Studies on the adrenal medulla

by

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PREFACE.

The work on which the present thesis is based was begun in Oct. 1952 in the Department of Pharmacology, University of Edinburgh. The studies were continued in the Physiological Department, Institute of Occupational Health, Helsinki, and completed in the Department of Anatomy, University of Helsinki.

Publication of many observations made during this work was rendered desirable by the appearance of reports on related subjects from other laboratories before the completion of the present thesis. Printed papers form, indeed, the main body of the thesis. They have been supplemented by typewritten sections and documentation data such as tables and original photomicrographs. A discussion of all the results obtained has also been added in the hope that it helps to tie together the information given in the separate papers.

Professor J.H. Caddum, F.R.S., and Dr. Marthe Vogt, F.R.S., have supervised this work. It is a pleasure to thank them warmly for their always willing guidance and readiness to place the resources of the Department of Pharmacology at the writer's disposal.
(II) INTRODUCTION.

The pressor effect of adrenal extracts was discovered already in 1894 by Oliver and Schäfer. Soon after that Abel and Crawford (1897) isolated the active principle of the adrenal medulla as a benzoic derivative, and Takamine (1901) isolated it in crystalline form and gave it the name adrenaline. Aldrich (1901), independently, also isolated adrenaline and determined its empirical formula 4 years later. Stolz (1904, 1906) and Dakin (1905) synthesized adrenaline, which was indeed the first hormone of the endocrine system to be isolated and synthesized.

Although these fundamental discoveries relating to the function of the adrenal medulla were made over fifty years ago, and although Stolz (1904), Biberfeld (1906) and Barger and Dale (1910-11) showed that peripheral sympathomimetic action is shared more or less by a large number of primary and secondary amines including noradrenaline, it was first in 1947 demonstrated by Holtz, Credner and Kroneberg pharmacologically and in 1948 by James chemically that the adrenal medulla contains noradrenaline in addition to adrenaline. The presence of noradrenaline in the adrenal medulla of several mammals has since then been confirmed with different methods by several investigators (for references, see Euler, 1956). Moreover, noradrenaline has
been demonstrated in the adrenal venous blood (Bülbring and Burn, 1949; Kaindl and Fuler, 1951; and others), also after denervation of the adrenal (Vogt, 1952), which shows that it is not only a precursor of adrenaline in the adrenal medulla but in itself a hormone.

Since the description by Henle (1865) of the chromaffin reaction, which renders the adrenal medulla brown, the adrenal gland has been a subject of a large number of histological studies. Most of the workers in this field have arrived at the conclusion that all the secretory cells of the adrenal medulla are in principle similar, although variations in the secretory state influence the microscopic properties of the cells so that some cells in the same gland may look different from the others (for references, see Bennett, 1941; Hillarp, 1946; Bachmann, 1954).

However, some investigators, who did not yet know of the two secretory products of the adrenal medulla, described two or more types of cells in it (Cramer, 1916, 1918, 1928; Kolmer, 1918; Hion, 1927; Ma, 1929). The studies made by Cramer are of special interest in this connection. He fixed mouse adrenals in osmic acid vapour, which is reduced by the medullary cells and forms a black colour. Supposedly this method demonstrated adrenaline. Cramer described two types of cells, one whose cytoplasm is filled with fine black granules, and the other which is clear grayish and contains
"globoid bodies" of varying size. Groups of cells forming secretory alveoli in the adrenal medulla were of the same type within each alveolus. The significance of these two types of cells remained unclear, and Cramer concluded that the differences may rather be in the secretory stage of the cells than in their basic properties, a view shared by most later workers (see Bennett, 1941; Hillarp, 1946; Bachmann, 1954).

Recently Bänder (1951) observed that some cells in the adrenal medulla of the mouse, the cat and the dog are stained after fixation in Orth's fluid in a different way than the remaining cells and suggested that these cells, "fuchsinophil" or "F-cells", produce noradrenaline and the others, "picrinophil" or "P-cells", adrenaline. In the adrenal medulla of the rat and the guinea pig, on the other hand, only "P-cells" were seen. Bänder tried to correlate the presence of the "F-cells" with the presence of noradrenaline in the medulla, and found that there is no noradrenaline in the adrenals of the rat, the rabbit and the guinea pig, while noradrenaline is present in the adrenals of the mouse, the cat and the dog. The method used by Bänder for differential determination of adrenaline and noradrenaline is based on the assumption that yohimbin completely inhibits the pressor effect of adrenaline on cat's blood pressure but does not inhibit that of noradrenaline. The accuracy of this
method seems however questionable. Indeed, many other workers have found a considerable proportion of noradrenaline in the adrenal medulla of the rat, in which Bänder observed neither noradrenaline nor two types of cells. Even supposing that his rats were of exceptional type the evidence he presented to show that there are special cells for the secretion of noradrenaline can be regarded insufficient.

Bränkö (1951, 1952) demonstrated two different types of cells in the adrenal medulla of the rat with the aid of three different histochemical methods. The main bulk of the medulla was found to be acid phosphatase positive, non-fluorescent after fixation in a calcium chloride-formol mixture and unstained by ammoniacal silver nitrate after the same fixation. Groups of medullary cells scattered as small islets over the medulla were, on the other hand, acid phosphatase negative, but fluoresced strongly in ultraviolet light and reduced ammoniacal silver nitrate after calcium-formol fixation. Stimuli such as injections of insulin, which clearly affected the adrenaline content of the adrenals without influencing the noradrenaline content, did not essentially change the histochemical reactions. On the other hand, treatment with thyroid powder, which caused a loss of noradrenaline without effect on adrenaline content, decreased the total volume of the silver-positive medullary tissue to less than a half. These observations were taken to suggest
that the presence of two histochemically different types of medullary cells in the adrenals of the rat may be related to the presence of the two different catechol amines in this gland. Although insufficient to prove that one of the two types of cells secretes adrenaline, the other noradrenaline, the results obtained seemed to justify a more thorough study of the problem and served as a starting point for the present work.

The purpose of the work to be described in this thesis is, then, to study the nature of connection between the two types of cells mentioned above and the production of adrenaline and noradrenaline. After description of the methods, microscopical observations on the adrenal medulla made together with determinations of the two catechol amines are reported. In a study like this, it is desirable to obtain material in which the relative amounts of the two catechol amines and/or of the two types of cells vary sufficiently. Therefore, adrenals of several species have been examined both microscopically and for the catechol amine composition. In some species small areas in neighbouring sections have been studied histologically and chemically to obtain direct information of the relations between the two types of cells and the distribution of the catechol amines within the medulla. In the same purpose, adrenals have been studied after treatment of the animals
with drugs affecting the secretion of the adrenal medulla.

The distributions in the adrenal medulla of the fluorescence test and a new histochemical reaction for the demonstration of noradrenaline have further been compared with each other.

Finally, the significance of the observations made is discussed and the conclusions are drawn.
METHODS.

(1) Histological methods.

a) Acid phosphatase.

As a first step of this investigation the three histochemical methods which were used to discriminate between two types of cells in the adrenal medulla of the rat (Eränkö, 1951, 1952) were applied to adrenals of the mouse, the cat, the dog, the guinea pig and the rabbit.

Acid phosphatase was demonstrated in fresh-frozen sections cut at 50 μ with a freezing microtome and subsequently fixed in Baker's (1944) calcium-formol fixative for some minutes. The sections were rinsed in water and incubated in a substrate mixture containing lead nitrate and sodium glycerophosphate and buffered to pH 4.7 with 1/10 acetate buffer (for details of the method, see Eränkö, 1952).

These studies showed that the intensity and the distribution of acid phosphatase are not related to the noradrenaline content of the adrenals of these species. Thus, adrenals of the cat, which contain c. 40 per cent of noradrenaline of the total amount of catechol amines, showed a weak and evenly distributed reaction for acid phosphatase in the medulla as did also adrenals of the guinea pig, which contain only very little noradrenaline (for the relative amounts of noradrenaline in the adrenals of various animals, see Chapter IV and Euler, 1956). On the other hand, all the
cells in the adrenal medulla of the mouse, which contains c. 30 per cent of noradrenaline, were strongly acid phosphatase positive.

It was furthermore observed that no such spatial correlation between the distributions of acid phosphatase and calcium-formol-induced fluorescence as was earlier demonstrated in the adrenal medulla of the rat could be found in the adrenals of the other species examined. Fluorescent cell islets were, e.g., seen in the adrenals of the cat, the dog and the mouse, of which the former two species showed a weak reaction for acid phosphatase in the adrenal medulla and the mouse a strong reaction in all medullary cells. That this observation was due to true species differences and not to imperfect technique was shown by confirming with the same histochemical set-up the earlier observations on the rat adrenal.

For the above reasons acid phosphatase was not studied in further work.

b) Calcium-formol-induced fluorescence.

This method proved promising from the beginning and was used throughout this study. The original technique, as described in an earlier paper (Eränkö, 1952), consisted of fixation of whole adrenals in Baker's (1944) calcium-formol fixative for 20-24 hours at 0°C in a refrigerator, sectioning at 40 μ with a freezing microtome, washing in distilled water
and mounting in glycerol. Thereafter the specific cell
islets were demonstrated by fluorescence microscopy in
ultraviolet light. The islets were also found to be visible
in fresh-frozen sections fixed after similar fixation in the
same way.

The use of fresh-frozen sections, instead of glands
fixed as a whole and subsequently sectioned, offered the
possibility of obvious advantage to examine one section of
the same gland for the fluorescence, and the neighbouring
section for the content of adrenaline and noradrenaline.
Therefore, fresh-frozen sections were used for studying the
fluorescence reaction. For practical reasons the fixation
was performed at room temperature instead of 6°C.

To determine the optimum conditions for obtaining the
fluorescence reaction in sections, rat adrenals were studied.

The adrenals were frozen immediately after removal on
the freezing microtome with a liberal stream of carbon
dioxide. Sections were then cut at 50 μ and these were
transferred frozen with a small brush in the fixative, where
they thawed. After varying periods of fixation the sections
were washed in distilled water, floated on slides and either
allowed to dry on it or mounted in glycerol. They were then
immediately examined for medullary fluorescence in a
fluorescence microscope. A carbon arc lamp or a mercury
vapour lamp was used as a source of ultraviolet light.
The visible light was filtered off with a copper sulfate filter and a Schott UG 1 filter. Ultraviolet light was stopped above the microscope ocular with a yellow filter of unknown origin which did not pass any light transmitted by the copper sulfate and UG 1 filters. The microscope magnification was about x100.

Different compositions of the calcium-formol fixative were examined. Two concentrations of formaldehyde were tried, i.e., 4 and 8 per cent (1 resp. 2 volumes of concentrated analytical formalin solution were diluted to make 10 volumes). Calcium chloride concentration was with both formaldehyde concentrations varied from 0.4 to 1.8 per cent, with intervals of 0.3 per cent. The fixation time in these mixtures was 4 hours, whereafter medullary fluorescence was examined.

The sections which were fixed in mixtures containing 0.4 per cent of calcium chloride did not show any medullary fluorescence, regardless of formaldehyde concentration. The fluorescent medullary islets were just visible in sections fixed in a solution containing 0.7 per cent of calcium chloride but the intensity of the fluorescence was weak. When the calcium chloride concentration was 1.0 per cent or more, bright fluorescence was seen in the medullary cell islets, although the main bulk of the medulla exhibited only a very weak fluorescence. Whether 4 or 8 per cent of
formaldehyde was used did not make any difference. All further studies were made using a mixture with 1 per cent of calcium chloride and 4 per cent of formaldehyde, which is the same composition as that used earlier and originally suggested by Baker (1944) for other purposes. Since the calcium content of the fixative was found to be of great importance, special care was taken to avoid using old "anhydrous" calcium chloride in making the solutions. Disappointing results had earlier been obtained with such material, probably because of hydration of the substance, which resulted in making of too dilute solutions.

Fixation in Baker's fixative for 10 min. at room temperature was sufficient to produce a weak fluorescence in the specific cell islets of rat's adrenal medulla. The intensity of the fluorescence increased with increased fixation time up to about 2 hours. In sections fixed for 2, 4, 6 or 8 hours the fluorescent medullary cell islets were brightly visible against a dull background. In further studies the length of fixation time was kept within 2 through 8 hours.

The fluorescence intensity was much brighter in sections allowed to dry on the slide than in sections mounted in glycerol. However, structural details were better preserved in glycerol-mounted sections. Therefore, mounting in glycerol was subsequently used as a routine. In cases of
doubt dry sections were also examined.

c) Ammoniacal silver nitrate test.

This test (Eränkö, 1952), which worked reproducibly in Helsinki, proved capricious in Edinburgh, in spite of some efforts to standardise the ammoniacal silver nitrate solution. This test was therefore abandoned.

(2) Methods of sampling and determination of catechol amines.

Since it was desired to examine both the extent of tissue which fluoresces after calcium-formol fixation and the catechol amine composition in small parts of adrenal medulla, a suitable sampling method and a sufficiently sensitive and accurate technique for the differential determination of adrenaline and noradrenaline had to be developed. While fixation in calcium-formol, which is necessary for the demonstration of the fluorescent cells, destroys the catechol amines and thus renders chemical analysis impossible in the same section, serially cut sections seemed to offer good chances of correlating the two kinds of variables in closely neighbouring areas of adrenal medulla. To be able to separate small medullary areas of the section to be analysed chemically, a technique of freeze-drying was adapted for the purpose.
A SIMPLE APPARATUS FOR FREEZE-DRYING OF ANIMAL TISSUES

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The freeze-drying technique is the method of choice for many histochemical studies. In the course of the last few years several improved designs of freeze-drying equipment have been published, by means of which the drying process can be done without the risk of artifacts and in a relatively short time (1, 4, 6-9). Many freeze-drying apparatus are provided with high-power pumps, vacuum gauges, and/or thermostatic devices. Such apparatus are undoubtedly efficient and convenient in use, but their fairly elaborate construction is necessarily combined with a high expenditure, for which reason many laboratories cannot afford constructing or purchasing such equipment. The present paper describes a simple and inexpensive freeze-drying apparatus for histochemical work, incidentally the first one used in Finland. This apparatus has been shown to give results comparable to those obtained with more complicated designs. The same apparatus can be conveniently used for freeze-drying of either small pieces of tissue or serially cut fresh-frozen sections.

THE APPARATUS

The apparatus is illustrated in Figs. 1-3. It consists of an inner and an outer Pyrex tube fitted together with a standard ground joint. The space between the two tubes is evacuated with a mechanical two-stage pump reported by the factory to give an ultimate vacuum of $5 \times 10^{-5}$ mm Hg.

When fresh-frozen sections are dried, a tissue holder (C) lying on the bottom of the outer tube is used. It is made of a brass disc 8 mm thick and 50 mm of diameter by drilling several holes of desired diameter ca. 6 mm deep and by inserting an arm of steel wire. In the

1 A part of this work was done in 1952-53 in the University of Edinburgh, where the writer stayed as a British Council Scholar. The writer is very grateful to Professor J. H. Gaddum, F.R.S. and to Dr. Marthe Vogt, F.R.S., for the laboratory facilities and for many advices.
writer's studies the sections were i.a. used for the estimation of minute quantities of adrenaline. As traces of metals such as copper may catalyse premature oxidation of adrenaline, the tissue holder was silver-plated.

The inner tube has several recesses made by pressing the heated glass with a conical carbon rod. When the inner tube is used as a cold trap these recesses increase the effective surface. On the other hand,

\[ \text{Fig. 1.} \]

The freeze-drying apparatus in a Dewar flask. When frozen sections are dried, the sections are in the holes of the holder C. The inner tube (A) is then filled with liquid air or with solid CO₂, and the Dewar flask (B) contains a mixture of solid CO₂ and diaethyl oxalate. When tissue pieces are dried, the inner tube contains the diaethyl oxalate mixture and the Dewar flask is filled with liquid air or with solid CO₂ and alcohol (see Fig. 2).

they may be half-filled with paraffin wax on which the pieces of tissue are placed for embedding in vacuum after the drying is completed (Fig. 2). In this case the inner wall of the outer tube serves as a cold trap.

**METHODS**

1. **Drying of fresh-frozen sections**

   The silvered tissue holder is kept during cutting on the bottom of a long beaker cooled from outside with solid CO₂ contained in a larger beaker. The piece of tissue to be cut is quickly frozen on the tissue holder of an ordinary freezing microtome by allowing liberal amounts of CO₂ flow through the microtome and by covering the tissue with
CO₂ snow. The microtome knife is also cooled in the same way, and that part of the knife edge which is used for cutting is cleaned from deposited ice with fingers. The temperature of the tissue and of the knife are critical. Too low temperature in either of them results in fragmentation of the sections. Too high temperature, on the other hand, causes gross ice-crystal-artifacts and dislocation of water-soluble substances. With some experience it is nevertheless possible to keep the temperature within an optimal range. Sections are cut and individually transferred from the knife edge into the small holes in the tissue holder. Straight sections can be obtained by slightly pressing the section during cutting against the knife with a fine camel-hair brush, which is also used for transferring the sections. To avoid undue warming and eventual thawing of the sections during transfer, the cold beaker with the tissue holder must be kept close at the microtome.

When the desired number of sections has been cut, the section holder is transferred onto the bottom of the outer tube in the apparatus, which has previously been immersed in a Dewar flask containing a cold liquid. A mixture of diaethyl oxalate and solid CO₂ is very satisfactory and gives a constant temperature of about -44° C as long as solid oxalate is present. The inner tube is then filled with solid CO₂ and some alcohol or acetone to facilitate the cooling of the glass. The joint is then closed and the pump started. Sections from adrenals of various animals cut at 20 to 60 µ are dry in less than 1 hour. After this period the Dewar flask is removed and after a few minutes' warming at room temperature the apparatus is immersed in water warmed to about 55° C. After further 5 min. the vacuum is broken and the pump

![Fig. 2.](image_url)

A detail of the apparatus showing the tissue piece (T) on paraffin wax (P) in a recess of the inner tube. A, solid CO₂ and diaethyl oxalate; B, liquid air or solid CO₂ and alcohol. The small points (W) in the evacuated space represent water molecules emerging from the tissue and forming a layer of ice (I) on the inner wall of the outer tube, which serves as a cold trap.
stopped. The inner tube and the section holder are taken out, and individual sections can be stored either in the holder, covered with a glass plate, or in small stoppered glass tubes. Frozen-dried sections keep well for months in tightly stoppered tubes at $-15^\circ$ C. The outer tube is again immersed in the CO$_2$-acetone mixture and a new tissue holder can be plunged in and another set of sections dried. To avoid deposition of ice from the atmospheric water vapour, the inner tube containing solid CO$_2$ must be kept during these manipulations in a tight tube containing some effective drying material, e.g., phosphorus pentoxide.

2. Drying of tissue pieces

The described metal holder can be similarly used for drying of tissue pieces. The tissue pieces are, nevertheless, preferably dried in the small recesses of the inner tube. In this case the inner tube is
filled with the diaethyl oxalate—CO₂ mixture. The outer tube, whose inner wall serves now as a cold trap, is immersed in either liquid air or in a mixture of alcohol and solid CO₂. One filling of the inner tube is enough for a run lasting 24 hours. If a sufficiently voluminous Dewar flask is used for cooling the outer tube it is neither necessary to refill it.

Tissue pieces are first quenched at a low temperature either by immersing in some organic fluid previously cooled to —150° C (1, 4, 6, 7, 9) or with the aid of cold metal surfaces (2). The quenched pieces are kept before drying at —78° C. For many purposes quenched pieces are conveniently stored in solid CO₂. However, direct contact with dry ice alters e.g. the autofluorescence of some tissues. Therefore, it is better to keep the pieces in small tubes cooled with solid CO₂, if longer storage is necessary. The recesses of the inner tube of the apparatus are half-filled with melted paraffin wax. The inner tube inserted the apparatus is then immersed in either liquid air or a mixture of acetone or alcohol and solid CO₂ contained in a Dewar flask. The inner tube is filled with a mixture of diaethyl oxalate, solid CO₂ and charcoal powder, which facilitates heat radiation. It is then lifted and the quenched tissue pieces are transferred on paraffin wax in the recesses. This must be done quickly to avoid deposition of ice on the outer surface of the inner tube. If the humidity of atmospheric air is high, the inner tube is better kept during this step in a perspex box with some drying agent on the bottom and with two side-holes for manipulation. The inner tube is then fitted in the apparatus and the pump started.

Drying times vary according to the kind of tissue and the dimensions of the pieces. As an approximate guide it may be mentioned that pieces of liver, spleen, and kidney about 1 mm thick have been adequately dried by running for 24 hours at ca. —40° C, after which the fluid in the inner tube was removed and the pump was further run overnight. For embedding in paraffin wax, water warm enough to melt the paraffin wax in the recesses is then poured in the inner tube and the vacuum is broken after 15 min.

DISCUSSION

Both theoretical and practical aspects of freeze-drying have been pertinently treated in recent reviews (1, 7). Therefore, they will not be exhaustively discussed in this paper. Some requirements will nevertheless be mentioned which an effective freeze-drying apparatus must fulfill.

(1) The temperature of the tissue must be kept below ca. —30° C, which is generally regarded sufficiently low for most purposes. In the described apparatus the tissue is certainly kept below —44° C during the process of drying. The danger of diffusion seems thus to be avoided for most purposes (1, 7).
(2) The moisture trap on which the water molecules released from the tissue are collected must be within the mean free path from the tissue \(1, 3, 4, 6, 7, 9\). If the ultimate vacuum in the apparatus described is \(5 \times 10^{-5}\), the mean free path is ca. 30 cm, which is about 30 times the tissue-trap distance.

(3) The effective surface area of the trap must be large enough to guarantee a sufficient drying capacity \(7\). Owing to the recesses the surface area of the inner tube is approximately equal to that of the outer tube, \(i.e.\) ca. 500 sq.cm. As the total surface area of 28 tissue pieces sized 1 cm \(\times\) 1 cm \(\times\) 1 mm is only about 67 sq.cm and as the average thickness of ice on the trap after completed dehydration would then be about 60 \(\mu\), the trap area must be regarded sufficient.

(4) The gradient in the water vapour pressure between the tissue and the trap must be sufficient to secure a maximal evaporation of water from the surface of the tissue. According to Flosdorff \(3\), a differential between the vapour pressure at the surface of the condenser and that at the ice surface within the piece where the latter is 55 per cent or less of the former would result in the maximal rate of flow obtainable. Applied to a tissue at \(-44^\circ\) C (pressure of saturated aqueous vapour \(6 \times 10^{-2}\) mm Hg) this would mean that a trap temperature of \(-50^\circ\) C (corresponding a vapour pressure of \(3 \times 10^{-2}\) mm Hg) would be sufficiently low. Thus, pressure gradients produced by dry ice and liquid air—reducing the pressure of saturated water vapour to 1 per cent and \(10^{-21}\) per cent of that at \(-44^\circ\) C, respectively—are amply sufficient.

Although the apparatus described theoretically fulfills the above conditions, its efficiency is somewhat inferior to that of more complicated designs using high-power oil diffusion pumps for the production of vacuum. While dehydration of small tissue pieces with the writer's apparatus takes 1–2 days, the drying is said to be completed in about 5 hrs. with freeze-drying systems having more efficient pumps \(4, 6, 9\). A possible reason to this difference may be an insufficiency of a low-speed vacuum pump to produce and maintain the desired vacuum in a kinetic system like that between the tissue piece and the trap wall, though it would produce a sufficiently low vacuum in a static system. Such failure would lower the mean free path, and collisions of water molecules with each other and with air molecules may consequently slow the rate of drying.—It may also be questioned whether the vapour-pressure differential between the tissue and the trap really is as high as calculated above, because the corresponding temperature gradient tends to decrease during the transfer of water molecules, which involves a transfer of heat, from the tissue to the trap.

Whatever the reasons are causing a lower than theoretical efficiency, the present apparatus is probably as effective as a freeze-drying unit can be at as low cost. Though it is naturally of advantage to have tissues embedded within such a short period as 5 hours from
the beginning of the freeze-drying procedure, prolongation of this time to 1–2 days is for many purposes not a decisive drawback, this time being less than is frequently required for paraffin embedding according to the classical alcohol-benzene technique. On the other hand, freeze-drying of freshly cut sections can be quickly done with the described technique along the same lines as with Linderstrom-Lang's (5) microtome cryostate.

SUMMARY

A simple and inexpensive freeze-drying apparatus is described. It consists of an inner glass tube connected with a standard ground joint to an outer tube, and of a two-stage mechanical pump for evacuating the space between the two tubes. The design is based on the principle of short-path condensation. Diaethyl oxalate and solid CO₂ are used to produce a constant tissue temperature of −44°C. The trap wall is cooled either with liquid air or with a mixture of solid CO₂ and ethyl alcohol or acetone. Freshly cut sections can be dried with the apparatus in less than 1 hour and small tissue pieces in 1–2 days.

REFERENCES

HISTOLOGICAL SAMPLING, CHROMATOGRAPHIC SEPARATION, AND DETERMINATION OF ADRENALINE AND NORADRENALINE IN THE ADRENAL MEDULLA

by

OLAVI ERÄNKÖ

(Received for publication October 25, 1954)

In some previous studies the present writer (6, 7, 8) found two histochemically different types of cells in the adrenal medulla of the rat. After fixation in formalin, most cells showed but a faint fluorescence, but there were groups of intensely fluorescing cells forming clearly delineated islets. The assumption was made that this morphological dualism is related to the presence of two kinds of catechol amines in the adrenal medulla. Subsequent studies (10) have confirmed the presence of similar islets in other species whose adrenal medulla contains noradrenaline. It was therefore found advisable to study whether the distribution of noradrenaline is similar to that of the fluorescing islets. The results, which suggest the presence of high concentrations noradrenaline in the islet cells, will be reported elsewhere.

