serotonin) and neuropeptides (endorphins and ACTH) specifically in the hypothalmus[24] of alcohol-preferring and non-alcohol-preferring animals will provide important information with regard to the chemical control of uncontrollable drinking.

Acknowledgments. The authors express their gratitude to L. DeLallo and S.F.A. Elston for expert technical assistance. This research was supported by a Grant to K.B. from Southwest Research Institute.

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0014-4754/87/040408-03$1.50 + 0.20/0

Tetanus intoxication causes an increment of serotonin in the central nervous system

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Summary. Mice injected with tetanus toxin (TTx) showed an increase of 5-hydroxytryptamine (serotonin, 5-HT) levels in the central nervous system. The increment was not uniform thoughout the central nervous system. Particularly significant were the 25% and 80% increases observed, respectively, in whole brain and spinal cord. The levels of dopamine and norepinephrine remained unchanged. The subsequent studies of 5-HT turnover revealed a synthesis rate in the tetanic animals that was almost double that of controls. The degradation rate of the amine as well as the levels of 5-hydroxyindolacetic acid were unaffected.
Key words. Serotonin; 5-hydroxytryptamine; tetanus toxin; pargyline; alphamethyldopa; indolamine.

Tetanus is a grave condition produced by tetanus toxin and characterized by muscular rigidity and recurrent spasms leading to death in a considerable percentage of cases. Several reviews have been published dealing with the chemistry[1,2], physiological effects[1,3] and pathophysiology[4] of the toxin. The toxin is integrated by two polypeptidic chains of 100,000 and 50,000 daltons linked by a disulfide bridge[5,6]. As in the case of other bacterial toxins, one of the chains may have the toxic properties while the other mediates the binding to the target cells[7]. To exert its effects on the central nervous system (CNS), the toxin has to be bound by the peripheral nerve endings, probably through membrane gangliosides[8], incorporated inside them, and then retrogradely transported through the axon to the spinal cord, which is the level at which the toxin impairs the release of inhibitory amino acids from interneurons controlling the activity of the motoneurons. Conclusive evidence for or against a subsequent transport to higher levels of the CNS is currently lacking. However, this mobility of the toxin has proved very useful for studying the connections between different cerebral structures[9].

Both in vitro and in vivo experiments show that the toxin affects preferentially the synapses which use glycine or GABA as a transmitter[10]. Thus, the inhibition of glycine release at the spinal level plays a key role in the symptomatology of tetanus, in man as well as in experimental animals[11,12]. Nevertheless, as we mentioned above, the effect of the toxin on supraspinal regions is less well known, and the encephalic symptoms of tetanus infection have not been satisfactorily explained.

However, several direct effects of the toxin on certain brain structures have already been demonstrated. Thus, the intranigral injection of tetanus toxin suppresses the bicucculine-sensitive inhibition of neurons in the substantia nigra evoked from the striatum[13]. Certain encephalic symptoms of tetanus such as insomnia, parkinsonism, hyperthermia and hypertension seem to suggest the implication of different monoaminergic systems[1]. Moreover, TTx has been detected in the cerebrum of rats 24 h

Table 1. 5-HT levels in brain and spinal cord of the mouse after injecting tetanus toxin

Dose	Controls (nmol/g wet tissue)	Treated
	Brain	
1 MLD	4.12 ± 0.48 (10)	5.03 ± 0.59 (14)**
2 MLD	3.63 ± 0.34 (11)	4.52 ± 0.22 (11)***
10 MLD	3.94 ± 0.40 (6)	4.87 ± 0.53 (6)*
	Spinal cord	
2 MLD	3.85 ± 0.90 (6)	6.94 ± 2.60 (6)*

The results are expressed as means ± SD for the number of animals between brackets. The results have been analyzed using Student's t-test; * = $p < 0.05$, ** = $p < 0.025$, *** = $p < 0.005$, in comparison to control value.

