

Exhibit 36

The Effect of Gramoxone on the Activity of the
Nervous System of Laboratory Animals.

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A continuous increase in the application of weedkillers and the danger of poisoning of humans and domestic animals⁽¹⁾ associated with it, arouses the interest of scientific workers to the need for accurate determination of the toxic properties of this group of compounds. The toxic influence of gramoxone on the activity of the central nervous system is investigated in this work. Only a brief note was found in the available literature that weedkillers - dipyridyl derivatives, can have a negative influence on the nervous system⁽²⁾. For this reason, it was considered justified to undertake an objective experimental work on this subject.

The method of investigation.

The objective of these investigations was gramoxone (ICI) which is a 20% aqueous solution of paraquat. Paraquat is a quaternary ammonium compound, dimethylchloride of 4,4'-dipyridyl of a structural formula

fig.1.

In addition, commercial gramoxone contains 4% of sodium nitrite⁽³⁾. White rats, males of the Vistar breed 4-5 months old, weighing 220-245g were used as experimental animals. The total number of rats was 98. Gramoxone was injected into the peritoneum, whereupon according to Swan's data⁽²⁾, 19 mgr of paraquat (cation) per kg of body weight was

taken as LD_{50} dose. The volume of the liquid introduced amounted to 0.5 cm^3 . The period of treatment with gramoxone for the individual groups was from 3 to 4 weeks. Therefore each animal received 15-20 single doses of the chemical. During this time, the activity of the nervous system was investigated. Separate groups of animals each of 9-10 individuals were treated with gramoxone in the following doses: 0.05, 0.1, 0.2 and 0.3 of LD_{50} . Each group of animals investigated had a parallel control group. This was necessary because of the variability of results in the control animals. These animals were injected with 0.5 cm^3 of physiological solution of NaCl into the peritoneum. All animals had mixed feeding and remained under similar conditions at a temperature of $17-20^\circ\text{C}$.

The activity of the nervous system was tested by the Warner method (4), modified by Aleksandrov and Tibina (5). This method in various modifications is used by many authors as particularly suitable for neurophysiopharmacology (6,7). It is based on inducing in rats, conditional flight reflexes, whereupon the conditional stimulus consisted of an electric current applied alternately to one of the nets forming the floor of two compartments of the experimental chamber, the conditional stimulus was the sound of a bell. Numeration of successively applied stimuli was carried out from the beginning of the experiments, whereupon the calculated percentage of positive conditional flight reflexes appearing in a given group of animals was recorded. 100% positive

reaction was considered as the formation of the habitual flight reflex for the whole group. Once such a habit is formed, it is permanent and difficult to remove. The speed and frequency of the formation of positive flight reaction is the measure of the efficiency of processes of central stimulation.

The method of differentiation was used for the investigation of the development of the inhibiting process. Animals with well established flight reaction initially reacted to other accoustic stimuli, e.g., noise of a buzzer (on the basis of so called generalisation of stimuli). A systematic buzzing without an additional stimulant of electric current, lead *and a steady decrease in the number of reactions to the buzzer. The degree of differentiation* to the differentiation of buzzing from the bell ringing by all animals of a given group was expressed in percent of lack of flight reaction to the subsequent differential stimulant (buzzer). This was actually used as a measure of the intensity of central inhibiting. Statistical evaluation of the numerical data was carried out as follows. The starting point for calculations was stimulus (e.g. No.A) after which the conditional flight reflex appeared for the first time (independent of the group). Subsequent stimulus (e.g.No.B) at which both groups of animals (investigated and control) attained maximum 100%, or stabilised at a low level, reflex reaction was considered as the end point. Next, the mean frequencies (p_1 and p_2) on A-B line were expressed in decimal fractions calculated for each group and mean frequency

together for both groups (p). The values obtained for "investigated" and "control" groups, p_1 and p_2 were compared with the aid of "Z test"; $Z = (p_1 - p_2) : \sqrt{pq/n_1 + pq/n_2}$, where $q = 1 - p$. n_1 and n_2 - number of single stimuli applied for the whole group of animals on AB line (n - number of stimuli multiplied by the number of rats in the group).

