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PIGMENT FORMATION FROM TRYPTAMINE AND 5-HYDROXYTRYPTAMINE IN TISSUES: A CONTRIBUTION TO THE HISTOCHEMISTRY OF AMINE OXIDASE

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Little information is at present available on the localization of amine oxidase in tissues. An attempt to devise a histochemical reaction for amine oxidase was made by Oster & Schlossman (1942); this was based on the reaction between Feulgen's reagent and the aldehyde formed when amine oxidase acts on an amine, e.g. tyramine. This reaction has not been found satisfactory by other observers (e.g. Gomori, 1950; Pearse, 1953).

Francis (1953) has recently reported that a reduction of neotetrazolium occurs in tissue slices incubated with tyramine, but as it is known that the amine oxidase system reduces tetrazolium compounds only very slowly (Blaschko, 1952*a*), it is not yet certain if this reaction will be of use for the histochemical study of amine oxidase.

The experiments described in this paper represent a different approach to the histochemistry of amine oxidase. They take their starting-point from the observation that in the enzymic oxidation of tryptamine a coloured compound is formed (Pugh & Quastel, 1937). It has recently been shown that a similar pigment formation occurs when 5-hydroxytryptamine (serotonin, enteramine) is oxidized by tissue preparations containing amine oxidase (Blaschko, 1952*b*). The work of Pugh & Quastel suggested that the pigment was not the direct product of enzymic attack on tryptamine, an inhibition of pigment formation was observed in the presence of cyanide without a reduction in the rate of oxygen uptake.

We have incubated sections of tissue with tryptamine and 5-hydroxytryptamine and studied the formation of pigment. We were interested in the

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question whether a reaction of this kind could be used in the analysis of the distribution of amine oxidase in the tissues, but we also wanted to know more about the conditions required for pigment formation as it is now known that tryptamine derivatives normally occur in the tissues.

METHODS

Animals (rats and guinea-pigs) were killed by a blow on the head and bled. The tissues were put into ice-cold 0·9% (w/v) sodium chloride and at once cut on the freezing microtome at 25μ thickness. The sections were then incubated in 0·067 M-sodium phosphate buffer of pH 7·4. In most of our experiments tryptamine hydrochloride was used as substrate of amine oxidase; unless otherwise stated, it was present in a concentration of 1 mg/ml. At the end of the incubation the sections were mounted on slides in Messrs G. T. Gurr's Water Mounting Medium.

RESULTS

It is known that the mammalian liver is an organ rich in amine oxidase, and it is for this reason that liver was the first tissue to be examined.

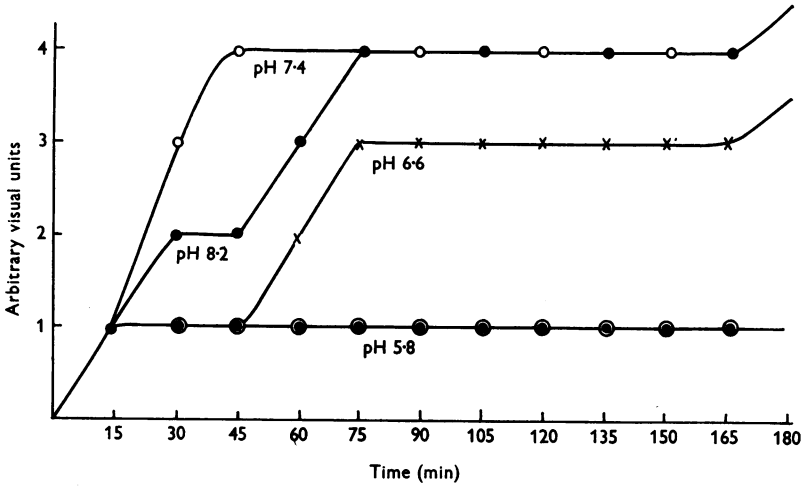
Sections of rat liver incubated in a solution of 0·007 M-tryptamine hydrochloride in 0·067 M-sodium phosphate buffer, so on developed ayellow colour which gradually became more intense and finally brown. The colour was similar to that seen when liver homogenates were incubated with tryptamine in manometric experiments. When the sections were examined under the microscope it was found that a dark brown pigment had accumulated in the parenchyma cells. The intensity and localization of the pigment in the relatively thick sections used made a detailed cytological study difficult. Nevertheless, the rapid development of colour made liver sections useful material for establishing conditions suitable for the tryptamine reaction, and in the tests to be described sections of rat liver were used.

