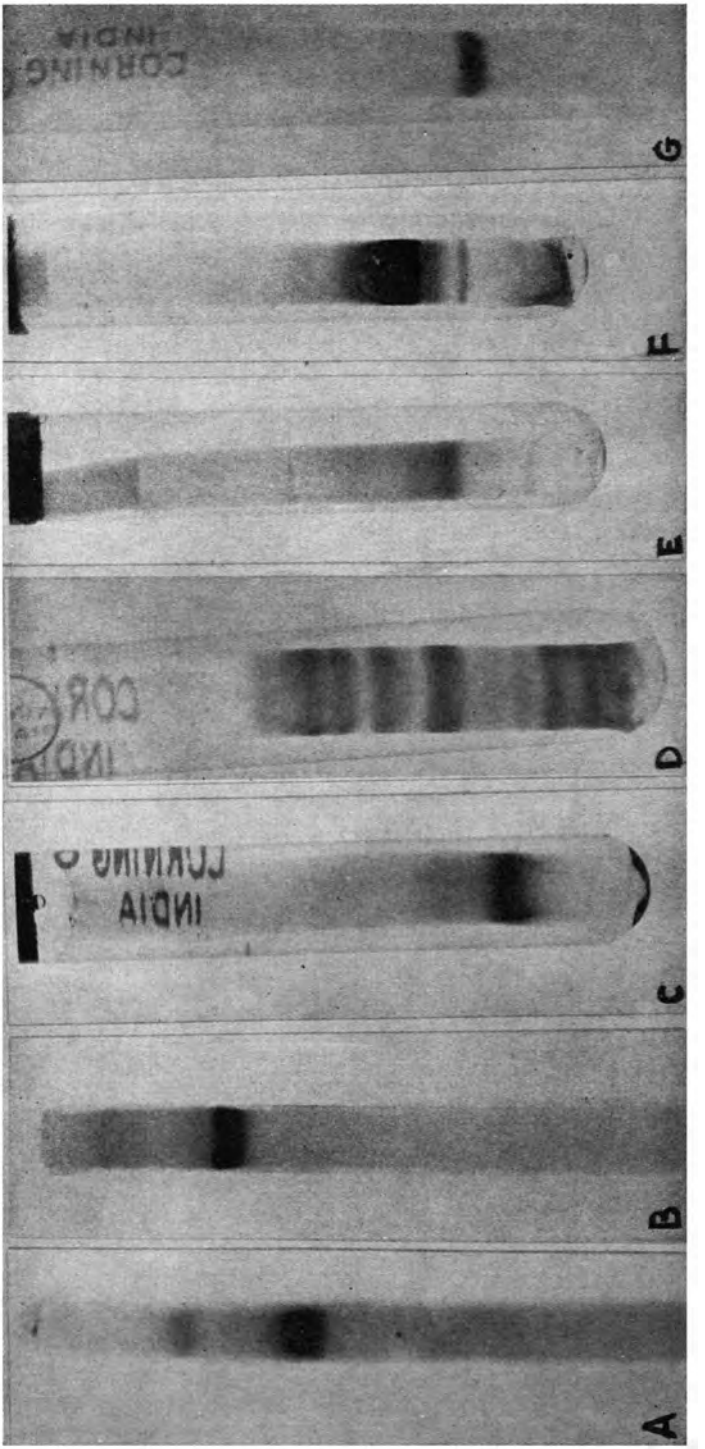
The idea that the pituitary may actually put out a complex of FSH and LH does not appear to be far-fetched. In a thoughtful review, Nalbandov stated: "Thus, in my view atleast, the independent *physiological* existence of FSH and LH has never been clearly established. Basically, then, I am raising the question whether it is biologically meaningful to study the secretion rates or the effects of injections of these two hormones separately. When one studies the intact male or female of any species, one quickly realizes that in neither sex is one of the two gonadotropins ever secreted without being accompanied by the other. It is impossible to cause spermatogenesis in either mammalian or avian males with FSH alone even though that hormone is usually stated to be the primary trophic hormone for seminiferous tubules. Spermatogenesis is readily induced if LH is added, probably because androgen is essential for this process. Similarly, both FSH and LH must be present if the ovarian follicle is to mature, to secrete estrogen, and to ovulate. Next, it should be pointed out that to my knowledge nobody has yet produced an FSH preparation which is totally free from some LH effects which further suggest that the pituitary gland does not secrete LH and FSH separately but secretes an *FSH–LH complex.*"

In a recent study of human hypophyseal gonadotropin-secreting cells, using immuno-histological techniques, Phifer, Midgley and Spicer (1972, 1973) found that the same cells of the anterior pituitary reacted to both FSH and LH antisera indicating that, in this species at least, FSH and LH are produced in the same adenohypophyseal cell. This appears to be true in the case of the rat pituitary also (Phifer Midgley and Spicer 1972, 1973). These observations lend further credence to the postulate by advanced Nalbandov.

