Acta Dermatovener (Stockholm) 50: 329–337, 1970

# HISTOCHEMICAL STUDIES ON THE MONOAMINERGIC NERVE IN THE SKIN CHIEFLY BY MEANS OF THE FLUORESCENCE METHOD OF FALCK AND HILLARP

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*Abstract.* With the use of the fluorescence method of Falck & Hillarp, El-Badawi-Schenk technique for AchE and the silver impregnation method of Suzuki, the author started experiments to elucidate the autonomic nerve system of the skin. The results obtained from the present study were as follows: 1) In the nose skin of dogs, green to yellow-green fluorescence developed in a plexus enclosing the smooth muscle layer of the arterial vessels and in a wide mesh around th sweat glands. In arterial walls the plexus was seen to be directly superimposed in a distance on the outer surface of the smooth muscle layer, not penetrating between the muscle cells. Most of these fibers had the characteristic varicose appearance. 2) From the data obtained from the histochemical criteria described by some investigators, it might be supported that the fluorescence is specific, i.e., due to the presence of the primary CA and that NA is the only primary CA present in the monoaminergic neuron in significant amounts. 3) The specific fluorescence completely disappeared after administration of reserpine. The administration of NA, DA or L-DOPA after pretreatment with nialmide caused a marked increase in intensity of the normally fluorescent nerve fibers, while the non-terminal axons and the mixture of the fluorescent nerves in a bundle of the sensory nerves could not be observed. 4) A new method for the consecutive demonstration of fluorescent nerves and argyrophilic nerves in the same sections clearly demonstrated that the fluorescent products were localized to the non-myelinated nerve fibers. From this view the fluorescent nerves were identified as monoaminergic nerves. The non-fluorescent nerves were morphologically recognized as sensory nerves. 5) The AchE staining and fluorescence method in adjacent cryostat sections revealed approximately similar innervation patterns around the cutaneous arteries. 6) The above-mentioned results showed a more limited distribution of the monoaminergic nerves in the skin than most previous studies have suggested.

Studies on the autonomic innervation of the skin have given many contradictory results, owing largely to the lack of a specific histochemical method for the localization of adrenergic and cholinergic nerves in tissues. However, the introduction of histochemical methods for acetylcholinesterase (AchE) (14) and fluorescence methods (7, 16, 17) for the direct demonstration of monoamines and some of their precursors at the cellular level have made possible the preferential demonstration of cholinergic and adrenergic (monoaminergic) components of the autonomic nervous system.

With the use of these techniques and silver impregnation method of Suzuki (24), the author has started experiments to elucidate the autonomic nervous system of the skin.

## MATERIAL AND METHODS

Nose skin of dogs is chosen for this present work because of its abundance of nerve fibers and presence of few elastic fibers. Untreated dogs and dogs pretreated with drugs interfering with catecholamine metabolism in monoaminergic nerves are used for the experiments. Skin specimens are taken from the nose of the dogs under intravenous anaesthesia of sodium pentobarbital. The specimens are rapidly frozen in isopentanol cooled by dry ice and dried in vacuo at $-35°C$ for 7 days. The freeze-dried tissues are exposed to gaseous formaldehyde with the humidity of 60% in a closed vessel at $+80°C$ for 1 hour. After this step, the tissues are embedded in paraffin in vacuo and sectioned for fluorescence microscopy. A fluorescence microscope (Zeiss) is used with a Zeiss 50 filter. The source of the activation lamp is an Osram HBO 200 high pressure mercury lamp with a Schott BG 12 filter. A darkfield condensor for oil immersion is used for examination and photography. Catecholamines (CA) and 5-hydroxytryptamine are in this way converted to intensely fluorescent 3,4-dihydroisoquinolines (green) and 3,4-dihydro-$\beta$-carboline (yellow), respectively.

