THE FATE OF DEVELOPMENT INHIBITOR MONSANTO® 0585 IN WATER, MOSQUITOES AND NON-TARGET HYDROBIONTS

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ABSTRACT. Development inhibitors, that are being worked on so intensively now, seem to be one of the most promising tools of insect control. Monsanto® 0585/2,6-di-*tert*-butyl-4 (α,α-dimethylbenzyl) phenol/ belongs to this group of compounds. It is highly active against mosquitoes *Anopheles, Aedes, Culex* and *Psorophora,* including species that are resistant to chlorinated and organophosphorus insecticides. The aim of this work was to get the initial data on absorption and excretion of this compound by insects that are needed for the further and more detailed study of action mechanisms of MON 585 and analogous development inhibitors. The present work is fulfilled using the compound Monsanto 0585, labelled by ^{14}C. The preparation was applied to an experimental reservoir with *Aedes cantans* (Mg.) larvae under simulated natural conditions.

INTRODUCTION

Though compounds of the MON 585 type differ absolutely in their chemical structure from insect juvenile hormones or their analogues, their action resembles the action of juvenile hormones in its formal manifestation: development of larvae is disturbed and, in the end, they die as pupae.

There is some evidence that MON 585 possesses many properties of an ideal insecticide (Sacher 1971a). It is highly active against mosquito larvae (i.e. mortality of *Aedes aegypti* larvae is equal to 95% at 24 hr exposure in water with 0.1 mg/l), while it has little effect on soil insects. Field trials of the compound at doses, causing 70–100% mortality, showed that the number of concomitant water insects (bugs, mayfly nymphs, chironomid larvae) remained unchanged, but the quantity of predacious insects (dragonfly and water-tiger larvae) was lowered to some extent, probably due to reduction in food resources (Steelman et al. 1975). MON 585 is nontoxic to fish and is quite safe to mammals in concentrations lethal to all mosquitoes. The LD_{50} was 2000 mg/kg for rats, when given *per os,* and when applied on rabbit skin—3000 mg/kg. The substance is sufficiently soluble in water and quickly (but not too quickly) decomposes in the environment. Its half-life in the soil is equal to 2 d. (Sacher 1971a), and in water the effectiveness of MON 585 was twice reduced in 30 d. (Jakob and Schoof 1972). As the structure does not contain other elements except C, H and O, environment pollution must be minimal. Finally, simplicity of the compound structure enables us to expect that industrial-scale manufacturing of this compound might be quite inexpensive.

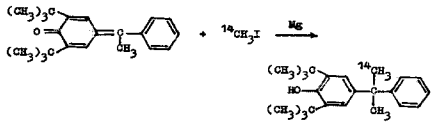
The mechanism of action of MON 585 has not been studied. It is supposed that it disturbs oxygen exchange in pupae (Sacher 1971b). Appearance of resistance to MON 585 is not found in *Culex pipiens quinquefasciatus* (Say) in 20 successive generations (Hsieh et al. 1974).

In field trials with *Aedes nigromaculis* (Lud.) their development was completely suppressed at dosage of active ingredient equal to 1.5 kg/ha (Schaefer and Wilder 1972).

MATERIALS AND METHODS

The synthesis of labeled compound 2, 6-di-*tert*-buytl-4 (α,α-dimethylbenzyl) phenol was carried out by ad-

ding methylmagnesiumiodide-^{14}C to α-methyl-α-phenyl-2, 6-di-*tert*-butyl-methylenequinone:



by the method Ostapetz-Sveshnikova et al. (1967) with altering of methylenequinone: methyl iodide ratio from 1:6 to 1:1.5. 0.08 ml of methyl-^{14}C iodide with specific activity of 4.3 Ci/mol was used in microsynthesis of the labeled compound. The end product was purified on the column of silicic acid with hexan as eluent.

In blank experiments end product purity was checked by gas chromatography: chromatograph Tzvet-2 (OKBA, Dzerjinsk); stainless steel column 3 mm i.d., 3 m length with 5% silicone SE-30 on support Chromaton N-AW-DMCS (Lachema, Czechoslovakia); column and injector temperature 220 and 240°C; flow rate of the carrier gas—nitrogen—80 ml/min. The compound MON 585 from Monsanto Research Corp. (St. Louis, Mo.) was used as a standard. The reaction product does not contain any contaminants.

