AUTORADIOGRAPHIC STUDIES ON ^3H -LEUCINE UPTAKE BY ADENOHYPHYSIAL CELLS *IN VITRO*

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Experimental evidence has clearly shown that the central nervous system plays a very essential role in the physiology of reproduction. The isolation of the pituitary gland from central nervous influences by lesions in the median eminence (Dey, 1943) or by stalk-section (Greep and Bernnett, 1951) as well as the autotransplantation of this gland to an ectopic site causes a complete cessation of the gonadotropic function of the pituitary, while luteotropin (LTH) secretion is maintained or rather accelerated under these conditions (Everett, 1954 and 1956). Thus, it has been shown that gonadotropin (FSH, LH) secretion by the pituitary is regulated neurohumorally by the hypothalamus via the hypophyseal portal vein (Green and Harris, 1947; Harris, 1955). On the other hand, it appears that the secretion of luteotropin is under an inhibitory influence of the hypothalamus (Haun and Sawyer, 1960; Rothchild, 1960; Meites *et al.*, 1962; Talwalker *et al.*, 1963).

The concept of neurohumoral regulation of hypophyseal gonadotropin secretion has become widely accepted, since hypothalamic extracts have been found to have gonadotropin (FSH, LH)-releasing action (Campbell *et al.*, 1961; McCann, 1962; Nikitovitch-Winer, 1962). McCann and Ramirez (1964) have recently reported a partial purification of LH-releasing factor (LH-RF) from beef stalk median eminence extracts on columns of Sephadex G-25 and suggest that the LH-RF is a small polypeptide different from the known posterior pituitary hormones. Guillemin *et al.* (1963) have presented similar data. The present authors (1961 and 1963a) have published that the hypothalamic extracts of rats can directly stimulate pituitary cells and augment their gonadotropic function *in vitro* when added to monolayer cultures of pituitaries, but that the extracts from brain cortex fail to show this action under the same conditions. From these findings *in vitro* it seems most likely that the active substance contained in the hypothalamus is specifically related to the biosynthesis of gonadotropin in pituitary cells.

In order to confirm this view, the cellular functions producing gonadotropin as well as luteotropin in the pituitary have been analysed autoradiographically using ^3H -leucine by the methods of organ culture or short term incubation. The results obtained so far will be described in this report.

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MATERIALS AND METHODS

Animals

Mature female rats of Wistar strain weighing 150 to 200 g were used.

Organ culture

The pituitaries were excised from rats immediately after decapitation under ether anesthesia and the posterior lobe was dissected out. Each anterior lobe was cut into 8 to 10 pieces. These were put into test tubes and attached to the side walls prior to adding culture medium. Each tube contained dissected pieces equivalent to 2 anterior lobes. One ml of Eagle's solution without leucine was added to each tube which was then put in a roller drum. Cultivation started at the rate of rotation 6 per hr. at 37°C. The cultures were divided into 2 groups. Ten μ C of ^3H -leucine (specific activity 215 mC/mM, The Radiochemical Center, Japan) were added to one group on the 2nd day and to the other one on the 4th day. Afterwards, the cultures were incubated for 4, 8, or 24 hrs. more. At the end of the incubation, they were subjected to autoradiography.

Short term incubation

Anterior lobe pieces which were obtained from normal rats and 10 days castrated rats were used. All the pieces from 3 rats of the same group were pooled and put into one flask which had 10 ml of saline F (Table 1) with 2 μ C of ^3H -leucine. Incubation was done in a shaker at 37°C for 2 hrs.

Table 1. Composition of saline F (g/l)

NaHCO_3	1.2
NaCl	7.4
KCl	0.285
Na_2HPO_4 .anhydrous	0.17
KH_2PO_4	0.083
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.154
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.016
Glucose	1.1
Phenol red	0.0012

In 1 l of double distilled water

Preparation of hypothalamic and brain cortical extracts

Spayed rats were used on the 7th day after castration. They were killed by decapitation under ether anesthesia. Small tissue fragments were taken out immediately from the basal portion of the hypothalamus and from the brain cortex. These, hypothalamic and brain cortical, were pooled, respectively, homogenized in 1/10 M acetic acid, and were centrifuged at 10,000 rpm for 10 mins. The supernatants obtained were defatted with petroleum ether several times and then frozen dried. These were designated hypothalamic extracts (HE) and brain cortical extracts (CE), respectively.

