rapidly removed, cleaned, weighed and then frozen. Each adrenal gland was homogenized in 1 ml. of ice-cold isotonic sucrose with glass Duall homogenizer and an aliquot taken for the determination of noradrenaline[7]. The remaining homogenate was centrifuged at 27,000*g*, tyrosine hydroxylase[8], phenylethanolamine N-methyl-transferase (PNMT)[9], and catechol-O-methyl transferase (COMT)[10] were assayed in the supernatant fraction and monoamine oxidase (MAO)[11] was determined in the particulate fraction.

Tyrosine hydroxylase, the initial and rate-limiting enzyme in catecholamine formation, is localized in sympathetic nerves and in the adrenal gland. PNMT is restricted to the adrenal medulla and certain areas of the brain and converts noradrenaline to adrenaline. In the socially deprived mice there was a significant decrease in adrenal tyrosine hydroxylase and PNMT activity (Table 1). The noradrenaline and adrenaline content was slightly but not significantly lower in these animals. No changes in MAO or COMT activity were observed. There was a marked elevation in tyrosine hydroxylase, PNMT, and MAO activity and in noradrenaline and adrenaline content in the adrenals of psychosocially stimulated mice, but no change in COMT activity. Adrenal gland weight was elevated in both deprived and stimulated mice.

Table 1. EFFECT OF PSYCHOSOCIAL STIMULATION ON THE ACTIVITY OF CATECHOLAMINE FORMING AND METABOLIZING ENZYMES IN MALE MOUSE ADRENALS

Treatment	Adrenal (weight/mg)	PNMT (units)	Tyrosine hydroxylase (units)	MAO (units)
Normal	1·05 ± 0·12	0·29 ± 0·013	1·8 ± 0·29	19 ± 3·0
Stimulus deprived	1·55 ± 0·05†	0·19 ± 0·026†	1·1 ± 0·21†	21 ± 1·5
Stimulus increased	2·5 ± 0·15*	0·48 ± 0·41*	4·3 ± 0·41*	36 ± 3·5†

Treatment	COMT (units)	Noradrenaline (μg)	Adrenaline (μg)
Normal	0·053 ± 0·006	0·42 ± 0·1	1·42 ± 0·20
Stimulus deprived	0·042 ± 0·005	0·36 ± 0·07	1·01 ± 0·17
Stimulus increased	0·057 ± 0·006	1·01 ± 0·15*	3·23 ± 0·25*

* $P < 0.001$. † $P < 0.01$. ‡ $P < 0.05$.

Results are expressed as mean ± *S.E.* per adrenal. One unit of enzyme activity is the pmole product formed per h.

These results demonstrate changes in enzymes concerned with the biosynthesis of noradrenaline and adrenaline in the adrenal glands of mice subjected to different social interactions. In socially deprived mice there was a fall in tyrosine hydroxylase and PNMT activity although adrenal weight was increased, while in socially stimulated mice there was a rise in both biosynthetic enzymes and one metabolizing enzyme (MAO). The elevation of these enzymes might be the result of increased discharge of corticoids from the adrenal cortex, or an increased stimulation of the adrenal gland by sympathetic nerves. We have previously shown that tyrosine hydroxylase and PNMT can be elevated by a reflex increase in sympathetic nerve activity, but not by pituitary or corticoid hormones[12]. Furthermore, it was found that the increase in enzyme activity was trans-synaptically induced, dependent on new protein synthesis, and required one to three days before an elevation was apparent[13]. Prolonged, forced immobilization of rats also produces an elevation of tyrosine hydroxylase and PNMT activity and this rise could be abolished by cutting the splanchnic nerve to the adrenal gland (personal communication of R. Kvetnausky, I. J. Kopin and V. Weise). These findings suggest that the increase in the catecholamine-forming enzymes in psychosocial stimulation may also be neuronally mediated and is not an immediate response, as in the case of a sudden discharge of noradrenaline and adrenaline in states of anger, fear or aggression[2,3].

A reduction of adrenal PNMT and tyrosine hydroxylase activity occurs after hypophysectomy and this decrease can be prevented with ACTH. Adrenal tyrosine hydroxylase activity decreases after cutting the splanchnic nerve to the adrenal[14]. Whether the reduced PNMT and tyrosine hydroxylase activity in psychosocially deprived animals is due to a depletion of ACTH, reduced sympathetic nerve stimulation or other factors remains to be established.

JULIUS AXELROD
ROBERT A. MUELLER
Laboratory of Clinical Science,
National Institute of Mental Health,
Bethesda, Maryland.