Since the methods used for histological sampling and for the

1 Present address: Institute of Occupational Health, Työterveyslaitos, Helsinki. An essential part of this study was done during the academic year 1952—1953, which the writer spent as a British Council Scholar in the University of Edinburgh. The writer owes many thanks to Professor J. H. Gaddum, to Dr. Marthe Vogt and to Dr. T. B.B. Crawford for laboratory facilities and willing guidance. Mr. E. Siltanen and Mrs. L. Räisänen have given technical assistance, which is gratefully acknowledged. Financial support has been obtained from the Sigrid Jusélius Foundation and from the Valtion Luonnon- tieteellinen Toimikunta.
HISTOLOGICAL SAMPLING, CHROMATOGRAPHIC SEPARATION

separation and determination of adrenaline and noradrenaline in the adrenal medulla may be of more general interest, they will be described in this communication. When the present work was in progress, Lowry (14) published an article on histological sampling for the study of the histochemistry of the brain. The sampling techniques used by Lowry and the present writer are in some respects similar, but the differences will be obvious from the following description.

EXPERIMENTAL

Comparison of the distribution of the fluorescing islets and that of the adrenal catechols is difficult because treatment with formalin, which is necessary for making the islets visible, destroys the catechols and because the islets cannot be demonstrated in sections from which the catechols have been extracted away. The only possibility is therefore to compare neighboring sections, one of which is treated with formalin for making the islets visible, the other one being studied for adrenaline and noradrenaline. The whole process is summarized in Table 1.

TABLE 1

1. FREEZING
   2. SERIAL CUTTING
     (ODD NUMBERS)       (EVEN NUMBERS)
       ↓                     ↓
     3a. FORMALIN FIXATION  3b. FREEZE-DRYING
       ↓                     ↓
     4. MOUNTING IN GLYCEROL
       ↓                     ↓
     Control               Control
     5a. FLUORESCENCE MICROSCOPY  5b. MICRODISSECTION
       ↓                     ↓
     8a. FLUOROMETRY  6. CHROMATOGRAPHY
       ←                         ↓
     8b. BIOASSAY  7. ELUTION

Preparation of Freeze-Dried Sections. — Fresh adrenals were frozen on the tissue holder of an ordinary freezing microtome. Rapid freezing was conveniently done by applying a thin layer of distilled water on the tissue holder, by pushing the gland into
the water and by cooling the holder quickly with a liberal stream of carbon dioxide. Sections cut serially with a cooled knife were then one by one transferred with a brush into individual holes in a section holder kept at -78°C with the aid of dry ice. After completed cutting the section holder was transferred into a simple short-path freeze-drying apparatus (9), and the sections were dried at -40°C.

Fig. 1. — Low-power photomicrograph illustrating pieces of freeze-dried adrenal medulla cut free-hand with small scalpels prepared of a razor blade. 15 x.

Dissection and Weighing. — The series of freeze-dried sections can of course be examined as such with chemical and histochemical methods. However, it was observed that the relative amounts of islet tissue and noradrenaline as a rule varied little from one whole section to another, thus allowing no conclusions about the relationships between the catechols and the islets. Therefore, dissection of small pieces from the adrenal medulla was indicated. The probable sites of fluorescing and non-fluorescing tissues in the freeze-dried section were determined by examining the two neighboring formalin-fixed sections for fluorescence. The corresponding parts in the freeze-dried section were then dissected out under a dissection microscope with the aid of a pair of small scalpels.
Preparation of pieces even less than 100 \( \mu \) each way can be done manually without particular difficulties (Fig. 1). The pieces were subsequently weighed with a steel wire balance (20) or, if very small, with a quartz fiber balance (14).

**Chromatographic Separation of Adrenaline and Noradrenaline.** — The chromatographic method used was in principle similar to that developed for quantitative determinations in the Department of Pharmacology, University of Edinburgh, (2, 3, 17, 18, 21) from James's (13) qualitative method. In this method extraction and several purification stages preceded the chromatography. As this preliminary treatment was tedious, it was examined whether it can be omitted in the study of adrenomedullary sections, containing high concentrations of catechols. To that end, freeze-dried pieces of the adrenal medulla were directly applied on the baseline of the paper previously moistened with a drop of 0.1 N hydrochloric acid. Whatman's No 1 paper washed overnight with 0.01 N hydrochloric acid was used. A cylinder was made by joining the lateral ends of the paper and the ascending chromatogram was developed at 26°C, using a mixture containing 85 per cent of phenol, freshly distilled in the presence of zinc powder, and 15 per cent (w/w) of 0.1 N hydrochloric acid. Chromatography was done under anoxic conditions by filling the tank with either nitrogen or carbon dioxide.

The paper cylinder was after development allowed to stay a few minutes on filter paper to drain off excess fluid. It was then cut to vertical strips, which were washed in several changes of analytical benzene until the smell of phenol vanished. One of the strips was then sprayed with a solution containing 0.44 per cent of potassium ferricyanide in a 0.4 M phosphate buffer and dried at about 100°C to develop fluorescence. The strip was examined in ultraviolet light, and the fluorescent spots were circumscribed with a pencil. When large quantities of catechols were present, the spots were visible in ordinary light but the fluorescence method is far more sensitive, making spots containing only some 0.1 \( \mu \)g of either catechol clearly visible. Narrow horizontal strips were then cut from the non-sprayed parts of the developed chromatogram, and these strips were eluted with a solution of 0.4 per cent sodium dihydrogen phosphate. The catechols were determined in the eluates either biologically or chemically (see below). Fig. 2
Fig. 2. — Above, distribution of adrenaline and noradrenaline in different parts of the chromatogram. Below, drawing prepared of the sprayed control strip showing the fluorescing spots as black. Note the localization of fluorogenic catechols exclusively in the regions characteristic to adrenaline and noradrenaline.

shows the result of a typical experiment. With sections 50 $\mu$ thick or thinner, practically all catechol activity was seen within a zone limited by lines some 0.5 cm from the extreme ends of the fluorescing spots in the sprayed strip. For routine estimations, 1.5 cm safety zones on both sides of the spots were used.

Both whole adrenal sections and their fragments can be treated in this way. As the presence of interfering substances in the section was doubted to influence the running speed, $R_f$ values obtained with pure solutions of adrenaline and noradrenaline and with sections were compared. Lower $R_f$ values were obtained with whole sections, while the running speed of the catechols from sections containing only the medullary part was the same as that of the catechols applied on the paper in pure solutions (Fig. 3). A third, clearly defined spot was always detected between the adrenaline and noradrenaline spots when cortical tissue was present in the section applied on paper; this spot was found to be due to ascorbic acid (Fig. 3), a fact of importance when fluorescence methods are
HISTOLOGICAL SAMPLING, CHROMATOGRAPHIC SEPARATION 397

RAT ADRENAL. HCl - Phenol 25 hrs. 26°C.

<table>
<thead>
<tr>
<th>ascorbic a.</th>
<th>cortex</th>
<th>medulla</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>A+N cortex</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>A+N</th>
<th>medulla</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5 cm.  front

Fig. 3. — Negative tracing of a chromatogram. The uppermost strip was sprayed with dichlorophenol-indophenol and showed a white spot against blue background, illustrating the location of ascorbic acid. The other strips were sprayed with a buffered potassium ferriyanide solution. Adrenaline and noradrenaline were visible as strongly fluorescing spots, ascorbic acid as a white spot against yellow background. Note the retardation of adrenaline and noradrenaline spots and the presence of ascorbic acid in the strips with cortical tissue and the lack of such retardation in the strip with pure medullary tissue.

used for the determination of the catechols (see below). Pure ascorbic acid simultaneously applied with the pure catechols on the paper was also found to retard the running speed of the latter.

**Biological Determination of Adrenaline and Noradrenaline.** — The rat blood pressure assay (3, 17, 18, 21) was the only biological method used for the determination of noradrenaline, which can be estimated with this method in as small quantities as 0.005 µg. As about ten to twenty times as much adrenaline is necessary to obtain a reliable blood-pressure increase, the amount of adrenaline must be accordingly larger.

The rat was anaesthetized by injecting subcutaneously 0.7 cc of 25 per cent urethane (w/v) solution per 100 gm body weight. After the injection and during the assay the animal was kept warm with the aid of a tungsten lamp. The femoral vein was cannulated and the animal was heparinized and atropinized intravenously. The neck was then opened, both vagal nerves cut and the carotid artery cannulated. The blood pressure was registered with Condon's (1) manometer, giving nearly twice as big responses as an ordinary
U-shaped mercury manometer. To sensitize the blood pressure response, 1 or 2 mg of hexamethonium was injected intravenously (17). The first assay doses of catechols were injected some 15 min. thereafter, when the blood pressure had reached a steady level. Adrenaline or noradrenaline were always injected at constant intervals in a total volume of 0.3 ml, including the volume used for washing the plastic cannula. Known and unknown doses were injected alternatively. The assay was continued until the magnitude of the blood pressure response to the unknown dose was at least twice between the responses obtained with two known doses sufficiently near to each other.

When the eluate contained less than ca. 0.1 μg of adrenaline, rat’s uterus assay was used. The assay (4, 5) is based on inhibition by adrenaline of uterine contractions induced by carbaminoyl choline. The sensitivity varies from one uterus preparation to another but 0.0001 μg can be assayed under favourable circumstances.

The biological methods are tedious and only few samples can be assayed on each preparation with sufficient accuracy. Whenever the amounts of catechols were large enough they were therefore determined chemically by the fluorescence method described below. Biological and chemical methods were found to give comparable results, the latter being nevertheless more accurate.

**Chemical Determination of Adrenaline and Noradrenaline.** — The method was based on the fluorescence method described by Lund (15, 16). This method makes use of the formation of a fluorescing compound, adrenolutine, from adrenochrome, which is an oxidation product of adrenaline. Lund (16) suggested differential determination of adrenaline and noradrenaline by oxidizing one half of the sample at pH 6 and the other half at pH 4 (see 12). Practically only adrenaline is oxidized at pH 4, while both adrenochrome and noradrenochrome are formed at pH 6. This kind of differentiation is too inaccurate in our experience (see also 19), particularly when the main object of the study is to register differences in the adrenaline/noradrenaline ratio. The determinations were therefore always done first after chromatographic separation of the catechols.

In earlier experiments, the eluates were oxidized according to Lund by shaking specially purified manganese dioxide with the
catechol solution for 30 sec. at pH 6, after which manganese dioxide was centrifuged off. Ascorbic acid was added to the clear supernatant to prevent further oxidation. The pH was then made strongly alkaline with sodium hydroxide and the fluorescence was measured with the Farrand fluorometer or with the Beckman photometer. For various reasons this method of oxidation was not entirely satisfactory. Manganese dioxide was therefore replaced with potassium ferricyanide, briefly mentioned by Euler (11) to give good results. The technique used in our laboratory at present is given below.

![Graph](image)

**Fig. 4.** Intensity of fluorescence as a function of time after addition of sodium hydroxide in oxidized solutions of adrenaline and noradrenaline. The fluorescence intensity is expressed as per cent of that of the quinidine standard. It is seen that adrenochrome is quickly transformed to adrenolutine, while the maximum fluorescence with the noradrenaline solution is reached first some 5 min. after the addition of sodium hydroxide.

Elute the paper with 0.4 per cent sodium dihydrogen phosphate. Adjust the volume with the same solution to 3 ml. Add 0.75 ml of a 0.98 per cent solution of disodium hydrogen phosphate, mix and add 0.05 ml of 0.5 per cent potassium ferricyanide and mix immediately. After exactly one minute add 0.05 ml of 0.5 per cent ascorbic acid and mix immediately. Add 0.6 cc of 25 per cent sodium hydroxide, mix and measure the fluorescence against a water blank and a quinidine standard. The fluorescence intensity reaches its maximum soon after addition of sodium hydroxide (Fig. 4). The maximum fluorescence intensity is linear to the catechol concentration and the accuracy is satisfactory even with amounts of ca. 0.2 μg when the fluorescence outfit of the Beckman photometer is used. Smaller amounts can be measured with the Farrand fluorometer. Studies on chromatographed extracts of the
adrenal medulla failed to demonstrate the presence of any other catechols or interfering substances. With extracts of the whole gland care must be taken not to elute the ascorbic acid off the paper, as this substance prevents the oxidation of adrenaline and noradrenaline.

Calibration lines were prepared in each set of determinations for both adrenaline and noradrenaline by measuring the intensities of fluorescence produced by 0, 0.5 and 2 μg of the catechols, respectively. The over-all standard error of the method, including errors in the pipetting of the mentioned amounts and those due to (negligible) deviation from the linearity amounted to ± 3.5 per cent for adrenaline and ± 2.8 per cent for noradrenaline, as calculated from 24 double determinations.

**SUMMARY**

A procedure is described for studying the quantitative distribution of adrenaline and noradrenaline in the adrenal medulla and for correlating the results with histochemical findings. Fresh organs are serially cut with a freezing microtome. Every other of the sections are examined histochemically after formalin fixation, the remaining sections being freeze-dried. From the dry sections are dissected small regions for chemical analysis. Adrenaline and noradrenaline are chromatographically separated from previously weighed small sections, which are as such applied on the base-line of the chromatography paper. Appropriate areas of the chromatogram are eluted and the amounts of adrenaline or noradrenaline are estimated either biologically or chemically. A sensitive fluorometric method suitable for this purpose is described.

**REFERENCES**


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*Mary Lockett (J. Physiol. 1954:124:67P) has recently reported the presence of a catechol in extracts of cat adrenals giving a fluorescent spot in the chromatogram at Rf 0.7 after spraying with potassium ferricyanide. The present writer, using identical techniques, has not been able to confirm this observation with sections of cat adrenal medulla.*
10. Eränkö, O.: In the course of publication.
TABLE 1.

Fluorometric readings obtained with the fluorescence attachment of Beckman spectrophotometer in 24 different days. The photometer reading was set at 0 with a water blank and at 100 with a quinidine standard.

<table>
<thead>
<tr>
<th>Adrenaline (0.5 μg. 2.0 μg. 4xa b-4a)</th>
<th>Noradrenaline (0.5 μg. 2.0 μg. 4xa b-4a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>41.5 172 166 + 6</td>
<td>47.5 200 190 +10</td>
</tr>
<tr>
<td>40 164 160 + 4</td>
<td>52.5 207 210 - 3</td>
</tr>
<tr>
<td>41 157 164 - 7</td>
<td>52 202 208 - 6</td>
</tr>
<tr>
<td>40 157 160 - 3</td>
<td>50 206 200 + 6</td>
</tr>
<tr>
<td>44 154 176 -22</td>
<td>51.5 191 206 -15</td>
</tr>
<tr>
<td>40 157 160 - 3</td>
<td>50 204 200 + 4</td>
</tr>
<tr>
<td>39.5 156 158 - 2</td>
<td>50.5 193 202 - 9</td>
</tr>
<tr>
<td>38 158 152 + 6</td>
<td>47 188 188 + 0</td>
</tr>
<tr>
<td>40 152 160 - 8</td>
<td>48 183 182 + 1</td>
</tr>
<tr>
<td>38 151 152 - 1</td>
<td>50.5 196 202 - 6</td>
</tr>
<tr>
<td>40 169 160 + 9</td>
<td>52 204 208 - 4</td>
</tr>
<tr>
<td>38.5 158 154 + 4</td>
<td>50 202 200 + 2</td>
</tr>
<tr>
<td>41.5 160 166 - 6</td>
<td>47 199 193 +11</td>
</tr>
<tr>
<td>40.5 157 162 - 5</td>
<td>50.5 204 202 + 2</td>
</tr>
<tr>
<td>41.5 159 166 - 7</td>
<td>50 203 200 + 3</td>
</tr>
<tr>
<td>39 160 156 + 4</td>
<td>52 194 208 - 14</td>
</tr>
<tr>
<td>41.5 162 166 - 4</td>
<td>51.5 198 206 - 8</td>
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<td>43.5 162 174 -12</td>
<td>49 196 196 +10</td>
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<td>44 162 176 -14</td>
<td>52 193 208 -15</td>
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<td>43 163 172 - 9</td>
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<td>40 166 160 + 6</td>
<td>52 200 208 - 8</td>
</tr>
<tr>
<td>40 166 160 + 6</td>
<td>51.5 210 206 + 4</td>
</tr>
<tr>
<td>39.5 165 158 + 7</td>
<td>53.5 216 214 + 2</td>
</tr>
<tr>
<td>40 163 160 + 3</td>
<td>52 210 208 + 2</td>
</tr>
</tbody>
</table>

Sum of differences $\Sigma d = -48$

Mean difference $\frac{\Sigma d}{n} = -2$

Sum of squares of differences $\Sigma d^2 = 1498$

Error of method $\sqrt{\frac{\Sigma d^2}{2n}} = 5.6$

$\Sigma d = -23$

$\frac{\Sigma d}{n} = -1$

$\Sigma d^2 = 1431$

$\sqrt{\frac{\Sigma d^2}{2n}} = 5.5$
ESTIMATION OF THE VOLUMES OF THE CORTEX AND MEDULLA FROM ADRENAL SECTIONS

by

OLAVI ERÄNKÖ

(Received for publication September 15, 1954)

A change in the adrenal weight may be due to a change in the weight of the cortex, in that of the medulla or in both. Since in most animals the medulla is small as compared to the cortex, the weight of the whole gland is in many instances a sufficiently accurate measure of the cortical weight. In the rat, e.g., the medullary volume is roughly one tenth of the cortical volume. A 50 per cent increase in the adrenal weight may then be due, e.g., to an increase by ca. 500 per cent of the medullary weight — which only occurs in very exceptional conditions, if ever — or to an increase in the cortical weight by some 50 per cent, an alternative mostly in question.

While frequently it thus is enough to estimate the size of the adrenal cortex by weighing the whole gland, an estimate of the medullary size cannot be obtained with the aid of a balance, wherefore its volume must be assessed by other means. The most accurate method of measuring the medullary volume is to measure the area of the medulla in a complete series of sections (6, 7). The sum of the medullary areas in all sections multiplied by the section thickness then gives the volume. This method is fairly tedious, which fact may explain the small proportion of recent papers dealing with the volume of the adrenal medulla, in comparison to those giving data of the weight of the whole gland.

Some less cumbersome methods for estimating the medullary volume have been used. One of the most popular ones is to measure
the size of the medulla in a »mid-section« only (e.g. 1, 3). This method is certainly convenient enough but its accuracy is open to doubt. Donaldson (2) has described a technique, in which the adrenal is made transparent by a clearing procedure, after which the outlines of the cortex and medulla are projected and drawn in two planes perpendicular to each other (see also 5). The volumes are then calculated from the areas measured. It is doubtful whether the real corticomedullary junction can be accurately seen in a cleared preparation. Moreover, even if that would be the case, the convex outer surface of the cortex acts as a lens distorting the outlines of the medulla.

Rather (8) has presented an interesting technique for estimating the corticomedullary ratio. Rat adrenals fixed in Bouin’s fluid were cut into three or four discs, embedded in paraffin wax, and cut serially. The magnified images of the medulla and cortex were drawn on paper, and the respective areas were measured with a planimeter. The ratio cortical area/medullary area was determined on one slide, and the ratio plotted against the whole area. The procedure was then repeated with the next slide and the ratios were cumulated and plotted against the area. The lines connecting the cumulating ratios tend to become flat when a sample large enough has been taken, and the sampling is stopped when the cumulative ratio is regarded sufficiently stable. While the accuracy of this method may approach that of a complete measurement in serial sections, it seems open to doubt whether it will in practice be more convenient than the latter, which can be much simplified without noticeable loss of accuracy (see below).

In view of the considerable number of publications using the information obtained by measuring the »mid-sections« and the convenience of this method, it was desired to study the adequacy of this kind of estimate in rat adrenals. Results obtained by comparing statistically the volume estimates calculated from mid-section areas with actual volumes measured from a complete series of sections are described in this paper.

MATERIAL AND METHODS

Altogether 60 adrenals from equally many animals were examined. 30 adrenals belonging to the first series were fixed in 10
per cent formalin for 24 hours and cut with a freezing microtome at 40 μ. The sections were projected through a low-power microscope on paper, and the outlines of the medulla were drawn in every section. The areas were then measured with a planimeter and the sum of areas in each gland, expressed in arbitrary units, was then used as a measure of the volume of the medulla. After the measurements this volume was plotted against the square root of the cube of the area of the section measured to have the largest medullary area in each gland. The regression was then analysed statistically.

Another series of 30 adrenals were fixed in a mixture of 1 part of concentrated formalin and 9 parts of 3.5 per cent potassium dichromate for 1 day and chromated in 3.5 per cent potassium dichromate for 3 days. This kind of treatment gives a suitable consistency to the glands so that they can be cut with a freezing microtome with little error in the section thickness. The fixed glands were weighed and sectioned serially at 50 μ. The sections were mounted unstained in glycerol. The magnified image of each section was then projected through the microscope on a horizontal glass tablet ground from the upper surface. The images were clearly visible from above and allowed direct planimetric measurements without drawing. The areas of the whole sections and those of the medullary parts were then measured by circumscribing each section in turn, always starting and finishing on the same point. The reading of the measuring wheel was read first after all sections in each gland were circumscribed. To get the volume in terms of mm³ the sum of the measured areas (mm²) was divided by the square of the linear magnification used (21.8 X) and multiplied by the section thickness (0.05 mm).

As the method of selecting the largest section was in the first series based on actual measurement, which will not be performed when the volume really is estimated only from one section, the largest section was chosen subjectively in the latter series. To get an idea of the additional variability thus introduced, three largest sections of each gland were selected and their whole and medullary areas measured. The first section thus selected of each gland is in the following text called mid-section, whether from the middle of the gland or not.

In some glands, planimetric measurements were repeated once or twice. As the differences between the values obtained in measure-
ments from the same gland always kept well below 0.5 per cent of the mean value, the rest of the glands were only once measured.

Some difficulties were met in the orientation of the adrenals for cutting. Fig. 1 illustrates the form of the rat adrenal. The mid-sections have different areas in different planes, and failure to recognize this fact results in a large variation entirely unrelated to the size of the whole gland or of the medulla. In the present study, painstaking efforts were made to cut the adrenals always in the same direction, i.e. the lower surface of the gland, which is against the kidney in situ, horizontal on the tissue holder of the microtome.

The volume estimates from the mid-sections were obtained, supposing the gland and the medulla to be rotation ellipsoids, by using the following equation:

\[ V = \frac{4}{3\sqrt{\pi}} \sqrt[3]{a^2}, \]

where \( a \) is the measured area.

Regression equations and correlation coefficients were calculated supposing a linear dependence between the variates. The explanation capacities of the regression equations thus obtained were studied by the usual methods of the analysis of variance (4).
RESULTS

Fig. 2. — Regression of the medullary volume measured from serial sections ($V_{ss}$) on the volume estimate ($V_{ms}$) from the medullary area of the mid-section.

TABLE 1

REGRESSION OF MEDULLARY VOLUME ON VOLUME ESTIMATE FROM MID-SECTION IN SERIES I

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Sum of Squares</th>
<th>D.F.</th>
<th>Variance</th>
<th>$r^2$</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
<td>3 669.26</td>
<td>1</td>
<td>3 669.26</td>
<td>43.44</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Residual</td>
<td>2 364.95</td>
<td>28</td>
<td>84.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>6 034.21</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Correlation coefficient = +0.78.
Residual standard deviation, per cent of original s.d. = 64%

1. Size of Medulla. — Figure 2 illustrates the dependence of the volume of the medulla, measured from a complete series of sections ($V_{ss}$), on the volume estimate obtained from the mid-section ($V_{ms}$), both expressed in arbitrary units, using different units for $V_{ss}$ and for $V_{ms}$. The middle line shows the regression of the measured volume on that estimated from the middle section. The upper and lower lines, parallel with the regression line, indicate the fiducial limits at the 5 per cent level, i.e. their vertical distance from the regression line is obtained by multiplying the residual variation of $V_{ss}$, after fitting of the regression line, by the value of Student's $t$ corresponding to $P = 0.05$ at 28 degrees of freedom. The width
of the belt bordered by these lines gives an idea of the goodness of the fit. Table 1 shows the corresponding analysis of variance.

It may be seen that, at the mean value of the volume estimate, \( V_{ms} \), the real volume lies in 95 cases out of 100 between 20.1 and 57.5 units, while in 5 per cent of cases the true value will be farther away from the mean, 38.8 units. The fiducial limits are thus very large, a fact which can also be observed by comparing the magnitude of the residual standard deviation, 9.2, with the original standard deviation, 14.4. After fitting of the regression line most of the original variability in the volume remains unexplained, the regression explaining only some 35 per cent of the original standard deviation. Still, the regression is highly significant, the probability that the regression is due to mere chance being far less than 1 out of 1,000 \((P < 0.001)\). The correlation coefficient is \( +0.78 \) signifying a close interdependence of the variates.

Figure 3 and Table 2 illustrate similarly the regression in the other series, both the measured volume and the volume estimated from the mid-section being now expressed in mm\(^3\), which allows direct comparison of the numerical values. Though the data are in this series obtained entirely independently of the first series and with slightly different methods, the results are closely similar: the

![Graph](image)

Fig. 3. — Similar regression as that shown in Fig. 2, from another set of 30 adrenals. In both cases the variations from the regression line are large.
ESTIMATION OF THE VOLUMES OF THE CORTEX AND MEDULLA

TABLE 2
REGRESSION OF MEDULLARY VOLUME ON VOLUME ESTIMATE FROM MID-SECTION IN SERIES II

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Sum of Squares</th>
<th>D.F.</th>
<th>Variance</th>
<th>$\nu^2$</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Regression ..........</td>
<td>8.1012</td>
<td>1</td>
<td>8.1012</td>
<td>39.52</td>
<td>&lt;0.001</td>
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<tr>
<td>Residual ...........</td>
<td>5.7390</td>
<td>28</td>
<td>0.2050</td>
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<tr>
<td>Total ..............</td>
<td>13.8402</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Correlation coefficient = 0.77.
Residual standard deviation, per cent of original s.d. = 65%.

correlation coefficients, the variance ratios ($\nu^2$), the relative fiducial limits and residual variations hardly differ from each other in the two series.

Though the regressions are in both series highly significant and a relatively high positive correlation exists between the variates, the above analysis shows clearly that the value of the medullary volume obtained from serial sections can only very roughly be estimated from the corresponding area in the mid-section.

