Spectrofluorometric and Chromatographic Characterization of a Butanol Extract from *Fasciola hepatica*

The possibility that 5-HT or a strictly related compound may be involved in the transmission of nerve impulses in *Fasciola hepatica* was first proposed by MANSOUR[1] and then confirmed through various investigations showing that the motility of these worms could be affected by different drugs such as 5-HT itself, amphetamine, LSD and reserpine[1–4]. The finding that the stimulant effects of amphetamine and 5-HT were identically antagonized by highly specific anti-5-HT drugs[5] provided additional evidence for the presence in *F. hepatica* of a single tryptamine receptor probably involved in the mechanisms of transmission of the motor impulses in these platyhelminthes.

Acetone liver-fluke extracts confirmed the presence of 5-HT or of a 5-HT-like substance in amounts of 60–70 ng/g wet weight when assayed on the rat uterus preparations in the presence of atropine[1]. However, similar and preliminary assays by us (unpublished data) showed that these acetone extracts contained also substances capable of modifying the responsiveness of the biological test, thus making it unsuitable for accurate determinations. These observations led us to undertake the research described in this paper, by employing fluorometric and thin-layer chromatographic methods in order not only to determine quantitatively the amount of the occurring indolealkylamine, but also to discriminate qualitatively whether this substance was really 5-HT.

Materials and methods. Liver flukes collected from bile ducts of bovine livers within 1 h of the death of the host, were immediately washed in Ringer solution (37 °C) in order to remove the coarse daubing material on their surfaces. Then they were either submitted to the extraction procedure or incubated in Ringer solution (37 °C) for a $1^1/_2$ h period and then extracted. Samples of 0.5–1 g fresh tissue were extracted with 10 ml of acid-butanol according to ANSELL et al.[6].

The extracts submitted to the various steps of the same method of ANSELL et al.[6] were then tested for their fluorescence on a 3000 CGA spectrophotofluorometer[7]. Both activation and fluorescence spectra were then recorded. All wavelengths were uncorrected.

Extracted and unextracted standards of 5-HT, as well as extracted samples of 6-hydroxytryptamine (6-HT), 5-hydroxytryptophane (5-HTP), 4-hydroxytryptophane (4-HTP), 5-methyltryptophane (5-MTP), 5-acetoxy-N-acetyltryptophane (5-acO-N-acTP), 5-metoxytryptamine (5-meOT), tryptamine (T), *F. hepatica* tissue and 5-HT added to *F. hepatica* tissue were similarly prepared and tested for their fluorometric spectra. The same extracted samples were tested on thin-layer chromatography (butOH, 4; acetic acid, 1; H_2O, 1) and compared for their characteristics at UV-light (254 and 366 nm) and for their colour reactions after development of the chromatographic plates with NNCD and *p*-dimethylaminobenzaldehyde reagents. Moreover, the coloured spots of *F. hepatica* tissue from various chromatographic plates were eluted with butanol and collected in order to obtain sufficient material to be tested biologically on rat uterus preparations.

Results. Qualitative analyses. The activation and fluorescence spectra of extracted and unextracted 5-HT, of extracted *F. hepatica* tissue and tissue added with 5-HT submitted to the extraction procedure are graphed in Figure 1. Moreover, the fluorometric data about the other indoles tested in our experiments are listed in Table I. The chromatographic characteristics of the same substances are reported in Table II. The results of chromatographic experiments with tissue extract added with known amounts of 5-HT, revealed that the spot of the tissue can always be clearly separated from that of 5-HT both for their different Rf values and for colour characteristics. It must, however, be pointed out that the migration of 5-HT alone is slightly faster than when it is added to tissue extract.

Quantitative determination. The results of the determinations on 14 samples of freshly collected worms and on a same number of samples of incubated liver flukes, showed that the fluorescent compound is present in amounts of 1640 and 855 ng/g of fresh tissue respectively when dosed against 5-HT base employed as a standard fluorescent compound. On the other hand, the chromatographic spots of *F. hepatica* tissue eluted and tested revealed no detectable biological activity, the amount extracted from 2 g of fresh tissue being less active than 10 ng of 5-HT on the rat uterus preparation.

Discussion. The results reported show that the extract from *F. hepatica* tissue contains a fluorescent compound clearly distinguishable from 5-HT and other indole compounds examined both for fluorometric and chromatographic properties. The closeness of the fluorometric spectra of this unknown substance to those of the known compounds tested by us suggests a strict resemblance to compounds with an indole structure. The results of thin-layer chromatography confirm this suggestion. In fact, according to HANSON[8], the positive reactions of the chromatographic spot developed by the NNCD and *p*-dimethylaminobenzaldehyde reagents, strongly support that it is an indole compound.

Since there is no indolealkylamine which is as potent as 5-HT in stimulating contractions of the rat uterus preparation[9], we can also explain the remarkable difference between our quantitative results obtained by means of

Table I. Fluorescence characteristics of the indoles tested

Substances	Activation maximum (nm)	Fluorescence maximum (nm)
Fasciola hepatica extract	392	487–492
5-hydroxytryptamine[a]	390	510–515
6-hydroxytryptamine	399	513–518
5-hydroxytryptophane	394	454–458
4-hydroxytryptophane	395	458–463
5-methyltryptophane	401	494–498
5-acetoxy-N-acetyltryptophane	389	457–462
5-methoxytryptamine	385	462–466
Tryptamine	396	457–463

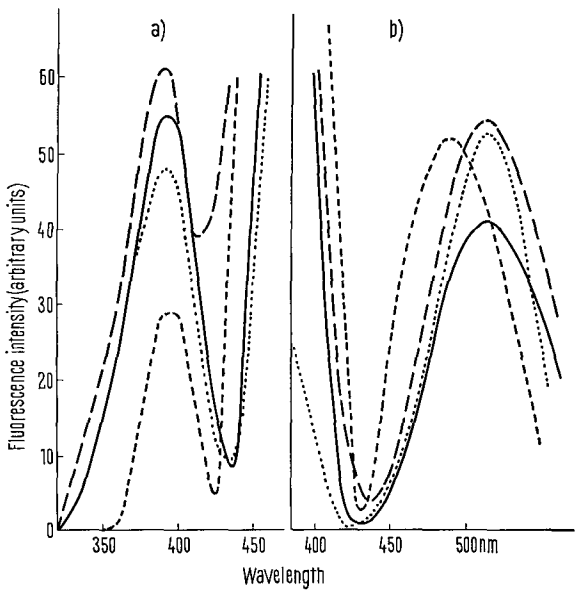
[a] 5-hydroxytryptamine creatinine-sulphate.

