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Uptake of 5-Hydroxytryptamine by *Schistosoma mansoni*

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SUMMARY

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In spite of the presence of high concentrations of 5-hydroxytryptamine (5-HT) in *Schistosoma mansoni*, synthesis of this amine by the parasite could not be demonstrated. The worm has a high- and a low-affinity uptake mechanism (below and above 2 μM, respectively) for 5-HT. The high-affinity uptake mechanism provides the parasite with a means to obtain 5-HT, even at the low concentrations of this amine prevailing in the physiological environment of the worm.

INTRODUCTION

Biochemical, histochemical, and pharmacological evidence suggesting that 5-hydroxytryptamine is a putative excitatory neurotransmitter in *Schistosoma mansoni* has been reported (1–3). The evidence from these investigations met two of seven postulated criteria (4) for the identification of a neurotransmitter: (a) 5-HT[2] was present in neuronal structures of the schistosome, as determined by a relatively specific histochemical method (combined with microspectrofluorometry) (3), and (b) cholinergic blockade unmasked the presence of an excitatory substance whose stimulatory effects were blocked by 2-bromolysergic acid diethylamide, and which were no longer demonstrable after the 5-HT stores of the worm had been depleted.[3] Two of the remaining five criteria are herewith examined. One of them is the occurrence of a biosynthetic pathway for 5-HT in *S. mansoni*; the second is the presence of a mechanism by which the effect of the neurotransmitter is terminated through reuptake into the nerve terminal from which it has been released.

MATERIALS AND METHODS

Adult schistosomes (*S. mansoni*, Puerto Rican strain) (average fresh weight of a male worm, 0.6 mg; of a female, 0.2 mg) were obtained from the mouse host as in previous studies (5). Immediately after their removal from the host, the worms were placed in 75% horse serum.

Blood from hamsters anesthetized with sodium pentobarbital (60 mg/kg administered intraperitoneally) was obtained by heart puncture, and from unanesthetized mice by

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[2] The abbreviation used is: 5-HT, 5-hydroxytryptamine.

[3] J. L. Bennett and E. Bueding, unpublished observations.

draining the suborbital sinus with a heparinized capillary tube (6).

Determination of Biogenic Amines

Schistosomes were removed from 75% horse serum, blotted, frozen in liquid nitrogen, and either stored at −60° or immediately homogenized in a solution of perchloric acid (0.4 N). The fluorometric method of Snyder *et al.* (7) was used for the determination of unlabeled 5-HT, with the following modifications. (a) The perchloric acid solution contained 0.02% ascorbic acid and 0.2% EDTA to prevent destruction of 5-HT by ferric ion (8). (This solution must be prepared freshly every 24 hr.) (b) The volume of the perchloric acid solution used for the extraction was never less than 2.2 ml. (c) The perchloric acid extract was neutralized at 2–4° with 0.25 ml of 0.5 M borate buffer (pH 9.3), followed by enough 2 N KOH to bring the pH of the extract to 9.0; no significant reduction occurred in the recovery of added known amounts of 5-HT when the extract was neutralized in this manner.

Norepinephrine and dopamine were determined by the methods of either Taylor and Laverty (9) or of Anton and Sayre (10). Standard deviations are indicated in Tables 1–3.

Incubation of Schistosomes in Vitro

All incubations of less than 6-hr duration were carried out in 75% horse serum at 37°. The number of worm pairs did not exceed 10/ml of horse serum for an incubation period of 2 hr, or 5/ml for 3–6 hr. All incubations lasting more than 6 hr were carried out in a medium whose composition (per 100 ml) was as follows: Earle's base (199-10X) (11), 6.0 ml; penicillin and streptomycin, 10,000 units each; glucose, 125 mg; sodium glycylglycine buffer, 0.05 M (pH 7.8); and horse serum, 20.0 ml. The number of worm pairs never exceeded 2/ml of this medium for incubations of 24 hr or less, or 1/ml for incubations longer than 24 hr. The worms were transferred to fresh medium every 48 hr. All incubations (regardless of the type of medium) were carried out in a temperature-controlled incubator with a rotating shaker (40 rpm).