Preparation of autoradiographs

When incubation with ^3H -leucine was completed, the media were discarded and the incubated pituitary fragments were rinsed twice with physiological saline. They were fixed in Bouin's fluid for 2 days. Then they were washed, dehydrated, embedded in paraffin, sectioned at 4 μ and

stained by the periodic acid-Schiff (PAS) method. The stained preparations were covered with Sakura's liquid emulsion by a dipping method (Kopriwa and Lebrond, 1962). Finally, they were stained again with methanil yellow and toluidine blue, after being developed and dried in the air (Siperstein, 1963).

RESULTS AND DISCUSSION

Several workers have recently carried out the autoradiographic studies using ³H-leucine, -glycine or -methionine (Caro, 1961; Siperstein, 1963; Warshawsky *et al.*, 1963). The use of tritium labelled amino acids for autoradiographic works seems rational, since the energy of tritium β particles is very small (maximum 17.9 Kev), reaches only the distance less than 2μ and gives localized spots on the photographic films.

It has been considered that the uptake of amino acid such as leucine by the cells is related to the biosynthesis of protein in general, because leucine is contained in all proteins. From these viewpoints, it is apparent that the incorporation of leucine to the hypophysial cells does not specifically represent the gonadotropin synthesis alone. However, the fact that leucine is a constituent of gonadotropin may allow the approach by use of ³H-leucine to the problem of the biosynthesis of this hormone.

Three types of cells could be differentiated from each other by the staining colors of their cytoplasm even in double-stained autoradiographic sections of the pituitaries. These were the PAS-positive type, the acidophil with yellowish color, and the chromophobe. Small black grains indicate the existence of ³H-leucine; therefore, the grains within the cells represent cellular uptake of the amino acid.

Intracellular grains were counted under a light microscope for the estimation of uptake rate by the 3 different types of cells. It was found that in organ-cultured pituitaries the PAS-positive type cell contained a significantly smaller number of grains than the other 2 types, irrespective of culture age and time of exposure to ³H-leucine (Table 2). Mean grain count per cell was only 2.9 ± 2.0 (S.E.) after 4 hrs. exposure on the 2nd day. On the other hand, the values of the acidophils and chromophobes were 8.8 ± 4.4 (S.E.) and 10.0 ± 3.2 (S.E.), respectively. These values were maintained almost unchanged until the 4th day. Grain counts were increased in all types with the time of exposure. However, the increase in the PAS-positive type was much less in comparison with that of the acidophil and the chromophobe. Such relations can be seen clearly in Figure 1.

Short term incubation of the pituitaries gave results similar to those which were found in organ culture (Table 3 and a part "without addition" of Table 4). Therefore, it appears that the PAS-positive type of cells in castrated rats as well as in normal rats lose the ability of incorporating ³H-leucine to a large extent at an early period of their *in vitro* life and that the abilities of the acidophils and the chromophobes are rather well maintained. When ³H-leucine had been injected intraperitoneally into rats and autoradiographs were prepared from their *in vivo* pituitaries, it seemed that the uptake by the PAS-positive cells was not lower than that by the other 2 types (Kobayashi *et al.*, 1964). This would suggest

Table 2. The rate of ^3H -leucine uptake by various types of pituitary cells in organ culture

Culture age	Incubation* time with ^3H -L (hrs.)	Mean grain counts per cell		
		PAS-positive cells	Acidophils	Chromophobes
2nd day	4	$2.9 \pm 2.0^{**}$	8.8 ± 4.4	10.0 ± 3.2
	8	5.4 ± 2.7	14.6 ± 4.2	13.2 ± 4.2
	24	14.0 ± 4.4	26.9 ± 5.2	24.6 ± 5.6
4th day	4	4.2 ± 2.0	10.1 ± 3.3	11.7 ± 3.4

* ^3H -L: ^3H -leucine

** Mean $\pm$ standard error

The total number of grains contained in 50 cells of the same type was counted and mean values per cell were calculated for each type.

Fig. 1. Changes in the uptake rate of ^3H -leucine by the various types of pituitary cells with respect to the time of incubation with ^3H -leucine

Table 3. The rate of ³H-leucine uptake by various types of pituitary cells is short term incubation* (2 hrs.)

	PAS-positive cells	Acidophils	Chromophobes
Mean grain counts per cell	1.9±1.5**	9.1±2.2	10.6±2.9

* In this experiment normal rats were used.

** Mean±standard error

The number of grains contained in 50 cells in the same type was counted and mean values per cell were calculated for each type.

Table 4. The rate of ³H-leucine uptake by various types of pituitary cells in short term incubation (2 hrs.)*

Series of incubations	Mean grain counts per cell		
	PAS-positive cells	Acidophils	Chromophobes
HE added	21.3±7.0**	7.4±2.7	11.6±3.2
CE added	8.8±5.1	8.9±3.6	12.1±3.7
Without addition	4.2±3.0	10.2±3.7	12.1±4.2

* In this experiment castrated rats were used.

