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Changes in the Blood of the Frog (*Rana temporaria* L.) after Different Doses of Ekatin

Zmiany we krwi żab (*Rana temporaria* L.) w zatruciu różnymi dawkami Ekatinu

The experiment with Ekatin demonstrated that this poison leads to considerable changes in the blood morphology of quails, these modifications appearing much earlier in males than in females (7). This difference is, in the Gromysz-Kałkowska and Szubartowska (7) opinion, connected with the high level of endogenous testosterone in males which conditions a higher rate of Ekatin metabolism to more toxic metabolites.

It may, therefore, be supposed that animals with a low metabolic rate will be more resistant to the toxic influence of Ekatin. The question arises, moreover, whether animals in which sexual activity is limited to the seasonal, thus a relatively short period of reproduction, show a different reaction to poisoning in dependence on sex. For confirming these supposition it was decided to evaluate the influence of Ekatin on the blood morphology of males and females of frog *Rana temporaria* L. in the autumn-winter season.

MATERIAL AND METHODS

Ekatin, the pesticide used for these studies, was obtained from Sandoz AG, Switzerland. It contains 25% of the active substance thiometon — 0.0-dimethylodithiophosphate-2(etylothio)ethyl and is classified as belonging to toxicity class II.

The studies were carried out on 110 frogs (55 males and 55 females), weighing from 22 g to 54 g, throughout November and the first half of December. The animals of each sex were divided into 4 experimental and 1 control group, each comprising 11 specimens. Those of the experimental groups were injected into the dorsal lymphatic sacs with Ekatin as suspension in 1 ml of amphibian Ringer's saline. The specimens of experimental groups I, II and III were given single doses of the pesticide in amounts of 50, 100, or 300 mg/kg b.w., respectively. The animals of group IV received 100 mg/kg b.w. of Ekatin daily for 3 days whereas the controls were injected with 1 ml of saline only.

Blood was sampled from the heart 3 hrs. after administering the preparation or physiological saline. The number of erythrocytes (RBC) and leucocytes (WBC) was determined by the chamber method in Natt-Herrick diluting fluid (11), haemoglobin level (Hb) by the cyanmethaemoglobin method and haematocrit value (Hct) by the micromethod. Mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated in accordance with the models given by Wintrobe (21). The percentage composition of leucocytes was calculated in preparations stained by the Pappenheim method. The number of erythroblasts per 1000 erythrocytes was determined in the same preparations. The number of reticulocytes per 1000 erythrocytes was determined in preparations stained *in vivo* with brilliant cresyl blue (15). The white blood cell index was calculated following the formula given by Stankiewicz (16).

The results obtained were analysed statistically using Student's t-test for independent data (Table 1). The value of $p<0.05$ was taken as statistically significant (13).

RESULTS AND DISCUSSION

The changes in the erythrocyte system of frogs intoxicated with Ekatin depended both on the dose applied and the sex of the animals (Table 2). The erythrocyte count fell after every dose applied in males, whereas a decrease was noted in females only after 2 doses — the lowest and the highest one. The remaining treatments increased this parameter (Table 2, Fig. 1).

It may be that the decrease of the erythrocyte content after Ekatin is due to their elimination from the circulation because of haemorrhages and formation of thrombi which were observed in all the examined animals, notwithstanding the pesticide dose. Sulik and Tyszkiewicz (18) in acute poisoning with Foschlor observed damage to the walls of blood vessels, with consequent leaking through them of serum with blood cells to the surrounding tissue. The mentioned authors also observed the swelling of the endothelial cells in capillary vessels, reducing their patency and leading to clotting.

Probably the haemolysing action of Ekatin plays an essential role in the reduction of the erythrocyte count. The cytolytic influence of this pesticide was acertained by Truchliński and Jabłoński (20). According to Gromysz-Kałkowska and Szubartowska (7) the depression of the erythrocyte parameters in quails poisoned with Ekatin is due to the haemolytic effect of the pesticide. Other organophosphorus pesticides also have a haemolytic influence on the morphotic blood elements of mammals (19), birds (4) and amphibians (6).

