A C T A U N I V E R S I T A T I S L O D Z I E N S I S
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Ryszard Wiaderkiewicz, Zofia Walter

PULSE-POLAROGRAPHIC CHARACTERISTIC OF DNA MODIFIED BY ORGANOPHOSPHORUS INSECTICIDES IN VITRO

Pulse-polarographic investigations of DNA isolated from calf thymus and modified by organophosphorus insecticides (OI) were carried out. Following standards of OI were used: malathion (0,0-dimethyl dicarboxyethyl dithiophosphate), DDVP (0,0-dimethyl-0-dichlorovinyl phosphate), methylparathion (0,0-dimethyl-0-4-nitrophenyl tionophosphate) and methylbromphenvinphos (0,0-dimethyl-0-1-2,4-dichlorophenyl-2-bromvinyl phosphate). We found the changes of pulse-polarographic properties of double stranded (ds) DNA after incubation with OI, especially at premelting temperature. There were differences between DDVP and malathion, methylparathion and methylbromphenvinphos. The structural changes caused by the latter three compounds were very insignificant. After thermal denaturation of ds DNA previously modified by OI we observed the presence of thermolabile regions in DNA molecule.

INTRODUCTION

Organophosphorus insecticides belong to a group chemicals most widely used in agriculture. This is most due to the fact that though they are very effective as insecticides they do not last long in aquous environment, they are not accumulated in the soil and hence are not dengerous for man. The examinations of biological activities of organophosphorus insecticides (OI) indicate, however, that many of them are harmful to living or-

ganisms damaging among others genetic material of the cell. It was found that some OI are carcinogenic (trichlorfon), teratogenic (malathion), and some induce sister chromatids exchange in the cultures of human lymphocytes (malathion, methylparathion) [1, 2, 3, 4, 6]. They also show mutagenic activity similar to that of weak mutagenes and carcinogenes such as methylmethansulphate or dichlorvos (DDVP) [5]. Our earlier experiments indicated that malathion added to human lymphocytes culture apart from evoking chromosomal aberrations significantly disturbs the metabolism of nucleic acids causing among others the changes of their synthesis rate and the decrease of DNA and RNA contents [7, 8].

The mechanisms of action of OI on DNA have not been fully elucidated yet. Alkylation of DNA by OI is surely one of the steps leading to its permanent damage. The other type of action of OI on DNA cannot be excluded i.e. the action of various, often very complicated, additional groups of particular OI. Though there exist a great number of literature data concerning structural changes of DNA after the reaction with OI they are mostly limited to the presentation of the chain breaks of DNA modified by OI using different methods (mostly centrifugation in $CsCl_2$ or sucrose gradient) [9, 10, 11].

Moreover our previous paper showed that some subtle DNA damages induced by OI can also be investigated by puls-polarography method [12]. In this paper to modify DNA we used a few organophosphorus insecticides different from each other not only in respect of their additional group but the structure of their central group as well. The obtained results were to help us to determine whether the classification basing on their central group can be useful for the estimation of the harmfulness of particular OI as the agents damaging DNA in vitro.

MATERIAL AND METHODS

DNA was isolated from calf thymus by Zamenhof's method [13]. The purity of DNA was checked by estimation of RNA contents by Schneider's method [14], protein content by Lowry's method [15],

and DNA content by Burton's method [16]. Particular values were as follows: <3%, <2%, >92%.

DNA was modified by the following standards of phosphoroorganic insecticides: malathion (0,0-dimethyl/dicarboetoxyethyl/dithiophosphate), DDVP (0,0-dimethyl-0-/dichlorovinyl/phosphate), methylparathion (0,0-dimethyl-0-/4-nitrophenyl/tionophosphate) and methylbromphenvinphos (0,0-dimethyl-0-1/2,4-dichloro/phenyl-2-bromovinyl phosphate). DDVP and malathion were obtained from the Institute of Physical Chemistry Polish Academy of Science in Warsaw, methylparathion from the Institute of Applied Radiation Chemistry Technical University of Łódź and methylbromphenvinphos from the Institute of Organic Chemistry Technical University of Łódź.