** Mean±standard error

The total number of grains contained in 50 cells of the same type was counted and mean values per cell were calculated for each type.

that the capacity of PAS-positive cells to incorporate the amino acid is particularly dependent on the central nervous system.

It has been generally accepted that gonadotropin is produced in and secreted from the PAS-positive cell and luteotropin in and from the acidophil. The present authors have reported that gonadotropic function of the pituitary was greatly reduced *in vitro* (1961 and 1963a). It is probable that the function of gonadotropin synthesis in the PAS-positive cell is suppressed significantly in a situation of complete isolation from hypothalamic influences. On the contrary, the luteotropic function is maintained or augmented under such a situation (Everett, 1954 and 1956; Kobayashi *et al.*, 1963b; Meites *et al.*, 1962). These findings would provide an explanation for the maintenance of the ability to incorporate radioactive leucine in the acidophil cells *in vitro*.

The effects of hypothalamic extracts (HE) were studied by short term incubation methods (Table 4). At this time the castrate pituitaries were used, since the uptake rate of pituitary cells of castrates was almost identical to that of normal rats *in vitro*. HE was added to incubation medium at the dose of 5 mg per ml (1 mg equivalent to 1~1/2 hypothalami). Brain cortical extracts (CE) were used as controls. It was demonstrated that the addition of HE markedly accelerated the uptake rate of PAS-positive cells; the grain count became 21.3±7.0 (S.E.). However, the values of chromophobes were unchanged even with the addition of HE. In acidophils a tendency of a slight decrease was observed

Fig. 2. Photomicrograph of a section of the pituitary in short term incubation (2 hrs.) with H^3 -leucine ($\times 1000$)

Small black grains show the uptake of radioactive leucine. Arrow indicates a PAS-positive cell in which the grains are not visible.

Fig. 3. Photomicrograph of a section of the organ cultured pituitary which was exposed to H^3 -leucine for 4 hrs. on the 2nd day of cultivation ($\times 1000$)

No grains can be seen in the PAS-positive cell, although they are rather diffusely distributed in many other cells.

Fig. 4. Photomicrograph of a section of the organ cultured pituitary which was exposed to H³-leucine for 8 hrs. on the 2nd day of cultivation ($\times 1000$)

The findings are similar to those of Figure 3. Arrow indicates a PAS-positive cell.

Fig. 5. Photomicrograph of a section of the pituitary in short term incubation with H³-leucine plus hypothalamic extracts ($\times 1000$)

A PAS-positive cell that contains a considerable number of grains is indicated by an arrow.

rather than an increase. Following the addition of CE a small rise was found in the PAS-positive cells, but the value only reached a level of 8.8 which was identical to the value of the acidophils under the same condition.

There are several reports to show that hypothalamic extracts exert gonadotropin-releasing actions *in vivo* or *in vitro*, although it is not always obvious whether such actions are related to gonadotropin synthesis, merely to its release or to both of them (Campbell *et al.*, 1961; McCann, 1962; Nikitovitch-Winer, 1962; Guillemin *et al.*, 1963). Our findings that the uptake rate in PAS-positive cells increases by the addition of HE would suggest that the HE-action is specifically related to the synthesis of this hormone in the adenohypophysis. Figures 2, 3 and 4 indicate the sections of the pituitaries incubated or cultivated without the addition of HE. Almost no grains were visible in PAS-positive cells while other cells contain considerable number of grains in their cytoplasm. As shown in Figure 5, however, it is apparent that HE causes a recovery or a further increase in the uptake rate of ^3H -leucine in the PAS-positive cell.

SUMMARY

^3H -leucine uptake of rat adenohypophysial cells was studied autoradiographically *in vitro*. The autoradiographs were stained with PAS, metanil yellow and toluidine blue. In organ-cultured pituitaries PAS-positive cells showed a significantly lower uptake rate than acidophils and chromophobes on the 2nd and 4th day of cultivation. The uptake of these three type cells was increased with the time of exposure to ^3H -leucine. However, the increase in the former was much less than that in the latter two.

The influence of acidic hypothalamic extracts was studied using short term incubation method. When hypothalamic extracts were added to incubation media, the uptake of ^3H -leucine by PAS-positive cells rose to a considerable higher level than that found in the other two types. The uptake rates of acidophils and chromophobes were unchanged by the addition of hypothalamic extracts.

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