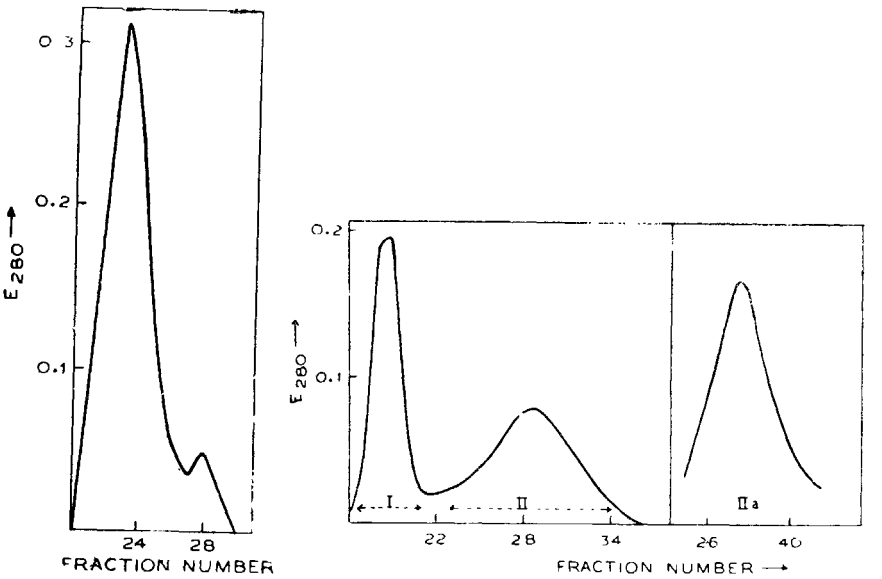
In the light of the preceding considerations, it would appear that Pl–2 is best regarded as a functional complex of the two gonadotropic activities, rather than as a unique molecular species of LH with intrinsic FSH activity. Such a point of view would also be tenable with the observed molecular weight and carbohydrate composition of the material.

## Further Studies on the Nature of Pl–2

If the glycoprotein isolated by us is indeed an FSH–LH complex showing monodisperse behaviour, it should be possible to dissociate it and recover the two gonadotropins as independent, homogeneous entities. In analogy with the experience of Li and Starman (1964) with ovine LH, Pl–2 was observed to dissociate when exposed to low *p*H (Plate XXVII Fig A). Attempts to dissociate and separate the 'subunits' using the counter-current distribution procedure of Papkoff and Samy (1967) were infructuous. Analysis by disc-gel electrophoresis (Plate XXVII, Fig. B) as well as gel chromatography on Sephadex G–100 (Fig. 1) indicated that incubation in 8 M Urea was not entirely successful in dissociating the protein. Treatment with β–mercaptoethanol in the presence of 8 M urea did not yield better results, nor did incubation with 6 M Guanidine-HCl. (Sreemathi and Duraiswami, unpublished observations).




FIGS. A–G. Disc electrophoretic patterns of : A, Pl–2 at *p*H 4.4; B, Pl–2 incubated in 8M urea for 24 hr and electrophoresed at *p*H 8.6 in 8M urea ; C, G II ; D, G III ; E, ASF–LH ; F, ASF–FSH; and G, reconstituted Pl–2 respectively run in the usual manner at *p*H 8.6. [In all cases, except A, migration was towards the anode. Samples in A and B were run on gels (15 per cent acrylamide) 21 cm long (Buchler apparatus). The others were run on gels (7.5 per cent acrylamide) 5.5 cm long (Canalco apparatus)].




