The Purification of Luteinizing-Hormone-Releasing Factor with some Observations on its Properties

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Bullock median-eminence tissue was used as a source of luteinizing-hormone-releasing factor for small-scale experiments to explore methods for its isolation. The presence of luteinizing-hormone-releasing factor was detected by the ovulation response in rabbits after intrapituitary infusion of the extract. Gel filtration was found to be suitable for the purification of these extracts. The releasing factor appeared to be a basic peptide of molecular weight in the range 1200–2500. On a larger scale, an extract of hypothalamic tissue from sheep was used to establish a multi-stage isolation procedure that resulted in a 200 000-fold purification of luteinizing-hormone-releasing factor. After the initial extraction the isolation process consisted of: (1) two cycles of gel filtration; (2) anion-exchange chromatography; (3) gel filtration in a partially organic medium; (4) thin-layer chromatography on cellulose. Stage (3) separated two zones of activity each containing peptides. One of these was purified further by stage (4) to give a preparation that was active at a dose of 6 μ g. of peptide/animal, although activity diminished seriously during storage. This preparation contained only five or six components, but the small amounts of peptides obtained at this stage of purity were insufficient for full characterization.

The concept of neurohumoral regulation (see Harris, 1955) of the function of the anterior pituitary gland was based on experiments involving electrical stimulation of the hypothalamus, interruption of the blood vessels between the hypothalamus and the pituitary by sectioning the pituitary stalk, and transplantation of the pituitary to various sites. Observations on the function of the target organs of the anterior-pituitary hormones (ovary, testis, adrenal cortex or thyroid) after these treatments revealed that regulation of pituitary hormone secretion was dependent on the integrity of its hypophyseal portal-blood supply. These vessels arise as capillaries in the median eminence and carry blood from this structure to the anterior lobe of the pituitary. It was deduced that these vessels carried humoral agents, now known as releasing factors, whose function was to translate neural impulses into biochemical responses by stimulating the secretion of the appropriate trophic hormone from the anterior pituitary (see Fig. 1).

The first report of the stimulation of gonadotrophic secretion by a humoral agent obtained from

bullock median-eminence tissue was given at the Endicott House Symposium in February 1960 (see Harris, 1961; Campbell, Feuer, Garcia & Harris, 1961). The extract was infused into the anterior pituitary of oestrous female rabbits and caused ovulation in nine out of 16 animals. Infusion of control substances (including other brain extracts) caused ovulation in only one out of 13 animals tested. Since ovulation in rabbits is attributable to the action of a sudden surge of luteinizing hormone from the pituitary, we have used this technique as a bioassay for the purification of LRF† from bullock and sheep tissue. In other Laboratories (McCann, Taleisnik & Friedman, 1960; Harris, Reed & Fawcett, 1966) the search for LRF has progressed with the aid of the ovarian ascorbic acid-depletion assay for luteinizing hormone.

The present paper describes the partial purification of LRF in which experience gained from small-scale experiments with freshly dissected bullock median-eminence tissue was used to develop a five-stage procedure with larger batches of commercially available sheep hypothalamic tissue. Preliminary

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† Abbreviation: LRF, luteinizing-hormone-releasing factor.

Fig. 1. Diagram of a mid-line sagittal section through the base of the hypothalamus and pituitary gland to show the structures concerned in the regulation of anterior-pituitary activity. Nerve fibres from the hypothalamus (stippled; *H*) enter the median eminence (*ME*) of the tuber cinereum to end on the primary plexus of the hypophysial portal vessels (*PV*). It is postulated that releasing factors pass from these nerve terminals into the portal vessels and are carried to the anterior pituitary gland (*AP*) to regulate its secretory activity. This is probably a similar mechanism to that obtaining in the posterior pituitary gland (*PP*), where peptides are discharged from nerve endings into the general circulation.

accounts of some of this work have appeared (Charlton, Fawcett, Harris & Reed, 1965; Fawcett, Harris & Reed, 1965).

