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Influence of aethimizol on the incorporation of labeled methionine by brain structures

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Summary. – *The present paper reports the influence of aethimizol on the incorporation level of labelel aminoacids in proteins of brain structures closely connected with the regulation of pituitary–adrenal system.*

Riassunto. – *Vengono riportati gli effetti dell'etimizolo sull'incorporazione di aminoacidi marcati nelle proteine di zone del cervello connesse con la regolazione del sistema ipofisi–surrene. L'etimizolo aumenta l'incorporazione di metionina nell'ipotalamo anteriore e nell'amigdala, mentre la diminuisce nell'ippocampo dorsale e nell'ipotalamo posteriore.*

Introduction.

Aethimizol (bis–methylamide, 1–ethyl–imidazol–4,5–dicarbonic acid), possesses pronounced stimulating actions on the hypothalamus–hypophysis–adrenal system. In previous investigations it was demonstrated an effect of this drug on the central regulating ACTH–glucocorticoids secretion [1–3]. The present paper reports on the influence of aethimizol on the incorporation level of labelel aminoacids in proteins of brain structures closely connected with the regulation of pituitary–adrenal system (anterior and posterior hypothalamus, medial amygdala and dorsal hippocampus). Moreover, the distribution of labeled methionine in the aforementioned brain structures was studied in conditions of excess and deficiency of hypophysis–adrenal cortex hormones.

Material and Methods.

The experiments were carried out on 60 white inbred male rats weighing 200 g. The animals were divided into 6 group: 1st group (control): animals injected with physiological solution (0.3–0.4 ml, intraperitoneally, daily for a week); 2nd group: animals injected with aethimizol (10 mg/kg subcutaneously twice a day for a week); 3rd group: hypophysectomized animals; 4th group: adrenalectomized rats; 5th group: rats treated with ACTH–zinc–phosphate (10 UI/kg intramuscularly every day); 6th group: animals receiving dexamethasone (0,5 mg/kg, intraperitoneally). The experiment started one week after the operation or drug treatment.

Protein metabolism in hypothalamic and limbic brain structures was measured by means of an histoautoradiographic method using ^{35}S–methionine. The labelel aminoacid was injected to all animals (0.5 mC per g intraperitoneally). Histoautographs were performed 15–20 min and 3.5 hours after administration of the marker [4]. The brain sections (50 μ) serially preparared on the microtome were fixed on plates covered with photoemulsion and kept in the dark for 10 days. For the quantitative analysis of labeled methionine incorporation in cells of anterior and posterior hypothalamic nuclei, dorsal hippocampus and mediobasal amygdala the number of tracks in the photoemulsion was counted in an area of 100 μ^2, using a microscope carrying an ocular micrometre net. This value served as an index of marker incorporation by the cells of brain structures studied. Arithmetical means from each rat were subjected to statistics.

Results and discussion.

Analysis of the autographs of the control rats (Fig. 1) shows that the labelel methionine, 15 min after its introduction, is incorporated in neural cells of different brain regions with different intensity. The number of tracks in the anterior and mammilar hypothalamus and in the amygdala varies from 1.4 to 2.25 while in the dorsal hippocampus is 5. Therefore, the rate of protein metabolism in neural cells of the dorsal hippocampus is higher than that the other brain structures considered. It should be noted that the number of tracks in neural cells exceeds the number in glial elements. Moreover, the intensity of radiomethionine incorporation in supraoptical nuclei of the anterior hypothalamus exceeds that found in the small cellular hypothalamic formations.

After aethimizol the incorporation of labelel methionine is increased in the anterior hypothalamic brain region and in the amygdala, while it is decreased in the dorsal hippocampus and in the posterior hypothalamus. It is doubtful that the alterations of intensity of labelel methionine incorporation observed after aethimizol treatment are caused by changes in penetration of the hematoencephalic barrier. Novikova [5], as well as Manina [6] in experiments carried out in rats showed that the intensity of mark incorporation into the proteins was identical after subcutaneous and intracerebral administration of labeled methionine.

In further experiments the role played by the hormones in the effect of aethimizol was studied. Numerous investigations show that corticosteroids penetrate the brain, and accumulate in hypophysis and in other brain regions [7–10]. This fact indicates the possibility of a direct action of adrenocortical hormones on these structures. Because of their influence on protein metabolism, some investigators consider glucocorticoids as catabolic hormones [11, 12]. The supposition is based on the increase of protein decay, the intensification of aminoacids deamination and the appearance of negative nitrogen balance occurring after large doses of ACTH or glucocorticoids. Other authors observed an increase of methionine and glycine incorporation in liver proteins upon cortisone and hydrocortisone administration [13, 14]. The literature data on the incorporation of various aminoacids in discrete brain areas are contradictory and very difficult to compare, due to the differences in the techniques employed [5, 15–19]. According to McEwen et al. [7] corticosteron concentrates mainly in neuronal nuclei, less in citoplasm and it is almost absent in membranes; hormones increase RNA and protein synthesis, leading to specific functional changes in all brain regions.

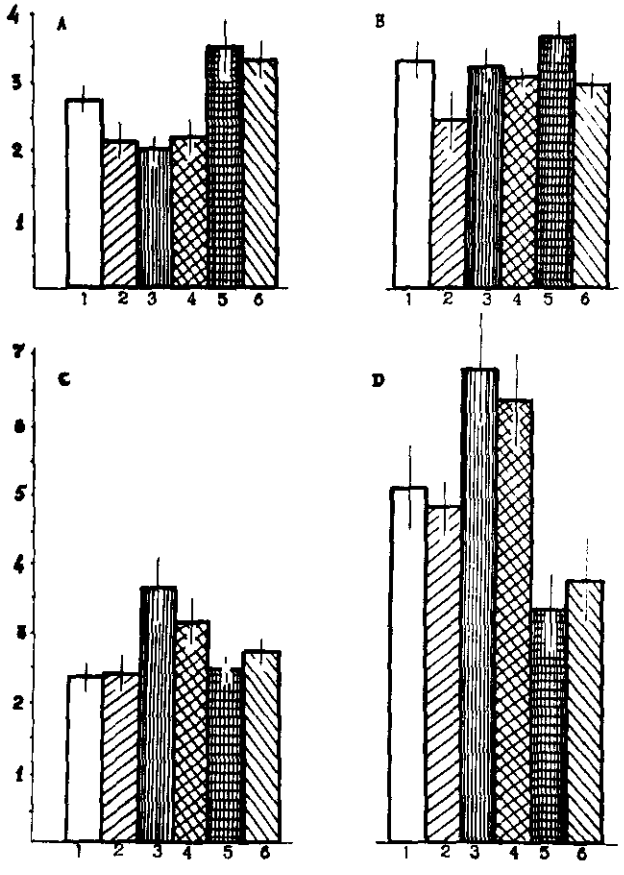

FIG. 1. – Intensity of labelel methionine incorporation in brain structures (tracks per 100 m^2). A = anterior hypothal amus; B = posterior hypothalamus; C = medial amygdala; D = dorsal hippocampus. 1 = control; 2 = hypophysectomy; 3 = adrenalectomy; 4 = ACTH (10 UI/kg); 5 = dexamethasone (0.5 mg/kg); 6 = aethimizol.

Allegedly, the alterations of intensity of methionine incorporation characterizing the protein metabolism level in nervous cells of different brain formations and visible under aethimizol influence depend on the functional state of these brain structures modulated by the hormones of pituitary–adrenal cortex system. In fig. 1 are shown the changes of ^{35}S–methionine incorporation in different brain structures caused by endocrine gland extirpation or by hormone treatment. In the anterior hypothalamus the intensity of incorporation is decreased in animals with removed adrenals or pituitary and after ACTH treatment; the incorporation is increased after dexamethasone or aethimizol. In the posterior hypothalamus the alterations are not so clearcut. Radiomethionine incorporation in dorsal hippocampus and in amygdala is increased in adrenalectomized rats and in animals treated with ACTH. In the dorsal hippocampus dexamethasone or aethimizol treatment diminishes the incorporation.

These results indicate that hypophysectomy leads to a diminution of incorporation in hypothalamic brain regions without essential changes in limbic structures. After adrenalectomy incorporation diminishes in anterior hypothalamus and rises in amygdala and hippocampus. A similar picture, but less pronounced, is observed after administration of ACTH. Repeated dexamethasone injections are accompanied by a raise of tracks number in hypothalamic brain region and by an inhibition of the intensity of incorporation in dorsal hippocampus.

The action of aethimizol in the anterior hypothalamus and in the hippocampus is similar to the glucocorticoids, while in the posterior hypothalamus and in the amygdala its effect resembles ACTH.

If changes in protein synthesis reflect alterations in excitability of brain structures under the action of hormones of pituitary–adrenal cortex system, the present data point to a diversified influence of ACTH and glucocorticoids on the brain structures controlling the activity of the pituitary–adrenal cortex axis.

REFERENCES

1. RYZHENKOV, V.E. 1962. The influence of alkylderivatives of diamides – imidazol dicarbonic acid on adrenocorticotropic hypophysis function. In: *Pharmacology of New Sedative Drugs and their Clinical Using.* S. V. Anickov (Ed.). Medgiz (Leningrad), pp. 175–185 (Russ.).

2. SAPRONOV, N.S. 1971. The influence of Aethimizol on the Hypothalamic–Hypophysial–Adrenal System of Rats in Dexamethasone Inhibition. *Bull. Exp. Biol. Med.* **6**: 58–61 (Russ.).

3. SAPRONOV, N.S. 1979. Participation of brain catecholamines in the pituitary–adrenal system activation caused by aethimizol. *Farmakol. Toxikol.* **3**: 216–221 (Russ.).

4. ZHINKIN, L.N. (Ed.) Radioactive Indicators in Histology. Medgiz, Leningrad, 1959.

5. Novikova, E. 1972. ^{35}S–methionine Incorporation in different Brain Structures of rats in Ontogenesis. In: *Proc. 6th All Union Conf. Neurochem.* Leningrad, Acad. Sci. USSR, p. 107.

6. Manina, A. 1959. The Incorporation and Discharge of radioactive methionine by Nervous System of white rats. In: *Radioactive indicators in histology.* L.N. Zhinkin (Ed.). Medgiz, Leningrad, pp. 139–154.

7. McEwen, B.S., Magnus, C. & Wallach, G. 1971. Biochemical studies of corticosterone binding to cell nuclei and cytoplasmic macromolecules in specific regions of the rat brain. In: *Steroid Hormones and Brain Function.* C.H. Sawyer & R.A. Gorski (Eds.). UCLA Forum Med. Sci., Univ. of California Press, Los Angeles. **15**: 247–258.

8. McEwen, B.S., Wallach, G. & Magnus, C. 1974. Corticosterone binding to hippocampus: immediate and delayed influences of the absence of adrenal secretion. *Brain Res.* **70**: 321–334.

9. Chytil, F. & Toft, D. 1972. Corticoid binding component in rat brain. *J. Neurochem.* **19**: 2877–2880.

10. Knizley, H. Jr. 1972. The hippocampus and septal area as primary target sites for corticosterone. *J. Neurochem.* **19**: 2737–2745.

11. Lebedeva, M. 1956. The influence of Cortisone and Desoxycorticosterone on Protein Metabolism. *Probl. Med. Chem.* (USSR). **4**: 278–286.