1 T. E. MANSOUR, Fedn. Proc. *16*, 319 (1957).
2 C. BERETTA, Fedn. Proc. *28*, 793 (1969).
3 A. M. LUXARDO, C. BERETTA and A. LOCATELLI, Veterinaria. *18*, 185 (1969).
4 K. D. BEERNINK, S. D. NELSON and T. E. MANSOUR, Int. J. Neuropharmac. *2*, 105 (1963).
5 C. BERETTA and A. LOCATELLI, J. Pharm. Pharmac. *20*, 744 (1968).
6 G. B. ANSELL and M. F. BEESON, Analyt. Biochem. *23*, 196 (1968).
7 CGA Dott. Ciampolini, Firenze (Italy).
8 A. HANSON, in *Handbook of Experimental Pharmacology* (Ed. V. ERSPAMER; Springer-Verlag, Berlin–Heidelberg–New York 1966), vol. 19, p. 77.

Table II. Chromatographic characteristics of the spots

Substances	UV-visualization 254 nm	366 nm	NNCD[a]	*p*-dimethylaminobenzaldehyde[b] 30 min after spray	24 h after spray	Rf
Fasciola hepatica extract	opaque immediately and fluorescent 24 h later	not fluorescent	orange/yellow	purple[c]	blue-green	0.58
5-HT[d]	opaque	not fluorescent	peach-red	blue/violet	blue-grey	0.70
6-HT	opaque	fluorescent	ruby-red	blue	blue-green	0.72
5-HTP	opaque	not fluorescent	flesh-colour	yellow	yellow/grey	0.50
4-HTP	opaque	not fluorescent	red-brown	grey/yellow	grey	0.55
5-MTP	opaque	not fluorescent	gold/yellow	grey	grey-blue	0.58
5-acO-N-acTP	opaque	not fluorescent	yellow/orange	purple	rose	0.74
5-meOT	opaque	not fluorescent	gold/yellow	violet	blue-green	0.72
T	opaque	not fluorescent	yellow	purple	blue-green	0.82

[a] 0.1 g of 2-chloro-4-nitrobenzenediazonium naphthalene-2-sulphonate were dissolved in 1 ml of concentrated HCl and the volume was brought to 100 ml with distilled H_2O. [b] 20 ml of *p*-dimethylaminobenzaldehyde 5% ethanolic solution were added to 10 ml of concentrated HCl and the volume was brought to 100 ml with absolute ethanol. [c] During the spray development the spot appears yellow and then becomes purple. [d] 5-hydroxytryptamine creatinine-sulphate.



Standard = 5-hydroxytryptamine creatinine-sulphate. (———), Fluorescence spectra of extracted standard; (- - - - - -), *F. hepatica* tissue extract; (– – – –), extracted standard added with *F. hepatica* extract; (.....), unextracted standard. (a) Activation spectrum. (b) Fluorescence spectrum.

fluorometric and biological tests. According to our present results, on the contrary, the quantitative data of MANSOUR[1] cannot be confirmed. From our experiments it can moreover be seen that the samples of incubated liver flukes show about 48% reduction ($P < 0.001$) in their content of the fluorescent compound, when compared with the samples of freshly collected worms. In our opinion this fact is worth noting, although, at present, we cannot explain this rapidly occurring decay.

Whether the compound described in this paper is itself the true neuromuscolar transmitter in *F. hepatica* or a precursor of the true transmitter we cannot yet say. However, the occurrence in *F. hepatica* tissue of a compound strictly related to the group of the indole compounds provides further evidence that these worms might account for it for their biological needs, and once more supports the suggestions of previous works[1-4] about the possible role of 5-HT or a related substance for the transmission of nerve impulses in *F. hepatica*[10].

Riassunto. Gli AA. descrivono le caratteristiche fluorimetriche e cromatografiche di un estratto butanolico dal tessuto di *F. hepatica* concludendo che la sostanza estraibile presenta caratteristiche indoliche ma non appare identificabile nè con la 5-HT nè con alcuni altri indoli saggiati.

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[9] V. ERSPAMER, in *Handbook of Experimental Pharmacology* (Ed. V. ERSPAMER; Springer-Verlag, Berlin–Heidelberg–New York 1966), p. 127.

[10] Ricerche svolte con il contributo del C.N.R.

Intermedin (MSH)-Like Effect of a Thermal Polymer on Vertebrate Chromatophores

The ability of small intermedin (MSH) peptides to stimulate dispersion of pigment granules within frog melanophores has been reported[1,2]. Iridophore contraction (reflecting platelet aggregation) is also induced by these peptides and this implies that their action is essentially like that of the parent hormone and suggests that mechanisms of both melanophore and iridophore stimulation have common features[3]. Recently, FOX and WANG[4] reported that thermal polymers of arginine, glutamic acid, glycine, histidine, phenylalanine, and tryptophan have melanophore-stimulating activity. The present study was undertaken to determine whether such thermal