2. Size of Cortex and Whole Gland. — In the second series of glands the accuracy of the estimate of the cortical volume was also studied. The results are given in Figure 4 and Table 3. If the estimate

![Figure 4](image_url)

**Fig. 4.** — Regression of the cortical volume measured from serial sections ($V_{ss}$) on the volume estimate ($V_{ms}$) from the area of the mid-section.
of the medullary volume from a mid-section was poor, the corresponding estimate of the cortical volume is entirely useless: the regression is capable of explaining only some 4 per cent of the original variation in the volume of the cortex, the 95 per cent fiducial limits nearly enclosing the whole variation range, and the correlation between the measured volume and the estimated volume being not even statistically significant.

As can be expected in view of the just-mentioned finding, the volume of the whole gland can neither be estimated with high accuracy from the area of the midsection. The results given in Figure 5, and Table 4 show closely the same characteristics as those given for the cortex.

Fig. 5. — Regression of the adrenal volume measured from serial sections (\(V_{ss}\)) on the volume estimate (\(V_{ms}\)) obtained from the area of the mid-section.
ESTIMATION OF THE VOLUMES OF THE CORTEX AND MEDULLA

TABLE 4

ANALYSIS OF VARIANCE

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Sum of Squares</th>
<th>D.F.</th>
<th>Variance</th>
<th>$v^2$</th>
<th>P</th>
</tr>
</thead>
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<td>1</td>
<td>26.49</td>
<td>3.91</td>
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<td>Residual ...........</td>
<td>189.84</td>
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<td>Total ..............</td>
<td>216.33</td>
<td>29</td>
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</tr>
</tbody>
</table>

Correlation coefficient = 0.35.
Residual standard deviation, per cent of original s.d. = 95%.

It may seem peculiar that the same method gives worse results when applied to the whole gland or to the cortex than when applied to the medulla. It must therefore be pointed out that the size of the medulla varied in the present series more than that of the whole gland or that of the cortex. While the largest value of the medullary volume was over 3 times as large as the smallest value, the ratios largest value/smallest value for the whole gland and for the cortex were smaller than 2. This difference at least partly explains the observed differences in the explanatory capacities of the regressions. In any case, the variability is too large to allow practical use of the mid-section estimate but under exceptional circumstances.

3. Inaccuracy of Mid-Section Estimate. — A poor correlation between the volume estimated from a mid-section and the volume measured with the aid of serial sections does not tell whether the former or the latter deviate more from the true volume. Consultation of data given in Figures 6 and 7 and in Tables 5 and 6 shows that there is no doubt about that the adrenal weight and thus, as there is no reason to suppose essential differences in the specific gravities of the glands, the adrenal volume are inaccurately estimated by the mid-section area.

On the other hand, the regression of the adrenal weight on the volume measured from serial sections is not only highly significant but shows only moderate residual variation after fitting of the regression line. Therefore, the volume obtained from serial sections can with some accuracy be used as a measure of the weight. The total residual variation in the regression of the weight on the measured volume is composed of two main components, of that due to error in the volume measurement and that caused by error in the
Fig. 6. — Regression of the adrenal weight (W) on the estimate of adrenal volume (V_{ms}) obtained from the area of the mid-section.

Fig. 7. — Regression of the adrenal weight (W) on the adrenal volume (V_{ss}) measured from serial sections.

<table>
<thead>
<tr>
<th>TABLE 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>REGRESSION OF ADRENAL WEIGHT ON VOLUME ESTIMATE FROM MID-SECTION</td>
</tr>
<tr>
<td>ANALYSIS OF VARIANCE</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Sum of Squares</th>
<th>D.F.</th>
<th>Variance</th>
<th>v^2</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression ..........</td>
<td>178.22</td>
<td>1</td>
<td>178.22</td>
<td>8.29</td>
<td>&gt;0.001</td>
</tr>
<tr>
<td>Residual ............</td>
<td>602.28</td>
<td>28</td>
<td>21.51</td>
<td>.</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Total ...............</td>
<td>780.50</td>
<td>29</td>
<td>.</td>
<td>.</td>
<td>.</td>
</tr>
</tbody>
</table>

Correlation coefficient = 0.48.

Residual standard deviation, per cent of original s.d. = 90%.
TABLE 6
REGRESSION OF ADRENAL WEIGHT ON VOLUME MEASURED FROM SERIAL SECTIONS
ANALYSIS OF VARIANCE

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Sum of Squares</th>
<th>D.F.</th>
<th>Variance</th>
<th>( \nu^2 )</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
<td>624.49</td>
<td>1</td>
<td>624.49</td>
<td>112.12</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Residual</td>
<td>156.01</td>
<td>28</td>
<td>5.57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>780.50</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Correlation coefficient = 0.89
Residual standard deviation per cent of original s.d. = 45%

Weighing the gland. Consequently, the real error of the volume measurement is smaller than could be expected on the basis of the regression analysis. The accuracy of the measurement of the medullary volume, moreover, is very probably higher than that of the whole volume as some cortical sections were occasionally lost in the course of sectioning, which was in both ends rendered difficult, as the glands were not embedded. All sections from those parts of the gland containing the medulla were, on the other hand, safely included in the series. It may thus be concluded that the poor regression of the volume measured from serial sections on the mid-section area is essentially due to inadequacy of the latter criterion and not to inaccuracy of the volume measurement.

The low discrimination power of the mid-section may have been caused mainly by the irregularity in the form of the gland (see Figure 1) or by a failure to select a representative section. The results obtained in the first series of glands, in which the largest section was selected after actual measurements of the medullary areas, suggests that the former source of error is of major importance. This view is further confirmed by the analysis of variance of the medullary and whole-section areas in triplicates, performed to get an impression of the magnitude of the error component introduced by the subjective selection of the largest section. Tables 7 and 8 show that this error can explain only a small fraction of the variability. Therefore, variations in the form, whose influence could not be prevented by trying to cut the glands always in the same direction, must be regarded as responsible for the failure of the mid-section estimate in guessing the adrenal or medullary volumes.
TABLE 7
ADRENAL AREAS IN MID-SECTION TRIPlicATES

ANALYSIS OF VARIANCE

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Sum of Squares</th>
<th>D.F.</th>
<th>Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between adrenals</td>
<td>472 592</td>
<td>29</td>
<td>16 365.2</td>
</tr>
<tr>
<td>Within adrenals, between sections</td>
<td>24 382</td>
<td>60</td>
<td>406.4</td>
</tr>
<tr>
<td>Total</td>
<td>498 974</td>
<td>89</td>
<td></td>
</tr>
</tbody>
</table>

Between-sections contribution to the standard deviation in a simple set of measurements

\[
\sqrt{\frac{406.4 \times 100.0}{16 365.2 + 406.4}} = 16\%
\]

TABLE 8
MEDULLARY AREAS IN MID-SECTION TRIPlicATES

ANALYSIS OF VARIANCE

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Sum of Squares</th>
<th>D.F.</th>
<th>Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between adrenals</td>
<td>157 497</td>
<td>29</td>
<td>5 430.9</td>
</tr>
<tr>
<td>Within adrenals, between sections</td>
<td>6 135</td>
<td>60</td>
<td>102.3</td>
</tr>
<tr>
<td>Total</td>
<td>163 632</td>
<td>89</td>
<td></td>
</tr>
</tbody>
</table>

Between-sections contribution to the standard deviation in a simple set of measurements

\[
\sqrt{\frac{102.3 \times 100.0}{5 430.9 + 102.3}} = 14\%
\]

DISCUSSION

The above results show that the mid-section areas of the whole adrenal, the cortex and the medulla are poor indices of the corresponding volumes. The accuracy of the volume estimate obtained from the mid-section is so low that statistically significant differences in the mid-section areas can only be expected when real differences in the volume are too large to be met but under extreme experimental conditions.

It is clear that some investigators are more skilled than the others. Therefore, it may well be possible that regressions better than those presented in this paper can be obtained by workers well-trained in the orientation of the adrenals for cutting. Moreover, the shape of the adrenal gland, which varies much from species to species and may be more ellipsoidal in some rat strains than in
others, is also of decisive importance in this respect. The above conclusions therefore apply only to animals with the same irregularities in the form of the adrenal than the rats used in this study. As the adrenal medulla of the mouse is very nearly ellipsiodical, at least in some strains, it is not surprising that Bullough (1) has apparently succeeded estimating its size from the area of the largest section. On the other hand, it seems rather unlikely that the shape of the adrenal medulla of the rat will vary between different strains much enough to influence essentially the goodness of a volume estimate obtained from the mid-section area. This casts some doubt on the value of those earlier studies on the variations of the size of the rat adrenal medulla, in which the medullary volume was assessed from the mid-section.

In some circles it is believed that only paraffin sections can be used for accurate measurements of tissue components. This opinion may in some specific instances be right but it is certainly not generally applicable. It may be possible that the accuracy of the volume measurement had been somewhat increased in the present study by using paraffin instead of frozen sections. Nevertheless, no difficulties were encountered in the serial cutting at a constant speed of the fixed glands with the freezing microtome, and the percentage error in the thickness of the relatively thick sections may not be excessive in comparison to paraffin sections. A satisfactory regression of the adrenal weight on the volume measured from serial sections was indeed obtained, and errors in the section thickness could anyway not explain the distinct inferiority of the mid-section area as a weight indicator, in comparison to the complete series of sections.

Frozen sections, particularly thin sections from an incompletely fixed tissue, tend sometimes to enlarge in water after the cutting. The sections were in this study cut from properly fixed glands and they were relatively thick; accordingly, no variable spreading was seen after the cutting. Even if such a factor had been present it seems very unlikely that the degree of area change had varied much from one section to another, remembering that the differences in the triplicated mid-section measurements were relatively small.

It thus seems that the size of the adrenal medulla can be measured with sufficient accuracy only from serial sections, which inevitably means that one whole adrenal must be offered for this
purpose. The labour can nevertheless be reduced by using thick sections and by summing up the areas in each section mechanically with the aid of a planimeter, as described in this paper. When sections 50 μ thick are used, some 30—40 sections need to be circumscribed for measuring the medullary area in the rat, a labour not intractable. After the measurement, the same sections can be stained with various stains including those for the demonstration of lipids.

The volume measured from serial sections of fixed glands will of course not give the true volume of the gland during the life. Consultation of Figure 7 shows that a volume of ca. 17 mm³ corresponds to a weight of ca. 22 mg. This would mean that the specific gravity of the gland would be 1.3 gm/cm³, a value probably too high even considering the increase in the weight of the gland due to chromatin. This seems to indicate that the fixation and chromation have caused some shrinkage. It is nevertheless reasonable to assume that the values obtained, though relative, are sufficiently informative for most purposes, large variations in the magnitude of the shrinkage seeming unlikely. Caution is necessary, however, in short-term experiments, in which the adrenal medulla is heavily stimulated. Such stimulation may cause a definite increase in the water content of the medulla, thus altering the readiness to shrinkage in various medulla, thus altering the readiness to shrinkage in various fixation fluids.

SUMMARY

The usefulness of the mid-section area as an indicator of the size of the adrenal medulla was studied in 60 serially cut adrenals.

A significant correlation was found between the estimate of medullary volume calculated from the mid-section area and the volume measured from a complete series of sections. However, in spite of the significant regression the residual variation left after fitting the regression equation was very large, indicating that the medullary area in a mid-section is a poor indicator of the medullary volume. Similar studies on the whole adrenal gland and the cortex gave similar results. Comparison of adrenal weights with the estimated or measured volumes showed moreover that area measurements in a complete series of sections give a reasonable estimate of the weight.
ACKNOWLEDGEMENTS

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REFERENCES

(IV)

Every now and then claims have been presented that there are two or more different kinds of secretory cells in the adrenal medulla (3, 4, 6, 23, 26, 27). However, these reports have not conclusively altered the general opinion that differences in the stainability or in the histochemical properties of the adreno-medullary cells are due to differences in the secretory state of otherwise similar cells, a conclusion arrived at in recent careful studies by Bennett, (2) and Hillarp (21). In 1951, Bänder (1) described a staining technique by means of which he claimed to be able to differentiate between the adrenaline-containing and the noradrenaline-containing cells of the adrenal medulla. The present writer (unpublished) has not been able to confirm Bänder’s histological observations; the results of Bänder’s adrenaline and noradrenaline determinations also diverge from those obtained by most other workers.

As the above-mentioned writers used either complicated staining techniques difficult to reproduce or histochemical methods giving...
positive reactions with both adrenaline and noradrenaline, it may not be surprising that they failed to demonstrate crucially two qualitatively different types of cells. In 1951, the present writer (7, 8) observed that some histochemical reactions clearly discriminated between two different kinds of secretory cells in rat's adrenal medulla. Such differences were equally well demonstrable in resting adrenals, whose medullary cells all gave an intense chromaffin reaction and whose contents of adrenaline and noradrenaline were normal, and in stimulated adrenals, in which a loss of adrenaline was experimentally induced (7, 8, 9, 10, 16). One of the histochemical methods used, i.e. fluorescence microscopy of formalin-fixed sections, has been shown to demonstrate two kinds of medullary cells also in the adrenals of the cat and the hamster (13, 14). Furthermore, both direct and indirect evidence is available suggesting that adreno-medullary cells fluorescing after treatment with formalin contain much noradrenaline and little or no adrenaline and that the other — non-fluorescing — medullary cells contain predominantly adrenaline (13, 14, 15, 16). Recent independent observations by Hillarp and Hökfelt (22) with a histochemical technique which according to these investigators selectively demonstrates noradrenaline fit well to the present writer's results.

In the present study the histochemical fluorescence technique has been applied conjointly with adrenaline and noradrenaline determinations on adrenals of some common laboratory animals. The results are in agreement with the view that the fluorescing medullary cells are the main site of storage of adrenal noradrenaline.

A brief preliminary communication on this subject has been published in Nature (10). Since then, more material has been collected. Therefore, the data presented in the preliminary note and in this paper differ somewhat from each other.

**MATERIAL AND METHODS**

The series comprised 86 animals: 10 cats, 9 dogs, 10 guinea pigs, 16 hamsters, 20 mice, 6 rabbits and 15 rats. The hamsters, mice and rats were killed by cutting the neck with sharp scissors without any previous treatment. Other animals were bled through the neck after a blow on the head (guinea pigs and rabbits) or under a short anaesthesia (cats and dogs). Animals of both sexes
amongst all these species have been examined but in the respects concerned in this study no sexual differences were observed.

The adrenals were freshly frozen on the tissue holder of a freezing microtome. Sections were cut at 50 μ with a cooled knife, a part of the sections being fixed in formalin for histochemical study, another part being freeze-dried for microdissection (see 11 and 12). Sections studied for fluorescence and with the earlier described silver method (9) were fixed for 2—8 hours in formalin. Sections fixed only for ca. 3 min. and washed in three rinses of distilled water were used for the histochemical demonstration of acid phosphatase, using a technique described in an earlier paper (9). Some formalin-fixed or freeze-dried sections of the rat adrenal were also studied for acid phosphatase with azo-coupling techniques (29).

Determinations of adrenaline and noradrenaline concentrations were made after chromatographic separation, with the aid of a fluorimetric method (12). In mice, these determinations were done using whole freeze-dried sections. In all other species the medullary area of each section was first dissected out and weighed, so that the catechol amines could be expressed in terms of per cent of dry medullary weight. A part of the adrenal sections from cats and hamsters were used for studying the correlation between noradrenaline and islet contents within each section (13, 14). As small pieces of either islet-rich or islet-poor areas were selected in these studies, sometimes without analyzing the whole medullary part of the section, some of these data were not suitable for the present purpose, the analyzed pieces being not representative samples of the whole medulla. All data used in the present study were obtained from several sections of each gland, the whole medullary area of each section being used. Even so, the estimates of catechol concentrations and of islet content obtained from some mid-sections of each gland are probably not representative of the whole medulla, particularly as the distributions of both the fluorescing islets and the catechols are not always even in the adrenal medulla. This is fortunately not serious from the point of view of the present problem, as the catechols and the islet content were estimated from neighbouring sections of each gland.

The relative amount of fluorescing tissue was assessed subjectively. Such kind of estimation is of course subject to considerable errors, particularly as the variations in the distribution and in
the size of the fluorescing islets tend to distort the subjective estimates. With experience in planimetric measurements of islets of varying size it is nevertheless possible to obtain reproducible area estimates in spite of variations of the mentioned kind. Thus, it will be possible after some training to state, e.g., that the area covered by the islets is with certainty more than one tenth and less than a quarter of the whole area of the medulla, or that the true value is between one third and three quarters. The subjective method, saving the enormous amount of work which had been necessary if planimetric measurements had been done on fluorescence photomicrographs, was — in spite of it's low accuracy — good enough to supply the information required in the present study.

RESULTS

Acid Phosphatase. — In normal rats, the fluorescing medullary islets are acid phosphatase negative, the rest of the medulla being strongly positive (7—9). This finding was now confirmed also with the azo-coupling techniques, in both formalin-fixed and in freeze-dried sections. In the cat, the dog, the guinea pig, the mouse and the rabbit, no inhomogeneity was seen in the medullary reaction. Adrenals of the hamster were not examined with this method.

Ammoniacal Silver Nitrate. — The fluorescing islets of the rat have been seen to darken selectively when treated with ammoniacal silver nitrate (9). Efforts to reproduce these observations with other species did not succeed. Indeed, it proved difficult to obtain the same reaction even in the adrenals of the rat, although the method worked earlier without particular difficulties. Brands of silver nitrate and/or ammonium hydroxide different from those used in the earlier work may be responsible.

Fluorescent Islets. — Fluorescence photomicrographs of adrenals from some species have been published in earlier papers (rat: 9, 15, 16; cat: 14; hamster: 13). Figg. 1 and 2 illustrate dog adrenals. The fluorescing medullary cells can be differentiated from groups of fluorescing cortical cells sometimes present in the medulla: in sections not previously exposed to ultraviolet light the cortical cells fluoresce either bright yellow or brick red while the medullary islets show a bright green fluorescence; in sections examined for
Fig. 1. — Fluorescence photomicrograph of a formalin-fixed section from the adrenal gland of a normal dog. Fluorescing materials are seen both in the cortex and in the medulla. Length of the white scale mark 300 μ.

Fig. 2. — The same section photographed again. The cortical fluorescence has largely disappeared during the exposure of Fig. 1.
Fig. 3. — Fluorescence photomicrograph of a formalin-fixed section from the adrenal gland of a normal mouse. The cortico-medullary junction is clearly outlined by strongly fluorescing lipid-rich cells. Medullary islets are clearly visible. Empty medullary blood vessels are dark.

Fig. 4. — Similarly taken picture of the adrenal of a rabbit. The medulla, free of fluorescing materials except for some lipid-rich cells, is in the centre. The length of the scale mark in both pictures 300 μ.
some time, the ultraviolet-sensitive yellow cortical fluorescence is no more visible but the green fluorescence of the medullary islets is unaltered. Medullary islets of the mouse are illustrated in Fig. 3. A section from the adrenal of a rabbit is shown in Fig. 4.

The fluorescing islets in the species examined are briefly characterized in Table 1. The estimation of the proportion of the medulla

<table>
<thead>
<tr>
<th>Species (Number of Animals)</th>
<th>Size</th>
<th>Distribution of Islets</th>
<th>Proportion of Islets of Whole Medulla (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat (10)</td>
<td>small to large</td>
<td>uneven</td>
<td>1/5–3/4</td>
</tr>
<tr>
<td>Hamster (16)</td>
<td>medium</td>
<td>in the periphery</td>
<td>1/4–1/2</td>
</tr>
<tr>
<td>Mouse (20)</td>
<td>medium</td>
<td>even</td>
<td>1/5–1/2</td>
</tr>
<tr>
<td>Dog (9)</td>
<td>small to large</td>
<td>uneven</td>
<td>1/4–1/2</td>
</tr>
<tr>
<td>Rat (15)</td>
<td>small (small)</td>
<td>even</td>
<td>1/10–1/4</td>
</tr>
<tr>
<td>Guinea pig (10)</td>
<td></td>
<td>?</td>
<td>0–1/20 (?)</td>
</tr>
<tr>
<td>Rabbit (6)</td>
<td></td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

covered by fluorescing islets was particularly difficult in the cat and in the dog because of the uneven distribution and the big variations in the size of the islets. In the guinea pig, no clear-cut islets were observed in the medulla but there were small areas fluorescing somewhat more strongly than the rest of the medulla. As the fluorescence of these areas was definitely weaker than that in the fluorescing islets of other species, it is questionable whether they are identical with the latter. In the rabbits examined, no fluorescing islets or anything resembling them were seen in the adrenal medulla, disregarding islets of cortical tissue.

Adrenaline and Noradrenaline. — Table 2 shows the concentration of both catechols as per cent of dry medullary weight and the proportion of noradrenaline as per cent of the total amount of both catechols. The over-all concentration of catechols is c. 5 per cent in the medulla of all species studied but there are marked species differences in the relative amount of noradrenaline. The figures of relative noradrenaline content in the medulla of the guinea pig and the rabbit are based on determinations on very few animals. As they do not deviate essentially from the figures re-
Fluorescing islets, adrenaline, and noradrenaline

Table 2
Medullary Catechol Amines

<table>
<thead>
<tr>
<th>Species (Number of Animals)</th>
<th>Adrenaline + Noradrenaline (% of Dry Weight of Medulla)</th>
<th>Noradrenaline (% of Both Catechols)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>Cat (9)</td>
<td>5.7</td>
<td>0.9</td>
</tr>
<tr>
<td>Hamster (11)</td>
<td>4.2</td>
<td>0.7</td>
</tr>
<tr>
<td>Mouse (9)</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>Dog (9)</td>
<td>5.0</td>
<td>0.9</td>
</tr>
<tr>
<td>Rat (15)</td>
<td>4.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Guinea pig (5)</td>
<td>3.6</td>
<td>0.8</td>
</tr>
<tr>
<td>Rabbit (2)</td>
<td>?</td>
<td></td>
</tr>
</tbody>
</table>

1 Cortical tissue present in the samples analyzed.

ported by earlier investigators (17, 19, 20, 30—33), and as sprayed chromatograms repeatedly proved the presence of small amounts of noradrenaline in the medulla of these species, the quantitative data may be sufficiently reliable for the present purpose. The corresponding figures for the other species are also in fair agreement with figures reported earlier (5, 9, 15—20, 22, 24, 25, 28, 30—34).

Noradrenaline and Fluorescent Islets. — The percentage of fluorescing medullary tissue is plotted in Fig. 5 against the percentage of noradrenaline. It must be emphasized that the limits of islet percentage (already given in Table 1) are wide enough to enclose with certainty the actual range in animals examined. The horizontal length of each rectangle, on the other hand, has been obtained by multiplying the standard deviation by the value of Student's \( t \) at the level \( P = 0.05 \) corresponding to the number of degrees of freedom in each group; thus, the true mean noradrenaline percentage is enclosed by each rectangle with 95 per cent probability.

In spite of the fairly large variations in both dimensions, examination of the diagram suggests a fairly good correlation between the noradrenaline content and the islet content. The significance of this correlation was tested by two methods. First, the correlation between the average noradrenaline percentage in each group and the mid-value of the group limits set for the islet content was cal-
Fig. 5. — Correlation of noradrenaline content (% of both medullary catechol amines) and islet content (% of whole medullary size) in different species. For significance of the rectangles, see text. The numbers in the brackets refer to the number of animals in which catechol determinations were made.

pulated. Thus, a correlation coefficient of 0.99 was obtained, the probability that such a high correlation is due to mere chance being far less than 0.000 01.

This way of calculating the correlation is, however, subject to criticism because the mean islet content need not be exactly in the middle of the extreme limits, assessed collectively for each group. Therefore, the interdependence of noradrenaline and islet percentages was tested by the rank correlation method. The animals were first arranged into the order of decreasing noradrenaline percentage according to the means of each species. Then the animals were arranged according to the islet content, and — to be on the safe side — this was done so that the order of the hamster, the mouse and the dog was set opposite to that according to the noradrenaline percentage, a precaution rendered desirable by the fact that the noradrenaline and islet contents of these species showed considerable overlapping. Further, the order of the guinea pig and the rabbit was reversed for the same reason. The following data were thus obtained.

According to noradrenaline percentage:
1) cat, 2) hamster, 3) mouse, 4) dog, 5) rat, 6) guinea pig, 7) rabbit.
According to islet percentage:
1) cat, 2) dog, 3) mouse, 4) hamster, 5) rat, 6) rabbit, 7) guinea pig.

Comparison of the ranks:

<table>
<thead>
<tr>
<th>Rank</th>
<th>Cat</th>
<th>Dog</th>
<th>Mouse</th>
<th>Hamster</th>
<th>Rat</th>
<th>Rabbit</th>
<th>Guinea Pig</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

Difference

| Difference | 0 | -2 | 0 | 2 | 0 | -1 | 1 |

Sum of squares of differences = 4 + 4 + 1 + 1 = 10

Rank correlation \( r_s = 1 - \frac{6 \times 10}{7(49 - 1)} = 0.821 \)

This correlation, although it is calculated according to the most pessimistic alternative, is significant at the 5 per cent level.

**DISCUSSION**

The observations described support the hypothesis that the fluorescing islets are composed of adreno-medullary cells containing predominantly noradrenaline. A positive correlation between the relative islet content and the relative noradrenaline content does, of course, not provide crucial evidence. However, there are hardly reasons to believe that species such as the dog and the mouse would in this respect differ essentially from the cat, the hamster and the rat, in whose adrenals the predominant localization of noradrenaline in the islet cells has been more directly proved (13—16, see also 22). Therefore it may be justified to regard the hypothesis correct until observations speaking against it have been presented.

The non-fluorescing medullary cells of the cat and the hamster have been seen to contain mostly the methylated variety of the adreno-medullary catechol amine, i.e. adrenaline, but besides it also small amounts of noradrenaline (13, 14). In the present study, the relative noradrenaline content in the adrenal medulla of the guinea pig was observed to be ca. 5 per cent although the presence of fluorescing islets was doubtful. Noradrenaline was clearly demonstrated also in the adrenal medulla of the rabbit, in which no islets were seen. These findings fit well together and show that noradrenaline is present in cells apparently specialized in production of adrenaline, the former substance being a prae-cursor of the latter.
On the other hand, it is as yet not clear whether the fluorescing cells contain exclusively noradrenaline of the two medullary catechol amines or also some adrenaline. This question can be answered with certainly first when pieces of the adrenal medulla have been analyzed containing only the fluorescing variety of the medullary cells. For such a study, foetal adrenals may offer better possibilities (cf. 31, 32). The regression demonstrated in the present study between the noradrenaline percentage and the islet percentage was apparently linear, and the regression coefficient was seemingly near 1, which would suggest that the islet cells contain but little adrenaline. Owing to wide variations within the species the evidence is however insufficient in this respect. That the adrenaline content of the islet cells is at least very small in comparison to the noradrenaline content in the same cells, has been more directly demonstrated in pieces of cat’s adrenal medulla with some 70 per cent of both fluorescing tissue and noradrenaline (14).

