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METHODS OF MEASURING EXPOSURE TO ANTICHOLINESTERASE INSECTICIDES

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Many methods for cholinesterase activity determination have been suggested and compared, but only a few of them have been found suitable for the routine monitoring of occupational exposure to anticholinesterase compounds. In the present paper the factors determining the choice and applicability of a method are discussed with particular reference to the differences between organophosphorus and carbamate insecticides.

Some results of own studies on volunteers and workers exposed to anticholinesterases are also presented.

The sequelae of the widespread use of pesticides can be classified into two categories: (1) toxicological effects being reflected in morbidity and mortality rates, and (2) storage of some compounds as expressed by the presence of residues in the environment, food of plant and animal origin and in human body itself. The former effects are definitely injurious, the latter are still the subject of many controversies.

The integral pesticide safety programme includes the knowledge of the actual situation, the legislation adequate for the country, effective control and fiscalisation, education and permanent monitoring of exposure.

The monitoring studies may include occupationally exposed workers in manufacture, formulation and field application of pesticides, as well as the general population. However, the most useful data on the effects of pesticide exposure can be obtained by scheduled trials on volunteers or from clinical case reports of accidental poisonings. Each group of the above mentioned studies does not give an all-round picture, but all together may provide sufficient data on the matter.

The exposure to pesticides may be measured *directly*, by means of absorbant pads and clothing, washes and similar techniques. The amount of the chemical trapped or removed is then a direct measure of the exposure. The results of direct measurements of exposure may be of help

in evaluating the relative hazards of different working procedures, protective devices and different routes of poison absorption. Total occupational exposure may also be estimated from direct measurement of the concentration of pesticides in the air.

The *indirect* methods of measurement include the detection of the pesticide compounds or their metabolites in body tissue or excreta, or the registration of some biochemical changes caused by the absorbed product. The fact that these changes may be discovered before the appearance of clinical effects offers the chance of preventing the over-exposure and accounts for a more accurate diagnosis. Thus, the data obtained by indirect measurements are preferentially used as indices of dosage at tissue level giving, as a rule, a reasonable correlation with the clinical picture of poisoning.

Until recently the determination of cholinesterase activity was used more than any other method for measuring the exposure to anticholinesterase pesticides, organophosphates and carbamates. It was feasible indeed to measure *p*-nitrophenol in the urine of people exposed to parathion, methyl-parathion and EPN, as well as metabolites, such as phosphorus, of a limited number of organophosphorus (OP) compounds. Undoubtedly the method developed by *Shafik* and *Enos* (1), by which the metabolites of the most common OP insecticides can be measured, permits the large scale monitoring of exposure to these insecticides. The metabolites of at least some carbamates can also be measured in the serum and urine of occupationally exposed persons (2). Regardless of these developments in analytical techniques, the cholinesterase activity determinations will continue to be used as a valuable index of exposure at least to organophosphorus insecticides.

It is generally accepted that the toxic action of *OP compounds* is closely linked with their ability to inhibit cholinesterases and thus to interfere with the proper functioning of the nervous system by allowing acetylcholine to accumulate. Their toxicity, however, depends on a number of factors, each of which may play a relatively different role in insects and mammals and so determine the selectivity of the compound. Such factors are: (1) the conversion of the original compound into an active inhibitor, (2) the relative affinity of inhibitor for different cholinesterases, (3) the speed of reactivation of the inhibited enzyme and (4) the hydrolysis of the inhibitor by independent enzyme systems.

The remarkably low toxicity of some OP compounds (e.g. bromophos, malathion, ronnel) may be explained by a combination of the slow rate of oxidation of these compounds to the active inhibitor and the relatively rapid recovery of inhibited enzyme. Consequently the rate of reversal of inhibition may keep pace with fresh inhibition by the newly formed inhibitor.

