

# SYNTHESIS AND RENEWAL OF PROTEINS IN DUCK ANTERIOR HYPOPHYSIS IN ORGAN CULTURE

A. TIXIER-VIDAL and D. GOURDJI

From the Laboratoire de Biologie Moléculaire, Collège de France, Paris V, France

## ABSTRACT

In cultures of duck anterior pituitaries, the synthesis and renewal of the specific secretory protein prolactin and of total newly synthesized tissue proteins were studied. As concerns prolactin, assay of the tissue and culture media hormone content demonstrates *de novo* synthesis of prolactin *in vitro* at a constant rate during at least 2 wk. The prolactin content after 1 wk and after 2 wk of culture is the same and is similar to the initial content. The renewal time of this prolactin can be estimated at 28 or 48 hr. As concerns total proteins, the use of a chase after a short pulse of 5 min in the presence of tritiated L-leucine demonstrated that newly synthesized proteins are excreted into the culture medium from 30 min to 1 hr after the beginning of the chase. Therefore, the synthesis and excretion of proteins are two discontinuous phenomena. The migration rate of the total proteins was slower than that of prolactin, indicating that this hormone does not represent more than about half of the newly synthesized proteins. These conclusions are in good agreement with those based on high resolution radioautographic data previously obtained on the same material.

## INTRODUCTION

In an earlier work (9) in which high-resolution radioautography was carried out on cultures of anterior hypophyses from male ducks, we drew certain conclusions relative to the movement of newly synthesized, intracellular proteins. These proteins follow the classic pathways already shown for other glandular cells. They move from the ergastoplasm towards the secretory granules after a phase of condensation in the Golgi zone. In addition, two peculiarities seem to distinguish hypophyseal cells: (a) The rate of labeling of the granules is slow, and (b) A larger portion of newly synthesized proteins (40–50%) remains localized in the cytoplasm outside of the granular elements.

These conclusions, based on radioautography, needed to be verified with biochemical and quantitative data relative to the renewal of proteins in

this material. Thus we studied: (a) The over-all synthesizing and secretory activity of hypophyseal tissue as concerns the specific secretory protein (prolactin) of this tissue in organ culture, and (b) The chronological and quantitative evolution of newly synthesized total proteins following a short pulse of tritiated L-leucine.

## MATERIAL AND METHODS

### *Culture of Hypophyses*

Anterior hypophyses of adult male ducks, sacrificed in a period of sexual inactivity, were first divided into halves in the sagittal plane. Each half was put into culture separately, after having been cut into some 20 small fragments. These fragments were placed on stainless steel grids and cultured in Falcon organ culture dishes (60 × 15 mm) (B.D. Lab. Inc., Los

Angeles, Calif.). The culture medium consisted of 199 synthetic medium supplemented with 10% duck serum and 5% 7-day chick embryo extract, as well as antibiotics (50 U of penicillin and 0.05 mg of streptomycin per ml). Each culture dish held half an anterior hypophysis (from 6 to 8 mg of tissue), and 0.7–0.8 ml of medium. The culture dishes were placed in hermetically sealed boxes in an I.G.R. Lequeux (Lequeux Co., Paris, France) oven. At each change of medium, the atmosphere of the box was saturated with a gas mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub> (3 liters/min for 10 min). The cultures were grown for 2 wk, and the media were changed every 2 or 3 days.

### *Study of the Rate of Synthesis and Secretion of Prolactin*

**EXPERIMENT A:** Six freshly sampled, duck anterior hypophyses were halved, one-half being immediately frozen on dry ice and stored at  $-45^{\circ}$  and the other cultured for 1 wk. The culture media, collected at each changing of the media, were stored at  $4^{\circ}\text{C}$  and then preserved at  $-45^{\circ}$ . At the end of the week, the cultures were frozen in acetone at  $-45^{\circ}$  and stored for assay.

**EXPERIMENT B:** Six other anterior hypophyses were subjected to the same protocol as in experiment A, but the six half-hypophyses were maintained in culture for 2 wk. The media from the 1st wk and from the 2nd wk were collected separately.