MATERIALS AND METHODS

Extraction of tissue. Median-eminence tissue from young bullocks was dissected at the slaughterhouse as soon as possible after the animals were killed. An extract of this tissue was prepared by using 160 mg. wet wt. of tissue/ml. of 0.15N-HCl. The homogenate was boiled for 10 min., as previously described (Campbell, Feuer & Harris, 1964). Sheep hypothalamic tissue, including the median eminence, was obtained from Pitco Biologicals (Long Island, N.Y., U.S.A.), and processed by the method of Guillemin, Schally, Lipscomb, Andersen & Long (1962) by using their procedure B. In common with other workers, we have found that large batches of tissue are more conveniently handled by freeze-drying at the outset and then homogenizing an acetone-dried powder prepared from the dry tissue. This latter step reduced the difficulty encountered when an aqueous-acid extract of the tissue, which has a high content of lipid, was being made. Care has been taken throughout this work to minimize the risk of contamination of the active material by artifacts arising from the procedures used. All reagents and water

were glass-distilled. Sephadex G-25 and A-25 were thoroughly washed, and the gel-filtration experiments were carried out at 3°. All glassware was rinsed with glass-distilled water immediately before use. Fractionating columns, flasks and storage tubes were treated with silicone to decrease irreversible adsorption of material. Thin-layer chromatography was performed on two types of micro-crystalline cellulose in butan-1-ol-acetic acid-water (4:1:5, by vol.; upper layer). Both types [Avicell (American Viscose Co. FMC Corporation, Marcus Hook, Pa., U.S.A.) and CC41 (British Drug Houses Ltd., Poole, Dorset)] gave very similar results. Plates were prepared from an aqueous suspension (16%, w/v) of the cellulose that had been blended in water and de-aerated under vacuum. They were dried at 50° for 4–6 hr., washed in the chromatography solvent and dried at least twice before use.

Examination of eluates from columns. The extinction of fractions was read at 275 mμ, except where otherwise stated. Samples amounting to 0.1–2% of the fraction volume, depending on the stage of purification, were used for determination of the peptide concentration by a modification of the Folin-Lowry method (Lowry, Rosebrough, Farr & Randall, 1951), in which the final volume was decreased to 0.35 ml. by suitably decreasing the volumes of each reagent used. This permitted the detection of microgram quantities of peptides.

When it was necessary to evaporate solvents from partially aqueous solution they were concentrated by rotary evaporation at 40° and then freeze-dried.

Proteolytic digestion. Pepsin (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) (30 μg. in 0.01N-HCl) was added to one of two identical samples that had been adjusted to pH 2.5 with HCl. After standing at room temperature for 22 hr. each sample was neutralized and made iso-osmotic for infusion.

Biological test. Solutions were tested for LRF activity by the method of Campbell *et al.* (1964). They were infused directly into the anterior pituitary of female rabbits through a platinum cannula whose lower end had been positioned in the gland. Each solution was made iso-osmotic and neutralized not more than 3 hr. before infusion into groups of four to six rabbits. Each animal received 0.13 ml. of a solution over a 2 hr. infusion period. The dose of extract infused was based on the number of fragments of tissue from which the material to be tested had been obtained. The animals were killed 48 hr. after infusion, and their ovaries examined for the presence of ruptured follicles. Ovulation in three or more rabbits was taken as a positive result. This method was chosen as it involves a specific reaction for detecting gonadotrophic-hormone secretion and since it is based on a response of known physiological significance. However, it does not lend itself easily to quantitative measurements and by its nature rules out the possibility of testing large numbers of samples at each stage of purification. Therefore, where a particular treatment gave rise to several fractions, a sample consisting of portions from each fraction was first tested to ensure that activity had not been lost, before the individual fractions were examined.

RESULTS

Experiments on extracts of bullock tissue. Treatment of the crude median-eminence extract with an equal volume of acetone provided a simple means

Table 1. *Experiments with extract from bullock median-eminence tissue*

Each rabbit received 0.13 ml. of the extract (two fragments of tissue/ml.). Details of the enzyme treatment appear in the text. Owing to the shortage of material and small numbers of rabbits, the results given of any particular stage are not statistically significant. The fractions that appeared to be the most active were used for the various stages of purification. CME, crude median-eminence extract.

Sample infused	No. of rabbits in group	No. of rabbits ovulating
CME	6	5
Acetone + CME (1:1), precipitate	6	1
Acetone + CME (1:1), supernatant	6	4
CME incubated with pepsin	5	2
CME incubated without pepsin	4	4

of removing inactive proteinaceous material, as shown in Table 1. LRF activity was found in the supernatant solution after centrifugation and removal of the acetone by rotary evaporation. Table 1 also shows the results of digestion of the crude extract with pepsin. The control sample retained all of its activity and the digested sample caused ovulation in two of the five rabbits used.