JAMES P. HENRY
PATRICIA M. STEPHENS
Department of Physiology,
University of Southern California,
Los Angeles, California.

Received December 17, 1969.

1 Schildkraut, J. J., and Kety, S. S., *Science*, **156**, 21 (1967).
2 Elmadjian, F., Hope, J. M., and Lamson, E. T., *J. Clin. Endocrinol.*, **17**, 608 (1957).
3 Levi, L., *Psychosomat. Med.*, **27**, 80 (1965).
4 Axelrod, J., Whitby, L. G., and Hertting, G., *Science*, **133**, 383 (1961).
5 Glowinski, J., and Axelrod, J., *Pharmacol. Rev.*, **18**, 775 (1966).
6 Henry, J. P., Meehan, J. P., and Stephens, P. M., *Psychosomat. Med.*, **29**, 408 (1967).
7 von Euler, U. S., and Lishajko, F., *Acta Physiol. Scand.*, **61**, 348 (1961).
8 Mueller, R. A., Thoenen, H., and Axelrod, J., *J. Pharmacol. Exp. Therap.*, **169**, 74 (1969).
9 Axelrod, J., *J. Biol. Chem.*, **237**, 1657 (1962).
10 Wurtman, R. J., and Axelrod, J., *J. Biol. Chem.*, **241**, 2301 (1966).
11 Wurtman, R. J., and Axelrod, J., *Biochem. Pharmacol.*, **12**, 1439 (1963).
12 Thoenen, H., Mueller, R. A., and Axelrod, J., *J. Pharmacol. Exp. Therap.*, **169**, 249 (1969).
13 Mueller, R. A., Thoenen, H., and Axelrod, J., *Mol. Pharmacol.*, **5**, 463 (1969).
14 Thoenen, H., Mueller, R. A., and Axelrod, J., *Endocrinology* (in the press).

Direct Postsynaptic Responses to Stimulation of Serotonin-containing Neurones

THERE is one giant serotonin-containing neurone in each metacerebral ganglion of the slug *Limax maximus* and of the snail *Helix pomatia*. These cells can be identified histochemically and their content of serotonin can be measured by bioassay of individually dissected neurones. The amine is localized in small electron dense granules in the cytoplasm of these cells and also, in certain conditions, in particles with the appearance of lysosomes (ref. 1 and unpublished data of G. A. C. and N. N. Osborne). Dye injection experiments with 'Procion Yellow', using the method of Stretton and Kravitz[2], show that each giant neurone in *Helix pomatia* gives off three main axonal branches. One branch passes across the cerebral commissure to the other group of cerebral ganglia, another can be traced to the ipsilateral cerebral-buccal connective, and the third passes into the ipsilateral exterior lip nerve.

Kandel and Tauc[3,4] have previously studied the membrane properties of these neurones in *Helix aspersa* and *Helix pomatia* and they have discovered that they show a high degree of anomalous rectification. They also showed, using electrophysiological methods, that each cell sends an axon to the ipsilateral exterior lip nerve and one to each cerebral-buccal connective.

In this study a search was made for cells directly innervated by these giant serotonin-containing neurones in *Helix pomatia*. The circum-oesophageal ganglionic complex, together with the buccal ganglia and connecting nerves, were dissected from active *Helix pomatia* and pinned to a nylon sheet fixed at the bottom of a small bath (volume 0·7 ml.). The preparation was perfused with snail saline[5]. Double-barrelled glass micro-electrodes were used to impale the giant cell and to explore possible postsynaptic cells. One barrel was filled with 3 M KCl and used for recording and the other, used for applying current, was filled with 0·6 M Na_2SO_4. Input leads from the electrodes were fed through cathode followers to a dual beam oscilloscope.

Giant serotonin cells usually fired a burst of spikes when impaled with a micro-electrode and then remained inactive. They were made to fire action potentials by pass-

ing a depolarizing pulse of 1 s or shorter duration through the second electrode. In each buccal ganglion a neurone was found which was stimulated by a burst of spikes in a giant serotonin cell. The cell was located close to the entry of the cerebral-buccal connective in each ganglion. The response recorded in some preparations was a slight depolarization at normal membrane potential (Fig. 1*a*) which was usually within the range of 50 to 60 mV. In other preparations, usually when the buccal cell showed some prior spike activity, a burst of spikes in a giant serotonin cell resulted in depolarization and a spike firing in the buccal cell. In a few preparations a small individual response was recorded with each spike fired in a giant serotonin cell. There was facilitation of such responses with repetitive stimulation and the potentials summed (Fig. 1*b*). There was a constant delay of about 100 ms in appearance of these potentials, suggesting a direct link between the giant serotonin cell and the buccal cell described.