**ASSAY OF PROLACTIN:** For assay, the three different culture media (Experiment A: 1st wk; experiment B: 1st and 2nd wk) were concentrated approximately three times according to our usual technique (4). The two lots of uncultured half-pituitaries, as well as the two lots of cultured half-pituitaries, were extracted by homogenization in Tyrode's solution according to our usual technique (3). Seven solutions were thus obtained which were assayed simultaneously, along with three reference solutions of ovine prolactin (NIH PS 7) titrating, respectively, 0.5, 1.5, and 4.5  $\mu\text{g}$  (24.3 IU/mg).

The micro-method pigeon crops was used (6). The pigeons were sensitized by a systemic injection of 100  $\mu\text{g}$  of ovine prolactin (NIH) (7). On each side of the crop sac (half-crop), the pigeons received three local injections of 0.01 ml in 3 days, the last injection being preceded by a local injection of hydrocortisone (0.5 mg in 0.1 ml) for preventing inflammation (2). For each solution we used five half-crops, each receiving three injections. The whole assay was organized in randomized blocks, each one including the seven unknown solutions and the three ovine prolactin references, and it involved a total of 25 white adult Paon pigeons. Each response was evaluated by taking the mean diameter (in mm) of the reactive zone.

The strict observation of Lison's instructions about experimental organization (5) allowed us to make

comparisons, statistically valid, between the mean values of diameters of the reactive areas of the various experimental groups. On the other hand, we obtained a log-dose response curve with the three ovin-prolactin dilutions, assayed in the same randomized blocks as the unknown solutions. Therefore, we may refer to that curve: (a) to calculate, by a  $1 \times 3$  test, the prolactin content of the unknown solutions, and (b) to appreciate the degree of relationship from one experimental group to another.

### *Study of the Incorporation of L-Leucine-4,5-<sup>3</sup>H into Tissue and Secretory Proteins*

L-Leucine-<sup>3</sup>H (specific activity of 25 Ci/mmmole)<sup>1</sup> was introduced in the amount of 200 or 250  $\mu\text{Ci/ml}$  into the incubation medium composed of Tyrode's solution enriched with 10% duck serum and 5% embryo extract. Incubation was carried out in the culture dishes in which the labeled medium was exchanged for the usual culture medium. The dishes were placed on ice for 10 min, then transferred into an oven at  $37^{\circ}\text{C}$  for 5 min. Immediately afterwards, the grids holding the tissues were rinsed in three changes of Tyrode's solution containing cold L-leucine at a concentration 220–275 times higher than in the tracer medium (2, 2  $\mu\text{mole/ml}$ ). They were then put back into new culture dishes containing fresh culture medium enriched with cold leucine at the same concentration. A chase was thus effectuated which was stopped after 15 and 30 min, and 1, 4, and 24 hr. For each time, one culture dish, i.e. one half-hypophysis and the corresponding medium collected, was used.

The cultured tissues were carefully homogenized in 1 ml of Tyrode's solution per half-hypophysis by using a ground glass Potter homogenizer. The labeled proteins were precipitated out with 10% trichloroacetic acid (TCA) according to the method of Allen and Schweet (1) after the addition of 1 mg of ovalbumin per half-hypophysis. The same technique was used for separating proteins from the media. The supernatants were collected after the first TCA precipitation in order to determine the amount of radioactivity not incorporated into the proteins. In one series (C.40), systematic study of all supernatants showed that the radioactivity of the supernatants corresponding to the later washes (TCA, alcohol, ether) was negligible. The proteins were digested with hyamine hydroxide (Packard) overnight at  $55^{\circ}$ , then counted in a Nuclear (Nuclear-Chicago Corp., Desplaines, Ill.) scintillation counter after the addition of 10 ml of POPOP toluene to each sample (composition of POPOP toluene: toluene Merck: 1,000 ml-PPO Packard (2–5-diphenyloxazole-scintillation grade): 4 g-POPOP Packard (1–4 bis-2-[4

<sup>1</sup> Centre d'Etudes Nucléaires, Saclay, France.

methyl 5-phenyloxazolyl]-benzene) 50 mg). Correction for quenching was made by introducing an internal standard of tritiated-toluene. The numerical results are expressed in counts per minute per half-hypophysis.

These experiments were repeated successively on three series of 10–12-day cultures, each involving either six duck hypophyses (C.39 and C.41) or three duck hypophyses (C.40). In series C.39 and C.41, a hypothalamic extract was added to the chase media of six half-hypophyses (series *a*) and a cerebral cortex extract, as control, to the six other halves (series *b*). In no case was significant modification of the curves ever observed. It thus seemed justified for us to pool the five chase experiments.

