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Regulation of gonadotropin secretion in the anterior pituitary

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[Plates 1–3]

Studies on the regulation of gonadotropin secretion in dissociated pituitary cell cultures are described. Initial studies employing a ferritin-labelled analogue of gonadotropin hormone releasing hormone (GnRH) to localize its receptor sites on the gonadotropin cell surface showed that while these receptor sites initially have a random monodisperse distribution, binding of the ligand causes coarse aggregation and internalization of the GnRH receptor. These events are not due to the multivalency of the ligand and probably reflect redistributive events *in vivo*. By using an octapeptide analogue GnRH that binds to the GnRH receptor but lacks gonadotropin releasing activity in conjunction with sequence-specific antisera it is shown that antibodies that bind the octapeptide can induce the octapeptide to release gonadotropin. These data suggest that receptor aggregation is important in GnRH stimulation. Finally immunocytochemical studies are described in which golg–protein-A-antibody complexes are used to identify gonadotropins on ultrathin frozen sections of porcine pituitary cells. These studies indicate that in porcine gonadotropin cells the majority of the secretory granules contain both luteinizing hormone and follicle-stimulating hormone.

Introduction

The regulation of gonadotropin secretion in the anterior pituitary is part of a complex system of negative and positive feedback information loops that control both short-term cyclical activity and longer-term developmental processes in the gonads. The participating neurendocrine and endocrine components of the system reside within the hypothalamus, the pituitary and the gonads, and most of the system's blood-borne peptide, polypeptide and steroid hormone mediators have been identified. The gonadotropin cells of the pituitary perform their regulatory role by integrating the information that they receive from the brain and the gonads and then secreting appropriate concentrations of the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) into the systemic circulation (figure 1) (McCann & Ramirez 1964; Bogdanove *et al.* 1975; Knobil 1974; Brown & Grant 1977; Fink 1979*b*).

The information currently available from studies on primates and rodents indicates that in spontaneously ovulating females ovulation depends upon a cyclic variation in steroid plasma levels (Knobil 1974; Fink 1979; Fink & Pickering 1980). Basal levels of oestrogen inhibit gonadotropin secretion but above threshold levels this steroid initiates a series of accumulative events that cause a surge in the rate of gonadotropin secretion from the pituitary. In the ovary this preovulatory gonadotropin surge brings the developing follicles to maturity and induces ovulation.

Against the background of cyclically varying steroid levels the hypothalamic peptide, gonadotropin hormone releasing hormone (GnRH), acts as the primary stimulus for gonadotropin release (Shally *et al.* 1971; Gallo 1980). The peptide is released from the hypothalamus

in regular pulses and transported directly to the pituitary by the hypothalamo-hypophysial portal system. It thus arrives at the gonadotropin cell as an intermittent signal that can be varied in both frequency and amplitude (Knobil 1980). The increase in oestrogen levels before ovulation enhances the sensitivity of the pituitary to GnRH (Fink 1979*a*). In addition, GnRH also exercises a self-priming effect, increasing the sensitivity of the gland to subsequent GnRH challenge (Aiyer *et al.* 1974).

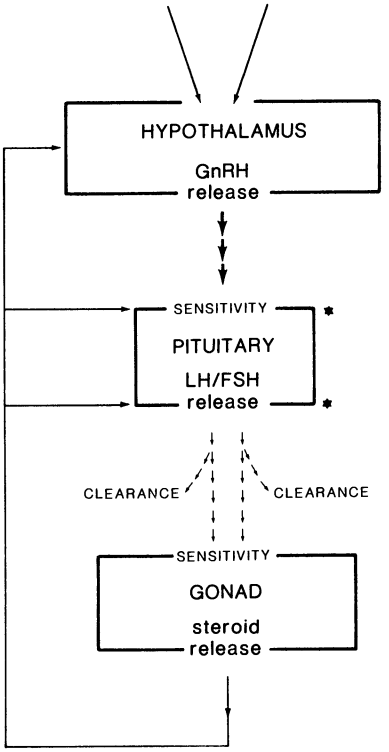

FIGURE 1. Outline of pathways concerned in the regulation of gonadotropin secretion. The hypothalamus receives inputs from higher centres, which in conjunction with circulating gonadal steroids modulate the pulsatile release of gonadotropin hormone releasing hormone (GnRH) into the hypothalamo-hypophysial portal system. The responsiveness of the pituitary to GnRH stimulation is modulated by both circulating steroid levels and by GnRH itself. GnRH stimulates the pulsatile release of gonadotropin. The amount of gonadotropin released by a given GnRH stimulus and the proportion of luteinizing hormone (LH) to follicle-stimulating hormone (FSH) in the circulation varies during the oestrous cycle and may thus also be modulated by the circulating steroid level. In the circulation, FSH has a much longer half-life than LH and both of these gonadotropins regulate the responsiveness of the ovary to subsequent gonadotropin stimulation.

The cellular mechanisms underlying the variations in pituitary responsiveness to GnRH during the reproductive cycle are not fully understood. However, for variations in the amplitude of the response, changes in GnRH receptor number are probably important since radioreceptor assays for the peptide have demonstrated changes in receptor number paralleling the regular fluctuations in responsiveness that occur during the cycle (Clayton *et al.* 1980; Savoy-Moor *et al.* 1980). Experimental manipulations such as castration and subsequent steroid replacement, which are able to modify responsiveness, have also been shown to induce predictable alterations in GnRH receptor number (Clayton & Catt 1981). So far no changes in the affinity of the receptor for the GnRH ligand have been found, although other changes within the vicinity of the receptor such as the desensitization of second messenger systems have been suggested (Smith & Vale 1981).

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Regulatory mechanisms operating at a more distal, effector, level within the gonadotroph also probably exist. Thus although GnRH provokes the release of both pituitary gonadotropins in many species, the relative concentrations of FSH and LH in the circulation vary during the cycle (Brown & Grant 1977; Fink 1979; Vandalem 1979). Additional regulatory mechanisms playing a role in varying the amplitude of the response may also operate at this level since it has been shown that the amount of gonadotropin available for secretagogue-stimulated release also fluctuates with the cycle (Speight & Fink 1981).

In our studies on the regulation of gonadotropin secretion we are considering two particular aspects of the secretory process in the gonadotroph: (1) the distribution and availability of GnRH receptors and (2) the distribution and availability of the two gonadotropins.