12. Miller, I., Peck, W. & Brandi, J. 1967. Hydrocortisone induced inhibition of protein synthesis and uridine incorporation in isolated bone cells in vitro. *Proc. Nat. Acad. Sci. USA.* **57**: 1959–1961.

13. Yudaev. N.A., Lebedeva, M. & Zavyalskaya, N. 1957. The effect of Cortisone and Desoxycorticosterone on inclusion of Glycine–1–C^{14} into the Proteins of Liver Sections. *Probl. Endocrinol.* **6**: 13–21.

14. Koike, K., Otaka, T. & Okui, S. 1967. Regulation of protein synthesis in mouse liver by steroid hormones. *J. Biochem.* (Tokyo). **61**: 679–684.

15. Semiginovsky, B., Jacoubek, B., Kraus, M. & Erdosova, R. 1974. Stress – induced changes in the brain cortex slices of rats: effects of adrenalectomy. *Physiol. Bohemoslov.* **23**: 503–510.

16. Warner, R.B. & Dick, A.R. 1974. Independence of glucocorticosteroids and blood brain barrier amino acid permeability. *Psychiat. Res.* **10**: 263–269.

17. Litteria, M. & Thorner, M.W. 1975. Inhibitory action of neonatal estrogenization on the incorporation of ^{3}H-lysine into proteins of specific hypothalamic nuclei in the adult male rat. *Brain Res.* **90**: 175–180.

18. Chasov, E.I., Veselova, S.P., Krivosheev, O.G. & Isachenkov, V.A. 1976. Interrelationship between the pineal gland and the hypothalamohypophysial complex. Report I. The effect of pinealectomy, blinding and continuous illumination of the ^{3}H-leucine incorporation into the nuclei of the anterior, middle and the posterior hypothalamus of the prepuberal and mature rats. *Probl. Endocrinol.* **1**: 33–39.

19. Mattock, J.M. & MacKinnon, P.C. 1976. An autoradiographic study of changes in relative incorporation of ^{3}H–methionine in nuclei of the amygdala and related brain areas of male, female and androgenized females prior to puberty. *Brain Res.* **114**: 166–170.

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Interaction of nicotinic and muscarinic cholinergic mechanisms with the specific and non-specific afferent brain systems

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Summary. – *In this work the interactions of M- and N-cholinergic mechanisms in specific (visual) and non-specific (reticular) afferent brain systems has been analyzed.*

Riassunto. – *Nel presente lavoro sono state studiate le interazioni tra i sistemi muscarinici e nicotinici nelle vie afferenti specifiche (visive) e non specifiche (reticolari).*

Introduction.

The division proposed by Anichkov of the central cholinergic system in muscarino- (M) and nicotino-(N) sensitive was a powerful stimulus for a series of researches on the transmission of impulses within the brain [1].

The study of the relationships between chemical structure and pharmacological action in a series of alkylderivatives of benactyzine and of trasentin synthetized in the department of pharmacology, allowed to formulate the hypothesis of an interaction of M- and N-cholinergic mechanisms within an unique cholinergic system [2].

Numerous literature data, as well as our investigations indicate that in the ascending reticular activating system (non -specific) a M-type transmission is involved while the character of mediation in the synapses of specific transmission it is not clear. In this work the interactions of M- and N-cholinergic mechanisms in specific (visual) and non-specific (reticular) afferent brain systems has been analyzed.

Methods.

Acute experiments were carried out in 17 non-anesthetized rabbits immobilized with d-tubocurarine with electrodes implanted in frontal and occipital cortex, in caudate nucleus, hippocampus and reticular formation of midbrain (RF). The EEG was recorded from the indicated brain structures. Reticulocortical responses (RCR) in frontal and visual cortex regions induced by bipolar electrostimulation of reticular formation were also recorded. Under light stimulation the primary responses (PR) were recorded in the visual and frontal cortex. The frequency of stimuli in both cases was 0.5 Hz; the voltage varied from 6-10 V. An impulse analyzer (Nokia) elaborated 100 responses in every test.

The following drugs were studied: an anticholinesterasic agent, nivalin (0.5-1.5 mg/kg); a M-cholinolytic, metamyzil (2-methyl-2-diethylaminoethylbenzilate, 0.1-1 mg/kg); two N-cholinolytics, gangleron (3-diethylamino-1-2-dimethilpropyl-p-isobutoxybenzoate, 1.5-3 mg/kg) and etherophen (1-ethyl-2-diethylaminoethyl-diphenylacetate, 5-7.5 mg/kg). All drugs were injected intravenously.

Results and discussion.

Upon light stimulation the PR recorded in the visual cortex consisted of an initial positive component followed by a negative one; in the frontal cortex the superficial negative wave was the initial component. In most experiments nivalin slightly decreased the amplitude of the positive component of PR and increased the amplitude of its negative component. This is probably connected with the endogenous acetylcholine accumulation. The last effect is realized through receptors of muscarinic type. The consequent blockade of M-cholinergic system by means of metamyzil leads to inhibition of the negative PR component and to an increase of the positive one. Compared to control the positive component of PR is increased while the negative component decreased. It can be supposed that after blockade of M-cholinergic synapses endogenous acetylcholine has still an action on N-cholinergic ones. Such a supposition is confirmed by the fact that administration of the N-anticholinergic drugs etherophen or gangleron decreases the positive PR component and increases the negative one, bringing the response to the initial level (Fig. 1).

Reticulocortical response in the frontal and visual cortex induced by the stimulation of reticular formation consists of a negative wave. When the stimulus is increased, a positive-negative RCR component appears. In most experiments nivalin increases the amplitude of the RCR negative component. Subsequent administration of metamyzil leads to a diminution of the amplitude of the RCR negative component, and sometimes to the appearance of a positive component before the negative one. Blockade of N-cholinergic synapses with etherophen or gangleron brings the RCR amplitude to the initial level.

Light stimulation brings about in the frontal cortex a weak negative wave whose amplitude in most cases

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Opiate mechanisms in the regulation of the cardiovascular system

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Summary. – *In the present paper the effects of enkephalins on central regulation of hemodynamics in unanaesthetized cats and the antagonism between the effects of clonidine and naloxone in hypertensive and normotensive rats are reported.*

Riassunto. – *Sono stati studiati gli effetti delle enkefaline sulla regolazione centrale della pressione arteriosa nel gatto. In ratti normali ed ipertesi è stato dimostrato un antagonismo tra naloxone e clonidina.*

Introduction.

There is some evidence for the participation of opioid peptides in the central regulation of the arterial pressure. High concentrations of opiate receptors and enkephalins in brain structures related to the central regulation of hemodynamics have been found [1, 2]. Enkephalins administered in the cerebral ventricles of anaesthetized animals decrease the arterial pressure and cause bradycardia [3, 5]. The participation of opiate receptors in the hypotensive effect of central antihypertensive agents clonidine and α-methyldopa has been demonstrated [6, 7].

In the present paper the effects of enkephalins on central regulation of hemodynamics in unanaesthetized cats and the antagonism between the effects of clonidine and naloxone in hypertensive and normotensive rats are reported.

Methods.

The experiments were carried out on 15 unanaesthetized cats. Under pentobarbital anesthesia, 3–5 days before the experiments, a canula was implanted in the IV ventricle; monopolar nichrome electrodes 0.15 mm diameter were implanted in the hypothalamus [8]. To record the arterial pressure a polyethylene catheter was inserted through the left common carotid artery into the aorta and a venous catheter was introduced in the jugular vein. The peripheral ends of the catheters were connected to a miniature titanium valve fixed to the skull [9]. In some cats the probes of an electromagnetic flowmeter were inserted in the ascending aorta in order to record cardiac output. Instant values of the heart rate were estimated with a digital cardiotachometer triggered by the pulse wave of the arterial pressure. To test the sensitivity of the baroreceptor reflex, phenylephrine was injected intravenously (40 μg/kg/min). The sensitivity of the reflex was determined as the ratio between the increase in beat-to-beat interval (msec) and the increase in systolic arterial pressure (mm Hg).

The hypothalamus was stimulated with rectangular pulses (100 Herz, 2 msec, 2–6 V) every 10 sec. All the parameters were recorded by means of a Mingograf-81.

The experiments dealing with the antagonism between clonidine and naloxone were performed in spontaneously hypertensive rats (SHR) and on normotensive Wistar-Kyoto rats (WHY) anesthetized with urethane-chloralose. Initial values of the blood pressure were 121 ± 3,1 mm Hg in SHR and 80 ± 3,3 mm Hg in WHY rats.

Met-enkephalin, leu-enkephalin and some analogs were synthetized in the Laboratory of Peptide Synthesis, All-Union Cardiological Scientific Center, Academy of Medical Sciences of the USSR and were kindly supplied by Professor M.I. Titov. Clonidine (Boehringer Ingelheim), naloxone (Endo Laboratories) and the peripheral M-cholinoreceptor blocker oxyphenonium bromide (Metacyn) were also used. Intracisternally administered drugs were dissolved in saline and single administration did not exceed 100 μl.

The results were statistically analyzed using paired « t » test.

Results and discussion.

10–15 sec after the intracisternal administration of leu- or met-enkephalin (100 μg), a transitory increase in arterial pressure and heart rate occurred (Fig. 1). Arterial pressure returned to normal after a few minutes, while the heart rate decreased below the initial level and remained low for 15 min. The intracisternal administration of 50–100 μg of naloxone completely abolished the effect of enkephalins, confirming that the changes in blood pressure and heart rate are caused by

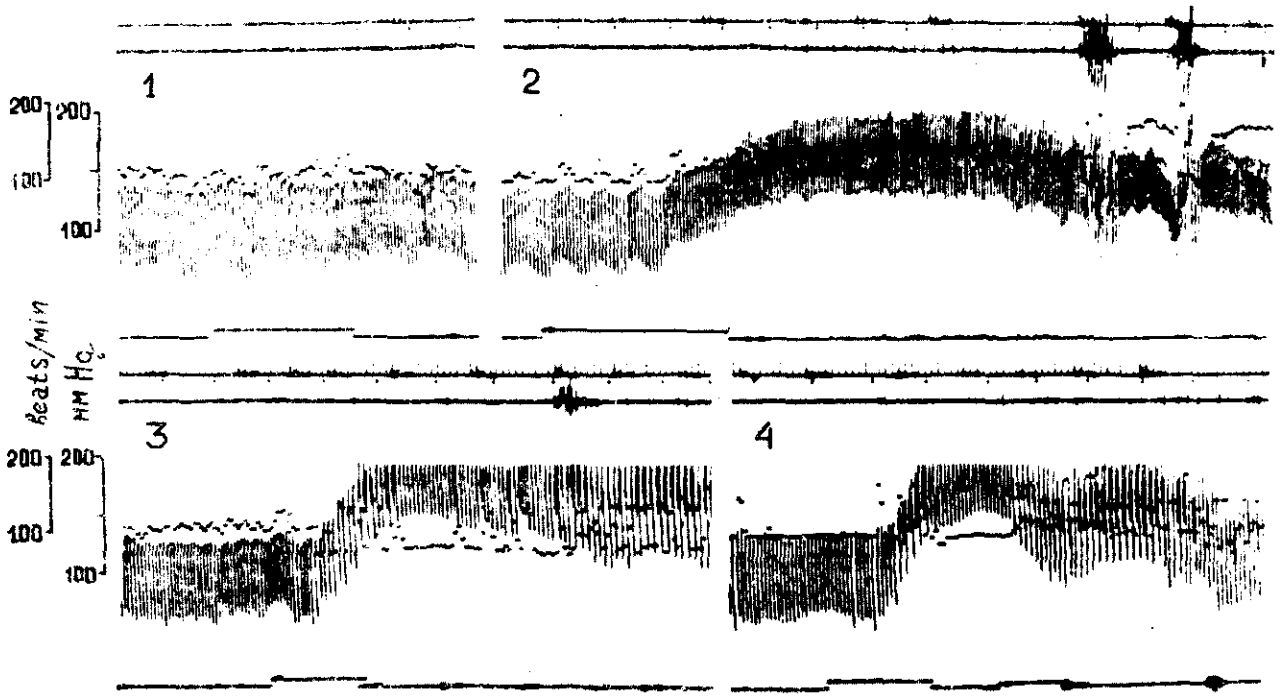

FIG. 1. – Changes of arterial pressure and baroreceptor reflex induced in the awake cat by the administration into the IV ventricle of leu-enkephalin 100 μg. 1 = control administration of normal saline 100 μl; 2 = effect of leu-enkephalin; 3 = control baroreceptor reflex to i.v. injection of phenylephrine; 4 = baroreceptor reflex after administration of leu-enkephalin. From top to bottom: time mark. 1 sec, actogram (signals from the strain-gauges of the floor-force platform), heart rate, arterial pressure, event mark.

