

STRUCTURE AND BIOLOGICAL ACTIVITY OF AVIAN
HYPOTHALAMIC LUTEINIZING HORMONE-RELEASING HORMONE

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ABSTRACT

In 1971 Schally and co-workers (Schally *et al.*, 1971) isolated gonadotropin-releasing hormone (now called luteinizing hormone-releasing hormone (LH-RH)) from sheep hypothalami and established that the hormone was a decapeptide with the amino acid sequence:

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂.

The peptide was subsequently synthesised (Matsuo *et al.*, 1971b) and shown to stimulate the release of gonadotropins (luteinizing hormone and follicle-stimulating hormone) in a wide range of mammalian species (Schally *et al.*, 1973, 1976). With the exception of amphibians, nonmammalian vertebrates have a poor gonadotropin response to synthetic mammalian LH-RH (for reviews, see Ball, 1981; Jackson, 1981; King and Millar, 1981a). Since there is considerable molecular heterogeneity in the related neurohypophysial nonapeptide hormones (oxytocin-vasopressin) amongst vertebrates (Acher *et al.*, 1972), we postulated that differences might exist in the structure of hypothalamic LH-RH in different vertebrate classes. Utilising a combination of region-specific antisera and chromatographic techniques, we established that amphibian hypothalamic LH-RH is identical to the mammalian peptide while avian, reptilian, and piscine hypothalamic LH-RHs differ structurally in the region Gly⁶-Leu⁷-Arg⁸ (King and Millar, 1979a, 1980). We have now conducted further studies on avian hypothalamic LH-RH, which indicate that the arginine residue in position eight of mammalian LH-RH is substituted by glutamine in this vertebrate class. Purification of LH-RH from chicken hypothalami and determination of the amino acid composition have confirmed that the structure of avian LH-RH is:

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly-NH₂.

For indirect structural analysis, chicken LH-RH was partially purified by immunoaffinity chromatography utilising a mammalian LH-RH

antiserum coupled to Sepharose. Binding of this partially purified chicken LH-RH with five different region-specific antisera raised against mammalian LH-RH was compared by radioimmunoassay. With antisera which require the NH₂- and COOH-termini for effective binding, quantitation was highest (denoted 100%) and parallel displacement curves were obtained. All other antisera yielded nonparallel displacement curves. With antisera which require the middle region of LH-RH (Trp³ to Pro⁹) for binding, relative quantitation was 17-30%; with a COOH-terminus-directed antiserum (requiring Arg⁸ to Gly¹⁰-NH₂) relative quantitation was only 6%. These data indicate a difference in the structure of chicken LH-RH at position eight (arginine). Studies comparing antiserum interaction with chicken LH-RH after chemical modification of specific amino acid residues and after cleavage with specific proteolytic enzymes supported this conclusion. On gel filtration chromatography (Sephadex G-25 and Bio-Gel P-2) chicken LH-RH eluted in an identical position to mammalian LH-RH. In cation exchange chromatographic systems (CM32 carboxymethyl cellulose chromatography and Partisil PXS 10/25 SCX high performance liquid chromatography) chicken LH-RH consistently eluted earlier than the mammalian peptide. These studies demonstrate that chicken LH-RH is of similar size but is less positively charged than mammalian LH-RH. The pI for chicken LH-RH was found to be 7.3 by isoelectric focusing. This is compatible with a neutral amino acid substitution for arginine at position eight. On the basis of conformational criteria and evolutionary probability of amino acid interchange for arginine, the most likely substitution is glutamine. We therefore synthesised [Gln⁸]LH-RH by the solid-phase method and established that the synthetic peptide elutes identically in a wide range of chromatographic systems, has an isoelectric point identical

to that of natural chicken LH-RH, and has similar immunological properties. Synthetic [Gln⁸]LH-RH also displayed similar activity to natural chicken LH-RH in stimulating the release of luteinizing hormone from dispersed chicken anterior pituitary cells. Both the natural and synthetic chicken peptides had similar low potency in stimulating luteinizing hormone release from mammalian (sheep) anterior pituitary cells in culture (approximately 10% of the potency of mammalian LH-RH).

In order to isolate pure chicken LH-RH, it was estimated that approximately 250,000 hypothalami would be required to obtain 50 µg of pure peptide. This number of hypothalami was collected, and extracted with 2 N acetic acid which yielded 33.7 µg immunoreactive LH-RH in 22.2 g of dry material. The extract was purified by immunoaffinity chromatography (yield was 24.4 µg immunoreactive LH-RH in 0.100 g dry weight) followed by cation exchange and reverse phase high performance liquid chromatography to give a final yield of 17.4 µg homogeneously pure LH-RH. This represents a purification of two million-fold and a recovery from starting material of 51%. Amino acid analysis of a 6 N HCl hydrolysate gave ratios Ser 1, Glu 2, Pro 1, Gly 2, Leu 1, Tyr 1, Trp 1 and His 1, confirming the indirect demonstration that arginine in position eight of mammalian LH-RH is substituted by glutamine.

A number of studies on the gonadotropin-releasing activity of chicken LH-RH in different vertebrates have been initiated. Results show that chicken LH-RH is equipotent with mammalian LH-RH in stimulating luteinizing hormone release from dispersed chicken anterior pituitary cells. Mammalian LH-RH has poor gonadotropin-releasing activity in

fish (Ball, 1981; Jackson, 1981; King and Millar, 1981a), and it is possible that the synthetic avian peptide will be more active in fish. Induction of spawning in fish is an important economic component of productivity in pisciculture. Attempts to substitute mammalian LH-RH (and analogues) for the expensive mammalian gonadotropins that have been used to induce spawning, have been relatively unsuccessful. However, synthetic avian LH-RH and, in particular, superactive analogues of the peptide, might be effective in stimulating reproduction in fish and may also find application in the poultry industry.

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1.0

PHYLOGENY OF LUTEINIZING HORMONE-RELEASING HORMONE

It is now generally accepted that in all vertebrates (with the possible exception of cyclostomes) the gonadotropic function of the anterior pituitary is regulated by the hypothalamus via the secretion of releasing and/or release-inhibiting factors into a hypothalamo-pituitary portal vascular system (for reviews, see Crim *et al.*, 1978; Goos, 1978; Jackson, 1978; Ball, 1981). However, the possibility that gonadotropin release is also, or independently, regulated via direct neural control or hormonal regulation via the general circulation has not been excluded.

The first definitive evidence for the existence of an anterior pituitary gonadotropin-releasing factor or hormone was provided by McCann *et al.* (1960), who showed that acid extracts of the mammalian median eminence could induce release of luteinizing hormone (LH) from the mammalian anterior pituitary. Subsequently, the LH-releasing hormone (LH-RH) was isolated from porcine (Schally *et al.*, 1971) and ovine (Burgus *et al.*, 1972) hypothalamic extracts and characterised as a decapeptide pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂ (Matsuo *et al.*, 1971a). The synthesised peptide (Matsuo *et al.*, 1971b) releases LH as well as follicle-stimulating hormone (FSH) in a wide variety of mammalian species (for reviews, see Schally *et al.*, 1973, 1976), suggesting that it is also the FSH-RH, so that this decapeptide is perhaps more correctly called gonadotropin-releasing hormone. For convenience, I shall refer to the gonadotropin-releasing hormone as LH-RH.

PHYLOGENETIC DISTRIBUTION AND FUNCTION OF LH-RH

Mammals

Mammalian LH-RH has been studied extensively and well reviewed, and will therefore be omitted from this review.

Birds

The secretion and release of gonadotropins from the anterior pituitary in birds is regulated by the hypothalamus (Dodd *et al.*, 1971). Evidence for this concept was first provided by experiments in which intrapituitary infusion of domestic fowl hypothalamic extracts evoked premature ovulation in domestic fowl (Clark and Fraps, 1967; Opel and Lepore, 1967, 1972). Subsequently, hypothalamic extracts from domestic fowl and quail have been shown to stimulate the release of LH from domestic fowl and quail pituitaries *in vitro* (Jackson and Nalbandov, 1969; Tanaka *et al.*, 1969; Follett, 1970; Casey *et al.*, 1971; Smith and Follett, 1972; Campbell and Wolfson, 1974; Harrison *et al.*, 1974; Bicknell and Follett, 1977; Hattori *et al.*, 1980; Kawashima *et al.*, 1981). LH-releasing activity has been demonstrated in hypothalamic extracts of domestic fowl (Jackson and Nalbandov, 1969; Jackson, 1971a, 1971b, 1972) and quail (Hattori *et al.*, 1980) tested on the rat anterior pituitary *in vitro*, and in extracts of domestic fowl tested on the sheep anterior pituitary *in vitro* (King and Millar, 1980, 1981a). The results of lesioning and deafferentation experiments in the turkey (Opel, 1979) and electrical stimulation and lesion studies in the quail (Davies and Follett, 1980) are consistent with the view that a gonadotropin-releasing factor is located in the hypothalamus.

Synthetic mammalian LH-RH (microgram rather than nanogram doses are necessary to evoke a response) elicits precocious ovulation in the domestic fowl (van Tienhoven and Schally, 1972; Tanaka *et al.*, 1974; Tanaka and Kamiyoshi, 1976; Johnson and van Tienhoven, 1981); induces ovarian growth in the domestic fowl (Reeves *et al.*, 1973) and turkey (Burke and Cogger, 1977); increases serum LH levels in the domestic fowl (Furr *et al.*, 1973; Bonney *et al.*, 1974; Wilson and Sharp, 1975; Williams and Sharp, 1978; Johnson and van Tienhoven, 1981; Tanabe *et al.*, 1981),

turkey (Burke and Cogger, 1977), sparrow (Wingfield *et al.*, 1979), bantam (Sharp and Lea, 1981), quail (Davies and Bicknell, 1976; Davies and Collins, 1979) and domestic duck (Balthazart *et al.*, 1980); increases (only very slightly) serum levels of FSH in the domestic fowl (Godden *et al.*, 1977; Scanes *et al.*, 1977), domestic duck (Balthazart *et al.*, 1980) and quail (Davies and Collins, 1979). *In vitro* studies have shown that synthetic mammalian LH-RH stimulates the secretion of LH (Bicknell and Follett, 1975; Gledhill, 1977) and FSH (Gledhill, 1977) from the quail anterior pituitary. In the laying domestic fowl, the biological effects of endogenous gonadotropin-releasing hormone can be blocked by injection of antiserum to synthetic mammalian LH-RH (Fraser and Sharp, 1978).

Further evidence for hypothalamic control of gonadotropic function in birds is provided by the following: in the domestic duck, pituitary responsiveness to synthetic mammalian LH-RH changes during the annual cycle (Balthazart *et al.*, 1980); in the turkey, sex hormones alter the sensitivity of the anterior pituitary to synthetic mammalian LH-RH (Godden *et al.*, 1977); anti-estrogen increases the responsiveness of the anterior pituitary of the domestic hen to an injection of synthetic mammalian LH-RH (Wilson and Cunningham, 1981); the sensitivity of the anterior pituitary to synthetic mammalian LH-RH is increased in quails at the beginning of gonadal maturation (Davies and Bicknell, 1976); synthetic mammalian LH-RH has a role in the reproductive behaviour of female ring doves (Cheng, 1977). Several analogues of synthetic mammalian LH-RH ([Phe⁵]LH-RH, [des-Gly¹⁰]LH-RH) have been shown to be more potent than the decapeptide in stimulating LH release from domestic fowl anterior pituitaries *in vitro* (Bonney and Cunningham, 1977), but they were less potent on rat anterior pituitaries (Rivier *et al.*, 1972; Coy *et al.*, 1973). A superactive analogue of LH-RH ([D-Lys⁶]LH-RH ethylamide) has been shown to elevate plasma testosterone levels in domestic

cocks (Pethes *et al.*, 1980).

In radioimmunoassays, antisera raised against synthetic mammalian LH-RH cross-react with hypothalamic and extrahypothalamic brain extracts of the domestic fowl (Jeffcoate *et al.*, 1974; King and Millar, 1979a, 1980, 1981a; Hattori *et al.*, 1980) and pigeon (King and Millar, 1979a, 1980, 1981a). Immunohistochemical staining for LH-RH has been reported in the median eminence of the domestic hen (Sterling and Sharp, 1982), cockerel (de Riviers and Dubois, 1974), greenfinch (Sharp *et al.*, 1975), duck (Calas *et al.*, 1973; McNeill *et al.*, 1976; Bons *et al.*, 1978), quail (Nozaki and Kobayashi, 1979; Hattori *et al.*, 1980) and pigeon (Weindl *et al.*, 1982).

Reptiles

In reptiles, regulation of gonadotropic function is less well understood. Early indications of hypothalamic regulation of gonadotropins in reptiles came from findings that implants of testosterone or estradiol in or near the median eminence of the lizard inhibited testicular and ovarian growth (Lisk, 1967), suggesting that the hypothalamus stimulates gonadotropin secretion and is sensitive to the suppressive action of sex steroids. The results of Hall *et al.* (1978) regarding the effect of terrapin hypothalamic extracts on the release of LH from terrapin pituitaries *in vitro* were inconclusive, although tortoise hypothalamic extracts have been demonstrated to stimulate release of LH-like gonadotropin from frog pituitaries *in vitro* (Thornton and Geschwind, 1974).

Synthetic mammalian LH-RH elevates plasma gonadotropin levels in the turtle (Callard and Lance, 1977), and Licht (1980) has recently shown that the synthetic mammalian hormone stimulates LH release, but

not the release of FSH, in the turtle *in vivo*. Injection of synthetic mammalian LH-RH (microgram doses), and of synthetic mammalian thyrotropin-releasing hormone, induces sexual receptivity in estrogen-primed, ovariectomised female lizards (Alderete *et al.*, 1980).

By radioimmunoassay, LH-RH-like immunoreactivity has been demonstrated in the hypothalamus and extrahypothalamic brain of the lizard and tortoise (King and Millar, 1979a, 1980, 1981a). Immunohistochemical studies have shown that immunoreactive LH-RH occurs in the median eminence and in other brain areas of the lizard, gecko, turtle and snake (Doerr-Schott and Dubois, 1978 ; Nozaki and Kobayashi, 1979).

Amphibians

Amphibians possess a well-developed hypothalamo-pituitary portal vascular system, and evidence from hypothalamic lesions, pituitary stalk section and pituitary transplantation suggests that the secretion of anterior pituitary gonadotropins is regulated by the hypothalamus (Jorgensen and Larson, 1967). Homogenates of frog hypothalami stimulate the release of bioassayable LH-like gonadotropin from frog hemipituitaries *in vitro* (Thornton and Geschwind, 1974), and immunoreactive LH-RH from the clawed toad hypothalamus is equipotent with mammalian LH-RH in stimulating LH release from the sheep anterior pituitary *in vitro* (King and Millar, 1980, 1981a).

Synthetic mammalian LH-RH (microgram doses) was shown to stimulate secretion of LH-like gonadotropin from frog hemipituitaries *in vitro* (Thornton and Geschwind, 1974). *In vivo* studies have shown that synthetic mammalian LH-RH induces ovarian growth, ovulation and vitellogenesis in the clawed toad (Thornton and Geschwind, 1974), and spermiation in the frog (Licht, 1974; Easley *et al.*, 1979). Synthetic

mammalian LH-RH thus seemingly stimulates the release of both LH (for ovulation) and FSH (for ovarian growth and vitellogenesis) in these anurans. This would accord with the action of synthetic mammalian LH-RH in mammals where release of both LH and FSH is brought about *in vivo* and *in vitro* (Blackwell and Guillemin, 1973; Schally, 1978). Recently, Daniels and Licht (1980) have confirmed that synthetic mammalian LH-RH stimulates release of both LH and FSH in the frog *in vivo*. Two analogues of synthetic mammalian LH-RH (an agonist and an antagonist) were shown to possess actions in the frog similar to those exhibited in mammals (McCreery *et al.*, 1982). The agonist ([¹imBzl-D-His⁶, Pro⁹-Net]LH-RH) was more potent and had a longer duration of action in stimulating *in vivo* LH and FSH secretion in frogs. Pretreatment with antagonist ([¹(Ac-dehydro-Pro¹, pCl-D-Phe², D-Trp^{3,6})-Na-MeLeu⁷]LH-RH) suppressed LH-RH-induced gonadotropin release in adult male frogs. In the clawed toad, hypothalamic LH-RH content varies seasonally in parallel with seasonal changes in ovarian weight (King and Millar, 1979b), suggesting a possible role for hypothalamic LH-RH in gonadotropic function.

Amongst urodele amphibians, perfusion of the newt pituitary *in vitro* by synthetic mammalian LH-RH induces ovulation (Vellano *et al.*, 1974), and synthetic mammalian LH-RH perfused into newts bearing ectopic pituitary autografts restores spermatogenesis (Mazzi *et al.*, 1974) and promotes oocyte recruitment (Mazzi, 1978).

Radioimmunoassay studies employing antisera raised against synthetic mammalian LH-RH have demonstrated the presence of immunoreactive LH-RH in the hypothalamus and extrahypothalamic brain of the frog, toad and clawed toad (Deery, 1974; Alpert *et al.*, 1976; King and Millar, 1979a, 1979b, 1980, 1981a, 1981b; Rivier *et al.*, 1981), and in the sympathetic ganglia of the frog (Jan *et al.*, 1979; Eiden

and Eskay, 1980). Immunohistochemical studies have demonstrated neurons reacting with anti-LH-RH in the median eminence and other brain areas of the frog, toad, clawed toad and newt (Doerr-Schott and Dubois, 1975, 1976, 1978; Alpert *et al.*, 1976; Dubois, 1976; Goos *et al.*, 1976; Goos and van Oordt, 1978; Doerr-Schott *et al.*, 1978, 1979; Kubo *et al.*, 1979; Nozaki and Kobayashi, 1979), and in the sympathetic ganglia of the frog (Jan *et al.*, 1980).

Fish

Evidence for hypothalamic control of the gonadal activity and gonadotropin secretion in fish has been reported (Peter, 1970, 1973; Peter and Crim, 1979). Gonadotropin-releasing activity has been demonstrated in hypothalamic extracts from various teleost fish (carp, trout, cichlid, goldfish) tested on the teleost pituitary *in vivo* and *in vitro* (Breton *et al.*, 1971a, 1971b, 1972, 1975a, 1975b; Breton and Weil, 1973; Holmes and Ball, 1974; Weil *et al.*, 1975; de Vlaming and Vodcnik, 1975, 1977; Crim *et al.*, 1976; Barnett *et al.*, 1979; Crim and Evans, 1980), on the rat pituitary *in vivo* (Barnett *et al.*, 1979), and on the sheep pituitary *in vitro* (Breton *et al.*, 1972; King and Millar, 1980, 1981a). Hypothalamic extracts from sheep stimulate gonadotropin release from carp pituitaries *in vitro* (Breton *et al.*, 1972), and rat hypothalamic extracts stimulate the release of gonadotropin from trout pituitaries *in vitro* (Crim and Evans, 1980).