Radiochemical purity of labeled compound was checked by thin layer chromatography on silica gel plates Silufol® (Kavalier, Czechoslovakia). Radiochromatograms (Fig. 1) were scanned by automatic apparatus consisting of collimated end-window Geiger-Müller counter, broach mechanism with electrical drive, automatic radiometer NP-227 (Gamma, Hungary) and numerical printer VA-G-24A (Vakutronik, DDR). Radiochemical purity of the compound (R_f 0.8) appeared to be equal to 95.3%, admixtures with R_f 0.05, 0.15 and

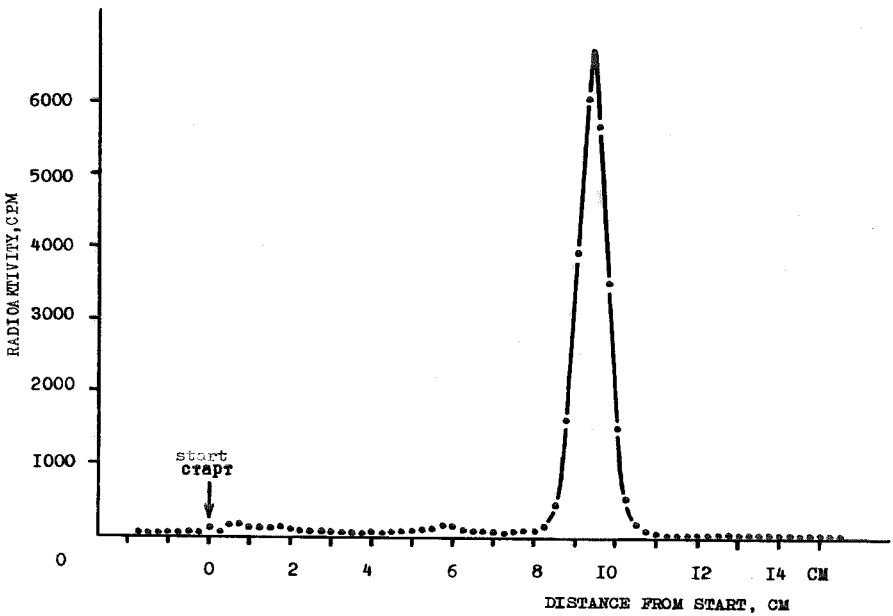


Fig. 1. Radiochromatogram of ^{14}C-labeled MON 585.

0.45 were correspondingly 1.3, 1.3 and 2.1% of total radioactivity. Recovery of pure substance determined by ^{14}C was equal to 16.2% from methyl iodide or 22.9% from methylenequinone.

Laboratory trials on biological activity of unlabeled but analogous synthesized samples were carried out on *Aedes aegypti* (L.) 4th-stage larvae at 24-hr exposure. The same species was used for preliminary laboratory experiments. In each of the 2 vessels with 200 ml of water (plus labeled compound) 100 larvae were kept from the beginning of stage IV up to emergence. Laboratory trials were carried out at temperature of 23–25°C.

Field trials were conducted near Moscow in May, 1974. An artificial reservoir, close to the natural conditions at most, was made to carry out this experiment. The bottom and edges of the reservoir were faced with turf, taken from lots with typical vegetation and flooded in the spring. The reservoir was isolated from subsoil waters by polyethylene film, placed under the turf. A day before the experiment began, 100 liters of water from natural habitation of *Aedes* mosquitoes were poured into the reservoir; 22 liters of water were soaked in the turf and 78 liters were left free. Approximately 9000 *Aedes cantans* (Mg.) larvae, collected from natural population the same day, were placed into the reservoir after water sediment. The larvae were at the 4th stage for the most part, but there were also larvae at the other stages. Pupae were placed at the same reservoir in a separate cage. Half the number of the larvae and half the volume of water were used in the control reservoir. Observations were carried out during a fortnight.

Radioactive preparations were placed in the experimental reservoir in the form of alcoholic solutions (hexane was removed from the initial solution by distillation under vacuum). In all 7.14 mg of MON 585-^{14}C with specific activity 12.1 mCi/g was introduced into the reservoir. Concentration calculated on 78 liters of water was 0.092 mg/l.