FIGS. 1–2. 1, Gel filtration of urea-treated Pl-2 on Sephadex G-100. Height of column — 35 × 2.5 cm. 2, Gel filtration on Sephadex G-100 of Pl-2 treated with 0.35 per cent TCA. Eluant 0.35 per cent TCA ; II a, Rechromatography of II on a NaCl (0.05M) equilibrated column of G-100.

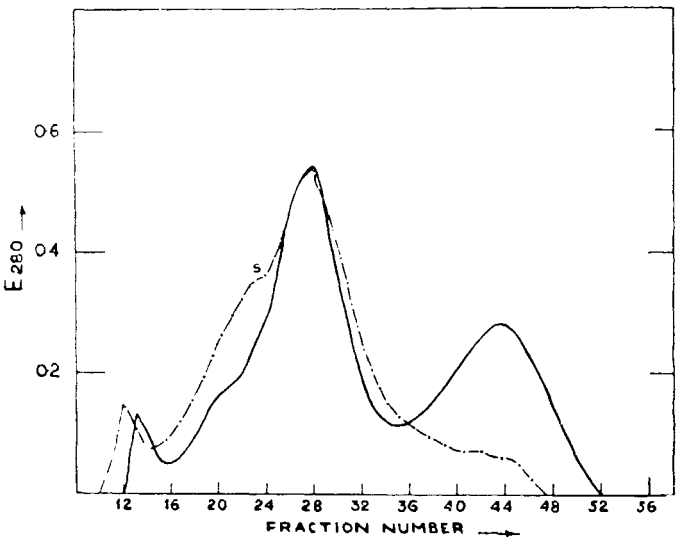



FIG. 3. Gel filtration of Pl-2 on G-100: —·—· Elution pattern of Pl-2 treated with 0.05M glycine buffer *p*H 2.2 (Column equilibrated with the same buffer), —·—· Recovery of reconstituted Pl-2. G II and G III were incubated in and chromatographed with 0.05M phosphate buffer (*p*H 7.7). The same results were obtained when 0.05M glycine buffer (*p*H 8.6) was substituted for the phosphate buffer. The shoulder marked S ($K_d$ = 0.25) represents reassociated material.

TABLE V

*HCG—Augmentation assay*

| Preparation | Dose ($\mu$g) | Mean response (ovary wt (mg)±S.E. |
|---|---|---|
| HCG-injected control | 50 I.U | 37.3±2.5 |
| Pl-2 | 140 | 55.6±1.8 |
| Pl-2 | 420 | 109.7±0.9 |
| G-II | 420 | 51.5±2.2 |
| | 1260 | 94.1±3.2 |
| G-III | 420 | 41.3±1.8 |
| | 1260 | 38.3±2.5 |

TABLE VI

*Ovarian ascorbic acid depletion assay*

| Preparation | Dose ($\mu$g) | Mean response (AA mg/100 mg ovary±S.E.) |
|---|---|---|
| Saline control | - | 80.4±3.6 |
| Pl-2 | 1.0 | 59.7±2.3 |
| Pl-2 | 4.0 | 29.2±2.8 |
| G II | 1.0 | 66.1±3.0 |
| | 4.0 | 36.8±2.8 |
| G III | 1.0 | 62.0±2.2 |
| | 4.0 | 65.2±3.1 |

Incubation with dilute trichloroacetic acid (0.35 per cent w/v, *p*H 2.8) had the effect of converting Pl-2 ($K_d$=0.25) into a 'polymer' ($K_d$=0.07) and a fraction of smaller size ($K_d$=0.35) (Fig. 2). Attempts to characterize the latter fraction were frustrated because any change in the *p*H towards the alkaline range resulted in reassociation of the components to regenerate Pl-2 (Fig. 2). As illustrated in Fig. 3, incubation of Pl-2 in 0.05 M glycine buffer (*p*H 2.3). followed by gel filtration on G-100, yielded two major peaks, G II ($K_d$=0.54) and G III ($K_d$=1.06). The material in these peaks could be recovered in stable form following nuetralization of the solutions to *p*H 7.5. Analysis by disc-gel electrophoresis (Plate XXVII Fig. C, D) showed that while G II consists of a major and a minor component, G III is made up of several components. On the basis of the data summarized in Tables V and VI, it is clear that G III is devoid of biological activity. While both FSH and LH activities were found to be associated with G II, recovery of biological activity has been disproportionate in that FSH potency has decrased by 50 per cent, whereas LH activity is comparable

to that of Pl-2. It is remarkable that, even though removal of some proteins in the G III fraction actually constitutes a purification step, the biological potency of G II is not greater than that of Pl-2. The carbohydrate composition of G II and G III has been compared with that of Pl-2 in Table VII. Attempts to reconstitute Pl-2 by reassociation of G II and G III at an alkaline *p*H were only partially successful (Fig. 3). The criterion for reassociation was the recovery of a component with a $K_d$ of 0.25 in samples subjected to gel chromatography on G-100.