## RESULTS

### *Synthesis and Renewal of Prolactin*

The results of the assay are summarized in Table I. The following facts become evident:

(*a*) Prolactin is present in the culture medium at a constant level during the 1st and the 2nd wk of culture, which confirms our previous results (8).

(*b*) The amount of prolactin liberated into the medium each week is clearly greater than the initial content of the gland: three times more in experiment A and six times more in experiment B.

TABLE I  
Results of Assay of Prolactin Expressed as the Average Diameter of the Reacting Zone in Pigeon Crop

Material	Crop response	Equivalence in prolactin
	mm ± SD	μg/½ A.H.
<i>Exp. A</i>		
Uncultured ½ A.H.*	26.6 ± 1.56	0.76
Cultured ½ A.H.	29.6 ± 1.56	1.0
1st wk medium	41.0 ± 1.82	2.3
<i>Exp. B</i>		
Uncultured ½ A.H.	25.4 ± 1.20	0.65
Cultured ½ A.H.	25.0 ± 1.47	0.60
1st wk medium	44.5 ± 0.64	3.50
2nd wk medium	44.5 ± 1.14	3.50
<i>Prolactin NIH PS 7</i>		
0/5 μg/0.4 ml	23.7 ± 0.62	
1/5 μg/0.4 ml	33.4 ± 2.06	
4/5 μg/0.4 ml	45.4 ± 1.24	

\* A.H. = anterior hypophysis.

(*c*) The amount of prolactin present in the tissues after 1 wk and after 2 wk of culture is constant and is equal to the initial amount in the uncultured gland.

From these facts, it can be concluded that *de novo* synthesis of prolactin occurs and that this synthesis is linear over a period of at least 2 wk. The renewal time of prolactin thus synthesized can be estimated at 48 hr in experiment A and 28 hr in experiment B. However, these times would be shorter if there were partial destruction of prolactin either in the media after excretion, or in the tissue before excretion. In other respects, the maintenance of a constant level of prolactin within the hypophysis reflects a state of equilibrium between the rate of synthesis and the rate of liberation of prolactin.

### *Synthesis and Renewal of Total Proteins*

The results of the five chase experiments are summarized in Fig. 1 and Table II.

It should be kept in mind that 10 days' cultured half-pituitaries are not strictly identical one to the other, which can explain some aberrant points on the curves.

These reservations being made, the following facts are noted:

(*a*) L-leucine-<sup>3</sup>H molecules taken up over a period of 5 min are incorporated very rapidly into tissue proteins.

(*b*) The radioactivity of these proteins is maximum at the beginning of the chase, that is, at time 0. In the case of series C.39, there was a certain delay which could be attributed either to a latency, or to a tapering halt in incorporation. This maximum is immediately followed by a decrease in the radioactivity of the tissue proteins.

(*c*) Inversely, the radioactivity of proteins in the culture medium increases as that of the tissue proteins diminishes. In the majority of cases, this increase becomes particularly evident after 30 min or 1 hr of chase. The radioactivity excreted into the medium represents an ever-increasing percentage of the total radioactivity (see Table III).

It can be concluded that there occur (*a*) neosynthesis of labeled proteins in the tissues and (*b*) excretion of newly synthesized proteins into the culture medium. Therefore, there is effectively a displacement of newly synthesized proteins, but this displacement is slow, for within 24 hr after their synthesis about half of the labeled proteins