DNA at the concentration of 1 mg/ml in 0.1 M buffer Tris-HCl, pH 7.4 was incubated with particular OI at the temperature 37°C. Before adding to DNA insecticides were dissolved in 0.3 ml of 50% ethanol (on 10 ml of incubation mixture). The final concentration of OI in incubation mixture was 4.5 mM. After a certain time of incubation the reaction was stopped by precipitation of DNA with two volumes of cold 96% ethanol. The DNA precipitate was dissolved in SSC/10 and dialysed against SSC/10 for 20h. In experiments with denaturated DNA the reaction was stooped by separation of the modyfing agent on Sephadex G-25 columns or by precipitation of ss DNA with cold 96% ethanol plus 0.075 M $MgCl_2$.

All measurements were carried out by differential pulse-polarography (DPP) method with an apparatus A 3100 Southern-Harwell Pulse Polarograph (Southern Analytical Ltd.) using mercury dropping electrode. DPP experiments were performed in a nitrogen atmosphere at the following settings: 1V in 15 min. amplitude 50 mV, delay time 2 sec., amplifier sensivity 1/8 to 1/64 dependently cn DNA concentration. 0.3 M ammonium formate with 0.1 M sodium phosphate pH 6.9 or 5.0 was used as a background electrolyte. The principles and details of the polarographic measurements were described by P a l e c e k et al. [17, 18, 19]. Thermal denaturation was carried out by heating DNA samples in SSC/10 to 100°C for 10 min., followed by quick cooling in an ice bath. Alkaline denaturation of DNA was carried

out by mixing 0.5 ml DNA in SSC/10 with 0.5 ml 0.2 M NaOH; after 5 min. the solution was neutralized with 1 M KH_2PO_4. Premelting changes of DNA were performed by measuring the hight of peak II during heating the modified DNA from 20°C to 60°C.

RESULTS AND DISCUSSION

The results obtained in this paper confirmed our earlier reports that organophosphorus insecticides react with DNA in vitro causing the damages of DNA native structure [12]. As we used OI from three different classes it was possible to draw conclusions regarding the supposed mechanism of action of OI with DNA.

The first changes of pulse-polarographic properties of ds DNA are already observed a few hours after the incubation with OI. The amount and character of these changes depend on the kind of insecticides. DDVP yields a small amount of ss DNA (peak III) after a few hours of incubation while in case of other OI it is possible to detect analogous amount of ss DNA only after 36 hours incubation. Earlier changes can be seen in peak II. This peak is formed at about 1.45 V i.e. 70 mV more positive than peak III characteristic for ss DNA. Peak II is characteristic for ds DNA and is caused by minor local disturbances of helical structure of DNA [18, 19]. The decrease of this peak a few hours after the reaction with methylparathion, malathion and methylbromphenvinphos can indicate the preferentialy action of the above mentioned compounds with these very regions. Similar effects were observed during the reaction of DNA with sodium bisulfate which selectively induces the changes consisting in deamination of polarographically active cytosine to nonreducible uracyl [20, 21]. It seems that in case of DNA modified by OI the decrease of peak II following alkylation is not only caused by the direct decrease of te amounts of nitrogen bases residues subject to polarographic reduction but also by other disturbances effecting indirectly the DNA reduction on electrode. They may among others consist in the local changes of the charge density in polynucleotide chain and thus in-

