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OXIDATION OF 5-HYDROXYTRYPTAMINE AND RELATED COMPOUNDS BY *MYTILUS* GILL PLATES

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Homogenates of gill plates of *Mytilus edulis* L. used oxygen when 5-hydroxytryptamine was added. The oxidation of 5-hydroxytryptamine was not due to the presence of an amine oxidase, but to that of an enzyme that catalysed the oxidation of other 5-hydroxyindoles (5-hydroxytryptophan, bufotenine). The oxidation was cyanide-sensitive, but was not inhibited by iproniazid. In the reaction a yellowish-brown substance was formed. The occurrence of an amine oxidase in the anterior retractor muscle of the byssus and in the digestive gland was confirmed.

A number of observations suggest that in molluscs 5-hydroxytryptamine acts as a hormone or mediator; these have been reviewed by Welsh (1955). In *Mytilus edulis,* the anterior retractor muscle of the byssus is inhibited by 5-hydroxytryptamine in very low concentrations (Twarog, 1954). Aiello (1957) reported that 5-hydroxytryptamine had a stimulating action on the ciliary movements in the gill epithelium of *Mytilus* and that extracts of gill plates had a similar effect.

Amine oxidase, a catalyst of the biological inactivation of 5-hydroxytryptamine, occurs in many molluscs. In *Mytilus,* it was not demonstrated in earlier studies (Blaschko and Hawkins, 1952), but more recently Blaschko and Hope (1957) found amine oxidase in homogenates of both the digestive gland and the anterior retractor muscle of the byssus, but no amine oxidase activity was found in the gill plates. Since in the work on *Mytilus* gill plates 5-hydroxytryptamine had not been used as substrate, it was decided to find out if gill plate extracts oxidized 5-hydroxytryptamine.

METHOD

Fresh specimens of *Mytilus edulis* L. were usually dissected immediately after arrival at our laboratory at Oxford. When necessary animals were kept aerated in sea water at 4°. Homogenates were usually prepared without adding fluid; they were dialysed, with frequent changes, against 0.067 M-sodium phosphate buffer of *p*H 7.4 for 2 to 4 hr.; dialysing against the phosphate buffer was then continued without changes overnight.

For the manometric experiments, two different types of conical manometer vessels were used. One had a capacity of 16 to 18 ml., the other 7.5 to 8.5 ml. In the larger vessels the volume of the reaction mixture was 2.0 or 3.0 ml., in the smaller vessels it was 0.7 ml. The substrates were tipped in from the side bulb to give an initial concentration of 0.5×10^{-2} M. The gas phase was either oxygen or, in all later experiments, air. The temperature of the manometer bath was 25°.

5-Hydroxytryptamine creatinine sulphate, 5-hydroxy-DL-tryptophan and iproniazid were obtained from Roche Products. We are grateful to Dr. R. B. Barlow and Dr. I. Khan for the sample of bufotenine hydrochloride.

RESULTS

When a dialysed homogenate of *Mytilus* gill plates was incubated with 0.5×10^{-2} M-5-hydroxytryptamine, a slow but steady uptake of oxygen occurred. No similar uptake of oxygen was found when the homogenate was incubated with tryptamine. In the presence of 5-hydroxytryptamine, a yellowish-brown colour soon developed and increased in intensity as the reaction proceeded.

To establish whether the oxidation of 5-hydroxytryptamine was due to the presence of an amine oxidase in the gill plate homogenates, a second experiment was carried out in which the homogenate was incubated with 5-hydroxytryptamine in the presence of either 10^{-3} M-iproniazid or 10^{-2} M-potassium cyanide, and also without any other substance. To ensure that the iproniazid was effective in inhibiting any amine oxidase present, all flasks were pre-incubated at 25° for 30 min. before tipping. In this experiment, the flask containing iproniazid showed the same

oxygen uptake, and the same development of brown colour, as that to which no inhibitor had been added. However, both the oxygen consumption and the development of colour were entirely suppressed in the presence of cyanide.