In these tests, the intensity of the colour developed was estimated visually according to an arbitrary scale; five steps of intensity were distinguished. This relatively crude method of assessment was found adequate for the purpose of these tests. Amine oxidase is known to require a high partial pressure of oxygen for optimum activity; we have therefore studied the effect of oxygenation on the rate of pigment formation. Sections were incubated in weighing bottles of 20 ml. capacity; in one bottle the incubation with tryptamine was carried out in air, in the other oxygen was bubbled through during the incubation. No difference in the rate of development of colour was observed. This was confirmed in several experiments. In subsequent experiments we therefore incubated the sections in air.

In another experiment sections of liver were incubated with tryptamine in an atmosphere of nitrogen, while control sections were similarly incubated in air. Very little colour appeared in the nitrogen-incubated sections, whereas the sections in air rapidly became brown. This suggests that the colour is formed in an oxidative reaction.

Incubation medium. The incubation was usually carried out in 0·067 M-sodium phosphate buffer at pH 7·4. Phosphate and bicarbonate buffered Krebs-Ringer solution was also used, but neither the amount of pigment formed nor the rate of its formation was increased in these media.

Effect of pH. Four weighing bottles were set up in which sections were incubated in 0·067 M-Sørensen phosphate buffers of different pH values in the presence of 0·007 M-tryptamine hydrochloride. The result is shown in Text-fig. 1. The time interval of the initial appearance of the colour was not noticeably affected by pH, but the subsequent rate of development of colour was most rapid at pH 7·4. Pigment formation was least at pH 5·8. In all later experiments the pH of the medium was 7·4.



Text-fig. 1. The effect of pH on pigment formation from tryptamine in sections of rat liver and in the presence of hydrogen peroxide. Abscissa: time in min. Ordinate: intensity of pigmentation in arbitrary units.

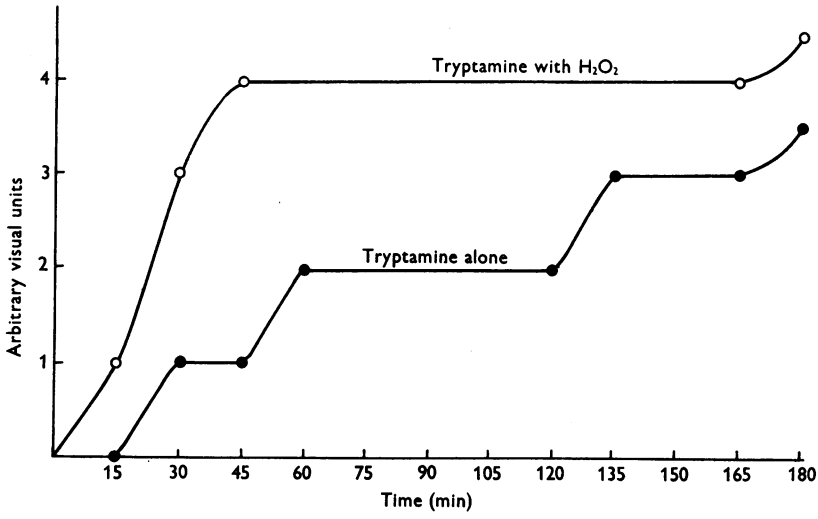
Substrate concentration. Liver sections were incubated at 37° C in three flasks which contained 0·0025 M-, 0·005 M- and 0·01 M-tryptamine hydrochloride respectively. The sections were inspected every 15 min. In 0·005 M- and in 0·01 M-tryptamine colour development became noticeable after 45 min; at that time no colour had appeared in the lowest concentration of tryptamine. In this flask the development of the colour remained slightly lower than with the other two specimens in which no difference could be seen. In most of the subsequent experiments a tryptamine concentration of 0·007 M was used. No colour appeared in sections which were incubated in the absence of tryptamine.

Temperature. When sections were incubated at 18 and 37° C respectively, the development of colour was somewhat slower at 18 than at 37° C.

When sections of fresh rat liver tissue were immersed in boiling buffer solution for 5 min they lost the ability to form pigment when incubated with

tryptamine. This suggests that pigment formation took place in a reaction catalysed by an enzyme.