activation of opiate receptors in the brain stem. After 100 μg of leu-enkephalin, the baroreflex elicited by the administration of phenylephrine was reduced from 6.7 to 1.7 msec/mm Hg (Fig. 1). Met-enkephalin was almost half as active as leu-enkephalin and decreased the baroreflex response from 5.38 to 2.34 msec/mm Hg. Smaller changes of the arterial pressure after intracisternal met-enkephalin as compared to leu-enkephalin were reported by Hughes et al. [12]. However, met-enkephalin was more potent than leu-enkephalin in dilating isolated pial arteries [10]. The results of our experiments with enkephalins are in good agreement with those of Freye and Arndt [11] who observed the suppression of the cardiac component of the baroreceptor reflex during perfusion of the IV ventricle with fentanyl.

The effects of met- and leu-enkephalin on the hemodynamic reactions caused by the stimulation of the hypothalamic regions involved in emotional states were then studied. After injection of leu-enkephalin, hypothalamic stimulation elicited an increase in blood pressure greater (169 % ± 22) than that obtained during pre-drug trials.

The increase in blood pressure was associated with a more intense « rage » reaction. Conversely, met-enkephalin failed to increase the hypertensive response.

Thus, the activation of some of the brain stem opiate receptors in the conscious cat was accompanied by the suppression of the cardiac component of baroreceptor reflex and by an increase in the hypertensive response evoked by hypothalamic stimulation. These results support an earlier hypothesis suggesting that endogenous opiates may play a role in the vegetative responses to emotional stimuli [12].

It has been shown [13] that naloxone injected in anaesthetized cats at the dose of 2 – 8 mg/kg does not affect blood pressure, heart rate, central venous pressure and blood flow in the superior mesenteric artery. In the present experiments naloxone (0.15 mg/kg i.v.) antagonized the hypotension induced by the i.v. administration of 30 μg/kg of clonidine, reducing also the concomitant bradicardia. It may be assumed that this effect is not dependent upon an influence of naloxone on blood pressure regulatory mechanisms but is due to the blockage of clonidine hypotension.

Fig. 2 shows one of the 3 experiments in which 5 μg of clonidine injected intracisternally prevented the increase in blood pressure elicited by hypothalamic stimulation. The intracisternal administration of naloxone (100 μg) restored in part the pressor effect of hypothalamic stimulation.

Fig. 3 shows that in the spontaneously hypertensive rats naloxone, at the dose of 1 mg/kg, completely antagonized the hypotension induced by clonidine (10 μg/kg) and caused a decrease in bradycardia to 60 % of the control. On the contrary naloxone did not affect bradycardia induced by clonidine in normotensive rats and reduced the hypotensive effect to 76 % of the control.

The administration of 2 mg/kg of oxyphenonium bromide did not influence the effect of naloxone on blood pressure but reduced its effect on heart rate.

At variance with the observation of Farsang et al. [6, 7] our findings and those of Karppanen [14] show

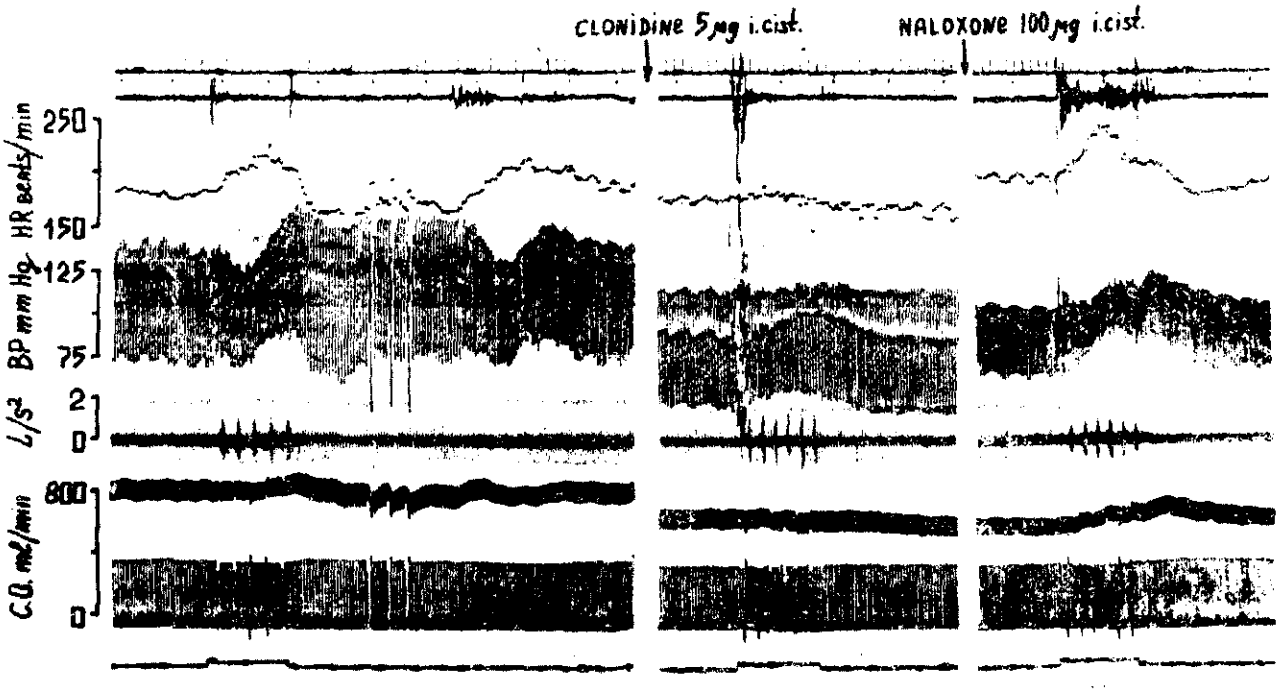

FIG. 2. – Effect of intracisternal administration of clonidine 5 µg and naloxone 100 µg on the pressor response induced by hypothalamic stimulation in the awake cat. Left: control response, middle after clonidine, right after naloxone. From top to bottom: time mark. 1 sec, actogram, heart rate, arterial pressure, first derivate of the blood flow velocity in ascending aorta - dF/dt max, mean cardiac output, blood flow in ascending aorta, event mark.

that an antagonism between clonidine and naloxone can be demonstrated not only in the spontaneously hypertensive rats but also in the normal rats.

The ability of naloxone to block the hypertensive effects induced by leu– and met–enkephalins and the hypotensive reactions induced by clonidine suggest that at least two types of opiate receptors are involved brain stem pressure regulation. The activation of one of them is accompanied by a stimulation of the sympathetic system, while the second inhibits sympathetic activity. Our data and the results of other authors [3, 15] suggest that enkephalins may be the endogenous activators of the « pressor » opiate receptors. During the development of experimental hypertension in rats an increase in the concentration of enkephalins in various brain regions and a decrease of pain sensitivity was found [16]. β–endorphin, which has a potent hypotensive effect [3], may be the endogenous ligand of « depressor » opiate receptors in the brain. A 2–3 fold increase in β–endorphin output from slices of rat medulla oblongata induced by clonidine [17] is a strong evidence for this possibility.

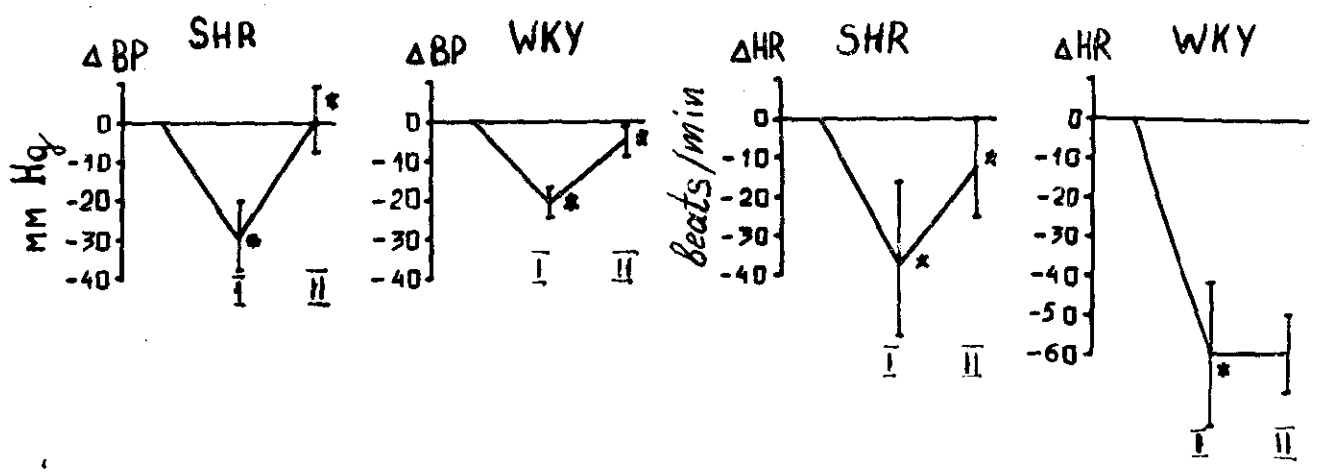

FIG. 3. – Changes of the blood pressure (BP) and heart rate (HR) in anesthetized spontaneously-hypertensive rats (SHR) and in normotensive Wistar-Kyoto rats (WKY) after sequential i.v. administration of clonidine 10 µg/kg and naloxone 1 mg/kg. I = max. effect of clonidine; II = max. effect of naloxone.

REFERENCES

1. Atweh, S.F. & Kuhar, M.J. 1977. Autoradiografic localisation of opiate receptors in rat brain. I. Spinal cord and lower medulla. *Brain Res.* **124**: 53–67.

2. Atweh, S.F. & Kuhar, M.J. 1977 (b). Autoradiografic localisation of opiate receptors in rat brain. II. The brain stem. *Brain Res.* **129**: 1–12.