Distribution of GnRH receptors on the surface of dissociated pituitary cells

For our initial studies in this area (Hopkins & Gregory 1977) we conjugated a superactive analogue of the GnRH peptide to a particulate tracer horse spleen ferritin. The analogue *P* Glu-His-Trp-Ser-Trp-D-Lys-Arg-Pro ethyl amide was prepared by classical methods, coupled via the ε-amino group of the D-lysine through hemisuccinate to ferritin. The total conjugate when finally separated by chromatography on Biogel A-5M had a 10% incorporation of peptide. For use with cells the ratio of peptide to ferritin was 1:1. The cells used for these experiments were derived from male rats. They were dissociated enzymically (Hymer 1972) and then incubated for 48 h under standard culture conditions. In response to synthetic GnRH these cells release LH and FSH in a dose-related manner with an $e.d._{50}$ of 10 nM, and by using similar primary cultures it was shown that the GnRH–ferritin conjugate had a similar biological potency. A previously prepared conjugate in which the ferritin was coupled directly to the D-Lys^6 amino group of the peptide lacked biological activity.

The GnRH–ferritin conjugate was incubated with 48 h cultured cells that had been fixed briefly with 0.1% paraformaldehyde (and quenched with borohydride) for 15 min. After rinsing thoroughly at 5 °C the cells were post-fixed in Karnovsky fixative (Karnovsky 1965) embedded in epoxy resin, sectioned and examined in the electron microscope. On the surface of cells recognizable as gonadotropin cells by virtue of their fine structural organization, a monodisperse distribution of ferritin particles (with a density estimated as $32 \pm 12/\mu m^2$) was observed (figure 2*a*, plate 1). In cells preincubated with 1 μM GnRH, binding of the ferritin conjugate to the free cell surface was inhibited.

On cells incubated with GnRH–ferritin for 15 min at room temperature (i.e. without prior treatment with formaldehyde) the distribution of ferritin particles was rapidly redistributed from the monodisperse distribution observed in prefixed cells into coarse polar aggregates (figure 2*b*). Within these aggregates there were frequent profiles indicating endocytic internalization and within the cell, in the vicinity of the Golgi complex, multivesicular bodies containing conjugate were commonly observed (figure 2*c*).

The polar aggregation, sloughing and internalization of the GnRH–ferritin conjugate observed in these experiments are very similar to those induced in other systems by multivalent ligands (Schreiner & Unanue 1976). We could not exclude the possibility that the ferritin conjugate contained a substantial proportion of multivalent complexes and we therefore concluded that the events we had observed were probably also caused by multivalent interactions. To overcome this complication we devised a two-step approach in which the

GnRH ligand was derivatized with biotin and allowed to bind and redistribute on the cell surface before a second step, avidin-bearing ligand (colloidal gold–avidin) was applied. The rationale behind this approach and its application in the surface localization of epidermal growth factor has been described elsewhere (Tolson *et al.* 1981). With pituitary cells the approach was less successful but it was possible to establish that essentially the same events as those observed with GnRH–ferritin occurred. No evidence of local aggregation before capping or local invagination was obtained.

Two recent studies have been published that are in agreement with these findings (Hazum *et al.* 1980; Naor *et al.* 1981). In both of these reports the same GnRH analogue coupled to a fluorescent rhodamine label have been used in conjunction with the video intensification microscopy of living, dissociated cells. Similar time courses for binding and polar aggregation of the GnRH receptor have been observed and some indication of internalization has been reported. These studies thus confirm the view that the binding of GnRH to the free surface of dissociated gonadotropin cells leads to a redistribution and probably the internalization of a substantial proportion of the GnRH receptor population. The gross redistribution observed suggests that in cultured pituitary cells there are few constraints on the mobility of aggregating GnRH receptors. The processing of bound ligand is thus probably similar to the ligand-induced patching and capping observed in free cells such as leucocytes. In the intact tissue, intercellular junctional elements can be expected to restrict such gross redistributive processes on the cell surface to more specifically defined domains. In future analyses it will thus be important to develop methods for applying the labelled GnRH to gonadotropins in which these restrictive domains are maintained.

The time course of the aggregation process is worthy of further consideration, however, since it suggests that major redistributive events and internalization are relatively prolonged (more than 15 min). It should thus be possible to relate them to the kinetics of GnRH–LH stimulus–release coupling. To obtain a more precise indication of the formation of coarse aggregates we have therefore repeated the study described above on dissociated porcine pituitary cells (Walker & Hopkins 1978) and by light microscopy in conjunction with immunofluorescence for LH counted the number of gonadotropin cells capped with GnRH–ferritin with time. As shown in figure 3 the data obtained in this study confirm our earlier observations and show that capping becomes maximal after about 15 min. Previous studies on the kinetics of GnRH-induced LH release in the porcine preparation (Walker & Hopkins 1978) have shown that the peptide induces a rapid increase in LH release within 2 min of stimulation, and later studies (Hopkins & Walker 1978) showed that this initial phase of release is preceded by a redistribution of Ca^{2+} within the cell (indicated in figure 3 as a 45 Ca^{2+} efflux). These initiating events clearly precede the gross redistribution of GnRH receptors described above and we therefore conclude that the functional significance of the polar aggregation and internalization of the GnRH conjugate must be related to a post-stimulatory mechanism. In a recent study in which a GnRH analogue coupled to agarose beads was shown to be able to stimulate LH release, Conn *et al.* (1981) have also concluded that internalization is not a prerequisite for GnRH-induced LH release.