Effect of hydrogen peroxide. Hydrogen peroxide speeded up the rate of production of the pigment. This is illustrated in the experiment shown in Text-fig. 2 in which rat liver sections were incubated in 5 ml. phosphate buffer containing tryptamine to which 0·35 ml. of hydrogen peroxide (20 vol.) had been added. It can be seen that in the presence of hydrogen peroxide the colour appeared very much more rapidly; throughout the period of observation the sections in the presence of peroxide were more deeply coloured.



Text-fig. 2. The effect of hydrogen peroxide on pigment formation in rat liver sections. Abscissa and ordinate as in Fig. 1.

Inhibitors of amine oxidase

We have tested the effects of a few compounds known to inhibit amine oxidase. Urea in 3 M concentration completely inhibits amine oxidase activity (Bhagvat, Blaschko & Richter, 1939); it also abolished the formation of pigment in incubated sections. A more specific inhibitor of amine oxidase is pentamidine; this was used in a 10^{-5} M concentration in which enzymic activity in the manometric experiments is known to be greatly reduced. Two experiments were carried out: in the first some of the sections were incubated in 10^{-5} M-pentamidine before they were transferred to the tryptamine solution; in the second experiment the incubation medium contained both 10^{-5} M-pentamidine and 0·007 M-tryptamine. In both experiments the phosphate concentration was reduced to 0·0067 M as in the 0·067 M-phosphate buffer a precipitate occurred when pentamidine was added. In the second experiment no colour appeared during the incubation. In the first experiment

some pigment was found, but its initial appearance occurred much later than in control sections, not treated with pentamidine, and the intensity of the final coloration was much less.

sec.-Octyl and *n*-octyl alcohol are inhibitors of amine oxidase, but they did not affect pigment formation. *sec.*-Octyl alcohol and *n*-octyl alcohol are not very soluble in water; 0·002 ml. of each were added to 10 ml. of incubation medium. This produced saturated aqueous solutions. No difference between the sections incubated with and without alcohol was noted.

The observation by Pugh & Quastel that cyanide inhibits pigment formation without interfering with oxygen uptake suggested that the pigment was formed in a further reaction of the aldehyde arising from the enzymic oxidation of tryptamine. This interpretation is supported by the study of compounds known to react with aldehydes. Hydroxylamine hydrochloride (10^{-3}M), sodium bisulphite (10^{-1}M) and hydrazine sulphate (10^{-2}M) abolished pigment formation. Semicarbazide hydrochloride (10^{-4}M) considerably reduced pigment formation, but did not abolish it completely.

These observations give support to the interpretation mentioned above that the coloured compound is a reaction product of the aldehyde produced in the deamination reaction.

The strong accelerating action of hydrogen peroxide on the development of colour suggested the possibility that the secondary reaction in which the pigment appeared was brought about by catalase. We therefore incubated rat liver sections with tryptamine in the presence of 10^{-5}M-sodium azide, a concentration in which the action of catalase is known to be strongly depressed (Blaschko, 1935); this concentration has no effect on amine oxidase activity. It did not affect the formation of pigment in liver sections. Similar results were obtained with even higher concentrations of azide, e.g. 10^{-4} and 10^{-2}M. These observations make it unlikely that catalase is involved in the formation of pigment from tryptamine.

Control experiments. That the colour formation depends on the action of an enzyme is supported by the absence of any colour development in boiled sections as already described. It finds further support in the observation that tryptamine did not form a coloured compound when incubated with hydrogen peroxide in the absence of tissue. We have incubated DL-tryptophan with liver slices; no colour was formed under these conditions.

Properties of the pigment

In the attempt to dehydrate the sections in order to clear and mount them, it was found that the pigment was rapidly dissolved in ethanol. Acetone, dioxane and *n*-butanol also acted as solvents. This made it impossible to use organic solvents for the dehydration of the sections, but it has made it possible to obtain information on some of the physical properties of the eluted pigment.

A characteristic bluish purple fluorescence was seen when solutions of the extracted pigment were examined under ultra-violet light. A similar fluorescence had already been noticed when liver extracts were incubated with tryptamine under aerobic conditions. Chromatographic examination of extracts from rat liver sections incubated with tryptamine showed that there were at least two compounds present: one gave the characteristic fluorescence, but was almost colourless; the other was yellowish brown and did not fluoresce.