The rise of the erythrocyte count in *Rana temporaria* females noted

Table 1. Statistical analysis of results

Parameters	Sex	Differences between groups									
		C/I	C/II	I/II	C/III	I/III	II/III	C/IV	I/IV	II/IV	III/IV
Erythrocytes	A	0.05	NS	NS	0.01	NS	NS	NS	NS	NS	NS
	G	NS	NS	0.05	0.05	0.01	NS	NS	NS	NS	0.01
Haematocrit	A	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
	G	NS	NS	NS	0.01	0.01	0.001	NS	NS	0.05	0.001
Haemoglobin	A	NS	NS	NS	0.05	NS	NS	NS	0.05	0.05	0.01
	G	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
MCV	A	0.01	0.01	NS	0.05	NS	NS	NS	NS	NS	NS
	G	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
MCH	A	NS	NS	NS	NS	NS	NS	NS	0.05	0.05	NS
	G	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
MCHC	A	0.01	0.01	NS	0.05	NS	NS	NS	0.01	0.001	0.05
	G	NS	NS	NS	NS	NS	NS	NS	0.05	0.05	0.01
Reticulocytes	A	0,001	0.01	NS	0.001	0.001	0.001	0.001	0.001	0.001	0.001
	G	0,001	0.01	NS	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Erythroblasts	A	NS	NS	NS	NS	NS	NS	0.05	NS	0.05	0.05
	G	NS	NS	NS	NS	NS	NS	0.001	0.01	0.01	0.01
Leucocytes	A	0.001	0.001	0.001	0.001	0.01	NS	0.001	0.001	NS	NS
	G	0.001	0.001	0.001	0.001	0.001	NS	0.001	0.01	0.01	0.01
Neutrophils	A	0.05	0.001	0.001	0.001	0.001	NS	0.001	0.001	0.05	NS
	G	0.001	0.001	0.001	0.001	0.001	NS	0.001	0.001	NS	NS
Eosinophils	A	0.001	0.05	0.001	NS	0.001	0.01	0.001	0.001	0.001	0.001
	G	0.001	0.001	0.001	0.01	0.001	NS	0.001	0.001	0.001	0.001
Basophils	A	NS	NS	NS	0.05	NS	NS	NS	NS	NS	NS
	G	NS	NS	NS	NS	NS	NS	0.01	0.001	NS	NS
Lymphocytes	A	0.05	0.001	0.001	NS	NS	0.01	0.05	NS	0.01	NS
	G	NS	0.001	0.001	0.05	0.05	NS	NS	0.05	0.001	0.01
Monocytes	A	0.01	0.05	NS	0.01	NS	NS	0.001	0.001	0.001	0.001
	G	0.05	0.05	NS	0.01	0.05	NS	0.001	0.001	0.001	0.001
White blood cell index	A	0.001	0.01	NS	0.01	0.05	NS	0.001	0.01	NS	NS
	G	0.001	0.001	NS	0.001	NS	NS	0.001	NS	0.05	NS

Explanation: C — control, I — 50 mg/kg b.w., II — 100 mg/kg b.w., III — 3×100 mg/kg b.w., IV — mg/kg b.wz., A — male, G — female.

in the present experiments is probably connected with blood concentration caused by dehydration of the animals. Patyra et al. (14) explain similarly the increase of the erythrocyte parameters by a concentration of the cellular components owing to dehydration.

In spite of the reduced erythrocyte count in males the haematocrit value is practically higher than in the group of control animals, this being connected with the greater erythrocyte volume (Table 2, Fig. 1). It seems that we are dealing here with the swelling of the blood cells which have a relatively high ability of water absorption (3). The large number of enlarged erythrocytes appears in the blood picture. Near them there is a large number of huge naked nuclei, indicating that enlarged erythrocytes easily burst. Similar changes were also observed by Gromysz-Kałkowska and Szubartowska (6) in frogs intoxicated with Trichlorfon.