The best evidence that we have been able to obtain in support of the idea that GnRH receptor aggregation may play a role in stimulating gonadotropin secretion is derived from studies in which GnRH-specific antisera have been shown to enhance the potency of the peptide. In these studies we have used a GnRH superagonist GnRH-D-Lys^6-Pro-ethyl amide and an antagonist

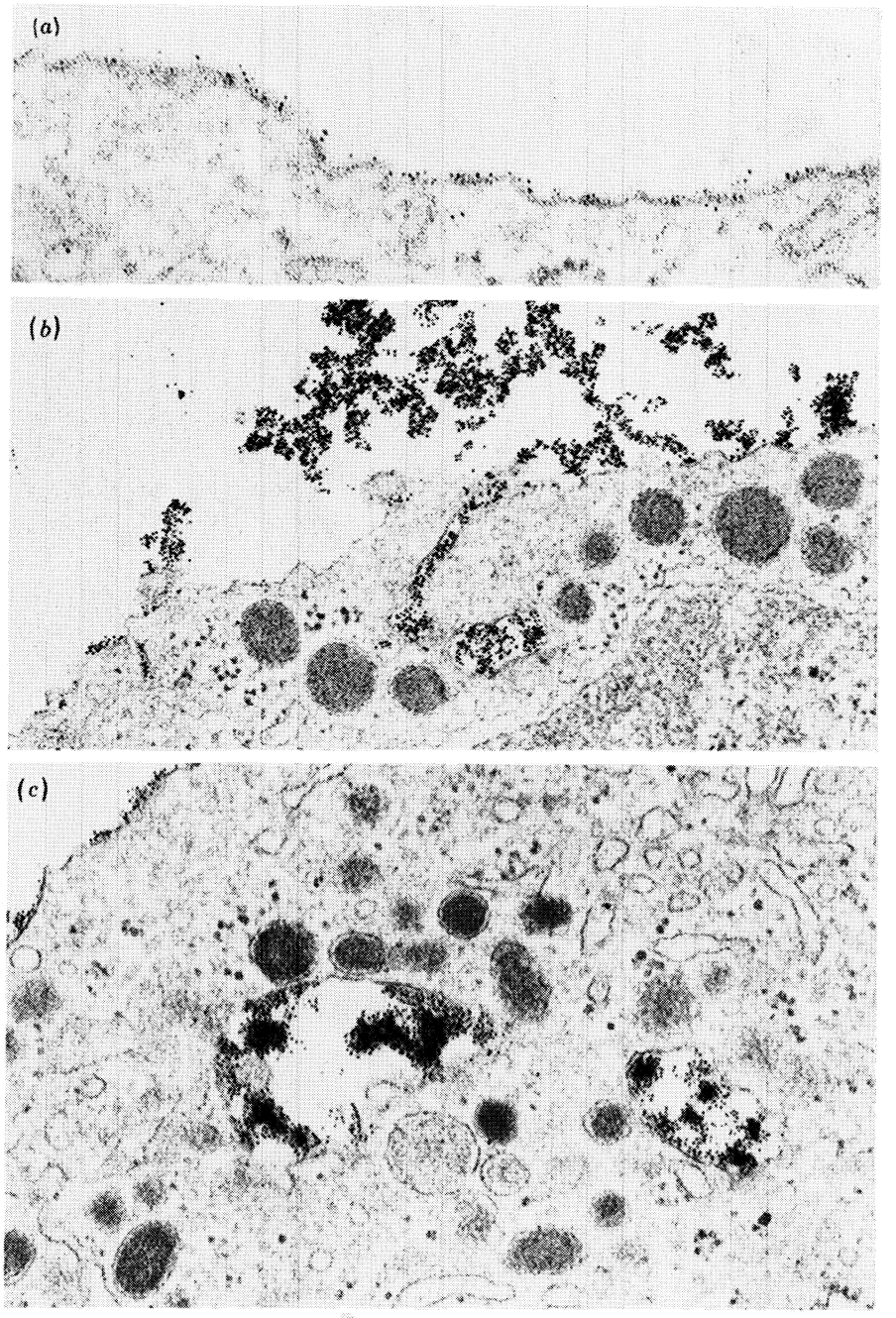

FIGURE 2. (*a*) Ferritin–GnRH analogue binding to prefixed gonadotropin cell surface. (Magn. ×50000.) (*b*) Ferritin–GnRH analogue binding to gonadotropin cell surface incubated for 15 min at ambient temperature. Coarse aggregation of conjugate and evidence of both sloughing and internalization. (Magn. ×50000.) (*c*) Ferritin–GnRH analogue in cells incubated for 15 min at ambient temperature. Conjugate distributed within multivesicular bodies in Golgi area. (Magn. ×50000.)

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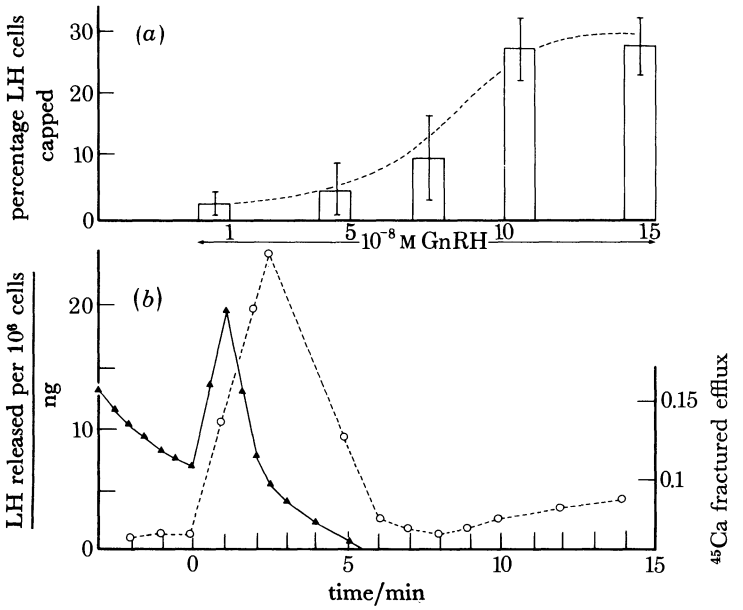

FIGURE 3. (*a*) Formation of coarse aggregates of ferritin–GnRH analogue on porcine gonadotropin cells with time. Aggregation is maximal at 10–15 min. (*b*) Kinetics of LH release and $^{45}Ca^{2+}$ efflux stimulated by continuous 10mM GnRH (details given in Hopkins & Walker (1978)). Maximum stimulation at 3 and 1 min respectively.