3. Bolme, P., Fuxe, K., Agnati, L.F., Bradley, R. & Smythies, J. 1978. Cardiovascular effects of morphine and opioid peptides following intracisternal administration in chloralose–anesthetized rats. *Europ. J. Pharmacol.* **48**: 319–324.

4. Feldberg, W. & Wei, E. 1978. Central cardiovascular effects on enkephalins and C–fragment of lipotropin. *J. Physiol.* **280**: 18P.

5. Laubie, M., Schmitt, H., Vincent, M. & Remond, G. 1977. Central cardiovascular effects of morphinomimetic peptides in dogs. *Europ. J. Pharmacol.* **46**: 67–71.

6. Farsang, C. & Kunos, G. 1979. Naloxone reverses the antihypertensive effect of clonidine. *Brit. J. Pharmacol.* **67**: 161–164.

7. Farsang, C., Ramirez–Gonzalez, M.L., Mucci, L. & Kunos, G. 1980. Possible role of an endogenous opiate in the cardiovascular effects of central alpha adrenoceptor stimulation in spontaneously hypertensive rats. *J. Pharmacol.* **214**: 203–208.

8. Snyder, R.S. & Niemer, W.T. 1961. A stereotaxic atlas of the cat brain. Chicago, Univ. of Chicago Press.

9. Medvedev, O.S. 1974. Method of blood pressure registration in chronic experiments on cats. *Sechenov Physiol. J. USSR.* **60**: 1473–1476, (Russ.).

10. Hanko, J.H. & Hardebo, J.E. 1978. Enkephalin–induced dilatation of pial arteries in vitro probably mediated by opiate receptors. *Europ. J. Pharmacol.* **51**: 295–297.

11. Freye, F. & Arndt, J.O. 1979. Perfusion of the forth cerebral ventricle with fentanyl induces naloxone–reversible bradicardia, hypotension and EEG synchronization in conscious dogs. *Naunyn–Schmiedeberg's Archiv. Pharmacol.* **307**: 123–128.

12. Valdman, A.V., Medvedev, O.S. & Titov, M.I. 1980. The study of the role of opiate receptors in blood pressure control. *Proc. Symp. Vasoactive Peptides*, 14–15 May 1980, Sofia, pp. 13, 14 (Russ.).

13. Curtis, M.T. & Lefer, A.M. 1980. Protective actions of naloxone in hemorragic shock. *Am. J. Physiol.* **239**: H416–H421.

14. Karppanen, H. 1979. Clonidine–opiate interaction in rats and chickens. In: *Abstracts of the Ist Soviet–Finnish Symposium on Neurotransmitters and the mechanism of action of neurotropic and cardiovascular drugs.* Moscow, pp. 59, 60.

15. Schaz, K., Stock, G., Simon, W., Schlor, K.H., Unger, T., Rockhold, R. & Ganten, D. 1980. Enkephalin effects on blood pressure, heart rate and baroreceptor reflex. *Hypertension.* **2**: 395–407.

16. Zamir, N., Simantov, R. & Segal, M. 1980. Pain sensivity and opioid activity in genetically and experimentally hypertensive rats. *Brain Res.* **184**: 299–310.

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Neuropharmacology of intraspecific aggression and sociability in isolated mice

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Summary. – *Experiments on isolated mice have demonstrated that GABA antagonists enhance aggression and reduce species-specific sociability (increase desocializing effects of isolation), while GABA-agonists within a certain dose range diminish aggression and favour a temporary recovery of sociability.*

Riassunto. – *Gli antagonisti del Gaba aumentano l'aggressione e riducono la sociabilità di topi isolati mentre i Gaba-mimetici hanno effetti opposti.*

Introduction.

The purpose of our experiments was to investigate the changes in intraspecific aggression and sociability in isolated mice as a function of the neuropharmacological modulation of the activity of gabaergic (GABA), noradrenergic (NA), dopaminergic (DA), or serotoninergic (5-HT) systems.

Prolonged isolation of animals seems to be a special kind of stress evoking pathological changes in behaviour [1-3]. These changes involved fixed aggression or timidity, and variations of other kinds of behaviour resulting, in particular, in intraspecific aggression. It is suggested to consider these changes in behaviour of animals as « desocializing » consequences of isolation. Psychotropic drugs may be screened for their ability to restore a normal intraspecific social behaviour or to potentiate the desocializing effect (DSE) of isolation.

Materials and methods.

A total of 200 random-bred male mice of the CC57W strain were used. The analysis of the behaviour of isolated mice and of their interaction with another mouse, its standard opponent, was based on the « Ethological atlas for pharmacological research » [4, 5] and the Ethograph-Computer ES 1022 System. This system allows to record the frequency, duration and sequence of 40 various items of behaviour (acts and postures). All these items were classified according to the motivational principle, namely: aggressive, defensive, explorative and sexual behavior, sociability [6-10]. A special program was compiled in Fortran IV for the statistical treatment of the data. The drug effects were evaluated by comparing average probabilities of appearance of all behavioural items possible in control mice (0,9 % saline) and in animals injected with drugs. Statistical significance between the control and each drug condition was assessed using the Wilcoxon matched-pairs signed-rank test. Examples of the evaluation of drug effects are presented in Tab. 1-3.

Results and discussion.

Aggression and sociability levels in isolated mice appear to be mainly mediated by changes in the activity of the gabaergic system. Activation of gabaergic receptors by muscimol (0,2 mg/kg) slightly inhibits aggression and does not affect the individual activity. Muscimol (0,5 mg/kg) suppresses attacks but not the entire spectrum of aggression: in fact, while threatening postures are not affected, the probability of ambivalent acts is decreased. In isolated animals muscimol slightly enhances the probability of social interactions directed towards body and genital parts. Mice injected with muscimol (1 mg/kg) do not show aggressive behaviour and present defensive reactions when matched with formely submissive opponents; moreover, the drug significantly reduces the individual activity of isolated mice. Another gabaergic agonist: gamma-acetylenic-GABA (GA-GABA, 50 mg/kg) decreases (though non significantly) the frequency of attacks an threatening displays, without changes in individual behaviour. GA-GABA (75 and 100 mg/kg) further decreases attacks and threats. This action is followed by a transient activation of social interactions and by an inhibition of the ambivalent forms of behaviour. Combined injections of GA-GABA (50 mg/kg) and diazepam (1 mg/kg) result in a considerable reduction of aggressive behaviour and in an activation of intraspecific social interactions, in absence of a marked decrement of spontaneous activity (Tab. 1). Phenibut and phenylpyrrolidon (GABA analogues) reduce aggression and in certain dose ranges increase intraspecific social interactions [3].

Table 1. – *Ethological profile of activity of GABA agonists.*

BEHAVIOURAL CATEGORIES AND ITEMS	C_m (*)	Muscimol (30 min) 0,2 mg/kg	0,5 mg/kg	1 mg/kg	C_{gg} (**)	Gamma-acetylenic-GABA (½hr) 50 mg/kg	75 mg/kg	100 mg/kg
Social behavior.								
Sociability:								
Sniff-body	0,009	(a) 0,051	(a) 0,108	0,062	0,010	(a) 0,103	(a) 0,168	0,016
Sniff-nose	0,004	0,001	—	—	—	—	—	0,012
Sniff-genitals	0,036	0,048	0,043	0,003	0,032	(a) 0,088	0,061	—
Aggression:								
Attack	(a) 0,210	(a) 0,111	0,019	—	(a) 0,322	(a) 0,174	0,042	0,238
Threat	(a) 0,066	(a) 0,065	0,051	0,023	(a) 0,116	0,037	0,024	(a) 0,065
Defence:								
Sideways postures	—	—	—	(a) 0,060	—	—	—	0,049
Upright postures	—	—	—	0,017	—	—	—	0,012
Ambivalent:								
Tail rattling	(a) 0,103	0,064	0,042	0,026	0,081	0,088	0,032	0,028
Individual Behavior.								
Dynamic:								
Locomotion	(a) 0,175	(a) 0,171	(a) 0,137	(a) 0,126	(a) 0,163	(a) 0,182	(a) 0,291	(a) 0,196
Rearing	0,071	(a) 0,170	(a) 0,120	0,010	0,091	0,083	0,095	0,036
Selfgrooming	(a) 0,110	0,037	0,006	—	0,085	0,074	0,038	0,008
Static:								
Sitting	(a) 0,192	(a) 0,272	(a) 0,488	(a) 0,665	(a) 0,102	(a) 0,163	(a) 0,241	ta) 0,549

(*) C_m = Control related to muscimol – probability of appearance of each item.
(**) C_{gg} = Control related to gamma-acetylenic-GABA.
(a) = Statistically significant.

Table 2. – *Ethological profile of activity of GABA antagonists.*

BEHAVIOURAL CATEGORIES AND ITEMS	$C_{tsk,p}$ (*)	Thiosemicarbazid (1hr) 1 mg/kg	3 mg/kg	Picrotoxin (15 min) 1 mg/kg	C_b (**)	Bicuculline (15 min) 0,5 mg/kg
Social behavior.						
Sociability:						
Sniff-body	0,007	0,007	0,002	0,007	0,012	0,011
Sniff-nose	0,009	0,008	—	—	0,007	0,002
Sniff-genitals	0,009	0,010	0,013	—	0,022	S —
Aggression:						
Attack	(a) 0,162	(a) 0,273	(a) 0,269	(a) 0,314	(a) 0,288	(a) 0,254
Threat	(a) 0,110	0,048	0,049	(a) 0,130	(a) 0,126	(a) 0,128
Sexual:						
Attempted mount	—	0,021	—	—	—	—
Mount	—	0,003	0,003	—	—	—
Ambivalent:						
Tail rattling	0,073	0,075	0,053	0,093	0,081	(a) 0,118
Individual Behavior.						
Dynamic:						
Locomotion	(a) 0,162	(a) 0,168	(a) 0,204	(a) 0,152	(a) 0,170	(a) 0,124
Rearing	0,092	(a) 0,103	(a) 0,114	0,067	0,089	0,064
Selfgrooming	0,098	0,067	0,078	0,065	0,076	0,010
Static:						
Sitting	(a) 0,164	(a) 0,141	(a) 0,130	(a) 0,134	(a) 0,185	(a) 0,278

(*) $C_{tsk,p}$ = Control related to thiosemicarbazid and picrotoxin –probability of appearance of each item.
(**) C_b = Control related to bicuculline.
(a) = Statistically significant.

Picrotoxin (1 mg/kg), an antagonist of GABA receptors, increases the probability of attacks and depresses all items of behaviour that are related to sociability (Tab. 2). Bicuculline (0,5 mg/kg) increases the number of attacks whereas in high doses depresses the aggressive behaviour and social interactions, i.e., this treatment produces desocialization (Tab. 2). Thiosemicarbazide, an inhibitor of GABA synthesis, also increases the probability of attacks (1 mg/kg) and activates attemps to mount other males. At the 3 mg/kg dose, thiosemicarbazide inhibits sociability and increases individual motor activity. The behavioural disinhibition of isolated mice, as reflected in increased aggression, hyperreactivity (but low level of sociability) in response to ordinary species-specific stimulation may be associated with a deficiency in central inhibitory mechanisms, in particular, gabaergic systems: in fact, GABA may play a key-part in the control of CA systems (NA and DA) and therefore in the control of aggression and sociability.