The evaluation of experiments with differentiation of stimuli was carried out in a similar way. The risk of error P was read off from Fisher and Yate's tables.

The influence of gramoxone on the spontaneous mobility of white mice was also investigated. Animals were divided into 4 groups of 8 individuals each. Two groups were injected with a single dose of 0.1 and 0.2 LD₅₀ respectively (used for rats) of gramoxone 15 minutes before the beginning of measurements. The remaining two groups were the corresponding control groups to which 0.25 cm³ and 0.8% NaCl were injected. The animals were placed singly for a period of 11 hours (8-19) in cages of the equipment for measuring mobility, based on the photoelectric principle. The counter was read off in 5, 15, 30 and 60 min from the beginning of measurements and then every hour. The corresponding rows of numbers were compared by the Wilcoxon test.

Own investigations.

1. In the first series of experiments, gramoxone was injected into rats in a dose of 0.05 LD₅₀. The first conditional reflexes in the group of animals treated with gramoxone appeared after 11 associations of stimuli, in the control

group after 10 stimuli. However the velocity of increase in the frequency of positive responses of flight in animals treated with gramoxone was higher. The 100% frequency was attained in this group after 39 applications of the stimuli, while in control animals, after 56 applications. The mean frequency of positive flight reactions from animals No.10 to stimuli No.56 was 49.6% for animals treated with gramoxone ($p_1 = 0.496$) and 30.5% for the control group ($p_2 = 0.305$). This difference is highly significant ($Z = 5.75$; $n_1 = 460$; $n_2 = 414$; $P < 0.001$) fig.2.

2. In the second series of experiments, the animals received 0.1 LD₅₀ of gramoxone. Reflex flight reactions appeared in this group at the 12th application of the stimuli; while in the control group at the 30th application. In the treated group, the maximum level of frequency was attained earlier (at stimuli No.54) than in the control group (stimuli No.76). The mean frequency of positive flight reaction between stimuli No.12 and No.76 was 49.2% ($p_1 = 0.492$) while for the control group it was 24.9% ($p_2 = 0.249$). The statistical significance of the difference was high ($Z = 11.95$; $n_1 = 630$; $n_2 = 567$; $P < 0.001$) (fig.3).

3. In the third series of experiments, the investigated group of animals received 0.2 LD₅₀ doses. In these animals the first symptoms of conditioned flight reflex reactions appeared late, after the application of stimuli No.89. While in the control group the first reflex reactions appeared during the application of stimuli No.27, and at stimuli No.79

the frequency of positive response reached 100%. The mean frequency of the flight reaction between stimuli No.27 and No.89 was 30.4% in the group treated with gramoxone ($p_1 = 0.304$) and in the control group in the same treatment period amounted to 79.2% ($p_2 = 0.792$). This difference is statistically highly significant ($Z = 30.12$; $n_1 = 1070$; $n_2 = 963$; $P < 0.001$) (fig.4).

4. 0.3 LD₅₀ of gramoxone was used in the 4th group of experiments. The first reflex reactions in this group appeared at stimuli No.73. In the control group the first reactions appeared at stimuli No.25, but the maximum of frequency between stimuli No.25 and 81 was 11.8% for the group investigated ($p_1 = 0.118$) and 59.6% in the control group ($p_2 = 0.596$).
The significance of this difference is high.
($Z = 22.87$; $n_1 = 1090$; $n_2 = 981$; $P < 0.001$) (fig.5).

5. The differentiation of stimuli was carried out on treated and control animals in which the reflex flight reaction to the bell was 100%. In agreement with the law of generalisation, there was a 100% response to the buzzer (0% differentiation) in these animals. It appeared that the process of differentiation was more efficient in animals treated with 0.05 and 0.1 LD₅₀ doses of gramoxone.

The mean frequency of differentiation in animals which received 0.05 LD₅₀ doses was 42.5% ($p_1 = 0.425$) in control animals 16.4% ($p_2 = 0.164$). This difference is statistically significant ($Z = 12.80$; $n_1 = 1070$; $n_2 = 963$; $P < 0.001$). The results for animals treated with 0.1 LD₅₀ doses of gramoxone were 34.4% and 25.4% respectively ($p_1 = 0.344$;

$p_2 = 0.254$; $Z = 4.35$; $n_1 = 1040$; $n_2 = 936$; $P < 0.001$).