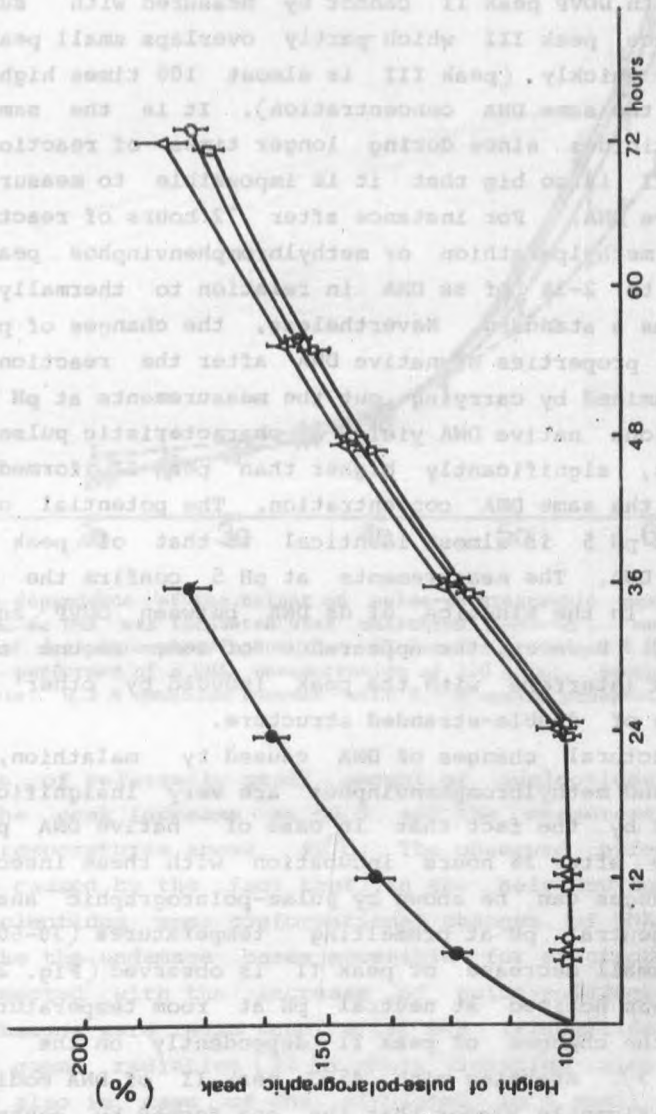

Fig. 1. Time course of the reaction of double-stranded DNA with malathion Δ——Δ, methylparathion ○——○, methylbromphenvinphos □——□, and DDVP ●——● as measured by means of DDP in 0.3 M ammonium formate with 0.1 M acetate buffer pH 5. DNA concentration was 200 ug/ml. The peak height of the control sample was taken

fluence the adsorption of a given polinucleotide chain and its orientation in the relation to negatively charged electrode, and in turn they can inhibit electroreduction of potentialy polarographically active bases residues. In case of DNA after the reaction with DDVP peak II cannot by measured with sufficient occuracy since peak III which partly overlaps small peak II is formed very quickly (peak III is almost 100 times higher than peak II at the same DNA concentration). It is the same with other insecticides since during longer times of reaction with ds DNA peak III is so big that it is impossible to measure peak II of native DNA. For instance after 72 hours of reaction with malathion, methylparathion or methylbromphenvinphos peak III corresponds to 2-3% of ss DNA in relation to thermally denaturated DNA as a standard. Nevertheless, the changes of pulse-polarographic properties of native DNA after the reaction with OI can be examined by carrying out the measurements at pH 5. Under such conditions native DNA yields a characteristic pulse-polarographic peak, significantly higher than peak II formed in neutral pH at the same DNA concentration. The potential of native DNA peak at pH 5 is almost identical to that of peak III of denaturated DNA. The measurements at pH 5 confirm the observed differences in the kinetics of ds DNA between DDVP and other OI (Fig. 1). However, the appearance of some amount of ss DNA at pH 5 can interfere with the peak induced by other possible disturbances of double-stranded structure.

