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## Quantitative determination of the gonadotrope polypeptide (GP 87) contained in and released by gonadotrope cells using a specific anti-rat GP87

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A polyclonal antibody has been raised against the gonadotrope polypeptide (GP87), a secretogranin II form released by rat gonadotrope cells under LHRH stimulation. GP87 was previously isolated through bidirectional polyacrylamide gel electrophoresis followed by electrotransfer onto nitrocellulose membrane. Using this antibody, it could be shown by immunoelectron microscopy that GP87 was localised in the secretory granules of several restricted cell types. A specific quantitative immunoassay was then devised to determine the cell content of GP87 or its release into culture medium. This assay involved an SDS-polyacrylamide gel electrophoresis of proteins followed by western blotting with anti-GP87 and densitometry of the nitrocellulose membrane. Combining this assay with preliminary separation of cell populations by centrifugal elutriation, it was demonstrated that GP87 is highly concentrated in gonadotropes. Moreover, the LHRH-stimulated minute-to-minute release of GP87 by rat pituitary cell aggregates in perfusion was shown to be closely correlated to that of LH, especially in its biphasic pattern. This anti-rat GP87 thus appears highly suitable to analyse the intracellular compartmentation of secretogranin II/GP87 and putative modification processes preceding or following LHRH-induced exocytosis.

### INTRODUCTION

Gonadotrope polypeptide (GP87) is released by cultured rat pituitary cells via the regulated pathway (Hubert et al. 1985; Duval and Hubert 1985; Chanat et al. 1986). Luteinizing hormone releasing hormone (LHRH) is a potent secretagogue and its was previously shown that GP87 release is closely related to the LH one (Chanat and Duval 1987), though its kinetics could not be monitored with a great accuracy using monolayer cell cultures. Protein labeling with [<sup>35</sup>S] methionine followed by two dimensional polyacrylamide gel electrophoresis (2D-PAGE) suggested that gonadotrope cells were particularly rich in GP87 (Chanat et al. 1986); nevertheless no antiserum was yet available to confirm this data.

It thus appeared essential to raise a specific antiserum for qualitative and quantitative analysis of kinetics of GP87 release from pituitary cells. Such an antiserum could help in elucidating the functional role of GP87 which still remains unknown. The anti-GP87 that will be described in this paper has recently brought about an interesting result: GP87 is the secretogranin II form released by gonadotrope cells (CHANAT et al. 1988). Secretogranin II is a sulfated and

phosphorylated acidic polypeptide found in secretory granules of numerous endocrine cells (Rosa et al. 1985a & 1985b; Hashimoto et al. 1988 and references therein). Yet, neither its concentration in the various cell populations of the pituitary nor the amount released under various stimuli could be determined due to a lack of dosage method. An immunoassay following electrophoresis and electrotransfer will be described in this report.

### MATERIALS AND METHODS.

Pituitary cell dispersion and separation.

14 day-old female rats from the Wistar strain (C.E.R.J., Le Genest, France) were used throughout this study. Pituitary cells were dispersed using trypsin according to Deneff et al. (1978) with some modifications introduced to allow subsequent separation of enriched populations using centrifugal elutriation (Thieulant and Duval 1985). Following elutriation, fractions were pooled on the basis of immunocytochemistry to constitute the gonadotrope-enriched population (usually 45-50% of LH-containing cells) and the gonadotrope-depleted one (subsequently named lactotrope population).

#### Cell culture and labeling.

Some experiments were performed using monolayer cell cultures. Briefly, cells from gonadotrope or lactotrope population were suspended in pH 7.5 DMEM buffered with 25 mM HEPES and supplemented with 0.035 % NaHCO<sub>3</sub>, 0.1 % BSA, 2.5 % fetal calf serum, 10% horse serum and antibiotic-antimycotic solution (1X). They were cultured ( $3 \times 10^5$  cells/0.5 ml of medium) at 37°C under a 5% CO<sub>2</sub> atmosphere. Medium was discarded and replaced by a fresh one devoid of methionine. [<sup>35</sup>S]methionine (N.E.N., 1000 Ci/mmol) was added (25 μCi/ well) to label proteins for 16 hours.