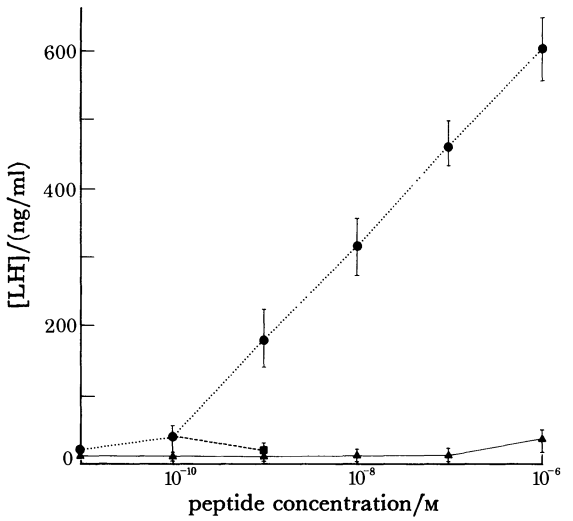

FIGURE 4. Stimulation of LH release from dissociated porcine pituitary cells by native GnRH (●) and octapeptide (▲). ■, Stimulation by 1 nM GnRH in the presence of 1 μM octapeptide.

the octapeptide *Z*-Gln-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-*N*-Et as an antagonist (Reel *et al.* 1980), and we have raised a variety of specific antibodies to them. Competitive assays against ^{125}I-labelled peptides have been used to select antisera that either bind both peptides or bind preferentially only to the octapeptide. These antisera have then been examined for their effect on GnRH-stimulated LH release.

Shown in figure 4 is the dose–response curve obtained with native GnRH. At concentrations below 1 μM the octapeptide has no effect on LH release in this preparation but it is able to

inhibit the effect of 10 nM GnRH competitively. We therefore presume that the octapeptide binds to the GnRH receptor but fails to elicit the LH release response. However, as shown in figure 5, in the presence of antibody to the octapeptide the octapeptide is capable of inducing LH release. Alone the octapeptide antibody has no affect on LH release, and antibody that cross-reacts with GnRH-D-Lys^6-Pro-ethyl amide and native GnRH but which fails to bind the octapeptide in the binding assay does not affect the agonist acitvity of the octapeptide. The GnRH-D-Lys^6 antibody can, however, enhance the potency of native GnRH (data not shown).

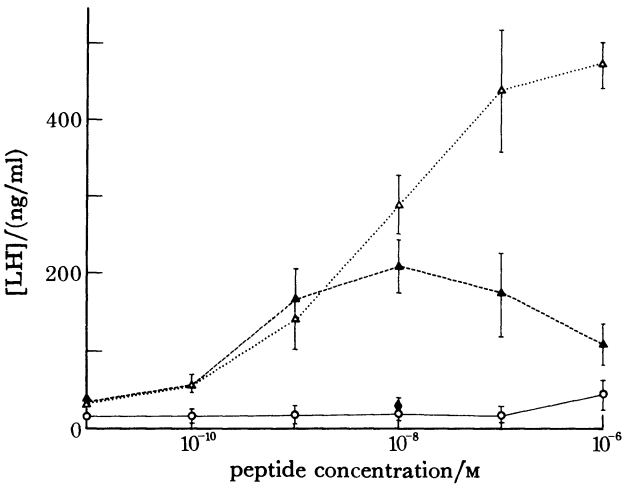

FIGURE 5. Stimulation of LH release from dissociated porcine pituitary cells by native GnRH (△), octapeptide (○) and octapeptide in the presence of 1:1000 dilution of octapeptide antibody (▲). ◆, Effect of antibody that binds native GnRH but has little detectable cross-reactivity with octapeptide.

The most reasonable interpretation of these data is that the antibodies that enhance the peptide-mediated response are able to crosslink peptide–GnRH receptor complexes. Studies are in progress to ascertain if the agonist effect of the octapeptide antibody depends upon its ability to bind the peptide multivalently.

DISTRIBUTION OF GONADOTROPINS IN THE PORCINE GONADOTROPIN CELL

Over the last two decades considerable efforts have been made to explain the differential release of LH and FSH in terms of their compartmentation within the gonadotropin cell population (reviewed in Batten & Hopkins (1978); see also Dacheux (1980)). The possibility that distinct cell populations or separate secretory granule populations for each of the two hormones has been considered but the most recent immunocytochemical studies have suggested that the majority of gonadotropin cells contain both gonadotropins and that both gonadotropins probably exist within the same secretory granules (Batten & Hopkins 1978; Dacheux 1979). The problem of localizing LH and FSH immunocytochemically presents difficulties because the two molecules share extensive homology (their α subunits are identical and their β subunits share a high proportion of common residues) (Pierce *et al.* 1976; Vandalem *et al.* 1979) and because their antigenicity is largely destroyed by glutaraldehyde-containing fixatives (Batten & Hopkins 1978). In our current studies we have largely overcome these difficulties by using antisera specific for the β subunits of FSH and LH (Batten & Hopkins 1978; Vandalem *et al.*

1979) in conjunction with ultrathin frozen sections Tokuyasu 1980) of tissue fixed in formaldehyde. To extend our earlier qualitative analysis we have employed a particulate complex of gold colloid coated with the staphylococcal coat protein A (Tolson *et al.* 1981), since this second-step reagent allows two different antigens to be identified within the same section and has the potential of being used for quantitative analyses.

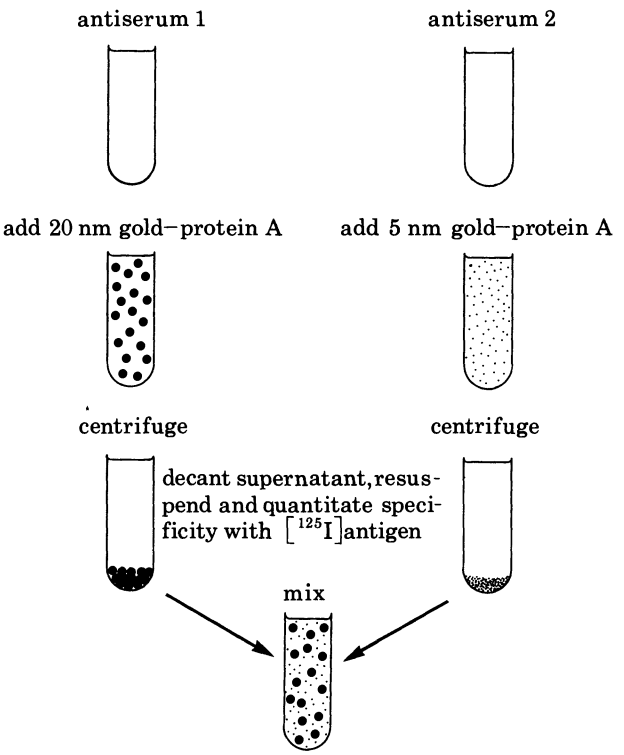

FIGURE 6. Outline of procedure for preparing gold–protein-A-specific antibody complexes. In this manner 20 nm complexes bearing LH antibody and 5 nm complexes bearing FSH antibody were prepared and characterized.