Determination of 5-HT Uptake

Incubation with nonlabeled 5-HT. Prior to exposure to 5-HT the schistosomes were incubated with or without added drugs at 37° for 15 min in 75% horse serum. Following incubation with 5-HT the worms were washed in a separate dish with the modified Earle's medium to remove any 5-HT adhering to their surface. Then the worms were blotted, frozen, and weighed.

Incubations with radioactive compounds. The schistosomes were incubated for 15 min before a solution of a labeled compound was added. After the appropriate incubation time the worms were removed from the incubation dish (by means of a Pasteur pipette with an enlarged opening) and transferred to a small polyethylene cup (1 × 1 cm). The bottom of the cup was covered with a fine nylon net which trapped the worms as they were released from the pipette. The medium described above (7.5 ml) was used to wash off labeled material adhering to the surface of the worms. The latter were then removed from the cup, blotted, frozen, weighed, and homogenized in a glass homogenizer (kept at 4°) containing 2.2 ml of 0.4 N perchloric acid. The homogenate was centrifuged for 10 min at 8000 × *g*. For the determination of 5-HT in the incubation medium, 1 ml of the latter was placed in a 15-ml centrifuge tube containing 1.2 ml of 0.72 N perchloric acid. After centrifugation for 10 min at 8000 × *g*, 2.0 ml of the schistosome supernatants and 1.7 ml of the medium supernatants were placed in vials containing 15 ml of scintillation fluid (0.55% 2,5-diphenyloxazole and 0.125% 1,4 - bis[2 - (4 - methyl - 5 - phenyloxazolyl)]benzene in a 2:1 mixture of toluene–Triton X) for counting in a Packard scintillation spectrometer (model 3,200). All samples were counted for a minimum of 10,000 counts. Tissue-to-medium ratios and uptake velocities were calculated according to Shaskan and Snyder (12).

Fluorescence Histochemistry

A previously described modification (3) of the fluorescence histochemical method of Falck and Owman (13) was used for the localization of catecholamines and of 5-HT

in whole mounts and cross-sections of *S. mansoni*.

RESULTS

The presence of 5-HT and of norepinephrine in *S. mansoni* (2) raises the question whether the worm can synthesize these two amines from their precursor amino acids, tryptophan and tyrosine. The pathway for the synthesis of these two amines *de novo* in mammalian nervous tissue has been well established (14, 15).

Several approaches have been used to determine the existence of these pathways in *S. mansoni*. There is evidence for the decarboxylation of 5-hydroxytryptophan and of L-dihydroxyphenylalanine. Incubation of schistosomes in 5-hydroxytryptophan (1 mM) for 2 hr gave rise to 5-HT levels 10 times higher than those of nonexposed controls (3). Furthermore, after incubation in L-dopa (1 mM) for 2 hr, the concentration of dopamine was 6.7 μg/g, wet weight. This amine was not found in worms incubated without L-dopa (Table 1).

No evidence could be found indicating that adult *S. mansoni* can hydroxylate tryptophan. For example, incubation of the worms in modified Earle's medium containing tryptophan failed to produce a significant increase in the concentration of 5-HT or in the motor activity of the worms. By contrast, stimulation of the motor activity of schistosomes is readily observable when the worms are incubated in a medium containing a low concentration of 5-HT (1). Since monoamine oxidase could have destroyed the newly synthesized 5-HT (16), inhibitors

TABLE 1

Dopamine and norepinephrine levels of S. mansoni before and after incubation with L-dopa (2 mM) and nialamide (1 mM)

Period of incubation	Norepinephrine	Dopamine
hr	*μg/g (wet wt)*	
Control	0.58 ± 0.2 (3)[a]	0.0 (3)
2	0.68 ± 0.2 (6)	6.6 ± 0.3 (6)
4	0.44 (1)	6.4 (1)

[a] Numbers of experiments are shown in parentheses.