Although according to their action *carbamates* belong to the group of anticholinesterase insecticides, their properties differ markedly from those of OP compounds. All monomethyl carbamates have been shown

so far to be »direct« inhibitors of cholinesterase. The spontaneous reactivation of carbamylated enzyme is much more rapid than that observed in the phosphorylated cholinesterases; thus the cumulative inhibitory effects on cholinesterase due to daily exposure to insecticidal carbamates would be extremely improbable. Rapid and complete recovery from illness is an outstanding feature of carbamate poisoning. Accordingly, routine cholinesterase determination has little or no practical value in assessing over-exposure to some carbamate insecticides (3). Furthermore, there is another important characteristic of this group of anticholinesterases which distinguishes them from OP compounds. In the case of over-exposure to carbamates the incapacitating symptoms (headache or nausea) would develop rapidly and prevent further exposure by stopping the operator working long before a dangerous dose had been absorbed. Owing to the existence of an »early warning system«, no severe poisonings after occupational exposure to carbamates have been reported (4).

Cholinesterase activity determination can be used as an index of absorption of anticholinesterases, but the interpretation of enzyme depression is complex and requires more information. The analysis of OP compounds or their metabolites in blood and urine will give a different kind of information than the measurement of cholinesterase activity, because excretion occurs rather rapidly, while enzyme activity recovers more slowly. Thus, the finding of a compound or metabolite indicates a very recent exposure, while the fall in enzyme activity, especially that of erythrocytes, represents the sum of the physiological effects of repeated exposure lasting sometimes for weeks or months.

Although the measurement of cholinesterase activity may give an indication of the dose of the inhibitor absorbed, it can not be used alone to predict the clinical sequence of events. A sudden slight enzyme depression is often associated with the moderate illness, while a gradual severe depression may be compatible with good health because the body has been adapted to the high levels of the accumulated acetylcholine. Therefore both, the degree and rate of enzyme depression should be taken into account. In almost all instances severe poisoning by an OP insecticide is accompanied by severe depression of blood cholinesterases, especially of that of the plasma. On the other hand, some widely used carbamate insecticides such as propoxur, show much greater activity against erythrocyte cholinesterase (5). This is relevant when making a decision about the type of cholinesterase to be measured and accordingly the choice of the adequate method (6).

The great distribution range of normal cholinesterase activity values, from one person to another (Fig. 1) (7), as well as the significant genetic differences between coloured, asiatic and white populations (8) makes the individual preexposure enzyme activity determination extremely important. The WHO Expert Committee on Pesticides (9) suggested that spray operators be withdrawn from the work if their blood cholinestera-

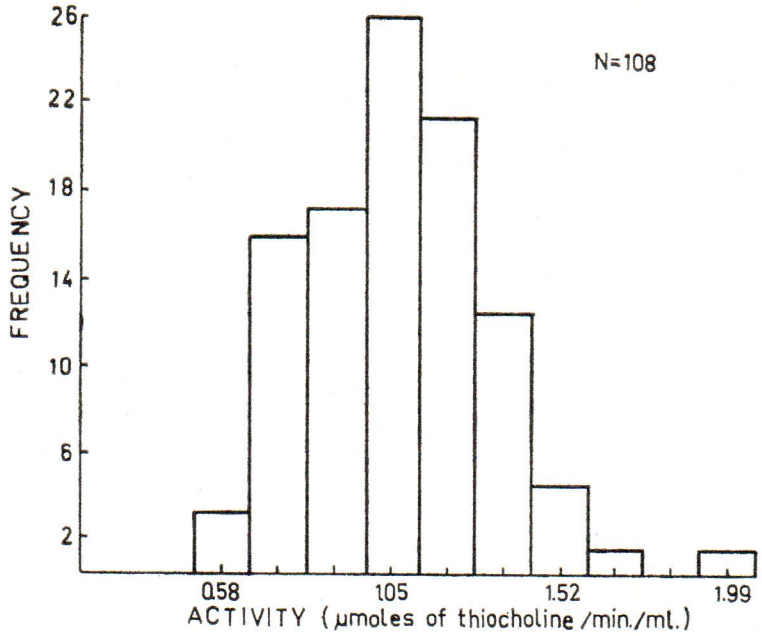


Fig. 1. *Distribution of plasma cholinesterase activity in 108 healthy persons (7)*

se activity decreases by 50% from well established preexposure value. It also seems advisable that workers who show the lowered enzyme activity from the beginning should not be recruited for the work in which the exposure is likely to occur.