The immediate precursor of catecholamines (CA) L-DOPA, was studied to establish to what degree the social behaviour of isolated mice and aggressive behaviour may change in relation to an increase of CA levels. L-DOPA (10 mg/kg) slightly enhanced the probability of aggression of isolated mice, their motor activity and gnawing reaction. High doses of L-DOPA (Tab. 3) produced dissociation of nonspecific hyperreactivity, defensive behaviour and aggression. While completely blocking aggression, L-DOPA markedly increased hyperreactivity and defensive behaviour at the approaches of a formerly submissive opponent. High doses of L-DOPA (200 mg/kg) transform the aggressive behaviour of isolated mice into inadequate

Table 3. – *Ethological profile of activity of catecholaminergic and serotoninergic drugs.*

DRUGS	Doses mg/kg	Time	Social Behaviour: Aggression	Sociability	Sexual	Defence	Ambivalent	Individual: Dynamic	Static
L-DOPA	10	0,5hr	▲	▼	▽		▽	▲	▽
	200	0,5hr	▼	▼	▽	▲	▽		△
L-DOPA + Fluoxetine	10 + 20	0,5hr	▽	▼	▽		▽		△
FD-008 + L-DOPA	100 + 10	1 hr + 0,5hr	▼	▼	▽		▽		△
Amphetamine	0,5	0,5hr	△	▼	▽		▽	▲	▽
	3	0,5hr	▼	▼	▽		▼	▲	▽
FD-008	100	1 hr	▼	▲	△	▽	▼	△	▲
	100	4 hr	▼	▲	▽	▽	▽	△	△
FD-008 + Amphetamine	100 + 3	1 hr + 0,5hr	▼	▽	▽	▽	▽	▲	▽
Disulfiram	100	1 hr	▽	▼	▽	▽	▽		△
	100	4 hr	▼	▼	▽	▽	▼		▲
Propranolol	2,5	0,5hr	▽	▼	▽		▽	▽	▲
Phentolamine	2,5	0,5hr	▽	▼			▽	▽	△
5-HT	50	1 hr	▼	▽	▼	△	▽	▽	△
Fluoxetine	10	1 hr	▼	▼	▽	△	▼	▽	▲
P-Chlorphenylalanine	300	24 hr		△	▲			▲	
PCPA	300 + 100 + 100	78 hr	▽	▽	△		▽	▽	▲
PCPA + L-DOPA	300 + 100 + 100 + 100	78 hr + 1 hr	▽	▽	▼		▽	▽	▲
PCPA + Fluoxetine	300 + 100 + 100 + 10	78 hr + 4 hr	▽	▽	▼		▽	▽	▲

Conventional signs: △ = increase; ▽ = decrease.
Black signs = statistically significant, $p < 0,05$. Conventional signs show the result of comparing average probabilities of appearance of items in control and after drug administration.

bizarre behaviour evident even if the opponent's threat or attack is not present (DSE of isolation appears to be increased).

Fluoxetine (20 mg/kg), an inhibitor of serotonin reuptake prevents the aggression induced by small L-DOPA doses. However, this action is not followed by selective RSE as the intraspecific sociability is suppressed. An analogue of fusaric acid, dopamine — β — hydroxylase (DBH) inhibitor, FD-008 (100 mg/kg) was even more active in preventing the aggression induced by L-DOPA. FD-008 (100 mg/kg) significantly decreases the probability of species-specific aggressive acts appearance while the sociability is maintained. The FD-008 effects stand in marked contrast to those of another non selective DBH inhibitor, disulfiram (100 mg/kg), that depresses aggression, but simultaneously inhibits the intraspecies sociability (Tab. 3).

The isolated mice were more sensitive to the effects of sympathomimetics than the grouped ones. Injection of small doses of amphetamine (0,5 mg/kg) into isolated mice potentiates DSE of isolation. Higher doses of amphetamine (3 mg/kg) resulted in a pathological « aggression-stereotypy » syndrome. Social isolation evokes an inversion of the effects caused by large doses of amphetamine, *e.g.*, fighting in grouped mice and suppression of attacks in isolated mice. These variations are attributable to an hypersensitivity of receptors to NA and DA induced by isolation. The use of adrenergic blockers (propranolol and phentolamine) does not antagonize this effect. The intraspecific contacts between mice injected with adrenergic blockers are characterized by passive responses.

Changes in turnover rate or decrease in brain 5-HT may account for the pathology of behaviour of isolated mice. A load of 5-HT results in reduction of aggression in isolated mice on one hand, and defensive escape items, on the other. Inhibition of 5-HT reuptake by fluoxetine reduced nonselectively the probability of species-specific attacks. A decrease of brain 5-HT associated to injections of p-chlorophenylalanin (PCPA) increases the hyperreactivity and the probability of the appearance of homosexual reactions (sexual mouting on male). The association of homosexual behaviour with attacks leads to a pathological behavioural syndrome — « aggression-homosexual behaviour ». Thus, the reduction in brain 5-HT contributes to increase DSE of isolation (Tab. 3).

Compulsive aggressive behaviour and deficient sociability of isolated mice don't depend merely on metabolic changes of NA, DA, 5-HT, hypersensitivity of NA and DA receptors systems, but also on the deficiency in GABA-ergic inhibitory system. To obtain a selective activation of intraspecies sociability with a simultaneous reduction of aggression, a complex pharmacological action on these systems seems to be required.

REFERENCES

1. Valdman, A.V. 1980. Experimental approaches to psychopharmacological treatment of animals' emotional pathological states. In: *Theoretical basis of pathological states.* N.P. Bekhtereva (Ed.). Nauka, Moscow, pp. 195–206 (Russ.).

2. Valdman, A.V., Zvartau, E.E. & Kozlovskaya, M.M. 1976. *Psychopharmacol. Emotions.* Meditsina, Moscow (Russ.).

3. Valzelli, L. 1973. Activity of benzodiazepines on aggressive behavior in rats and mice. In: *The Benzodiazepines*, S. Garattini et al. (Eds.), Raven Press, New York, pp. 405–417.

4. Poshivalov, V.P. 1974. Pharmacological and psychophysiological analysis of aggressive behavior in conditions of zoo-social interaction. In: *Neuropharmacological regulation of the system processes.* A.V. Valdman (Ed.). Leningrad, USSR, pp. 60–83 (Russ.).

5. Poshivalov, V.P. 1978. *Ethological atlas for pharmacological research on laboratory rodents.* VINITI, Moscow (Russ.).

6. Grant, E.C. & Mackintosh, J.H. 1963. A comparison of the social postures of some common laboratory rodents. *Behavior.* **21**: 246–259.

7. Silverman, P. 1965. Ethological and statistical analysis of drug effects on the social behavior of laboratory rat. *Brit. J. Pharmacol.* **24**: 579–590.

8. Adams, D.B. 1976. The relation of scent-making, olfactory investigation and specific postures in the isolation-induced fighting of rats. *Behavior.* **56**: 286–297.

9. Miczek, K.A. 1974. Intraspecies aggression in rats. Effects of d-amphetamine and chlordiazepoxide. *Psychopharmacol.* **39**: 275–301.

10. Krsiak, M. 1975. Timid singly-housed mice: their value in prediction of psychotropic activity of drugs. *Br. J. Pharmacol.* **55**: 141–150.

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RICERCHE E METODOLOGIE

Mutagenicity of trichloroethylene in Salmonella typhimurium TA100

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Summary. – *Trichloroethylene (TCE) sample of technical grade was directly mutagenic versus* Salmonella typhimurium *TA100 when tested as vapour in sealed desiccators. Purified TCE, free of epoxides, was borderline mutagenic only when tested with metabolic activation. Microsomal fractions from Aroclor induced male rats were effective in converting TCE into mutagenic metabolites, while preparations from uninduced rats, mice and hamsters were not. The metabolic activation of TCE turned out to be NADP dependent and insensitive to the addition of powerful epoxide hydrase inhibitor trichloropropane, 2-3 oxide (TCPO).*

Riassunto. – *Un campione di tricloroetilene (TCE) di grado tecnico è risultato direttamente mutageno nel ceppo TA100 di* Salmonella typhimurium *quando è stato saggiato in forma di vapori in essiccatori. Un campione di TCE purificato, privo di epossidi, è risultato debolmente mutageno solo in presenza di attivazione metabolica. Frazioni microsomiali ottenute da ratti maschi indotti con Aroclor sono risultate attive nella conversione del TCE in metaboliti mutageni, mentre quelle ottenute da ratti, topi ed hamster non indotti sono inattive. L'attivazione metabolica del TCE sembre essere un processo NADP-dipendente ed insensibile al tricloropropene, 2-3 ossido (TCPO), un potente inibitore delle epossidoidratasi epatiche.*

INTRODUCTION.

Trichloroethylene (TCE) is widely employed as vapour degreasing of fabricated metal parts and as industrial solvent; other applications include its use for the manufacture of decaffeinated coffee and as anaesthetic in surgical, dental and obstetrical procedures [1].

Owing the high extimated world wide production of TCE and the wide human exposure a number of investigations on the mutagenic and carcinogenic potential of TCE have been performed [2].

TCE has been reported as mutagenic in bacteria, yeast, mouse and produced a high incidence of hepatocellular carcinomas in mice but not in rats after administration by gavage in a N.C.I. study [3]. Most of these studies however, and particularly the long term one, are debated and inconclusive for the unspecified presence of highly mutagenic and carcinogenic epoxides and antioxidants in the commercial TCE samples assayed. For these reasons we have tried to compare the activity of a purified, epoxides free, sample with that of a TCE sample containing epichlorohydrin and 1-2 epoxybutane in the *Salmonella*/mammalian microsomes assay. The second sample is very similar in its composition to that employed in the N.C.I. long term study. Furthermore some efforts have been performed to clarify the role of the microsomal fractions in the *in vitro* mutagenicity of TCE.

MATERIALS AND METHODS.

Chemicals.

The two TCE samples assayed were kindly supplied by Montedison S.p.A. The irimpurities compositions as determined by gas-chromatography were the following:

Sample A

1,2-dichloroethylene: 2ppm
chloroform: 8ppm
carbon tetrachloride: 27ppm
1,1,2-trichloroethane: 21ppm
perchloroethylene: 2ppm
unknown: 2ppm
2,6-di-terzbutyl, 4-methyl, 1-hydroxybenzene (BHT): 20ppm

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Sample B has been prepared by addition to Sample A of the following chemicals according to the formula reported at page 3 of the N.C.I. technical report nº 2, NCI-CG-TR2:

1,2–epoxybutane: 1900ppm
ethylacetate: 400ppm
epichlorohydrin: 900ppm
N-methylpyrrole: 200ppm
diisobutylene: 300ppm

TCPO was purchased by the E.G.A. Chemie, West Germany; 2aminoantracene (2AA) was a gift of Dr. J. Ashby (I.C.I. Macclesfield, U.K.); G-6-P and NADP were purchesed from Boehringer, Mannhein.

Mutagenicity assay.

Salmonella typhimurium strain TA100 was a gift of Prof. B.N. Ames, University of Berkeley, California. Plates and S9 preparations as well as rats induction were as previously described [4].

Male Sprague–Dawley rats, Swiss mice and Syrian hamsters were used for S9 preparations.