Perifusion experiments required cell aggregates. The cells were suspended in pH 7.5 DMEM buffered with 15mM each of HEPES and TES, supplemented with 0.1% NaHCO<sub>3</sub>, 2% Ultrosor SF and antibiotic-antimycotic solution (1X). They were transferred to 35 mm Petri dishes (Falcon 1 008 ;  $2 \times 10^6$  cells/2.5 ml) which were sealed with silicone paste. Cells were submitted to continuous gyratory shaking to allow reassociation into aggregates, as described by Van der Schueren et al. (1982). Culture medium was renewed on day 3.

#### Perifusion of aggregates.

On day 7, aggregates (10 to  $12 \times 10^6$  cells) were placed in a chamber containing 200 μl of medium. The perifusion medium was free of Ultrosor SF but was supplemented with 0.001% BSA (BSA for SDS electrophoresis, Sigma). Flow rate was 15 ml/h and the effluents (1 ml fractions) were collected every 4 min. Up to 4 chambers could be used in parallel. Collection started after 90 min of equilibration since 1 hour was required to establish a baseline for LH and GP87 release.

#### Polyacrylamide gel electrophoresis (PAGE) and electrotransfer.

Medium proteins were precipitated by trichloroacetic acid (TCA, 10% final concentration) in the presence of protamine sulfate (0.001%) as carrier and Triton X100 (0.1%) to prevent adsorption of proteins on tubing. Pellets were washed with ethanol-ether 1 : 1 (v/v) and spun at 12,000xg for 20 min. They were dissolved in sample buffer containing 0.034 M SDS and 0.7 M β-mercaptoethanol. Proteins were analysed by SDS-PAGE according to Laemmli (1970). Cells from gonadotrope and lactotrope populations were rinsed with cold medium and then treated as previously described (Chanat and Duval 1987) prior to two-dimensional electrophoresis (2D-PAGE).

After SDS-PAGE and 2D-PAGE, proteins were either silver stained (Tunon and Johansson 1984) or transferred onto nitrocellulose sheet (HYBOND-C 0.45 μm ; Amersham) and immunostained as follows : the membrane was first saturated with a mixture of 1% BSA and 1% defatted milk (v/v); GP87 was probed using

anti-rat GP87 antiserum (dil. 1:400) for 2 hours at room temperature followed by peroxidase-labeled goat anti-rabbit immunoglobulins (Nordic Immunological Laboratories ; dil. 1:1000) for 2 hours. Substrate was 4-chloro 1-naphtol. Negative controls were performed with pre-immune rabbit serum or by omitting the first antiserum.

#### Preparation of anti-rat GP87 antiserum.

GP87 was isolated from the stimulation medium of cultured pituitary cells through 2D-PAGE followed by electrotransfer and detection with indian ink (Hancock and Tsang, 1983). The protein spot was cut up and dissolved in DMSO. An equivalent volume of Freund's adjuvant was added to the solution before injection to rabbit. 5 injections (one every 4 weeks) each containing an amount of GP87 equivalent to that released by  $2 \times 10^7$  cells stimulated for 8 hours by  $10^{-7}$  M LHRH were used for immunisation. Rabbit was bled after a booster injection containing twice as much of GP87. A crude fraction of immunoglobulins was prepared from serum using the acid precipitation method of Steinbuch and Audran (1969).

#### Immunoassay of GP87.

As was previously shown (Chanat et al. 1988), anti-GP87 is highly reactive with its antigen obtained both from the medium and from the cells. Nevertheless, it also slightly reacts with a few minor species that may be degradation products of GP87. It was thus decided to perform the immunoassays after SDS-PAGE and electrotransfer (45 V for 12 h) so as to determine specifically the amount of pure GP87 released after cell stimulation. Standard curves were established using a standard preparation (GP87-S1) obtained in the following way: aggregates ( $12 \times 10^6$  cells) were perifused for 4 hours with  $10^{-7}$  M LHRH at a flow rate of 1 ml/8 min. One unit of GP87-S1 was defined as the amount of GP87 released per  $10^6$  cells per min in these conditions. Proteins were precipitated as above and dissolved in the sample buffer. Four samples containing increasing amounts (10 to 100 units) of GP87-S1, each established in triplicate, were run in SDS-PAGE, transferred onto nitrocellulose and immuno-detected. The staining intensity of each band was determined by reflexion densitometry (Vernon densitometer) so as to establish the standard curve.

