Microspectrography of formaldehyde and fluorescamine-induced fluorescence in rabbit pulmonary neuroepithelial bodies: Demonstration of a new, probably polypeptide intracytoplasmic substance[1]

J. M. Lauweryns and M. Liebens

Katholieke Universiteit te Leuven School of Medicine, Laboratory of Pulmonary Histopathology, 12, Minderbroedersstraat, B–3000 Leuven (Belgium), 28 March 1977

Summary. The microspectrographic analysis of the fluorescence emitted by NEB's in gaseous formaldehyde-fixed lung tissue, posttreated with fluorescamine, revealed the presence of numerous primary amino groups which are clearly different from the serotonin identified in our earlier studies and correspond to a new, probably a polypeptide intracytoplasmic substance.

In earlier studies[2–6] we were able to demonstrate the presence of serotonin (5-hydroxytryptamin; 5-HT) in the intracytoplasmic type-1 dense cored vesicles of the granulated epithelial cells of neuroepithelial bodies (NEB's) of neonatal rabbit lungs (by the use of combined fluorescence, microspectrographical and ultrastructural cytochemical techniques). At that time we realized that the granulated cells probably contained and produced substances other than serotonin, whose metabolism may indeed be combined with a variety of cellular activities in the amine and peptide spectrum and suggested that the NEB's could possibly contain one (or more) polypeptide substance(s). In the present study the possible occurrence of such a polypeptide substance in the corpuscular cells of the NEB's was investigated, using fluorescamine (Fluram®). This recently introduced reagent is considered an interesting label for the histochemical demonstration of cells containing and secreting polypeptides[7–9]. A wide series of cells, such as pituitary growth hormone-producing cells, gastrin cells, pancreatic insulin cells and thyroid C cells, which are all known to contain polypeptide hormones, display a selective fluorescamine-induced fluorescence if the tissue in which they occur has been prefixed in formaldehyde gas.

In the studies here reported, a microspectrographical analysis of the fluorescence emitted by the NEB's in gaseous formaldehyde-fixed lung tissue posttreated with fluorescamine, revealed the presence of numerous primary amino groups which are clearly different from the primary NH_2-terminals of 5-HT. They do indeed not react upon treatment with formaldehyde gas (whereas the primary amine serotonin does react with formaldehyde gas) while becoming condensed with fluorescamine. These newly identified primary amino groups should indicate the presence of a polypeptide substance in the granulated epithelial cells of the NEB's. Further studies are undertaken to establish the precise nature of this newly identified substance.

10 neonatal rabbits from different litters were anaesthetized with pentobarbital (Nembutal®) after which the heart-lung preparations were removed. The lung tissues were cut into small pieces which were quenched in liquid nitrogen. Lyophilization was performed for 4 days at temperatures increasing from −80 °C to +30 °C. Next, most of the small tissue blocks were condensed for 1 h with formaldehyde gas with a relative humidity of 47% at 80 °C. Several tissue blocks (used as controls) were simply put at 80 °C for 1 h remaining unfixed. All biopsies were embedded in paraffin and cut at 6 µm. The sections were deparaffinized in toluol and placed in a 0.2 M phosphate-buffered saline (PBS) buffer, pH 8, for 5 min. Each section was drained and covered with a drop of the fluorescamine solution (2 mg fluorescamine-Fluram® – dissolved in 10 ml acetone) during 20 sec. Then, the sections were rinsed in the PBS buffer to be mounted in Entellan (slightly diluted with toluol). The spectral analysis of the fluorescence (both the formaldehyde- and the fluorescamine-induced fluorescence) emitted by the NEB's, was performed with the aid of a Leitz microspectrograph, equipped with an epi-fluorescent system. For the formaldehyde-induced fluorescence, we used an HBO 100 W Hg-lamp, excitation filters 2 mm KG 1, 4 mm BG 38, 3 mm BG 3, S 405 (Typ AL), a dichromic mirror with a cut-off of 455 nm and a barrier filter K 510. The fluorescamine-induced fluorescence was examined with the same excitation lamp, excitation filters 2 mm KG 1, 4 mm BG 38, 3 mm UG 1, a dichromic mirror with a cut-off of 400 nm and a barrier filter K 460. The emission spectra registered on a Hewlett-Packard 7004 B-XY recorder were corrected by comparison with the spectral curves of a Tungsten lamp of known color temperature (2850 °K at 5 A and 6 V). On the whole, the fluorescence from 20 NEB's randomly chosen out of several sections, was measured and analyzed microspectrographically.