Distribution of the tryptamine reaction in different tissues

The rapid development of colour in liver tissue is in keeping with the abundance of amine oxidase in this organ. If the tryptamine reaction is a valid test for amine oxidase, it appears that the enzyme is localized in the hepatic parenchyma cells.

Pigment formation would be expected to occur in those tissues which contain amine oxidase. A systematic survey of the tryptamine test in mammalian tissues has not been made, but we have found the pigment formation to occur in a number of tissues in which amine oxidase is present.

The kidney cortex has a high amine oxidase activity; the activity of the medulla is much lower. We have therefore incubated sections of kidney with tryptamine. Good staining was obtained in the sections of guinea-pig kidney, an organ rich in amine oxidase. The appearance of such a section incubated in tryptamine for 1 hr is shown in Pl. 1. The cortex shows much heavier deposition of pigment than the medulla, in which only little pigmentation is apparent. On closer inspection it can be seen that in the cortex staining is not uniform: it is absent from the glomeruli (Pl. 2A), in the tubules pigmentation is most intense in the proximal convoluted tubules. These details are seen in Pl. 2. If the proximal tubules are traced down into the medulla, it can be seen that the epithelium is pigmented, even in the straight segments.

From manometric experiments the rat kidney is known to be poor in amine oxidase. This finds its parallel in the poor staining seen when rat kidney sections were incubated with tryptamine.

Very little amine oxidase activity has been found in muscle by the use of the manometric method. We have only examined the diaphragm of the guinea-pig, and it is of interest that a small but definite development of colour occurred when sections of this tissue were incubated with tryptamine in the presence as well as in the absence of hydrogen peroxide. Colour was seen in the muscle on the surface of the fibres only; this could be seen on fibres that had been cut transversely. In these fibres only the area just beneath the sarcolemma had stained.

Observations on the occurrence of a positive tryptamine test in sweat and sebaceous glands and other parts of the skin will be fully described elsewhere (Hellmann, to be published).

5-*Hydroxytryptamine* (*serotonin*)

When sections of guinea-pig liver were incubated in a sodium phosphate buffer containing 5 mg of 5-hydroxytryptamine creatinine sulphate (serotonin) (Abbott) in 10 ml., a development of colour occurred. This was similar to that observed with tryptamine, but the colour appeared to be somewhat lighter. When sections were incubated with serotonin, the fluid acquired a yellowish brown colour; this had not been seen in incubations with tryptamine. No colour developed in solutions of serotonin which were allowed to stand without tissue for the same length of time under the same conditions. This observation suggests that the coloured product formed from serotonin is more readily soluble in water than the corresponding substance formed from tryptamine.

Sections of guinea-pig kidney and rat brain were also incubated with serotonin; in both tissues a yellow colour developed. In the kidney, the distribution of pigment was similar to that seen with added tryptamine.

DISCUSSION

The observations reported show that the colour reactions with tryptamine or serotonin depend upon the amine oxidase activity of the tissues. At present, we cannot say if a strict correlation exists between enzymic activity and colour development.

Liver and kidney, known to be rich in amine oxidase, showed the colour reaction strongly; moreover, in kidney the reaction was intense in the cortex and feeble in the medulla. This is also in agreement with the known distribution of the enzyme. The finding of a positive reaction with tryptamine in guinea-pig diaphragm is of interest in this connexion, as only a very small amount of amine oxidase has been reported in guinea-pig muscle (Bhagvat *et al.* 1939). The localization of the colour in the superficial part of the muscle fibre makes it possible that the pigment formation is connected with an enzyme present in nerve fibres or blood vessels.

The tryptamine test leads to formation of a compound which, being insoluble in water, is advantageous for histochemical analysis. On the other hand, our failure to find a tissue fixative in which the enzymic activity is retained, made a cytological study of the reaction difficult. Another difficulty is the solubility of the pigment in organic solvents which prevented their use as dehydrating and clearing agents.

The chemical nature of the pigment formed is unknown as is the mechanism of its formation. If we assume that β-indolylacetaldehyde is the first product of the enzymic oxidation of tryptamine, it seems likely that the coloured compound is a further reaction product of the aldehyde. It is possible that in the second reaction, not catalysed by amine oxidase, another enzyme is involved, but this is uncertain. The observation that hydrogen peroxide

speeded up the appearance of the colour suggested the possibility that catalase, a ubiquitous catalyst in mammalian tissues, was involved, but this is not likely as sodium azide, a strong inhibitor of catalase, neither prevented nor slowed down the appearance of pigment.