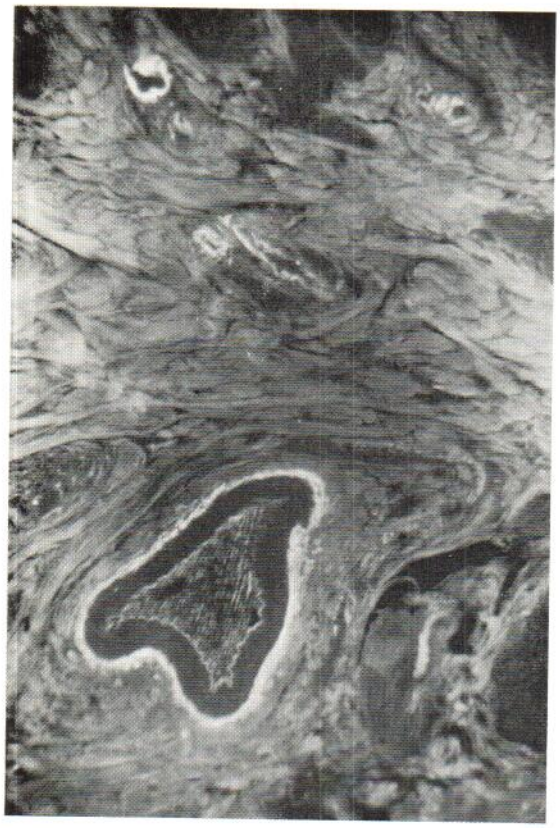

*Fig. 1.* Green to yellow-green fluorescence develops in a plexus, enclosing the smooth muscle layer of the arterial vessels through the entire dermis.

## RESULTS

### *Observation of the skin by the fluorescence microscope*

The specimens treated with formaldehyde gas according to the present method exhibited, principally, fluorescence of three different types. First, there were found different types of autofluorescence that were unaffected by reserpine pretreatment and occurred also in tissues not treated with formaldehyde gas. Secondly, collagenous fibers developed faint, dirty green, unspecific fluorescence, which also was unaffected by reserpine pretreatment. Thirdly, certain nerve fibers and epidermal melanocytes showed specific green to yellow-green fluorescence which did not develop after reserpine pretreatment.

Specific, green to yellow-green and intense fluorescence developed in a plexus around the arterial vessels through the entire dermis (Fig. 1). In cross sections the plexus was seen to be directly superimposed in a distance on the outer layer of the smooth muscle layer, not penetrating between the muscle cells (Figs. 1 and 4). Most of these fluorescent fibers had the characteristic varicose appearance (Fig. 2).

A widely meshed net work of the fluorescent fibers was found to be in contact with or lying in close proximity to the sweat glands, especially in the hairy portion of the dog nose (Fig. 3).

### *Histochemical and pharmacological criteria for specificity of fluorescence reaction*

To decide whether or not fluorescence observed in the specimens is specific, i.e., due to the presence of the reacting monoamines, the following histochemical criteria and pharmacological experiments were employed.

(A) Histochemical criteria (7, 23): Fluorescent tissue structures were analysed with respect to the following.