Samples of mosquitoes and some other concomitant invertebrates at different stages of development were taken daily. Radiometric analyses covered in all more than 400 samples, including approximately 4000 specimens of mosquitoes and more than 200 of other invertebrates. Biosamples in number from one to several specimens were crushed with a glass stick on the filter paper disks and were dried in the air, after that their radioactivity (together with the disks) was measured in 3 ml toluene-based scintillator (4 g/l *p*-terphenyl and 0.1 g/l POPOP). Radioactivity of water samples (0.5 ml) was estimated with 5 ml scintillation solution by Bray (methanol 100 ml, monoethyleneglycol 20 ml, naphthalene 60 g, PPO 4 g and POPOP 0.2 g in 1 l of dioxane). The measurements were carried out on a liquid scintillation spectrometer NZ-137 (Gamma, Hungary). Quench corrections were determined by a channel ratio method.

Radioactivity distribution in subcellular fractions, obtained from homogenates by differential centrifugation, was determined in several large samples of larvae, pupae and adults (200–500 insects in each sample). Fraction radioactivity was measured with Bray solution (0.5 ml of a suspension with 10 ml scintillation solution), and quenching was corrected by the channel ratio method. Amount of proteins in the fractions was determined according to Lowry.

To clear out the chemical form of MON 585 in water and in insects, extraction was used: from water samples (up to 200 ml)—with the help of hexane or diethyl ether, and from biosamples (up to 200–400 specimens)—with the help of diethyl ether, by grinding of insects in mortar and using several portions of solvent. The extracts were evaporated under vacuum and then transferred (in a minimum volume) on Silufol plates for thin layer chromatography with benzene/hexane (1:1). Radiochromatograms were registered by the scanning apparatus described above.

RESULTS AND DISCUSSION

In laboratory trials on *Ae. aegypti* MON 585 demonstrated high biological activity: the values of LC_{50} and LC_{95} were correspondingly 0.1 and 0.5 mg/l. In the treated reservoir mortality of *Ae. cantans* was 60% as compared to 15% in the control reservoir. Results of the field experiment will be given mainly because the results of preliminary laboratory experiment were generally confirmed in field trials.

Water radioactivity measurements indicate (Fig. 2) that in the first 1–2 days significant lowering of labeled substance concentration took place as a result of adsorption by bottom depositions. In the given conditions portions of the adsorption were equal to ¾ of all. Subsequently water radioactivity remained practically on the same level. Of course, the constant level of measured radioactivity cannot indicate the compound stability. As extract chromatograms showed (Fig. 3) on the 1st day, several (no less than 4) substances with radioactive labels, except the initial compound, were detected in the water. The number of radioactive products was reduced then, and by the 8th day (see below fig. 5d) only 2 zones clearly appeared on the chromatograms: the zones

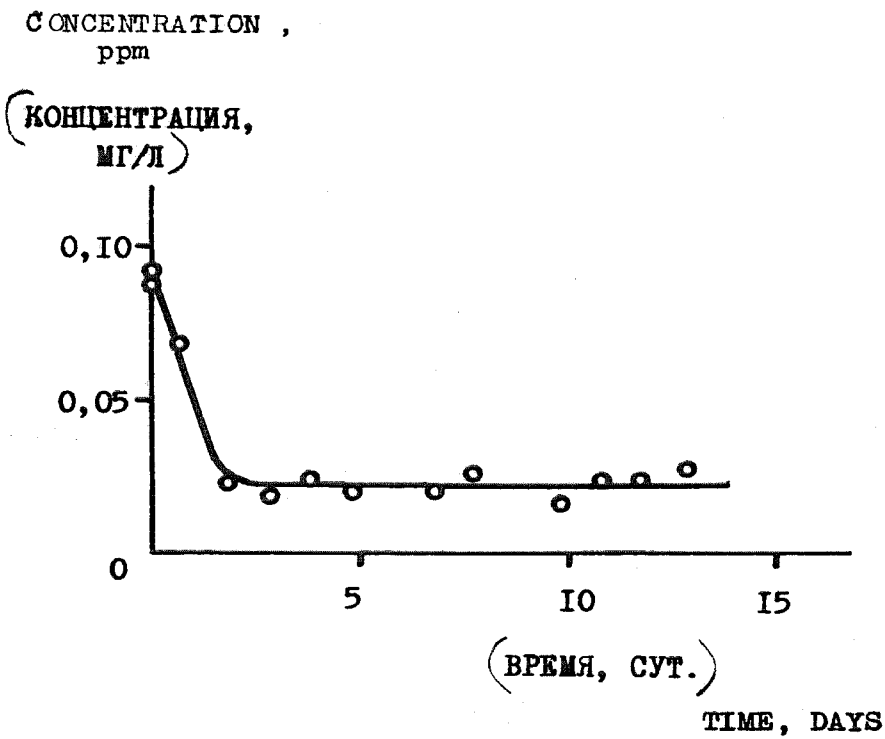


Fig. 2. Concentration of MON 585 and (or) its derivatives in the water of open air reservoir.