TCE was tested by adding a known volume of the chemical to a glass Petri dish suspended beneath the porcelain shelf of a 20 l desiccator; plates were incubated in it without their lids (Fig. 1, 2). The desiccator

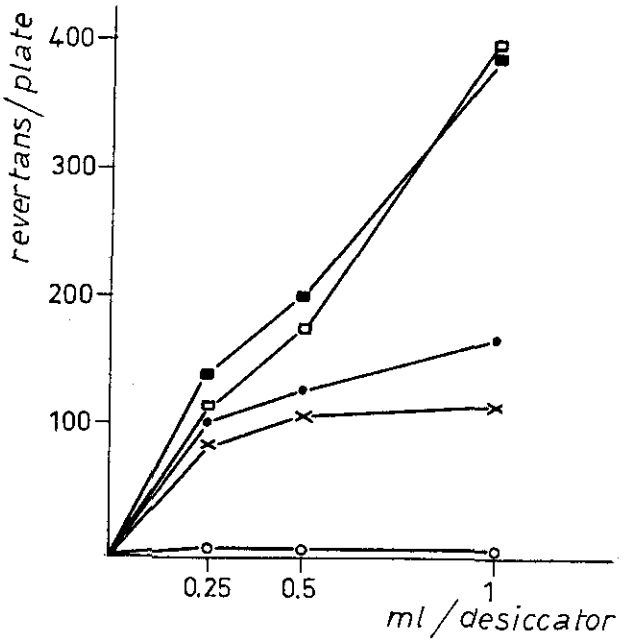

FIG. 1. – Mutagenicity of TCE in *S. typhimurium* TA100: (□) sample A; (■) sample A with S9 (150 µl/plate); (○) sample B; (●) sample B with S9 (150 µl/plate); (×) sample A with S9 (150 µl/plate) and TCPO (120 µg/plate). Pooled data from three experiments; S.E. did not exceed 10 % of observed values. The number of spontaneous *his*$^+$ revertants has been subtracted: it ranged from 160 to 199.

were placed at 37 °C for 7 hours. After this time the plates were removed from the desiccators, their lids replaced and incubated inverted 40 hours more at 37 °C before counting.

Protein concentrations for microsomal fractions were determined by the Lowry method [5]: they were

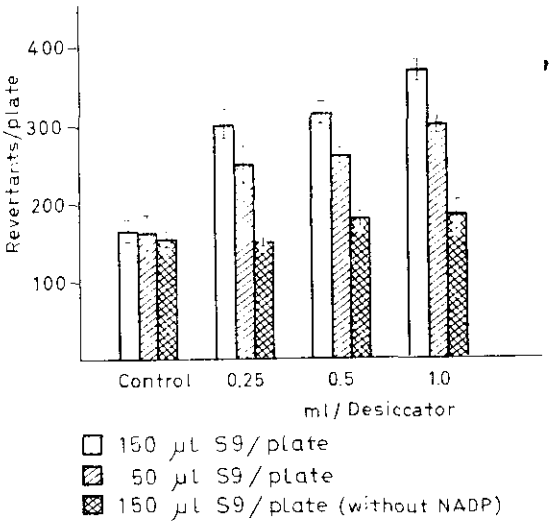

FIG. 2

adjusted at 30 mg/ml. 2AA was used as positive control of strain sensitivity and microsomal enzymes activity in every experiment. Routine controls for checking S9 sterility were also always included.

RESULTS.

In Tab. 1 and 2 is shown the different mutagenic activity of the two TCE samples assayed. The sample B containing epichlorohydrin and 1,2 epoxybutane was directly mutagenic, inducing a linear increase in the number of *his*$^+$ revertants in the range of the concentrations tested (Tab. 2). Its activity was not modified by the addition of S9 from Aroclor induced rats. The purified sample A on the contrary in repeated experiments never increased the number of *his*$^+$ revertants over the control value when tested alone, but was weakly mutagenic in the presence of S9 from Aroclor induced rats (Fig. 3), inducing low (nearly two fold) but reproducible and dose related increases in the number of revertants. This weak effect was slightly

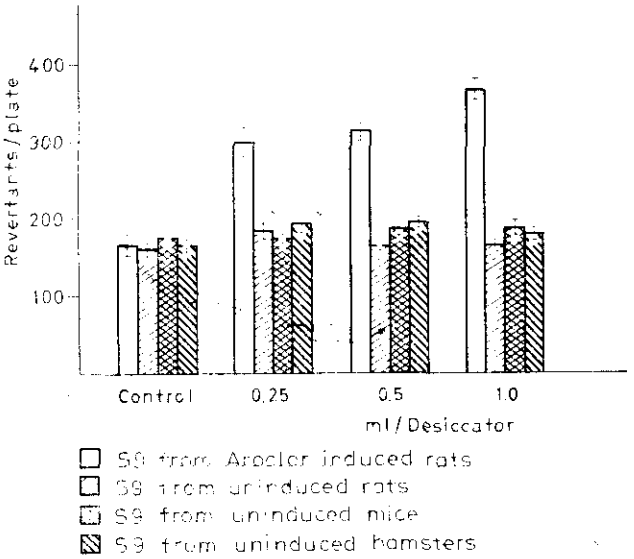

FIG. 3

Table 1. – *Mutagenicity of TCE sample A in S. typhimurium TA100.*

ml/Desiccator	— S9		
	I Exp.	II Exp.	III Exp.
0	194±5	184±17	167±12
0.25	190±19	203±14	163±16
0.50	190±7	175±30	165±12
1.0	183±1	178±2	172±15
Positive control .	(*b*)	(*b*)	(*b*)

ml/Desiccator	+ 50 µl S9/plate (*a*)		
	I Exp.	II Exp.	III Exp.
0	182±18	183±24	198±19
0.25	272±20*	239±23	260±22
0.50	282±20*	257±20	272±28
1.0	295±5*	290±14*	312±46*
Positive control .	927±132	954±136	1.070±27

ml/Desiccator	+ 150 µl S9/plate (*a*)		
	I Exp.	II Exp.	III Exp.
0	180±10	160±8	179±16
0.25	299±16*	225±32	301±30*
0.50	311±15*	295±11*	328±19*
1.0	350±9**	348±23**	379±44**
Positive control .	1,067±107	1,304±148	1,329±101

ml/Desiccator	+ 150 µl S9/plate (*a*) and 120 µg TCPO		
	I Exp.	II Exp.	III Exp.
0	199±9	174±7	187±13
0.25	291±12	205±9	269±21*
0.50	308±15*	247±20*	313±17*
1.0	319±11*	314±9**	310±13**
Positive control .	914±87	953±153	1,017±99

ml/Desiccetar	+ 150 µl S9/plate (*a*) withour NADP	
	I Exp.	II Exp.
0	156±18	167±21
0.25	151±3	170±32
0.50	175±5	149±4
1.0	177±16	170±26
Positive control .	(*c*)	(*c*)

In the Table $\bar{x} \pm$ SD from three plates.
(*a*) S9 from Aroclor induced male Sprague–Dawley rats.
(*b*) Experiments performed together with the assays with 50µl S9/plate, share their positive controls.
(*c*) Experiments performed together with the assays with 150µl S9/plate.
(*) P 1 %.
(**) P 0.1 % (t test).

Table 2. – *Mutagenicity of TCE sample B in S. typhimurium TA100.*

ml/Desiccator	— S9		
	I Exp.	II Exp.	III Exp.
0	172±12	161±4	187±17
0.25	280±43	356±38	304±5**
0.50	346±21*	482±44**	415±38*
1.0	567±61**	648±24**	651±53**
Positive control .	(*b*)	(*b*)	(*b*)

ml/Desiccator	+ 150 µl S9/plate (*a*)		
	I Exp.	II Exp.	III Exp.
0	188±18	172±16	195±14
0.25	329±36	414±18**	386±11**
0.50	387±27*	445±49*	446±10**
1.0	568±46**	648±62**	639±49**
Positive control .	942±36	878±95	974±142

In the Table $\bar{x} \pm$ SD from three plates.
(*a*) S9 from Aroclor induced male Sprague–Dawley rats.
(*b*) Experiments performed together with the assays with S9, share their positive controls.
(*) $P < 1\ \%$.
(**) $P < 0.1\ \%$ (t test).

Table 3. – *Mutagenicity of TCE sample A in S. typhimurium TA100 in the presence of S9 from uninduced male rats, mice and hamsters.*

ml/Desiccator	S9/rat	
	I Exp.	II Exp.
0	156±12	167±26
0.25	162±18	207±10
0.50	171±8	158±17
1.0	156±19	177±13
Positive control	1,010±88	1,317±79

ml/Desiccator	S9/mouse	
	I Exp.	II Exp.
0	176±16	172±6
0.25	185±1	191±7
0.50	181±33	193±20
1.0	189±28	193±19
Positive control	555±37	695±109

ml/Desiccator	S9/hamster	
	I Exp.	II Exp.
0	172±24	159±6
0.25	185±38	208±18
0.50	190±16	208±12
1.0	178±20	185±18
Positive control	1,853±116	1,521±67

In the Table $\bar{x} \pm$ SD from three plates.
Each plate was supplemented with 0.5 ml S9Mix containing 150 µl S9.

decreased modifying the S9 concentrations per plate (50 µl versus 150 µl/plate) and completely suppressed in the absence of NADP (Tab. 1). Also the addition of the powerful inhibitor of epoxide hydrase trichloropropene, 2-3 oxide (TCPO) at 120 µ9/plate slightly de reased the observed effect (Tab. 1). This concentration was chosen as the highest not mutagenic and proved to increase the mutagenicity of benzo(a)pyrene by us (data not shown) and others [6].

Furthermore, the activating capability of S9 fractions seems to be related to the induction procedure: S9 fraction from uninduced rats, mice and hamsters in fact were completely ineffective in activating TCE (Tab. 3). Part of these results are summarized in Fig. 1-3, which provide a clearer description of the observed phenomena.

Discussion.

The real genotoxic potential of TCE has been extensively debated in the past, in particular for what concerns the possible role of impurities present as antioxidants in determining false positive results [7]. Our study confirms that highly mutagenic impurities, as epichlorohydrin, may indeed extensively modify the results. In this connection it is particularly notable the different mutagenic activity of the commercial sample assayed by us in comparison to the purified one; this results is in good agreement with the recent observation that purified TCE is ineffective in inducing tumors formation in mice, rats and hamsters [8].

That notwithstanding it seems extremely likely that TCE has a weak genetic activity *per se*. None of the antioxidants employed in fact may account for the weak mutagenicity of the purified sample observed in this and other studies in the presence of metabolic activation [9-12]. The requirement of NADP in the S9 Mix demonstrated the involvment of oxidative activities in the formation of mutagenic TCE metabolites rather than a conjugation with glutathione, NADP independent as demonstrated for 1,2 dichloroethane [13].

It has been proposed that the metabolic conversion of TCE involves the formation of an electrophilic by the mammalian mixed function oxidase (MFO) [14] and the rearrangement of chloroethylene oxide into chloral, trichloroethanol and trichloroacetic acid [15, 16]. Hepatotoxicity and covalent binding of labelled TCE to DNA and to liver proteins appear to be increased by the phenobarbital induction of liver monooxygenated [17, 18] and by the inhibition of epoxide hydrase activity [17], thus supporting the crucial role of the oxirane in TCE reactivity.