The measurement of blood cholinesterases in the course of routine surveillance of individuals exposed to anticholinesterase insecticides may give the misleading results due to changes which occurred in the interval between sampling and measurement. The inhibitor present in the sample may favor the subsequent inhibition or the enzyme in the non-aged state of inhibition may show the spontaneous recovery. On the other hand, no change in cholinesterase activity was observed in blood samples of persons poisoned by OP compounds when stored dry at the temperature below 4°C (10). According to the *in vitro* findings of *Wilhelm* and *Reiner* (11), it is presumed that dilution of 1:300 into buffer of pH 5 and storage at 4°C is necessary to keep the cholinesterase activity unchanged in samples obtained from persons exposed to monomethyl carbamate insecticides.

Many methods for cholinesterase determination have been developed and critically evaluated, but only few of them have been found appropriate for the field purposes. *Holmstedt* reviewed briefly the most common groups according to the principles on which they are based (12).

In Table 1 is presented the process of acetylcholine decomposition due to the action of cholinesterase on alcohol and acetic acid as well as the methods based on the determination of the remaining substrate (acetylcholine or other esters) i.e. of the substrate which was left unreacted after incubation with the enzyme. The activity measured with these methods is calculated from the difference in the amount of the substrate at the beginning of the reaction and at the end of a fixed time interval.

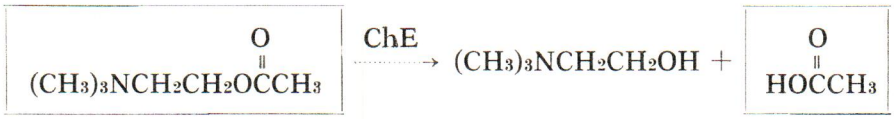
In the same figure are presented the methods based on acid formation by enzyme hydrolysis of acetylcholine or other choline esters.

Two methods *(Winteringham's* and *Reed's)* are based on the radiometric determination of cholinesterase activity, with radioactive acetylcholine as substrate.

In Table 2 presented by the same equation are the methods based on alcohol formation. They are used to measure the amount of alcohol (or thioalcohol , like the above mentioned methods) formed by enzyme hydrolysis of thiocholine and noncholine esters.

Table 1

»Remaining substrate« method and methods based on formation of acid



»Remaining substrate«:
Hestrin (1949)[13]

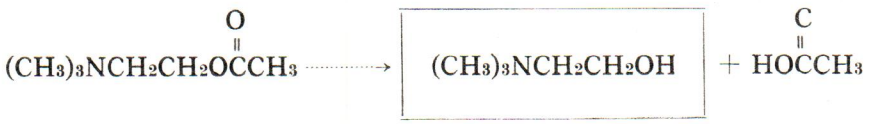
Winteringham and Disney (1962)[19]

Acid formation:
Ammon (1933)[14]
Michel (1949)[15]
Jensen-Holm (1961)[16]
Herzfeld and Stumpf (1955)[17]
Edson (1958)[18]

Reed et al (1966)[20]

Table 2

Methods based on formation of alcohol



Alcohol formation:
Ellman (1959)[21]
Fišerova-Bergerovà (1964)[22]
Guilbault and Kramer (1965)[23]

The spectrophotometric method described by *Ellman* and co-workers (25) provides adequate experimental conditions and yields reliable results. The method is based on the measurement of the enzyme activity by determining the rate of thiocholine formation due to enzyme hydrolysis of acetylthiocholine. The method is characterized by a relatively low substrate concentration, a comparatively short time required for measurement, a small quantity of enzyme preparation necessary for measurement and by the fact that the initial enzyme activity is being measured.