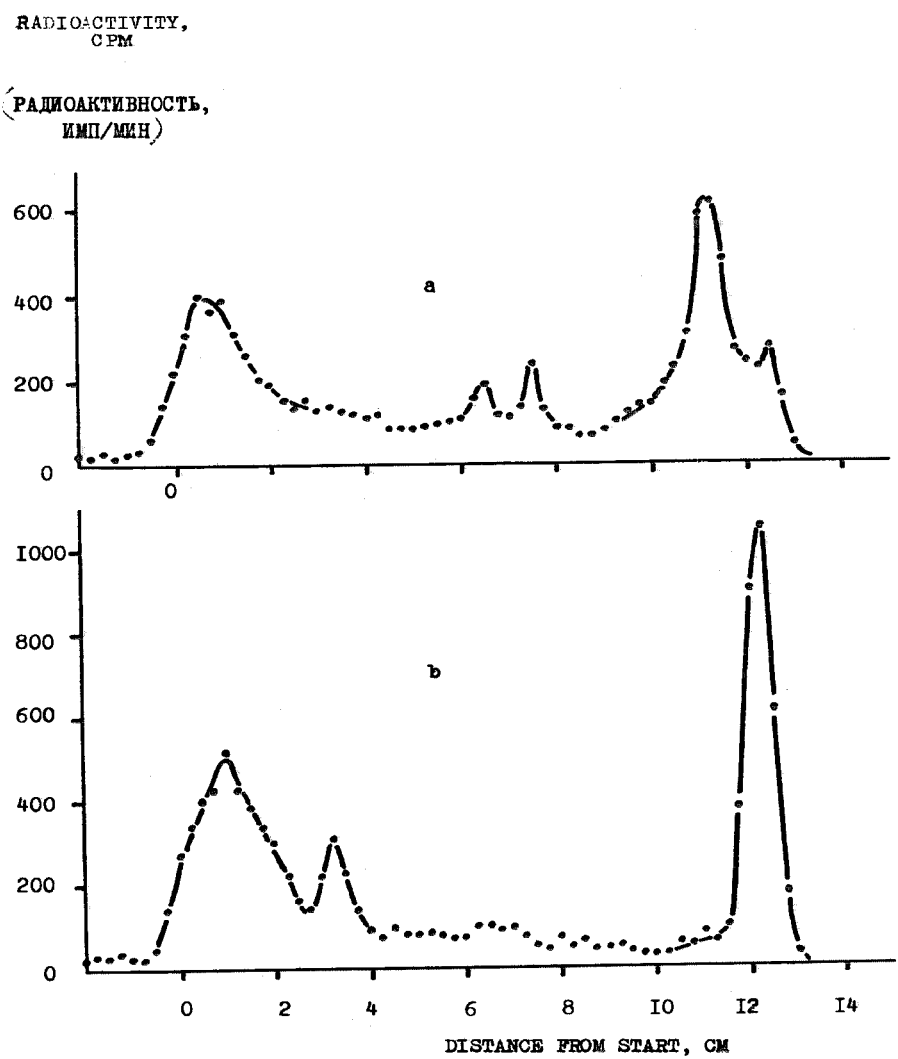


Fig. 3. Radiochromatograms of ether extracts from the water: a—day of treatment, b—next day.

of initial substance and of highly polar product(s) of its transformation. Relative quantity of the unchanged substance constituted 40–50% of the total quantity of labeled compounds in the water during the first 3 days, and it was reduced to 20–25% on days 5–8. Thus the half-life ($T_{1/2}$) of MON 585 in the water of the open reservoir is equal to 3–5 days. In the laboratory experiment in glass vessels without vegetation the part of unchanged substance constituted 26–36% of the general radioactivity of the hexane extract on the 11th day, i.e. the transformation without vegetation was slower. The polar products are probably oxidation products. The slow decomposition of MON 585 was marked in the absence of water too; radioactivity of the compound spot on the Silufol plate (in adsorbed state on silica gel) was decreased by 14% at 2.5 months and by 30% at 11 months storage in the air in darkness.