In spite of the fact that the fluorescing cells apparently contain some noradrenaline and the other medullary cells possibly some adrenaline, the evidence now available leaves little doubt that two distinctly different types of endocrine cells are present in the adrenal medulla of those mammals secreting noradrenaline besides adrenaline, an idea already put forward in the papers in which the medullary islets were first described (7–9). It is interesting that Hillarp and Hökfelt (22) have through other ways arrived at fairly similar conclusions. It would be of importance to compare their histochemical reaction for the demonstration of noradrenaline with the fluorescence methods used by the present writer.

**SUMMARY**

Cell groups fluorescing brilliantly in ultraviolet light after fixation in formalin have been demonstrated in the adrenal medulla of the cat, the hamster, the mouse, the dog and the rat. The presence of such fluorescing islets was questionable in the adrenal medulla of the guinea pig, and no islets were detected in the adrenal medulla of the rabbit. A statistically significant between-species correlation was observed between the percentage of noradrenaline of both medullary catechol amines and the percentage of fluorescing tissue of the whole medulla. The data, together with earlier
observations, are taken to indicate that the fluorescing medullary cells contain much noradrenaline and little or no adrenaline and that the non-fluorescing cells contain much adrenaline and little noradrenaline.

REFERENCES

Figure 1. Fluorescence photomicrograph of mouse adrenal. Fluorescent islets in the medulla. Lipid-containing, strongly fluorescent macrophages mark the cortico-medullary junction. x 75.
Figure 2. Mouse adrenal shown in Figure 1 at a higher magnification. x 120.

Figure 3. Mouse adrenal. Cortico-medullary junction with fluorescent macrophages in the upper part of the photograph. x 140.
Figure 4. Fluorescence photomicrograph of rat adrenal. Cortical fluorescence, although weak after exposure to ultraviolet light, is clearly visible, x 22.

Figure 5. Fluorescence photomicrograph of dog adrenal medulla. Note the small groups of fluorescent medullary cells, x 200.
Figure 6. Fluorescence photomicrograph of dog adrenal medulla. Taken immediately after cutting. x 115.

Figure 7. Same section rephotographed. Exposure to ultraviolet light has caused fading of the cortical fluorescence without any essential effect on the medullary fluorescence. x 115.
Figure 8. Fluorescence photomicrograph of rabbit adrenal. The cortical fluorescence is very strong. No clearly visible fluorescent islets in the medulla. x 25.

Figure 9. Same section at a higher magnification. Although the medullary fluorescence is not quite even, no clear cell islets can be seen. Lipid-containing macrophages fluoresce strongly. x 65.
### TABLE 2.
Cat adrenal medulla.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Adr. + Nor. M (% of dry med.wt.)</th>
<th>Noradrenaline (% of adr.+nor.)</th>
<th>Fluorescent tissue (% of med. tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>- x)</td>
<td>25</td>
<td>20 - 50</td>
</tr>
<tr>
<td>2</td>
<td>- x)</td>
<td>37</td>
<td>25 - 75</td>
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<td>3</td>
<td>- x)</td>
<td>21</td>
<td>20 - 75</td>
</tr>
<tr>
<td>4</td>
<td>5.6</td>
<td>60</td>
<td>25 - 75</td>
</tr>
<tr>
<td>5</td>
<td>6.5</td>
<td>47</td>
<td>25 - 75</td>
</tr>
<tr>
<td>6</td>
<td>4.2</td>
<td>38</td>
<td>25 - 75</td>
</tr>
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<td>7</td>
<td>6.9</td>
<td>46</td>
<td>20 - 75</td>
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<td>25 - 75</td>
</tr>
<tr>
<td>9</td>
<td>5.6</td>
<td>46</td>
<td>25 - 75</td>
</tr>
</tbody>
</table>

M = 5.7 SD = 0.9  M = 40.1 SD = 11.8  25 - 75

* x) sample not weighed.

### TABLE 3.
Hamster adrenal medulla.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Adr. + Nor. M (% of dry med.wt.)</th>
<th>Noradrenaline (% of adr.+nor.)</th>
<th>Fluorescent tissue (% of med. tissue)</th>
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</thead>
<tbody>
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</tr>
<tr>
<td>11</td>
<td>4.9</td>
<td>38</td>
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M = 4.2 SD = 0.7  M = 36.5 SD = 4.8  25 - 50

* x) sample not weighed
### TABLE 4.
Mouse adrenal medulla.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Adr. + Nor.</th>
<th>Noradrenaline</th>
<th>Fluorescent tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>N°</td>
<td>(% of dry med. wt.)</td>
<td>(% of adr.+nor.)</td>
<td>(% of med. tissue)</td>
</tr>
<tr>
<td>1</td>
<td>- x)</td>
<td>38</td>
<td>25 - 50</td>
</tr>
<tr>
<td>2</td>
<td>- x)</td>
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<td>25 - 50</td>
</tr>
<tr>
<td>3</td>
<td>- x)</td>
<td>34</td>
<td>20 - 50</td>
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<td>4</td>
<td>- x)</td>
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<td>20 - 50</td>
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<td>- x)</td>
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<td>18</td>
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<tr>
<td>9</td>
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<td>20 - 50</td>
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</table>

-x) Cortical tissue present in the samples.

### TABLE 5.
Dog adrenal medulla.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Adr. + Nor.</th>
<th>Noradrenaline</th>
<th>Fluorescent tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>N°</td>
<td>(% of dry med. wt.)</td>
<td>(% of adr.+nor.)</td>
<td>(% of med. tissue)</td>
</tr>
<tr>
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<td>6.9</td>
<td>34</td>
<td>25 - 50</td>
</tr>
<tr>
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</tr>
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</tr>
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M=5.0  SD = 0.9  M=29.1 SD=4.2  25 - 50

x) Sample not weighed.
### Table 6.

**Rat adrenal medulla.**

<table>
<thead>
<tr>
<th>Animal</th>
<th>Adr. + Nor.</th>
<th>Noradrenaline</th>
<th>Fluorescent tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(% of dry med. wt)</td>
<td>(% of adr. + nor.)</td>
<td>(% of med. tissue)</td>
</tr>
<tr>
<td>1</td>
<td>5.5</td>
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<td>10 - 25</td>
</tr>
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<td>3.6</td>
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</tr>
<tr>
<td>15</td>
<td>6.4</td>
<td>12</td>
<td>10 - 20</td>
</tr>
</tbody>
</table>

\[ \text{M}=4.9 \quad \text{SD}=0.9 \quad \text{M}=16.0 \quad \text{SD}=2.8 \quad \text{10} - 25 \]
### TABLE 7.
Guinea pig adrenal medulla.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Adr. + Nor.</th>
<th>Noradrenaline</th>
<th>Fluorescent tissue</th>
</tr>
</thead>
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<td></td>
<td>M (D of dry med. wt.)</td>
<td>(D of adr.+nor.)</td>
<td>(D of med. tissue)</td>
</tr>
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<td>1</td>
<td>- x)</td>
<td>5</td>
<td>?</td>
</tr>
<tr>
<td>2</td>
<td>4.3</td>
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<td>3</td>
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</tr>
<tr>
<td>4</td>
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<td>4</td>
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<td>7</td>
<td>?</td>
</tr>
</tbody>
</table>

M=3.6 SD=0.8 M=5.2 SD=1.1

x) Cortical tissue present in the sample

### TABLE 8.
Rabbit adrenal medulla.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Adr. + Nor.</th>
<th>Noradrenaline</th>
<th>Fluorescent tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M (D of dry med. wt.)</td>
<td>(D of adr.+nor.)</td>
<td>(D of med. tissue)</td>
</tr>
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<td>- x)</td>
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</tr>
<tr>
<td>2</td>
<td>- x)</td>
<td>2</td>
<td>0</td>
</tr>
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</table>

x) Cortical tissue present in the samples
DISTRIBUTION OF FLUORESCING ISLETS, ADRENALINE AND NORADRENALINE IN THE ADRENAL MEDULLA OF THE CAT

By

Olavi Eränkö

In previous reports (Eränkö, 1951 a, b, 1952) it was shown that cell islets can be demonstrated in the adrenal medulla of the rat which differ histochemically from the other medullary cells. These islet cells are acid phosphatase negative and exhibit a bright autofluorescence after fixation in formalin, while in contrast to this, the other medullary cells are strongly acid phosphatase positive and fluoresce feebly after treatment with formalin. Both types of cells are morphologically similar and give a positive chromaffin reaction.

The present study was undertaken to find out whether a similar dualism can be seen in the adrenal medulla of the cat. As this was found to be the case, the distribution of the fluorescing islets was compared with those of adrenaline and noradrenaline. The results described below suggest that noradrenaline is mainly located in the cells of the fluorescing islets and that adrenaline is to be found mainly in the non-fluorescing cells.

MATERIAL AND METHODS

Healthy cats of both sexes were used. They were killed by cutting the throat under nembutal anaesthesia. The adrenals were removed and frozen immediately after death.

1. An essential part of this study was done in the Department of Pharmacology, University of Edinburgh, where the author spent the Academic year 1952–1953 as a British Council Scholar. The author is grateful to Professor J. H. Gaddum and to Dr. Marthe Vogt for laboratory facilities and valuable guidance. Technical assistance given by Mrs. Liisa Räisänen and Mr. E. Siltanen is gratefully acknowledged. This study has been supported financially by grants from the Valtion Luonnontieteellinen Toinikunta and the Sigrid Jusélius Foundation.
Serial sections were cut from the fresh-frozen glands with a freezing microtome. Every other section was fixed for 2–5 hrs. in a mixture containing 1 part of commercial formalin, 4 parts of distilled water and 5 parts of 2 per cent calcium chloride. The remaining sections were freeze-dried at ca. –40° C. (Eränkö, 1954 a).

The sections fixed in the formalin mixture were quickly washed in distilled water and mounted in glycerol. The slides thus prepared were then examined in ultraviolet light for fluorescence.

The distribution of acid phosphatase was studied in six cats. Fresh-frozen sections quickly fixed in formalin were used for this purpose. Details of the histochemical method employed, which is a modification of Gomori's (1952) technique, have been described in a previous report (Eränkö & Lehto, 1954).

Pieces of the medulla were dissected under a dissecting microscope and weighed. The catechol amines were then separated chromatographically and determined quantitatively. Details of the technique have been described elsewhere (Eränkö, 1954 b).

RESULTS

1. Fluorescence microscopy

Both the cortex and the medulla showed a bright fluorescence. However, there was a fundamental difference in the fluorescences of these two adrenal zones in that the cortical fluorescence was unstable when exposed to ultraviolet light. After the section had been examined for a few minutes, the fluorescence of the cortex was weaker than that of the medulla. This suggests that most of the cortical fluorescence was due to the presence of vitamin A (see Popper, 1944), while the medullary fluorescence, which was not altered by even long exposures to ultraviolet light, was caused by some other substance.

Closer study revealed that the fluorescing cells were not uniformly distributed in the medulla but, in agreement with previous observations on the rat adrenal, concentrated to distinctly defined groups of medullary cells, the remaining cells showing a relatively weak fluorescence (Figs. 1–5). The presence of fluorescing medullary islets was a constant feature in all cat adrenals examined independently of the sex or age of the animal. In the following description these groups of fluorescing cells are referred to simply as `islets'. Occasionally, groups of cortical cells were seen in the medulla. Such cell groups were nevertheless easily distinguishable from the medullary islets, the former showing a yellow or reddish fluorescence while the latter fluoresced constantly bright green.

As can be seen from Figs. 1–3, a relatively high proportion – roughly one half – of the medulla is composed of fluorescing islets. The relative area of the islet tissue varied somewhat from one section to another and from animal to animal. In the whole material, the proportion of the islet tissue ranged from 20 to 60 per cent of the total area of the medulla. Although the shape and the size of the islets varied they were usually fairly uniformly scattered over the section. In a complete series of sections, some were nevertheless occasionally
found in which a relatively large area of the medulla was free of islets (Fig. 4).

Some experiments were carried out to study the nature of the fluorescing substance by examining freshly-frozen sections for fluorescence after various treatments. The islets were not visible in fresh or freeze-dried sections. The shortest period of treatment with calcium chloride-formalin capable of bringing up the islet fluorescence was 30 min. The intensity of the islet fluorescence increased with prolonged treatment in formalin up to ca. 4 hours, after which the fluorescence was altered very little for some hours. If the sections were left in formalin for longer than 24 hours, the intensity of the background fluorescence increased and the islets became difficult or impossible to detect.

If treatment in formalin was omitted or if the sections were allowed to remain in water for some minutes before immersion in formalin, no islets were seen. Previous treatment with alcohol likewise abolished the islet fluorescence. Short periods in pure acetone, ether or benzene before formalin treatment did not abolish the fluorescence of the islets.

2. Acid phosphatase and silver techniques

In contrast to the rat adrenal medulla (Eränkö, 1951 a, 1952) sections of the cat adrenal treated according to the histochemical procedure for acid phosphatase failed to differentiate between the two kinds of medullary cells. Moreover, sections immersed in ammoniacal silver nitrate after formalin fixation (see Eränkö, 1952) did not give consistent results. The silver technique also proved unreliable with rat adrenals.

3. Islets and the distributions of adrenaline and noradrenaline

Series of sections were prepared as described previously in this paper. The set of formalin-fixed sections was then examined for the distribution of medullary islets. In some early experiments attempts were made to measure planimetrically the islet content in every other (formalin-fixed) section and to determine the catechols in every other (freeze-dried) section. However, these efforts remained fruitless, as the islet content in each freeze-dried section can only be approximated by studying the fluorescence in the two neighbouring sections, and as the overall technical error was usually larger than the differences in the islet and catechol content of whole sections in the same adrenal.

In subsequent studies determinations of adrenaline and noradrenaline content were done only in freeze-dried sections in which observations on the neighbouring formalin-fixed sections suggested a definitely uneven distribution of islet tissue. The point is illustrated in Fig. 6. This shows tracings of 5 consecutive adrenal sections, of which sections A, C and E have been fixed in formalin and sections B and D freeze-dried. It is evident that the islets are distributed in sections A and C roughly in the same way, i.e. there is an islet-free area in the lower left part of the section and much islet tissue everywhere else.
Figs. 1-3.
Fluorescence photomicrographs of formalin-fixed sections of cat’s adrenal medulla. Figs. 1 and 2 are taken at the same magnification. Fig. 1 is constructed from 6 photographs of the same section. The fluorescing islets are clearly visible. Fig. 3 is a detail of the section shown in Fig. 2.

Figs. 4 and 5.
Fluorescence photomicrographs taken at a higher magnification. The cortico-medullary junction is indicated with a black line. A large area practically free of fluorescing cell groups is seen in Fig. 4.

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Further, the islet content is somewhat smaller in the extreme right end of the sections, as compared to the rest of the islet-containing parts. Evidently there are good reasons for believing that the islet tissue will be distributed roughly in the same way too in the freeze-dried section B. If the working hypothesis that noradrenaline is present only in the islet cells is correct, much noradrenaline ought to be found in part 1 of section B, somewhat less in part 2 and none in part 3. However, although no islets are seen in the areas of sections A and C corresponding to area 3 in section B, it is possible that some small islets may be located in the last-mentioned area. At any rate, if the hypothesis is at all correct, the relative amounts of noradrenaline should be highest in area 1 and smallest in area 3. Similarly, section D can be divided into two pieces, of which piece 1 can be expected to contain more noradrenaline than piece 2.

Sections from altogether 10 animals were thus examined, the experiments being numbered accordingly from 1 to 10. In some of these 10 experiments, 2 or 3 sections were dissected into 2 or 3 pieces, which were then analyzed. These tests with 2 or more sections from the same animal are denoted by a, b and c in the tables.

Table 1 shows that, in every analyzed section, the relative amount of noradrenaline was highest in the piece containing most islet tissue and lowest in the piece containing least islet tissue. The statistical significance of this and the other results was assessed with the aid of the binomial distribution. As the sections taken from the same animal cannot be regarded as independent of each other, the pooled result in each animal was denoted with (+) if the
Table 1.
Percentage of noradrenaline in pieces of adrenal medulla with varying islet content.

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Noradrenaline (% of both catechols) in piece with more islets</th>
<th>piece with less islets</th>
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</thead>
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<td>43</td>
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<tr>
<td>3a</td>
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<tr>
<td>3b</td>
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<td>4b</td>
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<td>10b</td>
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</table>

<table>
<thead>
<tr>
<th>Most islets</th>
<th>Less islets</th>
<th>Least islets</th>
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</tr>
<tr>
<td>47</td>
<td>35</td>
<td>12</td>
</tr>
</tbody>
</table>

¹) The piece with least islets in this experiment was between two sections in which the corresponding areas were virtually free of islets.

The finding was in agreement with the working hypothesis or with (—) if it was contrary to the hypothesis. The relative noradrenaline content was in 10 out of 10 independent experiments higher in the islet-rich piece, i.e. the finding was in favour of the working hypothesis. The probability (P) that such result or the exact opposite is obtained by mere chance is $2 \times (1/2)^{10}$ or ca. 0.002, which indicates that the observed difference is statistically highly significant.

Weights of the pieces were available for 6 independent experiments only. Table 2 reveals that the amount of noradrenaline expressed as per cent of dry weight of the piece was higher in the piece with more islets in 6 out of the 6 animals. The statistical significance of such an event attains the conventional 5 per cent level ($P \sim 0.03$), even when the possibility is considered that the opposite result is equally significant as the one observed.

Owing to the limited number of independent observations nothing can be said about the relations of islet tissue and the contents of adrenaline or of both
catechols, as expressed per unit of dry weight, the values of P being ca. 0.7 and 1.0, respectively. The lack of significant differences in the amounts of both catechols is indeed according to expectation. The failure in 2 out of 6 experiments to register a higher concentration of adrenaline in the piece containing few islets may well be ascribed to errors in weighing and in adrenaline estimation, particularly as the difference between the adrenaline concentrations of islet-rich and islet-poor areas is smaller than the corresponding difference in the noradrenaline concentration, even if the islets contained only noradrenaline and the non-fluorescing cells only adrenaline. Consider, e.g., a section with one half in which one quarter of the cells is of the fluorescing type; the relative amount of fluorescing tissue in the remaining half is, say, one half of the whole volume of this piece. Supposing the concentration of total catechols is constant, say 6 per cent of dry weight, then the concentrations of noradrenaline in the two halves are: 1.5 per cent in the islet-poor half and 3.0 per cent in the islet-rich half. The corresponding concentrations of adrenaline are then 4.5 and 3.0 per cent respectively. Thus, because relatively much non-fluorescing tissue was

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Adrenaline (% of dry weight)</th>
<th>Noradrenaline (% of dry weight)</th>
<th>Both catechols (% of dry weight)</th>
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<tbody>
<tr>
<td></td>
<td>More islets</td>
<td>Less islets</td>
<td>More islets</td>
</tr>
<tr>
<td>6a²)</td>
<td>3.3</td>
<td>2.9</td>
<td>2.7</td>
</tr>
<tr>
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<td></td>
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<td>Less</td>
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<td>9a²)</td>
<td>2.5</td>
<td>3.3</td>
<td>4.4</td>
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</tbody>
</table>

1) The figures for both catechols are not always exactly equal to the sum of the figures given for adrenaline and noradrenaline. These differences are due to rounding off.
2) The piece with least islets in this experiment was between two sections in which the corresponding areas were virtually free of islets.
present even in the islet-rich half – as was actually always the case in the present study –, the percentage difference in the noradrenaline concentrations – in the hypothetical example \( 100 \times (3.0 - 1.5)/1.5 = 100 \) per cent – is higher than the percentage difference in the adrenaline concentrations – in the example \( 100 \times (4.5 - 3.0)/3.0 = 50 \) per cent. Higher accuracy is therefore necessary to detect significant differences in the adrenaline concentration under conditions identical with those in the present study.

In four sections (Experiments 6a, b and 9a, b) pieces were analyzed in which, as judged from the neighbouring sections, very little or no fluorescing tissue was present. All these pieces contained 8 to 17 per cent noradrenaline of the total amount of both catechols or 0.3 to 0.6 per cent of dry weight of medullary tissue. This finding suggests that the feebly-fluorescing cells contain not only adrenaline but also a small proportion of noradrenaline.

The relative amount of noradrenaline was in several pieces (Nos. 1, 3a, b, 5 and 8b) about 70 per cent of the total content of both catechols. Although no accurate area measurements were carried out, it is quite certain that the relative area covered by fluorescing islets in any piece analyzed was not much higher than 70 per cent and almost certainly less than 80 per cent. It seems therefore that the fluorescing islets contain very little, if any adrenaline.

**DISCUSSION**

The main result of this study, i.e., that the fluorescing islets of the adrenal medulla of the cat contain much noradrenaline and little or no adrenaline and that the remaining medullary cells contain mostly adrenaline but also some noradrenaline, is in agreement with the observations obtained with a similar technique on the adrenal medulla of the hamster (Eränkö, 1955). Independent studies of the cat adrenal by Hillarp & Hökfelt (1953, 1954), who used a buffered solution of potassium iodate for the histochemical demonstration of noradrenaline, also led to the conclusion that »noradrenaline is apparently selectively stored only in certain specific cells of the adrenal medulla«. It seems that the same cells are demonstrable by fluorescence microscopy after fixation in formalin and by the iodate method, but this point requires further study.

The disappearance of the fluorescing islets after pre-treatment with water or with alcohol, which both dissolve noradrenaline, and the absence of a similar effect after treatment with pure acetone, benzene or ether are compatible with the view that noradrenaline is responsible for the fluorescence of the medullary islets. As the islets become visible only if the sections are treated with formalin, this substance must play an important role in the formation of the fluorescence. Formalin is well known as an agent promoting polymerization of both aromatic amines and phenolic compounds. Preliminary observations have indeed
shown that noradrenaline forms when mixed with a solution containing calcium chloride and formalin a poorly water-soluble fluorescing compound (Eränkö, unpublished). Though it is attractive to think that formalin fixation is one kind of histochemical method for differential demonstration of noradrenaline in the presence of adrenaline – an idea supported now by indirect evidence – it is well to bear in mind that the fluorescence in the islet cells may be due to some properties in these cells other than a high concentration of noradrenaline. That care is necessary is shown by the observation that the histochemical reaction for acid phosphatase, which sharply differentiates between the two types of cells in the adrenal medulla of the rat (Eränkö, 1951 a, b, 1952), fails to discriminate between these cells in the adrenal medulla of the cat.

What is the physiological significance of the localization of the two catechol amines in two different kinds of cells of the adrenal medulla? Theoretically, several alternatives present themselves. At present, the most likely explanation seems to be that adrenaline and noradrenaline are medullary hormones either of which can be secreted independently to meet the specific requirements of the organism. That the adrenaline content of the adrenal medulla can be experimentally decreased by insulin without significantly affecting the noradrenaline content has been firmly established by several investigators (Burn, Hutchison & Parker, 1950; Hökfelt, 1951; Outshoorn, 1951; Eränkö, 1952). Recent studies by Hillarp & Hökfelt (1954) and by the present author (1954 d) have further shown that the chromaffin reaction of the islet cells is in such adrenaline-depleted adrenals still positive in the medullary islets of both the cat and the rat. On the contrary, nicotine injection induces a selective loss of noradrenaline and a selective disappearance of the chromaffin reaction and of the fluorescence from the medullary islets of the rat (Eränkö, unpublished). These observations, along with the finding that stimulation of restricted areas in the hypothalamus may selectively increase either the adrenaline or the noradrenaline content of blood taken from the adrenal vein (Brücke, Kaindl & Mayer, 1952; Redgate & Gelhorn, 1953), strongly suggest that the medullary cells secreting adrenaline and those secreting noradrenaline are, in spite of their morphological similarity and close topographical relationship, fairly autonomous in function. Whether the cells may change their function when desirable, e.g., whether an islet cell can start storing and secreting adrenaline, is an interesting problem of which practically nothing is known at present.

**SUMMARY**

Freshly-frozen sections of the adrenal medulla of the cat show, after formalin fixation, groups of fluorescing medullary cells against a poorly fluorescing background of morphologically similar cells. Distribution of these fluorescing
islets was compared with distributions of adrenaline and noradrenaline by analyzing islet-rich and islet-poor areas of freeze-dried sections for the contents of these catechols. It was found that the fluorescent medullary cells contain much noradrenaline and little or no adrenaline and that the non-fluorescing cells contain much adrenaline and little noradrenaline. It is possible that the fluorescence of the islets is due to a fluorescent substance formed by formalin from noradrenaline present in the islet cells.

REFERENCES

Figure 10. Fluorescence photomicrograph of cat adrenal. x 70.

Figure 11. Similar micrograph of another section. x 70.
From the Physiological Department, 
Institute of Occupational Health, Työterveyslaitos, 
Helsinki, Finland

(V 2)

DISTRIBUTION OF FLUORESCING ISLETS, ADRENALINE AND NORADRENALINE IN THE ADRENAL MEDULLA OF THE HAMSTER

By

Olavi Eränkö

The present author has observed that two kinds of secretory cells can be demonstrated in the adrenal medulla of the rat, the cat, the dog, the mouse and the hamster (Eränkö, 1951 a, b, 1952, 1954 b, 1955). After fixation in formalin, some cell groups, called in the following description medullary islets, exhibit a bright green fluorescence in ultraviolet light, the other medullary cells fluorescing but faintly. The relative amounts of islet tissue and noradrenaline in the adrenal medulla vary in a parallel manner in different species. Thus, e.g., much islet tissue and a high proportion of noradrenaline is seen in the adrenal medulla of the cat; in the adrenal medulla of the rat, on the other hand, the islet content is low, and the amount of noradrenaline is much smaller than the adrenaline content (Eränkö, 1952, 1954 b, 1955). In rat and cat adrenals from which adrenaline has been selectively depleted by insulin injections the chromaffin reaction is shown to be present exclusively in cell groups morphologically identical with the fluorescing islets (Eränkö, 1954 c; Hillarp & Hökfelt, 1954). A large increase in the relative amount of the fluorescing medullary tissue and a parallel increase in the relative noradrenaline content has been observed in rats treated for several months with nicotine (Eränkö, 1954 d).