TABLE 2

5-HT levels of schistosomes following incubation at 37° in modified Earle's medium with or without tryptophan hydroxylase and monoamine oxidase inhibitors

The concentrations of tryptophan and of monoamine oxidase and tryptophan hydroxylase inhibitors were 0.1, 1, and 0.1 mM, respectively.

Additions	Incubation time	5-HT
	hr	*μg/g (wet wt)*
None	6	3.05 ± 0.68 (4)[a]
Tryptophan	6	3.31 ± 0.90 (2)
Tryptophan + iproniazid	6	3.45 ± 0.68 (4)
Tryptophan + nialamide	6	3.36 ± 0.5 (3)
None	48	2.60 ± 0.3 (3)
Tryptophan + iproniazid	48	2.91 ± 0.56 (2)
p-Chlorophenylalanine	48	2.78 ± 0.5 (2)
α-Propyldopacetamide	48	2.93 (1)

[a] Numbers of experiments are shown in parentheses.

(nialamide and iproniazid) of the activity of this enzyme were added to the incubation medium. This had no significant effect on 5-HT levels or muscular activity of the worms (Table 2). The rate-limiting enzyme in the synthesis of 5-HT in mammalian tissues is tryptophan hydroxylase, whose activity is inhibited by α-propyldopacetamide and by *p*-chlorophenylalanine (17, 18). Incubation of the worms in the presence of either of these inhibitors (0.1 mM) for 2 days produced no change in the concentration of 5-HT in the parasite (Table 2). However, this does not necessarily rule out the presence in schistosomes of a hydroxylase whose activity is not inhibited by high concentrations of these two compounds.

In two experiments 100–150 paired schistosomes were incubated with DL-[2-^{14}C]tryptophan (0.1 mM; specific activity, 10 μCi/μmole) for 4 days. After the schistosomes had been homogenized in the perchloric acid solution, determination of the radioactivity

of this mixture revealed that the number of counts per gram of worms was 4.5 times greater than the number of counts per milliliter of medium and that 0.9 μmole of tryptophan per gram of tissue had been taken up. One-fourth of the total radioactivity was present in the supernatant fluid. One-fifth of the latter was used to determine the endogenous concentration of 5-HT. To the remainder, 1 μg of unlabeled 5-HT was added. Following extraction by the method of Snyder *et al.* (7) and fractionation by column chromatography, as described by Anden and Magnusson (8), the eluate contained 3.6% of the radioactivity of the unfractionated supernatant. The eluate was lyophilized, extracted with methanol, and then co-chromatographed with a known amount of 5-HT (1 μg) and tryptamine (1 μg) by two-dimensional thin-layer chromatography (silica gel GF; thickness, 250 μ). A mixture of ethyl acetate, 2-propanol, and 58% ammonium hydroxide (4:3:1) was used in one dimension, and chloroform, methanol, and 58% ammonium hydroxide (12:7:1) in the other. The plate was then placed in a beaker containing formaldehyde and heated for 1 hr at 90° (19). The yellow spot on the plate, identified as 5-HT, contained only 0.36% of the radioactivity of the perchloric acid extract. Tryptamine accounted for 1%, while the remainder of the counts were not identified. The specific activity of DL-tryptophan in the incubation medium was 10 μCi/μmole, while that of the 5-HT isolated from the worms was only 0.06 μCi/μmole. Therefore, at the most, 1.2% of the L-tryptophan could have been converted to 5-HT in 96 hr. This is a maximum figure, because the chromatographed material may not have been identical with 5-HT, but merely have had the same chromatographic properties. Incubation of the worms for 2 hr in L-dopa resulted in the appearance of dopamine while there was no significant increase ($p > 0.5$) in the norepinephrine levels (Table 1). Fluorescence histochemical observations indicated that dopamine was entering the norepinephrine storage sites, since there was an increase in the intensity of the green fluorescence in these sites. If the parasite depends upon its host for a supply of 5-HT, its levels should reflect the concentrations of this amine in its habitat. This is consistent with the following observation. Whole blood of the hamster contains less 5-HT than mouse blood ($p < 0.01$). Similarly, the 5-HT levels of *S. mansoni* infecting hamsters were lower ($p < 0.01$) than those of worms from mice (Table 3).