The structural changes of DNA caused by malathion, methylparathion and methylbromphenvinphos are very insignificant. It is confirmed by the fact that in case of native DNA peak III appears only after 36 hours incubation with these insecticides. Earlier changes can be shown by pulse-polarographic analysis of ds DNA in neutral pH at premelting temperatures (30-60^{o}C). Up to 35^{o}C a small decrease of peak II is observed (Fig. 2). Similar effect was noticed at neutral pH at room temperature while analysing the changes of peak II dependently on the reaction time (Fig. 3). Starting with 45^{o}C peak II of DNA modified by OI is significantly higher than the one formed by control DNA (Fig. 2). It seems that the presented differences are due to

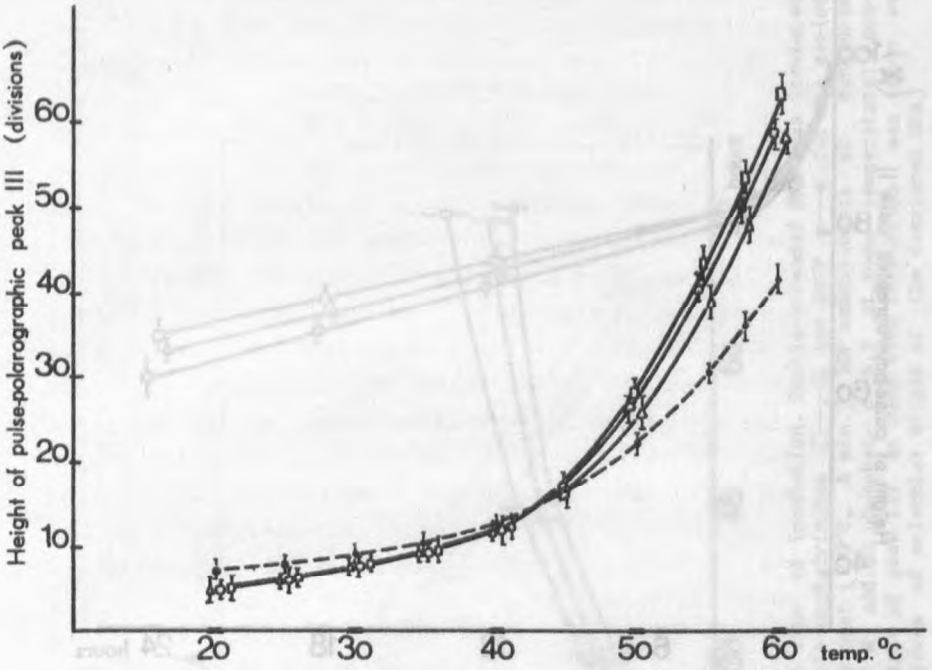

Fig. 2. The dependence of the height of pulse-polarographic peak II on the temperature. ds DNA was incubated with malathion Δ——Δ, methylparathion o——o, and methylbromphenvinphos □——□ for the period of 6 h. DDP measurements were performed of a DNA concentration of 200 μg/ml. Background electrolyte: 0.3 M ammonium formate with 0.1 M sodium phosphate pH 6.9

alkylation of relatively small amount of nucleotides both in case of the peak increase at pH 5 and the measurements at premelting temperatures above 40°C. The observed effect is most probably caused by the fact that in the neighbourhood of modified nucleotides some conformational changes of DNA take place which make the undamage bases accesible for electrode processes. It is connected with the increase of pulse-polarographic peak. Similar changes were also seen after DNA irradiation with small doses of gamma radiation [22], short digestion with DNase I [23] and also in case of DNA alkylated to a small extent by MNU [24].

Pulse-polarography measurements carried out after thermal denaturation of ds DNA previously modified by OI indicate the