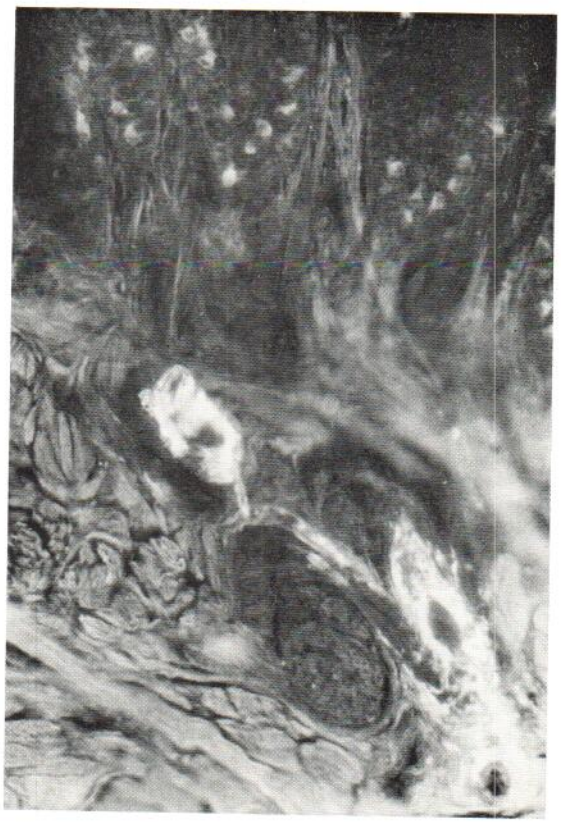

*Fig. 2.* Fluorescent nerves around the small arteries show the characteristic appearance and melanocytes exhibit yellow–green fluorescence.

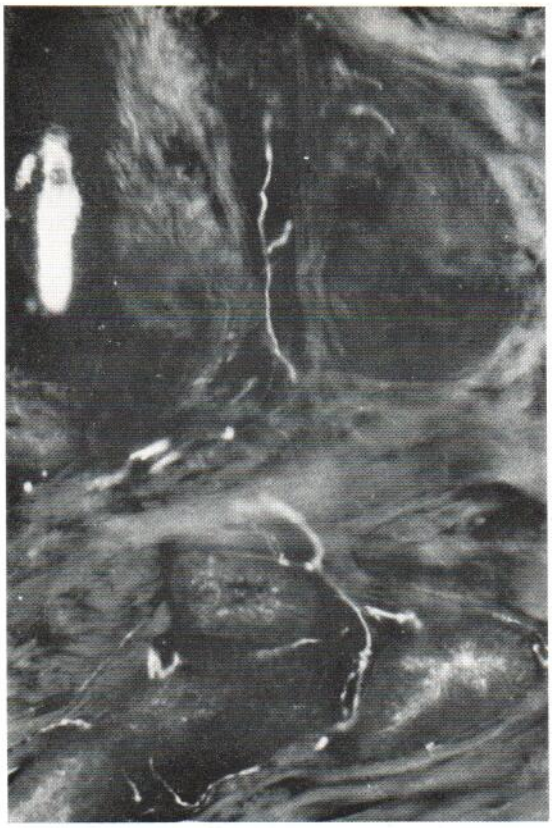

*Fig. 3.* Widely meshed fluorescent nerves around the sweat glands in the hairy portion of the nose skin.

1. Condition for the development of the fluorescence. When the formaldehyde treatment was performed at 80°C for 1 hour, the above-mentioned nerve fibers developed specific green to yellow-green fluorescence.

2. Sensitivity to irradiation with ultra violet light. When the fluorescent fibers were irradiated with UV light in the fluorescence microscope for a long time, green to yellow-green fluorescence decreased moderately in intensity.

3. Quenching of the fluorescence with water. The fluorescence products disappeared when the sections were mounted in 40% isopropanol in water.

4. Sodium borohydride reduction. According to the description of Corrodi et al. (8) the fluorescent compounds are very readily reduced by sodium borohydride to the corresponding, non-fluorescent 1,2,3,4-tetrahydro-compound. These can be converted again to the fluorescent 3,4-dihydro products on renewed formaldehyde treatment. Therefor, the section showing the fluorescent nerves enclosing the smooth muscle layer of an artery in the lower dermis was examined (Fig. 5 *a*). The fluorescence disappeared completely when the section was treated with a 0.1% sodium borohydride solution in 80% isopropanol for 2 min (Fig. 5 *b*). After the slide was thoroughly washed with solvents, the dried section was exposed to formaldehyde gas at 80°C for 3 hours. Fig. 5 *c* showed regeneration of the fluorescence.

The above-mentioned results leave little doubt that green to yellow-green fluorescence is due to the presence of the primary CA. Direct histochemical differentiation between the primary CA is difficult, since their fluorescent products have very similar chemical properties and practically identical absorption, excitation and emission characteristics (7).