TABLE 3

5-HT concentrations in hamster and mouse blood and of schistosomes removed from these hosts

Host	5-HT concentration	
	Blood	Schistosomes
	μg/g	μg/g
Mouse	2.98 ± 0.46 (6)[a]	2.88 ± 0.58 (6)
Hamster	0.75 ± 0.2 (8)	1.3 ± 0.3 (5)

[a] Numbers of experiments are shown in parentheses.

Uptake of 5-HT by S. mansoni

One of the mechanisms responsible for the inactivation of a neurotransmitter (e.g., 5-HT or norepinephrine) is its reuptake into the nerve terminal from which it has been released (20). In the mammalian central and peripheral nervous systems this reuptake operates against a concentration gradient, i.e., at tissue-to-medium ratios greater than 1.0. This has been demonstrated by incubating brain slices or perfusing intact organs with various biogenic amines which are thought to function as neurotransmitters in these tissues (11, 20, 21). This reuptake is slowed by a decrease in temperature, it is saturable, and it is dependent on metabolic energy and on the presence of sodium ions in the incubation or perfusion medium. In addition, reuptake is inhibited by certain drugs, such as amphetamine, imipramine, and protriptyline (21).

In an attempt to explore the presence of a specific uptake mechanism for 5-HT in *S. mansoni*, schistosomes were incubated *in vitro* at 37° in 75% horse serum containing various concentrations of 5-HT (1–50 μM). It was found that this parasite has the ability to accumulate 5-HT against a concentration gradient (Fig. 1). The tissue-to-

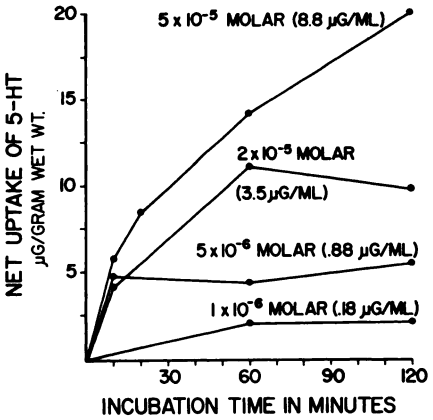

FIG. 1. *Uptake of 5-HT by S. mansoni exposed to various concentrations of 5-HT*

The tissue-to-medium ratios after 120 min were 13.8 at 1 µM, 7 at 5 µM, 3.5 at 20 µM, and 2.3 at 50 µM.

TABLE 4

Effect of temperature on 5-HT uptake by S. mansoni

Incubation in 5-HT (20 µM)	Net amount of 5-HT taken up		
	4°	17°	37°
min	*µg/g*	*µg/g*	*µg/g*
10	0.0 (1)[a]	1.0 (3)	3.2 (5)
60	0.9 (1)	3.4 (3)	7.7 (3)

[a] Numbers of experiments are shown in parentheses.

medium ratios were 2.3 or greater, depending on the 5-HT concentration in the medium. A marked reduction of the uptake rate occurred at lower temperatures (Table 4). 5-HT uptake was inhibited when the worms were incubated with a derivative of imipramine, chlorimipramine (5 µM). Reserpine proved to be a less potent inhibitor, and only a very slight inhibition of uptake was observed with ouabain (0.1 mM) (Table 5).