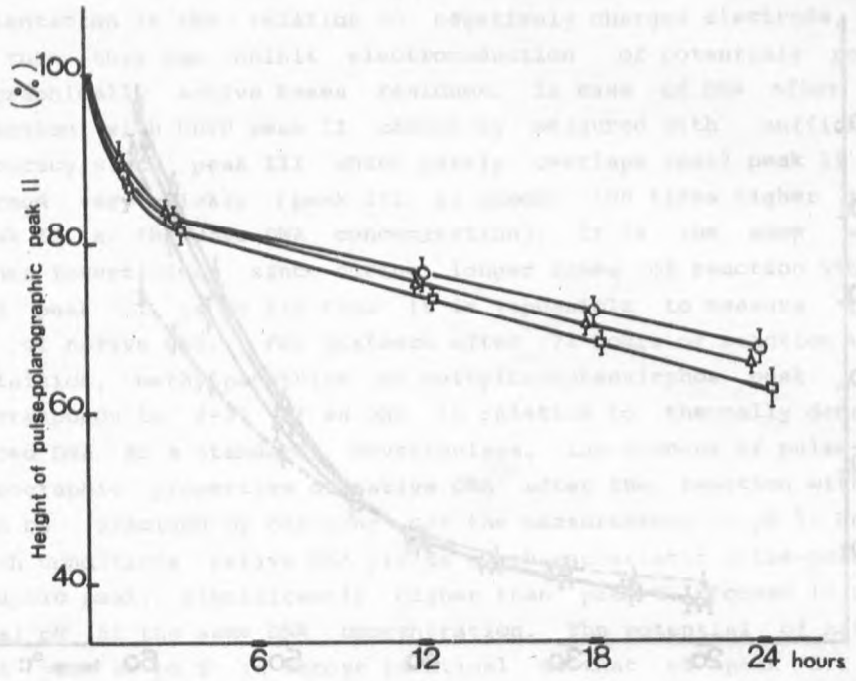

Fig. 3. Time course of the reaction of native DNA with malathion Δ——Δ, methylparathion o——o and methylbromphenvinphos □——□ as measured by means of DDP. DNA concentration 400 μg/ml. Background electrolyte: 0.3 M ammonium formate with 0.1 M sodium phosphate pH 6.9. The peak height of the control samples was taken as 100%

presence of thermolabile regions in DNA molecule (Fig. 4) while under analogous conditions of measurements in alkaline solution such regions were not found. The dependence of peak III of thermally denaturated DNA on the incubation time with OI (Fig. 2) is similar to that obtained for ds DNA not subject to thermal denaturation (Fig. 1, 5). This fact suggests the existence of direct dependence between the formation of a small amount of ss segments (about 2%) in ds DNA and the presence of thermolabile regions in molecule.

There are very few reports concerning the chemical nature or conformational changes induced by OI in DNA. It is likely that guanine alkylation by OI may lead to the appearance of ss