Synthetic mammalian LH-RH (microgram rather than nanogram doses are required to evoke a response) induces ovulation in the goldfish (Lam *et al.*, 1975, 1976), ayu (Hirose and Ishida, 1974), medaka (Chan, 1977), plaice and goby (Aida *et al.*, 1978), and various Chinese farm fish (carp) (Symposium Group for Hormonal Application in Pisciculture, 1975; Academia Sinica Cooperative Team, 1977), and stimulates

ovarian maturation in carp (Sokolowska *et al.*, 1978; Weil *et al.*, 1980). Other studies have shown that synthetic mammalian LH-RH stimulates the *in vivo* release of gonadotropin in carp (Breton and Weil, 1973; Weil *et al.*, 1975, 1980), trout (Crim and Cluett, 1974; Weil *et al.*, 1978; Crim *et al.*, 1981) and goldfish (Lam *et al.*, 1975; Crim *et al.*, 1976; Peter, 1980), and the *in vitro* release of gonadotropin from the trout pituitary (Crim and Evans, 1980; Crim *et al.*, 1981). Synthetic mammalian LH-RH also induces cytological activation of the gonadotropic cells of *Poecilia latipinna*, both *in vitro* and *in vivo* (Young and Ball, 1980), and of salmon (Ekengren *et al.*, 1978), goldfish (Lam *et al.*, 1976), medaka (Chan, 1977) and the grass carp (Academia Sinica Cooperative Team, 1978a, 1978b) *in vivo*. Several superactive analogues of synthetic mammalian LH-RH ([D-Ala⁶, des-Gly¹⁰]LH-RH ethylamide and other analogues with D-amino acid substitutions in position six and/or an ethylamide) have equal or greater biological activity than the synthetic decapeptide or the natural releasing hormone in various teleost fish (trout, goldfish, carp) *in vivo* and *in vitro* (Academia Sinica Cooperative Team, 1977; Cooperative Team for Hormonal Application in Pisciculture, 1977; Fukien-Kiansu Cooperative Group, 1977; Peter, 1980; Crim *et al.*, 1981).

Although the synthetic mammalian decapeptide activates gonadal function in teleost fish, it may not be identical to teleost hypothalamic LH-RH, since injection of hypothalamic extracts from the carp produces different profiles of carp gonadotropin secretion than does the synthetic mammalian LH-RH (Breton and Weil, 1973). Furthermore, higher doses of synthetic mammalian LH-RH are required to stimulate gonadotropin secretion in teleost fish than in mammals.

Amongst elasmobranch fish, dogfish median eminence extracts and synthetic mammalian LH-RH have been shown to activate the adenylyl cyclase

system of the gonadotropin-containing ventral lobe of the dogfish pituitary (Deery and Jones, 1975), and dogfish treated with synthetic mammalian LH-RH responded with elevated plasma gonadotropin levels in an experiment in which a heterologous radioimmunoassay was used to measure gonadotropin (Dodd, 1975). More recently, both dogfish hypothalamic extracts and synthetic mammalian LH-RH were shown to raise circulating levels of sex steroids in male and female dogfish (Jenkins and Dodd, 1980).

The existence of hypothalamic control of the anterior pituitary in cyclostome fish remains to be clearly established. An anatomical mechanism for the transfer of hypothalamic influences to the cyclostome anterior pituitary is lacking, or is very inefficient, as neither a portal system nor direct innervation of the anterior pituitary is present (for review, see Ball, 1981). There is no biological evidence indicating that gonadotropin release is stimulated by a hypothalamic gonadotropin-releasing hormone in cyclostomes.

Regarding the occurrence of immunoreactive LH-RH in the fish hypothalamus, Deery (1974) was unable to detect immunoreactive LH-RH in hypothalamic extracts of a teleost (goldfish) and elasmobranch (dogfish), and Crim *et al.* (1979a, 1979b) found no radioimmunoassayable LH-RH in the cyclostome (hagfish) brain. More recently, radioimmunoassay studies employing antisera raised against synthetic mammalian LH-RH have demonstrated the presence of immunoreactive LH-RH in the hypothalamus and in extrahypothalamic brain regions of teleosts (cichlid, codfish, carp, goldfish, catfish) (Peptide Hormone Group, 1977; Barnett *et al.*, 1979, 1982; Jackson, 1979; King and Millar, 1979a, 1980, 1981a), elasmobranchs (dogfish, ratfish) (Crim *et al.*, 1978; Jackson, 1978, 1979; King and Millar, 1979a, 1980, 1981a), and cyclostomes (hagfish, lamprey)

(Jackson, 1979; King and Millar, 1980, 1981a). By means of immunohistochemistry, immunoreactive LH-RH has been localised in the hypothalamus and extrahypothalamic brain of teleosts (trout, puffer, carp, platyfish) (Goos and Murathanoglu, 1977; Goos and van Oordt, 1978; Dubois *et al.*, 1979; Nozaki and Kobayashi, 1979; Schreibman *et al.*, 1979), and cyclostomes (lamprey, but not hagfish) (Crim *et al.*, 1979a, 1979b).

Other organisms

In addition to the widespread distribution of an LH-RH-like substance amongst vertebrates, immunoreactive LH-RH has also been described in more primitive organisms. In the tunicate *Ciona*, neurosecretory cells containing immunoreactive LH-RH occur in the cerebral ganglion of the adult but not in very young individuals (Georges and Dubois, 1980). A substance with LH-releasing activity has also been extracted from leaves of the plant *Avena sativa* (Fukushima *et al.*, 1976). These observations are of great interest, but any explanation for the occurrence of LH-RH in these organisms would be speculative at this stage especially since LH-RH sequences have been noticed in a number of functionally unrelated molecules (Stewart and Channabasavaiah, 1979).

STRUCTURE OF LH-RH

The foregoing evidence for the presence of a biologically active gonadotropin-releasing hormone in the hypothalamus of all vertebrate classes is thus substantial. However, the structural and biological nature of LH-RH in submammalian vertebrates has not been well investigated. There is, therefore, the need to undertake a thorough investigation of the structure, and to isolate and characterise hypothalamic LH-RH from submammalian vertebrates.

The structural nature of hypothalamic and extrahypothalamic brain LH-RH in submammalian vertebrates has been studied by radioimmunoassay and chromatography (for reviews, see Ball, 1981; Jackson, 1981; King and Millar, 1981a). Several reports indicated a structural identity between mammalian hypothalamic LH-RH and chicken and frog hypothalamic LH-RHs (Deery, 1974; Jeffcoate *et al.*, 1974; Alpert *et al.*, 1976), whilst others reported differences in the physico-chemical and biological properties of chicken and teleost fish hypothalamic LH-RHs (Jackson, 1971a, 1971b, 1972; Hattori *et al.*, 1980; Jackson, 1981; Barnett *et al.*, 1982) and frog sympathetic ganglion LH-RH (Jan *et al.*, 1979; Eiden and Eskay, 1980). On the basis of chromatographic properties and using region-specific antisera we demonstrated that avian, reptilian, and piscine hypothalamic LH-RHs differed structurally from mammalian hypothalamic LH-RH, the difference being in the region Gly⁶-Leu⁷-Arg⁸ (King and Millar, 1979a, 1980, 1981a). Jackson's group (Jackson, 1981; Barnett *et al.*, 1982) has recently identified two LH-RH molecules in teleost brain which appear to differ from one another and from synthetic mammalian LH-RH by a single amino acid substitution in position seven or eight, as well as a third immunoreactive material which appears to be a 'big' LH-RH. We have shown that amphibian hypothalamic LH-RH is identical to the mammalian hypothalamic decapeptide (King and Millar, 1979a, 1979b, 1980, 1981a, 1981b) and this has been confirmed by recent studies on the amino acid composition of frog brain LH-RH (Rivier *et al.*, 1981). Frog sympathetic ganglion LH-RH, however, differs from mammalian hypothalamic LH-RH in the COOH-terminal region of the molecule (Eiden and Eskay, 1980).

EVOLUTION OF LH-RH

A comment on the evolution of vertebrate hypothalamic LH-RH is appropriate at this stage. Although bird, reptile, and teleost fish hypothalamic LH-RHs are structurally different from mammalian decapeptide LH-RH, they behave identically to each other on CM32 carboxymethyl cellulose cation exchange chromatography and reverse phase high performance liquid chromatography as well as in their interaction with region-specific antisera (King and Millar, 1979a, 1980, 1981a). It is possible that the LH-RH in present-day birds, reptiles and teleosts represents the ancestral LH-RH molecule which occurred in the ancient osteichthyes and has persisted for about 400 million years. A single mutation in the ancestral 'avian-type' molecule might have occurred in a lineage common to amphibians and mammals in accordance with a contemporary phylogenetic scheme (Licht *et al.*, 1977), to give rise to the 'mammalian-type' analogue in present-day amphibians and mammals (King and Millar, 1979a, 1981a).

Synthetic mammalian LH-RH (and superactive analogues of the hormone) exhibit good gonadotropin-releasing activity in amphibians but have poor or no such activity in birds, reptiles and fish (for reviews, see Ball, 1981; Jackson, 1981; King and Millar, 1981a). It is likely that this phenomenon reflects structural differences in pituitary gonadotrope receptors related to the structural difference of LH-RH in these vertebrate classes.

The objective of the present study was to determine the structural and biological nature of avian hypothalamic LH-RH. The thesis comprises three fields of study:

- a) 'Indirect' studies on partially purified chicken hypothalamic LH-RH utilising radioimmunoassay with region-specific antisera, several chromatographic systems, and comparison of antiserum interaction with chicken LH-RH after cleavage with proteolytic enzymes and chemical modification of specific amino acids.
- b) Isolation of pure chicken hypothalamic LH-RH, and determination of the amino acid composition and sequence of the peptide.
- c) Synthesis of chicken hypothalamic LH-RH, and assessment of the biological activity of the natural and synthetic peptides in the mammal and in the bird, using anterior pituitary cell culture and receptor binding techniques.

2.0

STRUCTURAL DETERMINATION OF PARTIALLY PURIFIED CHICKEN

HYPOTHALAMIC LUTEINIZING HORMONE-RELEASING HORMONE

2.1

SUMMARY

Studies on partially purified chicken hypothalamic luteinizing hormone-releasing hormone (LH-RH) utilising chromatography, region-specific antisera, enzymic inactivation and chemical modification have established that the peptide is structurally different from mammalian hypothalamic LH-RH. Gel filtration chromatography demonstrated that chicken LH-RH is of the same molecular size as the mammalian decapeptide. Chicken LH-RH eluted earlier than mammalian LH-RH on reverse phase high performance liquid chromatography (HPLC). Cation exchange chromatography (CM32 carboxymethyl cellulose and HPLC) and isoelectric focusing established that chicken LH-RH is less positively charged than the mammalian peptide. Binding studies of chicken LH-RH utilising five different region-specific antisera raised against mammalian LH-RH demonstrated that the structural difference resides in amino acid residue eight (arginine). This finding was confirmed by measurement, with appropriate antisera, of changes in immunoreactivity after cleavage with proteolytic enzymes and chemical modification of specific amino acid residues. The lower isoelectric point of chicken LH-RH (7.3) relative to that of the mammalian peptide (9.1) is compatible with the substitution of a neutral amino acid for arginine at position eight. Structural studies on mammalian LH-RH have shown that the side chain of Arg⁸ is in close vicinity to the side chains of His² and Tyr⁵, and that these side chains are linked by hydrogen bonds and form a combined unit important for biological action (Shinitzky and Fridkin, 1976). A likely neutral amino acid substitution compatible with these structural requirements is glutamine. Moreover, of the neutral amino acids, the probability of glutamine replacing arginine (or vice versa) is high (Dayhoff *et al.*, 1972). We therefore synthesised [Gln⁸]LH-RH and found it to have identical chromatographic, immunological and biological properties to the natural chicken peptide.

The decapeptide LH-RH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂), originally isolated from porcine hypothalami (Matsuo *et al.*, 1971a) and later synthesised (Matsuo *et al.*, 1971b), is biologically active in a large number of mammalian species. Synthetic mammalian LH-RH appears to have lower activity in birds, reptiles, amphibians and fish. Crude hypothalamic extracts from these submammalian vertebrates, nevertheless, contain significant gonadotropin-releasing activity and LH-RH-like immunoreactivity has been demonstrated in the hypothalamus of these vertebrates by radioimmunoassay and immunocytochemistry (for reviews, see Ball, 1981; Jackson, 1981; King and Millar, 1981a). We have demonstrated that immunoreactive amphibian hypothalamic LH-RH is identical to the mammalian decapeptide, while immunoreactive LH-RHs from avian, reptilian, and piscine hypothalami are structurally different (King and Millar, 1979a, 1980, 1981a). These differences of LH-RH-like peptides in submammalian vertebrates have not been determined.

In view of the extremely small quantities of immunoreactive LH-RH present in hypothalamic tissue, several hundred thousand hypothalami and a purification of a similar magnitude (Matsuo *et al.*, 1971a) are required for structural determination. Since avian hypothalamic LH-RH appears to have considerable structural homology with mammalian decapeptide LH-RH (King and Millar, 1979a, 1980), we considered it feasible to determine the structural differences on a small quantity of partially purified material by indirect means utilising a combination of chromatographic analysis, binding of region-specific antisera, enzymic inactivation, and chemical modification of specific amino acid residues. This section describes the structural characterisation

of chicken hypothalamic LH-RH, and establishes that the peptide differs from mammalian hypothalamic LH-RH at residue eight, arginine being substituted by glutamine. Based on these findings we synthesised [Gln⁸]LH-RH (see section 4.3) for comparison with natural chicken LH-RH, and report here that chicken hypothalamic LH-RH has identical properties to [Gln⁸]LH-RH.

Extraction and partial purification of chicken hypothalamic LH-RH

Fragments of 15,000 chicken (*Gallus domesticus*) hypothalami consisting of the median eminence and pituitary stalk were dissected, lyophilised, defatted with petroleum ether (40-60°C), and extracted with 2 N acetic acid at 4°C. The supernatant extract was lyophilised, reconstituted in 0.5 M ammonium acetate, pH 7.0, and applied to an immunoaffinity column (2 x 22 cm) prepared by coupling rabbit gamma globulin (anti-LH-RH 1076) to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals). The column was washed with 0.5 M ammonium acetate to remove unbound peptides, and immunoreactive LH-RH was eluted with 1.5 N acetic acid. Aliquots of fractions were assayed for LH-RH immunoreactivity, and for total peptide using bovine serum albumin as standard (Lowry *et al.*, 1951). The active fractions were pooled and lyophilised, and used in all studies described below.

Chromatographic procedures

In each chromatographic system the elution volume of synthetic mammalian LH-RH (Ayerst Laboratories) was determined for comparison with that of chicken LH-RH. Aliquots of column fractions were lyophilised and assayed for LH-RH immunoreactivity.

Gel filtration chromatography - Immunoreactive chicken LH-RH was incubated at 50°C for 3 h in 2 ml 2 N acetic acid containing 6 M urea, and eluted on Sephadex G-25 superfine (1.6 x 90 cm; Pharmacia Fine Chemicals) and Bio-Gel P-2 200-400 mesh (1.6 x 90 cm; Biorad Laboratories) columns with 2 N acetic acid.

High performance liquid chromatography (HPLC) - HPLC analysis was on a Model SP 3500B liquid chromatograph (Spectra-Physics)

equipped with a Model 770 spectrophotometric detector (Spectra-Physics). A Spherisorb ODS reverse phase column (0.4 x 25 cm; 5 μ m particle size; Phase Separations) was eluted isocratically for 40 min with 22% acetonitrile in 0.01 M ammonium acetate, pH 4.0, followed by a 20-min linear gradient from 22% to 80% acetonitrile in 0.01 M ammonium acetate, pH 4.0, and then isocratically for 5 min at 80% acetonitrile. A Partisil PXS 10/25 SCX cation exchange column (0.4 x 25 cm; Whatman) was eluted isocratically with 10% ethanol in 0.2 M ammonium acetate, pH 4.6. Immunoreactive chicken LH-RH was reconstituted in the starting HPLC buffer and eluted at a flow rate of 1.5 ml/min.

Chymotrypsin-generated fragments of immunoreactive chicken LH-RH and synthetic mammalian LH-RH (described below), and the synthetic fragment LH-RH (6-10), were eluted from the cation exchange column using the conditions described above.

Cation exchange chromatography - Immunoreactive chicken LH-RH was reconstituted in 0.002 M ammonium acetate, pH 4.5, and eluted on a CM32 carboxymethyl cellulose column (1 x 18 cm; Whatman) with 0.002 M ammonium acetate for the initial 20 ml and then 0.06 M ammonium acetate, pH 4.5.

Isoelectric focusing - Immunoreactive chicken LH-RH was reconstituted in 50 μ l H₂O, and chromatofocused on polyacrylamide gel rods (0.4 x 7 cm) with either Ampholine pH 3.5 - 9.5 (LKB) or Pharmalyte pH 3-10 (Pharmacia Fine Chemicals) plus Ampholine pH 9-11 with a potential of 550 V at 4°C for 2.5 h. Buffers used were 0.01 M HEPES (Sigma) (upper reservoir) and 0.01 M ethanolamine (BDH) (lower reservoir). Gels were sliced, and the slices were disrupted and eluted with 300 μ l 0.2 N acetic acid: ethanol (1 : 1) for 16 h. Aliquots were assayed for LH-RH immunoreactivity. The pH was

determined on gels run in parallel, after slicing and addition of 0.45% NaCl.

Radioimmunoassay of LH-RH

LH-RH was measured as described by Hendricks *et al.* (1975), with modifications. Synthetic mammalian LH-RH (Ayerst Laboratories) was used as standard. Synthetic mammalian LH-RH was labelled with ^{125}I using the chloramine T method (Greenwood *et al.*, 1963) and purified on a CM32 carboxymethyl cellulose cation exchange column (1 x 20 cm; Whatman) eluted with 0.002 M ammonium acetate, pH 4.5, for the initial 20 ml and then 0.06 M ammonium acetate, pH 4.5. Antisera were generated in rabbits against synthetic mammalian LH-RH. Antiserum 1076 was produced to LH-RH conjugated with *Haliotis* sp. haemocyanin by carbodiimide condensation (Hendricks *et al.*, 1975). Antisera 743 and 744 were generated against [Glu^1]LH-RH conjugated with human serum albumin through predominantly Glu^1 by carbodiimide condensation (Schally *et al.*, 1980; gift from A. Arimura). Antiserum 422 was produced to LH-RH adsorbed on polyvinylpyrrolidone (Schally *et al.*, 1980; gift from A. Arimura). Antiserum R-42 was produced to LH-RH conjugated with bovine serum albumin by bis-diazotized-benzidine conjugation (Nett *et al.*, 1973; gift from T.M. Nett and G.D. Niswender). Radioimmunoassay characteristics are given in Table 1. The cross-reactivity of antisera 1076, 743, 744 and 422 with fragments and analogues of LH-RH is presented in Table 2; for antiserum R-42 see Nett *et al.* (1973) and Copeland *et al.* (1979).