All these observations suggest that the fluorescing islets contain all or most of the medullary noradrenaline. A study of cat adrenals (Eränkö, 1955), in which medullary pieces with varying islet content were dissected out and analyzed for the contents of adrenaline and noradrenaline, has provided more direct support for this view. The present paper describes similar experiments on the hamster, which show that the fluorescing islets in the adrenal medulla of this animal also contain much noradrenaline and little adrenaline and that the rest of the medulla contains much adrenaline and little noradrenaline.
Figs. 1-3. Fluorescence photomicrographs of unstained, formalin-fixed sections of the adrenal medulla of the hamster. All photographs are taken at the same magnification. The peripheral distribution of the fluorescing medullary islets is evident. The left side of the cortex in Fig. 1 has been exposed before photography to ultraviolet light and shows a reduced fluorescence intensity. The section in Fig. 3 is not exactly from the middle of the medulla, and hence the peripheral band of islets is fairly broad. The black areas in the medulla are empty blood vessels.

Figs. 4 and 5. Fluorescence photomicrographs taken at a higher magnification than those in the foregoing figures. The sharp cortico-medullary junction is evident in both figures.
MATERIAL AND METHODS

Healthy hamsters of both sexes were used. The animals were killed by rapid decapitation without any previous treatment. The adrenals were quickly frozen, and fresh sections were cut at 50 μ with a cooled knife. A part of each section was fixed in a mixture containing 5 parts of 2 per cent calcium chloride, 4 parts of distilled water and 1 part of concentrated formalin neutralized over calcium carbonate. After 4–6 hours' fixation, the sections were quickly rinsed in distilled water, floated on slides and mounted in glycerol. They were then examined in ultraviolet light for fluorescence.

Another part of the sections was freeze-dried at ca. -40° C (Eränkö, 1954 e). Small pieces of the dry sections were dissected, weighed and analyzed after paper chromatography for adrenaline and noradrenaline content as described in a previous paper (Eränkö, 1954 f). Adrenaline and noradrenaline were the only catechol amines detected.

RESULTS

Figs. 1–5 illustrate the distribution of fluorescent materials in formalin-fixed adrenal sections of the hamster. The cortex showed a bright yellow-green fluorescence. This fluorescence was localized in all cortical zones except the zona glomerulosa, and faded after a short exposure to ultraviolet light (Fig. 1, on the left), a phenomenon generally believed to be characteristic of vitamin A (Popper, 1944). Most of the medulla fluoresced faintly. In the peripheral parts there were nevertheless always cell groups which fluoresced with a bright green colour. This fluorescence was stable in spite of prolonged exposure to ultraviolet light. No doubt these cell groups are analogous with fluorescing islets previously observed in the adrenal medulla of other species (see the introduction). The cortico-medullary junction was always clearly outlined, and the medulla never contained any cortical cell groups. The above description applies for all sections cut through the middle of the medulla. Sections from the periphery of the medulla showed fluorescing islets all over the section.

The peripheral distribution of the fluorescing islets was observed in all hamsters examined, independently of the age or sex of the animal. The center in mid-sections was as a rule free of fluorescing tissue. In comparison with the medulla of other animals hitherto examined, the adrenal medulla of the hamster is unique in this respect and offers a good opportunity for separating islet-free and islet-containing parts of the gland.

Fig. 6 is a schematical drawing of the adrenal medulla of the hamster. The medulla is arbitrarily divided into three areas, A, B and C. If noradrenaline were present only in the fluorescing islets, the small central area (A) should be free of noradrenaline, and the remaining peripheral area (B+C) should contain reasonable amounts of both adrenaline and noradrenaline. Further, a narrow peripheral ring (C) should contain roughly equal amounts of adrenaline and noradrenaline and the remaining central area (A+B) principally adrenaline.
but also some noradrenaline. To test this hypothesis, freeze-dried mid-sections from 15 adrenals were each dissected into two pieces, one central and one peripheral. In six experiments only a small piece from the very center was taken, leaving a thick peripheral ring. In a further six experiments the central area was cut to comprise up to one half of the whole medullary area. In three experiments the peripheral ring was cut very thin. About 6 sections of each gland were used for each experiment.

The results are presented in Table 1. It shows that the relative amount of noradrenaline, expressed as per cent of the amount of both catechols, was always smaller in the central piece than in the peripheral piece. Further, in every experiment the amount of noradrenaline per dry weight of medullary tissue was smaller in the central piece than in the peripheral one. In all experiments but one the reverse was true for the adrenaline content. All these differences between the central and the peripheral pieces are statistically highly significant when tested with the aid of the binominal theorem, the probability (P) that the results obtained are due to mere chance being less than 0.001. There were no significant differences in the total contents of both catechols between the central and peripheral parts of the medulla. In 5 out of the 6 experiments in which the central piece was very small the total content of both catechols was higher in the periphery than in the center, a suggestive but statistically not significant finding (P ~ 0.2).

Further study of the data in Table 1 shows that the noradrenaline content of the central piece tends to increase with increasing size of the piece. To analyze further this tendency the figures of the first group (Expts. 5, 8, 9, 11, 13, 15) were compared with those of the last group (Expts. 6, 10, 12). As application of Student's t-test presumes normal distribution of the means to be compared – a condition probably not fulfilled in the present series – the significance of the differences was tested by means of Wilcoxon's (1954) test, using significance
Table 1.
Adrenaline and noradrenaline in the center and the periphery of the adrenal medulla of the hamster.

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Size of central piece (% of whole medulla)</th>
<th>Adrenaline (% of dry weight)</th>
<th>Noradrenaline (% of dry weight)</th>
<th>Both catechols</th>
<th>Noradrenaline (% of both catechols)</th>
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<tr>
<td></td>
<td>Periphery</td>
<td>Center</td>
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<tr>
<td>5</td>
<td>10-20</td>
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<td>2.9</td>
<td>1.5</td>
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</tr>
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<td>1.8</td>
<td>1.9</td>
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</tr>
<tr>
<td>11</td>
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<td>0.8</td>
<td>0.4</td>
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</tbody>
</table>

1) The figures for both catechols are not always equal to the sum of the figures given separately for adrenaline and noradrenaline content. These differences are due to rounding off.

Tables designed by van der Vaart (1952). It was thus shown that the content of noradrenaline, whether expressed as per cent of the total content of both catechols or as per cent of dry weight, was significantly higher in the latter group both in the central (P < 0.01) and in the peripheral (P < 0.02) pieces. This is exactly what can be expected according to the presence of some islet tissue in a large central piece and to the high islet content in a narrow peripheral piece. These differences therefore further support the view that noradrenaline is located in the islet cells.

**DISCUSSION**

The presented observations may justify the conclusion that the fluorescing islets in the adrenal medulla of the hamster contain mostly noradrenaline and the remaining medullary cells mostly adrenaline. As the very central areas of
the medulla were free of fluorescing islets but still contained ca. 10 per cent of noradrenaline of the catechol content, it can be further surmised that the feebly fluorescing cells also contain noradrenaline. Similar observations have been made in the cat (Eränkö, 1955). Although the two different kinds of medullary tissues should be regarded as independent of each other, this finding is not very surprising as adrenaline is probably formed in the adrenal medulla by methylation of noradrenaline (Bülbring & Burn, 1949). In the adrenal medullas of the guinea-pig and the rabbit, indeed, no fluorescing islets are seen though a small but definite proportion of noradrenaline is present (Eränkö, 1954 b).

Do the fluorescing islets contain only noradrenaline or do they also contain some adrenaline? The present study cannot give a certain answer to this question. Nevertheless, if the faintly-fluorescing cells in the periphery of the medulla contain 9 times as much adrenaline as noradrenaline, as they do in the center, the fluorescing cells must contain only or almost only noradrenaline and no or very little adrenaline.

The conclusions derived are in full agreement with the present author’s observations mentioned in the beginning of this paper. It is of great interest that Hillarp & Hökfelt (1953), using different techniques, have also arrived at similar results. They observed that noradrenaline forms an insoluble pigment when treated with potassium iodate at pH 6 while adrenaline does not. Iodate-treated sections of adrenal medulla from species whose adrenals contained noradrenaline showed distinctly delineated areas of darkened cells while the remaining medullary cells were but weakly stained. Similar iodate-positive islets were seen in the adrenal medulla of cats injected with insulin to deplete selectively adrenaline. The photomicrographs published by Hillarp & Hökfelt (1953) of the iodate-positive islets in cat’s adrenal medulla suggest that these cell groups are morphologically identical with the fluorescing islets of the same animal (Eränkö, 1954 a). As the series of studies on the fluorescing islets was completed before the present author was aware of Hillarp & Hökfelt’s histochemical technique, direct comparison of the iodate reaction and the fluorescence reaction has not as yet been done. Nevertheless, there seems to be hardly any doubt that they demonstrate the same medullary cells.

Whether the fluorescence of the islet cells after treatment with formalin is due to noradrenaline present in these cells or to some other property, is not quite clear. The facts that noradrenaline forms with formalin a water-insoluble, fluorescing substance in vitro, which adrenaline does not, and that nicotine, which depletes the medullary noradrenaline in an acute experiment, also abolishes the fluorescing islets (Eränkö, unpublished data), rather suggest that formalin-fixation may be a useful histochemical technique for the demonstration of noradrenaline in the presence of adrenaline. Further studies are in progress.

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SUMMARY

Fresh-frozen adrenal sections of the hamster show after treatment with formalin two kinds of cells in the medulla. The majority of the medullary cells fluoresce weakly in ultraviolet light but clearly delineated groups of morphologically similar cells exhibit a marked green fluorescence. These fluorescing islets are located in the periphery of the medulla, the center being free of fluorescing cells. Direct chemical analyses of mechanically separated central and peripheral areas of freeze-dried medullary sections revealed that the non-fluorescing cells contain much adrenaline and little noradrenaline and that the fluorescing cells contain much noradrenaline and little, if any, adrenaline.

ACKNOWLEDGMENTS

Valuable technical assistance has been given by Mrs. Liisa Räisänen and Mr. E. Siltanen, to whom the author is very grateful. This study has been financially supported by grants from the Valtion Luonnontieteellinen Toimikunta and the Sigrid Jusélius Foundation, which support is gratefully acknowledged.

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Figure 12. Fluorescence photomicrograph of hamster adrenal. x 25.

Figures 13 and 14. Similar micrographs taken at a higher magnification. x 75.
(VI)

EFFECT OF INSULIN ON CHROMAFFIN REACTION, FLUORESCING ISLETS AND CATECHOL AMINES IN THE ADRENAL MEDULLA OF THE RAT

By

Olavi Eränkö

Received 22.xi.54

It is well known that insulin causes a decrease in the adrenaline content of the adrenal gland (1, 2, 5, 14–19). This loss of adrenaline occurs without a significant decrease in the noradrenaline content (5, 14–18). The chromaffin reaction is not negative in all medullary cells after insulin treatment, some cell groups still retaining their chromaffin properties (12, 14, 20).

Simultaneous changes caused by insulin in the chromaffin reaction and in the catechol amine concentrations have been recently studied in the cat by Hillarp & Hökfelt (14). A nearly complete loss of adrenaline was accompanied by disappearance of the chromaffin reaction from all but some clearly delineated cell groups, which also gave a positive histochemical test for noradrenaline (13). To the present writer's knowledge studies comparing in the rat the chromaffin reaction with the adrenaline and noradrenaline contents have not been published, though separate chemical or histochemical observations have been made on insulin-injected rats (see above).

The presence of two kinds of histochemically different cells in the adrenal medulla of the rat was demonstrated in 1951 (3, 4). At present, the evidence available suggests that one of these cell types, which exhibits a strong fluorescence after fixation in formalin, stores predominantly noradrenaline, the other one containing mostly adrenaline (8–11). The fluorescence of the presumably noradrenaline-carrying cells is not affected by insulin shock (5). Data are presented in the present paper suggesting that the fluorescing medullary islets in the adrenal medulla of the rat are identical with the noradrenaline-containing cell groups retaining their chromaffin reaction after the medulla has been depleted of adrenaline by injections of insulin.

1 This study has been supported by grants from the Valtion Luonnontieteellinen Toimikunta and the Sigrid Jusélius Foundation.
2 With technical assistance of Mrs. Liisa Räisänen and Mr. E. Siltanen.
MATERIAL AND METHODS

Two series of experiments were carried out. Preliminary experiments were made in 1952-53 at the University of Edinburgh. Six rats were allowed to fast overnight, whereafter they were subcutaneously injected with 1 international unit of insulin per 100 gm body weight at 9 a.m. The same dose was repeated at 12 o'clock, and the animals were killed at 4 p.m. Corresponding controls were killed without any treatment. In these experiments, the left adrenals were examined for the distribution of the chromaffin reaction and the right adrenals for the distribution of the fluorescing islets.

The second series of experiments was made in Helsinki. Nine male rats were treated as described above, with the exception that the dose of insulin used was 0.6 units instead of 1 unit. Nine untreated controls were killed with the injected animals. The left adrenal of each animal was freshly frozen and cut at 50 μ with a freezing microtome. A part of the sections was fixed in formalin for ca. 6 hours to develop the islet fluorescence. The remaining sections were freeze-dried (6). Medullary parts of these sections were mechanically separated, and the concentrations of adrenaline and noradrenaline in these medullary pieces were determined fluorimetrically after chromatographic separation, as described earlier (7). The right adrenal was studied for the chromaffin reaction (for details of the technique used, see 10).

RESULTS

The histochemical findings were essentially similar in both experiments. All cells of the adrenal medulla of the controls gave a strong chromaffin reaction (Fig. 1). In formalin-fixed sections of the untreated animals, typical fluorescing islets were seen (Fig. 3). After treatment with insulin, most animals showed a pronounced loss of chromaffin materials from the medulla; clearly delineated small cell groups nevertheless always retained a strong chromaffin reaction (Fig. 2). These cell groups were of similar size, shape and distribution.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Adrenaline (% of dry weight)</th>
<th>Noradrenaline (% of dry weight)</th>
<th>Noradrenaline of both catechols</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (9 rats)</td>
<td>Mean 4.86 Standard deviation 0.77</td>
<td>Mean 1.10 Standard deviation 0.35</td>
<td>Mean 17.3 Standard deviation 4.1</td>
</tr>
<tr>
<td>Insulin-injected rats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. 1</td>
<td>0.7</td>
<td>1.0</td>
<td>58</td>
</tr>
<tr>
<td>2</td>
<td>0.7</td>
<td>1.0</td>
<td>61</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>0.8</td>
<td>60</td>
</tr>
<tr>
<td>4</td>
<td>0.6</td>
<td>0.8</td>
<td>58</td>
</tr>
<tr>
<td>5</td>
<td>0.9</td>
<td>1.2</td>
<td>58</td>
</tr>
<tr>
<td>6</td>
<td>1.3</td>
<td>1.1</td>
<td>54</td>
</tr>
<tr>
<td>7</td>
<td>1.4</td>
<td>1.6</td>
<td>47</td>
</tr>
<tr>
<td>8</td>
<td>1.4</td>
<td>0.9</td>
<td>39</td>
</tr>
<tr>
<td>92</td>
<td>4.0</td>
<td>1.3</td>
<td>26</td>
</tr>
</tbody>
</table>

1 Calculated directly from absolute amounts of adrenaline and noradrenaline found in each sample. Slight discrepancies between this and the other two columns are due to rounding off.

2 This animal did not show any signs of shock.
All figures have been taken at the same magnification.

**Fig. 1.** Adrenal medulla of an untreated control. The chromaffin reaction is positive in all medullary cells. Empty blood vessels are white.—**Fig. 2.** Adrenal medulla of an insulin-injected rat. Only some of the medullary cells show a positive chromaffin reaction.—**Figs. 3 and 4.** Fluorescence photomicrographs of formalin-fixed sections from a control and an insulin-treated rat. Cell groups similar to those seen in Fig. 2 exhibit a bright fluorescence. Empty blood vessels are black. The background fluorescence tends to be somewhat weaker in adrenaline-depleted medullas.

as the fluorescing islets (cf. Figs. 2, 3 and 4). The fluorescence of the medullary islets was not affected by insulin (Fig. 4).

Results of adrenaline and noradrenaline determinations are presented in Table 1. The insulin injections caused a marked loss of adrenaline from the medulla of most animals, while the noradrenaline concentration was not affected at all. One of the injected animals did
not show any signs of shock during the experiment. This animal (No. 9) had a normal adrenaline concentration, and the chromaffin reaction was positive in all medullary cells.

The intensity of the chromaffin reaction was in the injected animals to a certain degree dependent on the adrenaline content of the adrenal medulla. Thus, with the exception of the strongly chromaffin islet cells, no yellow colour was seen in the medullary cells of rats No. 1–4, while a faint yellow background tint was seen in the medullas of animals No. 6–9, which showed a higher adrenaline content.

**DISCUSSION**

Localization of the chromaffin reaction in specific groups of cells in adrenals of which most of the medullary adrenaline was depleted strongly suggests that these cells are specific carriers of most medullary noradrenaline. In experiments mentioned earlier in this paper, Hillarp & Hökfelt (14) came to the same conclusion.

Similarity of these noradrenaline-carrying cell islets with the fluorescing islets demonstrable after formalin fixation favours the view that the latter are identical with the former. Theoretically one might of course claim that the fluorescence may depend on some property of the medullary cells which has nothing to do with their content of adrenaline or noradrenaline. The simplest explanation, i.e. that they are identical, is nevertheless rendered rather likely by other experiments on the rat, in which a marked increase in the relative amount of fluorescing medullary tissue was associated with a proportional increase in the noradrenaline content (10). High noradrenaline amounts have been demonstrated in medullary parts rich in fluorescing islets in adrenals of the cat and the hamster (8, 9). Furthermore, there are good reasons to suppose that the fluorescence of the islets is due to formation of a poorly water-soluble, fluorescing compound from noradrenaline and formalin.

**SUMMARY**

Insulin caused a loss of most medullary adrenaline but did not influence the noradrenaline content. The chromaffin reaction was in the adrenaline-depleted adrenals selectively localized in small groups of cells scattered through the medulla, the other medullary cells being essentially colourless. The chromaffin-positive cell groups were similar of form, size and shape to the fluorescing cell groups demonstrable in the adrenal medulla after formalin fixation, as described earlier. It is concluded that noradrenaline is selectively localized in these fluorescing islets, whose fluorescence was not affected by insulin.
REFERENCES

Figure 15. Chromaffin reaction in the adrenal medulla of an untreated control rat. All cells positive. x 75.

Figure 16. Chromaffin reaction in the adrenal medulla of an insulin-injected rat. Only some cell islets positive. x 75.

Figure 17. Fluorescence photomicrograph of the adrenal of a control rat. x 75.

Figure 18. Similar micrograph of an insulin-injected rat. No essential difference as compared with Figure 17. x 75.
NODULAR HYPERPLASIA AND INCREASE OF NORADRENALINE CONTENT IN THE ADRENAL MEDULLA OF NICOTINE-TREATED RATS

By

OLAVI ERÄNKÖ

Received 19.xi.54

The chromaffin cells of the adrenal medulla of the rat can be differentiated into two distinctly different categories (1-3, 8, 9). Most cells are non-fluorescent after formalin fixation and possess a strong acid phosphatase activity. Amongst them, groups of cells are seen which fluoresce brightly in ultraviolet light after treatment with formalin and which give a negative histochemical reaction for acid phosphatase. There are good reasons to suppose that the fluorescing cell islets contain much noradrenaline and little adrenaline and that the rest of the medulla contains predominantly adrenaline besides small amounts of noradrenaline (6-9, 14, 15).

In 1935, Staemmler (17) described a nodular hyperplasia in the adrenal medulla of rats injected for a year or more with nicotine. Apparently this finding has drawn little attention since then. It seemed therefore to be of interest to reproduce Staemmler's observation and to study which of the two kinds of medullary cells is responsible for the medullary enlargement. Considering the presumptive significance of these two types of cells in the selective storage and release of adrenaline and noradrenaline, the question was also investigated whether the medullary hyperplasia is associated with an increase of either, neither or both of these catechols. The present paper describes the results of such a study.

MATERIAL AND METHODS

The material consisted of 32 male Wistar rats, of which 16 rats were injected with nicotine, the others serving as untreated controls. Injections were given subcutaneously every work day for ca. 6½ months. The dose of the 10 first injections

1. This study has been supported by grants from the Valtion Luonnontieteellinen Toimikunta and from the Sigrid Jusélius Foundation.
2. With the technical assistance of Mr. G. Amnell, Mr. R. Fordell, Mrs. Liisa Räisänen and Mr. E. Siltanen.
was 2.5 mg of nicotine bitartrate (B.D.H.) per animal, whereafter the daily dose was increased to 4 mg, which dose was then maintained. Because of the fairly high mortality, the dose was reduced to 2 mg after ca. 4 months. The surviving animals were killed by decapitation 9 days after the last injection together with the same number of controls.

The left adrenal was frozen fresh and cut at 50 µ with a freezing microtome. A part of the sections was fixed in a calcium chloride-formalin mixture (3). The other sections were freeze-dried at ca. -40°C (4). The formalin-fixed sections were after fixation for ca. 6 hours examined in ultraviolet light for fluorescence. The medullary part of the freeze-dried sections was dissected out, and the catechols in these pieces were chromatographically separated and determined as described in an earlier paper (5). Some freeze-dried sections were used for studying the distribution of acid phosphatase (1).

The right adrenal was fixed in a mixture containing 1 volume of commercial formalin and 9 volumes of 3.5 per cent potassium dichromate for 24 hours, whereafter the dichromate-formalin mixture was replaced with 3.5 per cent dichromate, which was daily changed three times. The glands were then serially sectioned with a freezing microtome at 50 µ, and the volume of the whole gland and that of the medulla were planimetrically measured (for details see 10). For microscopic examination, some of the already measured sections of each gland were stained first with ponceau-fuchsin (12) and then with Mallory's (16) aniline blue-orange G mixture.

Pituitary, thymus, spleen, heart, right kidney and right testis were weighed. Pieces of the kidney, pancreas and brain were fixed in Bouin's fluid and embedded in paraffin wax through alcohol and butyl alcohol series. Haematoxylin-eosin was used as a routine stain, besides which Gomori's (13) chrom-haematoxylin stain was used for sections from the pancreas and the hypothalamus.

Differences between the control group and the nicotine group were tested with Student's t-test (11). As no accurate estimations were made of the proportional amount of islet tissue in the adrenal medulla, this test was not applicable, and Wilcoxon's non-parametric test (18, 19) was used. Besides the significance levels thus calculated, the means and the standard deviations are shown in the tables.

RESULTS


Every injection of nicotine caused convulsions ca. ½–1 min. after the injection. The convulsions lasted some ½–2 min., after which the animals were unconscious for about half an hour. A few days after the beginning of the experiment the animals became easily irritable and jumpy, fighting with each other frequently. This irritated state lasted until the end of the experiment. Otherwise, no definite signs of illness were observed.

The body weights of all rats were ca. 100 gm in the beginning of the experiment. The growth of the injected animals was somewhat retarded during the first few weeks but thereafter they grew at the same rate as the controls. However, 10 out of the 16 injected animals died during the experiment. Only the adrenals of some of these spontaneously died animals were histologically examined. Organs of the remaining 6 injected animals and of the same number of controls were subjected to a detailed study. The final body weights were 302 ± 24 and 278 ± 27 gm (mean ± standard deviation) in the control and experimental groups, respectively, the difference of the means being not statistically significant.
2. Weights and Histology of Various Organs.

Weights of some organs are given in Table 1, which shows that no significant differences were found between the two groups. Histological examination likewise failed to detect changes in the organs studied, with the exception of the adrenals. The amount of "Gomori-positive" material in the cells of the nucleus supraopticus was roughly the same in both groups.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Control group</th>
<th>Nicotine group</th>
<th>Significance of difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Standard deviation</td>
<td>Mean</td>
</tr>
<tr>
<td>Pituitary</td>
<td>8.67</td>
<td>0.98</td>
<td>8.02</td>
</tr>
<tr>
<td>Adrenal</td>
<td>23.9</td>
<td>5.4</td>
<td>26.5</td>
</tr>
<tr>
<td>Thymus</td>
<td>366</td>
<td>82</td>
<td>284</td>
</tr>
<tr>
<td>Spleen</td>
<td>854</td>
<td>114</td>
<td>765</td>
</tr>
<tr>
<td>Heart</td>
<td>974</td>
<td>122</td>
<td>973</td>
</tr>
<tr>
<td>Kidney</td>
<td>948</td>
<td>81</td>
<td>989</td>
</tr>
<tr>
<td>Testis</td>
<td>1365</td>
<td>107</td>
<td>1337</td>
</tr>
</tbody>
</table>


The planimetrically determined volumes of the whole adrenal, adrenal cortex and adrenal medulla are presented in Table 2. The figures obtained for adrenal volumes agreed well with the corresponding figures for weights, although the volume estimates, though fairly accurate relatively, may not be correct in terms of mm³, a fact having little significance in this connexion. The mean weight and volume of the adrenals of the nicotine group did not differ significantly from the corresponding means of the control group. The volume of the cortex was not influenced by the experiment.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Control group</th>
<th>Nicotine group</th>
<th>Significance of difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Standard deviation</td>
<td>Mean</td>
</tr>
<tr>
<td>Whole Adrenal (mm³)</td>
<td>16.8</td>
<td>3.3</td>
<td>18.9</td>
</tr>
<tr>
<td>Cortex (mm³)</td>
<td>15.3</td>
<td>3.1</td>
<td>15.8</td>
</tr>
<tr>
<td>Medulla (mm³)</td>
<td>1.51</td>
<td>0.17</td>
<td>3.10</td>
</tr>
</tbody>
</table>

Proportion of islet tissue (% of whole medulla)   ca. 1/6            ca. 1/2          P > 0.0011

1 Calculated using Wilcoxon's test, see text.
Fig. 1.
Adrenal section from a control rat. Fixation in formalin-dichromate, counterstain with ponceau-fuchsin and aniline blue—orange G.