2-Bromolysergic acid diethylamide, a potent blocking agent of 5-HT receptors of schistosome muscle, had virtually no inhibitory effect on 5-HT uptake, while another 5-HT-blocking agent, 8β[(carbobenzoxyamino)methyl]-1,6-dimethyl-10α- ergoline (22), exerted an inhibitory effect on 5-HT uptake. The specificity of the uptake mechanism of the worms was tested by incubating them with [2-^{14}C] 5-HT for 5 min and comparing the amount of label taken up with that of worms which were incubated in ^{14}C-labeled 5-hydroxytrytophan, tryptamine, and *dl*-norepinephrine. The results are summarized in Table 6. The presence of the carboxyl group on the indole-ethylamine molecule (5-hydroxytryptophan) markedly decreased the rate of uptake. Furthermore, the absence of the 5-hydroxyl group (tryptamine vs. 5-HT) decreased the rate of uptake, although to a much lesser degree. The other amine (norepinephrine) stored by the nervous system of the schistosome was taken up, but not as rapidly as 5-HT.

When schistosomes were incubated in a 5-HT-containing medium, the 5-HT concentrations in the worms reached a constant level after 60 min, except at the highest concentration (50 µM) (Fig. 1). Therefore the uptake of 5-HT might be mediated by a saturable membrane transport process similar to that described by Dengler *et al.* (23) for the uptake of norepinephrine by brain slices and by cat heart. Iverson (21) measured the initial rates of norepinephrine uptake and found that they followed the classical Michaelis-Menten equation for saturable enzyme-substrate interactions. In an attempt to determine the presence of a similar uptake mechanism, schistosomes were incubated in various concentrations of 2-^{14}C-labeled 5-HT (Table 7). The velocity of the 5-HT uptake between 1 and 5 min was not proportional; i.e., it declined with time. Even during a 2-min period, the velocity of uptake was not constant (Table 7). A linear uptake rate was obtained when the temperature of the incubation medium was lowered to 27°. At this temperature the uptake velocity remained constant within 2 min (Table 7). The amount of nonspecific binding of 5-HT was determined by incubation of the worms at 4°. The values recorded at this temperature (less than 4–5% of those recorded at 27°) were substracted from those obtained at 27°. Between 50 nM and 1 µM the uptake velocity was nearly proportional to the 5-HT concentration in the medium (Fig. 2). This trend began to decline at concentrations above 1 µM. When

TABLE 5

Inhibition of 5-HT uptake by S. mansoni

Drug	Concentration	Incubation time	5-HT	Net uptake	Inhibition of uptake
	μM	*min*	*μM*	*μg/g*	%
Chlorimipramine (15 min)[a]	0	60	20	7.7 (4)[b]	
	5	60	20	3.9 (2)	50
	10	60	20	2.7 (2)	65
	30	60	20	1.6 (2)	79
Reserpine (60 min)	0	60	20	7.0 (2)	
	10	60	20	7.5 (1)	0
	30	60	20	3.0 (2)	57
	100	60	20	2.1 (2)	70
Ouabain (30 min)	0	20	50	18.7 (2)	
	10	20	50	18.3 (1)	2
	50	20	50	17.8 (1)	5
	100	20	50	16.8 (2)	15
BOL[c] (60 min)	0	60	20	8.3 (2)	
	10	60	20	7.7 (2)	7
MCE[c] (120 min)	0	60	50	17.0 (1)	
	2	60	50	18.0 (1)	0
	10	60	50	7.7 (1)	60
	100	60	50	0.2 (2)	99

[a] Preliminary incubation times.

[b] Numbers of experiments are shown in parentheses.

[c] The abbreviations used are: BOL, 2-bromolysergic acid diethylamide; MCE, 8β-[(carbobenzoxyamino)methyl]-1,6-dimethyl-10α-ergoline.

TABLE 6

Uptake of ^{14}C-labeled 5-HT, 5-hydroxytryptophan, norepinephrine, and tryptamine by S. mansoni during a 5-min incubation at 37°

The amines and 5-hydroxytryptophan were each present at 1 μM.