Table 2. Daily cycle variation of the levels of 5-HT, DA, and NE as influenced by tetanus toxin

Monoamines (nmol/g wet tissue)	Group of animals	Time after toxin injection (h) 30 (17.00)	36 (23.00)	42 (05.00)	48 (11.00)
5-HT	Tetanus	4.39 ± 0.39	3.13 ± 0.37*	3.69 ± 0.41**	3.52 ± 0.48**
	Control	4.36 ± 0.49	2.74 ± 0.36	3.00 ± 0.24	2.95 ± 0.26
DA	Tetanus	7.03 ± 0.94	6.96 ± 0.68	6.91 ± 0.36	6.86 ± 0.66
	Control	7.04 ± 0.71	6.81 ± 0.65	7.33 ± 0.56	6.62 ± 0.79
NE	Tetanus	2.62 ± 0.21	2.23 ± 0.14	2.26 ± 0.25	2.13 ± 0.09
	Control	2.35 ± 0.30	2.32 ± 0.32	2.39 ± 0.11	2.19 ± 0.21

Evolution of the brain levels of 5-HT and catecholamines during the period of 5-HT increase subsequent to the injection of 2 MLD tetanus toxin. The results are expressed as mean ± SD of 6–8 mice per group. The data have been analyzed using Student's t-test; * = $p < 0.05$ and ** = $p < 0.005$.

after intramuscular injection of the toxin[14]. Since TTx cannot cross the blood-brain barrier, this observation has been explained in terms of the well known ability of the toxin to ascend to the axons and migrate trans-synaptically[15]. How far the toxin can ascend in this way before the animal dies is still a matter of controversy.

Certain studies have also shown an 'in vitro' inhibitory effect of TTx on dopamine (DA)[13] and norepinephrine (NE)[16] release from, respectively, slices and particles of striatal tissue.

As another approach to the study of TTx effects at supraspinal levels, the concentrations of norepinephrine, dopamine and serotonin were measured in the whole brain and spinal cord of mice injected with the toxin. Subsequently, we determined the effect on the turnover of serotonin, the only one of these amines whose levels were found to be altered by the toxin.

Material and methods. Tetanus toxin was provided by Wellcome Research Laboratories (batch AWX 400). We considered 1 MLD as the minimal dose killing all mice (weighing about 20 g) within 96 h after an i.p. injection of the toxin. Our experimental MLD was 1.3 ± 0.1 times the DL50 and 1.8 ± 0.1 times the highest non-lethal dose, an equivalence which agrees with Wellhonner's data[1]. The main symptoms observed before death in mice treated with doses of TTx higher that the MLD were the following: – a reduction of the ocular space between the lids; – abnormal walking, with stiffness and functional loss of the hindlimbs; – lateral twitching; – seizures; – stiffness of the jaw; – scrotal skin retraction.

Mice were sacrificed by decapitation and their brains, or spinal cords, were rapidly removed, weighed and homogenized individually in ethanol-water (74% v/v) to obtain a 10% w/v homogenate. The homogenate was centrifuged at 1500 × g for 30 min and the supernatant removed and diluted with the same volume of distilled water. After adding 0.4 ml of 2% EDTA per gram of tissue, and adjusting the pH to 6.8, the diluted supernatant was transferred to a cation exchange column (Amberlite CG-50, H +, type II, 200–400 mesh, Prolabo). The amines were retained at neutral pH. All acidic and neutral compounds were eluted in the first eluate and first water washing and were concentrated subsequently by lyophilization.