The method for preparing gold–protein-A–antigonadotropin complexes is outlined in figure 6. The gold colloid–protein A complexes are prepared by standard methods (Horrisberger 1979) and incubated with IgG fractions of either FSH β or LH β antisera. Since the complexes are dense they are rapidly separated by centrifugation and their ability to bind radiolabelled gonadotropins is thus readily characterized by conventional competitive binding assay.

When their specificity at a given dilution had been determined, the complexes were mixed and incubated with the ultrathin sections prepared from paraformaldehyde-fixed tissue. Figure 7 shows a representative binding assay in which 12 nm gold–protein-A–anti-LH β complexes were incubated with [^{125}I]LH in competition with unlabelled LH and FSH, which demonstrates that in binding to the gold complex, LH competes much more effectively than FSH.

When a mixture of 5 nm gold–anti-FSH β and 12 nm gold–anti-LH β complexes were incubated with pituitary sections, only one cell type characterized by *ca.* 150 nm granules bound gold particles above background levels. Localization was largely restricted to secretory granules although there is some evidence of labelling over the rough endoplasmic reticulum (figure 8, plate 2). Attempts to employ mixtures of gold complexes for quantitative studies have not been

successful because the density of labelling seems to be determined primarily by the size of the gold particles rather than the density of antigen. To explore the possibility that a population of secretory granules may contain subpopulations in which the proportions of LH and FSH vary, we have optimized the binding of a single particle size (5 nm) and compared its distribution and density throughout the granule population.

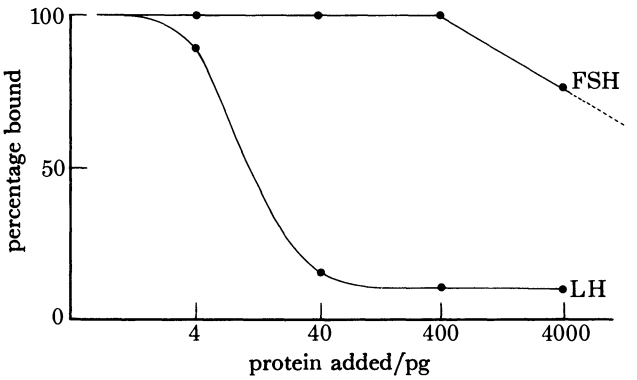

FIGURE 7. Competitive binding assay in which LH and FSH compete with [^{125}I]LH for binding to 20 nm gold–protein-A–anti-βLH antibody complexes. [^{125}I]LH (total count added: 10^5 min^{-1}) was incubated for 18 h with gold–protein-A–anti-βLH in the presence of LH or FSH at the concentrations indicated. Free and bound [^{125}I]LH was separated by centrifugation at 12000 *g*.

So far in glands from both male and female animals we have failed to find a population of cells that binds only one gonadotropin complex, and with rare exceptions we have found that all of the granules within a given cell also bind both FSH β and LH β antisera. We thus conclude that while the proportions of LH and FSH within a granule population may vary, neither gonadotropin is packaged exclusively within a separate granule population.

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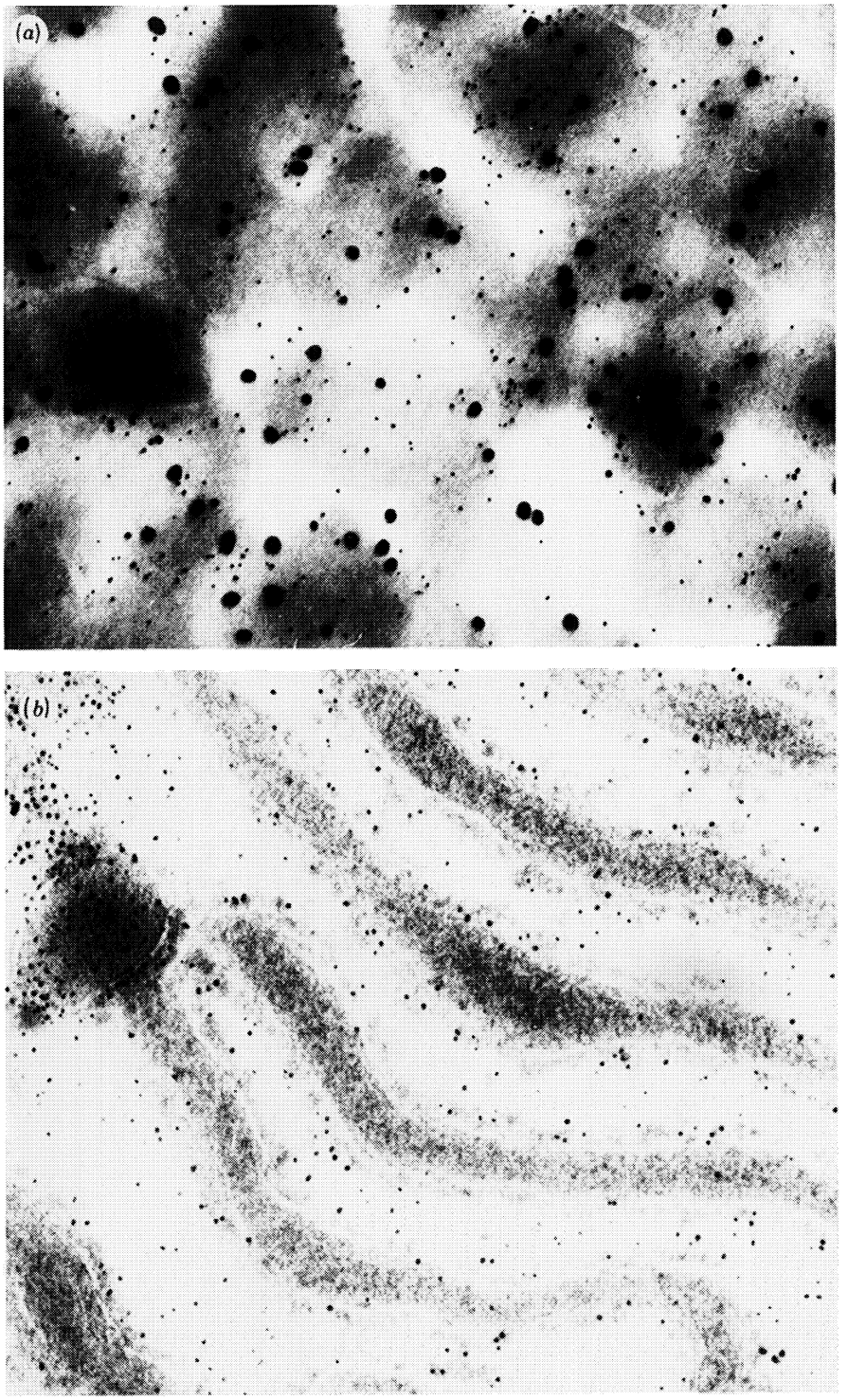