Gel filtration on Sephadex was chosen to separate the components of the extract according to molecular size. A reproducible pattern of ultraviolet absorption was obtained when the crude extract was passed through a column (35 cm. $\times$ 2 cm.) of Sephadex G-25 in 0.3N-acetic acid. This enabled the high-molecular-weight material that was not retarded by the column to be tested as a single sample separate from the lower-molecular-weight material, which was eluted over a large number of fractions and which was tested as two separate samples. Significant LRF activity was found in only one of these lower-molecular-weight samples, and no trace was found in the high-molecular-weight sample. The resolution of the components of the active sample was improved by increasing the dimensions of the Sephadex column to 65 cm. $\times$ 4.2 cm. and this enabled the active region to be subdivided into four distinct fractions for further testing. Two of these samples contained material having ovulation-inducing activity. The first fraction corresponded to the elution position of substances having a molecular weight in the range 1000–4000 that were only moderately retarded by the column and contained most of the peptide material and sodium salts. Antidiuretic activity (vasopressin) was found to occur in this fraction. The second active region contained material that was unusually strongly retarded by the column. The only detectable components of this region were ammonium acetate and cupric acetate. The presence of the copper salt was suspected, as a green

colour was visible after concentration of the fraction, and confirmation was obtained by means of its positive reaction with rubeanic acid.

A single attempt to achieve further purification by cation-exchange chromatography of the sample containing most of the peptide material that was obtained after gel filtration was unsuccessful. The sample was adjusted to pH 4 and applied to a column (33 cm. $\times$ 2 cm.) of CM-cellulose. A gradient of increasing pH (4.4–7.5) was applied by using 0.01M-ammonium acetate solution as the eluting agent. Most of the LRF activity was displaced from the column only after the pH of the eluate had reached 7.0. It was found that the high salt content of the original sample greatly decreased the efficiency of the ion-exchange process. Therefore this method of purification was not pursued further, although the increased activity in the eluate obtained at high pH indicated the basic nature of LRF.

Experiments with sheep tissue. About 3000 hypothalamic fragments (660 g.) gave 7 g. of the final dry precipitate when processed as described in the Materials and Methods section. Of this, 95% was soluble in 0.3N-acetic acid and was active at a dose of 1.5 fragments of tissue/rabbit. Gel filtration was carried out on 35 ml. of this extract (from 1500 fragments) on a column (65 cm. $\times$ 4.2 cm.) of Sephadex G-25 in 0.3N-acetic acid. A single filtration through this column was insufficient to produce a well-defined region of activity (Table 2) as had been obtained previously on a smaller scale with the bullock material. Activity was spread through the part of the filtrate that emerged between elution volumes 2 and 7. The active regions from two such columns were concentrated to 35 ml. and the gel-filtration process was repeated by a second passage through the same column (Fig. 2). The distribution of ultraviolet absorption and of Folin-positive material indicated that the second passage had achieved reasonable separation of components and three regions of the eluate were selected for testing. The significant activity was confined to the part of the filtrate that emerged between elution volumes 2.2 and 4 (R_1 ; Fig. 2). Identical results have been achieved on a larger scale with an improvement in the initial treatment of the tissue.

A 1 kg. sample of tissue (5000 fragments) was freeze-dried and the dry tissue blended with dry acetone (5:1, w/v) and washed with peroxide-free ether. The acetone-dried powder was then processed by the method referred to above and yielded 9.5 g. of final dry precipitate. Inclusion of the freeze-drying and acetone treatments was not detrimental to the recovery of activity, as this material was active when tested at the same concentration as the material obtained without the

Table 2. *Experiments with sheep hypothalamic extract*

These results show the effect of various treatments of the active fraction obtained at successive stages of the purification. Complete results of the infusions at each stage are given with the diagrams of the particular fractionation process (Figs. 2, 3 and 4). The concentration of the active fraction that was necessary to give a positive result is expressed in terms of the number of fragments of tissue/ml. Owing to the shortage of material and small numbers of rabbits, the results given of any particular stage are not statistically significant. The fractions that appeared to be the most active were used for the various stages of purification.