All these advantages have led us to choose this particular method as a reference method for the determination of cholinesterase activities in our laboratory experiments.

Useful as they are, experiments on laboratory animals are of limited use in evaluating a method for measuring exposure to anticholinesterase insecticides. There exist at first the species differences in cholinesterase activities, then different conditions of exposure and different factors of absorption to a given compound. Besides, the data recorded in the course of accidental poisonings, field studies on persons occupationaly exposed to insecticides, manufacturers, formulators and applicators, as well as studies on volunteers may provide the answer to many practical problems.

During the last few years, several studies have been carried out (some of them are still going on) in our laboratory related to human exposure to anticholinesterase insecticides. These include volunteers, occupationally exposed workers and accidental poisonings.

Some studies in volunteers have been carried out with the particular objective of evaluating methods for measuring blood cholinesterase levels in persons exposed to carbamate insecticide — propoxur (24, 6). Propoxur (95% pure, recrystallized before use) was given orally to healthy subjects, — investigators actively involved in the study — either in a single or repeated dose, the latter imitating the occupational exposure to insecticides. Two methods for cholinesterase assay were compared; signs and symptoms if any, were correlated with enzyme activity; the persistence of the inhibitor in the blood was studied; and the excretion of phenyl derivatives in the urine was determined.

In spite of a marked fall in erythrocyte cholinesterase activity down to 27% of the normal value after ingestion of 1.5 mg of propoxur/kg of body weight, no depression in plasma cholinesterase was observed. Both enzymes were determined spectrophotometrically. This was found consistent with the differences observed in the affinity of propoxur for the two enzymes *in vitro*, the I_{50} values being 4.6×10^{-7} M (erythrocyte), and 2.3×10^{-5} M (plasma).

The symptoms such as blurred vision and nausea appeared 20 min after ingestion. Within the next 10 min pronounced nausea with repeated vomiting and profuse sweating developed. Pulse rate and blood pressure

rose. One hour after ingestion the subject felt better. The rapid disappearance of symptoms was consistent with the further rapid recovery of erythrocyte cholinesterase activity.

In a subject who was given 5 doses of 0.20 mg of propoxur per kg of body weight, at 30 min intervals, erythrocyte cholinesterase activity decreased continually down to 60% of the normal value. Plasma cholinesterase was practically unaffected. By receiving the total dose of 1 mg/kg the volunteer complained of only slight mental confusion and difficulties in the pronunciation of some words. Spontaneous reactivation of cholinesterase started after the ingestion of the last dose.

The studies in volunteers have clearly shown that, while a single, relatively small oral dose (0.36 mg/kg) of propoxur may produce symptoms of short duration, higher doses may be tolerated without symptoms, if they are divided into fractions and taken within a relatively brief period.

It may also be noted that whole blood cholinesterase levels determined by the tintometric method, a field method designed originally for measuring the exposure to OP compounds (18), were in reasonably good agreement with the erythrocyte cholinesterase levels determined by the *Ellman's* spectrophotometric method (about 11% higher). Similar results were obtained when the tintometric and Acholest methods were compared with the spectrophotometric method under laboratory conditions (26).

In order to determine the condition which precedes clinical poisoning we assessed cholinesterase activities in blood of workers employed in the production, formulation and application of pesticides.

The measurements of blood cholinesterase activity were performed in the laboratory, while the sampling was done either in the laboratory or in the field when samples were kept cool and transferred to the laboratory. Cholinesterase activities were measured by the spectrophotometric method (25).

Over a period of two years, the number of workers engaged in the production of pesticides was 291 and of those applying them was 63 (spraymen and aircraft personnel). The workers handled the following compounds: dimethoate, thiometon, phosalon, dichlorvos, bromophos, demeton, parathion and malathion. Besides anticholinesterases, the workers were also in contact with some herbicides, rodenticides, chlorinated hydrocarbons and fertilizers.