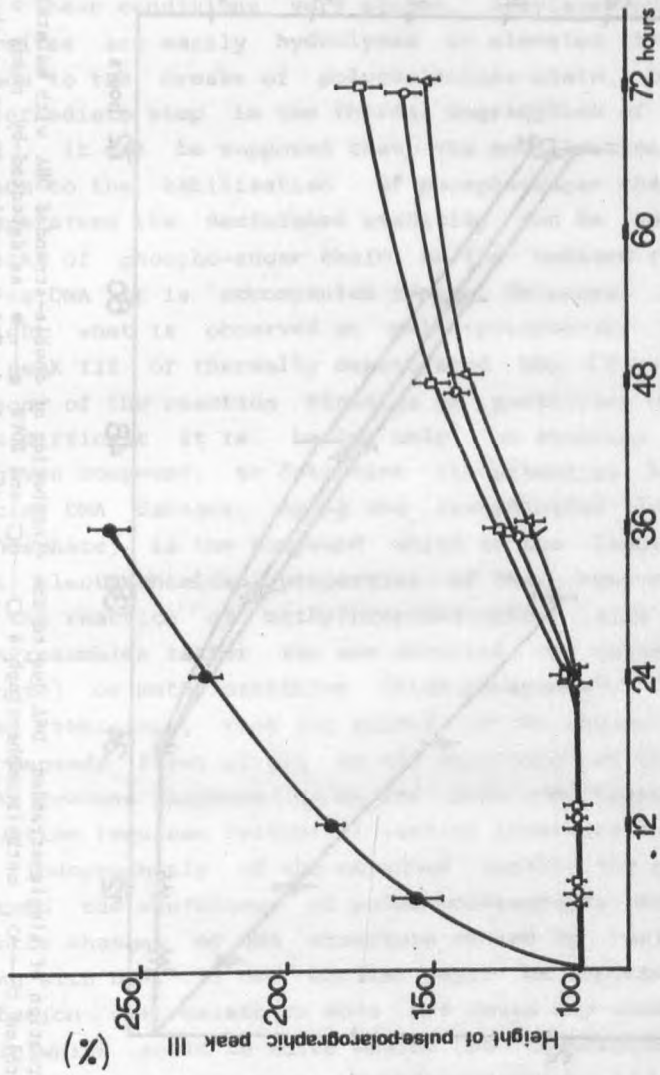

Fig. 4. The dependence of the height of peak III on the time of incubation. Double-stranded DNA was incubated with malathion Δ——Δ, methylparathion ○——○, methylbromphenvinphos □——□ and DDVP ●——● for the period indicated in the graph and subsequently denaturated by heat (100°C, 6 min.). DDP measurements of denatured DNA were performed in 0.3 M ammonium formate with 0.1 M sodium phosphate ph 6.9 at room temperature: concentration of the denatured DNA was 25 μg/ml. The height of peak III at zero time incubation was taken as 100% (peak heigher than 100% indicates a decrease of molecular weight of the denatured DNA)

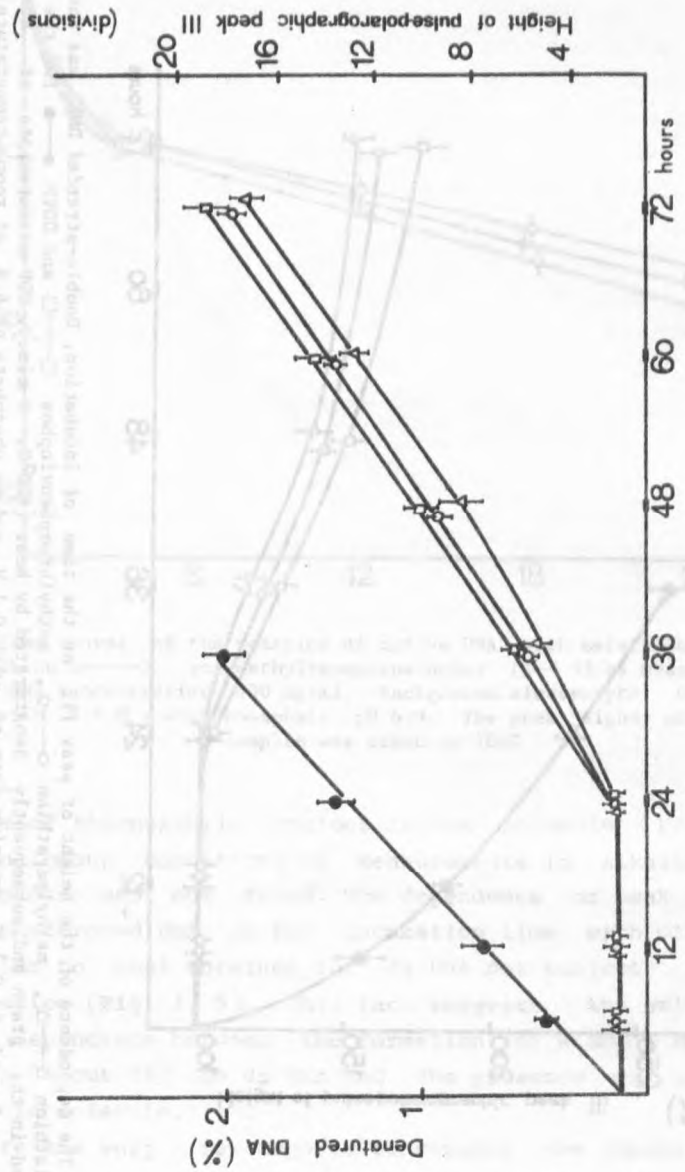

Fig. 5. Formation of single-stranded DNA due to the incubation of double-stranded DNA with malathion Δ——Δ, methylparathion ○——○, methylbromphenvinphos □——□ and DDVP ●——● as detected by means of DDP. DDP measurements were performed at a DNA concentration of 400 µg/ml in 0.3 M ammonium formate with 0.1 M sodium phosphate pH 6.9

