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OUR INVESTIGATIONS IN THE FIELD OF PESTICIDE TOXICOLOGY, WITH PARTICULAR REFERENCE TO ANTICHOLINESTERASE COMPOUNDS

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Based on a selection of work of the Institute in the field of toxicology of pesticides, the review deals with the development of methods, the toxicology of organophosphorus compounds, the study of the reactivators of the inhibited cholinesterase, carbamate toxicology, and field investigations. This work has been done either from the point of view of fundamental research or with a view to promoting the health protection of people exposed to pesticides.

First steps in the field of pesticide toxicology made at the Institute about 14 years ago were at the same time the beginning of sistematic research in experimental toxicology in Yugoslavia. The development of this relatively recent branch in experimental medicine in this country has been associated, especially in its first stage, with a close collaboration of our Institute with the Toxicology Research Unit of Medical Research Council at Carshalton. The support received from the World Health Organization, Geneva, in the way of fellowships and laboratory equipment, as well as the assistance received from the Commission for Medical Research, Belgrade, were also of great significance at that stage.

Today there are about 10 specialists at the Institute working either full-time or part-time on pesticide toxicology. Their activities relating to the analysis and detection of pesticides, kinetic biochemical studies, toxicity studies and investigations into the mode of action of pesticides in mammals, the therapeutic effect of antidotes, and the problems of the safe use of pesticides have been published in more than 100 articles. Although it will be possible in this paper to review only a fraction of the published articles, it is hoped it will none the less adequately illustrate our activity during the last 14 years.

1. DEVELOPMENT OF METHODS

The skin is considered the most important route of entry of a pesticide in the course of occupational exposure, and consequently dermal toxicity data on a compound are a better indicator of its potential hazard than those obtained by other routes of application. However, experimental procedures for evaluating dermal toxicity are not standardized and data obtained in different laboratories differ markedly. In this connection we have investigated the effect of the preparation of the rat skin area and the effect of the way of dermal application on the toxicity of some organophosphorus compounds. The removal of the fatty layer from the clipped skin 24 hours before the application of poison did not influence the parathion toxicity. The covering of the contaminated area by a polyethylene sheet or leukoplast diminished, however, the toxicity of parathion 2 and 3.5 times, respectively. In these experiments the possibility of ingestion of the poison was excluded (1). We hawe also found that the rate of absorption of the poison through the skin depends on the size of the contaminated area and the concentration of the poison. The penetration rate of paraoxon was estimated on the basis of the blood cholinesterase inhibition (2). By comparing blood cholinesterase inhibition in rats after percutaneous application with that after intravenous infusion the actual penetration rate of the poison could be determined. The rate of absorption of paraoxon from a 4 cm^2 surface corresponded to the rate of the i.v. infusion from 50–75 μg/kg/h, while the absorption rate of the same dose of poison from an area 4 times larger to the infusion rate of 100 – 150 μg/kg/h (3).

Since it is possible by intravenous infusion to imitate, to a certain extent, the entry of a poison through the skin or lungs, with the advantage that the exact amount of the poison which entered the circulation is known at every moment throughout the infusion period, the method for cannulating the rat's jugular vein has been developed. This method is also employed when repeated sampling of the rat's blood is required (4).

The determination of the degree of exposure to anticholinesterase insecticides is of great importance in protecting the workers from potential hazards in handling these materials. Thus, part of our activities was focused on the development of a suitable field method for measuring blood cholinesterase activity, especially in persons exposed to insecticidal carbamates (5, 6, 7, 8).

A method has also been developed for the determination of the free inhibitor in the circulating blood. By measuring the effect of a given amount of the plasma obtained from a poisoned animal on the erythrocyte cholinesterase *in vitro* the information on the inhibitor persistence in the body may be obtained.

2. ORGANOPHOSPHORUS COMPOUNDS

The beginning of our studies on the toxic properties of organophosphorus insecticides coincided with a rapid increase in the use of these compounds in Yugoslavia. Apart from a number of minor studies associated with the compounds being introduced into practice, more detailed studies were carried out on the isomers of Systox and Metasystox and their derivatives. The aim of these investigation was to find out the cause of discrepancy between *in vivo* and *in vitro* recovery rates of the inhibited enzyme.Using a group of dimethylphosphate inhibitors it was found that those compounds that persisted in the body either in their original form or as active metabolites produced the almost completely irreversibly inhibited enzyme, whereas the compounds that did not persist gave the inhibited enyzme which recovered spontaneously relatively rapidly. Using a prolonged intravenous infusion in order to maintain a steady level in the blood of a normally nonpersistent inhibitor (methylparaoxon) the effect was the same as that obtained with a single injection of a persistent inhibitor. These experiments have clearly shown that the persistence is an important variable upon which the character of the biological effect of dimethylphosphate esters depends. Consequently, the LD_{50} doses of two compounds with a different persistence, although equilethal by definition, cannot be regarded as equitoxic; the animals surviving the LD_{50} will be more susceptible to future exposure to poison in the case of a persistent inhibitor which yields the irreversibly inhibited enzyme (9).