Sample infused	Concn. (fragment equiv./ml.)	No. of rabbits in group	No. of rabbits ovulating
Crude extract	10	10	10
Gel filtration, first passage:			
Elution vol. 2-3	12	3	3
Elution vol. 4-7	12	4	2
Gel filtration, second passage:			
Elution vol. 2-2-4	14	4	4
Same sample, stored frozen for 8 weeks	14	4	4
Same sample, incubated with pepsin	18	5	1
Same sample, incubated without pepsin	18	5	4
Chromatography on Sephadex A-25:			
Short column (10cm. $\times$ 5cm.)	16	8	8
Gel filtration on Sephadex G-25 in ethanol-0.2N-acetic acid (1:5, v/v):			
Sample E ₁	22	9	6
Same sample stored frozen for 6 weeks	25	5	0
Thin-layer chromatography of sample E ₁ :			
R _F 0-0.3	{ 33	4	1
	{ 75	4	0
R _F 0.3-0.75	{ 33	5	4
	{ 75	4	3

Fig. 2. Results of gel filtration of median-eminence extract and of infusions of samples from adjacent regions of the eluate. In this Figure and also in Figs. 3 and 4, the ovulatory response is represented by a fraction in which the numerator is the number of rabbits that ovulated after infusion of a sample of the eluate corresponding to the section of the diagram delineated by the arrows. The denominator denotes the number of rabbits used for each test. This Figure shows the results of the second passage of material (obtained between elution volumes 2 and 7 from the first passage) through the same column (65cm. $\times$ 4.2cm.) of Sephadex G-25 in 0.3N-acetic acid of the extract from 3000 sheep hypothalami. Fractions of volume 40ml. were collected and their E_{280} values (—) and E_{750} values (---) measured, and 0.2ml. samples used for the Folin-Lowry procedure (E_{750} , ····). Elution volume = volume of eluate, V_e /void volume of column, V_0 .

use of these procedures. Further, satisfactory gel filtration of this material was obtained without dividing it into several portions. All 9.5 g. of the ether precipitate was dissolved in 70 ml. of 0.3 N-acetic acid and gel filtration was carried out as above by using repeated passage through the same column (95 cm. $\times$ 6 cm.) of Sephadex G-25 having a void volume of 1030 ml. Only the eluent that emerged between elution volumes 2 and 7 was subjected to the second passage and activity was again recovered only in the region corresponding to R_1 in Fig. 2. A sample of this region caused ovulation in all rabbits in a group of four when each received an amount equivalent to 1.8 fragments of tissue. Pepsin digestion of a similar sample decreased the response to one ovulating rabbit out of five, as compared with an incubated control sample (four out of five) (see Table 2). This indicates that the activity of the extract may be dependent on the integrity of peptide bonds, in agreement with the results of the experiments with material from bullock tissue.

At this stage of the purification the active sample contained a large proportion of the peptides and inorganic material from the original tissue. Its ultraviolet extinction at 260 and 280 $m\mu$ suggested that it still contained some nucleotide material. Therefore the next stage of the procedure was designed to remove any acidic components from the LRF preparation. The active preparation

(region R_1 ; Fig. 2) corresponding to 1500 fragments of the original tissue was freeze-dried to 7 ml. and adjusted to pH 6 with 0.1 N-sodium hydroxide. The solution was put on a short column (10 cm. $\times$ 5 cm.) of DEAE-Sephadex A-25, which was used in preference to DEAE-cellulose because of its higher flow rate and capacity. The column was washed through with water until no more Folin-positive material emerged (tube 26) and then developed with 0.1 N-acetic acid. This resulted in the further elution of ultraviolet-absorbing material and a small amount of peptide-like material (Fig. 3). Tests showed that LRF activity was confined to the constituents of the peak that were not retarded by the column, as expected from the results of the preliminary cation-exchange experiments with bullock material, which indicated the basic nature of LRF. A method was then devised to remove the inorganic contaminants, mainly sodium salts, from the LRF preparation before the application of a method of separating the individual peptides. It was not possible to do this by further gel filtration on Sephadex G-10, G-15, G-25 or G-50 in dilute aqueous acetic acid; in each case a single peptide peak was obtained that overlapped with the elution position of sodium salts. Separation was achieved when the filtration was carried out in ethanol-0.2 N-acetic acid medium (1:4, v/v), and this method has been applied successfully as the next stage in the purification procedure. The active

Fig. 3. Anion-exchange treatment of the active material in sample R_1 (Fig. 2), corresponding to 1500 fragments of tissue. The active solution (7 ml.) was adjusted to pH 6 and put on a short column (10 cm. $\times$ 5 cm.) of DEAE-Sephadex A-25. The column was washed with water (270 ml.) and, after fraction no. 26, as indicated by the arrow, developed with 0.1 N-acetic acid. Fractions of volume 9 ml. were collected and their E_{260} values measured (----), and 0.1 ml. samples used for the Folin-Lowry procedure (E_{750} , —).