regions in the neighbourhood of modified bases similar to the reaction on DNA with N-acetoxy-N-2-acetylaminofluoren [25, 26]. Many papers state that depurinated DNA is rather easily hydrolyzed in alkaline solutions while alkylated DNA itself is under these conditions very stable. Alkylated purines and apurine sites are easily hydrolyzed at elevated temperature what leads to the breaks of polynucleotide chain. Depurination is an intermediate step in the thermal degradation of alkylated DNA [27]. It can be supposed that the modification of DNA by OI leads to the labilisation of phospho-sugar chain. At elevated temperature the deminished stability can be seen due to the breaks of phospho-sugar chain at the damaged regions. In case of ss DNA it is accompanied by the decrease of its molecular weight what is observed on pulse-polarograms as the increase of peak III of thermally denaturated DNA (Fig. 2). The comparison of the reaction kinetics of particular OI with DNA shows how difficult it is, basing only on chemical composition of a given compound, to determine its potential harmfulness in inducing DNA damages. Among the investigated insecticides DDVP (phosphate) is the compound which to the largest extent effects the electrochemical properties of DNA. However, the kinetics of the reaction of methylbrophenvinphos (also phosphate) with DNA resambles rather the one obtained for malathion (dithiophosphate) or methylparathion (tionophosphate). It seems improbable, therefore, thet the extent of DNA damages by particular OI depends first of all on the structure of their central group. This process appears to be far more complicated and its elucidation requires further intensive investigations.

Independently of the obtained results the present paper confirmed the usefulness of pulse-polarography method in examining subtle changes of DNA structure evoked by various factors reacting with DNA. In our earlier paper we reported that 72h incubation with malathion dose not cause any changes in DNA structure which could be noted using the temperature melting curve method or fractionation on hydroxyapatite [12]. Using pulse-polarography method we found significant changes of electrochemical properties of DNA oven after shorter time of reaction.

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Department of Molecular Genetics
University of Łódź, Poland

Ryszard Wiaderkiewicz, Zofia Walter

CHARAKTERYSTYKA PULS-POLAROGRAFICZNA DNA
ZMODYFIKOWANEGO INSEKTYCYDAMI FOSFOROORGANICZNYMI IN VITRO

Przeprowadzono badania metodą puls-polarograficzną DNA izolowanego z grasicy cielęcia i zmodyfikowanego związkami fosforoorganicznymi. Zbadano wpływ następujących związków: malationu (0,0-dimetylo-S-1-2-dikarboetoksyetyloditiofosforanu), DDVP (0,0-dimetylo-0-1-2-dichlorowinylofosforanu), metyloparationu (0,0-dimetylo-0-4-nitrofenylotionofosforanu) i metylobromfenwinfosu (0,0-dimetylo-0-1-2,4-dichlorofenylo-2-bromowinylofosforanu). Zaobserwowano zmiany własności pulspolarograficznych po inkubacji dwuniciowego DNA ze związkami fosforoorganicznymi. Zmiany dotyczyły szczytu II i III. Szczyt II jest charakterystyczny dla dwuniciowego DNA i jest wywołany niewielkimi zaburzeniami helikalnej struktury. Szczyt III jest charakterystyczny dla jednoniciowego DNA. Po 72-godzinnej inkubacji z malationem, metyloparationem lub metylobromfenwinfosem szczyt III odpowiadał 2-3% zawartości jednoniciowego DNA w odniesieniu do zdenaturowanego termicznie DNA jako wzorca. Pomiary w pH 5 wykazały

różnice w kinetyce zmian dwuniciowego DNA między DDVP i innymi badanymi insektycydami. Zmiany strukturalne DNA wywołane przez malation, metyloparation i metylobromfenwinfos były bardzo nieznaczne. Obserwowano zmiany w zakresie temperatur przedtopnieniowych (30-60°C). Polarograficzne pomiary przeprowadzone po termicznej denaturacji dwuniciowego DNA, zmodyfikowanego uprzednio związkami fosforoorganicznymi, wykazywały obecność regionów termolabilnych.