A pure GP87 reference preparation (GP87-RP1) was also prepared from the perifusion medium of a specific batch of aggregates. Protein were separated using SDS-PAGE (preparative gel) ; successive 1 mm-wide bands in the 87 K zone (estimated through the use of coloured Rainbow protein molecular weight markers; Amersham) were cut and proteins eluted overnight at room temperature by a pH 7.5 1 M Tris-HCl buffer containing 1 % SDS. An aliquot of each fraction was re-analysed in SDS-PAGE followed by electrotransfer

and immunodetection to determine which one contained the pure GP87 that could be eluted and subsequently used as the reference preparation. Various amounts of GP87-RP1, each in triplicate, were run in the same gels as was GP87-S1 so as to determine the intra-assay and inter-assay coefficients of variation.

#### Hormone assays.

IH radioimmunoassays were performed using the RIA kits from the NIADDK. Relative proportions of the various cell types were determined both on freshly dispersed cells and on aggregates cultured for 7 days as previously described (THIFULANT and DUVAL 1985). In the latter case, cells from aggregates were dispersed before immunocytochemistry as for whole glands. All the antisera were from the N.I.A.D.D.K. except anti-LH and anti-PRL which were gifts from Dr M. Dubois).

#### Immunoelectron microscopy.

Pituitary cells that had been cultured in monolayer for 5 days were fixed for 1.5 h at 0°C with a mixture of 2% paraformaldehyde and 0.05% glutaraldehyde. Cells were scraped and pelleted before embedding in Lowicryl K4M at low temperature (-35°C) basically as described by Glättli et al. (1987). Ultra-thin sections (silver) were reacted with anti-GP87 (dil 1:80) for 2 hours followed by Immunogold (Auroprobe™ EM GAR G15, Janssen, dil 1:40) for 2 hours. Controls were performed either by omitting the first antibody or by using pre-immune immunoglobulins.

## RESULTS AND DISCUSSION.

#### GP87 immunoassay.

The anti-rat GP87 antiserum clearly allows quantitative determination of the antigen from medium of LHRH-stimulated pituitary cells (fig. 1). The intensity of coloured bands on nitrocellulose sheet is closely correlated to increasing amounts of antigen (correlation coefficient : 0.988). Yet, the standard curve is slightly curvilinear which suggests that the interaction between adsorbed GP87 and soluble antibodies slightly decreases when the local concentration of antigen on nitrocellulose increases. To assess preciseness and reproducibility, purified GP87-RP1 was used at two concentrations, each in triplicate, in three different assays using distinct standard curves. As reported in table 1, the intra assay coefficient of variation is 7% whatever the position of the assay in the standard curve. The inter assay coefficient of variation is about 6%.

An immunoassay following SDS-PAGE and electro-transfer may appear too complex and time-consuming. An easier way could be a direct immunoblotting of aliquots of perfusion medium on nitrocellulose without

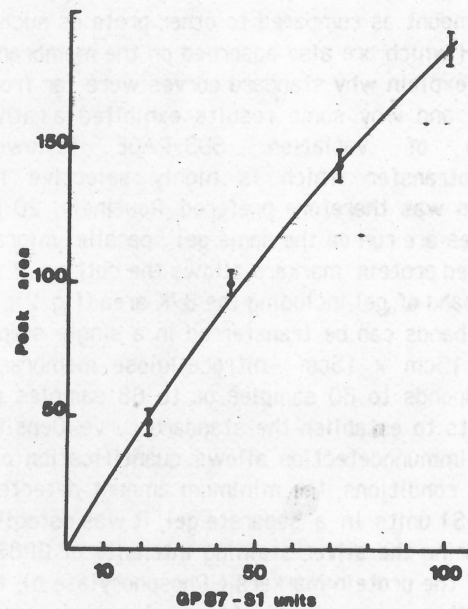


Fig. 1. Standard curve for GP87 determination. Peak area after densitometry is in arbitrary units. Results are expressed as mean  $\pm$  standard error of the three independent assays for each concentration (photographs of nitrocellulose GP87 areas are reported under each corresponding value).