Further information on the connexions between the cells was obtained in other preparations by hyperpolarizing one of the buccal cells described and repeatedly stimulating a giant serotonin cell. The size of the responses varied slightly from one preparation to another, but they were increased in amplitude by hyperpolarization. The potentials summed with repeated stimulation of a giant cell and in most cases facilitation of the responses could be observed. Increasing the level of hyperpolarization did not result in a loss of the potentials. Depolarizing pulses of different durations which did not result in spike firing in a giant cell did not cause any potential changes in the buccal cells.

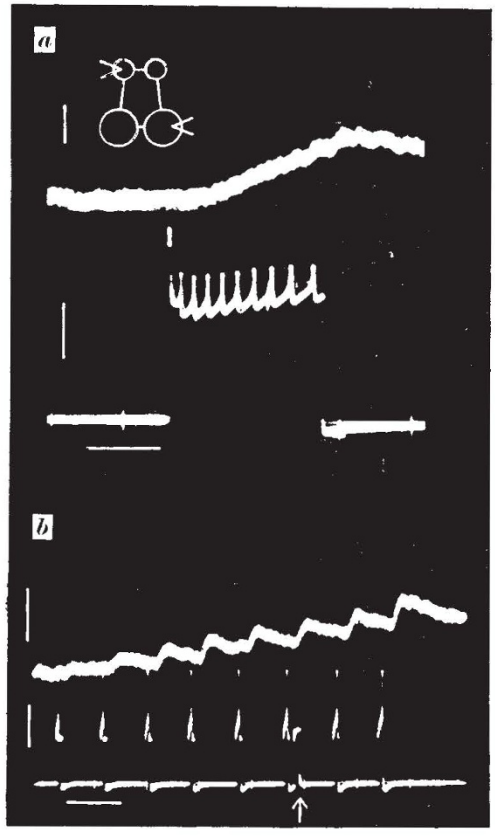

Fig. 1. Responses of a cell of the left buccal ganglion to spike firing in the right giant serotonin cell. The records are from two preparations. The buccal cell response is recorded on the upper beam in each case. (Sometimes, as in *a*, a giant serotonin cell would not fire spikes without relatively intense depolarizing pulses. Frequently axon spikes alone were recorded in these conditions.) In *b*, 50 ms depolarizing pulses, each resulting in only one spike, were applied to the giant serotonin cell. Each spike was followed by a small depolarizing potential in the buccal cell. The responses summed and were facilitated with repeated stimulation. A square wave alone did not affect the buccal cell (arrow). Calibrations: upper beam 2 mV; lower beam 20 mV; 0·5 s.

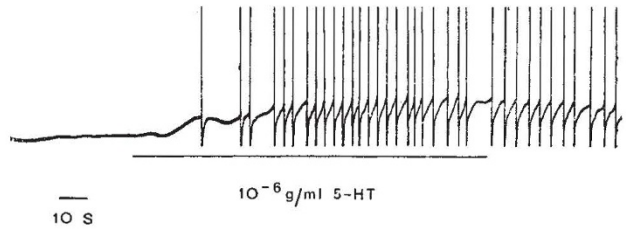

Fig. 2. Response of a cell, of the left buccal ganglion, which was previously shown to respond to spikes in the left giant serotonin cell, to perfusion with serotonin (5-HT). (The pen recorder did not record the full amplitude of the spikes.)

The data suggest that the small potentials observed are chemically mediated excitatory postsynaptic potentials (EPSPs). The view that the linkage was monosynaptic was supported by the observations that the EPSPs could not be lost by repeated stimulation, and the delay in appearance of EPSPs was always constant in a given experiment at about 100 ms.

The delay in response of the buccal cell was about what would be expected from information on conduction velocity of spikes in gastropod nerves. Conduction velocity along the intestinal nerve of *Helix pomatia* is at 5 cm/s (ref. 7). The distance between the giant serotonin cells and the buccal ganglia is about 0·7 cm.

Each of the two giant serotonin cells had an input on to a cell in each buccal ganglion and the conduction time for ipsilateral and contralateral connexions was observed to be the same. In some preparations both giant serotonin cells were stimulated while recording from the same buccal cell. No consistent difference in responses was observed depending on whether one or the other of the giant serotonin cells was stimulated.