Immunoreactive chicken LH-RH was reconstituted in phosphate-buffered saline containing gelatin (PBS-gel) (0.04 M phosphate, 0.15 M NaCl, 0.01 M disodium ethylenediaminetetra-acetic acid and 0.015 M NaN_3 , pH 7.0, with 0.1% gelatin) and assayed in serial doubling

TABLE 1

Radioimmunoassay characteristics

Antiserum	Final dilution	Half-maximal displacement (pg)	Limit of sensitivity (pg)	Intra-assay coefficient of variation (%)
1076	1:100,000	15.7	<1.2	3.5
743	1:30,000	23.1	1.6	6.0
744	1:25,000	217.0	6.0	5.6
422	1:5,000	147.0	41.0	3.1
R-42	1:200,000	13.1	<1.2	5.5

TABLE 2

Cross-reactivity of four LH-RH antisera with fragments and analogues of LH-RH, expressed as percentage

	Antiserum			
	1076	743	744	422
pGlu ¹ His ² Trp ³ Ser ⁴ Tyr ⁵ Gly ⁶ Leu ⁷ Arg ⁸ Pro ⁹ Gly ¹⁰ NH ₂	100	100	100	100
pGlu ¹ Gly ¹⁰ OH	-	56	0	0
pGlu ¹ Pro ⁹ Ethylamide	53	102	0	-
pGlu ¹ Pro ⁹ NH ₂	-	165	0	0
pGlu ¹ Leu ⁷ OH	-	0	0	0
pGlu ¹ Gly ⁶ OH	0	0	0	0
pGlu ¹ Trp ³ OH	-	0	0	0
pGlu ¹ His ²	0	-	-	-
Glu ¹ Gly ¹⁰ NH ₂	-	33	121	1
His ² Gly ¹⁰ NH ₂	24	23	109	0
pGlu ² Gly ¹⁰ NH ₂	-	11	40	0
pGlu ³ Gly ¹⁰ NH ₂	12	0	65	0
Trp ³ Gly ¹⁰ NH ₂	14	20	95	0
Ser ⁴ Gly ¹⁰ NH ₂	-	0	35	0
Gly ⁶ Gly ¹⁰ NH ₂	0	-	16	-
Leu ⁷ Gly ¹⁰ NH ₂	0	0	12	0
Arg ⁸ Gly ¹⁰ NH ₂	-	-	0.8	-
His ² Leu ⁷ OH	-	0	0	0
Trp ³ Pro ⁹ OH	-	1.5	0	0
Trp ³ Pro ⁹ NH ₂	-	19	0	0
Trp ³ Arg ⁸ NH ₂	-	0	0	0
Trp ³ Leu ⁷ OH	-	0	0	0
Ser ⁴ Leu ⁷ OH	-	0	0	0
pGlu ¹ Ala ² (Pyr(3)) Gly ¹⁰ NH ₂	103	94	111	31
pGlu ¹ Phe ³ (pNO ₂) Gly ¹⁰ NH ₂	0.3	2	137	2
pGlu ¹ Thr ⁴ Gly ¹⁰ NH ₂	137	79	101	8
pGlu ¹ Ile ⁶ Gly ¹⁰ NH ₂	0.1	0.3	45	8
pGlu ¹ D-Leu ⁶ Gly ¹⁰ NH ₂	0	105	56	0
pGlu ¹ D-Leu ⁶ Pro ⁹ Ethylamide	0	-	-	-
pGlu ¹ D-Trp ⁶ Gly ¹⁰ NH ₂	0	0	55	0
pGlu ¹ Phe ⁷ Gly ¹⁰ NH ₂	130	<.03	154	41
pGlu ¹ Lys ⁸ Gly ¹⁰ NH ₂	80	1	1	62
pGlu ¹ Ala ⁹ Gly ¹⁰ NH ₂	0.4	0.5	0.3	26

The cross-reactivities were calculated from the molar concentrations of the fragments and analogues which decreased the binding of ¹²⁵I-LH-RH to the antisera by 50%. Some of these data are from Hendricks *et al.* (1975) and Millar *et al.* (1978).

dilutions in duplicate, using the five antisera (1076, 743, 744, 422 and R-42) directed toward specific regions of the LH-RH molecule. Appropriate antisera were used in assaying chromatographic column eluates and samples from the enzymic inactivation and chemical modification experiments.

Enzymic inactivation experiments

One to 20 ng immunoreactive chicken LH-RH was digested with 2 µg of enzyme in a total volume of 0.1 - 1 ml buffer. The digestions with subtilisin (Sigma), pronase P (Sigma), thermolysin (Sigma), carboxypeptidase A (Boehringer Mannheim) and carboxypeptidase B (Merck) were carried out in 0.02 M ammonium acetate, pH 7.5. Digestions with neuraminidase (Sigma), beta-glucuronidase (Miles) and papain (BDH) were in 0.02 M ammonium acetate, pH 5.0. Digestions with pyroglutamate aminopeptidase (Boehringer Mannheim) (100 µg enzyme) and protease from *S. aureus* V8 strain (Miles) were in 0.05 M potassium phosphate, pH 7.8. Digestion with alkaline phosphatase (Miles) was in 0.02 M ammonium acetate, pH 10.0. Digestion with pepsin (Sigma) was in 0.01 N HCl. Digestion with trypsin (2X recrystallised; BDH) was in 0.01 M ammonium acetate, pH 8.2. Digestion with chymotrypsin (Seravac) was in 0.01 M ammonium acetate containing 0.002 M ethylenediaminetetra-acetic acid, pH 8.4. Digestions with aminopeptidase M (Boehringer Mannheim) and aminopeptidase K (Sigma) were in 0.02 M ammonium acetate, pH 8.0. Digestion with post-proline-cleaving enzyme (gift from K. Bauer) was in 0.1 M phosphate containing 0.001 M ethylenediaminetetra-acetic acid and 0.002 M dithiothreitol, pH 7.4. Digestions were carried out in triplicate at 37°C for 24 h, and terminated by boiling for 15 min.

All enzymes were shown to be active under the conditions employed by reaction with appropriate substrates containing sensitive

cleavage points (synthetic mammalian LH-RH, analogues and fragments of LH-RH, and other peptides of known structure). Control samples of inactivated and active enzyme alone, and substrate with inactivated enzyme, were included in the studies. Samples were lyophilised, reconstituted in PBS-gel, and assayed for LH-RH immunoreactivity with appropriate antisera. For example, samples digested with post-proline-cleaving enzyme were assayed with antiserum R-42 to show whether there was cleavage post proline, and with antiserum 743 to show that the fragment LH-RH (1 - 9) remained intact. Chymotrypsin-digested samples for cation exchange HPLC analysis were lyophilised and reconstituted in HPLC buffer.

Chemical modification experiments

One to 10 ng immunoreactive chicken LH-RH was used in all reactions, which were carried out in triplicate. In the reaction with Pauly's reagent, 20 μ l diazotized sulfanilic acid (10 μ l 1% sulfanilic acid in 1 N HCl plus 10 μ l 0.5% aqueous sodium nitrite) was reacted with substrate in 100 μ l H₂O at 21°C for 30 min, followed by addition of 20 μ l 5% Na₂CO₃. In the reaction with ninhydrin, 10 μ l 2% ninhydrin in ethanol : 4 M sodium acetate, pH 5.0, (1 : 3) was reacted with substrate in 100 μ l H₂O at 37°C for 24 h. For the reaction with Sakaguchi reagent, 100 μ l of 0.01% α -Naphthol and 5% urea in ethanol was added to substrate in 100 μ l H₂O, followed by addition of 50 μ l 5% aqueous sodium hypobromite. The reaction mixture was incubated at 21°C for 24 h. Two mg of 1,2-cyclohexanedione (Koch-Light Laboratories) was reacted with substrate in 250 μ l 0.2 M aqueous KOH at 21°C for 24 h. Two mg of benzil (Sigma) was reacted with substrate in 250 μ l 0.2 M KOH in 80% ethanol. Eighteen μ g of nitrophenylsulphenylchloride (Sigma) was reacted with substrate in 250 μ l 50% acetic acid at 21°C for 24 h. 0.17 M ethoxyformic anhydride (Sigma) was reacted with substrate in

250 μ l H₂O at 21°C for 24 h.

All chemicals were shown to be active under the conditions employed by reaction with appropriate substrates containing sensitive amino acid residues (synthetic mammalian LH-RH, analogues and fragments of LH-RH, and other peptides of known structure). Control samples of reagents alone, and substrate alone, were included in the studies. Samples were lyophilised, reconstituted in PBS-gel containing an excess of the amino acid residue which is specifically modified by the particular chemical, and assayed for LH-RH immunoreactivity with appropriate antisera. For example, samples treated with Sakaguchi reagent were assayed with antiserum 1076 to show whether there was modification at Arg⁸, and with antiserum R-42 to show that the chemical did not alter the peptide in a nonspecific way.

Comparison of natural chicken LH-RH with synthetic [Gln⁸]LH-RH

[Gln⁸]LH-RH was synthesised by the solid-phase method of Merrifield (1963) and its structure was verified by chemical analyses (see section 4.3). The chromatographic and immunological properties of synthetic [Gln⁸]LH-RH were compared with those of natural chicken LH-RH, using the methods described above.

Partial purification of chicken hypothalamic LH-RH

The hypothalamic extract contained 128.3 mg total peptide and 998 ng immunoreactive LH-RH (antiserum 422). Non-immunoreactive peptides were eluted from the affinity column with 0.5 M ammonium acetate (Fig. 1) and immunoreactive LH-RH was then eluted with 1.5 N acetic acid (Fig. 1). Total yield was 969 ng immunoreactive LH-RH (antiserum 422) in 1.8 mg total peptide, representing a 70-fold purification.

Chromatographic analyses

Sephadex G-25 (Fig. 2A) and Bio-Gel P-2 (Fig. 2B) chromatography of 6 M urea-treated immunoreactive chicken LH-RH revealed a single peak of immunoreactivity which co-eluted with the synthetic mammalian decapeptide, indicating a similarity in molecular size. Since a single immunoreactive peak was obtained when Sephadex G-25 column fractions were assayed with both the middle - directed antiserum 1076 and the NH₂- and COOH-terminus-directed antiserum R-42 (Fig. 2A), it would appear that higher molecular weight forms of chicken LH-RH (if present) do not cross-react with these antisera. Reverse phase HPLC of immunoreactive chicken LH-RH revealed a single peak of immunoreactivity eluting earlier than synthetic mammalian LH-RH (Fig. 3A). In cation exchange HPLC (Fig. 3B) and CM32 carboxymethyl cellulose chromatography (Fig. 3C) immunoreactive chicken LH-RH eluted earlier than synthetic mammalian LH-RH, indicating that the chemical structure of chicken LH-RH is different and the peptide is less positively charged. Isoelectric focusing confirmed that chicken LH-RH (pI = 7.3) is less basic than synthetic mammalian LH-RH (pI = 9.1) (Fig. 4A).

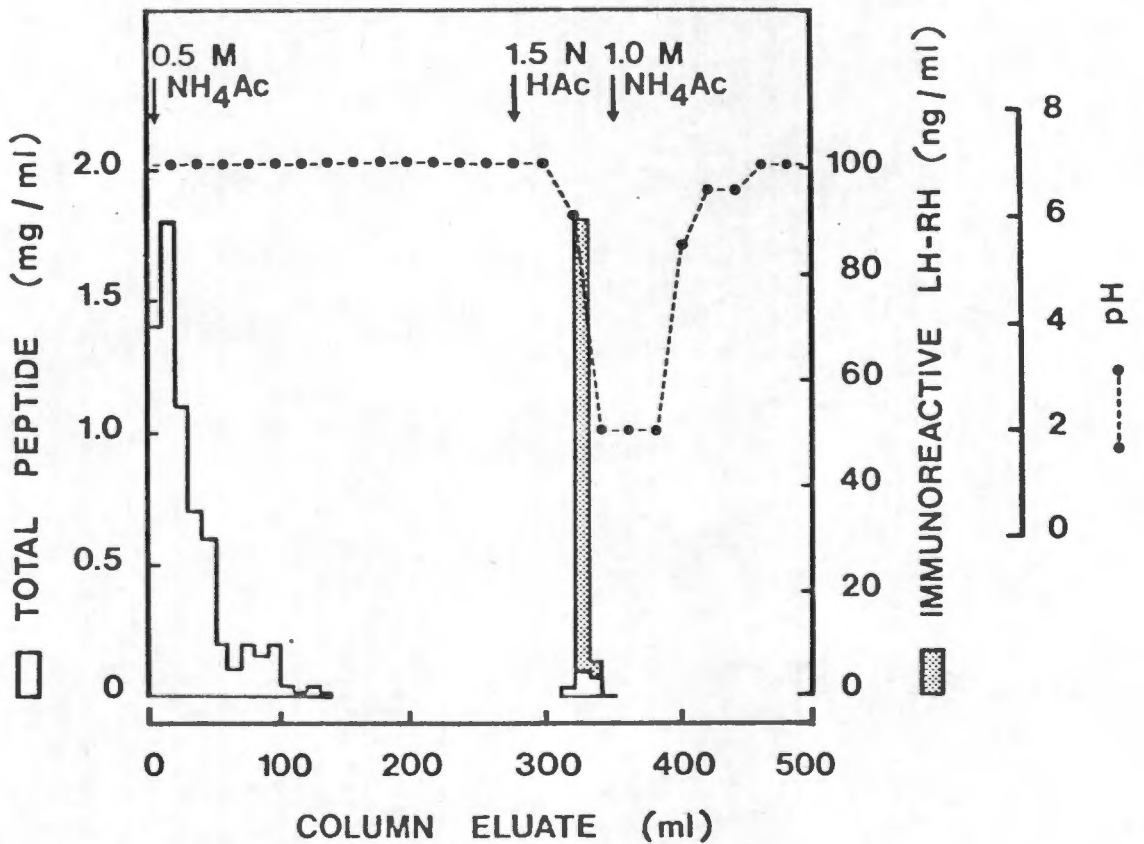


FIGURE 1

Affinity chromatography of immunoreactive chicken LH-RH on a Sepharose-anti-LH-RH 1076 column. Fractions were assayed for LH-RH immunoreactivity with antiserum 422, and for Folin-Lowry peptide immunoreactivity with bovine serum albumin as standard. NH₄Ac, ammonium acetate; HAc, acetic acid.

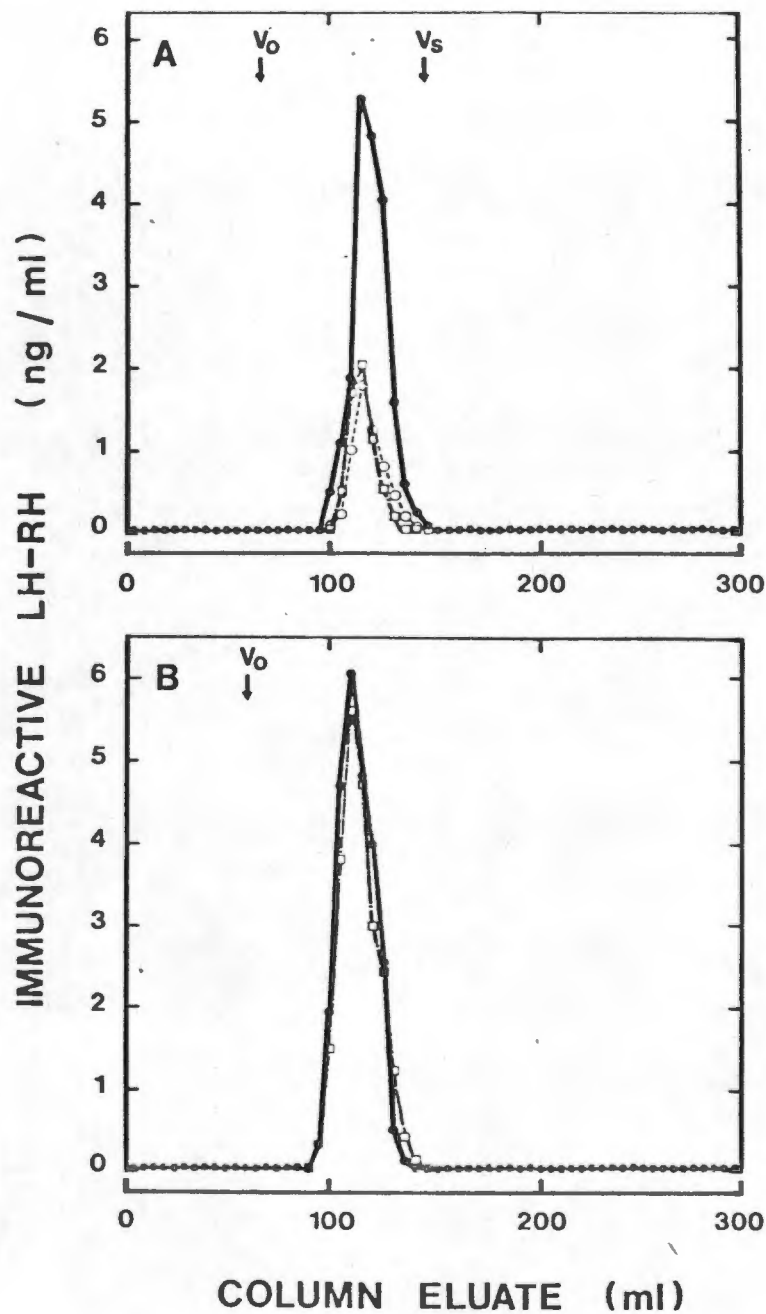
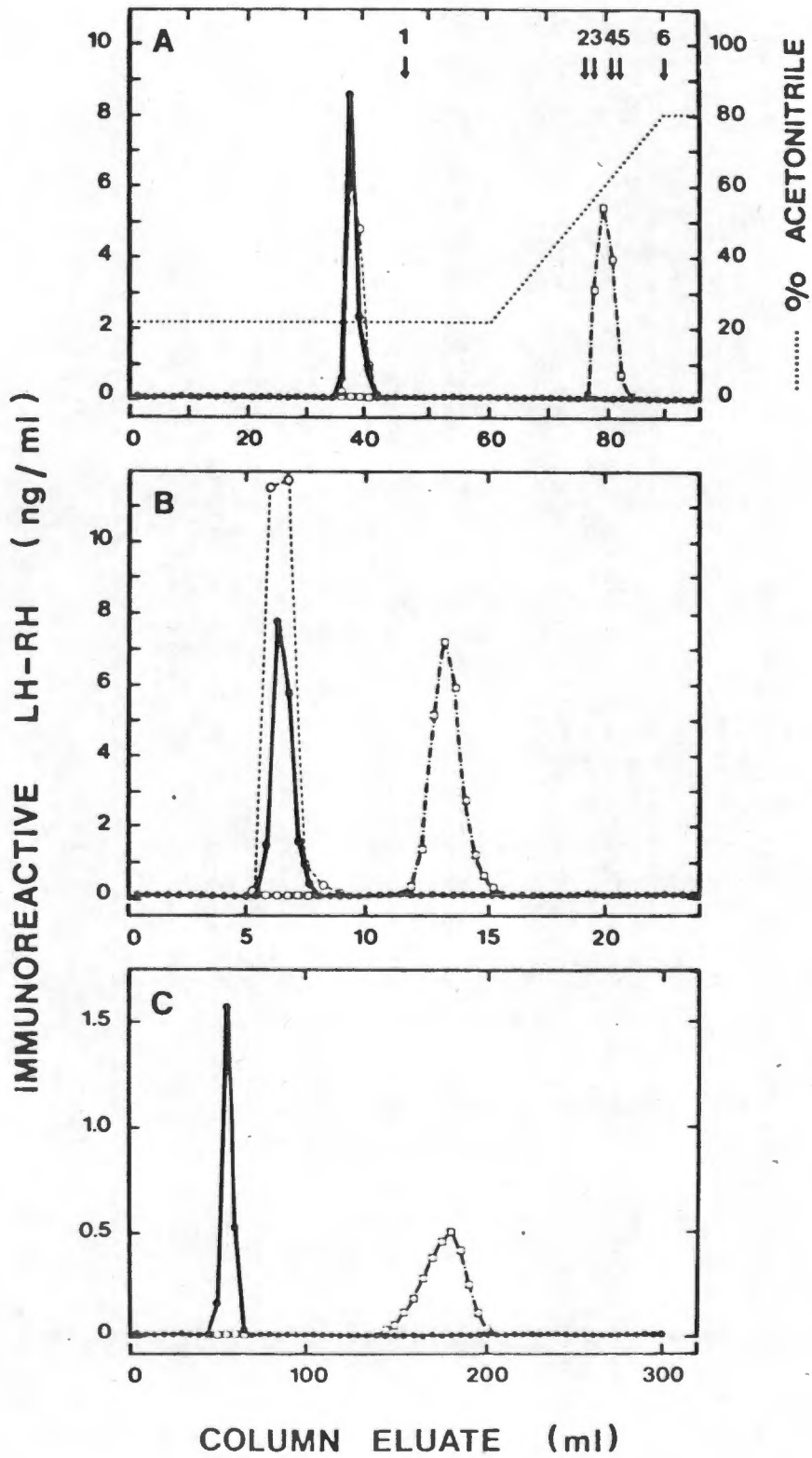