TABLE 1:

Reliability of GP87 determination. GP87 RP1 was used at two dilutions (1:1 and 1:2). Each sample was run in triplicate (intra-assay) and three independent assays were performed (inter assay). Results are expressed in GP87 S1 units as mean  $\pm$  standard deviation. cv = coefficient of variation.

	dil 1:2		dil 1:1	
	m $\pm$ $\sigma$	cv %	m $\pm$ $\sigma$	cv %
intra assay (n = 3)	38.6 $\pm$ 2.7	7.1	76.8 $\pm$ 5.5	7.1
inter assay (n = 3)	37.3 $\pm$ 2.2	5.8	75.5 $\pm$ 1.3	1.7

SDS-PAGE. That was our first approach but several problems then arose : 1. as seen in fig. 1, anti-GP87 also reacts with a minor band of about 80K ; this one was previously named B2 (Chanat and Duval 1987) ; it is not yet known if this B2 species is or not a by-product of GP87, but it promotes an interfering coloured reaction

that had to be avoided; 2. medium GP87 is present in a low amount as compared to other proteins such as PRL and GH which are also adsorbed on the membrane; this could explain why standard curves were far from being linear and why some results exhibited a rather high degree of variation. SDS-PAGE followed by electrotransfer which is highly selective for the antigen was therefore preferred. Routinely, 20 medium samples are run on the same gel; parallel migration of coloured protein markers allows the cutting of a 3 cm-wide band of gel including the 87K area (fig. 2). Four of these bands can be transferred in a single step on the same 15cm x 15cm nitrocellulose membrane; this corresponds to 80 samples or to 68 samples plus 12 aliquots to establish the standard curve. Densitometry after immunodetection allows quantification of GP87. In our conditions, the minimum amount detected is 10 GP87-S1 units. In a separate gel, it was established by comparing the silver staining intensity of GP87 and of one of the protein markers ( Phosphorylase b), that one S1 unit corresponds to 44 ng of protein equivalent. Phosphorylase b was chosen as a standard since its staining intensity was proportional to its loaded amount whereas that of BSA was not, an observation previously reported by Switzer et al. (1979).

#### Immunoelectron microscopy.

Our antibody was raised again a protein released into culture medium. Nevertheless, as shown in fig.3, it labels the intracellular antigen. This antigen is localized in the secretory granules of some specific cells whereas it is absent from other cell populations. Though the identity of GP87-containing cells was not determined using immunoelectron microscopy, it may be suggested that they represent mostly gonadotropes since GP87 is released under LHRH stimulation. As GP87 was identified as a SgII form, our antibody may also detect any SgII-containing cell and particularly thyrotropes that are also rich in this antigen (Rosa et al. 1985).

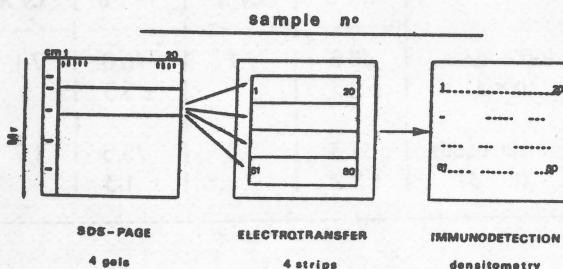


Fig.2. Schematic representation of the quantitative analysis process for GP87. Numbers refer to individual samples. Mr : relative molecular weight estimated through the use of coloured protein markers (cm).

GP87 content of cells.

Cells from gonadotrope and lactotrope populations were labeled with [<sup>35</sup>S] methionine and their proteins analysed by 2D-PAGE (fig. 4). Autoradiography revealed the same pattern for both populations except for GP87, heavily labeled on the former and quite undetectable in the latter though the same number of cells was used. Immunodetection with anti GP87 produced an intense coloured spot in gonadotropes accompanied by a small

TABLE 2:

Relative proportions of specific cell populations in freshly dispersed cells and in 7 day-old aggregates. Results are expressed as mean ± standard deviation.