5. Exposure to thionyl chloride. A method for

*Fig. 4.* Administration of DA after pretreatment with nialmide causes a marked increase in intensity of fluorescence.

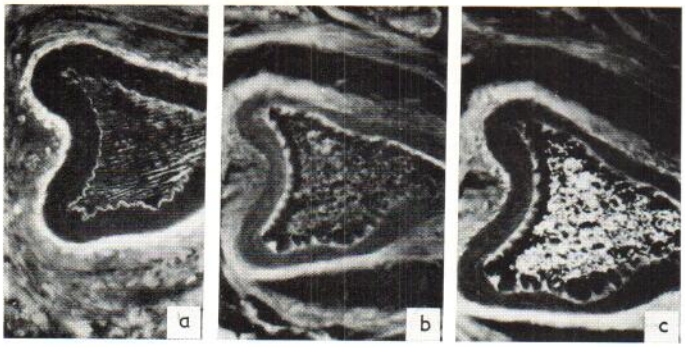


*Fig. 5.* Sodium borohydride reduction. (*a*) Before borohydride reduction. (*b*) After treatment with a 0.1% sodium borohydride solution in 80% isopropanol for 2 min, fluorescence disappears completely. (*c*) Regeneration of fluorescence by renewed formaldehyde treatment at 80°C for 3 hours.

differentiation between depamine (DA) and noradrenaline (NA) has been studied by Corrodi et al. (9) in model on the basis of the finding that the fluorescent product of NA, 4,6,7,-trihydroxy-3,4-dihydroisoquinoline has a labile hydroxy group in position 4. This can be split off by thionyl chloride, to form the fully aromatic 6,7-dihydroxyisoquinoline whose chemical and spectral characteristics are different from those of 3,4-dihydroisoquinoline formed from DA and NA. The 6,7-dihydroisoquinoline is also stable against sodium borohydride treatment, in contrast to the 3,4-dihydroisoquinolines. After thionyl chloride treatment the dopamine product becomes non-fluorescent on borohydride treatment, while 6,7-dihydroisoquinoline does not change its fluorescence.

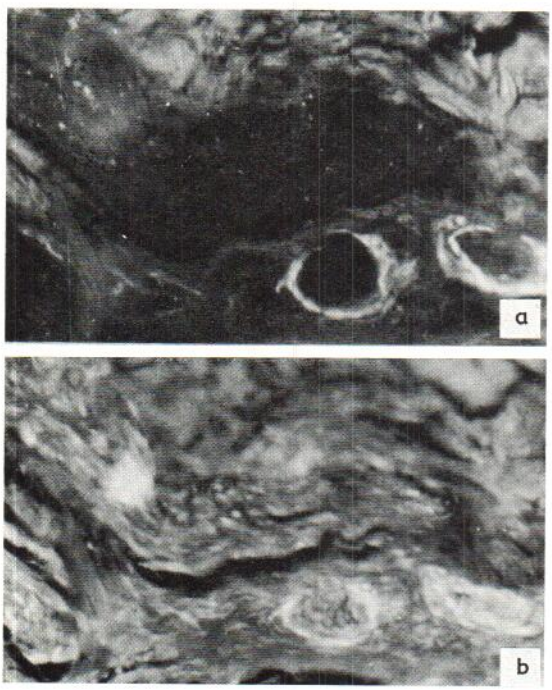


*Fig. 6.* Thionyl chloride test. (*a*) Before thionyl chloride test. (*b*) After exposure to thionyl chloride vapour at 50°C for 45 min the section is treated with a 0.1% sodium borohydride solution in 80% isopropanol for 2 min. Fluorescence remains moderate in intensity.

The section showing the fluorescent fibers around small arteries (Fig. 6 *a*) was treated with thionyl chloride vapour at 50°C for 45 min. After this step the section was treated with a 0.1% sodium borohydride solution in 80% isopropanol for 2 min. Fig. 6 *b* showed that the fluorescence remained moderate in intensity.