That tryptophan may be connected with pigment formation in the body has often been discussed. It seems of interest, therefore, that 5-hydroxytryptamine, a metabolite of tryptophan found in the organism, is converted to a coloured product under the influence of an enzyme widely distributed in mammalian tissues. We must therefore consider the possibility that this reaction may not only occur *in vitro*. According to Erspamer & Asero (1952), 5-hydroxytryptamine is present in the enterochromoffine cells of the alimentary tract. These cells are also called 'yellow' cells because, even unstained, they contain pigment. Amine oxidase is present in high concentration in the gastro-intestinal mucosa; thus, both substrate and catalyst of the enzymic reaction here described occur in this tissue.

SUMMARY

1. Tissues containing amine oxidase develop a brown colour when incubated with tryptamine or 5-hydroxytryptamine; this pigment formation has been studied in fresh frozen sections.

2. Liver gives a strong pigment formation under these conditions.

3. In sections of kidney the cortex is more heavily stained than the medulla; pigment deposition is most intense in the proximal convoluted tubules.

4. Pigment formation has also been seen in some other tissues (rat brain; guinea-pig diaphragm).

5. The development of pigment is speeded up in the presence of hydrogen peroxide.

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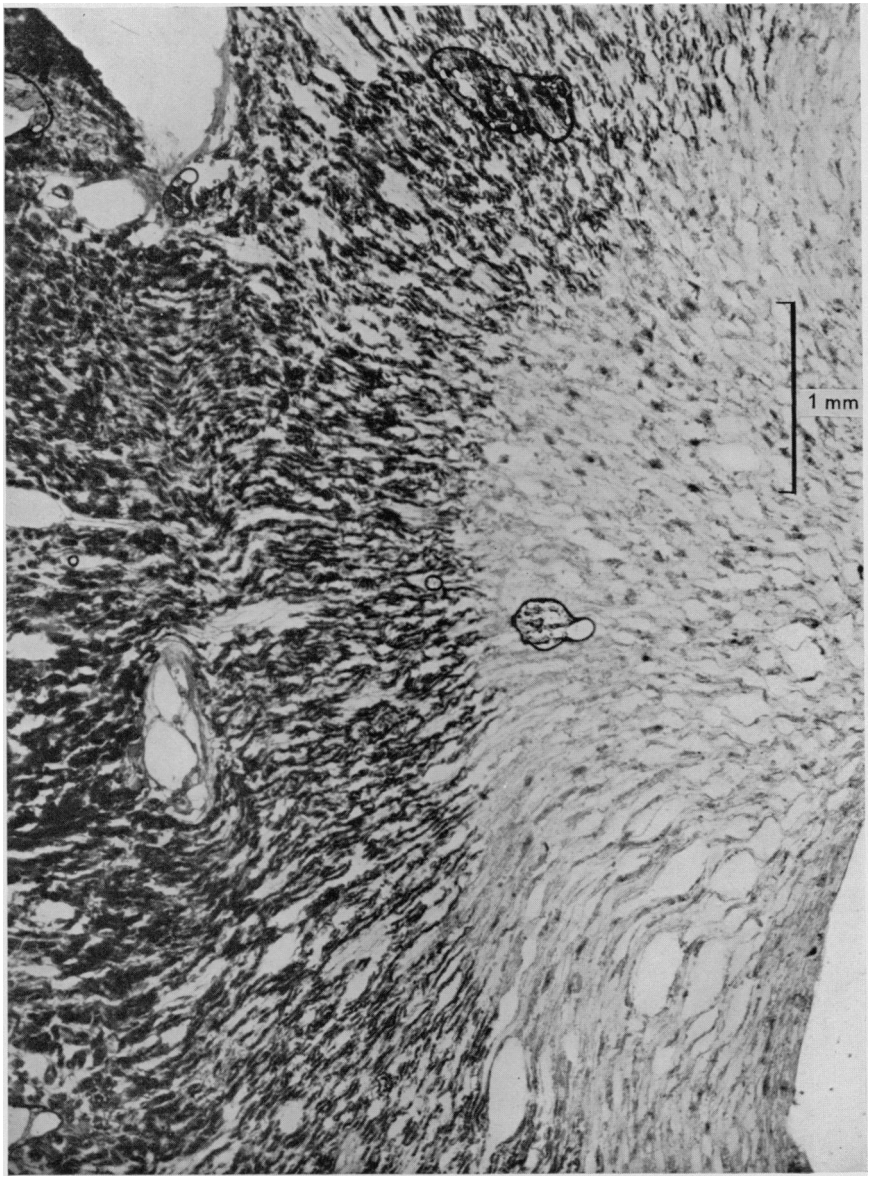