n = number of experiments

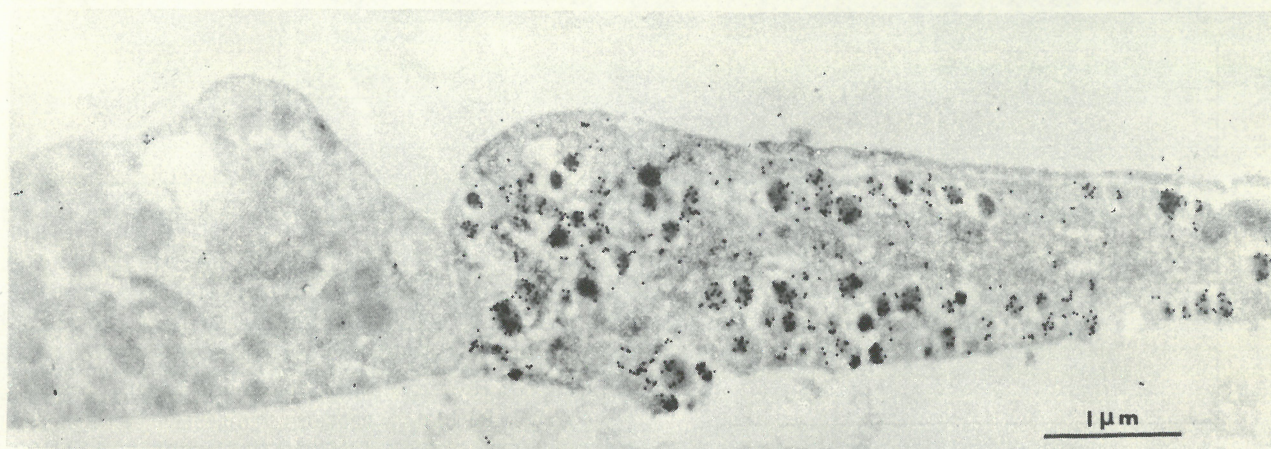
	cell population (%)				
	LH	PRL	ISH	GH	ACTH
freshly dispersed	12.4	17.0	3.9	26.0	8.2
	(n=6)	(n=6)	(n=6)	(n=2)	(n=2)
aggregates	12.6	23.8	4.1	22.0	9.4
(n=2)					

spot corresponding to B2. On the opposite, detection was extremely low in lactotropes. These data confirm that intense [<sup>35</sup>S] methionine labeling in gonadotropes does not result from a higher turnover rate of GP87 synthesis in this population but corresponds to a true higher cell content.

A quantitative estimation was then attempted after a new elutriation run. The GP87 content of the gonadotrope, lactotrope, and average cell population was respectively: 4 116, 710 and 933 GP87-S1 units per 10<sup>6</sup> cells. As expected, the amount of GP87 was the highest in LH-containing cells. Moreover, as the lactotrope population still contains about 4% of gonadotropes (Chanat et al., 1986), part of the GP87 amount found in that population may belong to these contaminating LH cells. As suggested in electron microscopy section, the antigen may also be contained within thyrotropes; as this cell category is distributed between gonadotrope and lactotrope populations, its content cannot be responsible for the quantitative differences reported above.

#### Release of GP87 by perfused aggregates.

As was demonstrated by Van der Schueren et al. (1982), pituitary cell aggregates allow precise minute-to-minute determination of LH release during a

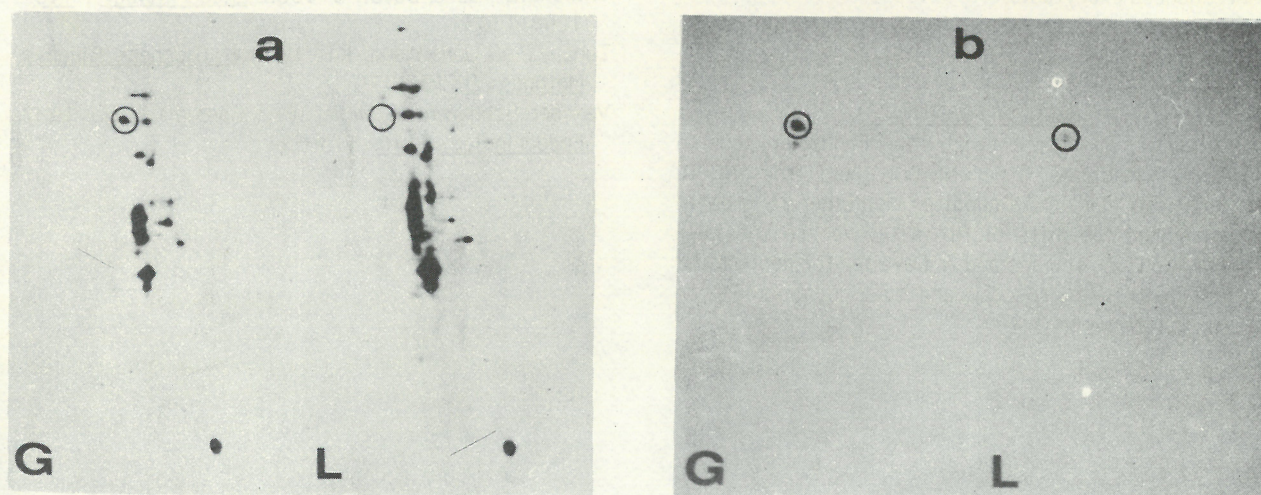


**Fig.3.** Detection of GP87 by electron microscope immunogold in cultured pituitary cells embedded in Lowicryl K4M. IgG-gold particules are present in all secretory granules of a cell; in the other cell, immunoreactivity is absent.  $\times 25,000$ .