Out of 354 subjects, 56 showed an activity of blood cholinesterases below 50% of their control value. In only 14 of these workers, the following cholinergic symptoms were observed in addition to blood cholinesterase inhibition: weakness, salivation, sweating and vomiting. These cases came from a pesticide production plant where it was observed that some work places are particularly exposed and certain technological procedures inadequate. All cases were observed during the first year of our survey, while during the second year only one subject had blood cholinesterase activities less than 50% of the normal value, and this coincided with improved work conditions.

Four monomethyl carbamate insecticides were used as inhibitors. The plasma cholinesterase values were found the same when measured by the Acholest or spectrophotometric method. The degree of the whole blood cholinesterase inhibition was found up to 11% lower when measured by the tintometric method compared with the level registered spectrophotometrically by the *Ellman's* method. The results obtained with one carbamate insecticide — propoxur are presented in Fig. 4.

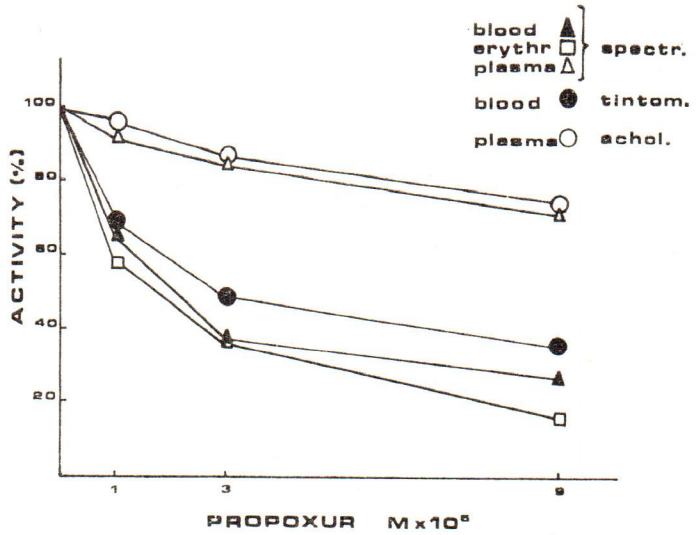


Fig. 1. *Activity of human blood cholinesterases measured by the acholest, tintometric and spectrophotometric methods after inhibition by propoxur. Each point represents the mean value of four determination*

Svetličić et al. (27) reported the results of the trial in a group of formulating workers exposed alternatively to OP and OP plus DDT component of the product. The fall in the enzyme activity during OP compound exposure and the reverse to the normal values during the exposure to the combined product was attributed to the hepatic enzymes induction.

The fact that it is possible to follow the biochemical lesion produced by anticholinesterase agents before the critical fall in the enzyme activity, i. e. before the appearance of clinical picture of poisoning, offers the following practical advantages within the security scheme of occupationally exposed personnel:

(1) to verify the efficiency of protective devices,
(2) to classify the working places according to the health hazards for workers,
(3) to select the individuals »chronically« careless as regards the safety measures and
(4) to prevent the intoxication by stopping the exposure and rotating the worker to the safe job.

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Sažetak

METODE ZA MJERENJE EKSPOZICIJE ANTIKOLINESTERAZNIM INSEKTICIDIMA

Od mnogih metoda za mjerenje aktivnosti kolinesteraze razmjerno je malo prikladnih za rutinsku kontrolu profesionalne ekspozicije antikolinesteraznim spojevima. U radu su prikazani činioci od kojih zavisi izbor i primjenljivost pojedine metode s osobitim obzirom na razlike između organofosfornih i karbamatnih spojeva. Također su izneseni rezultati vlastitih istraživanja na dobrovoljcima i radnicima zaposlenim u proizvodnji spomenutih insekticida.

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