FIGURE 2

Gel filtration chromatography of immunoreactive chicken LH-RH and synthetic mammalian LH-RH. Void volume (V_0) and salt volume (V_s) are marked with arrows. A, Sephadex G-25 chromatography. Immunoreactive chicken LH-RH in fractions from a single column run was measured with both antiserum 1076 (36 ng; recovery was 89%) (○---○) and antiserum R-42 (118 ng; recovery was 92%) (●—●). Synthetic mammalian LH-RH in fractions was measured with antiserum 1076 (30 ng; recovery was 114%) (□-----□). B, Bio-Gel P-2 chromatography. Immunoreactive chicken LH-RH in fractions was measured with antiserum R-42 (143 ng; recovery was 87%) (●—●). Synthetic mammalian LH-RH in fractions was measured with antiserum 1076 (123 ng; recovery was 97%) (□-----□).

FIGURE 3

Reverse phase HPLC, cation exchange HPLC and CM32 carboxymethyl cellulose chromatography of immunoreactive chicken LH-RH, synthetic mammalian LH-RH and synthetic [Gln⁸]LH-RH. Immunoreactive chicken LH-RH (●—●) and synthetic [Gln⁸]LH-RH (O-----O) in fractions was measured with antiserum R-42. Synthetic mammalian LH-RH (□-----□) in fractions was measured with antiserum 1076. A, reverse phase HPLC separation of immunoreactive chicken LH-RH (20 ng; recovery was 91%), synthetic mammalian LH-RH (20 ng; recovery was 98%) and synthetic [Gln⁸]LH-RH (20 ng; recovery was 91%). Elution peaks of synthetic LH-RH analogues (detected by UV absorbance at 280 nm) are marked with arrows: 1 LH-RH-OH, 2 [Lys⁸]LH-RH, 3 [Ala⁹]LH-RH, 4 [Thr⁴]LH-RH, 5 [Phe⁷]LH-RH, 6 [Ile⁶]LH-RH. B, cation exchange HPLC separation of immunoreactive chicken LH-RH (7.5 ng; recovery was 87%), synthetic mammalian LH-RH (11 ng; recovery was 89%) and synthetic [Gln⁸]LH-RH (18.5 ng; recovery was 95%). C, CM32 carboxymethyl cellulose chromatography of immunoreactive chicken LH-RH (14 ng; recovery was 81%) and synthetic mammalian LH-RH (14 ng; recovery was 98%). In each of these chromatographic systems the relative elution volumes of chicken LH-RH, synthetic mammalian LH-RH and synthetic [Gln⁸]LH-RH were confirmed by chromatographing the three peptides separately and in combination in different runs. When column fractions were assayed with both antiserum R-42 and 1076, only a single immunoreactive peak was obtained; the elution volume was the same as those described above.



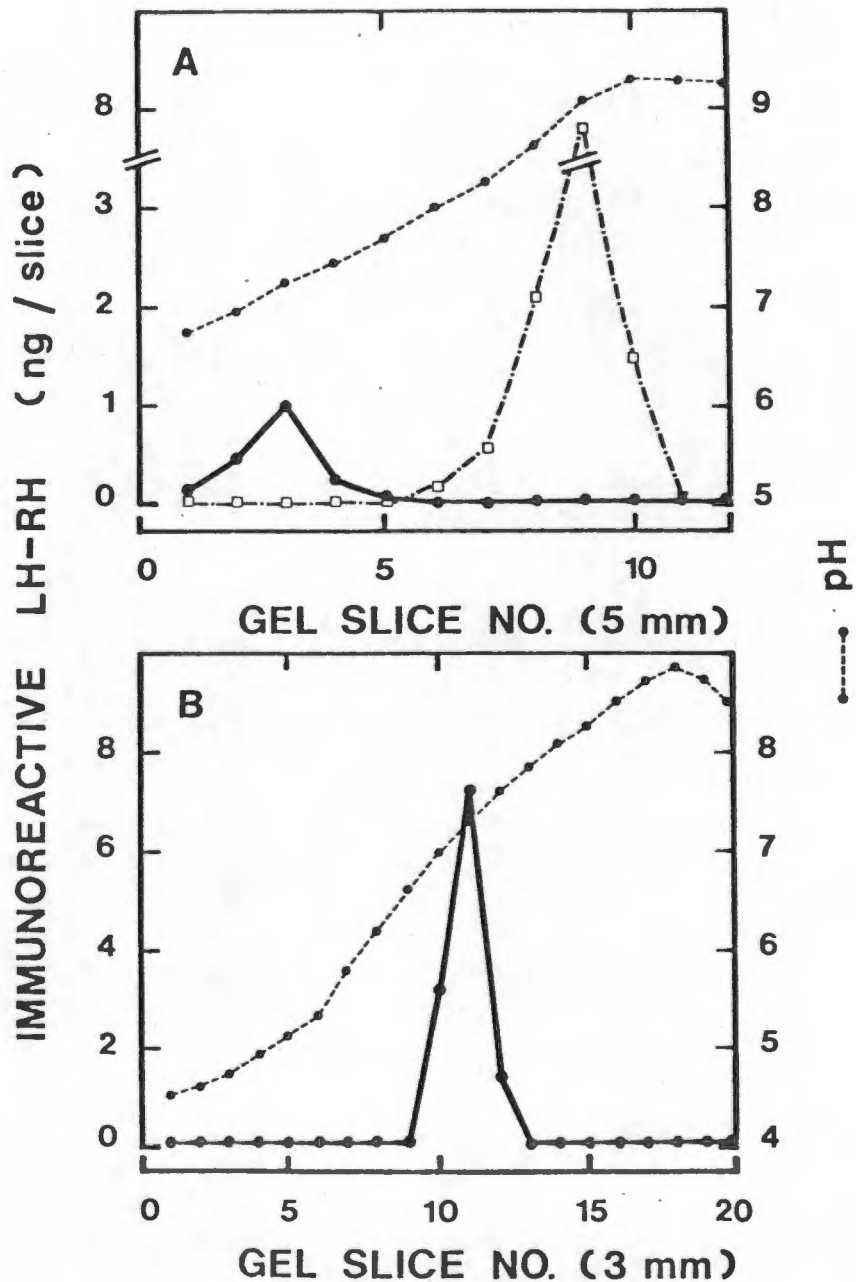


FIGURE 4

Isoelectric focusing of immunoreactive chicken LH-RH, synthetic mammalian LH-RH and synthetic [Gln⁸]LH-RH on polyacrylamide gel rods. A, immunoreactive chicken LH-RH (2.4 ng; recovery was 69%) (●—●) and synthetic mammalian LH-RH (15 ng; recovery was 81%) (□—□) eluted with Pharmalyte pH 3-10 plus Ampholine pH 9-11. Gel slices were assayed with antiserum 1076. The pI of synthetic mammalian LH-RH is lower than the theoretical value, because high pH conditions were not produced when using the pH range suitable for chicken LH-RH. B, immunoreactive chicken LH-RH spiked with synthetic [Gln⁸]LH-RH (7.5 ng each; recovery was 79%) (●—●) eluted with Ampholine pH 3.5-9.5. Gel slices were assayed with antiserum R-42.

Radioimmunoassay employing different antisera

For effective binding, antisera 1076 and 743 require residues within the region Trp³ to Pro⁹, antiserum 744 requires residues Arg⁸ to Gly¹⁰-NH₂, and antisera R-42 and 422 require both NH₂- and COOH-termini and tolerate certain alterations in the middle of the decapeptide (Fig. 5).

In radioimmunoassays employing antisera R-42 and 422, immunoreactive chicken LH-RH yielded displacement curves parallel to those of synthetic mammalian LH-RH (Fig. 6). However, in radioimmunoassays employing antisera 1076, 743 and 744 the immunoreactive chicken LH-RH displacement curves were clearly nonparallel to those of synthetic mammalian LH-RH, indicating nonidentity of chicken LH-RH with the mammalian peptide in those regions of the molecule which are required for binding by these antisera (Fig. 6). Furthermore, the relative quantitation of immunoreactive chicken LH-RH varied in radioimmunoassays employing the different antisera. Antisera R-42 and 422 consistently yielded higher values than did the other antisera (Fig. 6). Considering the regions of the LH-RH molecule which are required for binding by these antisera (Fig. 5), these data are consistent with an alteration in chicken LH-RH at position eight (arginine).

The possibility that nonparallelism was caused by contaminants in the affinity-purified immunoreactive chicken LH-RH (such as peptidases and substances binding LH-RH) was excluded by the demonstration that ¹²⁵I-LH-RH was neither degraded nor bound by the affinity-purified material. Moreover, when the affinity-purified immunoreactive chicken LH-RH was chromatographed in the systems described above and the column fractions assayed with different antisera (some data are not shown), only a single immunoreactive peak was obtained and the same relative quantitation was obtained with the different antisera. This emphasizes

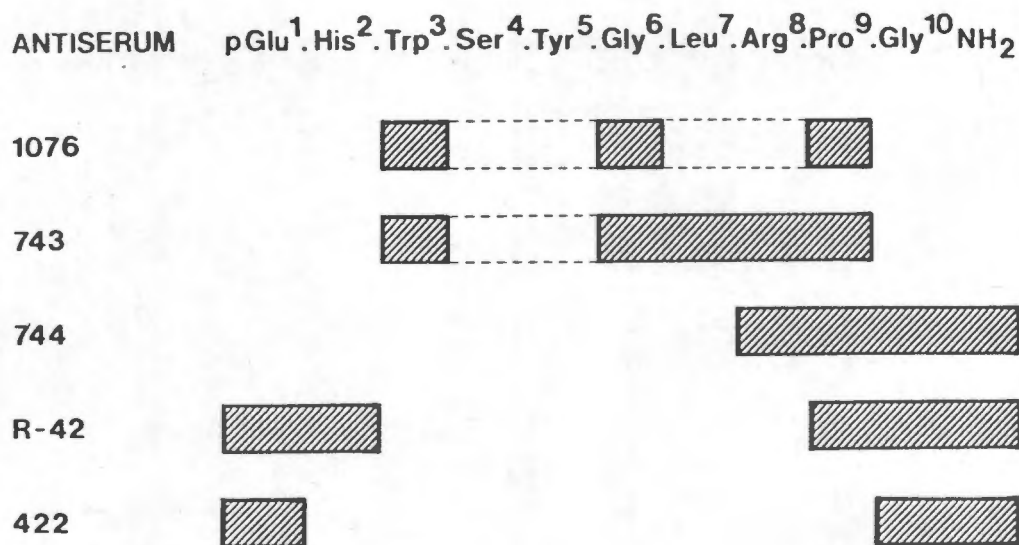


FIGURE 5

Regions of the LH-RH molecule which are essential for full immunoreactivity with each of five antisera, determined from cross-reactivity studies (see Table 2 and Copeland *et al.*, 1979). LH-RH is represented in linear form here; the NH₂- and COOH-termini are closely apposed in the three-dimensional conformation of the molecule (Momany, 1978).

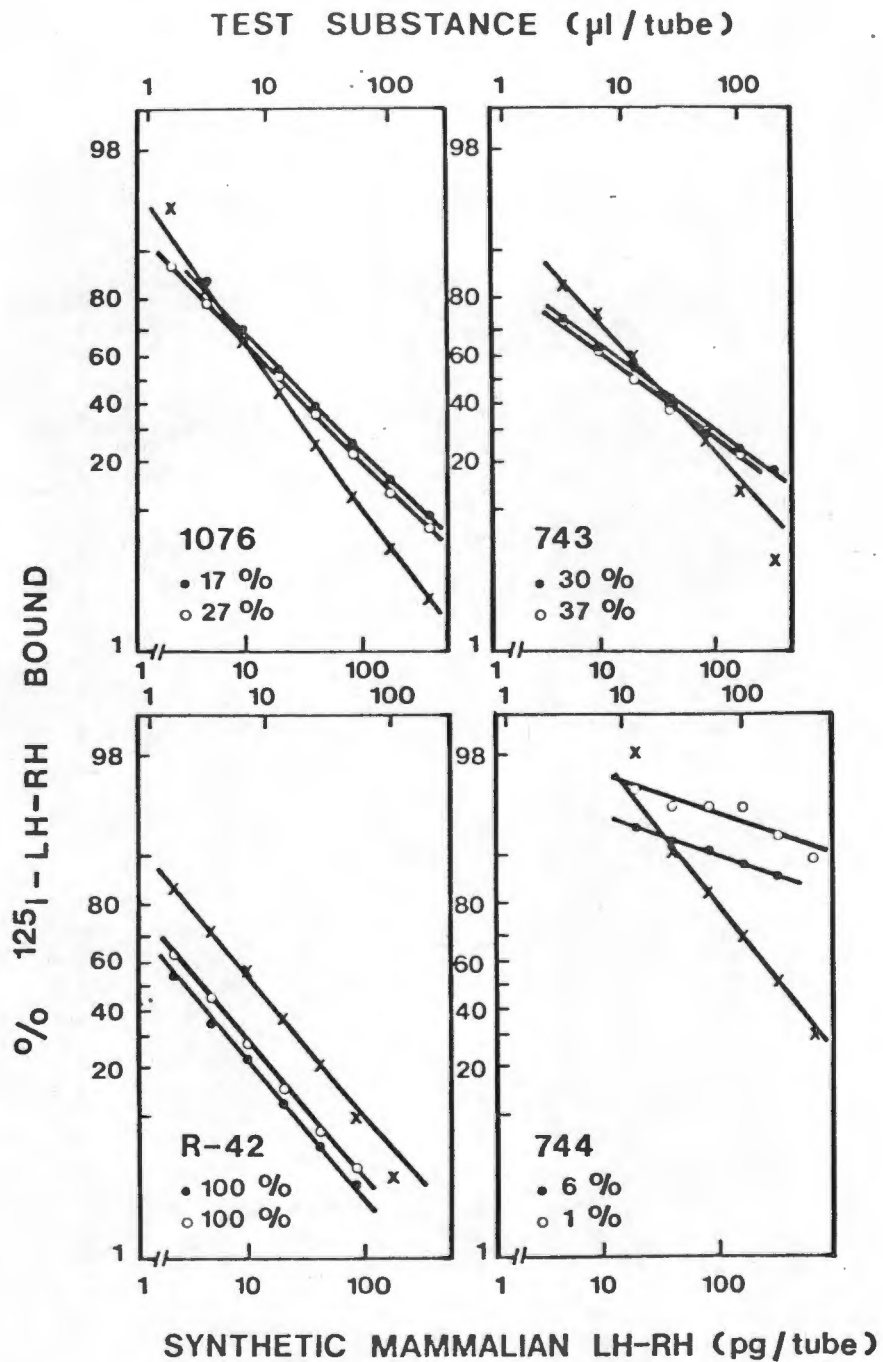


FIGURE 6

Comparative displacement of ^{125}I -LH-RH from antisera 1076, 743, R-42 and 744 by immunoreactive chicken LH-RH (●—●), synthetic mammalian LH-RH (x—x) and synthetic $[\text{Gln}^8]\text{LH-RH}$ (○—○). Quantitation of immunoreactive chicken LH-RH (●) and synthetic $[\text{Gln}^8]\text{LH-RH}$ (○) with the different antisera is expressed relative to the value obtained with antiserum R-42. The displacement curve of immunoreactive chicken LH-RH in radioimmunoassay with antiserum 422 was parallel to that of synthetic mammalian LH-RH, and quantitation was identical to that obtained with antiserum R-42 (data not shown).

that the different quantitation with various antisera is due to different interactions with the same molecule.

Enzymic inactivation experiments

The effect of various enzymes on the immunoreactivity of chicken LH-RH and synthetic mammalian LH-RH is shown in Table 3. Immunoreactivity of both substrates was unaffected by alkaline phosphatase, neuraminidase, beta-glucuronidase, pepsin, trypsin, carboxypeptidase A and B, and aminopeptidase M and K. However, incubation with papain, subtilisin, pronase P, thermolysin, chymotrypsin, post-proline-cleaving enzyme, protease from *S. aureus* V8 strain, and pyroglutamate aminopeptidase reduced or abolished the immunoreactivity of both substrates. Immunoreactivity of post-proline-cleaving enzyme-treated chicken and mammalian LH-RH was unaltered by carboxypeptidase A and B. Cation exchange HPLC of the chymotrypsin-generated fragments of immunoreactive chicken LH-RH and synthetic mammalian LH-RH revealed no peak of immunoreactivity (antiserum 744) of fragment (6-10) of chicken LH-RH, while chymotrypsin-generated fragment (6-10) of synthetic mammalian LH-RH co-eluted with the synthetic fragment LH-RH (6-10), indicating that chicken LH-RH differs from synthetic mammalian LH-RH in the region Gly⁶ to Gly¹⁰-NH₂.