Fig. 4. Gel filtration in a partially organic medium of the active material obtained after anion-exchange chromatography. An active sample (5 ml.), corresponding to 1500 fragments of tissue, was passed down a column (120 cm. $\times$ 2 cm.) of Sephadex G-25 in ethanol-0.2 N-acetic acid (1:4, v/v). Fractions of volume 5 ml. were collected only after 250 ml. had emerged. Their E_{275} values (----) were measured, and 0.01 ml. samples used for the Folin-Lowry procedure (E_{750} , —). Inorganic material was found in fractions 60–70.

sample was adjusted to the composition described above, and passed through a column of Sephadex G-25 in the ethanolic medium (Fig. 4). Fractions were examined for their content of peptides and sodium salts and the pattern shown in Fig. 4 was used as a guide to separate the eluate into four regions for testing (E_1 – E_4). The first of these (E_1) contained almost all the peptides of the sample and was active at an amount of 27 μ g. (equivalent to about 3 hypothalamic fragments/animal). The third region (E_3) contained some of the sodium salts, a small quantity of peptide and a component having an extinction maximum at 275 m μ whose identity remains unknown. This region was also found to have LRF activity at a dose of only 3 μ g. of peptide/animal. This stage of the purification has consistently separated these two active regions. The activity in each was destroyed by proteolytic treatment with pepsin.

Further attempts to isolate LRF were restricted to fraction E_1 , since it appeared to contain only peptides whereas fraction E_3 was a mixture of peptides and salts. At least ten components were distinguishable when a sample was chromatographed on micro-crystalline cellulose in a butanol-acetic acid-water solvent. The routine screening of fractions was carried out by the use of thin-layer chromatography on 0.25 mm. layers of cellulose spread on standard microscope slides. The sample had to be applied to the cellulose in a narrow band, but the slides required less material than larger plates and took only 20–30 min. to run. After the

slides had been dried in a current of air at room temperature and examined under ultraviolet light, they were placed in an atmosphere of iodine vapour for up to 30 min. The iodine-positive bands were marked and their variations in colour noted. The cellulose was freed of iodine by mild steaming over a hot-water bath and finally sprayed with ninhydrin-cadmium reagent (Heilmann, Barollier & Watzke, 1957). This highly sensitive reagent usually revealed more components than the ultraviolet light or iodine vapour, although some ninhydrin-positive bands may be due to amino acids rather than peptides (see Fig. 5). Unfortunately, the small amount of material available precluded the use of further tests for specific amino acids. The 0.5 mm. layers have been found to give the most reproducible results when this technique was adapted to preparative experiments.

A 0.5 mg. sample of the active mixture of peptides was chromatographed on a 20 cm. $\times$ 10 cm. plate for a development period of 4 hr., during which time the solvent rose 20 cm. Resolution of components was improved appreciably without lengthening the development time by interrupting the development after the solvent had risen some 2 cm., drying rapidly in a current of air at room temperature and then restarting the chromatography. The sections of the cellulose to be eluted were marked while the plate was being examined under ultraviolet light, and scraped into a sintered-glass funnel before the cellulose was completely dry. The cellulose was triturated with 0.5 N-acetic acid

Fig. 5. Analytical thin-layer chromatography on cellulose (CC41; British Drug Houses Ltd.). The 0.25mm. layers of cellulose were spread on microscope slides (7.5cm. long) and the cellulose was removed from each edge of the slide to facilitate handling. (1) The active peptide fraction (E_1) obtained by gel filtration in ethanol-0.2N-acetic acid (1:4, v/v). (2) Components of fraction E_1 obtained by preparative thin-layer chromatography (R_F 0.0-0.3) that were found to be inactive. (3) The active fraction obtained by preparative thin-layer chromatography of fraction E_1 (R_F 0.3-0.75). The lines at the edge denote bands visible under ultraviolet light. In each case the solvent was butan-1-ol-water-acetic acid (4:1:5, by vol.) and the slides were developed with the ninhydrin-cadmium reagent.

and the filtrate from each section of cellulose concentrated for further examination and for testing. This technique allowed a recovery of approx. 80% of the peptide applied to the plate. A sample corresponding to R_F 0.0-0.3 was found to be inactive. LRF activity was recovered from the region corresponding to R_F 0.3-0.75. This region contained 40% of the total peptide recovered and rechromatography showed that it still contained at least five components (Fig. 5). It was active at a dose of 6 μ g. (about 4 hypothalamic fragments)/animal. Repetition of this process in order to obtain a homogeneous preparation was precluded by the finding that at neither this stage of the purification, nor indeed at the previous one, would a sample retain its activity during storage in frozen aqueous solution at about pH4 for any length of time. This instability was not encountered during earlier stages of purification (see Table 2). We have, however, in agreement with other workers

(Endroczi & Hilliard, 1965), noticed a loss of activity in the neutralized samples prepared for testing.