The results gained in laboratory and field experiments showed that MON 585 penetrates quickly in the larva; in several hours the radioactivity reached almost equilibrium (Fig. 4ab). Then, for a certain period of time larval radioactivity remained on a constant level that was characteristic for each stage of development (Table 1). Considering these data we should bear in mind that measured radioactivity refers to MON 585, as well as to the possible products of its transformation; in all the cases, conditionally, calculation is made on the initial labeled compound.

In the larvae that reached the given stage at 3–4 days after treatment and later, radioactivity became lower and lower in the meantime, which corresponded to (and was caused by) the decrease of active substance concentration in water. Nevertheless it is of interest that this was observed only in the larvae of the 1st, 2nd and 3rd stages. In the 4th-stage larvae, captured from water between the 3rd and 13th days, radioactivity was practically one and the same (with regard to statistical dispersion). Consequently, accumulation of labeled substance by the 4th-stage larvae was approximately the same irrespective of its concentration in water, which became almost an order lower by the end of the mentioned period. Also taking into consideration that there are practically no other labeled compounds in the larvae, except the initial (see further), the fact of the same accumulation of the matter at its high and low concentration in the water testified to the existence of an active mechanism of MON 585 absorption by the 4th-stage larvae.

Table 1. Mean amounts of absorbed MON 585 in *Aedes cantans*.

Stages	Post-treat time (days)	Number of specimens analysed	Content* of MON 585 (ng/specimen)
A. Treatment at larval stage			
1st-stage larvae	1	53	2.7±0.1
2nd-stage larvae	1+3	80	5.9±0.5
3rd-stage larvae	1+4	80	18±2
4th-stage larvae	2+12	92	67±5
Young pupae (unpigmented), alive	3+13	67	65±7
Young pupae (unpigmented), dead	3+13	43	69±8
Pupae (pharate adults)	7+16	75	39±2
Imago	13	9	9.6±1.3
Pupal exuvia	12	14	2.9
B. Treatment on pupal stage			
Pupae	2	20	5.7±0.6
	4	9	11.8±1.5
Imago	4	13	10.4±1.6
Pupal exuvia	4	36	2.6

* Means ± S.D.