It is thought that serotonin may be a transmitter substance in molluscs[7]. If this is the case with the giant serotonin cells, it would be expected that buccal cells would be activated by the amine. This was, indeed, shown to be so. The cells were depolarized and started firing spikes when serotonin, at a concentration of 10^{-7} g/ml. or greater, was added to the perfusion liquid (Fig. 2), whereas the giant serotonin cells were not depolarized and generally did not spike when the amine was added. Reduction in the potential, produced by applying a constant hyperpolarizing current, when serotonin was added to the bath showed that the amine increases membrane conductance of the specified buccal cells. Acetylcholine also stimulated the postsynaptic buccal neurone (as well as the giant serotonin cells). With respect to this observation, it may be important that each of the buccal cells receives at least one excitation input other than those from the giant serotonin cells. Dopamine, in concentrations of about 10^{-7} g/ml., hyperpolarized the postsynaptic cells. In the case of each of these drugs the responses of the specified buccal cells resemble those of the "CILDA cells" of gastropods which have been shown to have specific serotonin receptors[8,9].

The response of the cells in the buccal ganglia to activation of the giant serotonin cells was blocked with relatively high concentrations of the serotonin antagonists lysergic acid diethylamide (5×10^{-5} g/ml.) and "Methysergide" (10^{-4} g/ml.) but not by perfusing lower concentrations of these drugs for periods up to about 15 min. Further studies, however, need to be completed in order to evaluate the significance of the experiments with high concentrations of the blocking drugs in view of the observations[10] that the closely related compound 2-bromolysergic acid diethylamide can interfere with axonal conduction in another gastropod mollusc, *Aplysia*.

These results show that two symmetrically arranged serotonin-containing neurones form excitatory synaptic links with other neurones in the snail brain. They also provide evidence that serotonin is a transmitter in the

central nervous system of the snail. The system that I have described may provide a favourable preparation for further pharmacological studies with drugs which modify the effects, or levels, of serotonin in nervous tissue.

I thank the MRC for financial support and Mr C. J. Roemmélé and Mr J. Brown for technical assistance.

G. A. COTTRELL
Wellcome Laboratories of Pharmacology,
Gatty Marine Laboratory,
University of St Andrews,
Fife, Scotland.

Received November 7; revised December 29, 1969.

[1] Cottrell, G. A., and Osborne, N. N., *Nature*, **225**, 471 (1970).
[2] Stretton, A. O. W., and Kravitz, E. A., *Science*, **162**, 132 (1968).
[3] Kandel, E. R., and Tauc, L., *J. Physiol.*, **183**, 269 (1966).
[4] Kandel, E. R., and Tauc, L., *J. Physiol.*, **183**, 287 (1966).
[5] Meng, K., *Zool. Jber.*, **68**, 539 (1960).
[6] Krijgsman, B. J., *Z. vergleich. Physiol.*, **28**, 286 (1941).
[7] Cottrell, G. A., and Laverack, M. S., *Ann. Rev. Pharmacol.*, **8**, 273 (1968).
[8] Stefani, E., and Gerschenfeld, H. M., *J. Neurophysiol.*, **32**, 64 (1969).
[9] Ascher, P., *J. Physiol.*, **198**, 48 P (1968).
[10] Gerschenfeld, H. M., and Stefani, E., *J. Physiol.*, **185**, 684 (1966).

Acute Toxicity of Aflatoxin B_1 in Wethers

THE original objective of this study was the preparation of milligram quantities of aflatoxin M_1. Holzapfel *et al.*[1] and Masri *et al.*[2] had collected sufficient quantities of the toxin from the urine of sheep which had eaten food containing aflatoxin B_1 for structural studies. We administered an exploratory dose of 4 mg of aflatoxin B_1/kg, a conservative value compared with a reported single dose LD_{50} of approximately 500 mg/kg (ref. 3). This was lethal to our wether. In subsequent correspondence Purchase advised that an (intraperitoneal) dose of 2 mg/kg had killed a Merino sheep, and Masri noted that a repeated oral dose of 0·38 mg/kg per day for seven days was lethal to both a Hampshire ewe and a crossbred western whiteface wether. If the toxin is to be given over a period of time, a sublethal dose must be used. We undertook a toxicological study to satisfy this requirement and now report the results of our LD_{50} study.

The aflatoxin B_1 was obtained from extracts of cultures of *Aspergillus flavus* (FDA, DM Isolate No. M141) grown on sterile moist rice and purified by the procedure of Rodricks[4]. Recrystallization from chloroform yielded aflatoxin B_1 of 98 per cent purity based on thin layer chromatography and ultraviolet analysis (λ_{max} (361 nm) in methanol, ε 21,500). The required quantities of aflatoxin B_1 were weighed into gelatine capsules which were completely filled with powdered sucrose and sealed.