Results. Unfixed (not condensed with formaldehyde gas) lung tissue treated with fluorescamine, yields an intense and widespread fluorescamine-induced fluorescence. The fading of this fluorescence occurs very rapidly, since it has disappeared completely in the selected microscopical field already after 1 min. In gaseous formaldehyde-fixed lung tissue, prepared according to Falck's histochemical fluorescent amine technique[10] and posttreated with fluorescamine, an intense and selectively fluorescamine-induced fluorescence is microscopically observed in cell groups which may be identified without doubt as NEB's, while the remaining lung tissue reveals only a markedly reduced fluorescence. In order to detect whether the fluorescence observed in the NEB's with the filter combination suitable for fluorescamine is not merely due to an important amount of serotonin, a microspectrographical analysis was performed of the formaldehyde-induced fluorescence on the one hand, and the fluorescamine-induced fluorescence on the other hand.

1 Supported by a grant from the Council for Tobacco Research (USA) and the National Fonds voor Wetenschappelijk Onderzoek (Belgium). We thank C. Armee, M.-R. Van Hamme and E. Swinnen for technical, G. Pison for photographical and N. Tyberghien for secretarial assistance.

2 J. M. Lauweryns, M. Cokelaere and P. Theunynck, Z. Zellforsch. *135*, 569 (1972).

3 J. M. Lauweryns and M. Cokelaere, Z. Zellforsch. *145*, 521 (1973).

4 J. M. Lauweryns, M. Cokelaere and P. Theunynck, Science *180*, 410 (1973).

5 J. M. Lauweryns and M. Cokelaere, Experientia *29*, 1384 (1973).

6 J. M. Lauweryns, M. Cokelaere, P. Theunynck and M. Deleersnyder, Chest *65*, 22S (1974).

7 R. Håkanson, L. I. Larsson and F. Sundler, Histochemistry *39*, 15 (1974).

8 F. Sundler, L. I. Larsson, R. Håkanson and O. Ljungberg, Virchows Arch. path. Anat. Histol. *363*, 17 (1974).

9 L. I. Larsson, F. Sundler and R. Håkanson, Histochemistry *44*, 245 (1975)

This analysis of the fluorescence emitted by all the NEB's investigated which were treated with gaseous formaldehyde and posttreated with fluorescamine, revealed before correction an average maximum of 550–560 nm for the formaldehyde-induced fluorescence and an average maximum of 510–520 nm for the fluorescamine-induced fluorescence (figure 1). After correction of the emission curves, 2 clearly separated curves are again obtained, revealing an emission maximum of 510–520 nm for the formaldehyde-induced fluorescence and of 470–480 nm for the fluorescamine-induced fluorescence (figure 2). These data indicate unequivocally the occurrence within the corpuscular cells of the NEB's as well of serotonin (with its emission maximum of 510–520 nm) as of another substance which has reacted with fluorescamine (with an emission maximum of 470–480 nm).

Discussion. A series of cells which all characteristically contain polypeptide hormones, exhibit a strong and selective fluorescamine-induced fluorescence though these cells were prefixed in formaldehyde gas. It has been proposed that condensation with gaseous formaldehyde is blocking only a certain number of primary amino groups in the cell, the remaining NH_2-terminals being able to react with fluorescamine[7–9]. As we observed that NEB's following a fixation with gaseous formaldehyde react in a very positive way to a fluorescamine treatment, one may logically conclude that the NEB's contain – besides serotonin – another substance which must be provided with a rather large amount of primary NH_2-terminals. This newly identified product could possibly represent a polypeptide principle, being stored in the secretory granules, which could protect one or more of its free amino groups to react with formaldehyde, while being accessible for fluorescamine[9]. It may be mentioned that it has also been suggested that the fluorescamine-induced fluorescence could correspond directly to fluorogenic components occurring together with the presumed peptide or polypeptide substance within the cytoplasmic granules.

Fluorescamine may also react with amino-phospholipids of lipoproteins and amino-sugars of glycoproteins[11, 12]. Our earlier observations, however, never revealed the presence of e.g. important fat accumulations or large amounts of glycogen within the granulated cells of the NEB's. A contribution of such substances to the fluorescamine-induced fluorescence hence appears negligible.