Fig. 2.
Similarly treated section from a rat injected daily for ca. 6 months with nicotine. Note the presence of less intensely chromaffin nodules in the medulla. Photographed at the same magnification as Fig. 1.

On the other hand, the volume of the medulla had roughly doubled in the nicotine-treated animals, this change being statistically highly significant.


In control animals the adrenal medulla showed a typical picture. All cells were chromaffin (Fig. 1) and typical fluorescing islets were seen after formalin fixation (Figs. 3 and 5).

The adrenals of the nicotine-treated animals were distinctly different from those of the controls. Small nodules were observed in the periphery of the medulla of all injected animals. These nodules were less intensely stained after dichromate treatment than the rest of the medulla (Fig. 2). They were also more scarcely vascularized than normal medullary tissue and contained more nuclei per unit of area. In formalin-fixed sections they were seen to fluoresce brightly in ultraviolet light, the colour of the fluorescence being exactly the same as that of the islets normally found in the medulla (Figs. 4 and 6). The nodules were as a rule sharply demarkated from the adjacent chromaffin cells, giving the impression of a fairly rapidly proceeding ex-
Fluorescence photomicrographs of formalin-fixed adrenal sections. Fig. 3 and 5 are from controls, Figs. 4 and 6 are from nicotine-injected animals. Figs. 3 and 4 on the one hand, and Figs. 5 and 6 on the other, are taken at the same magnification. The cortico-medullary junction is drawn black in Figs. 5 and 6. The increase in the relative amount of fluorescing tissue in the medulla of nicotine-treated animals is obvious. Empty blood vessels are shown as black areas.

Pansive process. However, they were frequently found to go over smoothly to normal medullary cells belonging to the category of fluorescing islets.

Histochemically demonstrable acid phosphatase activity, which was
very weak in the normal fluorescing islets, was moderate in some of the medullary nodules of the nicotine rats.

No accurate measurements were made of the proportional area covered by the fluorescing cells in the medulla. It was nevertheless quite clear that the amount of fluorescing tissue was much higher in all experimental animals than in any of the controls. In the former, roughly one half of the medulla was fluorescing, the corresponding figure for the controls being ca. \( \frac{1}{6} \) (see Table 2 and Figs. 3–6). The significance of this difference was calculated with the aid of Wilcoxon's test (18, 19). It was thus shown that the probability of such a difference being obtained by change only is ca. 0.001, thus indicating that the difference between the two groups is highly significant.

### Table 3

**Medullary Catechols.**

<table>
<thead>
<tr>
<th>Catechol</th>
<th>Control group</th>
<th>Nicotine group</th>
<th>Significance of difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Standard deviation</td>
<td>Mean</td>
</tr>
<tr>
<td>Both catechols (% of dry medullary weight)</td>
<td>4.18</td>
<td>0.50</td>
<td>4.36</td>
</tr>
<tr>
<td>Adrenaline (% of dry medullary weight)</td>
<td>3.42</td>
<td>0.58</td>
<td>2.40</td>
</tr>
<tr>
<td>Noradrenaline (% of dry medullary weight)</td>
<td>0.76</td>
<td>0.13</td>
<td>1.96</td>
</tr>
<tr>
<td>Noradrenaline (% of both catechols)</td>
<td>18.5</td>
<td>4.5</td>
<td>45.5</td>
</tr>
</tbody>
</table>

5. **Adrenal Catechols.**

Table 3 indicates that there was an enormous, highly significant increase in the content of medullary noradrenaline in the nicotine-injected animals, both in terms of per cent of dry weight and in terms of per cent of total catechols. The reverse was true for adrenaline, i.e. both the absolute concentration and the relative amount of this catechol were significantly lower in the nicotine group. There was no significant difference in the summed amount of both catechols between the two groups.

6. **Correlation of Histochemical and Chemical Changes.**

To facilitate direct comparison of the observed histochemical and chemical changes, the amounts of fluorescing and non-fluorescing tissues as well as of adrenaline and noradrenaline in each gland were computed. The former figures were obtained by multiplying the medullary volume by the estimated percentage of islets tissue. As both the relative amount of fluorescing tissue and the size of the adrenal medulla increased significantly in the injected animals, it is clear that
the absolute amount of islets tissue in each gland increased equally significantly. The amounts of the catechols were calculated by assuming a water content of 80 per cent in the medulla of both the experimental animals and the controls. Although the values thus obtained may for various reasons not be entirely correct in absolute terms, they certainly reflect with sufficient accuracy the changes caused by nicotine injections (Table 4).

**TABLE 4**

*Medullary Cells and Catechols.*

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control group</th>
<th>Nicotine group</th>
<th>Significance of difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescing islets (mm³/gland)</td>
<td>ca. 0.25</td>
<td>ca. 1.55</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Noradrenaline (µg/gland)..........</td>
<td>2.6 ± 0.2</td>
<td>12.0 ± 2.2</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>Non-fluorescent tissue (mm³/gland)</td>
<td>ca. 1.25</td>
<td>ca. 1.55</td>
<td>—</td>
</tr>
<tr>
<td>Adrenaline (µg/gland)............</td>
<td>10.5 ± 2.8</td>
<td>14.8 ± 4.0</td>
<td>—</td>
</tr>
</tbody>
</table>

It will be seen that the mean amount of fluorescing tissue and the mean amount of noradrenaline per gland increased by ca. 400 per cent. The volume of the non-fluorescing tissue and the amount of adrenaline, on the other hand, both showed a slight, statistically not significant increase. In other words, the ratios (noradrenaline/fluorescent tissue) and (adrenaline/non-fluorescent tissue) were equal in both groups separately and, moreover, these ratios were not altered by treatment with nicotine.

In some freeze-dried sections from the injected animals, medullary nodules bulging towards the cortex could be distinguished without any treatment such as fixation or staining. Such nodules, dissected out and applied on filter paper for chromatography (see 5) were observed to contain noradrenaline, indicated by the presence of a strongly fluorescing spot in the sprayed chromatogram in a position typical of noradrenaline, but little or no adrenaline, as judged by the absence of any other spot. This observation is so far the only direct proof of the predominant presence of noradrenaline in the fluorescing medullary tissue of the rat.

**DISCUSSION**

The present study confirms Staemmler's (17) observations on the appearance of nodular hyperplasia in the adrenal medulla after nicotine injections. It also agrees with his finding that large doses of nicotine seem to exert little influence on organs other than the adrenal medulla. Furthermore, the growth of the injected animals was in the present study observed to be not significantly affected by this treatment.
Increase in the volume of the fluorescing medullary tissue, associated with an increase in the noradrenaline content, provides further support to the view that the fluorescing tissue is the main site of storage of noradrenaline in the adrenal medulla. Indirect evidence of the presence of a high concentration of noradrenaline in the medullary islets of the rat has been presented earlier (8, 14, 15). The directly proved presence of noradrenaline in the hyperplastic nodules of nicotine-injected rats is however of special interest in so far that the cytophological picture and the phosphatase content of these cells differed from the normal islet-cells of the same animal. This brings up the question whether the nodules can be regarded as hypertrophied islets. There are some points in favour of this view. First, the nodules were frequently found to turn gradually over to cytologically normal medullary islets. Secondly, nicotine not only induced the appearance of nodules but also an increase in the relative amount of islets cytologically and histochemically similar to those seen in normal rats. Thirdly, nicotine seems to exert a selective, stimulating influence on the islet cells in an acute experiment, abolishing the fluorescence which can be normally observed after formalin fixation and depleting the islets of noradrenaline (Eränkä, unpublished).

Whether the hyperplastic fluorescing nodules originate from the normal fluorescing islets or not, the present study shows that even in these nodules, which are not in all respects similar to the normal islets, high noradrenaline content is associated with fluorescence after formalin fixation. This seems to support the assumption that the fluorescence is due to a fluorescing compound formed by formalin of noradrenaline, suggested by in vitro—evidence on mixtures of noradrenaline and formalin (unpublished).

The presence of histochemically demonstrable acid phosphatase in the predominantly noradrenaline-containing nodules shows that this histochemical reaction is not directly related to the presence or absence of noradrenaline, although little acid phosphatase is seen in the islet cells and much acid phosphatase in the other medullary cells of normal rats. The absence of similar correlation in other species (7, 9) also points to the same direction. This fact may be taken to indicate either that acid phosphatase has little bearing in the production of the medullary catechols or that adrenaline is in different species synthetized through different metabolic mechanisms. It would be attractive to think that the intense phosphatase activity in the adrenaline-producing medullary cells of the rat is somehow energetically coupled with the transmethylation process, necessary in the synthesis of this amine (cf. 3). However, no direct proof of the participation of acid phosphatase in transmethylation has to the present writer's knowledge been presented.

The doses of nicotine used in this study were very high. Therefore it is not possible to draw any conclusions of the effect of smaller doses
of nicotine on the adrenal medulla, a subject of considerable interest
in view of the popularity of tobacco smoking. It would nevertheless
not be surprising if some of the effects of smoking could be ascribed
to a release of noradrenaline from the adrenal medulla—and perhaps
from the sympathetic ganglions.

**SUMMARY**

Male rats were injected daily with 2–4 mg of nicotine tartrate for ca.
6 months. This treatment was observed to cause no demonstrable
changes in the adrenal cortex but a doubling of the volume of the me-
dulla. This volume increase was partly due to the appearance of hyper-
plastic nodules in the medulla. These nodules showed a weakly or
moderately positive chromaffin reaction, and they fluoresced with
green colour in ultraviolet light. Noradrenaline was the only catechol
detected in the nodules. Approximately one half of the hyperplastic
medulla of the nicotine-injected animals was composed of cells fluores-
cing after formalin fixation, and the relative amount of noradrenaline
was also ca. 1/2 of the total amount of medullary catechols. In normal
rats, the corresponding figures were ca. 1/6 for the content of fluores-
cing islets and ca. 1/6 for the relative content of noradrenaline. It is
concluded that nicotine causes a hyperplasia of the fluorescing medul-
lar cells, which have previously been seen to be the main carriers of
noradrenaline in the adrenal medulla.

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   Fenniae, in press, 1954.
9. - (Medullary islets in different species) Ann. med. exp. biol. Fenniae, in
18. Vaart, H. R. van der: Gebruiksaanwijzing voor de toets van Wilcoxon. Mathe-
   matisch Centrum, Amsterdam, 1952.
Figure 19. Adrenal of a control rat. Chromaffin reaction. Counterstain with ponceau-fuchsin and aniline blue-orange G. x 45.

Figure 20. Adrenal of a nicotine-injected rat. Similar histological technique. Note the medullary nodules, which are less intensely chromaffin than the rest of the medulla. x 45.
<table>
<thead>
<tr>
<th>Animal No.</th>
<th>(a) Medullary volume (mm³)</th>
<th>(b) Adrenaline (% of dry med. wt.)</th>
<th>(c) Noradrenaline (% of dry med. wt.)</th>
<th>Noradrenaline (% of adr. + nor.)</th>
<th>Adrenaline per gland (a x b)x</th>
<th>Noradrenaline per gland (a x c)x</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>3.02</td>
<td>3.38</td>
<td>2.22</td>
<td>40</td>
<td>10.20</td>
<td>6.70</td>
</tr>
<tr>
<td>N2</td>
<td>3.02</td>
<td>2.85</td>
<td>1.72</td>
<td>38</td>
<td>8.61</td>
<td>5.19</td>
</tr>
<tr>
<td>N3</td>
<td>2.46</td>
<td>2.02</td>
<td>2.50</td>
<td>55</td>
<td>4.97</td>
<td>6.15</td>
</tr>
<tr>
<td>N4</td>
<td>3.25</td>
<td>1.70</td>
<td>1.37</td>
<td>45</td>
<td>5.52</td>
<td>4.45</td>
</tr>
<tr>
<td>N5</td>
<td>3.69</td>
<td>1.81</td>
<td>2.06</td>
<td>53</td>
<td>6.68</td>
<td>7.60</td>
</tr>
<tr>
<td>N6</td>
<td>3.16</td>
<td>2.64</td>
<td>1.89</td>
<td>42</td>
<td>8.34</td>
<td>5.97</td>
</tr>
<tr>
<td>M &amp; SD</td>
<td>3.10</td>
<td>2.40</td>
<td>1.96</td>
<td>45.5</td>
<td>7.0</td>
<td>7.39</td>
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</table>

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>(a) Medullary volume (mm³)</th>
<th>(b) Adrenaline (% of dry med. wt.)</th>
<th>(c) Noradrenaline (% of dry med. wt.)</th>
<th>Noradrenaline (% of adr. + nor.)</th>
<th>Adrenaline per gland (a x b)x</th>
<th>Noradrenaline per gland (a x c)x</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co 1</td>
<td>1.55</td>
<td>3.57</td>
<td>0.72</td>
<td>17</td>
<td>5.53</td>
<td>1.12</td>
</tr>
<tr>
<td>Co 2</td>
<td>1.57</td>
<td>3.24</td>
<td>0.62</td>
<td>16</td>
<td>5.09</td>
<td>0.97</td>
</tr>
<tr>
<td>Co 3</td>
<td>1.23</td>
<td>2.74</td>
<td>0.97</td>
<td>26</td>
<td>3.37</td>
<td>1.19</td>
</tr>
<tr>
<td>Co 4</td>
<td>1.56</td>
<td>3.01</td>
<td>0.79</td>
<td>21</td>
<td>4.70</td>
<td>1.23</td>
</tr>
<tr>
<td>Co 5</td>
<td>1.42</td>
<td>3.57</td>
<td>0.80</td>
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<td>Co 6</td>
<td>1.73</td>
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<td>M &amp; SD</td>
<td>1.51</td>
<td>3.42</td>
<td>0.76</td>
<td>18.5</td>
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x) This figure multiplied by 2 gives the amount of catecholamine µg per gland, assuming that the water content of the medulla is 80 per cent.
HISTOCHEMISTRY OF NORADRENALINE IN THE ADRENAL MEDULLA OF RATS AND MICE

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INTRODUCTION

Histochecmical methods have recently been described demonstrating two different kinds of chromaffin cells in the adrenal medulla: 1) after fixation in formalin, some medullary cells exhibit a strong fluorescence in ultraviolet light and the remaining cells fluoresce but feebly (Eränkö, 1951, 1952); 2) treatment with potassium iodate selectively stains some medullary cells brown and the others remain almost colorless (Hillarp and Hökfelt, 1953). Determinations of adrenaline and noradrenaline in microdissected pieces of adrenal medulla, carried out after separation of these catechol amines by paper chromatography (Eränkö, 1954), showed that the noradrenaline content of those medullary cells of the cat and the hamster which fluoresce after formalin fixation is far higher than that of the remaining cells (Eränkö, 1955a, b). Strongly suggestive evidence of similar relations between the fluorescent medullary cells and noradrenaline has been obtained in studies with some other mammals (Eränkö, 1955c). Similar suggestive observations support the view that the medullary cells which give a positive iodate reaction also contain much noradrenaline (Hillarp and Hökfelt, 1953, 1954), but direct proof concerning the validity of this reaction has not been presented. The validity of the iodate reaction is nevertheless supported by the fact that both the fluorescence method and the iodate method have been seen to demonstrate selectively those medullary cells of the hamster's adrenal which are known to contain predominately noradrenaline (Eränkö, 1955d). The present paper describes a study in which the two histochemical methods were applied to adrenals of the rat and the mouse.

MATERIALS AND METHODS

Untreated adult rats and mice were decapitated without anesthesia. The adrenals were quickly but carefully removed and dissected free of the surrounding fat.

The fluorescence method. Fresh-frozen sections were cut at 50 µ with a freezing microtome. The frozen sections were allowed to thaw on the surface of a solution containing 1 volume of commercial formalin, kept over calcium carbonate, 5 volumes of 2% calcium chloride, and 4 volumes of distilled water. After 2-6 hours the sections were rinsed in

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distilled water, floated on slides and mounted in glycerol. They were then examined under a fluorescence microscope.

The iodate method. Attempts to apply the iodate reaction to fresh-frozen sections were unsuccessful. The medulla became immediately red in the iodate solution but the colored substances, probably adrenochrome and noradrenochrome, soon dissolved in the reagent and no further color appeared. Only whole adrenals were therefore used subsequently. These were plunged into the iodate reagent immediately after removal. After 2–72 hours the glands were transferred into 10% formalin, where they were allowed to stay for 1–24 hours. Thereafter, frozen sections were cut at 50 μ. The sections were rinsed in distilled water and mounted in glycerol.

In the beginning, buffered solutions containing 2.5% of potassium iodate were used (Hillarp and Hökfelt, 1953). Without regard to the acidity or the kind of buffer used, the results were unsatisfactory, wherefore a saturated solution of potassium iodate was then adopted (Hillarp and Hökfelt, 1955).

RESULTS

Earlier observations with the fluorescence method were confirmed. In the adrenal medulla of the rat, strongly fluorescing islets of cells were seen, the fluorescence being localized in the cytoplasm of these cells (Fig. 1). The adrenal medulla of the mouse behaved similarly (Fig. 3). However, the fluorescence of the islet cells was less intense in the mouse adrenal. Therefore, and because of the small size of the medullary cells in this species, the cytoplasmic localization of the fluorescence is not visible in the photograph, although there was no doubt about it upon visual examination. The medullary islets of the mouse were as a rule larger than those of the rat; in both species they seemed to be randomly distributed in the medulla.

As to the iodate reaction, immersion in saturated potassium iodate overnight and subsequent fixation in formalin for 2 hours yielded equally satisfactory results as longer periods of treatment (72 hours and 24 hours, respectively), which have been recommended by Hillarp and Hökfelt (1953, 1954 and 1955). Yellow-brown cell groups were demonstrated in the adrenal medulla of both species (Figs. 2 and 4). The intensity of the color produced was low and frequently variable even in different cells of each positive cell group. The fluorescence intensity, on the other hand, was always closely of the same order in all cells of the fluorescent islets demonstrated by the formalin treatment.

Although the weak and variable contrast rendered some difficulties in the study of the iodate-positive medullary islets, there was nevertheless little doubt that these islets were similar in shape, size and distribution to the fluorescent islets in the formalin-fixed sections from the contralateral adrenal of each animal, as can be seen by comparing Figures 1–4.

The intracellular distribution of the iodate reaction was different from that of the fluorescence in the islet cells. The whole cell was colored and, if there was any difference in the intensity of darkening between the nu-

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1 I am grateful to Dr. Hillarp who kindly suggested the use of a saturated solution in a letter to me in December 1954.
Fig. 1. Fluorescence photomicrograph of the adrenal medulla of a normal rat. Formalin fixation.

Fig. 2. Photomicrograph of the medulla in the contralateral adrenal of the same rat. Iodate-positive islets outlined with India ink. Iodate reaction.

Fig. 3. Fluorescence photomicrograph of the corticomedullary junction of a normal mouse. Cortex in the left upper corner. The junction is indicated by a black line. Formalin fixation.

Fig. 4. Photograph of the contralateral adrenal of the same mouse. Cortex above, medulla below; the corticomedullary junction is drawn black. Iodate reaction.

Magnification in all figures $\times 160$. 
cleus and the cytoplasm, the former seemed to be usually darker than the latter. Another difference between the results obtained with the two methods was that the iodate reaction was more intense in the medullary islets of the mouse than those of the rat, while the converse applied to the intensity of the formalin-induced fluorescence. The other two species hitherto examined, i.e. the cat and the hamster, show both an intense fluorescence after formalin fixation and an intense iodate reaction in the adrenomedullary islets.

**DISCUSSION**

The above observations further support the view suggested by previous work that both the fluorescence method and the iodate method selectively demonstrate the noradrenaline-containing cells of the adrenal medulla. Theoretically one might of course claim that the cell islets demonstrated by the former method are not necessarily identical with the islets stained brown by the iodate treatment, although the distribution, size, and shape of the respective islets were similar. In view of previous evidence (see the papers referred to in the introduction) such a possibility seems very unlikely.

On the other hand, one may ask whether both of these reactions are really due to noradrenaline and whether the intensity of either reaction can be regarded as an indicator of the relative noradrenaline concentration in these cells. These questions deserve serious consideration because the two methods gave discrepant results concerning the intensity of the reaction. If the fluorescence intensity of the medullary cells be a reliable guide to the noradrenaline concentration in these cells, fluorescent islets of the rat adrenal would contain more noradrenaline than the corresponding islets of the mouse adrenal. The opposite is suggested by the observation that the iodate reaction in medullary islets of the mouse is stronger than that in similar islets of the rat. The iodate reaction would furthermore indicate that the noradrenaline content of each individual cell in a single islet varies considerably in both rat and mouse adrenals, while the fluorescence reaction seemingly shows that all islet cells in each gland contain roughly equal concentrations of noradrenaline.

*In vitro*, mixing of pure noradrenaline and formalin produces a poorly water-soluble, fluorescent precipitate (Eränkö, 1955). A brown, poorly water-soluble precipitate is on the other hand obtained by mixing pure noradrenaline and potassium iodate (Hillarp and Hökfelt, 1953). This seems to show that noradrenaline is responsible for both reactions. However, it must be kept in mind that cells specialized in the production of noradrenaline may well differ in many respects from the cells producing adrenaline. Indeed, there is a sharp contrast in the acid phosphatase activities of these two types of cells in the rat (Eränkö, 1952). Therefore, also the fluorescence of the islet cells demonstrable after treatment with
formalin may be due to some substance other than noradrenaline. Still, all observations hitherto made, particularly the fact that nicotine injection, which causes a drop in the noradrenaline content of the medulla, abolishes the fluorescence of the islet cells, are compatible with the view that the fluorescence is directly caused by a reaction between formalin and noradrenaline. However, whether such a reaction can occur quickly enough to fix the fluorescent reaction product before noradrenaline has diffused out of the fresh-frozen section into the formalin solution, is questionable.

In all the species hitherto examined, the fluorescence has been observed exclusively in the cytoplasm of the islet cells. This agrees with studies of the intracellular distribution of adrenomedullary catechols by differential centrifugation (Blaschko and Welch, 1953; Hillarp, Lagerstedt and Nilson, 1953), which have unequivocally shown that these catechols are almost exclusively present in the cytoplasmic granules. Thus, the fluorescence—even if it is not directly due to noradrenaline—has a similar intracellular localization as noradrenaline. The iodate reaction, which is positive also in the nuclei, does on the other hand not truly indicate the distribution of noradrenaline in the cells—even if it is specifically due to this catechol amine. It seems likely that diffusion complicates the iodate method, as shown by the failure to give any permanent coloration in adrenal sections. Such diffusion might indeed explain the variations in the intensity of the iodate reaction observed in the present study. The smallest islets, i.e. those of the rat, give the weakest reaction; a stronger color is seen in the—somewhat larger—islets of mouse adrenals, and a strong reaction is observed in the—still larger—islets of cat and hamster adrenals.

In conclusion, it may be stated that the two histochemical methods are still incompletely known, wherefore more work is necessary to elucidate their significance. Although these methods cannot be recommended as substitutes for biological or chemical adrenaline and noradrenaline determinations, they may provide interesting information not obtainable with such determinations alone. Already at present it seems fairly well established that the two cell types demonstrable with them are independent of each other and that each of them is specialized in the secretion of one of the two adrenomedullary hormones.

SUMMARY

Two histochemical reactions, both discriminating between two different kinds of adrenomedullary cells, were applied to rat and mouse adrenals. One of the methods shows fluorescent cell islets in the medulla of formalin-treated adrenals against a non-fluorescent background and the other one brown islets against a yellow background in the medulla of iodate-treated adrenals. Distribution, size and shape of the cell groups demonstrable with the two methods are identical. It is believed that these cell groups contain and secrete noradrenaline. The fluorescence reaction is positive exclusively
in the cytoplasm, while the iodate reaction colors both the cytoplasm and the nuclei of the islet cells.

REFERENCES

Eränkö, O.: J. Histochem. and Cytochem., in press. 1955d.
Figures 21 and 22. Fluorescence photomicrographs of rat adrenal medulla. Note the non-fluorescent center corresponding to the nucleus in each fluorescent cell. x 190.

Figures 23 and 24. Sections from the contralateral adrenal of the same rat. Iodate reaction. x 190.
Figure 25. Fluorescence photomicrograph of mouse adrenal. Cortex in the left lower corner. x 190.

Figure 26. Section from the contralateral adrenal of the same mouse. Iodate reaction. x 190.
HISTOCHEMICAL DEMONSTRATION OF NORADRENALINE IN THE ADRENAL MEDULLA OF THE HAMSTER

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Two histochemically different kinds of chromaffin cells were four years ago described in the adrenal medulla of the rat (1, 2, 3). After treatment with formalin, cell groups which fluoresce bright green in ultraviolet light were seen in the otherwise poorly fluorescing medulla. Similar fluorescent cells have since then been observed in adrenals of other mammals (4, 5). The histochemical findings have been directly compared with results obtained from chemical adrenaline and noradrenaline determinations in neighboring sections of normal or stimulated adrenals. These studies have shown that the fluorescent cells contain much noradrenaline and little or no adrenaline and that the nonfluorescent medullary cells contain much adrenaline and little noradrenaline (6, 7, 8). In vitro experiments revealed that noradrenaline forms with formalin a poorly water-soluble fluorescent compound, which adrenaline does not, a finding suggesting that the fluorescence of the medullary cell islets is indeed due to the high noradrenaline content (4).

While the above-cited studies were in progress, Hillarp and Hökfelt (9) described a histochemical technique for demonstration of noradrenaline in the adrenal medulla. Whole adrenals or slices of large adrenals were immersed in a buffered solution of potassium iodate and, subsequently, in formalin. In the medullary parts of sections cut from adrenals thus treated, brown areas were seen, which were claimed to indicate the distribution of noradrenaline. Although this claim has been significantly supported by parallel noradrenaline determinations in contralateral adrenals of untreated or insulin-injected animals (9, 10), direct chemical evidence similar to that presented in favour of the fluorescence method is lacking and, moreover, difficult to provide because the iodate method works only with whole glands or thick adrenal slices.