The erythrocytes in intoxicated female frogs are in general smaller than in control ones. The large percentage of erythrocytes, smaller and more rounded than in the control frogs, can be observed. The increase in the number of such erythrocytes may be considered as the organism's defensive reaction to the toxic effect of Ekatin. A similar fact was observed in *Rana temporaria* after poisoning with Trichlorfon in 50 and 100 mg/kg b.w. doses (6).

It may be assumed on the basis of the above presented data that changes in the erythrocyte count and haematocrit value after the applied Ekatin doses are the resultant of blood cell swelling, their rounding, various degree of haemolysis and also pathomorphological changes of various intensity such as congestion extravasation.

A decrease in the reticulocyte count in both sexes after all Ekatin doses (Table 2, Fig. 2) was noted. This may have resulted from the ready haemolysis of these blood cells. It is also an indication of the low efficiency of the haemopoietic organs of the frogs. As demonstrated, namely, in birds, Ekatin poisoning leads to a marked reticulocytosis and an increase in the number of erythroblasts (7).

The changes in the haemoglobin level are not unidirectional either in males or in females. Both an increase and a fall of these parameter values have been noted (Table 2, Fig. 3).

It is possible that in the individuals in which intensive haemorhages occurred, blood loss was compensated by its dilution, the haemoglobin level fell, whereas, when erythrocyte disintegration dominated, the haemoglobin level remained high.

The differences in the reaction of male and female frogs to poisoning with Ekatin may result from a somewhat different rate of biotransformation of the pesticide in both sexes. Biotransformation processes occur,

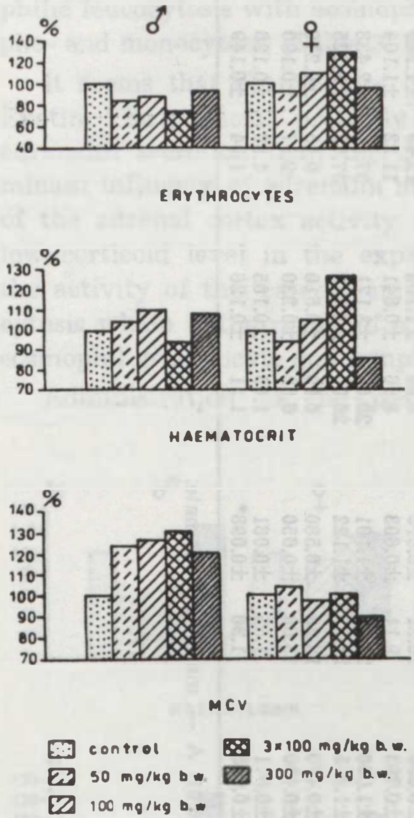

Fig. 1. The effect of different doses of Ekatin on the number of erythrocytes, haematocrit value, and mean corpuscular volume (MCV)

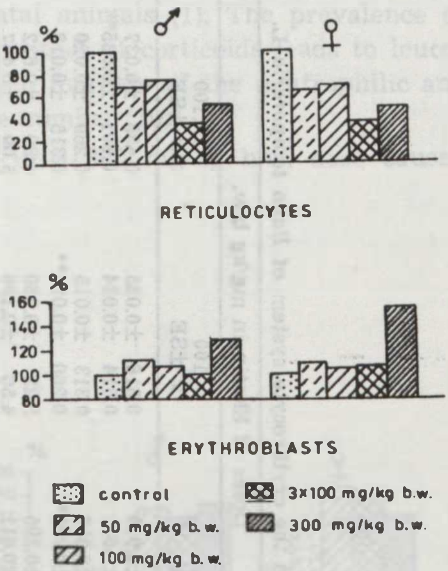

Fig. 2. The effect of different doses of Ekatin on the number of reticulocytes and erythroblasts

namely, with the participation of microsomal liver enzymes, and the activity of these enzymes is higher in males on account of the presence of testosterone (12).

It would seem, therefore, that in males, in spite of the lack of sexual activity in the experimental period, even a low level of this hormone ensures a higher enzymatic activity, and thus, a higher rate of transformation of the pesticide to more toxic metabolites. As demonstrated by Gromysz-Kałkowska et al. (8) testosterone is, thus, an essential factor modifying the rate of metabolism of the Ekatin and the toxicity of the pesticide to quails.