Chemical modification experiments

The effect of chemical modification of specific amino acids on the immunoreactivity of chicken LH-RH and synthetic mammalian LH-RH is shown in Table 4. Immunoreactivity of both substrates was unaffected by treatment with ninhydrin, while treatment with Pauly's reagent and nitrophenylsulphenylchloride reduced the immunoreactivity of both substrates. The results from chemical modification with Sakaguchi reagent, 1,2-cyclohexanedione, benzil and ethoxyformic anhydride were

TABLE 3

Effect of enzymes on the immunoreactivity of chicken LH-RH and synthetic mammalian LH-RH

Enzyme treatment ^a	Antiserum used in radioimmunoassay	Percentage residual immunoreactive LH-RH	
		Chicken LH-RH	Synthetic mammalian LH-RH
Alkaline phosphatase	1076	105	76
Neuraminidase	1076	84	124
Beta-glucuronidase	1076	94	100
Pepsin	1076	92	84
Trypsin	1076	100	101
Carboxypeptidase A	1076	101	89
Carboxypeptidase B	1076	92	79
Carboxypeptidase A plus B	R-42	98 ^b	95 ^b
	743	112 ^b	97 ^b
Aminopeptidase M	1076	92	94
Aminopeptidase K	1076	89	99
Aminopeptidase M plus K	R-42	108	95
Papain	1076	2	2
Subtilisin	1076	0	1
Pronase P	1076	0	1
Thermolysin	1076	0	1
Chymotrypsin	1076	0	2
Post-proline-cleaving enzyme	R-42	0	0.6
	743	111	127
Protease from <i>S. aureus</i> V8 strain	R-42	52	29
Pyroglutamate aminopeptidase	R-42	41	12

^a Control samples showed enzymes did not interfere in the radioimmunoassay.

^b Substrates were pretreated with post-proline-cleaving enzyme.

TABLE 4

Effect of chemical modification of specific amino acids on the immunoreactivity of chicken LH-RH and synthetic mammalian LH-RH

Chemical reagent ^a	Antiserum used in radioimmunoassay	Percentage residual immunoreactive LH-RH	
		Chicken LH-RH	Synthetic mammalian LH-RH
Pauly's reagent	1076	0	0
Ninhydrin	R-42	98	88
Nitrophenylsulphenyl-chloride	1076	19	6
	R-42	20	19
	744	^b	100

^a Data on reagents which were nonspecific in their modification or which interfered in the radioimmunoassay have been excluded.

^b Level of chicken LH-RH (before and after treatment) was below the sensitivity limit of the radioimmunoassay with antiserum 744.

inconclusive. Interference by these reagents in the LH-RH radio-immunoassay did not allow meaningful interpretation of the data.

Comparison of natural chicken LH-RH with synthetic [Gln⁸]LH-RH

Synthetic [Gln⁸]LH-RH was shown to have identical chromatographic and immunological properties to natural chicken LH-RH. The retention times of synthetic [Gln⁸]LH-RH and natural chicken LH-RH were the same under identical reverse phase (Fig. 3A) and cation exchange (Fig. 3B) HPLC chromatographic conditions. The synthetic peptide was indistinguishable from the natural material in isoelectric focusing (Fig. 4B). In radioimmunoassays employing four region-specific antisera, the immunological properties of synthetic [Gln⁸]LH-RH were very similar to those of natural chicken LH-RH in terms of slope of displacement curves and relative quantitation with different antisera (Fig. 6).

Gel filtration chromatography indicated a single species of immunoreactive chicken LH-RH, identical in size to synthetic mammalian decapeptide LH-RH. However, from reverse phase and cation exchange HPLC, CM32 carboxymethyl cellulose chromatography and isoelectric focusing it is apparent that immunoreactive chicken LH-RH is not identical to synthetic mammalian LH-RH, the chicken peptide being less positively charged as previously demonstrated (Jackson, 1971a, 1971b; King and Millar, 1979a, 1980, 1981a; Hattori *et al.*, 1980).

The structural difference in chicken LH-RH was investigated by radioimmunoassay employing region-specific antisera. The parallelism in the displacement curves of immunoreactive chicken LH-RH and synthetic mammalian LH-RH in radioimmunoassays with the NH₂- and COOH-terminus-directed antisera R-42 and 422, together with the higher quantitation with these antisera, suggests that the chicken LH-RH molecule has an NH₂-terminal pGlu¹ and a COOH-terminal Gly¹⁰-NH₂ as in synthetic mammalian LH-RH. Nonparallel displacement curves were yielded in radioimmunoassays employing the middle-directed antisera 1076 and 743, and the COOH-terminus-directed antiserum 744, and quantitation was much lower with these antisera. It is evident, then, that chicken LH-RH differs from synthetic mammalian LH-RH in that region of the molecule which antisera 1076, 743 and 744 require for binding but which is not required for binding by antisera R-42 and 422, i.e. amino acid residue eight (arginine). Significantly, antiserum 744 which requires residues Arg⁸ to Gly¹⁰-NH₂ for binding gave the lowest quantitation and the greatest deviation from parallelism. Alteration in a remote region of the LH-RH molecule could also conceivably cause conformational change in LH-RH at Arg⁸, but quantitation with antisera binding regions remote from Arg⁸ was not low.

Results from enzymic inactivation and chemical modification studies were consistent with an amino acid substitution in position eight (arginine). The possibility that the structural difference in chicken LH-RH represents a post-translational alteration such as phosphorylation of His², Ser⁴ or Tyr⁵, glycosylation of Ser⁴, or sulphation of Tyr⁵, was excluded due to the lack of effect of alkaline phosphatase, neuraminidase and beta-glucuronidase (containing aryl sulphatase). Studies with proteolytic enzymes also yielded information on the structural homology and differences between chicken LH-RH and mammalian LH-RH. The fragment LH-RH(6-10) was generated from synthetic mammalian LH-RH by chymotrypsin digestion, and shown to elute at 13.5 ml in cation exchange HPLC. Such a fragment was not detected (antiserum 744) among the chymotrypsin-generated fragments of immunoreactive chicken LH-RH, again indicating that the structural difference in the chicken peptide resides in the region Gly⁶ to Gly¹⁰-NH₂. Inactivation by Pauly's reagent and nitrophenylsulphenylchloride indicates the presence of Trp³ and possibly also His² and Tyr⁵, supporting our observation that the structural difference in chicken LH-RH is in the COOH-terminal half of the molecule. The lack of effect of aminopeptidase, carboxypeptidase and ninhydrin, and inactivation by pyroglutamate aminopeptidase, confirms that chicken LH-RH has a blocked NH₂- and COOH-terminus in accordance with the observation that antisera R-42 and 422, which have an absolute requirement for pGlu¹ and Gly¹⁰-NH₂, bind chicken LH-RH avidly. The presence of proline at position nine is indicated by inactivation of chicken LH-RH by post-proline-cleaving enzyme (immunoreactivity was abolished when measuring with antiserum R-42 but not with antiserum 743), and by the lack of effect of carboxypeptidase A and B on post-proline-cleaving enzyme-treated chicken LH-RH.

The foregoing data provide firm evidence that chicken LH-RH differs from mammalian LH-RH by an amino acid substitution at position eight (arginine). Attempts to confirm the absence of Arg⁸ in chicken LH-RH by attempting chemical modification (Sakaguchi reagent, 1,2-cyclohexanedione and benzil) were unsuccessful. Chicken LH-RH is clearly less positively charged than the mammalian peptide (as shown by chromatography), and the isoelectric point of 7.3 (Fig. 4) is compatible with a neutral amino acid substitution for Arg⁸ (Greenstein and Winitz, 1961). Structural studies on mammalian LH-RH have shown that the side chain of Arg⁸ is in close vicinity to the side chains of His² and Tyr⁵, and that these side chains are linked by hydrogen bonds and form a combined unit important for biological action (Shinitzky and Fridkin, 1976). A likely neutral amino acid substitution at position eight compatible with these structural requirements is glutamine. Moreover, of the neutral amino acids, the probability of a glutamine/arginine interchange is considerable (Dayhoff *et al.*, 1972).

In view of these firm indications that chicken hypothalamic LH-RH differs from the mammalian hypothalamic peptide by the substitution of glutamine for arginine at position eight, we synthesised [Gln⁸]LH-RH for comparison with the natural material. Synthetic [Gln⁸]LH-RH was found to have identical chromatographic and immunological properties to the natural chicken peptide in chromatographic systems capable of separating a wide range of LH-RH analogues (see Fig. 3A). Moreover, we have also established that synthetic [Gln⁸]LH-RH and natural chicken LH-RH have identical potency in stimulating luteinizing hormone release from dispersed chicken anterior pituitary cells and cultured ovine anterior pituitary cells (see section 4.4). We propose, therefore, that the structure of chicken hypothalamic

LH-RH is :

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly-NH₂.

The low biological activity of mammalian LH-RH in birds and fish may be due to a structural difference at residue eight of LH-RH in these vertebrate classes. Studies have, therefore, been initiated to investigate the gonadotropin-releasing activity and receptor binding characteristics of synthetic [Gln⁸]LH-RH in submammalian vertebrates.

3.0

ISOLATION AND CHARACTERISATION OF CHICKEN HYPOTHALAMIC

LUTEINIZING HORMONE-RELEASING HORMONE

3.1

SUMMARY

Avian luteinizing hormone-releasing hormone (LH-RH) has been isolated from 249,000 chicken hypothalami and shown to differ structurally from mammalian hypothalamic LH-RH. Purification was achieved by acetic acid extraction, anti-LH-RH affinity chromatography, and cation exchange and reverse phase high performance liquid chromatography (HPLC). The isolated peptide eluted as a single peak on reverse phase HPLC. Acid hydrolysis of the peptide yielded integral molar ratios of amino acids and a composition identical to that of mammalian decapeptide LH-RH, except for the presence of an additional glutamic acid residue and the absence of arginine. The isoelectric point of chicken LH-RH (7.3) is consistent with the glutamic acid representing a glutamine residue. We therefore synthesised [Gln⁸]LH-RH, and established that it has identical chromatographic properties to natural chicken LH-RH. These studies indicate that the structure of chicken hypothalamic LH-RH is:

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly-NH₂.

The decapeptide LH-RH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂), originally isolated from porcine hypothalamus (Matsuo *et al.*, 1971a) and later synthesised (Matsuo *et al.*, 1971b), appears to have lower gonadotropin-releasing activity in submammalian vertebrates than in mammals. Crude hypothalamic extracts from birds, reptiles, amphibians and fish, however, contain significant gonadotropin-releasing activity, and LH-RH-like immunoreactivity has been demonstrated in the hypothalamus of these vertebrates by radioimmunoassay and immunocytochemistry (for reviews, see Ball, 1981; Jackson, 1981; King and Millar, 1981a). We have shown that immunoreactive amphibian hypothalamic LH-RH is identical to the mammalian decapeptide in chromatographic properties and in its interaction with region-specific LH-RH antisera, whilst immunoreactive LH-RHs from avian, reptilian, and piscine hypothalamus are structurally different from mammalian hypothalamic LH-RH (King and Millar, 1979a, 1980, 1981a). These structural differences of LH-RH-like peptides in submammalian vertebrates have not been determined. We have characterised chicken hypothalamic LH-RH using partially purified material, and established that the peptide differs from mammalian hypothalamic LH-RH at position eight, arginine being substituted by glutamine (see section 2). This section describes the isolation and amino acid composition of homogeneously pure chicken hypothalamic LH-RH, and confirms that glutamine substitutes for the arginyl residue at position eight in mammalian hypothalamic LH-RH.

Extraction

Fragments of 249,000 chicken (*Gallus domesticus*) hypothalami consisting of the median eminence and pituitary stalk were dissected, frozen on dry ice, and lyophilised. Three batches of lyophilised hypothalami were defatted with petroleum ether (40-60°C) at 4°C, and extracted with 2 N acetic acid at 4°C using an ultraturrax homogeniser and then a glass homogeniser with a teflon pestle. The homogenate was centrifuged at 18,000 g for 1 h at 4°C, and the insoluble residue was re-extracted four times. The supernatants from the five extractions were pooled, and aliquots were taken for radioimmunoassay and for total peptide determination using bovine serum albumin as standard (Lowry *et al.*, 1951). The supernatant extract was then lyophilised.

Affinity chromatography

Antiserum 1076 was generated in a rabbit against synthetic mammalian LH-RH conjugated with *Haliotis* sp. haemocyanin by carbodiimide condensation (Hendricks *et al.*, 1975). The antiserum requires residues within the region Trp³ to Pro⁹ of synthetic mammalian LH-RH for effective binding (Millar *et al.*, 1978; Table 2). The gamma globulin fraction of antiserum 1076 obtained after ammonium sulphate precipitation was immobilised on cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals) at a concentration of 0.3 ml antiserum equivalents of gamma globulin per g of Sepharose. The retention capacity of the Sepharose-anti-LH-RH 1076 column (3.5 x 26 cm) was 8.3 µg of synthetic mammalian LH-RH. Due to this limited retention capacity it was necessary to process the supernatant extract in ten

separate runs. The lyophilised supernatant extract was reconstituted in 0.5 M ammonium acetate, pH 7.0, and cycled through the column for 6 to 8 h. The column was washed with 0.5 M ammonium acetate to remove unbound peptides, and the bound material was eluted with 1.5 N acetic acid. Aliquots of fractions were taken for radioimmunoassay and for Folin-Lowry total peptide determination. Fractions with LH-RH immunoreactivity from all ten runs were pooled and lyophilised.

High performance liquid chromatography (HPLC)

Two different systems were used on a Model SP 3500B liquid chromatograph (Spectra-Physics) equipped with a Model 770 spectrophotometric detector (Spectra-Physics). The first HPLC system consisted of a Partisil PXS 10/25 SCX cation exchange column (0.4 x 25 cm; Whatman) and a mobile phase of 10% ethanol in 0.05 M ammonium acetate, pH 5.6. Lyophilised affinity-purified immunoreactive LH-RH was reconstituted in HPLC buffer and eluted in twelve separate runs. Flow rate was 1.6 ml/min. Absorbance at 280 nm was monitored at 0.2 units full scale. Fractions of 1.6 ml were collected and aliquots of 5 μ l were taken for radioimmunoassay. Immunoreactive fractions from all twelve runs were pooled and lyophilised. The second HPLC system consisted of a Spherisorb ODS reverse phase column (0.4 x 25 cm; 5 μ m particle size; Phase Separations) and a mobile phase of 22% acetonitrile in 0.01 M ammonium acetate, pH 4.0. Lyophilised immunoreactive LH-RH from the cation exchange HPLC system was reconstituted in HPLC buffer and eluted in four separate runs. Flow rate was 1.5 ml/min. Absorbance at 280 nm was monitored at 0.04 units full scale. Fractions of 1.5 ml were collected and aliquots of 5 μ l were taken for radioimmunoassay. Immunoreactive fractions from all four runs were pooled and lyophilised.

Radioimmunoassay of LH-RH

LH-RH was measured as described in section 2.3. Antiserum R-42 (gift from T.M. Nett and G.D. Niswender) was used throughout the study since it yields chicken hypothalamic LH-RH displacement curves parallel to that of mammalian LH-RH and apparently estimates chicken LH-RH near quantitatively (see sections 2.3 and 2.4). For effective binding, the antiserum requires both NH₂- and COOH-termini and tolerates certain alterations in the middle of the decapeptide (Nett *et al.*, 1973; Copeland *et al.*, 1979).

Lyophilised aliquots of extracts and chromatographic column fractions were reconstituted in phosphate-buffered saline containing gelatin (0.04 M phosphate, 0.15 M NaCl, 0.01 M disodium ethylenediaminetetra-acetic acid and 0.015 M NaN₃, pH 7.0, with 0.1% gelatin) and assayed for LH-RH immunoreactivity.

Amino acid analysis

Purified immunoreactive LH-RH (0.5 µg) was hydrolysed in 100 µl constant boiling 6 N HCl containing 2% thioglycolic acid in sealed evacuated tubes at 110°C for 16 h. The hydrolysate was lyophilised, reconstituted in 0.15 M lithium citrate buffer, pH 2.2, and subjected to amino acid analysis (Beckman Instruments Model 121-MB).

Comparison of natural chicken LH-RH with synthetic [Gln⁸]LH-RH

[Gln⁸]LH-RH was synthesised by the solid-phase method of Merrifield (1963) and its structure was verified by chemical analyses (see sections 4.3 and 4.4). The chromatographic properties of synthetic [Gln⁸]LH-RH on cation exchange and reverse phase HPLC were determined using the methods described above for the purification of natural chicken LH-RH. The isoelectric points of natural chicken LH-RH and

synthetic [Gln⁸]LH-RH were determined on polyacrylamide gel rods as described in section 2.3.

Immunoreactive chicken hypothalamic LH-RH was purified two million-fold using the scheme outlined in Table 5. The hypothalamic acetic acid extract contained 7.5 g total peptide and 33.7 μ g immunoreactive LH-RH. In affinity chromatography, non-immunoreactive peptides were eluted with 0.5 M ammonium acetate and immunoreactive LH-RH was then eluted with 1.5 N acetic acid (Fig. 7). The affinity-purified immunoreactive LH-RH was subjected to cation exchange HPLC which revealed a major peak of immunoreactive LH-RH at 7 - 8 min (11.2 - 12.8 ml) and an earlier-eluting minor immunoreactive peak at 4 min (6.4 ml) (Fig. 8). The major immunoreactive LH-RH peak was further purified by reverse phase HPLC. A single peak of immunoreactive LH-RH co-eluted with a single sharp absorbance peak at 23 - 24 min (34.5 - 36.0 ml) (Fig. 9), indicating that the isolated peptide was essentially pure.

The LH-RH was homogeneous, as judged by amino acid analysis (Table 6) demonstrating integral molar ratios of amino acids. The total amount of LH-RH peptide determined by amino acid analysis (0.48 μ g) agreed closely with the value obtained by radioimmunoassay (0.51 μ g) of the same material. The amino acid composition, determined after acid hydrolysis, was : Ser 1, Glu 2, Pro 1, Gly 2, Leu 1, Tyr 1, Trp 1 and His 1 (Table 6).

The chromatographic properties of natural chicken LH-RH and synthetic [Gln⁸]LH-RH were identical on cation exchange and reverse phase HPLC, and their isoelectric points were the same (Table 7).

TABLE 5

Summary of purification scheme for chicken hypothalamic LH-RH

Purification step	Dry weight (g)	Total peptide (mg)	Immuno- reactive LH-RH (μ g)	Recovery of LH-RH at each step (%)
Lyophilised hypothalami	103.3	57,120.0	-	-
2 N acetic acid extract	22.2	7,461.0	33.7	100
Affinity chromatography	0.100	36.2	24.4	73
Cation exchange HPLC	-	-	19.5	80
Reverse phase HPLC	-	0.016 ^a	17.4	90

^a Determined by amino acid analysis.