The animals used were two year old wethers, crossbred Hampshire–Shropshire–Merino–Southdown and an experimental Morlam breed derived from crossbred Dorset–Rambouillet–Merino sheep. They were kept in individual metabolism stalls adapted from a design by Briggs and Gallup[5] which allowed separate collection of faeces and urine. A mixture of alfalfa, clover and timothy hay was freely offered, and a grain feed, Beltsville 20C, was restricted to 0·9 kg per animal each day. While the animals were in the stalls, rectal temperatures were taken, and the droppings and general clinical appearance were observed. Sheep showing irregular or questionable results and abnormal behaviour without treatment were rejected.

After dosing, the clinical signs and body temperatures were recorded at 2–5 h intervals. Laparotomy was performed on surviving sheep, usually at day 7 and again after several weeks, to follow the course of liver restitution. Dead animals were necropsied. If the sheep survived, faeces were collected for 5 days and urine for 3 days after dosing and analysed for the content of aflatoxins B_1 and M_1.

Another single exploratory dose was given at 1·28 mg/kg and multiple doses were given on successive days (shown in parentheses): 0·9 (2), 0·59 (2), 0·23 (2) and 0·14 (6) mg/kg to separate wethers. None of these died.

Table 1. ACUTE EFFECT OF AFLATOXIN B_1 ON 2 YEAR OLD WETHERS

Daily dose mg/kg body weight	Days	No. of animals	No. dead
4·00	1	3	3
2·84	1	3	3
2·00	1	3	1
1·42	1	3	0
1·28	1	1	0
0·90	2	1	0
0·59	2	1	0
0·23	2	1	0
0·14	6	1	0

The single dose LD_{50} for crossbred wethers, calculated by the Weil[6] procedure, was 2·0 with a 95 per cent confidence interval of 1·6 to 2·4 mg/kg, where, according to the notations used by Weil, $K=3$, $n=2$, $r=0$, 1, 2, 2, $d=0{\cdot}1505$ and $Da=1{\cdot}42$ mg/kg. When one Morlam wether was added to each group, the LD_{50} was 2·0 (1·7–2·6) mg/kg where $n=3$, $r=0$, 1, 3, 3, and the remaining constants are as before. The dosing and mortality data are summarized in Table 1.

Loss of appetite and diarrhoea occurred at 0·23 mg/kg and higher, and persisted several days after a sublethal dose. Excessive salivation, frothing at the mouth and increased rectal temperatures of 0·9° ($P<0{\cdot}025$) within 12 h were seen at 1·28 but not 0·59 mg/kg. Higher doses caused an increased rate of respiration and increased the temperature 1·5° C or more. Much mucus and some bloody rectal discharge accompanied the scours after days 1–2 among sheep dosed at 1·28–2·00 mg/kg.

All the sheep that died in 15–18 h had massive centrolobular necrosis of the liver. One animal, dosed at 4 mg/kg, which died on day 6, had marked centrolobular necrosis of the liver with necrotic bridges extending to contiguous central veins. This produced an apparent reversal of the normal lobular architecture in that the portal triads, and surrounding normal parenchyma, were now central to a new pseudolobular pattern.

In liver biopsies of surviving sheep on day 7 there was periportal congestion with widely dilated sinusoids and loss of liver cells in these areas. On day 53 there was still focal congestion, along with microfocal liver cell degeneration.

An oral dose of aflatoxin B_1 0·5 mg/kg followed by 1–2 weeks' rest and subsequent redosing at the same level was tolerated by several sheep. This schedule, however, may have contributed to the cause of later death in two wethers. Other schedules therefore need to be examined for converting aflatoxin B_1 to M_1 without endangering the animals.

The susceptibility of sheep to an acute aflatoxin poisoning is thus similar to that observed with other animal species[7]. A limited number of biopsy observations indicate that the animals require several months to recover from sublethal doses of aflatoxin B_1.

We thank Dr George M. Sidwell, Sheep Branch, ARC, USDA, Beltsville, for supplying several sheep and technical advice; and Mr James E. Jackson and Mr Harry E. Zimmerman, jun., for technical assistance.

BERNARD H. ARMBRECHT
WILLIAM T. SHALKOP
LARRY D. ROLLINS
Division of Veterinary Research,
Food and Drug Administration,
Washington DC 20204.

ALBERT E. POHLAND
LEONARD STOLOFF
Division of Food Chemistry
and Technology,
Food and Drug Administration,
Washington DC 20204.

Received August 5; revised December 9, 1969.

[1] Holzapfel, C. W., Steyn, P. S., and Purchase, I. F. H., *Tetrahedron Lett.*, 25, 2799 (1966).