Compound	Radioactivity in medium	Uptake	Tissue to medium ratio	Label taken up compared with 5-HT
	cpm/ml	*cpm/g (wet wt)*	*cpm/cpm*	%
5-HT	1.11×10^5	2.8×10^5 (3)[a]	2.58	100
5-Hydroxytryptophan	1.11×10^5	6.65×10^3 (2)	0.059	2
Tryptamine	1.12×10^5	1.16×10^5 (2)	1.03	40
dl-Norepinephrine	1.11×10^5	4.5×10^4 (3)	0.41	17

[a] Numbers of experiments are shown in parentheses.

the data reproduced in Fig. 2 were plotted according to Lineweaver and Burk (24), two slopes were obtained (Fig. 3), one at concentrations below and the other above 2 μM. This indicates the presence of two uptake mechanisms for 5-HT, one of which has a high affinity for this amine, while the other has a low affinity.

In a limited number of experiments it was found that *Schistosoma japonicum* removes 5-HT from the medium at rates at least as high as those found for *S. mansoni*. Since no 5-HT storage sites could be localized by fluorescence histochemistry in *S. japonicum*, the physiological role of 5-HT in this schistosome species may be different from that of *S. mansoni* (1, 3).

The ability of the worms to retain 5-HT

TABLE 7

Velocity of 5-HT uptake by S. mansoni at two temperatures during three time periods

Incubation time	Rate of uptake of 5-HT	
	37°	27°
min	*ng/min/g schistosomes*	
1	160 (4)[a]	98.9 (3)
2	109 (1)	95.5 (3)
5	70.2 (4)	

[a] Numbers of experiments are shown in parentheses.

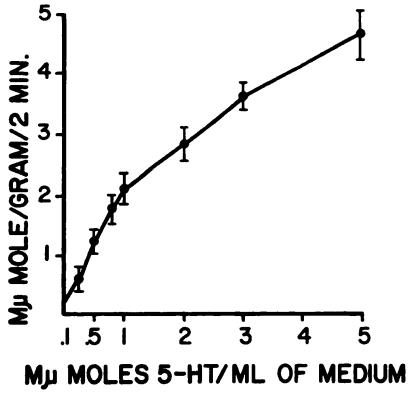

FIG. 2. *Velocity of 5-HT uptake at 27° by S. mansoni at various 5-HT concentrations*

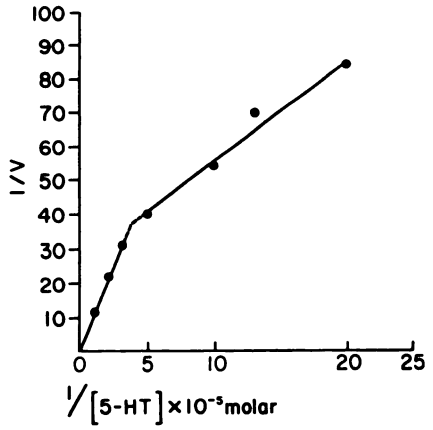

FIG. 3. *Double-reciprocal plot of 5-HT concentrations in the medium and in S. mansoni*
Incubation was carried out at 27° for 2 min.

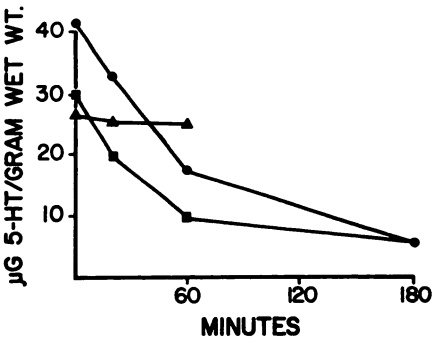

FIG. 4. *Release of 5-HT by S. mansoni following incubation for 2 hr at 37° in 20 μM 5-HT* (■——■) *and 50 μM 5-HT* (●——●) *and at 4° in 20 μM 5-HT* (▲——▲)