The data obtained indicate that the changes in the leucocyte system of frogs receiving Ekatin depend on the dose of this substance. Thus, after application of 50, 100 and 3×100 mg/kg b.w. of this agent neutro-

Table 2. The effect of Ekatin on the erythrocyte system of *Rana temporaria* L.

Parameters	Sex	Control $\bar{x}$	±SE	50 $\bar{x}$	±SE	100 $\bar{x}$	±SE	3×100 $\bar{x}$	±SE	300 $\bar{x}$	±SE
		Doses of Ekatin in mg/kg b.w.									
Erythrocytes in T/l	A	0.591	±0.036	0.496	±0.019	0.516	±0.035	0.435	±0.037	0.555	±0.055
	G	0.470	±0.040*	0.430	±0.031	0.514	±0.024	0.601	±0.035**	0.449	±0.033
Haematocrit in l/l	A	0.285	±0.016	0.296	±0.017	0.313	±0.015	0.269	±0.020	0.309	±0.023
	G	0.248	±0.017	0.234	±0.020*	0.260	±0.005**	0.315	±0.012	0.214	±0.019**
Haemoglobin in mmol/l	A	6.40	±0.572	5.01	±0.395	5.22	±0.360	4.39	±0.632	7.22	±0.723
	G	4.68	±0.531*	4.08	±0.411	4.56	±0.196	5.09	±0.447	5.17	±0.718
MCV in μm³	A	487.72	±20.15	602.97	±34.98	618.81	±29.53	636.81	±49.43	588.90	±53.89
	G	536.70	±25.03	556.75	±35.59	519.64	±30.48*	534.22	±23.05	481.49	±33.79
MCH in fmol	A	10.94	±0.853	10.22	±0.830	10.21	±0.610	9.99	±1.108	13.49	±1.269
	G	9.93	±0.665	9.73	±0.793	9.11	±0.603	8.66	±0.841	11.22	±1.104
MCHC in %	A	36.50	±2.817	27.13	±1.090	26.67	±1.101	26.05	±3.171	37.41	±2.438
	G	30.25	±2.344	28.08	±1.315	28.23	±1.122	26.04	±2.139	37.62	±3.225
Reticulocytes in %	A	17.69	±1.110	12.01	±0.470	13.23	±0.580	6.19	±0.510	9.45	±0.210
	G	18.82	±1.621	12.26	±0.490	13.08	±0.650	6.62	±0.230	9.37	±0.190
Erythroblasts in %	A	1.45	±0.090	1.62	±0.071	1.55	±0.061	1.45	±0.105	1.86	±0.126
	G	1.25	±0.037	1.36	±0.095*	1.30	±0.098*	1.31	±0.126	1.94	±0.149

Differences between sexes: * — $p<0.05$, ** — $p<0.01$, A — male, G — female.

philic leucocytosis with eosinophilia were noted together with slight lympho- and monocytosis (Table 3, Figs. 4—7).

It seems that changes are connected with the stressogenic action of Ekatin. The pesticide probably stimulates more the adrenal medulla to adrenalin secretion than the cortex cells to corticoid release. The dominant influence of adrenalin may also be conditioned by the retardation of the adrenal cortex activity in the autumn-winter period, thus, with low corticoid level in the experimental animals (1). The prevalence of the activity of this catecholamine over that of corticoids leads to leucocytosis which is the result of a uniform increase of the neutrophilic and eosinophil granulocyte and lymphocyte number (10).