From the data obtained from the histochemical criteria used here, it might be supported that NA is the only primary CA present in the monoaminergic neuron in significant amounts.

(B) Pharmacological criteria. The desirability of using pharmacological tests to evaluate the specificity of the fluorescence in all tissues studied has been repeatedly emphasized (2, 7, 11, 23). Such experiments, summarized in Table I were performed in the present work.

(*a*) Reserpine. A large dose of reserpine, which is known to deplete all stores of monoamines in

Table I

| Drug | Dose mg/kg body weight | Time before taking the specimens (hours) |
|---|---|---|
| Reserpine | 5 i.p. | 4–36 |
| Nialamide | 500 i.p. | 2–4 |
| Nialamide | 500 i.p. | 4 |
| + DA | 25 i.v. | 2 |
| Nialamide | 500 i.p. | 4 |
| + L- DOPA | 50–100 i.p. | $\frac{1}{2}$–1$\frac{1}{2}$ |
| Nialamide | 500 i.p. | 4 |
| + NA | 2.5 i.v. | 1 |

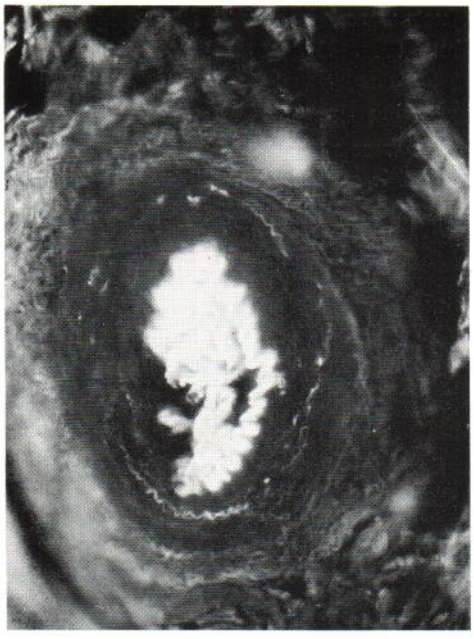

*Fig. 7.* Administration of reserpine. Fluorescence completely disappears, while autofluorescence of elastic fibers in the wall of artery remains unchanged.

the nervous system, resulted in a complete absence of specific fluorescence in the nerve fibers 4 hours after administration of the drug. No reappearance of the fluorescence in the nerve fibers was obtained 24 to 36 hours after administration of the drug. No obvious changes in the autofluorescence of the elastic fibers were observed (Fig. 7).

(*b*) Nilamide. The specific fluorescence remained unchanged 2 hours after administration of the drug but increased slightly at 4 hours.

It has been found that the entire monoaminergic neuron has a specific mechanism, localized to the level of the cell membrane, for the uptake and concentration of NA (23). Due to this mechanism amines accumulate rapidly to very high levels in the postganglionic axons and terminals after administration of NA and its precursor, especially when the animals are pretreated with monoamine oxidase inhibitor (nialamide).

(*c*) Nialamide-DA. The background fluorescence was markedly increased. The specific fluoresence became yellowish, probably due to the accumulation of the NA content in the nerve fibers (Figs. 4 and 8).

(*d*) Nialamide-L-DOPA and Nialamide-NA. The specific fluorescence showed a moderate increase in intensity. In contrast to administration of Nialamide-DA the increase in the background fluorescence was less pronounced.

Administration of NA, DA or L-DOPA after pretreatment with nialamide caused a marked increase in the intensity of the normally fluorescent fibers, while sensory nerves showed no specific fluorescence (Fig. 8).

*Correlations of fluorescent fibers developed by fluorescence method and nerve fibers stained with silver impregnation method* (*Suzuki*)

The sections prepared for the fluorescence method are examined and photographed in the fluores-

*Fig. 8.* Administration of DA after pretreatment with nialamide causes a marked increase of normally fluorescent nerves, while mixture of fluorescent nerves in a bundle of sensory nerves cannot be observed.