perifusion step. Nevertheless, it appeared necessary to control prior to perifusion that reaggregated cells in culture still contained the same relative amounts of the various cell populations. As shown in table 2, 7 day-old aggregates contain the same amount of gonadotropes, thyrotropes and corticotropes as freshly dispersed cells whereas lactotropes appeared more numerous and somatotropes slightly less abundant. Our system thus appeared particularly suitable for simultaneous monitoring of LH and GP87 output.

Aggregates were continuously submitted to a high concentration of LHRH ( $10^{-7}$  M) for 3 h. As shown in fig.5, the LH surge is intense during the first 20 min and it then declines for about 30 min. This first phase is followed by the mobilization of a second pool of hormone, the release of which appears highly pulsatile. The amount of released LH progressively decreases and

goes down to the basal level as soon as LHRH stimulation is suppressed. In this respect, this release pattern is in agreement with the data reported by others who were using perifusion of pituitary fragments (Kercret and Duval 1978) or perifusion of dispersed cells (Naor et al. 1982). As is evident from fig.5, GP87 release follows a quite similar pattern though the first transient decrease appears less important than that of the hormone. Interestingly, the ratio GP87/LH reveals some heterogeneity. This could be explained by the presence in gonadotrope cells of secretory granules of variable composition. To sustain this hypothesis, it must be mentioned that if LH was chosen as the standard for hormone secretion, FSH is simultaneously released in these conditions. Moreover, if GP87 exerts a role in granule maturation and particularly in hormone condensation to constitute the



**Fig.4.** 2D-PAGE and western blotting of intracellular GP87 (circled) contained within gonadotrope (G) and lactotrope (L) populations. Cells were previously cultured in the presence of [ $^{35}$ S]methionine. Autoradiography of the nitrocellulose membrane (a); immunodetection with anti-GP87 (b).

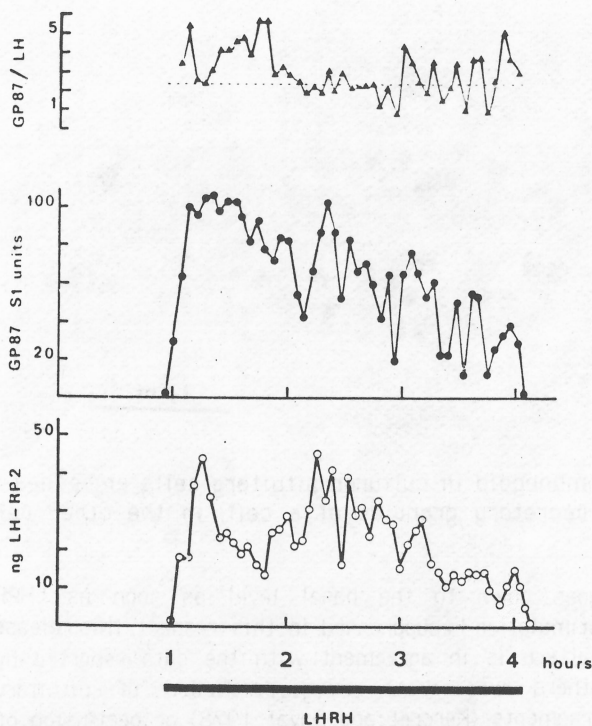


Fig.5. Simultaneous release of LH and GP87 by pituitary cells cultured in aggregates, during a continuous perfusion with  $10^{-7}$ M LHRH. (One out of three similar experiments is represented here).

granule core, stoichiometry may not be required between hormone and GP87/SgII.

To conclude, the anti-rat GP87 described in this paper appears highly suitable to analyse intracellular compartmentation of secretogranin II/GP87 and putative modification processes preceding or following LHRH-induced exocytosis.

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