That the oxygen consumption in the presence of 5-hydroxytryptamine was due to an enzyme was supported by the observation that no uptake of oxygen occurred when 5-hydroxytryptamine was added to a gill plate homogenate after this had been heated in a boiling water bath for 30 min.

The observations reported made it unlikely that the catalyst involved in the oxidation of 5-hydroxytryptamine was amine oxidase. First, there was an absence of oxidation of tryptamine, usually a good substrate for the study of amine oxidase, and second, there was the lack of inhibition by iproniazid and also the inhibition by cyanide.

In another experiment, both phenethylamine and tyramine were incubated with the gill plate homogenate. Neither amine was oxidized. With tyramine, observations were continued for 180 min. to establish whether there was a phenolase present which required a prolonged incubation of a monophenolic amine; no oxygen uptake occurred within the period of observation.

The findings suggested that the oxidation of 5-hydroxytryptamine by the gill plate homogenates depended upon the presence of the phenolic hydroxyl group in position 5 on the indole nucleus. An experiment was therefore carried out in which both 5-hydroxytryptamine and 5-hydroxytryptophan were incubated. Oxygen uptake occurred not only in the flask incubated with 5-hydroxytryptamine, but also in the flask which contained 5-hydroxytryptophan; the rate of oxidation with the latter was a little slower than with 5-hydroxytryptamine. As with 5-hydroxytryptamine, a brown colour developed when 5-hydroxytryptophan was oxidized, but the rate of development of colour was also a little slower than with 5-hydroxytryptamine.

That the enzymic reaction was, in fact, characteristic of 5-hydroxyindoles was supported by the observation that bufotenine was also oxidized. In Fig. 1 an experiment is shown in which the rates of oxidation with 5-hydroxytryptamine were compared with 5-hydroxytryptophan and with bufotenine. Development of colour was seen with each of these substances.

Although there had been no oxygen uptake with tyramine, dopamine (3,4-dihydroxyphenethylamine) was oxidized; its rate of oxidation in two experiments was slightly less than that of 5-hydroxytryptamine. The oxidation of dopamine was also accompanied by a development of colour, but the greyish-black colour was in marked contrast with the yellowish-brown one formed when the 5-hydroxyindoles were oxidized.

In the earlier experiments, the gas phase during incubation was 100% oxygen, but it was found that there was no significant difference in the rate of the enzymic reaction when the manometers contained air. For this reason, in all later experiments, the gas phase was air. The independence of the rate of the enzymic reaction from the partial pressure of oxygen is also in contrast to what is known of the properties of amine oxidase: the latter is less active in air than in 100% oxygen (Kohn, 1937).

On high-speed centrifugation the activity was found to be retained in the supernatant fluid. In a preliminary experiment, a homogenate was centrifuged at 18,000 *g*; the enzymic activity of the supernatant fluid did not significantly differ from that of the original homogenate.

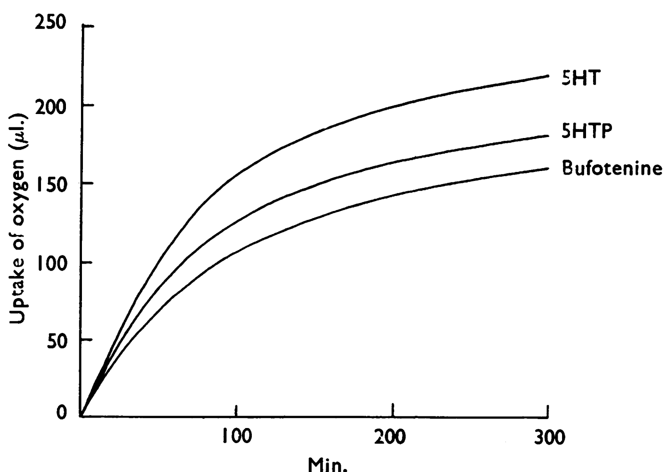


FIG. 1.—Oxidation of 5-hydroxytryptamine, 5-hydroxytryptophan and bufotenine by a preparation from gill plates of *Mytilus edulis* at 25° in air. Each flask contained 1.8 ml. of a resuspended sediment from a 25–55% ammonium sulphate precipitation. Substrate concentrations: 0.5×10^{-3} M. Abscissa: time in min. Ordinate: μl. oxygen used.