Administration of the pesticide in a 300 mg/kg b.w. dose caused

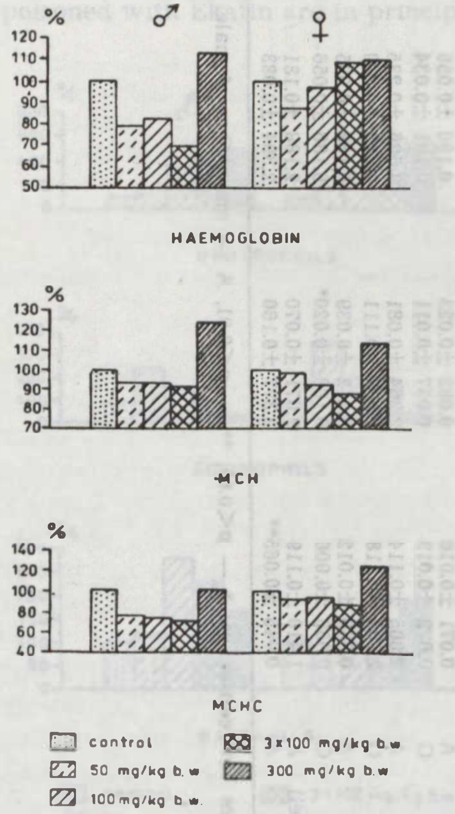

Fig. 3. The effect of different doses of Ekatin on the haemoglobin content, mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC)

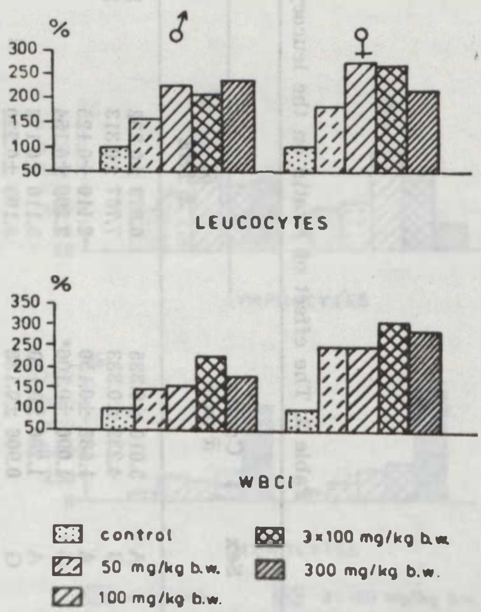

Fig. 4. The effect of different doses of Ekatin on the number of leucocytes, and on the white blood cell index (WBCI)

Table 3. The effect of Ekatin on the leucocyte system of *Rana temporaria* L.

Parameters in G/l	Sex	Control $\bar{x} \pm SE$	Doses of Ekatin in mg/kg b.w. 50 $\bar{x} \pm SE$	100 $\bar{x} \pm SE$	3×100 $\bar{x} \pm SE$	300 $\bar{x} \pm SE$
Leucocytes	A	5.010 ±0.335	0.878 ±0.232	11.434 ±0.469	10.383 ±0.804	11.899 ±1.015
	G	4.232 ±0.333	7.767 ±0.313	11.596 ±0.636	11.293 ±0.655	9.161 ±0.316*
Neutrophils	A	1.583 ±0.150	2.110 ±0.125	5.870 ±0.434	6.293 ±0.549	7.945 ±0.689
	G	1.096 ±0.106*	2.206 ±0.154	6.246 ±0.342	6.583 ±0.426	6.190 ±0.204*
Eosinophils	A	1.266 x̄0.130	3.110 ±0.178	1.674 ±0.116	1.120 ±0.128	0.425 ±0.048
	G	0.906 ±0.138	3.193 ±0.220	1.772 ±0.132	1.627 ±0.154*	0.344 ±0.036
Basophils	A	0.071 ±0.016	0.083 ±0.023	0.196 ±0.058	0.164 ±0.036	0.118 ±0.032
	G	0.072 ±0.013	0.057 ±0.011	0.101 ±0.034	0.094 ±0.026	0.136 ±0.017
Lymphocytes	A	2.005 ±0.114	2.364 ±0.081	3.526 ±0.235	2.455 ±0.289	2.577 ±0.228
	G	2.103 ±0.118	2.236 ±0.111	3.246 ±0.220	2.811 ±0.246	1.839 ±0.141*
Monocytes	A	0.085 ±0.012	0.212 ±0.039	0.168 ±0.035	0.262 ±0.059	0.834 ±0.105
	G	0.058 ±0.006	0.108 ±0.020*	0.186 ±0.056	0.222 ±0.043	0.653 ±0.044
White blood cell	A	1.410 ±0.119	2.071 ±0.070	2.188 ±0.181	3.167 ±0.456	2.537 ±0.109
index	A	0.945 ±0.065**	2.377 ±0.160	2.394 ±0.083	2.889 ±0.256	2.750 ±0.148