In the study of the toxic properties of Systox, Metasystox and related compounds, an increase in the toxicity of some of the compounds was observed after dilution with water or during the storage of undiluted samples. It has been shown that the increase in toxicity is associated with the formation of the corresponding sulphonium derivative caused by the autoalkylation process. It has also been found that the sulphonium derivates injected intravenously are 100 to 1000 times more toxic than the parent compounds, and that their anticholinesterase activity is about 1000 times greater (10, 11).

A pronounced anesthetic effect was observed in animals injected intravenously with sublethal doses of purified isomers of Metasystox. In connection with this phenomenon other organophosphorus compounds of low anticholinesterase activity were investigated as well as those with no anticholinesterase activity whatsoever. On the grounds of the results obtained we could conclude that the organophosphorus compounds with a low anticholinesterase activity produce a pronounced anesthetic effect, while those which are more powerful cholinesterase inhibitors cannot produce such an effect, since the dose required to induce anesthesia would be well above the lethal dose (12).

Studies on the specificity of the enzymes hydrolising organophosphorus compounds were performed employing human sera. It has been found that human sera contain at least two enzymes that hydrolise

armin and paraoxon. These enzymes are associated with globulin and albumin fraction. The globulin fraction hydrolises armin and paraoxon at a much slower rate than the albumin fraction. The enzymes of these two fractions differ also in their thermal stability: the enzyme present in the albumin fraction is much more stable than the enzyme in the globulin fraction (13).

3. REACTIVATORS OF PHOSPHORYLATED CHOLINESTERASE

Our studies on the therapeutic effect of antidotes in organophosphorus poisoning started in 1957, when the protective action of pralidoxime, diacetyl monoxime and monoisonitrosoacetone was compared. Only pralidoxime was found to be effective, increasing the intravenous LD_{50} value of parathion in mice and rats about 2 and 3 times, respectively. Monoisonitrosoacetone produced no therapeutic effect, while diacetyl monoxime, on the other hand even enhanced the toxicity of parathion (14). The synergetic toxic effect of diacetyl monoxime was studied in more detail by determining the brain cholinesterase activity in poisoned animals. While diacetyl monoxime injected alone had no effect on the enzyme activity, and a given dose of injected parathion produced only a slight inhibition, the same dose of parathion applied simultaneously with diacetyl monoxime produced a marked depression in cholinesterase activity. It was shown by experiments *in vitro* that this synergetic effect might be contributed to the accelerated conversion of parathion into paraoxon in the presence of diacetyl monoxime.

The therapeutic effect of pralidoxime observed in smaller laboratory animals was then tested in parathion poisoned rabbits, dogs, and horses. In these experiments it was possible to determine cholinesterase activity in poisoned animals by taking blood samples at frequent intervals. Pralidoxime produced reactivation of dictylphosphorylated erythrocyte cholinesterase in the horse even 24 hours after the enzyme had been inhibited, which means that within this period of time the ageing phenomenon did not occur to an appreciable extent. At the same time it was found necessary to repeat the oxime application, since – due to the relatively slow conversion of parathion into the active inhibitor neither the improved clinical signs nor the reactivation of erythrocyte cholinesterase could be considered definite (15). The experiments show clearly how important is to know the biotransformation of the antidote in relation to persistence of the poison and/or his active metabolites.

In a further series of experiments the biological effects of some new synthetized oximes, derivatives of pyridinium–4–aldoxime were tested and the results obtained were compared with the action of pyridinium –6–aldoxime methiodide and pralidoxime. The inhibitory power of individual oximes against cholinesterase varied significantly and their acute toxicity differed accordingly. The differences in the reactivatory effect on diethylphosphorylated cholinesterase *in vitro* were found too;

pralidoxime showed the most pronounced reactivatory properties. The difference in the reactivatory power *in vitro* was also reflected in their protective action *in vivo*. The simultaneous application of oximes and atropine resulted in a much greater therapeutic effect in parathion-poisoned rats. Studying the oxime distribution in these experiments no measurable quantities of oximes were found in the rat brain. Determining the persistence of oximes in the liver, muscle and blood of the rat, we concluded that the pronounced therapeutic value of pralidoxime in relation to other oximes is based on a prolonged persistence of this compound in the blood and muscle (16, 17, 18, 19, 20).

In an extensive study *in vitro* the kinetics of reactivation of ethyl–ethoxyphosphorylated cholinesterase by some dioximes was investigated. It has been found that the all-over reaction does not follow the second-order kinetics but the equation which postulates the inhibition of free cholinesterase by the phosphorylated oxime (21).

4. CARBAMATES

The study of the toxilogy of carbamates initiated in 1961 covers a series of monomethyl – and a few dimethyl derivatives. Most of these compounds have been included in the WHO programme of the evaluation of new insecticides. Some of them e. g. carbaryl, arprocarb and carbamult have already found a wide use in agriculture and public health. Investigating the biological effects of carbamates we have almost regularly compared their properties with those of organophosphorus compounds. In spite of the fact that both groups of compounds are characterized by producing the same biochemical lesion, the differences between them are of great importance when assessing the risk deriving from exposure to these compounds.