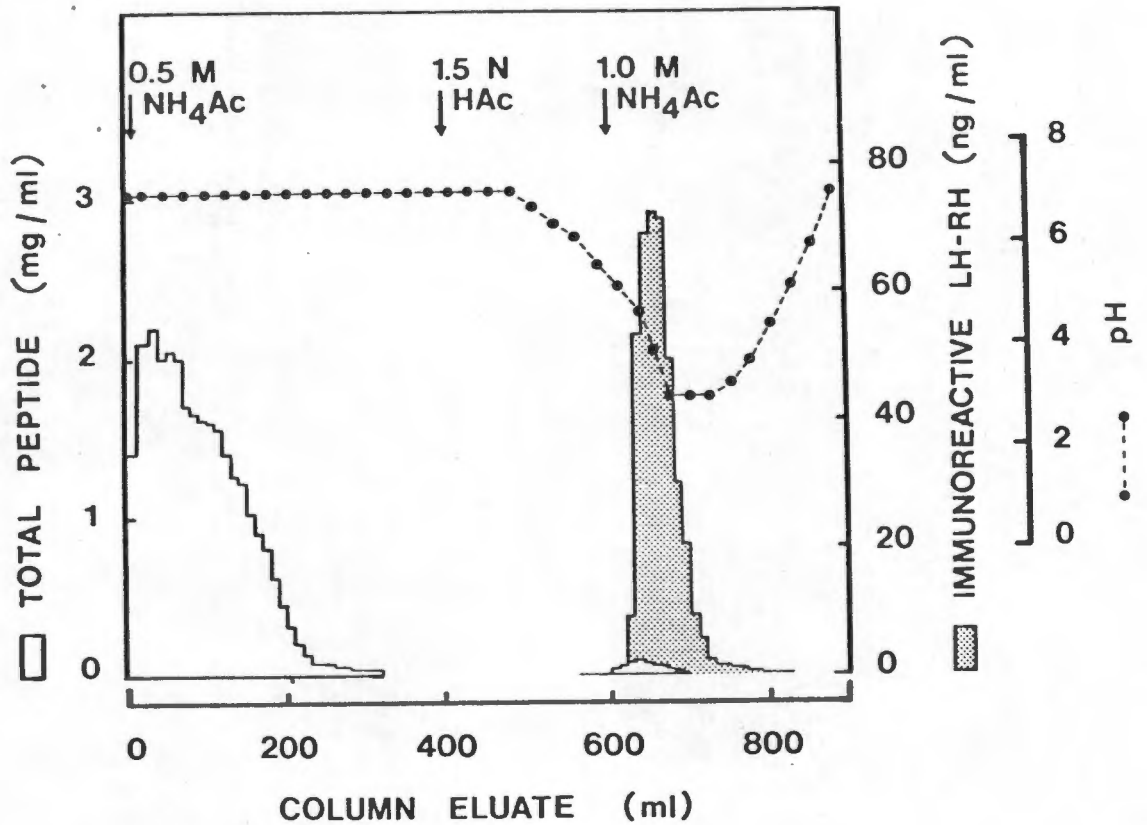


FIGURE 7

Affinity chromatography of immunoreactive chicken LH-RH on a Sepharose-anti-LH-RH 1076 column. Aliquots of fractions were assayed for LH-RH immunoreactivity with antiserum R-42, and for Folin-Lowry peptide using bovine serum albumin as standard. NH₄Ac, ammonium acetate; HAc, acetic acid. One of ten affinity runs is shown here; a similar pattern was seen in all column runs.

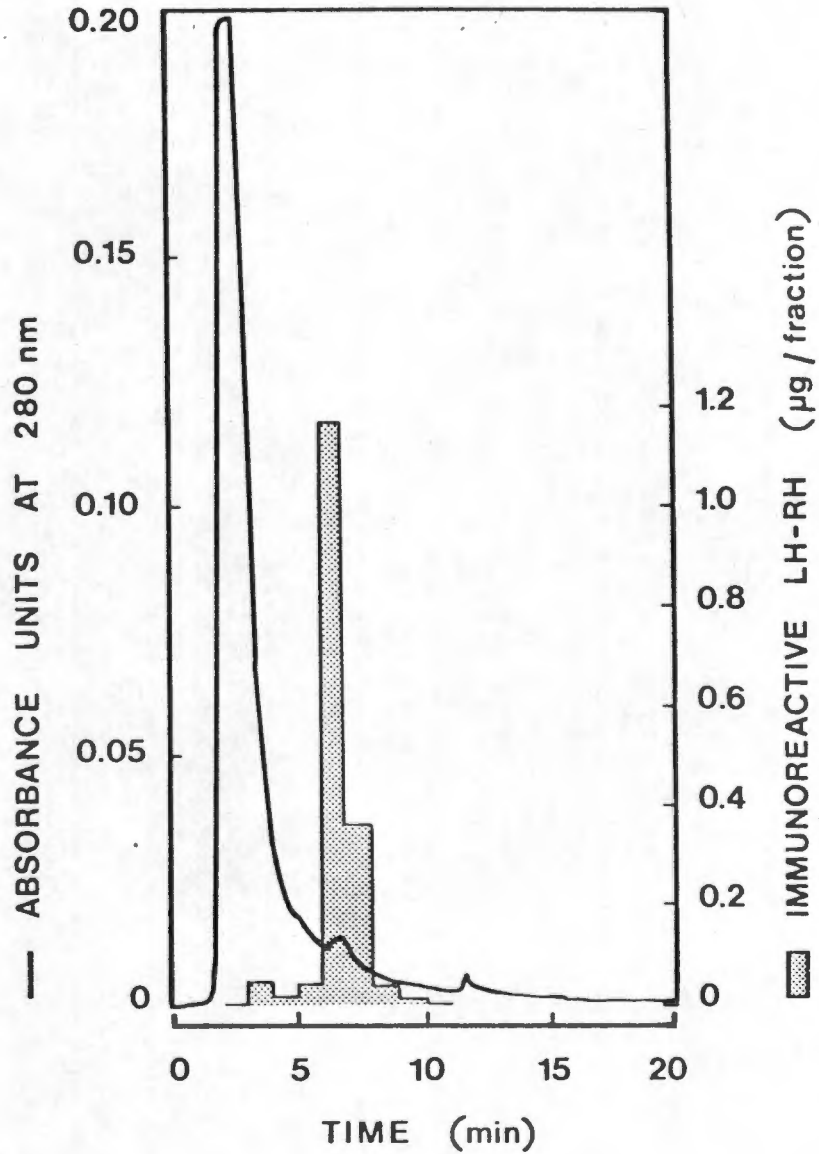


FIGURE 8

Cation exchange HPLC separation of affinity-purified immunoreactive chicken LH-RH using 10% ethanol in 0.05 M ammonium acetate, pH 5.6. Aliquots of fractions were assayed for LH-RH immunoreactivity with antiserum R-42. Absorbance at 280 nm was recorded at a setting of 0.2 units full scale. One of twelve cation exchange HPLC runs is shown here; all column runs generated similar UV absorbance peaks.

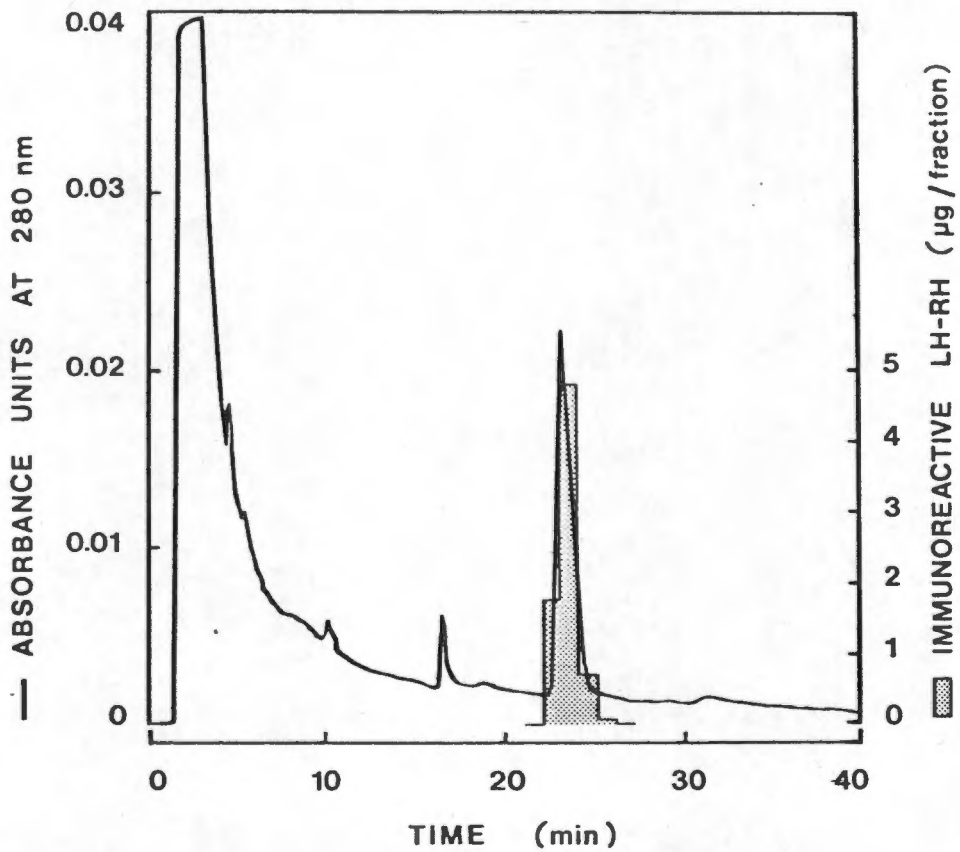


FIGURE 9

Reverse phase HPLC separation of cation exchange HPLC-purified immunoreactive chicken LH-RH using 22% acetonitrile in 0.01 M ammonium acetate, pH 4.0. Aliquots of fractions were assayed for LH-RH immunoreactivity with antiserum R-42. Absorbance at 280 nm was recorded at a setting of 0.04 units full scale. One of four reverse phase HPLC runs is shown here; all column runs generated similar UV absorbance peaks.

TABLE 6

Amino acid composition of acid hydrolysate
of chicken hypothalamic LH-RH

Amino acid	nmol/0.5 µg	Amino acid ratio ^a	Integral residues
Serine	0.44	0.92	1
Glutamic acid	1.00	2.08	2
Proline	0.53	1.10	1
Glycine	0.99	2.06	2
Leucine	0.48	1.00	1
Tyrosine	0.46	0.96	1
Tryptophan	0.47	0.98	1
Histidine	0.41	0.85	1

^a Molar ratios of amino acids were normalised to 1.0 for Leu.

TABLE 7

Comparison of the chromatographic properties of natural chicken LH-RH, synthetic [Gln⁸]LH-RH and synthetic mammalian LH-RH

Chromatographic system	Elution position		
	Natural chicken LH-RH	Synthetic [Gln ⁸]LH-RH	Synthetic mammalian LH-RH
Cation exchange HPLC	7	7	>20
(min)	4.2 ^a	4.2 ^a	8.5 ^a
Reverse phase HPLC	24	24	>40 ^b
(min)			
Isoelectric focusing	7.3 ^c	7.0 ^d	> 9.1 ^c
(pI)			> 8.9 ^d

^a Eluted with 10% ethanol in 0.2 M ammonium acetate, pH 4.6.

^b Synthetic mammalian LH-RH did not elute in a 40-min isocratic run of 22% acetonitrile in 0.01M ammonium acetate, pH 4.0, but eluted at 53 min when this was followed by a 20-min linear gradient of 22% to 80% acetonitrile in 0.01 M ammonium acetate, pH 4.0.

^c Ampholytes used were Pharmalyte pH 3-10 and Ampholine pH 9-11.

^d Ampholyte used was Ampholine pH 3.5-9.5.

This section describes the first biochemical characterisation of hypothalamic LH-RH from submammalian vertebrates. The isolation of LH-RH from hypothalami presents difficulties in view of the low total content and concentration of the peptide. This is particularly so in the avian hypothalamus in which the immunoreactive LH-RH content is considerably lower than in mammals. In the hypothalamus of the adult chicken and pigeon the content of immunoreactive LH-RH as measured with antiserum 422, which requires the NH₂- and COOH-termini of the molecule for binding, was 2.8-4.3 ng (King and Millar, 1980). In the present study using hypothalami from immature chickens and dissection of a more discrete region (median eminence and pituitary stalk) to simplify purification, the hypothalamus contained only 0.135 ng. It was, therefore, necessary to process several hundred thousand hypothalami in order to isolate sufficient material for structural characterisation. A number of chromatographic systems were tested in studies preliminary to the final purification scheme. From these we selected a combination of anti-LH-RH affinity chromatography followed by only two HPLC systems which were sufficient to yield homogeneously pure peptide totalling 17.4 µg. This represented a purification of two million-fold with an overall recovery from starting material of 51%. Contamination with non-LH-RH material as determined by amino acid analysis was less than 1%.

Amino acid analysis of the acid-hydrolysed peptide demonstrated the presence of the same ratio of amino acids present in mammalian LH-RH except for an absence of arginine and with an additional glutamic acid residue. The isoelectric point of chicken LH-RH (7.3) is consistent with a neutral amino acid replacing arginine at position

eight in the peptide. Thus glutamic acid is most likely represented as glutamine in the peptide.

In view of the structure of LH-RH (blocked NH_2 - and COOH - termini and the presence of a proline residue at position nine), attempts to demonstrate glutamine by amino acid analysis of enzymic hydrolysates of the peptide and by microsequencing techniques were unsuccessful and were not pursued further owing to scarcity of material. Microsequencing studies were in collaboration with K. Bauer and J. Salnikow, Technische Universität Berlin, West Germany. For solid-phase microsequencing, the COOH -terminal $\text{Gly}^{10}\text{-NH}_2$ and NH_2 -terminal pGlu^1 were first removed by incubation with post-proline-cleaving enzyme and pyroglutamate aminopeptidase. Various approaches to coupling the COOH -terminal Pro^9 to aminopropyl glass were attempted but all were unsuccessful. We therefore adopted the conventional liquid-phase sequencing technique after mild chymotrypsin cleavage of two μg of purified chicken LH-RH to generate presumed fragments LH-RH(1-5) and LH-RH(6-10). On Edman degradation residues six and seven, glycine and leucine, were identified in the first and second cycles respectively. The crucial position eight amino acid residue was undetectable in the third cycle.

Indirect studies strongly support the conclusion that the only difference between chicken and mammalian LH-RH resides in the replacement of glutamine for arginine in the eight position with the retention of all other structural features including pGlu^1 and $\text{Gly}^{10}\text{-NH}_2$. The interaction of region-specific antisera with the peptide, and studies on the effects of chemical modification of specific amino acid residues and cleavage of specific peptide bonds on immunoreactivity, all support this conclusion (see sections 2.4 and 2.5). It is also

pertinent that a common neutral amino acid substitution for arginine is glutamine (Dayhoff *et al.*, 1972). Glutamine is also an appropriate neutral amino acid substitution for arginine if some semblance of the combined unit of hydrogen bond interactions between the side chains of His², Tyr⁵ and Arg⁸ in mammalian LH-RH is to be retained (Shinitzky and Fridkin, 1976). These authors believe this combined unit is important for biological activity. Finally, the putative sequence of chicken LH-RH has been synthesised by conventional solid-phase methodology and shown to have identical physico-chemical properties to the natural chicken peptide (see sections 4.3 and 4.4). In contrast, other position eight-substituted LH-RH analogues had distinctly different properties.

The nature of hypothalamic LH-RH in submammalian vertebrates has been studied by radioimmunoassay and chromatography (for reviews, see Ball, 1981; Jackson, 1981; King and Millar, 1981a). Several reports indicated a structural identity between mammalian hypothalamic LH-RH and chicken and frog hypothalamic LH-RHs (Deery, 1974; Jeffcoate *et al.*, 1974; Alpert *et al.*, 1976) whilst others reported differences in the physico-chemical and biological properties of chicken and teleost fish hypothalamic LH-RHs (Jackson, 1971a, 1971b; Hattori *et al.*, 1980; Jackson, 1981; Barnett *et al.*, 1982). On the basis of chromatographic properties and using region-specific antisera we demonstrated that avian, reptilian, and piscine hypothalamic LH-RHs differed structurally from mammalian hypothalamic LH-RH, the difference being in the region Gly⁶-Leu⁷-Arg⁸ (King and Millar, 1979a, 1980, 1981a). Amphibian hypothalamic LH-RH was, however, identical to the mammalian hypothalamic peptide (King and Millar, 1979a, 1980, 1981a), and this has been confirmed by recent studies on the amino acid composition of frog brain LH-RH (Rivier *et al.*, 1981). It has now been shown in indirect studies

on partially purified material that chicken hypothalamic LH-RH differs structurally from the mammalian peptide by the substitution of glutamine for arginine in position eight (see sections 2.4 and 2.5), which accords with the findings presented in this section.

Since hypothalamic LH-RHs from birds, reptiles and teleost fish behave identically on CM32 carboxymethyl cellulose cation exchange chromatography and reverse phase HPLC as well as in their interaction with region-specific antisera (King and Millar, 1979a, 1980, 1981a), it is possible that a substitution of glutamine for arginine of mammalian LH-RH is the characteristic structural feature of the peptide in all of these vertebrate classes. However, the HPLC system used in our early studies may not have been adequate to resolve certain neutral amino acid substitutions in position eight from each other, and other position eight-substituted analogues may be present in reptiles and fish. Thus, [Gln⁸]LH-RH (or another analogue with a conservative neutral amino acid substitution in position eight) is probably the ancestral molecule, and a mutation to [Arg⁸]LH-RH occurred in a line common to amphibians and mammals in accordance with a contemporary phylogenetic scheme (Licht *et al.*, 1977; King and Millar, 1979a).

Synthetic mammalian LH-RH has been found to exhibit good gonadotropin-releasing activity in amphibians but has poor or no such activity in birds, reptiles and fish (for reviews, see Ball, 1981; Jackson, 1981; King and Millar, 1981a). It is likely that this phenomenon reflects structural differences in pituitary gonadotrope receptors related to the structural difference of LH-RH in these vertebrate classes.