Differences between sexes: * — $p<0.05$, ** — $p<0.01$, A — male, G — female changes typical of stress, namely, neutrophilic leucocytosis with marked eosino- and slight lymphopenia (Table 2, Figs. 4—6).

The depressed eosinophilia may be due to migration of these cells to the sites affected with destructive changes in the course of which histamine accumulates. Eosinophilia contributes, namely, to the capture and inactivation of histamine. This supposition seems correct since the highest dose of the insecticide produced the highest pathomorphological change. It also finds confirmation in the pronounced monocytosis (Table 2, Fig. 6) noted after the 300 mg/kg b.w. dose. Monocytosis is probably the result of the enhanced activity of the reticuloendothelial system as response to the pathomorphological changes in the tissues. In this situation mechanisms have to be set in operation which would remove the consequences of destruction.

To sum up, it may be affirmed that changes in the blood of frogs poisoned with Ekatin are in principle the result of the haemolysing action

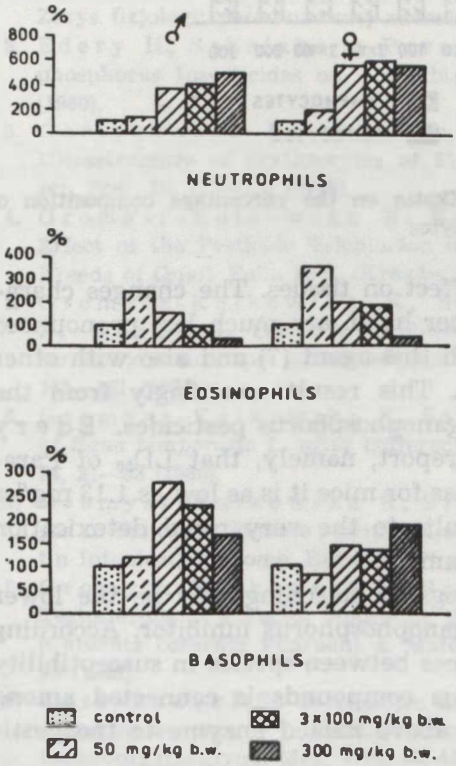

Fig. 5. The effect of different doses of Ekatin on the number of neutrophils, eosinophils, and on the basophils

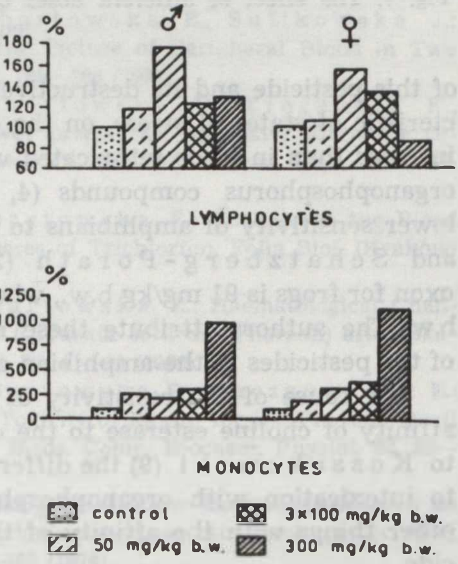

Fig. 6. The effect of different doses of Ekatin on the number of lymphocytes and monocytes