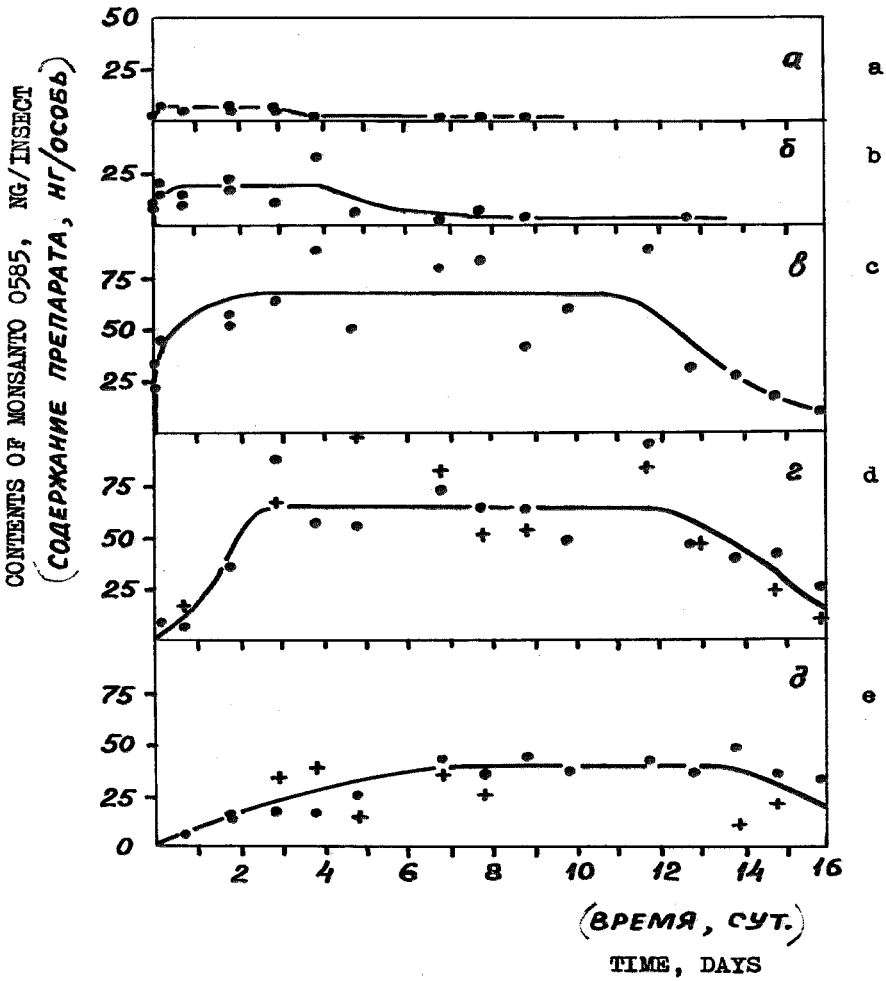


Fig. 4. Dynamics of the MON 585 contents in *Aedes cantans,* recaptured from the water on different days after treatment. a—2nd-stage larvae, b—3rd-stage larvae, c—4th-stage larvae, d—young pupae, e—pupae (pharate adults) (o—alive, +—dead).

The data given in Table 2 show that larval development is accompanied by progressive accumulation of the compound, counting on a mass unit. This also confirms the thesis of the active compound absorption, which increased from one stage to another. While transforming from stage II to III, and from III to IV relative content of MON 585 in the larvae increased in 1.5-2 times on the average. Thus, additional accumulation of MON 585 is timed to larval moulting periods.

The preparation is not absorbed by the pupae. This is confirmed by the cessation of further compound accumulation (as compared to its content in 4th-stage larvae), and by the results of radioactivity measurements in the pupae which were treated as pupae. After 2–3 days being in water with labeled MON 585 these pupae demonstrated very small radioactivity (see Table 1).

In the young pupae there was found approximately the same amount of MON 585 as in the 4th-stage larvae. However a rapid removal of labeled material occurs in pupae which remain alive. To the period of pharate adult the content of ^{14}C in pupae averaged 50%, and in adults, 15% of the mean content of ^{14}C in 4th-stage larvae. A statistically reliable difference in radioactivity between alive and dead pupae was not found.

Among the concomitant invertebrate hydrobionts we did not detect those which demonstrated extremely great differences from the mosquito larvae in labeled matter accumulation (see Table 2). Thus there is no reason to speak about preferable (as compared to other hydrobionts) absorption of MON 585 by the mosquito larvae, though its action on the latter is undoubtedly specific.

Thin layer radiochromatography (Fig. 5) permitted us to determine the form of the labeled matter which was absorbed by the larvae. The larvae and pupae contained almost exclusively (92% in both cases) the compound in its initial active form. On the contrary, no initial substance was found in surviving adults, and a small available radioactivity corre-

Table 2. Contents of MON 585 in mosquitoes and non-target invertebrates.

Hydrobionts	Number of specimens analysed	Mean content (ng/specimen)	Mean mass of one specimen (mg)	Relative content (ug/g)
ARTHROPODA				
Insecta				
Mosquitoes *Aedes cantans* (Diptera)				
2nd-stage larvae	80	5.9±0.5	1.8	3.3
3rd-stage larvae	80	18±2	3.2	5.6
4th-stage larvae	92	67±5	6.9	9.7
young pupae (unpigmented)	67	65±7	7.3	8.9
pupae (pharate adults)	75	39±2	7.3	5.3
Larvae *Dytiscus* (Coleoptera)	3	57.5	22.5	2.6
Imago *Noterus* (Coleoptera)	4	15.4	4.5	3.4
Larvae *Limnophilus* (Trichoptera)	2	4.2±4.5	30.2	0.15
Imago *Hydrometra* (Hemiptera)	1	24.0	14.8	1.6
Larvae *Cloeon* (Ephemeroptera)	7	56–112	11.2	5–10
Arachnoidea				
Water mites *Eulais* (Hydrochnellae)	5	5.7–5.9	1.2	5
Crustacea				
Cyclops (Eucopepoda)	140	0.8–1.8	0.1	8–18
Daphnia (Cladocera)	35	7–23	0.6	11–39
MOLLUSCA				
Gastropoda				
Pond snails *Limnaea*	8	15–29	30	0.5–1.0

