Methods for Assessing Exposure of Insects

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1 INTRODUCTION

Like other constituents of ecosystems, insects are exposed to the action of many man-made chemicals, some of which accumulate in live and inanimate parts of the environment. Insects constitute an important group of organisms, because of the diversity of their morphology and physiology, their abundance, economic importance, and numerous effects on human health. Insects seem to display the greatest diversity of biochemical and genetic responses to stress from exposure to exogenous chemicals. For that reason, adequate methods must be developed for qualitative and quantitative determinations of xenobiotics, and detoxication mechanisms in insect tissues must be understood. While some relatively reliable methods have been developed for diagnosing some of these mechanisms (e.g., insect resistance to some pesticides), methods to measure precisely biological responses to exposure need to be improved.

This survey attempts to summarise methods commonly used for assessing exposure:

(1) Direct determination of xenobiotics at the level of cells and organs;

(2) Qualitative and quantitative biochemical changes in some low-molecular and high-molecular substances;

(3) Genetic changes at individual and population levels as indicators of exposure; and

(4) Effects of xenobiotics at the population level.

2 METHODS TO DETERMINE XENOBIOTICS IN INSECT TISSUES AND ORGANS

Substances absorbed systemically may be either excreted in their original state or altered by various mechanisms and excreted as products of hydrolysis or conjugation. The most important target tissues are the body fat and haemolymph whose proteins and lipids bind chemicals and other organs, especially the reproductive and nervous systems.

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2.1 THE USEFULNESS AND LIMITATIONS OF MONITORING CHEMICALS IN INSECT TISSUES

Gas chromatography (GC) is a basic method for the detection of xenobiotics (e.g., pesticides) (Zweig and Sherma, 1972). The mobile phase is an inert gas, the stationary phase is most often a liquid fixed in a solid vehicle. The carrier gas drives the mixture of chemicals through the column, and substances leave the column separately after adsorption and desorption from the stationary phases. Elution chromatography is often used. It requires one initial application of the sample. The mobile phase is never selective, but separation of substances can be influenced by different temperatures.

One of the universal detectors used in GC for the detection of pesticides, is the thermal conductivity detector (katharometer). Xenobiotics in the carrier gas reduce its thermal conductivity, which translates into an electric signal in the detector and is recorded as a peak. The detector is not destructive, and its sensitivity is in the μg range.

The flame ionisation detector is another universal detector for measuring electric conductivity of gas after ionisation of a sample by burning in an oxygen or hydrogen flame. The detector is destructive and highly sensitive. The detector responds to almost all compounds except for some inorganic gases. It can be modified to be an alkali flame detector, highly sensitive for compounds containing phosphorus (e.g., 2×10^{-12} g for parathion), sulphur or nitrogen (10^{-10} g).

The electron capture detector is highly sensitive to compounds containing the atoms of halides (e.g., Cl and Br) and to conjugated carbonyls (about 5×10^{-12} g of compound). A microwave emission detector can also be used to analyse compounds containing halides and phosphorus; it is sensitive to the subnanogram level.

Spectrophotometric detectors are also selective. They are used for analyses of compounds containing phosphorus, halides, and sulphur, and for the detection of metal chelates. Their sensitivity to compounds containing phosphorus is 10^{-13} g.

Another method widely employed for detection of xenobiotics is thin layer chromatography (TLC) (Zweig and Sherma, 1972), often used to confirm residues identified preliminarily by GC, or alternatively, samples for analysis by gas chromatography can be prepared by TLC.

Silver nitrate/phenoxyethanol is an agent to detect chlorinated insecticides and methylated chlorophenoxy herbicides. Chlorinated insecticides are distinguished by their colour either in the visible part of the spectrum or under longwave UV light if diphenylamine/zinc chloride is applied (sensitivity 0.01 to 0.1 μg). Spraying with 0.005 percent aqueous Rhodamine B solution and 10 percent sodium carbonate followed by scanning with UV light at 254nm enables detection of 0.1 to 0.3 μg of pesticide.

The reagent mercuric nitrate/potassium ferrocyanide—without interference

from carbamates, chlorinated insecticides, and lipids—is used for detection of organophosphorus pesticides (sensitivity 1 to 10 μg). Other systems are tetrabromophenolphthalein/silver nitrate/citric acid (sensitivity = 0.05 to 0.1 μg), bromide/silver nitrate,2,6-dibromobenzoquinone-4-chloroimide (sensitivity = 0.1 to 0.5 μg), or cholinesterase/indoxyl acetate (sensitivity = 1 to 10 μg, method also applicable to carbamate insecticides). *p*-nitrobenzene/diazonium fluoroborate/KOH (detection limit on polyamide = 0.02 to 0.05 μg) can be used for detection of *N*-methylcarbamates with *O*-aryl substitution.