TABLE VII

*Carbohydrate composition of Pl-2 and 'Subunits'*

| Constituent | Pl-2 | G II | G III |
|---|---|---|---|
| Neutral hexoses | 22.0* | 24.3 | 14.9 |
| Sialic acid | 3.0 | 3.9 | 0.6 |

*All values are expressed as μg/mg total solids

A characteristic property of FSH that sets it apart from all other pituitary hormones is its solubility in 50 per cent saturated ammonium sulphate solution (Li and Evans 1948). In the next series of experiments, dissociation of Pl-2 was attempted on the basis of this observation. An aqueous solution of Pl-2 was brought to 50 per cent saturation with ammonium sulphate and the resultant precipitate (ASF-LH) was recovered by centrifugation. The supernatant was brought to 90 per cent saturation to yield another precipitate (ASF-FSH). The two fractions were dissolved in 0.05M glycine buffer (*p*H) 8.6 and dialyzed against the same buffer to remove the salt. Samples were then subjected to gel chromatography on G-100 (Fig. 4) and to disc-gel electrophoresis at *p*H 8.6 (Plate XXVII, Figs. E, F). From the electropherograms, it is apparent that both ASF-LH and ASF-FSH are heterogeneous. Gel filtration does not reveal this heterogeneity. However, it is of interest that ASF-FSH is retarded to a significantly greater extent than ASF-LH on the column (Fig. 4). Also presented in the same figure is the typical result of attempts to reconstitute the original material (Pl-2) by dialyzing ASF-LH and ASF-FSH together in glycine buffer (*p*H 8.6). Judging by the fact that virtually all of the samples loaded on the column are recovered in the major protein peak ($K_d$=0.25) it is clear that a significant amount of recombination of the two fractions to give Pl-2 like material has occurred. This is confirmed by the results of disc-gel electrophoresis (Plate XXVII, Fig. G). That the reconstituted material is indistinguishable from Pl-2 is also demonstrated by the data on carbohydrate composition (Table VIII).

The biological properties of the two ammonium sulphate fractions as well as of the 'reconstituted Pl-2' have been compared with Pl-2 in Tables IX and X. It is remarkable that, with respect to biological activity, the reconstituted material appears to be identical to Pl-2. As is to be anticipated, ASF-FSH has the follicle-stimulating activity. However, its potency is not different from that of Pl-2 — a rather

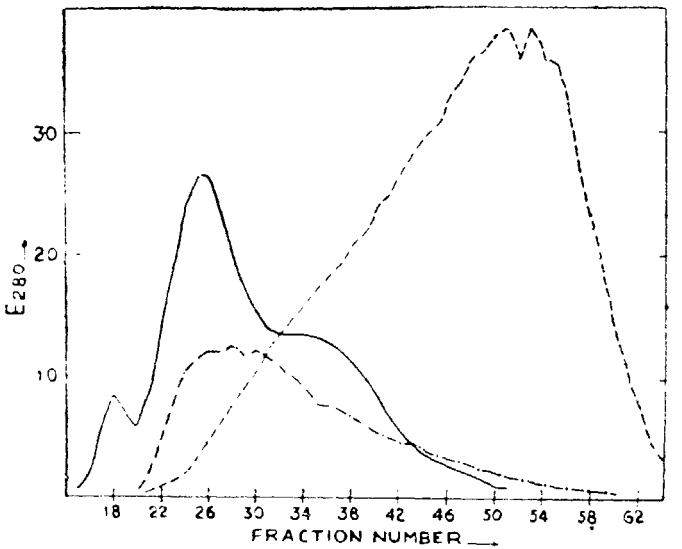



FIG. 4. Elution patterns on G-100 of : - - - ASF-FSH, —·—. ASF-LH, and —··— ASF-FSH + ASF-LH. Material was dissolved in and eluted with 0.05M glycine buffer (*p*H 8.6). For the reassociation experiment (Continuous line) ASF-LH and ASF-FSH were dissolved together in 0.05M glycine buffer (*p*H 8.6), incubated for 18 hr and chromatographed.