Our data demonstrate, irrespective of the species employed, the requirement of microsomal enzymes induction in the *in vitro* TCE mutagenicity. It is not clear however the lack of a positive response in conditions of epoxide hydrase inhibition: in this case the cytotoxic effect of the increased oxirane concentration might mask the higher mutagenicity. As an alternative hypothesis the reduced overall response might be due to the block of TCE metabolic transformation into chloral hydrate. This compound, which was recovered in the plasma of men exposed to TCE vapours [19], was found mutagenic in *Salmonella typhimurium* TA100 by us [20] and others [21].

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REFERENCES

1. IARC Monographs on the evaluation of the carcinogenic risk of chemicals to humans. 1979. **20**: 545-572.

2. Baden, J.M. & Simmon, V. 1980. Mutagenic effects of inhalation anesthetic *Mutat. Res.* **75**: 169-189.

3. N. C. I. 1976. Carcinogenesis bioassay of TCE Techn. Rep. Ser. N° 2, NCI CG, TR2, U.S., D.H.E.W., Washington.

4. Ames, B.N., McCann, J. & Yamasaki, E. 1975. Methods of detecting carcinogens and mutagens with the *Salmonella*/mammalian microsomes mutagenicity test. *Mutat. Res.* **31**: 347-364.

5. Lowry, O.H., Rosembrough, N.J., Farr, A.L. & Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265-275.

6. Bentley, P., Oesch, F. & Glatt, H. 1977. Dual role of epoxide hydratase in both activation and inactivation of B(a)P. *Arch. Toxicol.* **41**: 249-277.

7. Henschler, D., Eder, D., Neudecker, T. & Metzler, M. 1977. Carcinogenicity of TCE: fact or artifact? *Arch. Toxicol.* **37**: 233-236.

8. Henschler, D., Romen, W., Elsasser, H.M., Reichert, D., Eder, E. & Radwan, Z. 1980. Carcinogenicity studies of TCE by long term inhalation in three animal species. *Arch. Toxicol.* **43**: 237-248.

9. Bartsch, H., Malaveille, C., Barbin, A. & Planche, G. 1979. Mutagenic and alkylating metabolites of haloethylenes, chlorobutadienes and dichlorobuten produced by rodent or human liver tissues. *Arch. Toxicol.* **41**: 249-277.

10. Bronzetti, G., Zeiger, E. & Frezza, D. 1978. Genetic activity of TCE in yeast. *J. Env. Patol. Toxicol.* **1**: 411-418.

11. Callen, D., Wolf, C.R. & Philpot, M. 1980. Cytochrome P450 mediated genetic activity and cytotoxicity of seven hydrogenated aliphatic hydrocarbons S. cerevisiae. *Mutat. Res.* **77**: 55-63.

12. Simmon, V. 1977. Structural correlation of carcinogenic and mutagenic alkyl alides. Proc. Second FDA Office of Science, Annapolis, Maryland.

13. Rannug, U., Sundvall, A. & Ramel, C. 1978. The mutagenic effect of 1,2-dichloroethane on *S. typhimurium*. Activation through conjugation with glutathion in vitro. *Chem. Biol. Interact.* **20**: 1-16.

14. UEHLEKE, H., TABARELLI-POPLAWSKI, S., BONSE, G. & HENSCHLER, D. 1977. Spectral evidence for 2,2,3-trichlorooxirane formation during microsomal trichloroethylene oxidation. *Arch. Toxicol.* **37**: 95–105.

15. HENCHLER, D. & BONSE, G. 1977. Metabolic activation of chlorinated ethylene dependence of mutagenic effects on electrophilic reactivity of the metabolically formed epoxides. *Arch. Toxicol.* **39**: 7–12.

16. VAN DUUREN, B.L. & BANERIJE, S. 1976. Covalent interaction of metabolites of the carcinogen TCE in rat hepatic microsomes. *Cancer Res.* **36**: 2419–2422.

17. BANERIJE, S. & VAN DUUREN, B.L. 1978. Covalent binding of the carcinogen TCE to hepatic microsomal proteins and to exogenous DNA in vitro. *Cancer Res.* **38**: 776–780.

18. CARLSON, G.P. 1974. Enhancement of the hepatotoxicity of TCE by inducers of drug metabolism. *Res. Comm.*, in: *Chem. Pathol. and Pharmacol.* **7**: 637.

19. SCANSETTI, G., RUBINO, G.F. & TROMPEO, G. 1959. Studio sulla intossicazione cronica da trielina. *Med. Lav.* **50**: 743–765.

20. BIGNAMI, M., CONTI, G., CONTI, L., CREBELLI, R., MISURACA, F., PUGLIA, A.M., RANDAZZO, R., SCIANDRELLO, G. & CARERE, A. 1980. Mutagenicity of halogenated aliphatic hydrocarbons in *Salmonella typhimurium*, *Streptomyces coelicolor* and *Aspergillu nidulans*. *Chem. Biol. Interact.* **30**: 9–23.

21. WASKELL, L. 1978. The study on the mutagenicity of anhestetics and their metabolites. *Mutat. Res.* **57**: 141–153.

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In vitro mutational studies with trifluralin and trifluorotoluene derivatives

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Summary. – *An environmental contamination by Trifluorotoluene derivatives (4-chlorotrifluorotoluene (CTT), 4-chloro-3-nitro-trifluorotoluene (NCTT) and 4-chloro-3,5-dinitro-tri2uorotoluene (DNCTT) was detected in 1977 in the water bearing stratum in the area of Vicenza (North eastern Italy). Responsible of this pollution was a plant producing several fluoro-toluene derivatives among which DNCTT, an intermediate in the synthesis of dinitroaniline herbicides, such as Trifluoro-2,6-dinitro-trifluorotoluene (Trifluralin).*

In this paper we report results about the genotoxic potential of the three pollutants and Trifluralin: CTT, NCTT and DNCTT failed to exhert any mutagenic or recombinogenic activity when tested in Salmonella/*mammalian microsome assay and in the* Aspergillus nidulans *mitotic segregation system respectively; CTT and NCTT stimulated unscheduled DNA synthesis. A technical grade sample of Trifluralin turned out to be unable to induce point mutations and stimulation of UDS, but induced a significative increase of mitotic crossing-over in* A. nidulans; *a purified sample however showed no genetic activity when rested in the same genetic system.*

Riassunto. – *Nel 1977, nella zona di Vicenza (5 Italia Nord-Orientale) è stata segnalata la presenza di contaminanti ambientali derivati del trifluorotoluene nella falda idrica (4-chloro-trifluorotoluene (CTT), 4-chloro-3-nitrotrifl uorotoluene (NCTT) e 4-cloro-3,5-dinitrotri2uorotoluene (DNCTT).*

La responsabilità della contaminazione fu attribuita ad un impianto che produceva diversi derivati fluorotoluenici, tra cui DNCTT, un intermedio nella sintesi di erbicidi dinitroanilinici del tipo del Trifluralin.

Sono qui riportati i risultati di studi sul potenziale genotossico dei tre contaminanti e del Trifluralin. CTT, NCTT e DNCTT non hanno mostrato attività mutagena o ricombinogena in Salmonella typhimurium *in presenza di frazione microsomiale e in* Aspergillus nidulans, *rispettivamente; CTT e NCTT hanno dato luogo a sintesi non programmata del DNA (UDS). Il Trifluralin di grado tecnico non ha indotto mutazioni puntiformi e stimolazione di UDS, ma ha determinato un aumento significativo di crossing-over mitotico in* A. nidulans. *Tuttavia un campione di maggiore purezza (99,5 %) saggiato successivamente non ha mostrato alcuna attività nello stesso sistema genetico.*

INTRODUCTION.

In 1977 an environmental contamination by trifluorotoluene derivatives was detected in a large area in north-eastern Italy, near Vicenza. The results of gas-chromatographic and mass-spectrometric analyses indicated [1] three main pollutants as responsible of the contamination: 4-chloro-trifluoro-toluene (CTT), 4-chloro-3-nitro-trifluoro-toluene (NCTT) and 4-chloro-3,5-dinitro-trifluoro-toluene (DNCTT) in the ranges of 0.05-90 μg/lt, 0.6-450 μg/lt and 0.7-56 μg/LT respectively in the water from some wells in the polluted area. Traces of NCTT (0.6 μg/lt) were found in drinkable water from the water-pipe system of Creazzo city.

Responsible of this pollution was a chemical plant located in Trissino (Vicenza); this plant had been producing for many years several fluorotoluene derivatives among which, on pilot-scale, DNCTT, an intermediate in the synthesis of dinitroaniline herbicides such as trifluoro-2,6-dinitro-N,N-dipropyl-p-toluidine (Trifluralin). Due to the lack of information about the genotoxic potential of the three pollutants we decided to test their activity in three *in vitro* short term tests previously set up in our laboratory in order to detect gene mutations (*Salmonella*/mammalian microsome test system) and unscheduled DNA synthesis (UDS).

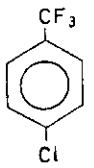
Afterwards we decided to study the herbicide Trifluralin, not only for its structural analogy with the three pollutants, but also for what is known on its genetic and carcinogenic effects [2].

MATERIALS AND METHODS.

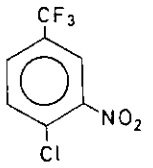
Organisms and test procedure.

1) *Salmonella typhimurium.* – All the strains employed in this research were kindly supplied by Prof. B.N. Ames, University of Berkley, CA U.S.A. The selection of induced *his*$^+$ revertants was performed by the procedure of Ames et al. [3]. Liver microsomal fractions were obtained from males Sprague-Dawley of about 250 g pretreated with Aroclor 1254. The procedures followed in rats induction and S-9 fractions preparations were as described by Ames et al. [3].

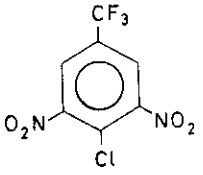
Each compound was tested in repeated experiments till the higest not toxic concentration. Positive controls were always included to check enzymic activity and strains sensitivity as well as routine controls of S-9 sterility.



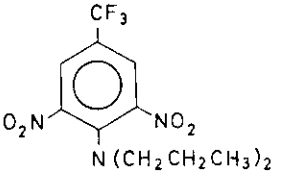
CTT
(I)



NCTT
(II)



DNCTT
(III)



TRIFLURALIN
(IV)

2) *Aspergillus nidulans.* – Strain P, heterozygous for p-fluoro-phenylalanine resistance (FPA^r) and coulor markers was used in spot test experiments to detect mitotic crossing-overs by selecting FPA^r as described in Bignami et al. [4]. The same strain was used in liquid test procedure in which 5×10^6 conidia/ml suspended in phosphate buffer were incubated 1 hour at 37 °C in a shaking bath with known amounts of tested chemical. After treatment conidia were washed twice by centrifugation, resuspended in buffer and seeded at proper dilutions on both selective and supplemented plates.