A further purification of the oxidase responsible for the enzymic reaction was carried out on a larger batch of homogenate as follows:

Ten dozen specimens of *Mytilus* were dissected to give 129 g. of gill plates. This material was stored at – 12° overnight. After homogenizing, it was dialysed for 2 hr. against distilled water and for a further 2 hr. against the phosphate buffer. The homogenate was then centrifuged for 1 hr. at 40,000 r.p.m. in the angle head No. 40 of the Spinco ultracentrifuge. The supernatant fluid from this centrifugation—100 ml.—was used as starting material for two ammonium sulphate precipitations. In the first experiment, 50 ml. was used and three ammonium sulphate precipitates and a final supernatant fluid were obtained. The first sediment was the precipitate from adding ammonium sulphate to bring saturation to 25% ; the second from 25 to 55%, and the third from 55 to 100%. Each of these sediments was suspended in 5 ml. of phosphate buffer and they, as well as the final supernatant fluid, were dialysed against the buffer for two hours.

No activity was present in the final supernatant fluid, but all three sediments contained enzymic activity. Most of the activity was recovered in the precipitate from the 25 to 55% saturation. The recoveries, in terms of the supernatant fluid obtained by high-speed centrifugation which had been the starting material for the ammonium sulphate precipitation, were as follows:

High-speed supernatant (starting material)		800 μl. O_2/hr.
Sediment from 0 to 25%	saturation	80 „ „
„ „ 25 to 55%	„	600 „ „
„ „ 55 to 100%	„	437 „ „

The second batch of the same high-speed supernatant fluid was used for another ammonium sulphate precipitation at 30, 60, and 90% saturation ; in this experiment the protein content of the various fractions was also determined.

The results summarized in Table I show that the greater part of the activity was recovered in the precipitate from the 30 to 60% saturation. The specific activity of this fraction was 2.09, as compared with 0.64 for the high-speed supernatant fluid, the starting material for the ammonium sulphate precipitation. In other words, the sediment from the 30 to 60% saturation contained 77% of the enzymic activity of the starting material, and at the same time a 3.3-fold purification had been achieved. The overall purification from the original homogenate must have been considerably greater, but this fraction still contained too much fibrous material to make the determination of protein useful.

The more highly purified enzyme preparations were used to find out how much oxygen was consumed in the oxidation reaction. For this experiment, 1.35 ml. of the resuspended sediment from the 30 to 60% ammonium sulphate precipitation, plus 0.45 ml. phosphate buffer, was incubated with 5.0 μmoles of 5-hydroxytryptamine (total reaction volume 2.0 ml.). The oxidation reaction, which proceeded rapidly at first but with a slowly declining rate later, was followed for 5 hr. It came to a standstill after 4 hr., when 100.5 μl. oxygen corresponding to 4.5 μmoles of oxygen had been taken up. In other words, 0.9 moles of oxygen had been consumed per mole of substrate added.

TABLE I

ENZYMIC ACTIVITY AGAINST 5-HYDROXYTRYPTAMINE, AND PROTEIN CONTENT OF THE HIGH-SPEED SUPERNATANT FLUID OBTAINED FROM HOMOGENATES OF THE GILL PLATES OF *MYTILUS EDULIS* AND OF FRACTIONS OBTAINED BY AMMONIUM SULPHATE PRECIPITATION

Protein content determined according to Layne (1957). One enzyme unit (E.U.) is defined as the amount of enzyme that causes uptake of 1 μl. oxygen in 30 min. The readings for the final supernatant were too small to allow an accurate measurement of enzymic activity and protein content.