4.0

SYNTHESIS AND BIOLOGICAL ACTIVITY OF CHICKEN HYPOTHALAMIC
LUTEINIZING HORMONE-RELEASING HORMONE

4.1

SUMMARY

Chicken hypothalamic luteinizing hormone-releasing hormone (LH-RH), the isolation of which was described in section 3, was synthesised by solid-phase methodology, purified, and chemically characterised. The biological activity of the synthetic chicken peptide was compared with that of natural chicken LH-RH and synthetic mammalian LH-RH by assessment of luteinizing hormone (LH) release from dispersed chicken anterior pituitary cells and cultured ovine anterior pituitary cells, and binding to chicken and rat anterior pituitary cell membrane receptors was tested. Synthetic chicken LH-RH exhibited identical properties to the isolated natural chicken hypothalamic LH-RH in cation exchange and reverse phase high performance liquid chromatography (HPLC), and the isoelectric points of the synthetic and natural peptides were identical. Synthetic chicken LH-RH displayed identical activity to natural chicken LH-RH in stimulating LH release from dispersed chicken anterior pituitary cells, and synthetic mammalian LH-RH was equipotent in this system. Both the natural and synthetic chicken peptides had similar low potency in stimulating LH release from cultured ovine anterior pituitary cells (approximately 10% of the potency of synthetic mammalian LH-RH), and the ED₅₀ of binding to rat anterior pituitary cell membrane receptors was $>10^{-5}$ M (ED₅₀ for synthetic mammalian LH-RH was approximately 10^{-7} M).

4.2

INTRODUCTORY STATEMENT

Previous sections (2 and 3) provided strong evidence that chicken hypothalamic LH-RH differs from the mammalian hypothalamic peptide at amino acid residue eight, glutamine substituting for the arginyl residue of mammalian LH-RH. However, studies showing an identity of synthetic chicken LH-RH with the natural material in biological properties provide more conclusive evidence. This section describes the solid-phase synthesis of chicken hypothalamic LH-RH, and studies on the biological properties of the peptide. The LH-releasing activities and receptor binding properties of natural chicken LH-RH, synthetic chicken LH-RH and synthetic mammalian LH-RH in the bird and in the mammal were compared, with a view to obtaining further information on the evolution of the peptide and its receptor.

Peptide synthesis

Chicken LH-RH was synthesised by R. P. Millar using the solid-phase method of Merrifield (1963). The resin employed was chloromethylated polystyrene crosslinked with 1% divinyl benzene (Pierce). Amino acids of the L-configuration (gift from S. Jacobson) were coupled as their N- α -tert-butyloxycarbonyl (Boc) derivatives, and side chain functional groups were protected as follows: para-nitrophenyl for Gln; benzyl for Tyr and Ser; tosyl for His. Boc-Gly was attached to the resin by refluxing in ethanol and triethylamine for 24 h at 70°C. Four equivalents of each amino acid in CH₂Cl₂ (or 10% dimethylformamide in CH₂Cl₂ for Trp) were then successively coupled by mixing with four equivalents of dicyclohexylcarbodiimide in CH₂Cl₂ for 2 h. Completion of coupling was monitored at each step by the ninhydrin test or by the chloranil test when Pro was at the NH₂ terminus. The Boc protection residue was removed at each step by treatment with 25% trifluoroacetic acid in CH₂Cl₂ for 30 min. Dithioethane (5%) was included after the addition of the Trp residue. The NH₂-terminal pGlu was coupled using the pentachloride phenyl ester dissolved in dry dimethylformamide and mixed for 48 h. Simultaneous cleavage of the peptide from the resin and amidation of the COOH-terminal Gly was achieved by stirring the peptide resin in dried liquified ammonia at 4°C in a sealed flask for 48 h. Protective residues on side chains were removed by treatment with anhydrous liquid hydrogen fluoride containing 10% anisole for 30 min at 0°C. Hydrolysis of peptide coupled to resin was achieved using propionic acid/concentrated HCl (1 : 1).

Peptide purification and chemical characterisation

HPLC was performed on a Model SP 3500B liquid chromatograph (Spectra-Physics) equipped with a Model 770 spectrophotometric detector (Spectra-Physics). Small aliquots (0.02 - 10.30 mg) of the crude peptide preparation were purified on a Spherisorb ODS reverse phase column (0.4 x 25 cm; 5 µm particle size; Phase Separations) using a mobile phase of 22% acetonitrile in 0.01 M ammonium acetate, pH 4.0. Flow rate was 1.5 ml/min. Absorbance was monitored at 280 nm. Peptide purity was assessed by amino acid analysis after hydrolysis in 100 µl constant boiling 6 N HCl containing 2% thioglycolic acid in sealed evacuated tubes at 110°C for 16 h. The hydrolysate was lyophilised, reconstituted in 0.15 M lithium citrate buffer, pH 2.2, and subjected to amino acid analysis (Beckman Instruments Model 121-MB).

Release of LH from anterior pituitary cells

The ability of natural chicken LH-RH, synthetic chicken LH-RH and synthetic mammalian LH-RH to stimulate the release of LH was studied using dispersed chicken anterior pituitary cells and cultured ovine anterior pituitary cells.

The dispersed chicken anterior pituitary cell bioassay was based on the method of Bonney and Cunningham (1977). Anterior pituitaries were dissected from chicken heads (South African Poultry Company, Cape Town, South Africa) collected on ice within 2 h of death, and placed in ice-cold, sterile Romanoff's avian Ringer's Albumin buffer (RRA) (147 mM NaCl, 4.15 mM KCl, 1.8 mM CaCl₂, 0.49 mM MgCl₂.6H₂O, 1.5 mM KH₂PO₄, 2.2 mM Na₂HPO₄.12H₂O, 11.1 mM glucose, and 0.5% bovine serum albumin (Pentex bovine albumin, fraction V; Miles Laboratories), pH 7.2). The anterior pituitaries were washed with RRA, diced, and incubated with stirring in HEPES buffer (137 mM NaCl, 5 mM KCl, 0.7 mM

Na₂HPO₄, 25 mM 4-2-hydroxyethyl piperazine ethanesulfonic acid, 0.36 mM CaCl₂, 10 mM glucose, and 1% bovine serum albumin, pH 7.2) containing 0.9% collagenase (155 U/mg, Worthington) and 1.8 mg% deoxyribonuclease (Miles Laboratories) for 1 h at 37°C. After incubation the suspension was passed up and down a pipette to aid dispersion, centrifuged at 50 g for 5 min, and the pellet was resuspended in RRA. The cells were washed twice in RRA, counted, and distributed to polypropylene test-tubes (12 x 75 mm) at a concentration of approximately 2.5 x 10⁵ cells/tube. The tubes were pre-incubated in 1.5 ml RRA for 20 min at 37°C with gentle shaking under a 90% water-saturated atmosphere of 4% CO₂ - 96% air. The cells were then centrifuged at 50 g for 5 min, the supernatant was poured off, and multiple dilutions of test substances were added in quadruplicate, to give a final incubation volume of 1.0 ml. The tubes were incubated for 2 h, centrifuged at 50 g for 5 min, and the supernatant was poured off into polypropylene vials. The supernatant was recentrifuged at 50 g for 5 min and an aliquot of the final supernatant was assayed for LH immunoreactivity.

Radioimmunoassay of chicken LH was as described by Follett *et al.* (1972), using the double-antibody technique. Chicken LH (gift from B.K. Follett) was used as standard, and for preparation of ¹²⁵I-LH using the chloramine T method (Greenwood *et al.*, 1963) followed by purification by cellulose CF 11 chromatography (Whatman). Antiserum (gift from B.K. Follett) was raised against chicken LH. Diluted aliquots of cell medium were assayed in duplicate.

Culture of ovine anterior pituitary cells was according to the method of Vale *et al.* (1972), with modifications. Pituitaries dissected from sheep heads (Stanley Abattoir, Cape Town, South Africa) within 20 min of death were placed in ice-cold, sterile HEPES buffer

and the posterior pituitaries were removed. The anterior pituitaries were diced, rinsed with HEPES buffer, and incubated in HEPES buffer as described for chicken anterior pituitaries. At 10-min intervals coarse fragments were allowed to settle, and half of the supernatant was removed and centrifuged at 50 g for 2-5 min. The supernatant was returned to the incubation flask and the process was repeated until the bulk of the cells were dispersed. The cell pellets were pooled, washed with HEPES buffer, and suspended in Minimum Essential Medium (MEM, with Hank's salts) containing 10% donor calf serum and 0.1 mM glutamine. Approximately 10^5 - 10^6 dispersed cells were distributed to tissue culture dishes (15 x 60 mm, Falcon Plastics) containing 3 ml medium, and incubated at 37°C under a 90% water-saturated atmosphere of 4% CO₂ - 96% air. After 4-6 days in culture, the cells were washed six times with serum-free medium, and multiple dilutions of the test substances were added in triplicate. After 3 h of incubation, the medium was removed, centrifuged at 400 g for 5 min, and the supernatant was assayed for LH immunoreactivity.

Radioimmunoassay of ovine LH was as described by Millar and Aehnelt (1977), using the double-antibody technique. Ovine LH (gift from L.E. Reichert) was used as standard, and for preparation of ¹²⁵I-LH using the chloramine T method followed by purification by cellulose CF 11 chromatography. Antiserum GDN 15 (gift from G.D. Niswender) was raised against ovine LH. Diluted aliquots of cell medium were assayed in duplicate.

Potency of the peptides was estimated from parallel linear regressions (Rodbard and Lewald, 1970).

Anterior pituitary cell membrane receptor binding

Binding of natural chicken LH-RH, synthetic chicken LH-RH and synthetic mammalian LH-RH to chicken and rat anterior pituitary cell membrane receptors was compared.

A superactive, degradation-resistant analogue of mammalian LH-RH, [D-Trp⁶,des-Gly¹⁰]LH-RH ethylamide (gift from W. Vale), was iodinated using chloramine T and purified on a CM32 carboxymethyl cellulose cation exchange column (1 x 30 cm; Whatman) eluted with a gradient of 0.06 M to 0.18 M ammonium acetate, pH 4.5. Specific activity of the ¹²⁵I-labelled peptide, assessed by the self-displacement method using an antiserum to [D-Trp⁶]LH-RH (Barron *et al.*, 1981), ranged from 800 - 1,000 μ Ci/ μ g.

Fresh anterior pituitaries from chickens of mixed sex and age (South African Poultry Company, Cape Town, South Africa) and adult male rats (Long-Evans) were homogenised in a Dounce homogeniser in ice-cold 10 mM Tris-HCl, pH 7.4, containing 1 mM dithiothreitol, 1.5 mM ethylenediaminetetra-acetic acid and 0.1% bovine serum albumin, and centrifuged at 1,000 g for 10 min at 4°C. The supernatant was centrifuged at 10,000 g for 20 min at 4°C to yield a crude membrane pellet which was washed twice and resuspended in the same buffer. Membrane fractions were assayed immediately after preparation.

The binding assay procedure was as follows: approximately 70,000 cpm ¹²⁵I-labelled [D-Trp⁶,des-Gly¹⁰]LH-RH ethylamide was incubated for 75 min at 4°C with the membrane preparation (2-4 chicken pituitary equivalents/tube or 0.5 rat pituitary equivalents/tube) in the presence of increasing concentrations of the test peptides in glass test-tubes (12 x 75 mm) in a total volume of 0.5 ml of 10 mM Tris-HCl, pH 7.4, containing 0.1% bovine serum albumin. Incubation was terminated

by dilution with 3 ml ice-cold phosphate-buffered saline, pH 7.4, containing 1% bovine serum albumin (PBS-BSA), followed by immediate filtration under vacuum through glass fibre filters (GF/C; Whatman) presoaked in PBS-BSA. The filters were washed twice with 3 ml PBS-BSA, and the retained radioactivity was counted. All samples were assayed in triplicate.

Synthetic peptide

Reverse phase HPLC separated a single peak of synthetic chicken LH-RH from salts and contaminating peptides (Fig. 10), which co-eluted with natural chicken LH-RH. Amino acid analysis indicated the peptide was homogeneous, comprising the following ratios of amino acids : Ser 0.74, Glu 2.04, Pro 1.00, Gly 2.01, Leu 1.03, Tyr 1.04, Trp 0.57 and His 1.01. Synthetic chicken LH-RH and natural chicken LH-RH had identical retention times on reverse phase and cation exchange HPLC, and their isoelectric points were the same (see Table 7 in section 3.4). In radioimmunoassays employing four region-specific antisera, the immunological properties of the natural and synthetic materials were very similar in terms of slope of displacement curves and relative quantitation with different antisera (see Fig. 6 in section 2.4).

LH-releasing activity

Natural and synthetic chicken LH-RH were equipotent in stimulating LH release from dispersed chicken anterior pituitary cells (Fig. 11A), and synthetic mammalian LH-RH displayed a similar LH-releasing activity (Fig. 11B).

Natural and synthetic chicken LH-RH stimulated LH release from cultured ovine anterior pituitary cells in a similar manner (Table 8). Relative to synthetic mammalian LH-RH, the biological potency of synthetic chicken LH-RH in the ovine anterior pituitary cell bioassay was approximately 10% (Fig. 12).

Receptor binding

Binding of synthetic chicken LH-RH and synthetic mammalian LH-RH to chicken anterior pituitary cell membrane receptors was low,

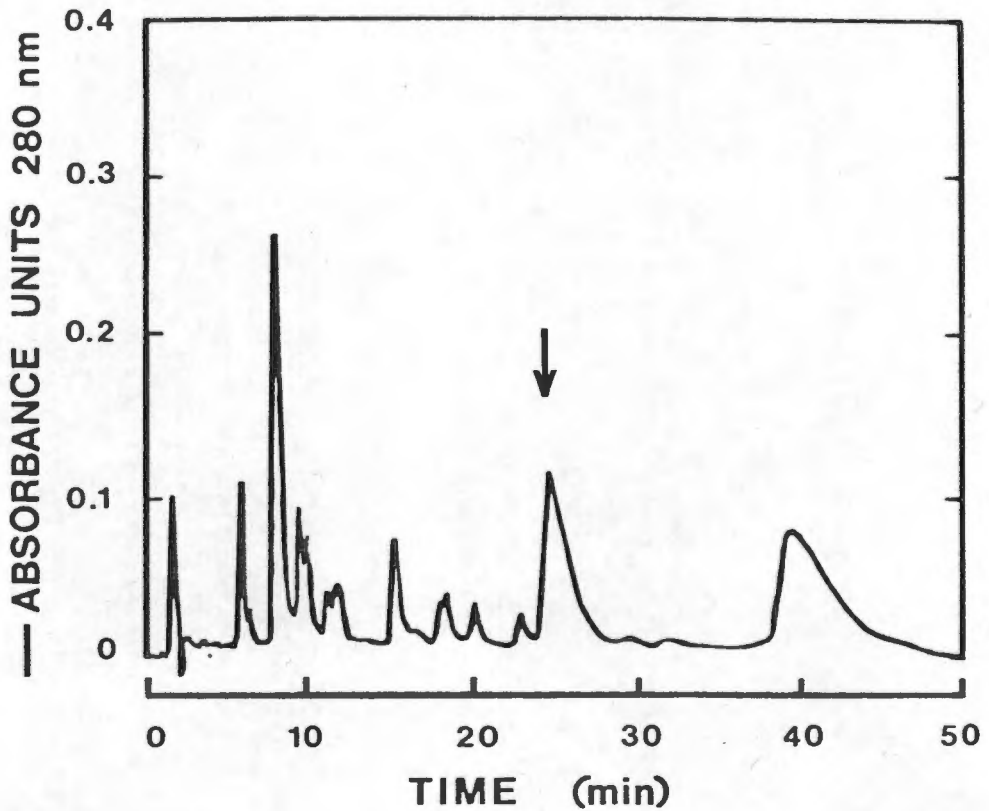


FIGURE 10

Reverse phase HPLC purification of 0.5 mg of crude synthetic chicken LH-RH using 22% acetonitrile in 0.01 M ammonium acetate, pH 4.0. Absorbance at 280 nm was recorded at a setting of 0.4 units full scale. One of several reverse phase HPLC runs is shown here; all column runs generated similar UV absorbance peaks. The elution position of natural chicken LH-RH is marked with an arrow.

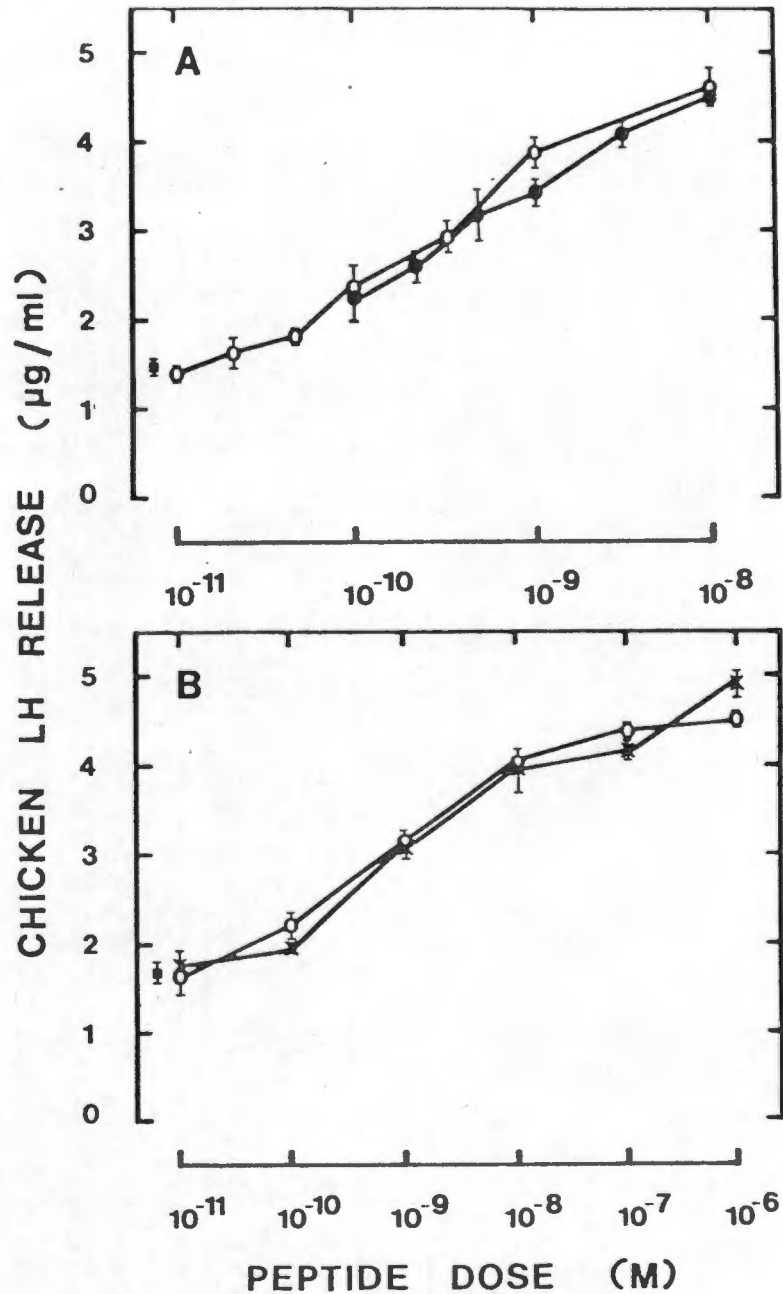


FIGURE 11

Effect of natural and synthetic chicken LH-RH, and synthetic mammalian LH-RH, on LH release from dispersed chicken anterior pituitary cells. Peptide doses were determined by amino acid analysis. Points represent mean \pm S.D. (n = 4). A, medium alone (■), natural chicken LH-RH (●—●) and synthetic chicken LH-RH (○—○). B, medium alone (■), synthetic chicken LH-RH (○—○) and synthetic mammalian LH-RH (×—×). Natural chicken LH-RH, synthetic chicken LH-RH and synthetic mammalian LH-RH were equipotent in stimulating LH release (estimation of potency from parallel linear regressions).