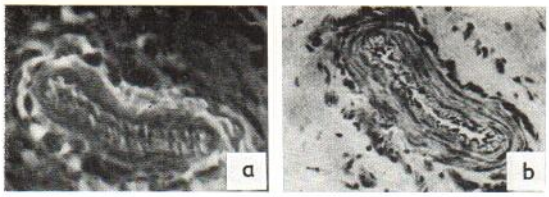


*Fig. 9.* Fluorescence method (*a*) and AchE staining (*b*) in the adjacent cryostat sections reveal approximately a similar innervation pattern around the small artery.

cence microscope. After this the sections are deparaffinized in xylene-alcohol series and washed in distilled water. To increase the affinity for silver the sections are fixed in a 10% formalin solution for 7 days. Subsequently, the sections are stained by the silver impregnation method of Suzuki described by Okumura (24). The same positions as previously are again photographed under ordinary microscopy.

On observing the fluorescent fibers and consecutively argyrophilic nerve fibers in the same section, it was clearly demonstrated that cutaneous nerves consisted of the fluorescent and non-fluorescent nerve fibers (Figs. 10 and 11). The fluorescent products were localized to the nonmyelinated nerve fibers (Fig. 11). From this viewpoint the fluorescent fibers were identified as monoaminergic nerves. On the other hand, non-fluorescent nerves which were observed to be darkened in the fluorescence microscope, probably due to the lack of the connective tissues, were morphologically recognized as a bundle of the sensory nerves. The fluorescent nerve fibers did not intermingle with a bundle of the sensory nerves. No other fluorescent nerve fibers could be observed besides the monoaminergic innervation of the arterial vessels and sweat glands.

*Histochemical correlations of AchE and CA in the autonomic nerve of the skin*

According to the hypothesis of Burn & Rand (3–6), the liberation of NA by postganglionic sympathetic impulse is mediated at the periphery by acetylcholine. To clarify this problem the following histochemical methods for separate demonstration of NA and AchE in cryostat sections are performed. The specimens taken from the nose skin of the dog are cut into two serial sec-

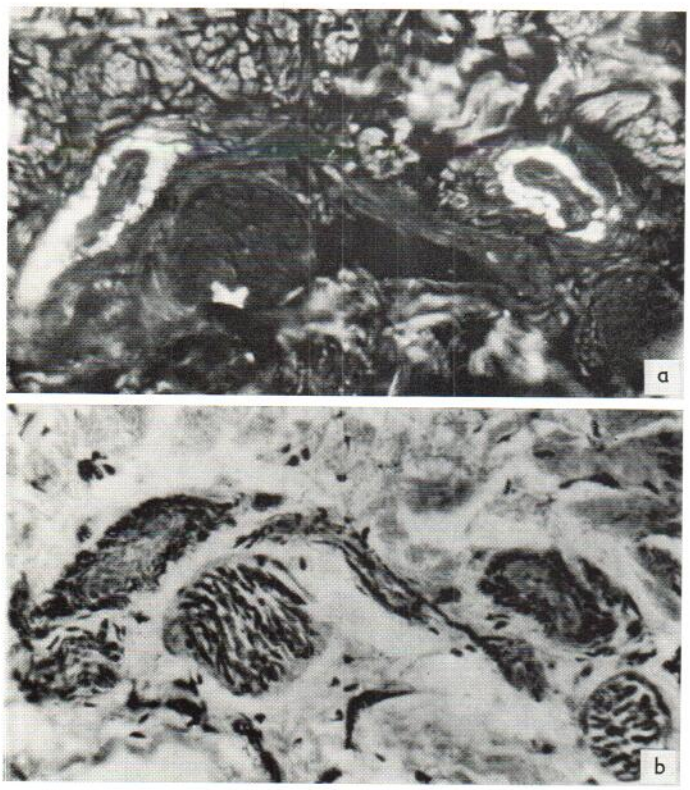


*Fig. 10.* Observation of the fluorescent nerves and argyrophilic nerves in the same section. Cutaneous nerves consists of the fluorescent (monoaminergic) and non-fluorescent (sensory) nerves.