5-HT, DA and NE were eluted with 5 ml 0.2 N acetic acid and quantified by independent fluorescent procedures. NE and DA were measured by the dihydroxyindol assay of Anton and Sayre[17]. This technique uses iodide as oxidant, and sulfite/EDTA as reducing/stabilizing agent. 5-HT was assayed following the modification of Curzon and Green[18] of the orthophthaldialdehyde condensate assay of Maickel and Miller[19]. In this method, the samples are treated with orthophthaldialdehyde (OPT) in an acid medium (HCl) during 15 min, at 100 °C, and fluorescence subsequently read at 470 nm, the wavelength of the incident light being 360 nm. The use of L-cysteine in the assay results in a marked increment in the amplitude of the fluorescence and the reproducibility. 5-hydroxytryptophan (5-HTP) and 5-hydroxyindoleacetic acid (5-HIAA) were separated by the Fisher and Aprison method[20]. The indolic groups were quantified by development of their native fluorescence in strong acid (HCl 3N).

In order to determine total tryptophan levels, an independent process was developed: Samples were homogenized in trichloroacetic acid (TCA) and centrifuged at a moderate speed. Subsequently, the levels of tryptophan were determined by a spectrofluorometric method based on the conversion of this amino acid to norharmane using formol and hydrogen peroxide[21].

Results. Increase of 5-HT. Following i.p. injection of tetanus toxin, an increase of serotonin was detected in the central nervous system (table 1). The concentrations of dopamine and norepinephrine remained unchanged (table 2). The increase of 5-HT was not uniform throughout the central nervous system. Increments of up to 25% and 80% were detected, respectively, in the brain and spinal cord. The evaluation of the encephalic levels of serotonin observed over 6-h periods after the administration of 2 MLD of TTx showed that the increases of 5-HT were not statistically significant until a latency period of 36 h had elapsed. Nevertheless, the 5-HT variations followed the circadian pattern of this amine (table 2). The latency period decreased when we increased the toxin dose. With 10 MLD the increase of serotonin was apparent within 24 h after the injection of the toxin; with 1, 2, and 3 MLD of TTx the increase in serotonin levels became apparent within 72, 42, and 30 h, respectively (see tables 1–4).

5-HT turnover. Table 3 shows the 5-HT values together with those of its precursor, 5-HTP, and its degradation product,

Table 3. Brain and spinal cord 5-HT, 5-HIAA, Trp and 5-HTP in tetanus intoxication

Dose (nmol/g wet tissue)	Brain Control	Tetanus	Spinal cord Control	Tetanus
5-HT	4.14 ± 0.82	5.03 ± 0.81*	6.63 ± 1.54	11.95 ± 4.49**
5-HTP	0.67 ± 0.04	0.58 ± 0.09*	0.71 ± 0.10	0.77 ± 0.14
5-HIAA	2.27 ± 0.34	2.15 ± 0.48	4.25 ± 0.60	4.27 ± 0.85
Trp	31.10 ± 2.33	31.83 ± 3.54		

Values are expressed as mean ± SD of 10–18 mice after injection of 3 MLD. The results have been analyzed using Student's t-test; * = $p < 0.01$, ** = $p < 0.001$.

Table 4. Effect of tetanus toxin on the turnover of 5-hydroxytryptamine

Rate of biosynthesis: monoaminoxidase inhibition by pargyline	Time after the injection of pargyline 0	15	30	45 min
Control (A)	2.49 ± 0.26	2.79 ± 0.42	3.01 ± 0.35	3.09 ± 0.44
Tetanus (B)	3.10 ± 0.69	3.36 ± 0.44	3.92 ± 0.64	4.55 ± 0.79**
Rate of metabolism: 5-hydroxytryptophan inhibition by alphamethyldopa	Time after the injection of alphamethyldopa 0	1	2	3 h
Control (C)	3.36 ± 0.48	2.82 ± 0.26	2.46 ± 0.51	2.21 ± 0.31
Tetanus (D)	4.21 ± 0.22	3.39 ± 0.27	2.67 ± 0.19	2.70 ± 0.47

The results are expressed mean ± SD (nmol/g wet tissue) of 6–10 mice after injection of 2 DLM of TTx in the intoxicated animal or physiological serum in the control animal groups. The doses of enzyme inhibitors are for pargyline: 75 mg/kg i.p., for alphamethyldopa: 400 mg/kg s.c. The different slopes (pmol/g/min): A = 13.50, B = 31.90, C = −5.93, D = −8.81. In the pargyline experiment the comparison of the two regression lines/using Student's t-test shows that the respective slopes are significantly different. ** = $p < 0.005$.