It seemed obviously of great interest to compare the iodate method with the fluorescence method. Hamster's adrenals were selected for the comparative study for two reasons. Firstly, the periphery of hamster's adrenal medulla contains much and the center very little noradrenaline (6). Secondly, the fluorescent islets are present only in the peripheral parts of hamster's adrenal medulla (6).

Untreated hamsters were killed by rapid decapitation. One adrenal of each animal was frozen fresh and cut with a cooled microtome knife at 50 micra. The frozen sections were allowed to thaw in 10 per cent formalin with 1 per cent of calcium chloride. After 2-6 hours the sections were rinsed, mounted in glycerol and examined for fluorescence. The contralateral adrenal was plunged in saturated potassium iodate (11), where it was allowed to
stay overnight. It was then fixed for 2-6 hours in formalin and cut with a freezing microtome at 50 micra.

Fig. 1 illustrates typical sections of hamster's adrenal medulla. The fluorescent islets were always seen in the periphery, and the same applied to the iodate-positive islets. Comparison of full series of formalin-fixed sections, examined for fluorescence, with similar series from iodate-treated adrenals showed a close similarity in the form and size of the fluorescent and the iodate-positive islets. Moreover, the islet patterns were surprisingly similar in formalin-fixed and iodate-treated sections taken from topographically corresponding parts of two adrenals from two different animals.

Some attempts were made to adapt the iodate method for fresh-frozen or freeze-dried sections, so that direct comparison of the two methods in neighboring sections would become possible. These efforts remained fruitless owing to the slow formation of the brown pigment from iodate and noradrenaline and the ready solubility of the latter substance in the reagent.

The fluorescence of the medullary islets was always observed exclusively in the cytoplasm of the cells, and the nuclei remained non-fluorescent. This observation is in good agreement with studies on cellular fractions separated by differential centrifugation, which have demonstrated that the medullary catechols are almost exclusively present in the cytoplasmic granules (12). The iodate reaction, however, was positive both in the nuclei and in the cytoplasm of the islet cells. This fact may probably be ascribed to diffusion, which complicates the iodate method and which also renders necessary the use of whole glands instead of sections (see above).

The present observations clearly show that the fluorescence method and the iodate method give positive reactions with the same medullary cells and that these medullary cells contain principally or exclusively the non-methylated variety of medullary catechols. This is the more interesting as the two methods are entirely different chemically. In practice, the iodate reaction is somewhat simpler because no fluorescence equipment is necessary. On the other hand, the fluorescent islets can be demonstrated in fresh-frozen or in freeze-dried sections, wherefore other sections from the same gland can be used for studies with other histochemical or chemical techniques.

SUMMARY

Formalin-induced fluorescence and a positive iodate reaction are both present exclusively in the peripheral adrenomedullary cells of the hamster. These cells contain principally noradrenaline.
REFERENCES

Figure 27. Fluorescence photomicrograph of hamster adrenal, x 60.

Figure 28. Section from the contralateral adrenal of the same hamster. Iodate reaction, x 60.
(VIII 3)

Distribution of Adrenaline and Noradrenaline in the Hen Adrenal Gland

Recent studies have indicated that the mammalian adrenal medulla contains both adrenaline and noradrenaline, which can be demonstrated both chemically and histochemically. In the adrenals of several mammalian species, specific chromaffin cell groups have been shown to be carriers of most of the adrenomedullary noradrenaline, while the other cells contain predominantly adrenaline. Studies on vertebrates other than mammals are rather few, but a similar secretory duality has been demonstrated in the adrenal medulla of the lizard. In this animal the cord of chromaffin cells embracing the cortical tissue peripherally is apparently specialized in the secretion of noradrenaline, whereas islets of chromaffin tissue intermingling with the cortical cells themselves apparently contain predominantly adrenaline. To my knowledge no report of the distributions of noradrenaline and adrenaline in the adrenals of birds has been published.

In the course of an investigation of the adrenals of various domestic animals, I observed that the chromaffin tissue of the hen adrenal gland has some particularly interesting features, which differ from those of all other medullary tissues hitherto examined. Adrenals of adult, egg-laying hens were used. Slices of fresh adrenals were examined for (1) the chromaffin reaction, which is positive for both noradrenaline and adrenaline, (2) the formalin-induced fluorescence, and (3) the iodate reaction. Methods (2) and (3)

Fig. 1. Camera-lucida drawing of the adrenal gland of a hen. The cortical tissue is shown black, the chromaffin tissue white. Each black dot indicates one noradrenaline cell. Blood vessels are marked with vertical lines.
selectively demonstrate the cells containing nor-
adrenaline. Fluorometric determinations of adrenaline
and noradrenaline, made after chromatographic sep-
oration of these catechol amines, showed that about
one-third of the total catechol amines of the hen
adrenal is noradrenaline.

The well-known fact was confirmed that the
chromaffin tissue is intermingled with the cortical
cells in the adrenals of birds (Fig. 1). The intensity of
the chromaffin reaction was closely the same in all
chromaffin cells. The cells containing noradrenaline
were demonstrated either by the fluorescence method
or by the iodate method with identical results. The
distribution of these cells is indicated by dots in
Fig. 1. The size of each dot corresponds approx-
imately to the size of each cell. Most of the cells
containing noradrenaline were solitary in the chrom-
affin tissue of the hen adrenal and surrounded by
cortical cells or chromaffin cells which were essen-
tially iodate-negative and which did not show
appreciable fluorescence after fixation in formol-
calcium. Such histochemical properties apply to cells
which contain and secrete predominantly adrenaline.
The noradrenaline cells were seemingly distributed
at random in the tongues and islets of the chromaffin
tissue. Certainly there was no tendency of the
noradrenaline cells to predominate in the peripheral
layers of chromaffin tissue, in contrast to conditions
in the adrenals of the lizard.

It is of interest to note that although the cells
containing noradrenaline tend to gather into groups
or cell islets in the mammalian adrenal medulla,
solitary, randomly distributed noradrenaline cells
have been seen in autografts of the rat adrenal med-
ulla\(^5\). Both in the hen adrenal and in the adrenal
autografts of the rat, there seems to be no correlation
between the sites of cells secreting the methylated
variety of catechol amines and the site or amount
of cortical tissue. This fact, together with the observa-
tion that the ratio of noradrenaline to adrenaline
remains constant during the postnatal development
of the rat adrenal gland, in spite of a considerable
increase in the relative size of the cortex (unpublished
work), suggests that the two kinds of adrenomedullary
cells are independent of each other, and that the
quality of the catechol amine they secrete is not
decisively dependent on the concentration of cortical
hormones in the blood surrounding them, as has been
supposed\(^6\).

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Figure 29. Fluorescence photomicrograph of hen adrenal. Cortical cell acini are faintly fluorescent. In the medullary tissue between cortical acini only some solitary cells are fluorescent. x 70.

Figure 30. Section from the contralateral adrenal of the same hen. Iodate reaction. Cortical acini unstained. Medullary tissue weakly stained throughout. Solitary medullary cells strongly iodate positive. x 70.
Adrenaline and Noradrenaline in Adrenal Autografts

Both cortical and medullary cells survive in adrenal grafts months after transplantation into the anterior chamber of the eye. It is, however, not known whether the two medullary hormones, adrenaline and noradrenaline, are present in the grafts. Moreover, all previous work has been without regard to the fact that there are two different types of cells in the adrenal medulla. A substantial body of evidence is now available indicating that one of these cell types contains and secretes adrenaline, and the other noradrenaline. It seemed therefore to be of interest to investigate adrenal grafts by chemical and histochemical methods which differentiate between adrenaline and noradrenaline.

Adult Wistar rats were used. The left adrenal of each animal was removed, and a piece of the medulla was inserted into the anterior chamber of the left eye. Five months later the animals were killed and the grafts removed.

The adrenaline and noradrenaline contents of some grafts were estimated chemically after separation of these amines by ascending paper chromatography, using a mixture of phenol and 0.1 N hydrochloric acid. Both adrenaline and noradrenaline were detected in the grafts (Fig. 1). The adrenaline content was 5–10 times the noradrenaline content.

Other grafts were plunged into a 3.5 per cent solution of potassium dichromate to produce the chromaffin reaction, or into a saturated solution of potassium iodate to demonstrate the noradrenaline-containing cells. These grafts were afterwards fixed in formalin and cut with a freezing microtome at 50μ. Some sections were mounted unstained in glycerol, others were stained with hematoxylin and sudan red to facilitate the detection of adrenocortical cells.

![Base line and Front line](image)

**Fig. 1.** Chromatogram of an adrenomedullary autograft smeared directly on the base line of the paper (Whatman No. 1). Phenol and hydrochloric acid were used for development, and the strip was sprayed with potassium ferricyanide to develop fluorescence in the adrenaline and noradrenaline spots. Fluorescence photograph.
Fig. 2. Unstained frozen section of an adrenomedullary autograft in the anterior chamber of the eye. The graft is attached to the cornea (above) and the iris (below). Chromaffin cells are brown after dichromate fixation. Red blood cells in the vessels of the iris (lower left corner) are also brown.

Fig. 2 shows a typical graft, mainly composed of strongly chromaffin cells, and attached to both the cornea and the iris. The chromaffin cells were closely packed together in the grafts. However, regular cell acini, such as can be always seen in the normal adrenal medulla, were replaced by irregular groups of cells. This may be indicative of cellular migration within the graft.

The iodate reaction, which stains the noradrenaline-containing cells brown, was positive in some graft cells, which were usually solitary and randomly distributed in the graft. The majority of graft cells, although chromaffin, remained colourless after iodate treatment. This is in good agreement with the chemical observations made, and suggests that the grafted adrenomedullary cells have retained their ability to make and secrete the same catechol amine which they were making and secreting in the adrenal before grafting.

Although efforts had been made to include only medullary tissue in the grafts, typical sudan-positive cortical cells were detected in all grafts which were examined histologically. The cortical cells were fewer than the medullary ones and usually apart from these. It is therefore most likely that cortical hormones were equally available to both the iodate-positive, noradrenaline-secreting cells and the adrenaline-secreting cells of the graft, in which the two types of cells were present in proportions similar to those in the normal rat’s adrenal medulla. If cortical
hormones really have a specific influence on the quality of the secretory products of the adrenomedullary cells, as has been claimed, such an influence could not be observed in the present study.

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Figure 31. Autograft of rat adrenal medulla in the eye. Chromaffin reaction, which stains brown both the medullary cells and the erythrocytes. x 65.

Figure 32. Similar autograft. Iodate reaction. Only some solitary cells are stained intensely the background being weakly positive. x 130.

Figure 33. Fluorescence photograph of a chromatogram. Adrenal autograft was smeared on the base line. Two spots corresponding to adrenaline and noradrenaline clearly visible.
(IX)

RADIOAUTOGRAPHIC DEMONSTRATION OF NORADRENALINE AND ADRENALINE IN THE ADRENAL MEDULLA WITH I^{131}-LABELLED IODATE AND IODATE-FORMALIN

OLAVI ERÄNKÖ
RADIOAUTOGRAPHIC DEMONSTRATION OF NORADRENALINE AND ADRENALINE IN THE ADRENAL MEDULLA WITH I\textsuperscript{131}-LABELLED IODATE AND IODATE-FORMALIN

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Fixation with a solution containing potassium dichromate and formalin is the classic method for the study of the adrenal medulla, which is stained brown by this reagent (8, 11, 12). It has long been believed that the brown color is due to the presence of chromium, and, after Kohn (11, 12), the reaction is generally called the “chromaffin” reaction.

However, Gerard, Cordier & Lison (7) observed that the medulla can be rendered colored by immersion of the adrenal into a mixture of potassium iodate and formalin. From this and other observations Gerard et al. concluded that this color and that obtained with a mixture of potassium dichromate and formalin are both due to oxidation products of adrenaline, rather than to the presence of iodine or chromium in the treated medulla. They therefore suggested that the term “chromaffin” should be replaced by “pheochrome”. Bennett (1) confirmed the observations made by Gerard et al., and proposed the term “fusogenic”.

The subject was reinvestigated by Coupland (2). He determined chemically the chromium content in the medulla of adrenals fixed in dichromate-formalin and prepared radioautographs of adrenal sections fixed in a mixture of Cr\textsuperscript{6+}-labelled dichromate and formalin. He was thus able to demonstrate that chromium is fixed by the cells of the adrenal medulla during fixation in dichromate-formalin. He suggested that a chrome-adrenochrome is produced, and that this compound is responsible for the brown color reaction, which is thus truly “chromaffin”.

As compared with the chromaffin technique, fixation of adrenals with iodate-formalin has less frequently been used for the demonstration of adrenomedullary catechols. Coupland (2) mentions that the color produced in the medulla by iodate-formalin is less intense and without the yellow tinge of the chrome-fixed material; hence iodate-formalin would be of less value in routine histological work.

Potassium iodate is, however, of considerable interest as a histochemical reagent, since Hillarp and Hökfelt (9) observed that only certain clearly delineated groups of medullary cells become brown in adrenals which are first immersed in a solution of potassium iodate and then fixed in formalin. These workers (9, 10) suggested that iodate “stains” specifically the noradrenaline-containing cells, leaving the adrenaline-containing cells almost uncolored. Eränkö (5, 6) showed that the iodate-positive medullary cells are identical with the cells shown earlier to fluoresce intensely in ultra-violet light after formalin fixation (3), which cells have been directly demonstrated to contain predominantly noradrenaline (4).
It seemed to be of interest to investigate whether iodine is present in the brown reaction products seen in adrenals treated with potassium iodate and in adrenals fixed with a mixture of potassium iodate and formalin. Results of such a study are described in this paper, together with some observations on the “chromaffin” reaction.

**MATERIALS AND METHODS**

Healthy adult hamsters, mice and rats were killed by rapid decapitation with sharp scissors. The adrenals were rapidly removed and plunged into the reagent.

Radioactive iodate solution was prepared by mixing 1 volume of a carrier-free solution of I\(^{131}\) ion with 9 volumes of a saturated potassium iodate solution. The radioactivity of the mixture varied from 20 to 200 μcuries/cc. The mixture was allowed to stay in a tightly stopped bottle at 50°C for three days or more. It was thought that a considerable proportion of I\(^{131}\) had after this period become oxidised to I\(^{127}\)O\(_2\). Because both adrenaline and noradrenaline in is essential improvement in resolution actual chemical fixation, only the glands were transferred in 10% formalin (c. 4% HCHO), fixed in this solution for 1/2—24 hours, and sectioned as above.

Whole adrenals were plunged into the radioactive solution thus prepared, in which they were allowed to stay for 1/2, 3, 12, 72 or 96 hours. A number of the glands were thereafter cut at 50 μ with a freezing microtome, and the sections were collected in distilled water. The remaining glands were transferred in 10% formalin (c. 4% HCHO), fixed in this solution for 1/2—24 hours, and sectioned as above.

Some adrenals, as a rule from the same animals whose contralateral adrenals were plunged in the radioactive iodate solution, were fixed in a mixture containing 1 volume of commercial formalin and 9 volumes of the radioactive iodate solution. After 1/2—96 hours' fixation, the glands were sectioned as described above.

Sections from the iodate-treated glands were mounted together with the sections from the corresponding iodate-formalin-fixed glands on thoroughly cleaned glass slides and allowed to dry. The slides were then immersed for 5 min. in a 1% solution of celloidin dissolved in equal parts of ether and absolute alcohol, and for another 5 min. in 70% alcohol. Thereafter the slides were thoroughly rinsed in running tap water and in distilled water, and allowed to dry.

Radioautographs were prepared by placing the emulsion surface of Gevaert “Duplo Copy” 35 mm film against the sections. The film was sandwiched between the slide carrying the section and another, clean slide. The exposure time was determined after measurement of the radioactivity of the section with a Geiger counter. A total of 5—10 million beta particles per sq. cm. were allowed to strike the emulsion to obtain an optimal exposure. Actual exposure times ranged from a few hours to a few days.

After the exposure, the film was removed, developed, fixed, washed and dried. No essential improvement in resolution was observed when a fine-grain developer was used instead of the much more rapid ordinary paper developer.

The sections of the autographed slides were then mounted in glycerol or Canada balsam. The sections and the corresponding radioautographs were photographed under identical conditions, either through a microscope or through an ordinary enlarging apparatus, and positive prints were then prepared.

To be able to compare the effects of iodate-formalin and dichromate-formalin, a series of mouse adrenals were immersed for 1/2—96 hours into a solution containing 9 volumes of 3.5% K$_2$Cr$_2$O$_7$ and 1 volume of concentrated formalin. Thereafter sections were cut at 50 μ. These were rinsed in water and mounted in glycerol.

**RESULTS**

1. Iodate-treated adrenals: Brown, clearly delineated groups of medullary cells were observed in the adrenals treated with the radioactive iodate solution (Figs. 1 and 3). The
**FIG. 1.** Photomicrograph of an unstained section from a mouse adrenal cut after treatment with the radioactive potassium iodate solution and fixation in formalin. The noradrenaline-containing cell islets are dark brown, other medullary cells faintly yellow, the cortex colorless. Photographed through a blue filter.

**FIG. 2.** Radioautograph of the section shown in Fig. 1. The distribution of the radioactivity closely corresponds to that of the iodate reaction. Some radioactivity is seen in the cortex.

**FIG. 3.** Iodate reaction in the adrenal medulla of a hamster. Technique as in Fig. 1. Typical peripheral distribution of the noradrenaline-cells.

**FIG. 4.** Radioautograph of the section shown in Fig. 3.

**FIG. 5.** Photomicrograph of an unstained section from a mouse adrenal fixed in the mixture of radioactive iodate and formalin for 3 hours. The medulla is salmon red but there is a brown spot in the upper part of the medulla. The cortex is colorless.

**FIG. 6.** Radioautograph of the section shown in Fig. 5. There is little radioactivity in the medulla, except for the brown spot. The cortex is strongly radioactive.

**FIG. 7.** Radioautograph of a section from a mouse adrenal immersed overnight in the mixture of radioactive iodate and formalin. Both the cortex and the medulla are strongly radioactive.
rest of the medulla was pale yellow, and the cortex was entirely colorless. No differences were observed between the glands immersed in iodate for 3 hours and those treated longer. Similarly, fixation in formalin after the iodate treatment was without effect on the intensity and distribution of the reaction. The reaction was strongest in the iodate-positive medullary cells of the hamster adrenals and least intense in those of the rat adrenals.

Radioautographs showed that the distribution of radioactivity closely corresponded to the distribution of the brown reaction (Figs. 2 and 4). However, some radioactivity was present also in the cortex, though this activity was small as compared with the activity of the iodate-positive islets.

2. Iodate-formalin-treated adrenals: No reaction at all or a weak color in the peripheral parts of the medulla was seen in adrenals fixed in the iodate-formalin solution for 3/4 hour. Glands immersed for 3 hours in this reagent showed a salmon red color throughout the medulla. This red color resembled closely that of an adrenochrome solution. Occasionally, brown spots were seen in the periphery of the medulla, particularly in such medullary areas as were covered by a thin layer of cortex (Fig. 5). The medulla of adrenals fixed for 12 hours or more was brown with a yellow tinge throughout.

Distribution of radioactive materials in adrenals fixed with the radioactive iodate-formalin solution was entirely different from that seen in the iodate-treated adrenals. The cortex exhibited an intense radioactivity both in briefly fixed glands (Fig. 6) and in glands fixed for several days (Fig. 7). On the other hand, a strong radioactivity was seen only in those parts of the medulla which were stained yellowish-brown. Thus, the whole medulla was active in adrenals fixed for 12 hours or more (Fig. 7), while the radioactivity was restricted to the brown areas in the periphery of the briefly fixed glands, although the rest of the medulla in these glands gave a red reaction (Fig. 6).

3. Dichromate-formalin-treated adrenals: Glands immersed in the dichromate-formalin solution showed a brown coloration of the medulla which closely resembled the coloration seen in the iodate-formalin fixed adrenals. After a short immersion in the reagent the medulla was red. However, the peripheral areas of the medulla were sometimes brown. A brown color was observed in all medullary cells of the glands immersed in the dichromate-formalin mixture for 12 hours or more. The intensity of the brown color seen in the medulla of the dichromate-formalin-fixed glands was perhaps somewhat stronger than that in adrenals fixed for an equally long period in iodate-formalin, but the difference was not marked and subjectively the color seemed the same in both instances.

DISCUSSION

In the present study the distribution of radioactive iodine in the medulla of the iodate-treated adrenals closely corresponded to the distribution of the brown color. It seems reasonable to conclude that the brown color is indeed due to an iodine-containing compound. Knowing that iodo-adrenochrome and iodo-noradrenochrome are formed when adrenaline and noradrenaline are oxidised by iodate in vitro (10, 13), this observation does not seem surprising. However, it may be that the brown compound is, considering its poor solubility, a polymeric iodine-containing oxidation product of noradrenaline, rather than iodo-noradrenochrome. That only a weak yellow color and but little iodine was observed in medullary cells other than those forming the positive islets is readily explained by the fact that these cells contain little noradrenaline and much adrenaline, whose reaction product with iodate is too soluble in water to be retained in situ (see 5, 6, 9, 10).

If, as it seems, the iodate-positive cells specifically bind radioactive iodine after treatment with I\(^{131}\)-labelled iodate because of their noradrenaline content, the described radioautographic method might prove useful for the demonstration
of noradrenaline in such sites where the color produced by the iodate reaction is too weak to be detected visually.

The present work suggests that the brown color seen in all medullary cells after a sufficiently long treatment in the iodate-formalin solution is also due to oxidation products of adrenaline and noradrenaline into which iodine is linked, quite as chromium has been seen to be linked in the brown substance of dichromate-formalin-treated adrenals (2). It also shows that the brown iodine-containing reaction product is formed from an originally red product which contains little or no iodine.

It is quite possible that this initial red color is due to the same substance as the red color observed in the adrenals briefly treated with dichromate-formalin. Therefore, Gerard et al. (7) and Bennett (1) were seemingly right in maintaining that a positive color reaction is given by the adrenal medulla without linkage of iodine or chromium in the colored reaction product. On the other hand, the presence of iodine or chromium seems to be essential for the brown reaction, which can be seen in adrenals fixed overnight or longer in iodate-formalin or dichromate-formalin. Coupland’s (2) failure to obtain a brown color with iodate-formalin may perhaps be ascribed to the lower concentration (3%) of iodate in his iodate-formalin mixture. In the present work, in which saturated KIO₃ was used, the intensity of the medullary reaction was fully satisfactory even for routine work.

Why both adrenaline and noradrenaline give positive reactions in adrenals immersed in an iodate-formalin mixture, although only noradrenaline gives it in iodate, is an interesting question whose further evaluation is outside the scope of the present article. It may be that red oxidation products of adrenaline, such as adrenochrome and iodo-adrenochrome, which are soluble in water, are polymerised and linked with tissue proteins under the strong link-building influence of formalin.

Another difference between the results obtained with the iodate treatment and the iodate-formalin treatment was the fixation of iodine by the cortex in the iodate-formalin-treated but not in the iodate-treated adrenals. An explanation could be that iodine, which is formed by reduction of iodate by the tissues, is fixed by unsaturated compounds of the cortex. However, it could be expected that immersion in the alcohol-ether-celloidin mixture had dissolved away most cortical lipids. Lipids insoluble in alcohol-ether and substances other than lipids may therefore be responsible for the fixation of iodine. However that may be, variations were observed in the radioactivity of the cortex in the adrenals fixed in radioactive iodate-formalin. This suggests that differences in the functional state of the cortex may influence its iodine-binding capacity.

**SUMMARY**

Adrenals of the hamster, the mouse and the rat were studied. Radioactive iodate solution was prepared by mixing carrier-free I¹³¹ with a saturated solution of potassium iodate. In adrenals immersed in this solution for 3–96 hours and subsequently fixed in 10 per cent formalin, a typical brown reaction was seen in some, presumably noradrenaline-containing, cells of the medulla.
Radioautographs prepared of the same sections indicated that the distribution of the brown color and that of the radioactivity were identical. This suggests that the brown color produced by treatment with iodate is due to an iodine-containing compound. The cortex exhibited only a weak radioactivity and no color.

Adrenals immersed in a mixture of 1 volume of commercial formalin and 9 volumes of the radioactive iodate solution for 12–96 hours showed a brown color in all medullary cells. The medulla was demonstrated by radioautographs to contain large quantities of iodine. However, the cortex was also strongly radioactive in spite of previous treatment with an alcohol-ether mixture.

Some observations on the chromaffin reaction were also reported.

REFERENCES

Figure 34. Mouse adrenal. Iodate reaction obtained by using radioactive iodate. x 30.

Figure 35. Radioautograph of the same section. x 30.

Figure 36. Mouse adrenal fixed in the mixture of radioactive iodate and formalin for 3 hours. x 30.

Figure 37. Radioautograph of the same section. x 30.

Figure 38. Radioautograph of mouse adrenal fixed overnight in the mixture of radioactive iodate and formalin. x 30.
The observations already described are well compatible with the hypothesis that the fluorescent cells in the adrenal medulla are specialised in the production of noradrenaline. However, comparison of the fluorescence test and the iodate reaction in more numerous species seemed to be desirable for the reason that the fluorescence observed after calcium-formol fixation in the cell islets of the adrenal medulla of the rat was much more intense than that in the fluorescent medullary of the mouse, although the iodate reaction was clearly stronger in the mouse adrenal than in the rat adrenal.

The species additionally studied with both of these two histochemical methods were man, the pig, the cow, the sheep, the horse, the cat, the hedgehog, the rabbit and the guinea pig. Pieces of adrenals of all these species were immersed immediately after removal in the calcium-formol fixative, in a saturated solution of potassium iodate and in a mixture of 1 volume of 3.5 per cent potassium dichromate and 19 volumes of concentrated formalin. Human adrenals were obtained from surgical operations; and to ensure freshness of the specimen the surgeon cut out a piece before ligation of the adrenal arteries. Adrenals of the domestic animals were obtained from the slaughter-house, where they were by
special arrangement removed immediately after killing of the animals.