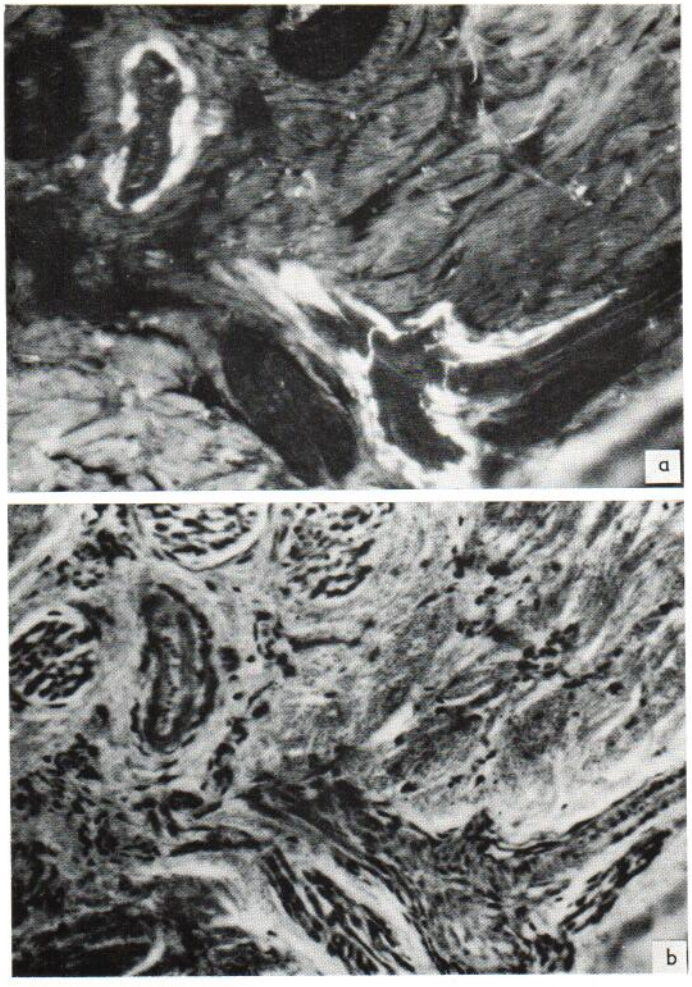


*Fig. 11.* Correlation of the fluorescent fibers developed by means of the fluorescence method and nerve fibers stained with the silver impregnation method of Suzuki. (*a*) Fluorescent nerves observed in the fluorescence microscope. (*b*) Argyrophilic nerves stained with the silver impregnation method of Suzuki in the same portion as (*a*). Fluorescent products are localized to non-myelinated nerve fibers.

tions in a cryostat. The one is stained by the El-Badawi-Schenk technique for AchE (14) and the other examined by the above-mentioned fluorescence method.

AchE staining showed that the cholinergic nerves were present only around the arterial vessels and sweat glands. There was some diffusion of the fluorescent products here because of the water content of the cryostat sections. However, Fig. 9 might suggest that duplication of the fluorescence and enzyme localization was present around the small artery.

## DISCUSSION

Histologic studies on the autonomic nervous system in the skin had been based upon silver impregnation and supravital methylene blue techniques (24–26). These techniques are non-specific and do not allow differentiation between the two functional types of peripheral autonomic neural elements, namely, the cholinergic and monoaminergic (14). The fluorescence method of Falck & Hillarp now available makes it possible to demonstrate the localization of the stores of the adrenergic transmitter NA in the tissues.

Examinination of the nose skin of the dogs with this technique shows green to yellow-green fluorescence in a plexus around the arterial vessels and in a wide mesh around the sweat glands. The results obtained from the histochemical and pharmacological experiments permit the definite conclusion that the fluorescent products are specific, i.e. due to the presence of the primary CA and that NA is the only primary CA in the monoaminergic neuron in significant amounts. This view

is supported by the data of earlier biochemical study (22) of CA in the skin.