5-HIAA, in the brains of treated and control animals. The increase of 5-HT was associated with a decrease of 5-HTP in the brain, but not in the spinal cord. 5-HIAA levels remained unchanged. 5-HT turnover in the brain was studied by using enzymatic inhibitors. The modification of 5-HT levels consequent to the administration of these agents is illustrated in table 4. When the degradation is inhibited with pargyline (75 mg/kg, i.p.), the brain of the animals treated with tetanus toxin accumulated 5-HT faster than controls. In contrast, the administration of alphamethyldopa (400 mg/kg, s.c.) showed a similar degradation velocity in both groups of animals.

Discussion. The tetanus toxin does not cause a general increase of brain neurotransmitters. Among the neurotransmitters which have been investigated, only 5-HT was significantly altered as a result of the injection of tetanus toxin. The effect is very marked in the spinal cord, where the levels of serotonin are almost twice those found in controls. This heterogeneous distribution of 5-HT increment is probably due to the uneven distribution of the serotoninergic terminals which are especially abundant in the spinal cord. The increase of 5-HT is maintained and parallels the daily variations of the amine levels, thus ruling out the possibility that they are brought about by simple displacement of the circadian pattern.

The notable increase in 5-HT synthesis that we have described in the intoxicated animal, combined with the maintenance of the degradation rate, supports and explains the high levels of this amine found in the central nervous system. The effect of tetanus intoxication on the synthesis of 5-HT cannot be explained by a possible hypoxia due to the spasticity of the respiratory muscles as experiments carried out in rats demonstrate that hypoxia, if anything, causes a reduction of 5-HT levels as well as a decrease of its turnover[22]. On the other hand, an increase of serotonin as a consequence of the eventual fasting of the intoxicated animals has been ruled out since no effect was seen in control animnals kept without food for periods of comparable time. Moreover, an increment of serotonin secondary to an increase of tryptophan levels in plasma has also been discarded[23]. The brain tryptophan concentration is related to the levels of free tryptophan in plasma, and this in turn is determined by the fasting state since non esterified fatty acids displace this amino acid from albumina[24]. However, we failed to find any significant variation of brain tryptophan levels in tetanic animals with respect to controls (table 3).

It has been repeatedly demonstrated that the variations of 5-HT are correlated with the variations in the flux of nervous impulses in the serotonergic cells. The stimulation of the serotonergic fibers increases the synthesis of 5-HT[25,26] and there is a positive correlation between the synthesis of 5-HT and the serotonergic functions[27]. The drugs which inhibit the spontaneous discharges of the 5-HT cells also decrease the metabolic turnover of 5-HT in the brain[28–30].

The experiments with alpha-methyldopa seem to rule out an increase of 5-HT metabolism in the intoxicated animals, and the levels of 5-HIAA observed suggest the same conclusion. Such results apparently contradict the hypothesis of a greater serotonergic activity related to 5-HT metabolism, although the possibility of an increased activity of the system without a concomitant increase of the 5-HT degradation detectable in the brain cannot be dismissed, since factors such as the uptake rate and the reutilization of the amine affect the relation between neural activity and neurotransmitter metabolism.

One possible mechanism to explain the above results would be an impairment of 5-HT release because of the tetanus intoxication; this, in turn, would induce an increase of the synthesis of this amine by a feed-back mechanism[31]. It would explain the presence of encephalic symptoms of tetanus disease such as insomnia and parkinsonism. This hypothesis would also agree with the general inhibitory effect that TTx is supposed to have on neurotransmission[10–13].

Acknowledgment. This study has been sponsored by the 'Fondo de Investigaciones Sanitarias' (FIS) 559/81.

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