The calcium-formol-fixed pieces of adrenals were sectioned at 50 micra 4-6 hours later, mounted in glycerol and examined with the fluorescence microscope. The pieces immersed in the iodate or the dichromate solution were in the next day transferred in 4 per cent formaldehyde solution, fixed in it for 1 or 2 days, sectioned at 50 micra, mounted in glycerol and examined in transmitted light. Adrenals of at least three animals of each species were examined in this way. Seven human adrenals and six pig adrenals were studied.

In all adrenals examined there was a good agreement between the results obtained with the two histochemical methods: the pattern of the fluorescent medullary cells was in all species similar to that of the iodate-positive cells in the same adrenal or in the contralateral adrenal of the same animal.

In man, most of the medullary tissue showed only a dull fluorescence, excepting the blood vessels and the islets of cortical cells. In several sections cut through the gland, no medullary cells with green fluorescence were seen. However, in some sections of most human adrenals examined, groups of medullary cells exhibiting a green fluorescence were observed (Figures 39 and 40). In the adrenals immersed in the iodate solution, similar groups formed by iodate-
Figure 39. Fluorescence photomicrograph of human adrenal medulla. Strong fluorescence in the connective tissue fibers of a vein (left upper corner). Medullary fluorescence weak throughout. In the center a group of cells with a somewhat more intense fluorescence. x 100.

Figure 40. Similar section. A small group of fluorescent cells visible in the center. Horizontal cortico-medullary junctions visible in the upper and lower parts of the picture. x 100.
positive cells were seen against a pale yellow background of the main bulk of medullary cells (Figures 41 and 42). The intensities of both the fluorescence and the iodate reaction in these cell islets were weak as compared with those in the adrenals of the species earlier examined. All medullary cells were strongly chromaffin in the adrenals of man and the other species examined.

In the pig, the cow, the sheep and the horse, large groups of strongly fluorescent and strongly iodate-positive cells were observed in the adrenal medulla (Figures 43-47). In all these animals the intensity of the iodate reaction in these specific cells was stronger than in any other species studied. The fluorescence, which in each species showed a distribution pattern similar to that demonstrated by the iodate reaction, was however not essentially more intense than that seen in most other animals. Differences were seen in the intensity of the iodate reaction outside the specific medullary cell islets. Thus, a pale yellow colour was seen in these cells in the pig adrenal medulla (Figure 44), while the background was brownish in the horse adrenal medulla (Figure 47).

The cat adrenals contained iodate-positive medullary cell islets similar to those demonstrable by fluorescence microscopy (Figure 48). A weak iodate reaction was detected in all medullary cells of the rabbit and the guinea pig but
Figure 41. Section from human adrenal. Iodate reaction. A part of the cortico-medullary junction marked with ink lines. Iodate-positive islet in the medulla indicated by arrow. Taken with large condenser aperture to avoid light refraction by cortical cells. x 45.

Figure 42. Same section. The ratio of condenser aperture to objective aperture smaller, which renders islets of cortical tissue (C) visible in the medulla. x 110.
Figure 43. Section from pig adrenal. Iodate reaction. A part of the cortico-medullary junction marked with ink lines. Note the very strongly positive medullary cell islets. x 45.

Figure 44. Same section. x 110.
Figure 45. Section from cow adrenal. Iodate reaction. x 110.

Figure 46. Section from sheep adrenal. Iodate reaction. A part of the cortico-medullary junction marked with ink lines. x 110.

Figure 47. Section from horse adrenal. Iodate reaction. Cortico-medullary junction lined with ink. Note the dark background due to a relatively intense iodate reaction. x 110.
Figure 48. Sudan-stained section from cat adrenal. Iodate reaction. A part of the cortico-medullary junction marked with ink. Sudan-positive cortical cells appear black, iodate-positive medullary cells dark. x 110.

Figure 49. Section from hedgehog adrenal. Iodate reaction. A part of the cortico-medullary junction marked with ink. Erythrocytes black. Note the variation in the intensity of the iodate reaction in different parts of the medulla. x 110.
neither distinct iodate-positive cell groups nor fluorescent islets were seen.

The adrenal medulla of the hedgehog was unique in showing considerable variation in the intensity of the iodate reaction in different parts of the medulla but no distinctly delineated iodate islets (Figure 49). Similar variation was also seen in the fluorescence intensity of the medullary cells, although the intensity differences were less marked. However, the chromaffin reaction was fairly even in all medullary cells.

Figure 50 shows the distribution of the iodate reaction in whole sections of adrenal medulla. The fairly regularly negative peripheral zone in the medullas of the pig, the cow, the sheep and the horse was a phenomenon typical of these species.

Results of the few quantitative determinations made are given in Table 10. Data presented in this table are obtained in the same way as those in Tables 2-3.

Attempts were made to determine the proportions of adrenaline and noradrenaline in small pieces (50 x 50 x 50 micra) of human and pig adrenal medulla. Preliminary experiments after chromatographic separation of the amines on large sheets of chromatography paper were disappointing because the blank readings obtained from eluates without any adrenal tissue showed fluorescence intensities approaching
Figure 50. Camera-lucida drawings of the distribution of iodate-positive cell islets (black) in the adrenal medulla of different mammals. Note the iodate-negative periphery in the medulla of the sheep, the pig, the horse and the cow. In the two drawings of human adrenal medulla islets of cortical tissue have been outlined.
<table>
<thead>
<tr>
<th>Species</th>
<th>Adr. + Nor. (% of dry med wt.)</th>
<th>Noradrenaline (% of adr. + nor.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Man 1</td>
<td>6.4</td>
<td>17</td>
</tr>
<tr>
<td>2</td>
<td>4.9</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>4.8</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>6.2</td>
<td>13</td>
</tr>
<tr>
<td>5</td>
<td>7.2</td>
<td>11</td>
</tr>
<tr>
<td>6</td>
<td>3.7</td>
<td>15</td>
</tr>
<tr>
<td>Pig 1</td>
<td>x)</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>x)</td>
<td>33</td>
</tr>
<tr>
<td>Cow</td>
<td>7.4</td>
<td>28</td>
</tr>
<tr>
<td>Sheep</td>
<td>4.9</td>
<td>36</td>
</tr>
<tr>
<td>Horse</td>
<td>9.6</td>
<td>36</td>
</tr>
<tr>
<td>Hedgehog 1</td>
<td>5.5</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>8.3</td>
<td>33</td>
</tr>
<tr>
<td>3</td>
<td>11.3</td>
<td>33</td>
</tr>
<tr>
<td>4</td>
<td>12.4</td>
<td>49</td>
</tr>
<tr>
<td>5</td>
<td>7.8</td>
<td>20.7</td>
</tr>
</tbody>
</table>

x) Samples not weighed.
closely the sample figures. The size of the strips was therefore diminished and the experiments renewed.

It was found that an approximate idea of the relative amounts of the two catechol amines in each sample could be obtained by spraying the whole chromatogram with potassium ferricyanide and by examining the fluorescence of the adrenaline and noradrenaline spots, which could be differentiated from each others with the aid of their colour even when no complete separation had occurred after a short period of chromatography.

Figures 51-55 are fluorescence photographs of such "microchromatograms" of adrenomedullary pieces of the pig (Figures 51-53) and of man (Figures 54 and 55). They suggest that, as could be expected according to the histochemical observations (e.g. Figure 50), the relative proportions of adrenaline vary from piece to piece in the sections of pig adrenal medulla but keep relatively constant in pieces of human adrenal medulla. The same variability and agreement with histochemical observations are evident from Table 11, which shows the results obtained from the first successful quantitative determinations of this kind. Thereafter, unfortunately, the writer lost access to the equipment by which such small amounts of catechol amines could be determined.
Figures 51-55. Fluorescence photographs of "microchromatograms" of pig (Figures 51-53) and human (Figures 54 and 55) adrenal medulla. Note that in the pig the intensity of the upper (adrenaline) spot is low when the intensity of the lower (noradrenaline) spot is high, and vice versa, while in man the intensity of the two spots shows no such reverse relationship.
TABLE II.

Adrenaline and noradrenaline in freeze-dried square pieces of pig adrenal medulla.

<table>
<thead>
<tr>
<th>Piece</th>
<th>Adrenaline (ng.)</th>
<th>Noradrenaline (ng.)</th>
<th>Adr. + Nor. (ng.)</th>
<th>Noradrenaline (% of adr. + nor.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>99</td>
<td>7</td>
<td>106</td>
<td>7</td>
</tr>
<tr>
<td>A2</td>
<td>26</td>
<td>38</td>
<td>64</td>
<td>59</td>
</tr>
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<td>A3</td>
<td>33</td>
<td>31</td>
<td>64</td>
<td>49</td>
</tr>
<tr>
<td>A4</td>
<td>73</td>
<td>37</td>
<td>110</td>
<td>34</td>
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<tr>
<td>A5</td>
<td>112</td>
<td>6</td>
<td>118</td>
<td>5</td>
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<tr>
<td>B1</td>
<td>95</td>
<td>5</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td>B2</td>
<td>35</td>
<td>53</td>
<td>88</td>
<td>60</td>
</tr>
<tr>
<td>B3</td>
<td>16</td>
<td>51</td>
<td>67</td>
<td>81</td>
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<td>B4</td>
<td>49</td>
<td>29</td>
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<td>37</td>
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<tr>
<td>B5</td>
<td>93</td>
<td>13</td>
<td>106</td>
<td>12</td>
</tr>
<tr>
<td>C1</td>
<td>78</td>
<td>15</td>
<td>93</td>
<td>16</td>
</tr>
<tr>
<td>C2</td>
<td>11</td>
<td>70</td>
<td>88</td>
<td>80</td>
</tr>
<tr>
<td>C3</td>
<td>27</td>
<td>64</td>
<td>91</td>
<td>70</td>
</tr>
<tr>
<td>C4</td>
<td>98</td>
<td>23</td>
<td>121</td>
<td>19</td>
</tr>
<tr>
<td>C5</td>
<td>16</td>
<td>35</td>
<td>51</td>
<td>69</td>
</tr>
<tr>
<td>C6</td>
<td>26</td>
<td>14</td>
<td>30</td>
<td>16</td>
</tr>
<tr>
<td>C7</td>
<td>76</td>
<td>12</td>
<td>88</td>
<td>14</td>
</tr>
</tbody>
</table>

Order of pieces in the section:

| A1 A2 A3 A4 A5 | periphery | B1 B2 B3 B4 B5 | periphery | C1 C2 C3 C4 C5 C6 C7 | center |

Note the low noradrenaline content of the peripheral pieces of adrenal medulla (A1, A5, B1, B5, C1, C6 and C7).
(XI) DISCUSSION.

It was observed in the present study that there is a significant correlation between the relative noradrenaline content and the relative amount of fluorescent medullary tissue in the adrenal medullas of different species. It was also shown with adrenals of the cat and the hamster that medullary areas with much fluorescent tissue contain much noradrenaline and little adrenaline, while much adrenaline and little noradrenaline is found in virtually non-fluorescent regions of the medulla. Quasi-quantitative and quantitative observations on the adrenal medulla of man and the pig, although few, were also similar.

These observations suggest that noradrenaline is stored in those cells of the adrenal medulla which fluoresce after fixation in calcium formol. Adrenaline, on the other hand, would seem to be present mainly, if not exclusively, in the non-fluorescent medullary cells, which however contain also some noradrenaline, presumably as a precursor of adrenaline.

Independent work by Hillarp and Hökfelt (1953, 1955) strongly supports the view that adrenaline and noradrenaline are formed by separate cells in the adrenal medulla. Hillarp and Hökfelt (1953) found, firstly, that potassium iodate produces a brown precipitate with noradrenaline but forms
a soluble compound with adrenaline and, secondly, that the extent and the intensity of the iodate reaction – localised in distinctly delineated cell islets – is in the adrenal medullas of different species in a fair correlation to the relative noradrenaline content.

Although the specificity of the iodate reaction to noradrenaline were accepted, its validity in the accurate localisation of noradrenaline in tissues is rendered somewhat questionable by some observations. Potassium iodate has earlier been used as a histochemical reagent in a study on the rat adrenal medulla (Fränkô, 1952). In fresh adrenal sections treated with potassium iodate only a transient red colour was seen over the whole medulla but no permanent staining was obtained. In vitro experiments show indeed that the formation of the brown precipitate in a mixture of potassium iodate and noradrenaline is relatively slow. Therefore, when fresh adrenal sections are immersed in, or floated on, a solution of potassium iodate, all catecholamines dissolve out of the section before the brown precipitate is formed. Although a precipitate is obtained in the adrenal medulla when fresh pieces of, or whole, adrenal glands are immersed in a solution of potassium iodate, it cannot be taken as granted that some dislocation of noradrenaline does not occur before precipitation of the brown reaction product in the medulla.
No iodate reaction is obtained in a briefly frozen adrenal although a fresh gland of the same animal would show a strong reaction in the medulla. This fact together with the observation, to be discussed later, that both adrenaline and noradrenaline are in the intact adrenal medulla packed in small granules covered with a membrane which is broken by freezing explains why the iodate reaction is only transient in fresh-frozen sections. Still, the possibility remains that, even if the diffusibility of noradrenaline is in a fresh gland restricted by its storage in membrane-covered granules, the membranes may be broken by the iodate reagent with the resulting diffusion of noradrenaline within the gland before the formation of the brown precipitate.

Although these theoretical objections can be made against the iodate reaction as a histochemical test, usual signs of diffusion cannot be seen in sections cut from adrenals immersed fresh in a solution of potassium iodate, unless the solution is made too acid (see Hillarp and Hökfelt 1953, 1955); there is no colour at all in the cortical cells and the reaction is clearly delineated in the specific groups of medullary cells.

Both the studies made using the fluorescence method and those with the iodate reaction independently lead to the postulation of specific, separate adrenaline-containing and noradrenaline-containing cells in the adrenal medulla. The
observation made in the present work that evidently the fluorescence test and the iodate reaction demonstrate the same cells in the adrenal medulla further supports this view. Although direct comparison of these two tests cannot be made in neighbouring sections, because the iodate reaction demands the use of whole glands or large pieces, the adrenals of the species examined showed such a variation in the distribution patterns of the fluorescent and the iodate-positive medullary cells that a discrepancy in the distributions of the two tests compared, had it been present, should have been noticed.

All the observations referred to hitherto have been made by studying adrenals obtained from intact animals, and accordingly the conclusions drawn only apply to adrenals with a normal content of catechol amines and a relatively slow secretion rate. The question therefore arises whether the fluorescence test and the iodate reaction similarly demonstrate the noradrenaline-containing cells in an adrenal medulla whose output of adrenaline and/or noradrenaline is experimentally changed.

It was observed in the present study that insulin, which in a short-term experiment causes a great loss of adrenaline from the adrenal medulla of the rat but does not affect its noradrenaline content, has no detectable effect on the distribution or intensity of the calcium-formol-induced fluorescence. On the other hand, the chromaffin reaction was
in the adrenal medulla of the insulin-injected animals positive only in cell groups whose shape and distribution were similar to the fluorescent cell islets. Hökfelt and Hillarp (1954) observed that the iodate reaction of the cat adrenal medulla remains unchanged under similar conditions.

On the other hand, it has been shown that a single injection of nicotine, which causes a loss of noradrenaline from the rat adrenal medulla, sometimes accompanied with a loss of adrenaline, sometimes not, also weakens the fluorescence of the medullary islets or induces its complete disappearance (Eränkö, 1956). Likewise, a single injection of reserpine causes both a selective depletion of noradrenaline and a disappearance of both the fluorescence and the iodate reaction from the adrenal medulla of the rat, in the mouse, repeated injections of reserpine also result in a loss of noradrenaline and disappearance of the two histochemical reactions (Eränkö and Hoppe, 1958).

Daily injections of nicotine for several months were in the present work found to increase the volume of the fluorescent medullary tissue of the rat adrenal medulla and, in the same proportion, its noradrenaline content. Later work (unpublished) has shown that the same treatment equally increases the amount of iodate-positive medullary tissue.

These studies are so far the only ones in which the fluorescence test, the iodate reaction and quantitative
determinations of adrenaline and noradrenaline have been applied to adrenals whose catechol amine content has been experimentally altered. In all of them, the change in the noradrenaline content, increase or decrease, was accompanied by corresponding changes in the fluorescence and in the iodate reaction of the adrenal medulla. This would seem to indicate that these two histochemical methods either really demonstrate noradrenaline or a substance whose site and amount are closely correlated with those of noradrenaline.

In view of the behaviour of mixtures of noradrenaline and iodate in vitro, the former alternative seems more likely, as far as the iodate reaction is concerned. It is less certain that the fluorescence test really depends directly on noradrenaline. In vitro experiments of the present study showed, it is true, that addition of formalin into a solution of noradrenaline results in the formation of a precipitate which fluoresces green, while no precipitate appears in mixtures of formalin and adrenaline. However, the fact that the fluorescence of the adreno-medullary cell islets can be brought about by treating fresh-frozen sections, in which noradrenaline is presumably in a freely diffusible form, with the formalin fixative, presumes an instantaneous precipitation reaction between formalin and noradrenaline, for the fluorescence thus produced in the specific cells to be due to their noradrenaline content. Since such a rapid
reaction does not occur in vitro, it is more likely that the fluorescence test is caused by a substance other than noradrenaline in the specific cells. It is difficult to guess the nature of this substance but its poor diffusibility in fresh-frozen sections might be an indication of its being a protein.

Support to the assumption that the fluorescence test does not demonstrate the same substance in the noradrenaline-containing cells as the iodate reaction is given by the observation made in this study that the fluorescence intensity and the intensity of the iodate reaction do not always run parallelly. If the calcium-formol-induced fluorescence of the specific cells were due to a substance other than noradrenaline, it would be natural that its amount, reflected in the intensity of the fluorescence, does not necessarily follow variations in the noradrenaline content of these cells, supposedly demonstrable with the iodate reaction. On the other hand, it is well known that the fluorescence intensity of many substances is essentially dependent not only on the amount of the fluorescent substance itself but also on the presence of even minute quantities of interfering substances (Pringsheim, 1949). Therefore, even if the fluorescence is due to noradrenaline, its intensity need not be a reliable indicator of its concentration.
Recent investigations have shown that several histological and histochemical methods not mentioned in this work can be used to demonstrate differences in the cells of the adrenal medulla. Thus, Picard and Vitry (1954); Picard, Vitry and Chambost (1955) found that the peripheral parts of the adrenal medulla of the horse and the cow exhibit an intense reaction for acid phosphatase, while the center reacts but weakly. These writers assumed that the peripheral acid phosphatase positive, cells were specialised in the production of adrenaline and the central cells in that of noradrenaline. They did not provide any data to prove this assumption but the observation made in the present study that the periphery of the adrenal medulla of these animals contains predominantly adrenaline and the center predominantly noradrenaline (Figure 50) is in favour of it.

In these two species there does then seem to be a similar relationship between the content of adrenaline and the acid phosphatase activity as in the rat adrenal medulla (Brünkös, 1952), although the same does not apply to the adrenals of the cat, the dog and the mouse (the present study). Picard and Vitry (1956) observed also that the peripheral medullary cells in the adrenals of the horse and the cow are metachromatically stained by toluidine blue, while the centrally situated cells are, as a rule, not.

Histological staining methods were reported recently
by both Bänder (1954) and Kracht and Klein (1958) for the
differentiation of two types of medullary cells in the
adrenal medulla. While Bänder's new report was an elaboration
of his earlier paper (1951) in which he described
"fuchsinophil" and "piorinophil" cells, Kracht and Klein
used in addition to their histological methods also the
fluorescence test and the iodate reaction. They found that
the fluorescent, iodate-positive cells are stained dark with
Weigert's haematoxylin in adrenals fixed in dichromate-
containing fixatives, while the non-fluorescent, iodate-
negative cells remained only lightly stained. This applied
to adrenals of the mouse, the rat, the hamster, the cow, the
cat, the monkey and man.

In a study of the adrenal medulla of the mouse, Allen
(1956a) compared the distribution of the chromaffin reaction,
the iodate reaction and alkaline phosphatase. He observed
that insulin shock and inanition caused a loss of the
chromaffin reaction from most medullary cells and an increase
in the alkaline phosphatase activity of these cells. Cold, on
the other hand, induced disappearance of the iodate and
chromaffin reactions from the specific medullary cell islets,
which, on the other hand, became intensely alkaline
phosphatase positive. Coutinho, Baker, Kent, Flisbe and Van
Duke (1955) subdivided the adrenomedullary cells of the rat
into two classes, esterase positive and esterase negative.
Allen (1956b) confirmed this observation. Allen, Bränkö and Hunter (1957, 1958) further showed that the positive esterase reaction was localised in nerve trunks, ganglion cells and the fluorescent parenchymal cells, the non-fluorescent parenchymal cells showing only a weak reaction. The esterase concerned was found to be acetylcholine-sensitive. Further studies by Bränkö (to be published) with specific inhibitors demonstrated that specific acetylcholinesterase was equally present all over the medullary parenchyma but nonspecific cholinesterase was selectively localised in the fluorescent cell islets.

The studies just cited demonstrate clearly that the two types of cells in the adrenal medulla do not differ only with regard to the nature of the catechol amine they contain but also in many other respects. The significance of these histochemical differences is as yet obscure but it is obvious that histochemical techniques can be expected to be fruitful in further study of the adrenal medulla. On the other hand, care is necessary when histochemical techniques are used for the differentiation of the adrenaline-containing and the noradrenaline-containing cells, unless the reaction can be proved to be specific to either of these amines. As is clearly shown by Allen's (1956a) work, changes in the secretory state of the adrenal medulla may be accompanied by
a loss of the catechol amines and an increase in the enzymatic activity.

It is of interest to note that the presence of two types of specific adreno-medullary cells can well explain recent observations on the adreno-medullary secretion of adrenaline and noradrenaline into the blood stream. Thus, Brücke, Kaindl and Mayer (1952) observed that the amount of adrenaline secreted in rest was about 10 per cent of the total catechol amine secretion but it increased to about 50 per cent during hypothalamic stimulation. Redgate and Gellhorn (1953) and Folkow and Euler (1954) furthermore found that excitation of the hypothalamus caused an increased secretion of either adrenaline or noradrenaline or both, depending on the site of stimulation.

Studies on the intracellular distribution of the catechol amines are also of interest in this connexion. Several investigators have been able to show that adrenaline and noradrenaline are in the adrenal medulla contained in small cytoplasmic granules which can be separated by differential centrifugation (Blaschko and Welch, 1953; Hillarp, Lagerstedt and Nilson, 1954; Blaschko, Hagen and Welch, 1955). Moreover, it has proved possible to separate granule fractions which contain almost only noradrenaline, and other fractions which contain predominantly adrenaline.
(Bade, 1956; Schümann, 1957; Blaschko, Hagen, Hagen and Schümann, 1957). Electron microscopic studies have confirmed the presence in the adreno-medullary cells of specific chromaffin granules which are distinct from mitochondria and which are covered by a membrane (Lever, 1955; Sjöstrand and Wetzstein, 1956).

All these recent observations enlarge our knowledge of the structure and function of the adrenal medulla and create new, interesting problems. It is likely that significant new observations will be made by trying to unsolve these problems and that combination of morphological, physiological and chemical methods offers the best possibilities for making them.
(12) CONCLUSIONS

(1) The calcium-formol-fluorescence test demonstrates in the adrenal medulla cells which contain much noradrenaline and little, if any, adrenaline. The remaining cells, which show only a faint fluorescence, contain much adrenaline and little noradrenaline.

(2) The iodate reaction demonstrates the same adrenal medullary cells as the fluorescence test.

(3) Experimentally induced changes in the noradrenaline content of the adrenal medulla are accompanied by corresponding changes in the distributions and intensities of both the fluorescence and the iodate reaction.

(4) It is likely that the iodate reaction is a specific histochemical test for noradrenaline, but the fluorescence induced by fixation in calcium formol may depend on substances other than noradrenaline in the noradrenaline-containing cells.

(5) Observations made in the present study, together with those made independently by other workers with entirely different methods, strongly suggest that adrenaline and noradrenaline are produced, stored and secreted by two independent, histochemically different types of adreno-medullary cells.
(1) The purpose of the work is to study the significance of the two types of adreno-medullary cells which can be differentiated with the aid of fluorescence microscopy after fixation in calcium formol.

(2) Methods for histological examination and sampling, as well as for chromatographic separation and determination of adrenaline and noradrenaline have been described.

(3) A correlation was observed between the amount of fluorescent medullary tissue and the relative noradrenaline content of adrenals of different species.

(4) In the adrenal medulla of the cat and the hamster, areas containing much fluorescent tissue were found to contain much noradrenaline and little adrenaline, while areas with little or no fluorescent tissue contained much adrenaline and little noradrenaline.

(5) Insulin shock caused a pronounced loss of adrenaline from the adrenal medulla of the rat without altering the noradrenaline content. A positive chromaffin reaction was observed only in such medullary cell groups which were identical of shape and distribution with the groups of fluorescent cells. The fluorescence was not affected by insulin shock.
(6) Prolonged administration of nicotine caused a hyperplasia of the adrenal medulla due to increase in the amount of fluorescent parenchymal tissue. This increase was accompanied by a corresponding increase in the noradrenaline content. The hyperplastic nodules of fluorescent tissue were observed to contain only noradrenaline.

(7) The distribution of the fluorescence test was found to be similar to that of the iodate reaction (a histochemical method for the demonstration of noradrenaline) in adrenals of 14 different species and in adrenal autografts of the rat.

(8) It was demonstrated with the aid of radioautography that iodine is fixed by the specific cell islets in adrenals treated with a saturated solution of $\text{I}^{131}$-labelled potassium iodate. All medullary cells were stained brown and contained iodine after fixation in a mixture of formalin and radioactive iodate.

(9) It is concluded that both the fluorescence test and the iodate reaction demonstrate in the adrenal medulla those chromaffin cells which contain noradrenaline, and presumably produce and secrete it, while the remaining chromaffin cells contain, produce and secrete adrenaline.
(XIV) REFERENCES.

Only the references relating to the typewritten parts are given in this list.


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