Fraction	Ammonium Sulphate Percentage Saturation	Volume of Fraction ml.	E.U./ml.	Protein mg./ml.	Specific Activity (E.U./mg. Protein)	Total Enzyme Units
High-speed supernatant ..	—	37·5	8·9	13·8	0·64	334
Sediment I	0–30	6·7	4·4	30·0	0·15	29
„ II	30–60	7·5	34·3	16·4	2·1	257
„ III	60–90	12·7	6·4	3·4	1·9	81
Final supernatant	—	36	1·4 (?)	0·14 (?)	—	—

The most active sediment from the first ammonium sulphate precipitation, that from the 25–55% saturation, was used for a comparison of the rate of oxidation of 5-hydroxytryptamine and L-dopa (β-3,4-dihydroxyphenyl-L-alanine). With 0.63 ml. of the fraction in each flask and a total reaction volume of 0.7 ml., the rates of oxidation were:

	After 15 min.	After 30 min.
With 5×10^{-3} M-5-hydroxytryptamine	8.5 μl. O_2	19 μl. O_2
With 5×10^{-3} M-L-dopa ...	12 μl. O_2	26 μl. O_2

The experiment of Fig. 1, in which the rate of oxidation of different 5-hydroxyindoles is shown, was also carried out using the sediment from the 25–55% saturation with ammonium sulphate.

A few experiments were carried out on other *Mytilus* tissues. In one experiment with 37 specimens, the anterior retractor muscles of the byssus were dissected (weight of muscles 4.0 g.) and a dialysed homogenate in phosphate buffer was prepared (total volume of homogenate 10 ml.). Of this 1.6 ml. was used in each manometer flask. Upon incubation for 1 hr. with 10^{-2} M-tryptamine and 0.5×10^{-3} M-5-hydroxytryptamine, 12.5 μl. oxygen was consumed with tryptamine and 11.5 μl. oxygen with 5-hydroxytryptamine. The corresponding figures after 2 hr. incubation were 23.5 μl. and 21 μl. respectively. Here also a brown colour appeared, but, in contrast to the experiments with the gill plate homogenates, a strong bluish-white fluorescence was seen, particularly in the flask incubated with tryptamine. This experiment confirmed the occurrence of amine oxidase in the anterior retractor muscle, as already reported by Blaschko and Hope (1957). Both amines were also oxidized by a homogenate of the posterior retractor muscle of the byssus.

A homogenate was also prepared of the digestive glands of *Mytilus* (1 part of fresh tissue plus 2 parts of phosphate buffer) ; this homogenate also contained parts of the intestine and other adjoining tissues. Each manometer flask contained 1.6 ml. of the dialysed homogenate. Both tryptamine and 5-hydroxytryptamine were oxidized. In the same experiment, 10^{-3} M-iproniazid was also tested. The amounts of oxygen used in the first hour of incubation were:

With tryptamine	36 μl. oxygen
„ 5-hydroxytryptamine	19 μl. „
„ tryptamine plus iproniazid ...	1 μl. „
„ 5-hydroxytryptamine plus iproniazid	9.5 μl. „

In the experiment, the oxidation of tryptamine was more strongly inhibited by iproniazid than that of 5-hydroxytryptamine, suggesting that the oxidation of tryptamine was only due to amine oxidase but that the oxidation of 5-hydroxytryptamine was due in part to an iproniazid-resistant enzyme. Thus, it is possible that the digestive gland contains not only amine oxidase, but also the catalyst of the oxidation of 5-hydroxytryptamine found in the gill plates.

Homogenates of the gill plates, the gonads and the digestive glands of *Chlamys* (*Pecten*) *opercularis* were also prepared. Tryptamine was readily oxidized by all three homogenates, but an oxidation of 5-hydroxytryptamine occurred only with the gill plate homogenate.