It is well known that the skin is supplied with sensory and autonomic nerves. To clarify correlations between the monoaminergic and sensory nerves a new method for the consecutive demonstration of the fluorescent fibers and argyrophilic nerve fibers in the same sections was used in the present work. By this method, it is clearly demonstrated that the fluorescent products are localized to the non-myelinated nerve fibers. From this viewpoint the fluorescent fibers are identified as monoaminergic nerve fibers. Also, the non-fluorescent myelinated nerves correspond to the sensory nerves. The monoaminergic nerves do not intermingle with a bundle of the sensory nerves. It has not usually been possible to visualize the monoaminergic non-terminal axons running from the ganglia to the innervated structures, due to too low a content of NA (12–13, 23). However, they can be easily demonstrated after axotomy or administration of monoamines and monoamineoxidase inhibitor, which result in the rapid accumulation of monoamines in the nerve fibers. Therefor, such experiments were carried out in the present study. Other fluorescent nerve fibers except for the monoaminergic innervation of the arterial vessels and sweat glands can not, however, be observed in the dermis.

The above-mentioned results in this study show a more limited distribution of the monoaminergic nerves in the skin than most previous studies have suggested (24–26).

Similar results have been obtained by Falck et al. (18) and Möller (21). Falck & Rorsman described how the adrenergic nerves in the human skin were only present in the arrectores muscle and around the arterial vessels mainly in the deeper layer of the corium. Also, in the cat skin, Möller noted that strong green fluorescence developed in fine varicose nerve fibers situated in the arrectores muscles and around some vessels located in the deeper layer of the dermis.

As regards the innervation of the cutaneous blood vessels, it has been described how the autonomic nerves form plexus nervosus perivascularis in the adventitia, from which the nerve fibers ramify into the tunica media and even tunica interna (19). However, the present study shows that the monoaminergic and cholinergic nerves are present only on the outer surface of the smooth muscle layer, the muscle layer itself being devoid of innervation. Studies on the innervation of various arteries made by means of the electron microscope (1) and the fluorescence method of Falck & Hillarp (23) might support this view. The absence of special nerve endings, the characteristic appearance of the terminal and intimate connections between the effetcor cells and plexus strongly support that the varicose axon ramifications are true terminals (23).

According to the hypothesis of Burn & Rand (3, 4), the release of CA from the adrenergic fibers is initiated by a first jet of acetylcholine, which according to the recent interpretation of Burn & Gibbons (5) and Burn (6), results in an influx of Ca-ions that finally release NA. Such a system would require an AchE activity of the adrenergic fibers.

To elucidate this problem, histochemical methods for separate demonstration of NA and AchE in adjacent cryostat sections were performed. The results obtained from this present work might suggest that a close topographical correlation of the monoaminergic and cholinergic fibers is present around the cutaneous arterial vessels. Similar correlations of the adrenergic and cholinergic axons in some species of animals has already been suggested by Jacobowitz & Koelle (20) and Eränko (15). Recently, Csillik et al. (10) examined AchE activity of the autonomic nerve fibers in the dilator area of the rat iris with the electron histochemical method for AchE and demonstrated that AchE active and AchE inactive nerve fibers ran together, sometimes even within the common boundaries of the same Schwano-cellular envelope. They stated that the opinion that all postganglionic nerve fibers, both parasympathetic and sympathetic ones, would contain AchE should be rejected. In order to obtain more direct evidence regarding the function of AchE in the monoaminergic neurons of the skin, it will be necessary to develop electron microscopic techniques or others for the definite identification and precise localization of the transmitters or their related enzyme within individual nerve fibers.

## ACKNOWLEDGEMENT

The author wishes to express his thanks to Professor Yutaka Sano, Department of Anatomy, Kyoto Pref. University for valuable suggestions.

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*Received March 31, 1970*

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