It thus appears logical to propose that the hereby newly identified primary amino groups form part of a (poly)-peptide substance which occurs – besides serotonin – in the cytoplasmic granules of the epithelial cells of the NEB's. It should, moreover, be noted that the occurrence of more than one substance in a cell or in a group of cells is not an exceptional finding. Indeed, catecholamines as well as indolamines were identified in the cells of the carotid body which shares many structural characteristics of the NEB's[13–16]. Our earlier studies also revealed the occurrence of 2 types of dense-cored vesicles (DCV's) in the NEB's. While the occurrence of serotonin was demonstrated in the DCV's of type 1, no primary amine could be identified in the DCV's of type 2. Thus we suggested at that time that the DCV's of type 2 could contain another active product not yet demonstrable[2–6].

Finally one cannot exclude the possibility that the role of the serotonin present could consist in increasing the transudation capacity of the capillaries[17], thus facilitating the uptake in the pulmonary vascular bed of the polypeptide substance present within the granulated cells of the NEB's. This point of view could be compared with observations concerning the ultimobranchial body of the chicken, where dopamine is presumed to be required to permit the secretion of calcitonine in the blood[18, 19].

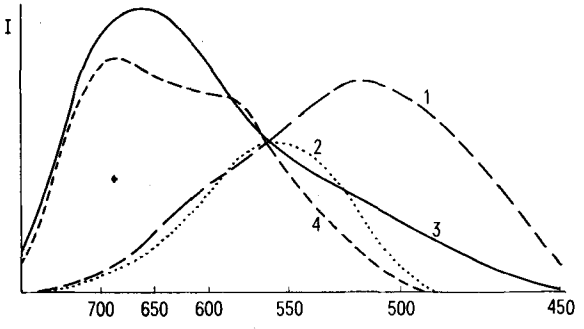

Fig. 1. Microspectrographical analysis of the uncorrected emission curves (nm) of the fluorescamine-induced fluorescence (1) and the formaldehyde-induced fluorescence (2) of NEB's in neonatal rabbit lungs. Matching tungsten curves for the correction of the fluorescamine-induced fluorescence (3) and the correction of the formaldehyde-induced fluorescence (4). I, emission intensity.

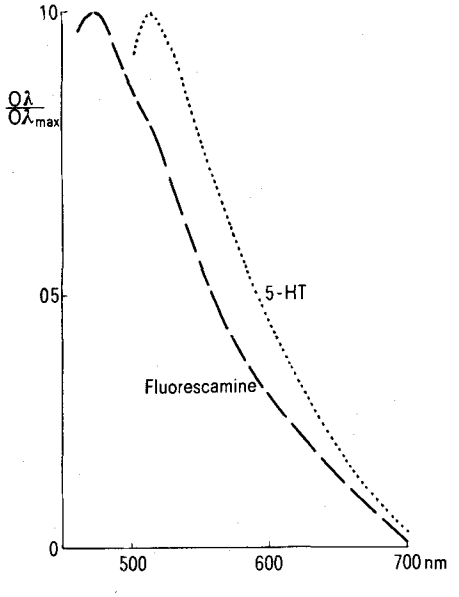

Fig. 2. Corrected microspectrographical analysis of the emission curves (nm) of figure 1 revealing the simultaneous occurrence of serotonin (5-HT) and of another substance reacting with fluorescamine (Fluram®). $Q\lambda/Q\lambda$ max, relative fluorescence yield.

10 B. Falck and C. Owman, Acta Univ. Lund, sectio II, 7, 1 (1965).
11 M. Naoi, Y. C. Lee and S. Roseman, Analyt. Biochem. *58*, 571 (1974).
12 M. Naoi and Y. C. Lee, Analyt. Biochem. *57*, 640 (1974).
13 G. Battaglia, Z. Zellforsch. *99*, 529 (1969).
14 S. R. Chiocchio, A. M. Biscardi and J. H. Tramezzani, Science *158*, 790 (1967).
15 P. M. Grimley and G. G. Glenner, Circulation *37*, 648 (1968).
16 E. Morita, S. R. Chiocchio and J. H. Tramezzani, J. Ultrastruct. Res. *28*. 399 (1969).
17 V. Erspamer, Handb. exp. Pharmak. *XIX*, 245 (1966).
18 A. Melander, C. Owman and F. Sundler, Histochemie *25*, 21 (1971).
19 A. Melander, C. Owman and F. Sundler, Histochemie *25*, 32 (1971).