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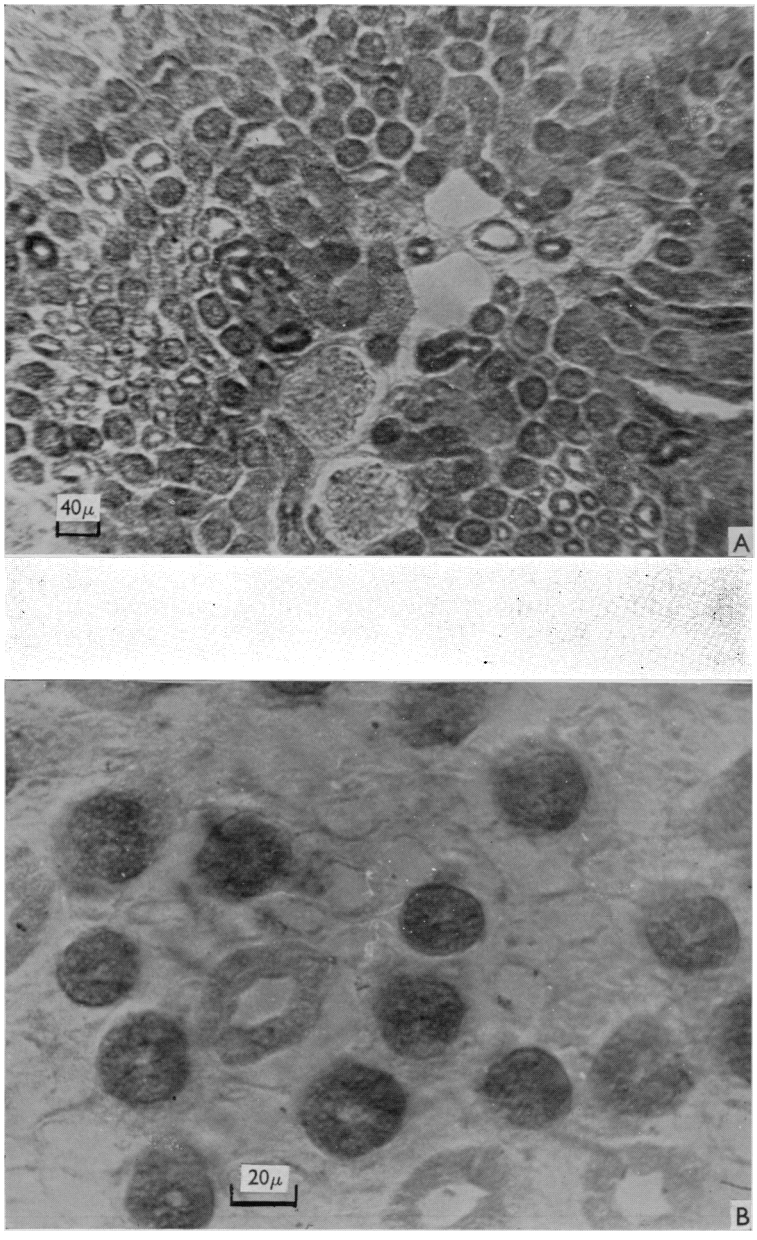
40μ
A
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EXPLANATION OF PLATES

PLATE 1

Radial section of guinea-pig kidney incubated with tryptamine. ×25.

PLATE 2

A. Radial section of guinea-pig kidney cortex incubated with tryptamine. ×125. Two adjacent glomeruli can be seen at the centre of the bottom edge of the section. They are unstained.

B. Guinea-pig kidney cortex section incubated with tryptamine. ×400. The proximal tubules stained intensely, whereas the distal tubules gave only a weak reaction.