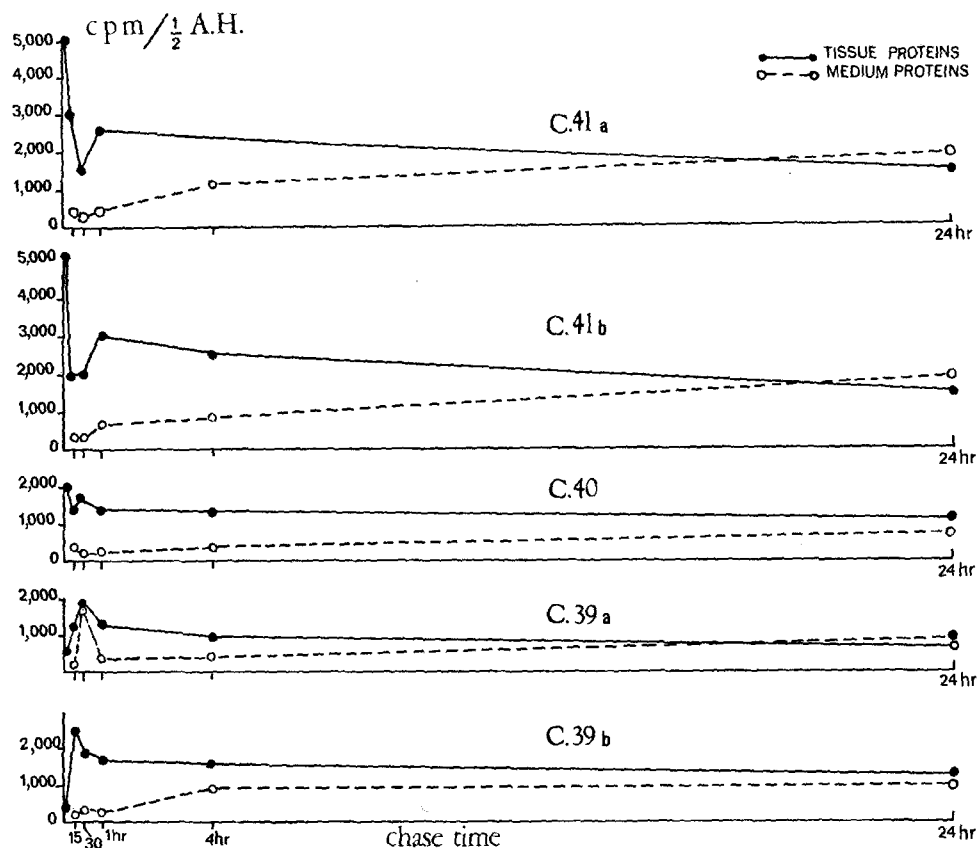


FIGURE 1 Evolution of the radioactivity of proteins in the tissues and in the culture media as a function of the length of chase (counts per minute per half-hypophysis). *A.H.*, anterior hypophysis.

still persist in the tissues, the other half having been excreted.

For all three series, Fig. 2 shows the evolution of nonprotein radioactivity in the tissues (first TCA supernatant). This radioactivity falls sharply between time 0 and 15 min, indicating that transfer to chase medium resulted in a rapid wash-out of cold TCA-soluble radioactivity from the culture and that there is no continuous contribution of new amino acid molecules by the external medium. Moreover, the radioactivity of the last rinse was checked and found to be negligible ( $1 \times 10^{-5}$ ) as compared to that of the labeled medium.

For the same three series, Fig. 3 and Table IV show the change in the ratio between protein radioactivity and nonprotein radioactivity of the tissues. This ratio increases up to 4 hr of chase, then decreases slightly. Again, it can be concluded that during the chase there is no continuous incorporation of labeled amino acid molecules from

the medium. However, during the first 4 hr of chase, incorporation of new L-leucine- $^3\text{H}$  molecules continues actively during the 1st hr, becoming slower later on. This indicates that a particularly important pool (probably in the extracellular spaces) of free, labeled leucine persists in the tissue after the incorporation period.

## DISCUSSION

### *Nature and Properties of the System Used*

Previous research (8) showed that the duck anterior hypophysis, when maintained in organ culture for 1 wk, undergoes a simplification in structure. The anterior hypophysis then contains only two cell types out of the seven present *in situ*. Of these two types, the prolactin cell prevails, and bioassays show the presence of prolactin in the medium at a constant level during each of the 2

TABLE II

Evolution of the Radioactivity of Proteins in Cultured Tissues and in Culture Media as a Function of the Length of the Chase Period after a 5-min Pulse with 200-250  $\mu$ Ci of L-Leucine- $^3$ H/ml

Counts per minute per  $\frac{1}{2}$  hypophysis.