3) *Epitelial-like human cells (EUE).* – The induction of unscheduled DNA synthesis (UDS) was essentially the same as described by San and Stich [5], except no arginine deprivation of the cells was performed. EUE cells established *in vitro* from skin and muscle explants of human embryo, were grown in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10 % fetal calf serum (Flow). The tested compounds were dissolved in Hanks buffer balanced solution plus 1 % DMSO, then added to the cell cultures for 1 hr. After the treatment the cells were kept for 4 hours in DMEM with 5 μCi (H^3) thymidine/ml. Radioactive

Table 1. – *Absence of mutagenicity of trifluorotoluenes and Trifluralin* (**) *in* Salmonella typhimurium.

TESTED COMPOUND	His revertants/plate (a)							
	TA 1535		TA1537		TA98		TA100	
	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
CTT								
0 μl	21	35	10	10	39	69	152	154
0.1	23	34	10	9	25	64	133	126
0.2	19	30	10	13	13	45	103	125
0.4	16	29	10	17	13	48	83	108
NCTT								
0 μl	19	12	22	19	29	56	170	141
0.05	16	12	30	11	25	34	179	88
0.1	16	8	21	10	26	26	161	73
0.25	25	7	28	12	15	33	120	93
DNCTT								
0 μg	21	19	22	25	54	65	163	208
10	17	20	14	14	28	46	136	176
25	21	17	17	17	43	39	157	167
50	18	15	17	13	38	40	154	126
Trifluralin								
0 μg	22	10	17	12	40	30	186	100
100	30	11	15	8	21	23	156	75
200	27	11	11	11	29	27	152	88
500	23	13	12	9	20	30	134	79
Positive control (*)	1932	178	791	80	1754	779	1543	688

(a) Mean of three plates (SE never exceeded 10 %).
(*) EMS 5 μl for TA1535; MMS 1 μl for TA100; 9-AA 50 μg for TA1537; 4-n-o-phenylendiamine, 10 μg for TA98; 2-AA, 1 μg for all the strains in the presence of S9.
(**) Technical grade sample (95 %).

incorporation was detected by autoradiography. The cells in S-phase appeared heavily and easily discriminable from G_1 or G_2 phase cells scored for UDS.

Chemicals.

CTT (I), NCTT (II) and DNCTT (III) were purchased from Rimar S.p.a., Vicenza, Italy. Trifluralin (IV), technical grade (95 %) and pure grade (99.5 %) were kindly supplied by the Ely Lilly Research Centre Ltd. Surrey, UK. (Fig. 1). 9-aminoacridine and FPA were purchased from Sigma Chem. Co.; 8-azaguanine from Calbiochem, San Diego, CA; nitroso-guanidine from Koch-Light Laboratories, Ltd.; 2-aminoanthracene from Fluka, ethyl methansulphonate and methyl methansulphonate from Merck Schuchard.

RESULTS.

The data in Tab. 1 show the results obtained in *Salmonella typhimurium* by assaying the three trifluoro-derivatives and Trifluralin of technical grade both with and without microsomal activation. These chemicals were tested till the highest not toxic dose with no appreciable mutagenic activity. The cytotoxic effect of CTT and NCTT however did not allow to assay more than quite low doses.

In Tab. 2 are reported the results obtained by assaying with a selective method the three trifluorotoluene and both tecnical and pure samples of Trifluralin to induce mitotic recombination in *Aspergillus nidulans:* in repeated experiments only Trifluralin of technical grade turned out to be active. The pure sample, moreover, was tested in liquid test experiments confirming the negative results obtained in spot tests (Tab. 3). This procedure was chosen because the incubation of conidia in liquid medium allows a more drastic treat-

Table 2. – *Induction of mitotic recombination (FPA resistance) in* Aspergillus nidulans *– spot test.*

TESTED COMPOUND	amount/plate	FPA resistants/ plate (*)
CTT	0 µl	2.6±0.7
	0.25	3.0±0.4
	1.0	2.0±0.9
	2.5	2.0±0.4
NCTT	0.25 µl	1.7±0.5
	0.5	2.2±0.7
	1.0	1.5±0.6
DNCTT	10 µg	3.7±0.5
	20	2.7±0.5
	50	2.0±0.9
Trifluralin (*a*)	0.1 mg	14.2±5.7
	0.5	12.0±1.1
	1.0	10.0±1.6
	5.0	17.2±3.5
Trifluralin (*b*)	0 mg	4.3±0.2
	0.1	4.5±0.2
	0.5	4.0±0.6
	1.0	4.3±0.3
	5.0	4.8±0.9

(*) $\bar{x} \pm$ SE from three plates.
(*a*) Technical grade (95 %) sample.
(*b*) 99.5 % purity sample.

Table 3. – *Absence of recombinogenic activity of purified Trifluralin in* Aspergillus nidulans *– liquid test.*

DOSE (µg/ml)	srv %	Treated conidia $\times 10^8$	FPA resistants	FPAr/ 10^8 srv
0	100	1.2	22	1.8
5	53	0.63	11	1.7
25	30	0.36	8	2.2
125	8	0.096	1	1.0
625	2	0.024	0	—
0	100	1.6	19	1.2
5	53	0.85	9	1.1
25	23	0.37	2	0.5
75	7	0.11	1	0.9

Table 4. – *Detection of UDS as measured by autoradiography.*

COMPOUND	Concentration	Mean net number of grains per nucleus ± S.E.
Trifluralin	0	2.54±0.51
	0.1 µg/ml	5.00±1.21
	1.0 µg/ml	0.80±0.30
	10.0 µg/ml	3.26±0.80
	100.0 µg/ml	3.02±0.48
DNCTT	0	1.76±0.80
	10.0 µg/ml	1.96±0.45
	50.0 µg/ml	1.38±0.46
	100.0 µg/ml	1.28±0.57
	500.0 µg/ml	1.96±0.48
CTT	0	1.78±0.53
	0.2 µl/ml	3.08±1.70
	1.0 µl/ml	10.02±2.21 *
	2.0 µl/ml	19.82±2.18 *
	10.0 µl/ml	11.94±1.33 *
NCTT	0	1.78±0.53
	0.2 µl/ml	5.90±1.07
	1.0 µl/ml	34.28±3.00 *
	2.0 µl/ml	12.68±1.37 *
	10.0 µl/ml	1.66±0.40
MNNG	0	0.00±1.11
	0.15 µg/ml	7.20±2.19 *
	1.5 µg/ml	8.10±1.33 *
	15.0 µg/ml	17.50±1.34 *

The mean net number of grains per nucleus at different concentrations of compounds is reported. Data were compared by a t'test, at p 0.01 significance level (* : p 0.01). The data shown refer to typical experiments, repeated two or more times.

ment in cases in which no toxicity is detectable on agarized medium.

At least the four compounds were tested for the induction of UDS at the concentrations shown in Tab. 4; two of them, CTT and NCTT, induced detectable levels of ^{3}H-TdR incorporation, while Trifluralin of technical grade and DNCTT resulted negative.

Discussion.

In our experimental conditions CTT, NCTT and DNCTT failed to exhert any mutagenic or recombinogenic activity when tested in *Salmonella*/mammalian microsome assay and in the *A. nidulans* mitotic segregation system respectively.

The lack of mutagenicity in the Ames test was also demonstrated by Monti-Bragadin (University of Trieste) (unpublished data), who studied also the effect of *in vivo* activation by administration of the three chemicals by gavage to Wistar rats (100 mg of each compound/kg b.w.) and by testing the concentrates of urines collected in the three days following the treatment with the *S. typhimurium* strains: also in this way the three pollutants turned out to be non mutagenic. Up to now no published data are avaible on the genotoxic activity of these chemicals and so it is particularly remarkable the significative increase in the stimulation of UDS by two of them (CTT and NCTT) notwithstanding the absence of a clearcut dose-response curve. This evidence of a DNA-damaging activity should be taken into account for an evaluation of the potential harmful effects of these pollutants.

For whath concerns the genotoxic potential of Trifluralin, some information is available in the literature: as a matter of fact, this herbicide has been reported to be positive (NCI technical report nº 34, 1978) in the induction of liver carcinoma after oral administration to female mice; in that case, the commercial sample assayed contained some impurities in the range of 0.1-0.2 %, among which the well known carcinogen N-nitroso-dipropylamine. As far as the mutagenicity studies are concerned, Trifluralin was found to be negative in the following tests: UDS in human fibroblasts (WI 38 line), reverse mutation in *S. typhimurium* and *E. coli*, mitotic recombination in *S. cerevisiae* (D3 strain) and in the preferential cytotoxicity assays in DNA repair proficient and deficient strains of *E. coli* and *B. subtilis* [6].

In our experimental conditions a technical grade sample of Trifluralin resulted unable to induce both reverse mutations in *Salmonella* and UDS in FUE cells but gave a significative and reproducible increase of mitotic crossing-overs in qualitative spot test experiments performed in *A. nidulans* (Tab. 2). Following experiments with a sample of higher purity (99.5 %) however failed to confirm the observed recombinogenic activity (Tab. 2 and 3), thus indicating a possible involvement of mutagenic impurities in the previous response.

Previous cytological studies [7-10] indicated the spindle as the main target for Trifluralin toxicity in plants and brain cells: these studies suggest that a possible genetic activity may be observed mainly at chromosomal level. The weak activity observed by Murnik [2] in *Drosophila* for the induction of chromosomal damage confirms this hypothesis: in this connection some work is now in progress to evaluate the possible induction of mitotic non-disjunction in *A. nidulans*.

REFERENCES

1. Belsito, F., Bonifort, L., Dommarco, R. & Laguzzi, G. 1979. Mass spectra, fragmentation patterns and mass fragmentography of 4-chlorobenzotrifluoride, 4-chloro-3-nitrobenzotrifluoride and 4-chloro-3,5-dinitrobenzotrifluoride. *Ann. Chem.* **69**: 259.
2. Murnik, R. 1978. Mutagenicity of the herbicide Trifluralin in *Drosophila melanogaster*. *Mutation Res.* **53**: 235.
3. Ames, B.N., McCann, Y. & Yamasaki, E. 1975. Methods for detecting carcino gens and mutagens with the *Salmonella*/mammalian microsome mutagenicity test. *Mutation Res.* **31**: 347.
4. Bignami, M., Morpurgo, G., Pagliani, R., Carere, A., Conti, G. & Di Giuseppe, G. 1974. Non-disjunction and crossing-over induced by pharmaceutical drugs in *Aspergillus nidulans*. *Mutation Res.* **26**: 159.
5. San, R.H. & Stich, H.F. 1975. DNA repair synthesis of cultured human cells as a rapid bioassay for chemical carcinogens. *Int. J. Cancer.* **16**: 284.
6. Simmon, V.F., Mitchell, A.D. & Jorgenson, T.A. 1977. EPA Report 600/1-77-023.
7. Bartels, P.G. & Hilton, J.L. 1973. Comparison of Trifluralin, Oryzalin, Propham and Cochicine treatments on microtubules *Pesticide Biochem. Phisyol.* **3**: 462.
8. Jackson, W.T. & Stetler, D.A. 1973. Regulation of mitosis. IV. An in vitro and ultrastructural study of effects of Trifluralin. *Can. J. Bot.* **51**: 1513.
9. Hess, F.D. 1979. The influence of the herbicide Trifluralin on flagellar regeneration in *Chlamydomonas*. *Exp. Cell Res.* **119**: 99.
10. Hess, F.D. & Bayer, D.E. 1977. Binding of the herbicide Trifluralin to *Chlamydomonas* flagellar tubulin. *J. Cell Scie.* **24**: 351.