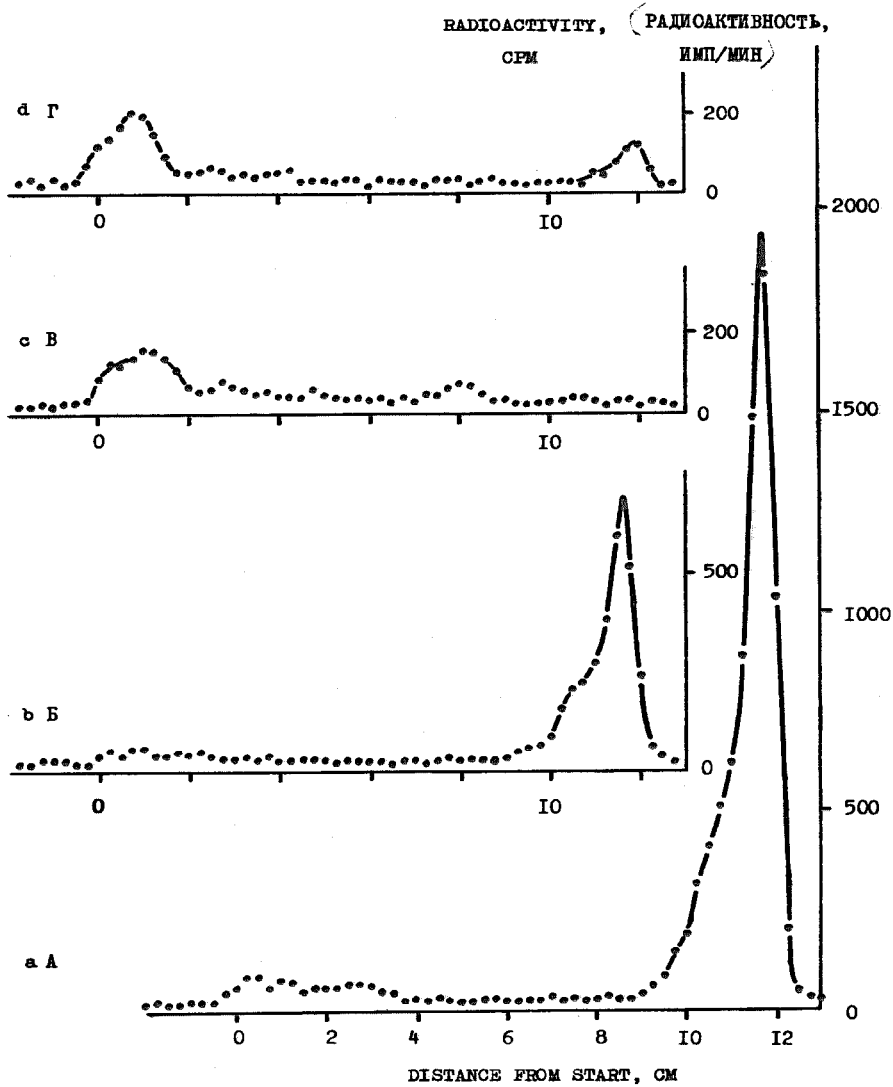
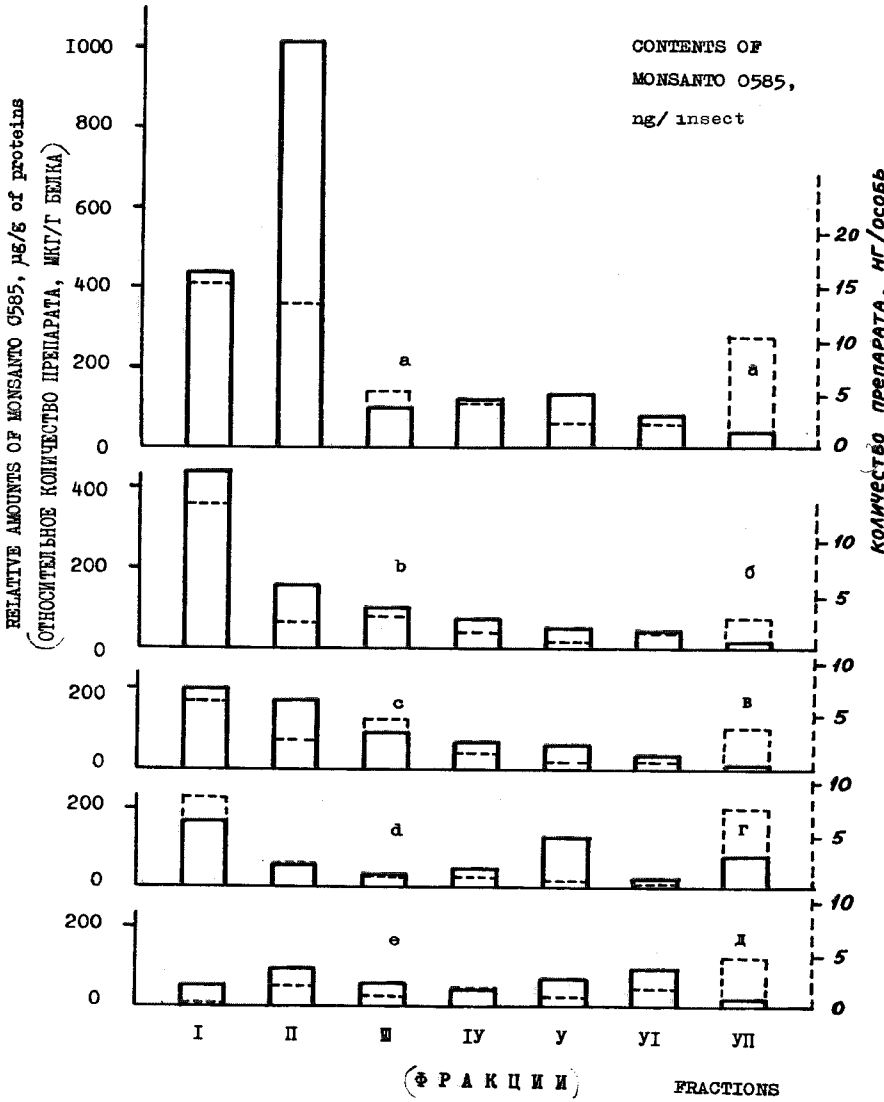