Material	Length of chase	C.39		C.40	C.41	
		a	b		a	b
Tissue proteins	0	464	464	2021	5118	5118
	15 min	1208	2387	1473	2996	1995
	30 "	1843	1870	1641	1537	2068
	1 hr	1246	1680	1394	2591	3011
	4 "	955		1337		2625
	24 "	697	1241	1122	1537	1692
Medium	15 min	164	223	351	384	302
	30 "	1892	348	234	329	313
	1 hr	336	266	243	485	619
	4 "	454	938	319	1047	866
	24 "	829	982	665	1884	1887
	Total proteins	0	464	464	2021	5118
15 min		1372	2610	1824	3380	2297
30 "		3735	2218	1875	1866	2381
1 hr		1582	1946	1637	3076	3630
4 "		1409		1656		3491
24 "		1526	2223	1787	3421	3579

TABLE III

Evolution during the Chase of the Protein Radioactivity of the Medium Expressed in Per Cent of the Total Radioactivity of the Culture (Tissue + Medium)

Length of chase	C.39		C.40	C.41	
	a	b		a	b
	%	%	%	%	%
15 min	11.9	8.5	19.2	11.3	13.1
30 min	50.6	15.6	12.4	17.6	13.1
1 hr	21.2	13.6	14.8	15.7	17.0
4 hr	32.2	—	19.2	—	24.8
24 hr	54.2	44.1	37.2	55.0	52.7

wk of culture. The second, less frequent, type of cell may be the melanophorotropic cell (MSH cell).

The present results allow us to define the biosynthetic capacities of our system.

(a) As concerns prolactin, its renewal time can be estimated at 28 hr at the most, if the possibility of eventual destruction of the hormone in the culture medium or within the cells is elim-

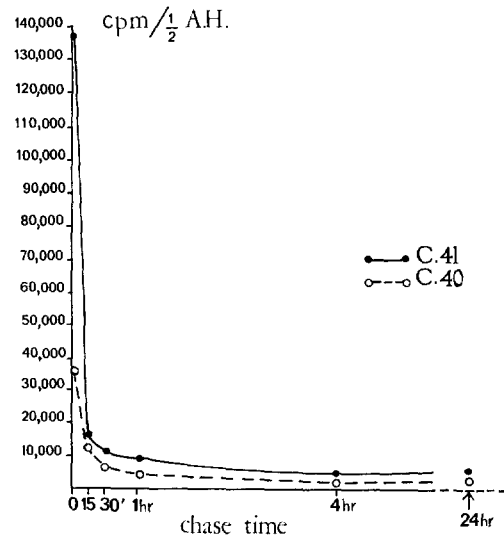


FIGURE 2 Evolution of the nonprotein radioactivity in the tissues as a function of the length of chase.

inated. About 3.5% of the prolactin cell content should be thus renewed in 1 hr. In addition, the tissue content of prolactin remains constant during the course of any one experiment. Thus, from the

point of view of synthesis and secretion of this hormone, our system is in equilibrium during the 2 wk studied. Accordingly, when undertaking the detailed chronological study of neosynthesized protein displacement, we chose 10–12-day cultures in order to be sure we were studying the balanced phase.

Although prolactin cells predominate in our cultures, prolactin is probably not the only hormone elaborated. Thus, using the same experimental design, we made an attempt to determine by in vitro assay on *Anolis carolinensis* the activity of our cultures in synthesizing melanocyte-stimulating hormone (MSH). Unfortunately, comparison of the initial content of the gland with that of

the culture media and the cultured tissues showed a considerable loss of hormone that can only be attributed to an important destruction of MSH in the medium. In addition, although MSH was still apparent in the tissues after 28 days of culture, it was always infinitely less abundant than in the original tissues (0.2% after 1 wk; 0.02% after 28 days) (10). Therefore, prolactin secretion plays a quantitatively preponderant role in the biosynthetic phenomena studied here.

(b) As concerns total proteins, the use of a labeled precursor affords a much finer study of kinetics than is obtained by using bioassays, which necessitate much material.

Two facts characterize our kinetic data: (a) the radioactivity of tissue proteins is maximal at the beginning of chase and decreases immediately afterwards; and (b) inversely, the radioactivity of proteins in the culture media, at first weak, increases very slowly. The neosynthesis of proteins is, therefore, followed by their displacement into the culture medium. Hence, there is excretion of newly synthesized proteins. The first parts of these curves, from 0 to 1 hr, show a drop, while there is no complementary rise on the curves representing excreted proteins. During this period there is, therefore, a certain proteolysis. This proteolysis might correspond to the degradation of the second hormone (MSH ?) liberated by our cultures, resulting in small molecules which cannot be precipitated by 10% TCA. During the second part of the chase (1–24 hr), the curve of tissue protein radioactivity is practically symmetrical to that of the excreted proteins. This indicates that the synthesis and excretion of proteins are two discontinuous phenomena. The formation of a heterogeneous pool of labeled and unlabeled molecules would explain this discontinuity, but this hypothesis needs further experiments.