TABLE 8

Effect of natural and synthetic chicken LH-RH on LH release from
cultured ovine anterior pituitary cells

Peptide dose ^a	LH release ^{b,c} (ng/ml)
Control (medium)	42 ± 8
Natural chicken LH-RH (10 ⁻⁸ M)	81 ± 1
Synthetic chicken LH-RH (10 ⁻⁸ M)	75 ± 11

^a Determined by amino acid analysis.

^b Mean ± S.D. (n = 3).

^c Compared to the control value, stimulation of LH release by natural chicken LH-RH (P < 0.005) and synthetic chicken LH-RH (P < 0.01) was significant. Natural and synthetic chicken LH-RH were not significantly different from each other in their ability to stimulate LH release.

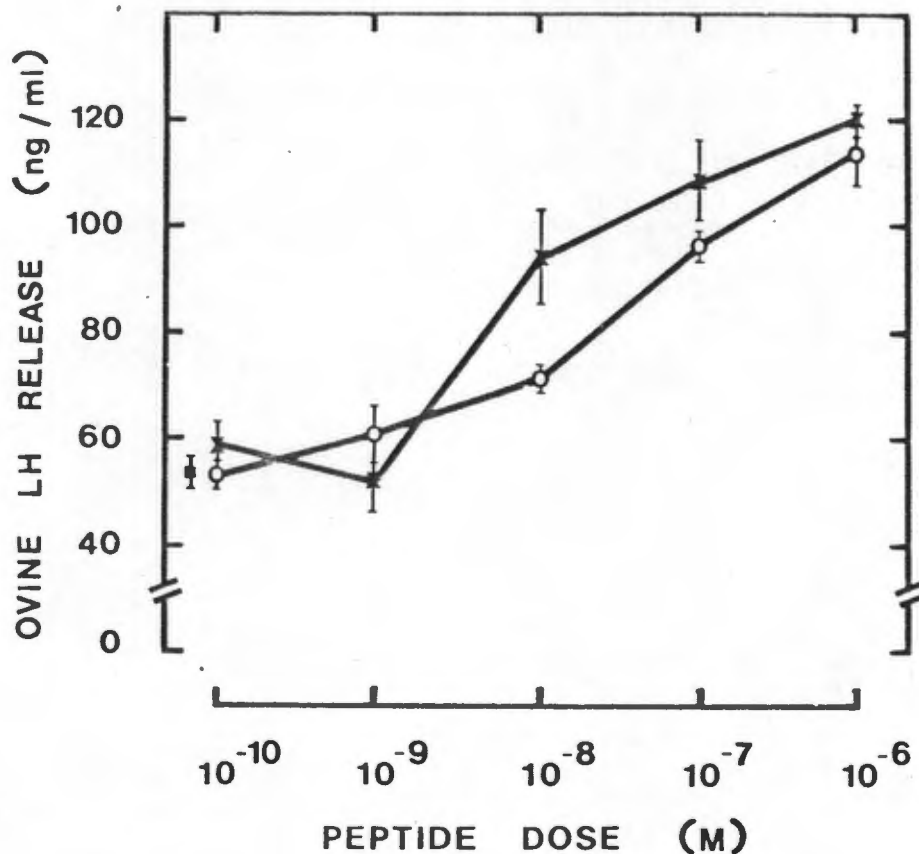


FIGURE 12

Effect of medium alone (■), synthetic chicken LH-RH (○—○) and synthetic mammalian LH-RH (×—×) on LH release from cultured ovine anterior pituitary cells. Peptide doses were determined by amino acid analysis. Points represent mean ± S.D. (n = 3). The potency of synthetic chicken LH-RH in stimulating LH release was 14% relative to that of synthetic mammalian LH-RH (estimation of potency from parallel linear regressions). From three such experiments, the average potency of synthetic chicken LH-RH was 10%.

presumably due to the low concentration of LH-RH receptors in the chicken anterior pituitary (approximately 0.1 fmol/pituitary). The experiment was repeated five times, but the data were not satisfactory and no conclusions could be made from them except to say that the concentration of receptors in the anterior pituitary was low.

Synthetic chicken LH-RH bound to rat anterior pituitary cell membrane receptors with a lower affinity ($ED_{50} > 10^{-5}$ M) than synthetic mammalian LH-RH ($ED_{50} \sim 10^{-7}$ M) (Fig. 13).

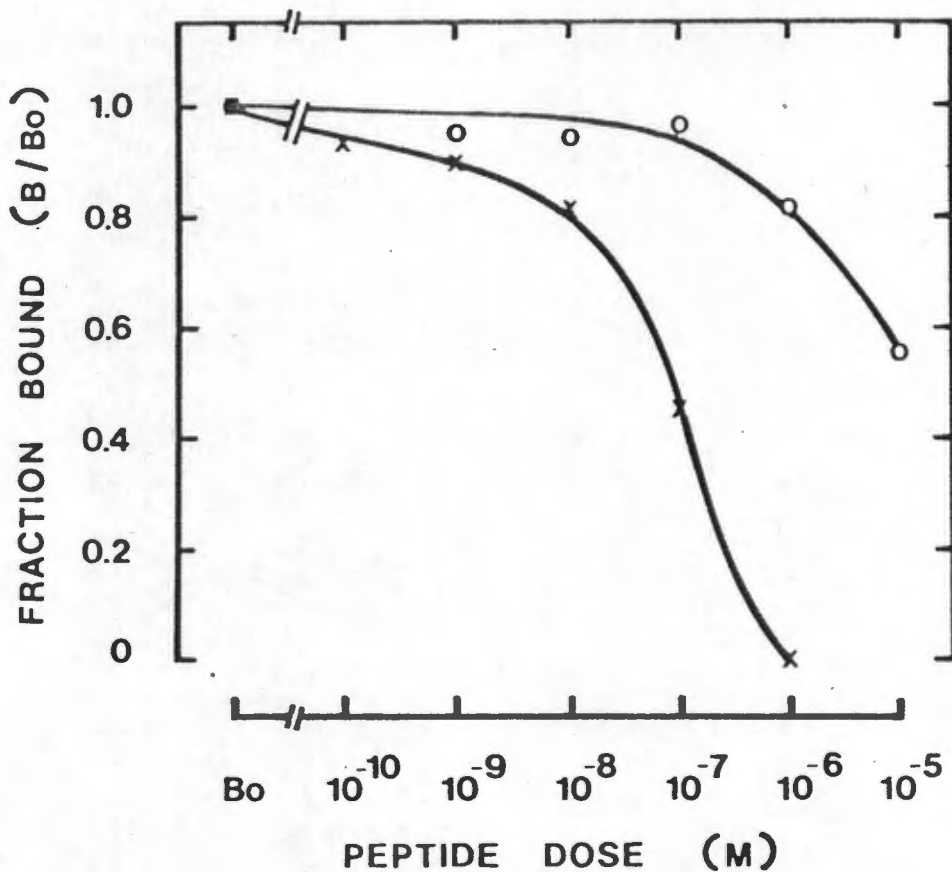


FIGURE 13

Inhibition of ¹²⁵I-labelled [D-Trp⁶,des-Gly¹⁰]LH-RH ethylamide binding to rat anterior pituitary cell membrane receptors by synthetic chicken LH-RH (O—O) and synthetic mammalian LH-RH (X—X). The fraction of initial tracer bound ($\frac{B}{B_0}$) is plotted on the ordinate. Peptide doses were determined by amino acid analysis.

This section describes the synthesis of chicken hypothalamic LH-RH, the isolation of which was described in section 3. In addition to providing confirmation of the proposed primary structure of the natural compound, the synthesis has allowed more detailed biological characterisation of the peptide which was not possible on the minute quantities of natural chicken LH-RH available.

Synthetic and natural chicken LH-RH had identical chromatographic properties and amino acid compositions, suggesting a correct assignment of structure of natural chicken LH-RH and demonstrating homogeneity of the synthetic compound. This was further emphasised by the similarity in their interaction with region-specific LH-RH antisera. Both peptides gave lowest quantitation and displacement curves nonparallel to synthetic mammalian LH-RH displacement curves with antiserum 744 which requires residues Arg⁸ to Gly¹⁰-NH₂ for effective binding; intermediate quantitation and nonparallel displacement curves with the middle region-directed antisera 1076 and 743; and highest quantitation and parallel displacement curves with antiserum R-42 which requires NH₂- and COOH-termini for binding.

Previous studies have indicated that mammalian LH-RH has poor biological activity in birds (for reviews, see King and Millar, 1981a; section 1). We anticipated, therefore, that in birds chicken LH-RH would have greater biological activity than mammalian LH-RH. However, chicken LH-RH has similar properties to mammalian LH-RH in stimulating LH release from dispersed chicken anterior pituitary cells.

Synthetic and natural chicken LH-RH had relatively poor potency in stimulating LH release from ovine anterior pituitary cells in culture (approximately 10% relative to synthetic mammalian LH-RH)

and in binding to rat anterior pituitary cell membrane receptors ($ED_{50} > 10^{-5}$ M). This finding is in accordance with data showing poor biological activity of position eight-substituted analogues of mammalian LH-RH in releasing LH from the mammalian pituitary (Yanaihara *et al.*, 1972; Sandow *et al.*, 1978).

Mammalian LH-RH has extremely low gonadotropin-releasing activity in fish (for reviews, see Ball, 1981; King and Millar, 1981a; section 1). Using a number of high resolution chromatographic systems and radioimmunoassay with region-specific antisera we have shown that piscine LH-RH differs from mammalian LH-RH in the region Gly⁶-Leu⁷-Arg⁸ (King and Millar, 1979a, 1980, 1981a). Avian LH-RH might be a more potent stimulant of gonadotropin release in fish, and could be of economic importance in pisciculture.

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CONCLUDING DISCUSSION

The structure of chicken hypothalamic luteinizing hormone-releasing hormone (LH-RH) was determined independently using two different approaches. Firstly, an 'indirect' approach was taken since it was anticipated that sufficient material for conventional structural determination might not be obtained. A small quantity of partially purified material (immunoaffinity chromatography only) was studied using a combination of chromatographic analysis, binding of region-specific antisera, enzymic inactivation, and chemical modification of specific amino acid residues. This study established that the chicken peptide differs from mammalian hypothalamic LH-RH at residue eight, arginine being substituted by a neutral amino acid. On the basis of conformational criteria and evolutionary probability of amino acid interchange for arginine, we postulated that glutamine is the most likely substitute for arginine in position eight. The putative chicken LH-RH, [Gln⁸]LH-RH, was then synthesised, and found to have identical chromatographic and immunological properties to the natural chicken LH-RH, thus confirming the proposed primary structure of chicken hypothalamic LH-RH.

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Although we obtained substantial evidence that the structure of chicken LH-RH had been correctly assigned, direct determination of the amino acid composition and sequence of the pure peptide was desirable.

In order to isolate pure chicken LH-RH, 249,000 hypothalami were collected, extracted with acetic acid, and purified by immunoaffinity chromatography followed by cation exchange and reverse phase high performance liquid chromatography to yield 17.4 µg of homogeneously pure peptide. Acid hydrolysis of the peptide showed an amino acid composition identical to that of mammalian decapeptide LH-RH,

except for the presence of an additional glutamic acid residue and the absence of arginine. In order to ascertain whether the glutamic acid represented a glutamine residue as we had proposed in the 'indirect' study, amino acid analysis of enzymic hydrolysates and sequence analyses were performed, but were unsuccessful. The sequence -Gln⁸-Pro⁹- presents problems in that there is apparently resistance to cleavage and/or derivative formation during enzymic hydrolysis and in sequence analysis. The same difficulties in amino acid composition and sequence determination were encountered with synthetic [Gln⁸]LH-RH. In contrast, synthetic mammalian LH-RH was successfully analysed by these techniques.

In spite of the inability to obtain definite proof of the structure of chicken LH-RH by sequence analysis, the demonstration of the amino acid composition (acid hydrolysis), and the identity of *pure* natural chicken LH-RH with synthetic [Gln⁸]LH-RH in immunologic, chromatographic and biological properties, together with the 'indirect' studies, provide strong evidence for the proposed structure of chicken hypothalamic LH-RH:



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In order to provide more conclusive evidence for the identity of synthetic [Gln⁸]LH-RH with the natural chicken peptide, their biological properties were compared. Comparison with synthetic mammalian LH-RH was also made, with a view to obtaining further information on the evolution of the peptide and its receptor. Natural and synthetic chicken LH-RH, and synthetic mammalian LH-RH, were equipotent in stimulating luteinizing hormone (LH) release from dispersed chicken anterior pituitary cells. Both the natural and synthetic chicken peptides had similar low potency in stimulating LH release from cultured ovine anterior

pituitary cells (approximately 10% of the potency of synthetic mammalian LH-RH), and the ED₅₀ of binding to rat anterior pituitary cell membrane receptors was >10⁻⁵ M, compared to an ED₅₀ of approximately 10⁻⁷ M for synthetic mammalian LH-RH.

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Regarding the evolution of vertebrate hypothalamic LH-RH, we have previously demonstrated that avian, reptilian, and piscine hypothalamic LH-RHs are structurally different from mammalian decapeptide LH-RH, but are indistinguishable from each other in chromatographic properties and in their interaction with several region-specific antisera (King and Millar, 1979a, 1980, 1981a). It is possible, therefore, that a substitution of glutamine for arginine of mammalian LH-RH is the characteristic structural feature of the peptide in these vertebrate classes. However, the HPLC system used in our early studies may not have been adequate to resolve certain neutral amino acid substitutions in position eight from each other, and other position eight-substituted analogues may be present in reptiles and fish. Amphibian hypothalamic LH-RH is identical to the mammalian decapeptide (King and Millar, 1979a, 1980, 1981a; Rivier *et al.*, 1981). Thus, [Gln⁸]LH-RH (or another analogue with a conservative neutral amino acid substitution in position eight) is probably the ancestral molecule, and a mutation to [Arg⁸]LH-RH occurred in a line common to amphibians and mammals in accordance with a contemporary phylogenetic scheme (Licht *et al.*, 1977; King and Millar, 1979a).

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Comments on the evolution of the pituitary gonadotrope receptor for LH-RH are largely speculative since there are not yet sufficient data to allow any definite proposals. A number of studies on the gonadotropin-releasing activity of chicken LH-RH in different vertebrates have been

initiated by us, and the results of these studies might allow a more complete understanding of the evolution of the receptor.

Synthetic mammalian LH-RH stimulates release of both LH and follicle-stimulating hormone (FSH) in mammals and in amphibians. In birds, reptiles and fish, however, higher doses of synthetic mammalian LH-RH are required to stimulate release of LH (in birds and reptiles) or gonadotropin (in fish), and the FSH response (in birds and reptiles) is very low even with extremely high doses of synthetic mammalian LH-RH (for reviews, see Ball, 1981; King and Millar, 1981a; section 1). Synthetic chicken LH-RH has 10% of the potency of synthetic mammalian LH-RH in stimulating LH release in the mammal (present study); in the bird, synthetic chicken and mammalian LH-RHs are equipotent in stimulating LH release (present study); in amphibians, recent results indicate that synthetic chicken and mammalian LH-RHs are equipotent in stimulating release of both LH and FSH (unpublished; studies in collaboration with P. Licht, University of California, Berkeley, California). Data from studies initiated on the gonadotropin-releasing activity of synthetic chicken LH-RH in reptiles and fish are not yet available. Crude preparations of hypothalamic LH-RH from amphibians and fish have LH-releasing activity in mammals (for reviews, see Ball, 1981; King and Millar, 1981a; section 1), but the potency of the peptides can obviously not be estimated in these studies.

Based on the above data, the following comments can be made on the receptor for LH-RH. The mammalian receptor binds mammalian LH-RH, but binding of chicken (avian) LH-RH is low. The avian receptor binds both mammalian and avian LH-RH, as does the amphibian receptor. Since [Gln⁸]LH-RH (or another analogue with a conservative neutral amino acid substitution in position eight) appears to be the natural peptide in

reptiles and fish, it is probable that the receptor in these vertebrate classes binds avian LH-RH well. Mammalian LH-RH, however, has low activity in reptiles and in fish. These speculations are summarised in Table 9.

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An interesting concept which has emerged from our studies on the phylogenetic and anatomical distribution of LH-RH in vertebrates is consonant with 'Haeckel's Law' that ontogenesis recapitulates phylogenesis. On the basis of immunologic and chromatographic properties, it would appear that the LH-RH molecules in the hypothalamus of the bird, reptile and fish (King and Millar, 1979a, 1980, 1981a), in the amphibian sympathetic ganglion (Eiden and Eskay, 1980) and in the mammalian pineal gland (King and Millar, 1981c) all differ from mammalian hypothalamic LH-RH, a conservative neutral amino acid substituting for the arginyl residue in position eight. LH-RH in the mammalian extrahypothalamic brain (where LH-RH has effects but binds poorly to receptors) and in amphibian extrahypothalamic brain might also have this structural difference. [Gln⁸]LH-RH (or another analogue with a conservative neutral amino acid substitution in position eight) which occurs in the hypothalamus of extant birds, reptiles and fish, but not in the hypothalamus of amphibians and mammals, is probably the ancestral molecule. Elaboration of this ancestral LH-RH has apparently been retained in some nerve tissues of amphibians and mammals, while the hypothalamic LH-RH in these vertebrate classes (i.e. [Arg⁸]LH-RH) is more recently evolved.

A similar evolutionary pattern is emerging for the neurohypophysial hormones (Acher, 1978): the more primitive neurohypophysial hormone, arginine vasotocin, is present in the pineal gland of adult mammals (Milcu *et al.*, 1963; Pavel, 1971).

TABLE 9

Biological activity of avian and mammalian LH-RHs
in different vertebrate classes

Hormone	Biological activity in different vertebrates				
	Mammal	Bird	Reptile	Amphibian	Fish
Avian LH-RH	+	+++	(+++)	+++	(+++)
Mammalian LH-RH	+++	+++	(++)	+++	(+)

Plus signs indicate relative biological activity of LH-RH in different vertebrates: +, low activity; ++, intermediate activity; +, low activity. Brackets indicate predicted values-data not yet available.

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PUBLICATIONS

Papers published during the period of registration for the Ph.D. degree are listed below.

1. King, J.A., and Millar, R.P. (1980) Comparative aspects of luteinizing hormone-releasing hormone structure and function in vertebrate phylogeny. *Endocrinology* 106, 707-717.
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