was determined by measuring the rate at which they lost 5-HT following incubation with this amine. Worms were incubated at 37° for 2 hr in 20 and 50 μM 5-HT. They were then transferred to an incubation medium containing no 5-HT for 20, 60, and 180 min (Fig. 4). 5-HT release was temperature-dependent, since the decay rate was slower when the temperature of the medium was lowered to 4°. The loss of 5-HT was also monitored by the fluorescence histochemical method. The intensity of the diffuse fluorescence, generated by the parenchymal cells, progressively decreased with time; the rate of decay was slower in those cells which store 5-HT. After 180 min, increased fluorescence of the 5-HT storage sites was clearly distinguishable, whereas the fluorescence intensity of the parenchymal cells returned to that of control worms which had not been exposed previously to 5-HT. This indicated that a measurable amount of 5-HT was retained in the nervous tissue.

DISCUSSION

A recent paper by White and Paton (25) emphasizes the need to determine initial uptake velocities under conditions such that the uptake rates are linear with time. In determining the kinetics of 5-HT uptake by *S. mansoni* we found it necessary to reduce the temperature by 10° and to shorten the incubation period to 2 min; under these conditions the rates of 5-HT uptake were linear with time. The affinity of schistosomes for 5-HT was found to be 100 times greater than that reported by Woodward and Read (26) for the uptake of histidine by the ces-

tode *Hymenolepis diminuta*, and by Haynes (27) for the uptake of tyrosine by *Taenia crassiceps*. The affinity of *S. mansoni* for 5-HT is closer to the K_m (0.5 μM) obtained by Shaskan and Snyder (12) from brain slices known to contain 5-HT terminals. These authors also found a second, low-affinity uptake mechanism for 5-HT.

One of the characteristics associated with the presence of a neurotransmitter in a given neuronal tissue is the occurrence of enzymes which catalyze the synthesis and degradation of the transmitter. Enzymes catalyzing the degradation of 5-HT and norepinephrine (monoamine oxidase) and of acetylcholine (acetylcholinesterase) as well as the synthesis of acetylcholine (choline acetylase) have been demonstrated in *S. mansoni* (15, 28). On the other hand, the results reported in this paper have failed to indicate the presence of pathways *de novo* for 5-HT and norepinephrine in *S. mansoni*. This negative evidence does not exclude this possibility. The lack of an increase in 5-HT after prolonged incubation with tryptophan and with a monoamine oxidase inhibitor might merely reflect saturation of the tryptophan hydroxylase with endogenous tryptophan. Furthermore, the lack of a decrease in 5-HT levels during incubation with tryptophan hydroxylase inhibitors and the extremely low rate of incorporation of [^{14}C]tryptophan into 5-HT could be accounted for by a similarly low turnover of 5-HT. On the other hand, the dependence of schistosomes on their host has been demonstrated for several essential constituents of the worm; for example, adenine, steroids, and fatty acids are not synthesized *de novo* by these parasites (29–31).

The only major site for the synthesis of 5-HT outside the central nervous system of mammals is the enterchromaffin cell (32). Part of the 5-HT secreted by these cells reaches the mesenteric veins. Approximately 90% of the 5-HT released into the blood is taken up by platelets (33), while the rest remains in the plasma. Erspamer and Testini (32) found that the concentration of plasma 5-HT is highest in the hepatic veins, i.e., the veins which receive the blood from the mesenteric veins. Thus it appears that there is a gradient of 5-HT within the veins of the host and that the concentration of 5-HT increases as the source of 5-HT is approached. Therefore the establishment of adult schistosomes in the mesenteric-portal venous system, where the 5-HT concentration is relatively high, could be ascribed to the dependence of the worm upon this amine.

Histochemical evidence indicates that neuron-like structures are capable of storing 5-HT. Even at the low concentrations of unbound (platelet-free) 5-HT in blood plasma of the mouse (estimated to be approximately 1 μM) (32), the high-affinity 5-HT uptake mechanism of *S. mansoni* can provide an effective means of supplying the worm with a neurotransmitter which it may not be able to synthesize. Since schistosomes ingest blood cells of their hosts, the 5-HT stored in the platelets represents an additional source of this amine for the parasite.

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