6. Measurements of spontaneous mobility in ~~rats~~^{white mice} after treatment with ~~doses of~~ gramoxone in doses corresponding to 0.1 and 0.2 LD₅₀ for rats (single doses) and in control mice showed no statistically significant changes.

Discussion of results.

In rats treated for a long period with gramoxone in doses of 0.05 and 0.01 LD₅₀, the conditioned flight reflexes were established faster than in the corresponding control groups. Doses of 0.2 and 0.3 LD₅₀ acted in the opposite direction: i.e., delayed and weakened the appearance of reflex reactions in comparison with the control animals. A dose of 0.4 LD₅₀ acted obviously toxically, causing a general deterioration in the state of the animals. Doses of 0.05 and 0.1 LD₅₀ acted positively not only on the establishment of conditioned reflexes, but also had an improving influence on differentiation processes.

It follows from the above results, that gramoxone acts on the central nervous system, in two phases. In small doses it amplifies processes of stimulation and inhibition in the central nervous system, in larger doses it acts in the opposite way, weakening these processes. As was pointed out earlier, the main constituent of gramoxone is paraquat. Ammonium nitride constitutes an insignificant admixture. In view of the generally known pharmaco- and toxicological properties of nitrides and their insignificant content in the preparation, it can be assumed that the observed changes in the activity of the

nervous system can be ascribed to the dipyridyl component of gramoxone.

Two-phase activity of various factors on living matter is a frequent phenomenon. It will be enough to mention as examples, the influence of narcotics, two-phase action of hypoxia as well as an increase in the concentration of hydrogen ions in body liquids. Undoubtedly, some biochemical changes caused by gramoxone are at the basis of action of this substance on the central nervous system. Unfortunately, the influence of dipyridyl compounds on the biochemistry of tissues is as yet little known. The weedkilling action is most probably based on the formation of free radicals and hydrogen peroxide (8). The mechanism of the influence on cells of mammals is known even less well (2). Attention was directed to the fact that their action is similar to curare (3), which most probably is associated with their structure, as these are quarternary ammonium derivatives. The inhibiting action of dipyridyls on healing of wounds was also observed. This lead to views that the these compounds interfere with the synthesis of collagen which is indicated by a decrease in the incorporation of S^{35} into chondroitin sulphate. On the other hand, the content of mucopolysacharides in the joining tissues of animals treated with paraquat is twice higher than in control animals (2).

Conclusions.

1. Gramoxone supplied in doses corresponding to 0.05 and 0.1 LD_{50} of paraquat, increases in white rats, the processes of

stimulation and inhibition in the central nervous system. The latter was measured by the method of conditioned flight reflexes.

2. Doses corresponding to 0.2 and 0.3 LD_{50} of paraquat inhibit and decrease the frequency of appearance of conditioned reflexes.

3. Doses corresponding to 0.1 and 0.2 LD_{50} of paraquat for rats applied to white mice do not cause any changes in their spontaneous mobility.

Bibliography.

- 1, 2, 3 and 4 - as in original.
5. LAZAREW, N.W. Causing illnesses in animals for medical experiments. PZWL, Warsaw, 1957.
- 6, 7, and 8 - as in original.

Figures.

fig.2. The formation of conditioned flight reaction in rats treated with 0.05 LD_{50} of gramoxone.

Continuous line - animals investigated

Broken line - control animals.

ordinate : % positive reaction.

abscissa : number of successive stimuli.

fig.3. The formation of conditioned flight reaction in rats treated with doses of 0.1 LD_{50} of gramoxone.

designations as in fig.2.

fig.4. The formation of conditioned flight reaction in rats treated with doses of 0.2 LD₅₀ of gramoxone. designations as in fig.2.

fig.5. The formation of conditioned flight reflexes in rats treated with doses of 0.3 LD₅₀ of gramoxone. designations as in fig.2.