DISCUSSION

The method used for LRF detection required that the extracts tested should be iso-osmotic, of neutral pH and non-toxic. Included among toxic substances commonly used in extraction procedures are heavy-metal ions, protein precipitants, organic bases often used in buffer solutions, such as pyridine or degradation products thereof, and high concentrations of ammonium salts. They are difficult to remove, particularly from small amounts of peptides. For this reason extensive use has been made of fractionation on Sephadex in conjunction with volatile eluents, such as dilute acetic acid, alone or with ethanol. Water alone was used as the solvent in one stage of the procedure.

Methods of separation have been used that initially yielded many successive fractions. Since the intrapituitary infusion test for LRF gave results of a qualitative nature, it was necessary to ensure that subsequent steps of purification did not result in fractions to be tested in which the LRF activity was dispersed in sub-threshold amounts. The risk of thereby obtaining false negative results was diminished by recombining successive fractions on the basis of their ultraviolet-absorption properties and their reaction in the Folin-Lowry procedure.

Though the final goal of this work was the identification of LRF, a subordinate purpose was the biochemical verification of the humoral regulation of luteinizing-hormone secretion. The starting point of the present study was the observation of Campbell *et al.* (1961) that LRF activity could be found in crude extracts of bullock median-eminence tissue. Evidence has since been obtained that some of the activity in hypothalamic extracts was due to traces of the gonadotrophic hormone rather than to its releasing factor (Guillemin, Colonge, Jutisz & Sakiz, 1962; Courrier, Jutisz & Colonge, 1963). The gel-filtration experiments demonstrate that LRF activity is associated with a much smaller molecule than luteinizing hormone. They also show that LRF activity can be separated from copper salts (which are known to evoke ovulation when injected into rabbits). The results of the experiments reported here verify the existence of LRF and also indicate that its nature may be a basic peptide of molecular weight in the range 1200-2500. While this work was in progress several other independent groups of workers, using biochemical techniques related to those described here, have arrived at similar conclusions (Guillemin, Jutisz & Sakiz, 1963; Schally & Bowers, 1964; Dhariwal, Antunes-Rodrigues & McCann, 1965).

The isolation of a homogeneous preparation of LRF has not been accomplished, though amino acid compositions of purified preparations have been reported for LRF from bullock and sheep (Schally & Bowers, 1964; Guillemin, 1965). In agreement with other workers, we find that the amount of active peptide that survives the later stages of our isolation procedures is insufficient for the complete isolation of LRF, even when the process is conducted on a scale involving several thousand hypothalamic fragments. A further hindrance has been the loss of activity that occurs during storage of the more purified preparations. Real or apparent loss of activity has been experienced in the isolation of other biologically active peptides, particularly when a high degree of purification has been achieved. In some cases, the explanation has proved to be adsorption to surfaces such as glass (with adrenocorticotrophic hormone; Stouffer & Lipscomb, 1963) or ion-exchange materials (with bradykinin; Elliott, Horton & Lewis, 1961). Inactivation by chemical modification, such as oxidation, has been observed with adrenocorticotrophic hormone (Li, 1962) and the parathyroid hormones (Rasmussen & Craig, 1962). No information is yet available to explain the inactivation of LRF, although causes other than oxidation may be suspected as neither of the analyses claimed for LRF peptides revealed any sulphur-containing amino acids. Indeed, in view of the varied conditions under which a loss or diminution of LRF activity has been observed (in neutral aqueous solution and during storage either in a frozen weakly acidic solution or as a freeze-dried powder), it is not yet possible to distinguish whether the loss of activity is of a physical or chemical nature.

The discovery of two separate regions of activity after gel filtration in the partially organic medium was unexpected; the activity in each appeared to be due to a peptide on the basis of their lability to pepsin. The significance of these findings is far from clear, and will probably remain so until a homogeneous preparation has been obtained.

Our experience amply confirms the earlier finding that the hypothalamus is not rich in peptides (Winnick, Winnick, Acher & Fromageto, 1955). Our purest preparation of LRF was obtained in an approximate yield of 0.5 mg. of peptide/1000 fragments processed, yet this still contained five or six components. This suggests that LRF may be present in hypothalamic tissue only to the extent of about 1 p.p.m. (by wt.).

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