FIGURE 8. (*a*) Ultrathin frozen section of formaldehyde-fixed tissue incubated with a mixture of 20 nm gold–protein-A–βLH antibody and 5 nm gold–protein-A–βFSH antibody. Secretory granules bind both 20 and 5 nm gold complexes. (Magn. × 110000.) (*b*) Tissue prepared as in (*a*) incubated with 5 nm gold–protein-A–LH antibody. Gold complexes associated with rough endoplasmic reticulum cisternae. Membrane boundaries appear in negative relief. (Magn. × 150000.)

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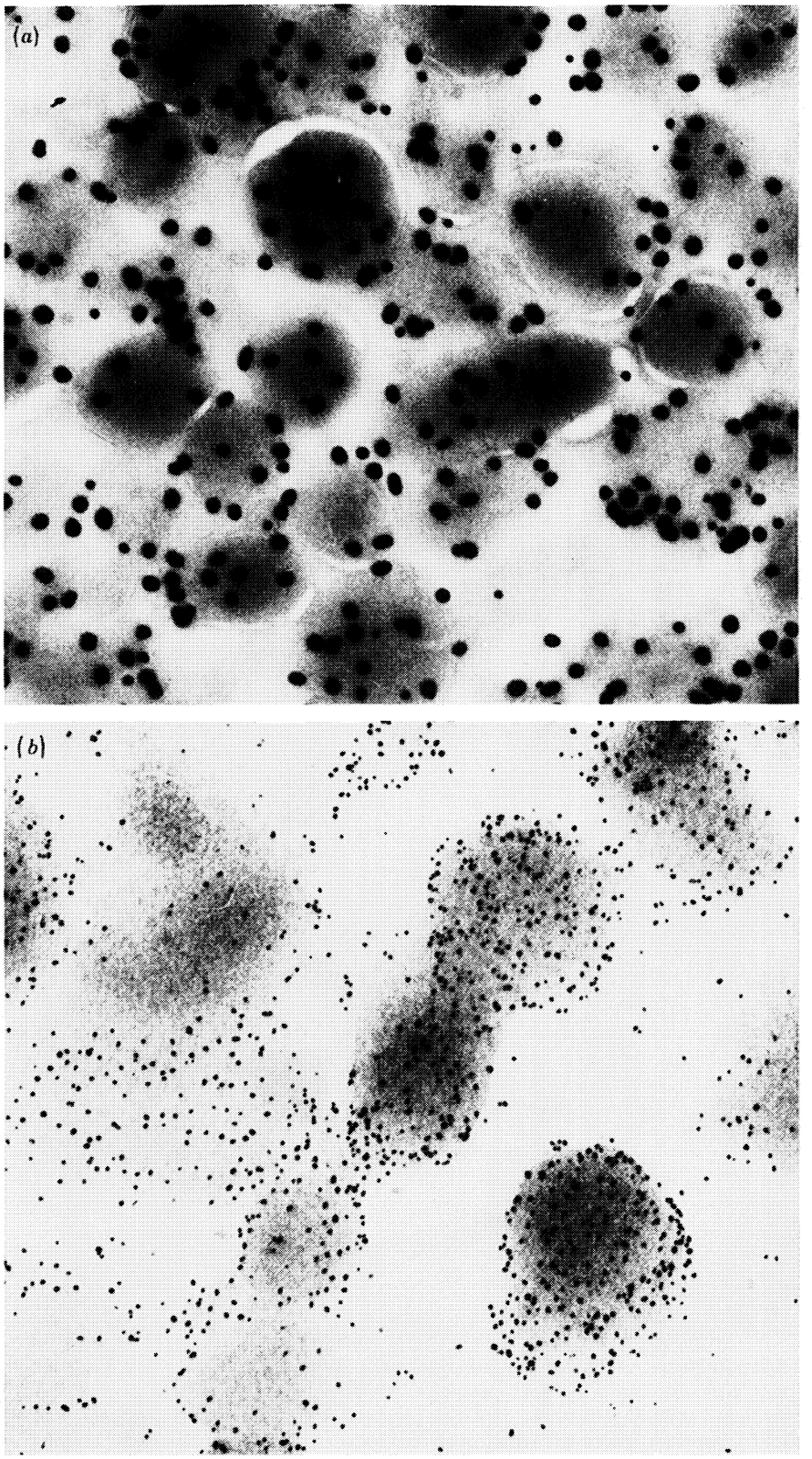

FIGURE 9. Tissue prepared as in figure 8*a* incubated under optimum conditions with 20 nm gold–protein-A–anti-βLH complexes. (Magn. ×150000.) (*b*) As (*a*) but prepared with 5 nm gold–protein-A–anti-βLH complexes. With smaller particulate complexes the density of labelling is significantly increased and a distinctive patterning of the granule matrix is evident. (Magn. ×150000.)