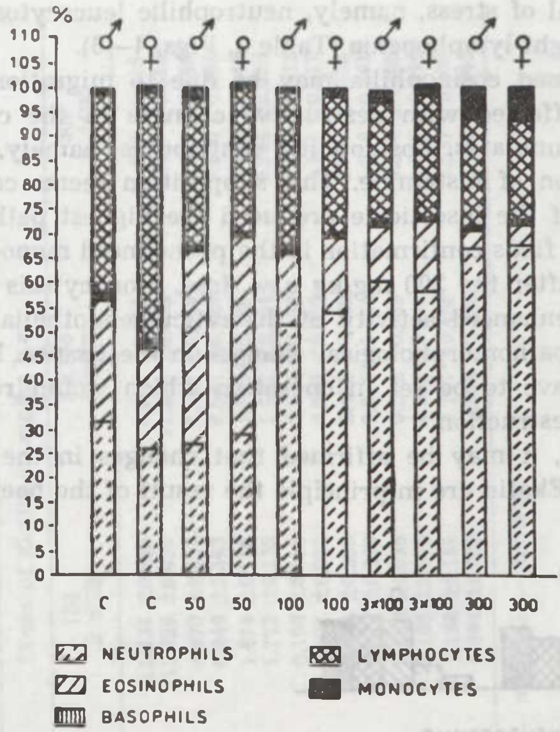

Fig. 7. The effect of different doses of Ekatin on the percentage composition of leucocytes

of this pesticide and its destructive effect on tissues. The changes characteristic of states of stress, on the other hand, are much less pronounced in frogs than in birds intoxicated with this agent (7) and also with other organophosphorus compounds (4, 5). This results seemingly from the lower sensitivity of amphibians to organophosphorus pesticides. E d e r y and S c h a t z b e r g - P o r a t h (2) report, namely, that LD_{50} of Paraoxon for frogs is 91 mg/kg b.w., whereas for mice it is as low as 1.13 mg/kg b.w. The authors attribute these results to the very rapid detoxication of the pesticides in the amphibian organism.

The cause of the resistivity of frogs to poisoning may be the lower affinity of choline esterase to the organophosphorus inhibitor. According to K o s s a k o w s k i (9) the differences between species in susceptibility to intoxication with organophosphorus compounds is connected among other things with the affinity of the above named enzyme to the pesticide.

It should also be noted that in frogs anoxia of the organism characteristic of poisonings with organophosphorus pesticides is probably less acute on account of their respiration through the skin.

The high intensity of changes in the blood revealed in the present investigations seems to indicate either a slower Ekatin decomposition than that reported by Edery and Schatzberg-Porath (2) or, if we assume that the metabolic changes are rapid, the products of pesticide decomposition are, however, excreted slowly and it is that they have an unfavourable influence on the organism. As demonstrated by Studnicka (17), Trichlorfon excretion from the organism of the carp lasts about two weeks.

It seems, thus, that the haematological changes in frogs poisoned with Ekatin are mainly the effect of its destructive effect and of the mechanisms removing the consequences of destruction, whereas the general systemic influence of this agent is not very well pronounced.

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STRESZCZENIE

Badania wykonano na samcach i samicach żab (*Rana temporaria* L.) zatrutych jednorazowo Ekatinem w dawkach 50, 100 i 300 mg/kg m.c. i 3-krotnie dawką 100 mg/kg m.c. Zmiany w układzie czerwonokrwinkowym intoksykowanych zwierząt uzależnione były zarówno od dawki stosowanego pestycydu, jak i od płci i stanowiły wypadkową pęcznienia krwinek, ich zaokrąglania się, różnego stopnia hemolizy, a także różnego nasilenia zmian patomorfologicznych typu przekrwień, krwawych wylewów i krwinkotoków. Zmiany w układzie białokrwinkowym zależały od dawki Ekatinu. Mniejsze dawki nie dawały objawów typowych dla stresu, dopiero dawka najwyższa powodowała charakterystyczne zmiany stresowe.

Brak reakcji stresowej może wynikać z małej wrażliwości płazów na zatrucie, uwarunkowanej prawdopodobnie niewielkim powinowactwem esterazy cholinowej do inhibitora fosforoorganicznego. Może on również być związany z uwstecznieniem kory nadnerczy u żab w okresie jesienno-zimowym, a tym samym z przewagą wpływu katecholamin nad kortykoidami.