TABLE VIII

*Carbohydrate composition* of Pl-2 and of reconstituted Pl-2*

| Component | Pl-2 | Reconstituted Pl-2 |
|---|---|---|
| Neutral hexoses | 2.20 | 2.30 |
| Hexosamine | 0.50 | 0.48 |
| Fucose | 0.20 | 0.23 |
| Sialic acid | 0.30 | 0.35 |

*Expressed as percentage of total dry weight of sample

surprising result since removal of 'inert' protein (with respect to FSH activity) in the ASF-LH fraction, constituting as it does a purification step, should have resulted in increased potency. What is of even greater interest is that ASF-LH exhibits no biological activity. LH activity, as judged by the ovulation test is, surprisingly enough, found in ASF-FSH; potency, however, is approximately only 50 per cent of Pl-2. That these results are not due to loss of biological activity during handling would be evident from the observation that on recombination of ASF-FSH and ASF-LH to yield 'reconstituted Pl-2', full biological potency is restored.

TABLE IX

*FSH-activity of Pl-2, ASF-FSH, ASF-LH and of reconstituted Pl-2 (HCG-augmentation assay)*

| Sample | Dose ($\mu$g) | Response (mg ov. wt) |
|---|---|---|
| Pl-2 | 140 | 80.2±4.8 (10) |
| | 420 | 148.4±6.2 ( 8) |
| ASF-FSH | 140 | 78.1±5.3 ( 8) |
| | 420 | 148.4±3.8 ( 9) |
| ASF-LH | 140 | 43.2±4.7 (10) |
| | 420 | 47.8±6.8 (10) |
| Reconstituted Pl-2 | 140 | 78.5±5.8 (10) |
| | 420 | 149.5±4.9 (10) |
| HCG-injected Control | – | 41.2±2.8 (15) |

TABLE X

*Ovulation inducing ability of Pl-2, ASF-FSH, ASF-LH and Reconstituted Pl-2*

| Sample | Dose ($\mu$g) | Average No. ova shed/rat | % rat ovulated |
|---|---|---|---|
| NIH-LH-$S_{14}$ | 1.0 | 0.0 (6) | 0 |
| | 2.0 | 0.2±0 (8) | 25 |
| | 4.0 | 21.2±6.6 (10) | 80.0 |
| Pl-2 | 1.0 | 2.2±1.2 (6) | 50 |
| | 2.0 | 26.6±15.3 (10) | 80 |
| | 4.0 | 63.6±18.5 (10) | 90 |
| ASF-FSH | 2.0 | 3.7±1.2 (8) | 75 |
| | 4.0 | 25.6±8.8 (10) | 100 |
| ASF-LH | 2.0 | 15.8±2.2 (10) | 90 |
| | 4.0 | 14.0±3.1 (10) | 60 |
| Reconstituted Pl-2 | 2.0 | 30.2±10.8 (10) | 80 |
| | 4.0 | 62.8±20.2 (10) | 80 |
| PMS | 15 I.U | No response | |

## CONCLUSIONS

Taken together, the evidence is strongly suggestive of a rather complex structure for the monodisperse Pl–2. That it is not similar to any of the pituitary or urinary gonadotropins reported in the literature, is evident. This protein appears to be more analogous to a multienzyme complex (Reed and Cox 1970). It is perhaps pertinent to emphasize here that the isolation of Pl-2 has involved minimal processing of the crude extract and that too is based on the gentle procedure of gel filtration.

In view of the heterogeneity of both ASF–LH and ASF–FSH, it is tempting to speculate that Pl–2 is a gonadotropic complex consisting not only of biologically active peptides, but also of other peptides which function as "allosteric effectors". The biological activity of the ammonium sulphate fractions, compared to that of reconstituted Pl–2, would tend to support such a concept. However, studies aimed at the isolation and characterization of the constituents of ASF–LH and ASF–FSH are clearly necessary, before any firm conclusions can be reached as to the role of these individual components in the functional complex (Pl–2). Investigations along these lines are in progress.

The data strongly support the idea that monodisperse, functional complexes of FSH and LH may be secreted by the pituitary. The physiological significance of the existence of such complexes needs no elaboration.

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