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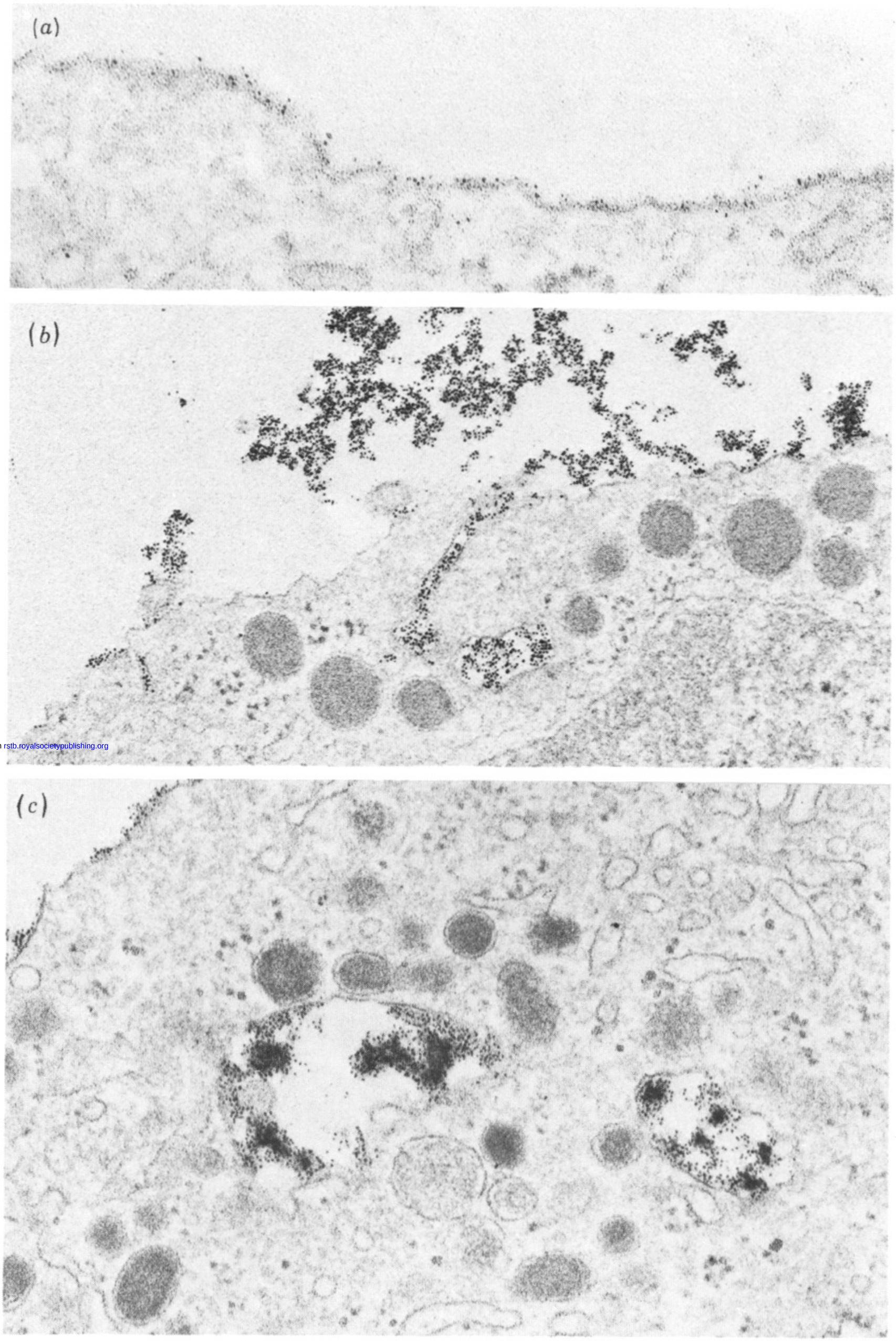

FIGURE 2. (*a*) Ferritin–GnRH analogue binding to prefixed gonadotropin cell surface. (Magn. ×50000.) (*b*) Ferritin–GnRH analogue binding to gonadotropin cell surface incubated for 15 min at ambient temperature. Coarse aggregation of conjugate and evidence of both sloughing and internalization. (Magn. ×50000.) (*c*) Ferritin–GnRH analogue in cells incubated for 15 min at ambient temperature. Conjugate distributed within multivesicular bodies in Golgi area. (Magn. ×50000.)