Fig. 5. Radiochromatograms of ether extracts from mosquitoes and from water (in brackets—time after treatment): a—from 200 larvae (2 d.), b—from 200 pupae (2 d.), c—from 400 imago (15 d.), d—from 170 ml of water (8 d.).


RELATIVE AMOUNTS OF MONSANTO 0585, µg/g of proteins
(ОТНОСИТЕЛЬНОЕ КОЛИЧЕСТВО ПРЕПАРАТА, МКГ/Г БЕЛКА)
CONTENTS OF
MONSANTO 0585,
ng/ insect
КОЛИЧЕСТВО ПРЕПАРАТА, НГ/ОСОБЬ
1000
800
600
400
200
0
20
15
10
5
a
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д
I
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VII
(ФРАКЦИИ)
FRACTIONS

sponded to the polar transformation products, analogous to those found in the water.

Removal of the compound was not directly connected with the moulting. Radioactivity measured in pupal exuviae appeared to be very small: it averaged 4% in the field experiment, and in the laboratory trial—1% in 4th-stage larvae. Absence of MON 585 in the pupal exuviae indicates either that it was removed from the organism before moulting, or that cuticle is not the site of activity. It is of interest that Schmialek et al. (1973) found out that the cuticle exactly is the target for the insect juvenile hormone (in the experiments with its analogue). Thus evidently there is a difference in mechanisms of action of the given development inhibitor and of the real juvenile hormone.

Distribution of ^{14}C-matter in the fractions from mosquitoes is represented in Fig. 6. The largest part (60–70%) of the radioactive substance in larvae and pupae—here available, as shown above, mostly in the initial form—was found in the fractions isolated at the rates up to 2000 rpm. In the next three fractions (4000–16000 rpm) only 11–17% were found, and about 20%—in the supernatant. Thus MON 585 in the unchanged state concentrated mainly in heavy fractions—in cell wall fragments, nucleus, etc. A distribution of the radioactive matter in subcellular fractions from the imago (containing only transformation products of MON 585) was not specific. According to Ferkovich et al. (1974), the binding of *Cecropia* juvenile hormone was the greatest on proteins of light fractions, i.e., it was of a character directly opposite to that found for MON 585.

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Fig. 6. Distribution of ^{14}C-labeled matter in subcellular fractions from *Aedes cantans:*
a—4th-stage larvae (3 d.), b—late 4th-stage larvae (4 d.), c—pupae (3 d.), d—pupae (4 d.), e—imago(22 d): Fractions (speed and time of centrifugation): I—500 rpm, 20 min; II—800 rpm, 20 min; III—2000 rpm, 10 min; IV—4000 rpm, 10 min; V—9000 rpm, 10 min; VI—16000 rpm, 30 min; VII—supernatant.