The inhibition of erythrocyte and serum cholinesterase by monomethyl- and dimethyl carbamates follows the kinetics of a bimolecular reaction with one component, the inhibitor, in excess (22, 23). Because of a spontaneous hydrolysis of carbamoylated enzyme the study state is reached between the free and inhibited cholinesterase. From the equilibrium constant at the steady state and the inhibition rate constant the spontaneous reactivation rate constant can be calculated. The rate constant of the spontaneous reactivation which presents the turn-over number is in the order of magnitude of 10^{-2} min^{-1} for erythrocyte cholinesterase and 10^{-3} min^{-1} for serum cholinesterase.

Measuring the influence of pH on the acylation of erythrocyte cholinesterase by phosphates and carbamates it has been confirmed that only one group of the enzyme is needed for acylation with pK 10.25 and that the group of the enzyme having pK 6,2 takes part only in the binding of the positively charged compounds. Two groups of the enzyme take part during the deacylation of erythrocyte cholinesterase and pH functions for carbamates and organophospates are equal (24).

Only one group of the enzyme (pK 7.7) is needed for the acylation of serum cholisterase regardles of whether carbamates or phosphates or uncharged and charged compounds are involved in the reaction. In was impossible to determine the number of the groups of the enzyme that take part in the deacylation of serum cholinesterase: pH functions for dephosphorylation differ from pH functions for decarbamoylation (25).

On the basis of some theoretical postulates the medium doses producing initial symptoms (ED_{50}) and medium lethal doses (LD_{50}) were compared of five monomethyl carbamates and two organophosphorus compounds after intravenous and intramuscular application to rats. Regardless of the route of application of the poison the LD_{50}/ED_{50} ratio of monomethyl carbamates was far greater than that of organophosphorus compounds. This is in agreement with the difference in the kinetics of cholinesterase inhibition of these two groups of compounds. The experimental results have also indicated that the ED_{50} value assessment can provide useful quantitative data for comparing the toxicity of new anticholinesterase compounds (26). Continuing the study of the tolerance of three monomenthyl carbamates at various rates of slow intravenous infusion was examined. While a reduction of the rate of infusion was followed by a significant increase in tolerance regarding the lethal dose, the first noticeable symptoms appeared after the same quantity of the poison applied, regardless of the rate of infusion. For all the carbamates tested the ratio between the lethal doses and the doses producing the first noticeable symptoms amounted, at the slower infusion rates, to 50. In similar experiment with paraoxon this ratio remained consistent no matter what the infusion rate was, while the first symptoms were noticed only after half the lethal dose was injected (27). The conclusion drawn from these results was that – in contrast to organophosphorus compounds – the first symptoms of poisoning after occupational overexposure to carbamates might be expected long before a lethal dose was absorbed.

A comparative toxicity of 10 monomenthyl carbamates as well as the study of the persistence of the inhifitor after the intravenous application of equitoxic doses to rats has shown evident differences in the character and duration of cholinergic symptoms. The observed differences of the duration of symptoms were consistent with the results of the inhibitor persistence determation in the blood (28). Comparing the inhibitory power (I^{50}) and intravenous toxicity (LD^{50}) of carbamates the correlation coefficient was found to be 0.89 which suggests that the acute toxicity of carbamates corresponds to their anticholinesteratic activity and that the toxicity of these compounds may be predicted on the basis of their inhibitory power *in vitro* (29).

Comparing the symptoms on the one hand and brain and plasma cholinesterase activity in rats poisoned by arprocarb on the other an evident correlation has been found, by which the brain cholinesterase appears as a rule, more inhibited than the plasma cholinesterase (30).

The *in vitro* experiments on human blood have shown that some carbamates (e. g. carbaryl and arprocarb) possess a greater affinity for erythrocyte cholisterase than for plasma, the others (e. g. carbamult), however, inhibit both enzymes equally. For this reason the characteristics of compound should be taken into consideration when selecting the method for the assessment of exposure to a given carbamate. The study on volonteers, as well as field experience have confirmed the erythrocyte cholinesterase assay being a far more sensitive index for the assessment of exposure to arprocarb (32).

5. FIELD STUDIES AND OTHER CONTRIBUTIONS TO THE SAFE USE OF PESTICIDES

In the last five years the specialists at the Institute have repeatedly taken part in the field trials of new insecticides covered by the WHO Programmes. Their goal has been to evaluate the degree of safety in the use of insecticides for exposed workers and villagers by using clinical and laboratory methods. At the same time these trials were used for the evaluation of methods for measuring the exposure of a given insecticide as well as the effect and applicability of various protective measures in unfavorable climate conditions (33, 34, 35). A similar evaluation of the exposure of workers to an organophosphorus insecticide has recently been carried out in a chemical factory (36).

The contribution of the Institute's staff in the field of the safe use of pesticides in this country has been given in a series of review articles relating to methods for cholinesterase activity measurements (37, 28, 39), clinical manifestations and therapy of pesticide poisoning (40, 41, 42, 43, 44, 45, 46), and protection of agricultural workers exposed to pesticides (47).

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