If these results are compared with those concerning the synthesis and secretion of prolactin,

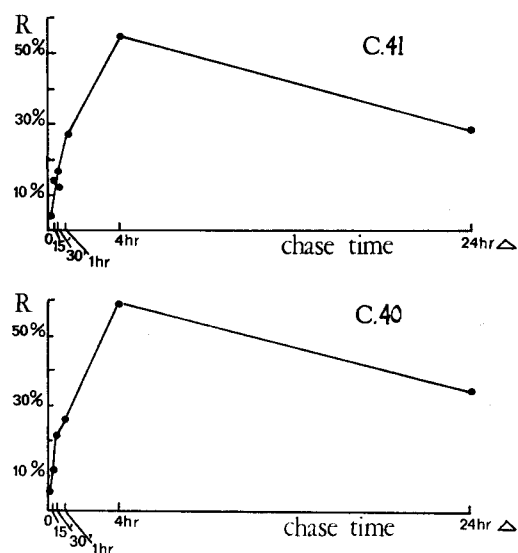


FIGURE 3 Evolution of the ratio:

$$\frac{\text{Radioactivity precipitated by TCA}}{\text{Radioactivity of the supernatant}}$$

in the tissues as a function of the length of chase. *R*, ratio.

TABLE IV  
Evolution during the Chase after a 5-Min Pulse with *L*-Leucine-<sup>3</sup>H of the Ratio:

Chase time	$\frac{\text{TCA-precipitable radioactivity}}{\text{Radioactivity of supernatant}} \%$					
	0	15 min	30 min	1 hr	4 hr	24 hr
C.41	3.71	12.97	16.15	27.56	54.32	28.76
C.40	5.22	11.94	21.49	25.81	58.58	34.47

marked differences appear. The migration rate of total proteins is inferior to that which should have been obtained if the majority of tissue proteins were represented by prolactin. This indicates that only a part of the newly synthesized proteins is represented by prolactin. The rest constitutes proteins which turn over more slowly.

We are well aware of the necessity of establishing the specific activity of purified prolactin labeled with L-leucine-<sup>3</sup>H as a function of time. Unfortunately, these data cannot be obtained with our material since purified avian prolactin is not available.

#### *Comparison with Radioautographic Data Previously Obtained with the Same Material*

This comparison reveals several common points. First of all, the marked drop in the level of labeled tissue proteins demonstrates that the intracellular pathway of labeled proteins detected by radioautography does correspond to a chase and not to a continuous incorporation of molecules from the medium. The second important point of agreement resides in the persistence, after 24 and 48 hr of chase, of the radioactivity in the nonsecretory granule part of the cytoplasm, which represents about 50% of the cytoplasmic radioactivity. The third point of agreement resides in the determination of the renewal time of prolactin. Radioautography allowed us to calculate that, for a 10–15 min period of incorporation, the proportion of labeled protein granules was about 2% after 1 hr of chase and attained a maximum of 3.1% after 4 hr of chase. Bioassays allowed us to calculate that in 1 hr at least 3.5% of the prolactin is renewed.

Lastly, our present results allow us to interpret one curious datum from the radioautographic study: prolactin cells, at least in organ culture, are characterized by the slowness with which the Golgi zone empties, this being achieved only after 4 hr of chase. This phenomenon might correspond to the curve in Fig. 3 showing the change in the ratio of protein radioactivity to nonprotein radioactivity of the tissues. It was seen that this ratio increases up to 4 hr of chase, which we interpreted as a continued incorporation of amino acids due to a particularly large pool of amino acids in the

tissue as a whole and which may be characteristic of organ cultures.

On the whole, the interpretations based on the radioautographic data are, therefore, confirmed and supplemented by the biochemical data. This shows that the artifacts connected with the radioautographic technique did not introduce any important error into the appreciation of this physiological phenomena.

In conclusion, the use of three different techniques—bioassay, kinetics of incorporation into total proteins, and high resolution radioautography—allows us to characterize the biosynthetic and secretory capacities of duck prolactin cells, at least in organ culture and with the use of L-leucine, one of the more common amino acids in tissue protein.

Further experiments with other hypophyseal cell types and other tracers are needed in order to generalize these results.

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