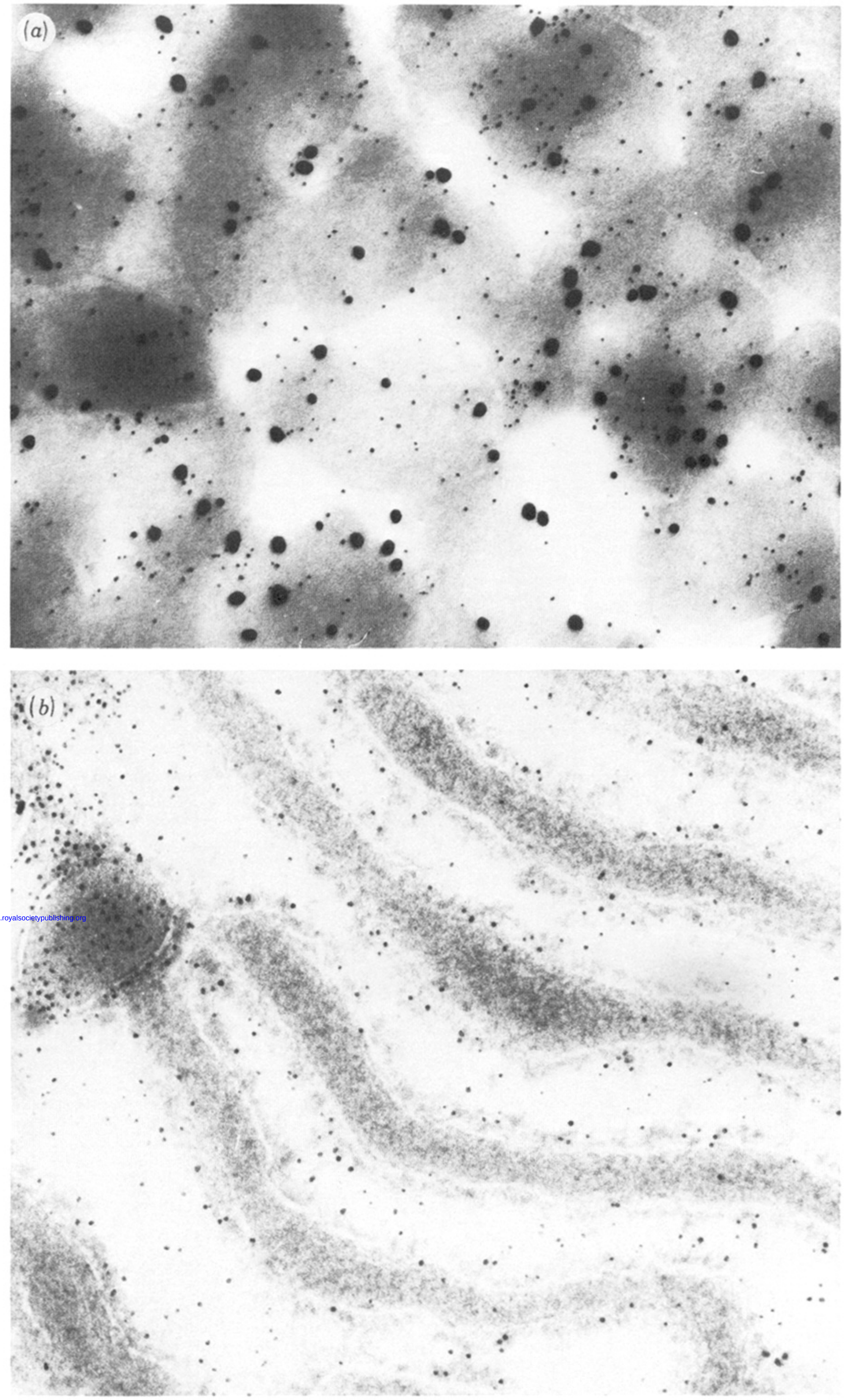

FIGURE 8. (*a*) Ultrathin frozen section of formaldehyde-fixed tissue incubated with a mixture of 20 nm gold–protein-A–βLH antibody and 5 nm gold–protein-A–βFSH antibody. Secretory granules bind both 20 and 5 nm gold complexes. (Magn. ×110000.) (*b*) Tissue prepared as in (*a*) incubated with 5 nm gold–protein-A–LH antibody. Gold complexes associated with rough endoplasmic reticulum cisternae. Membrane boundaries appear in negative relief. (Magn. ×150000.)

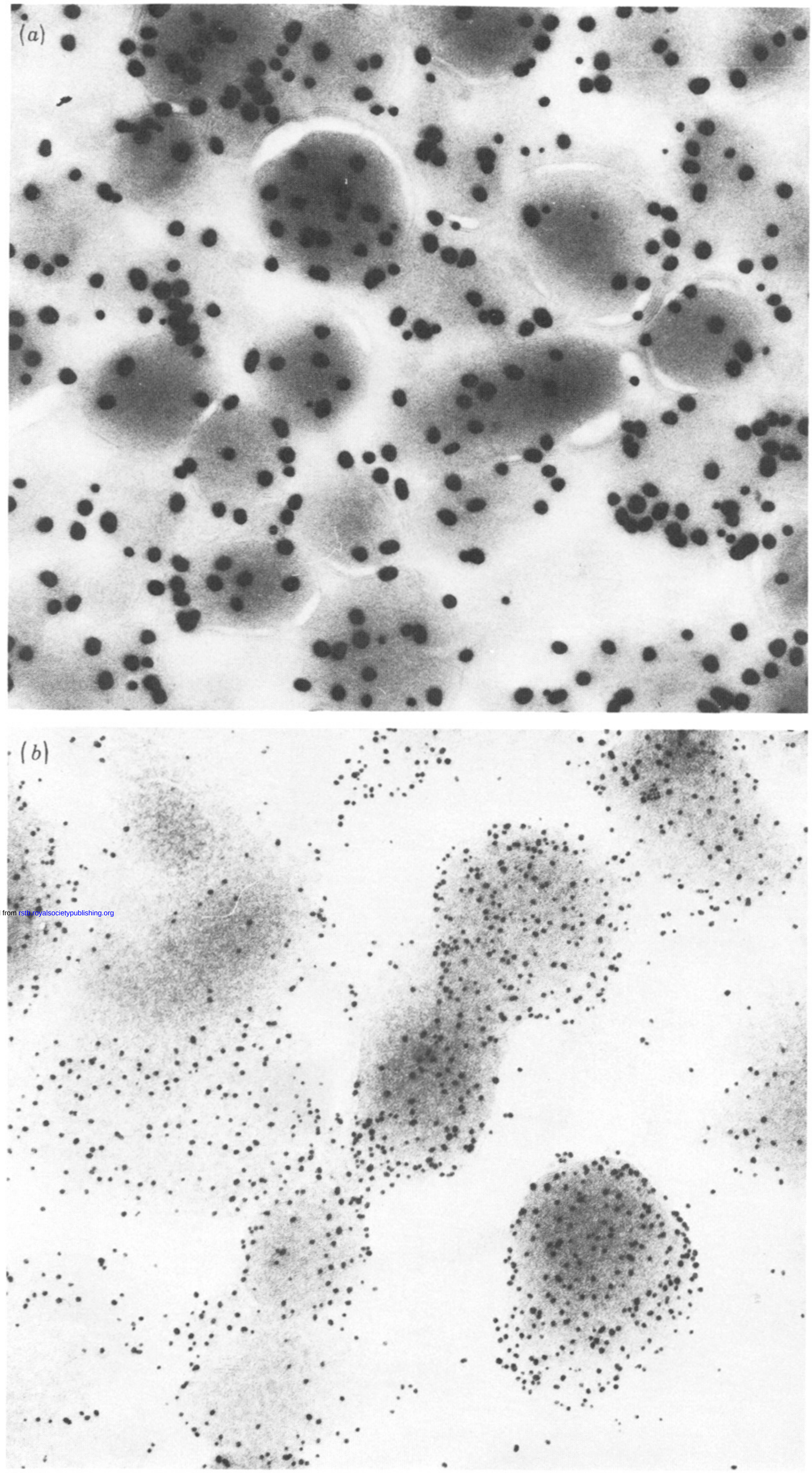

FIGURE 9. Tissue prepared as in figure 8*a* incubated under optimum conditions with 20 nm gold–protein-A–anti-βLH complexes. (Magn. ×150000.) (*b*) As (*a*) but prepared with 5 nm gold–protein-A–anti-βLH complexes. With smaller particulate complexes the density of labelling is significantly increased and a distinctive patterning of the granule matrix is evident. (Magn. ×150000.)