Discussion

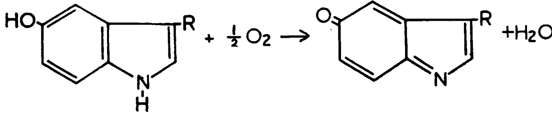
These experiments show that gill plates of *Mytilus edulis* contain an enzyme that oxidizes 5-hydroxytryptamine.

The absence of any oxygen uptake with tryptamine, phenethylamine and tyramine, the lack of inhibition by iproniazid, and the sensitivity to cyanide are all properties which show that the enzyme responsible for the oxidation is not amine oxidase. This view is supported by the observation that other 5-hydroxyindoles, 5-hydroxytryptophan and bufotenine, are similarly oxidized. All these compounds are characterized by the presence of the phenolic hydroxyl group in position 5 on the indole nucleus.

There is good reason to believe that the enzyme is of the phenolase type. First, there is the inhibition of the enzymic reaction by cyanide. Second, there is the fact that both dopamine and dopa are oxidized, the latter by a partly purified preparation of the enzyme. The lack of any uptake of oxygen with tyramine suggests that the enzyme is not a monophenoloxidase. It must be borne in mind that 5-hydroxytryptamine and other 5-hydroxyindoles are not strictly comparable to the monophenols ; they are substituted derivatives of *p*-aminophenol, and should therefore be more similar to the *p*-diphenols.

It has recently been shown by Mansour (1958) that 5-hydroxytryptamine has an affinity for the phenol oxidase of the liver fluke, *Fasciola hepatica*, and that of the Harding-Passey mouse melanoma. Both enzymes are inhibited by 5-hydroxytryptamine. There was no oxidation of 5-hydroxytryptamine by the *Fasciola* enzyme, but with the melanoma Mansour's data show a very slight oxidation of 5-hydroxytryptamine. However, the chief difference between these preparations and that containing the *Mytilus* gill plate enzyme is that the latter rapidly oxidizes the 5-hydroxyindoles.

The nature of the enzymic reaction catalysed has not been studied. The most likely reaction is the formation of a quinone-imine, as already suggested by Blaschko and Philpot (1953), thus:



This reaction would require 0.5 moles of oxygen per mole of substrate. In our experiments, the total oxygen uptake was nearer one mole of oxygen per molecule of substrate; this could either be interpreted as due to a further oxidation of the product of the enzymic reaction, or to the accumulation of hydrogen peroxide, or to peroxidatic reactions in which the latter was used up.

An alternative possibility should not be forgotten: the introduction of a second phenolic hydroxyl group in the indole nucleus. There is no evidence to suggest any particular position for such a group; it may be mentioned that Carlisle (1956) has discussed the occurrence of an *ortho*-dihydroxytryptamine in the pericardial organ of Crustacea.

The relatively rapid action of a phenolase type of enzyme of molluscan origin on 5-hydroxytryptamine and related substances is of interest. It has often been discussed whether melanin-like pigments can arise from tryptophan, and it seems that the reaction here described makes it possible that pigments may arise by the oxidation of a 5-hydroxyindole. The colour of the incubation mixture was different from that produced by dopa or dopamine; it was yellowish-brown, and it may be mentioned here that we have seen a similar colour when 5-hydroxytryptamine was oxidized by silver oxide.

Whether 5-hydroxytryptamine has a regulatory function in the ciliary epithelium of the gill plates is unknown. The work of Aiello (1957), who observed an effect of 5-hydroxytryptamine on the ciliary rhythm, has already been mentioned. There is evidence of an antagonism of 5-hydroxytryptamine and acetylcholine in the molluscan heart and in the anterior retractor muscle of the byssus. It is known that in the gill plates acetylcholine has an effect on the ciliary rhythm and that the gill plates contain all the catalysts for the synthesis as well as the inactivation of acetylcholine (Bülbring, Burn and Shelley, 1953; Milton, 1959). In the light of these findings, the presence in the gill plates of a catalyst for the inactivation of 5-hydroxytryptamine is of particular interest.

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