Pesticides of various chemical composition, herbicides, and fungicides can be detected using a spray reagent composed of 0.05 percent fisetin in isopropanol to produce fluorescent spots visible under longwave light on cellulose (detection limit about 10 to 100 ng).

Samples are prepared for chromatography by extraction with organic solvents (e.g., propylene carbonate), then purified in the column (Florisil), and concentrated (for GC or TLC). Samples for TLC require rigorous cleanup to prevent streaking during solvent development.

High performance liquid chromatography is a promising method for separation of heat-labile or poorly volatile materials. An advantage of this method in many cases is that there is no need for sample cleanup. A drawback is a lower sensitivity than that of GC (i.e., in the order of nanograms).

The use of atomic absorption spectrophotometry to assess exposure of insects is mostly limited to measuring the contents of heavy metals (in particular Cd, Cu, Hg, Pb, and Zn) and other trace elements in insect tissues. A specified amount of tissue (20 to 50 insects are usually used) is dried at 105°C up to constant weight. The sample (usually about 250 ± 0.1 mg) is then digested with 2 ml concentrated HNO_3 in a water bath until nitrous oxide fumes are no longer emitted (2 to 3 hours). For instance, in the case of mercury, concentrations of about 0.02 to 0.09 mg/kg, which roughly corresponds with the concentration of heavy metals in polluted biotopes, have been found in insect detritivores and herbivores. The concentration of heavy metals (Cu, Zn, Hg) in predators has been measured up to 10 times higher than in insects.

The analysis by electron microscopy of heavy metals and some other substances on the body surface, in tissues and excrements is a modern and most promising method.

2.2 DETECTION OF IMMEDIATE DAMAGE AT CELLULAR AND SUBCELLULAR LEVELS IN INSECTS

Chemically induced cell injury, cytotoxicity, may be initiated by the formation of a stable complex with an enzyme, receptor site, or via the formation of highly chemically reactive species (electrophiles, free radicals, carbenes,

nitrenes), or by provoking physicochemical changes (e.g., pH, redox, ionic composition) (Bridges *et al.*, 1983). Irreversible changes, due mostly to effects of either high or repeated and prolonged doses, are either qualitative or quantitative. Necrotic cells are easily identified and evaluated by cytological and histological techniques which focus on the following symptoms: mitochondrial swelling, disaggregation of polysomes, occurrence of aggregates of chromatin around the nucleus, cytoplasmic swelling, dissolution of organelles, plasma membrane rupture, and nucleus dissolution.

Histochemistry and cytochemistry are of special importance (Wachsmuth, 1981). When examining histological sections, the human eye registers contrast more readily than the intensity of colouring. Selective histochemical methods substantially enhance contrast by depositing stains at defined places, and enable not only qualitative but also semi-quantitative or quantitative evaluation of the changes occurring in tissues. The main advantage of histochemistry is the application of highly selective methods revealing changes in the specific functions of cells. Another advantage of histochemistry is its high sensitivity and preservation of the cell including membranes in a state which does not much differ from that in life.

Quantitative cytochemistry enables further methodological improvement (Chayen, 1984), which, of course, requires more sophisticated equipment, such as a microspectrophotometer combined with a microscope, or a scanning and integrating microdensitometer.

Basic cytochemical techniques include chromogenic reactions, fluorogenic interferometry, measurement of water content, and autoradiography. There are four types of chromogenic reactions:

(1) The production of colour in a structural component (e.g., DNA staining).

(2) Simultaneous capture reactions, when the reaction includes the release of a certain moiety by enzymatic activity and subsequent precipitation by another component of the reaction medium. Simultaneous determination of the activity of alkaline phosphatase, Na-, and K-ATPase is an example.

(3) Post-coupling reactions resembling the previous type are needed in cases when the reaction of an enzyme with substrate occurs under different conditions than the reaction of a chromogenic compound with the product of enzymatic reactions. An example of this is the measurement of β-glucuronidase.

(4) Tetrazolium reactions used for measurement of the activity of dehydrogenases.

Fluorogenic techniques are more sensitive than the more frequently employed chromogenic ones, but at present they are less well quantified and validated. Microscopic interferometry can be used to weigh intracellular organelles or whole cells. Its most valuable use has probably been the measurement of the water content of isolated cells or of specific regions of a tissue section.

3 INDIRECT EFFECTS ON INSECT CELL AND TISSUES

These methods of detecting biochemical and tissue changes are aimed at finding out the indirect effects of exposure on the whole insect. They include: methods to detect the effects of xenobiotics on low-molecular substances, on nucleic acids, on peptides and proteins, on haemocytes and defensive responses, and on insect metabolism in general.

3.1 ASSESSMENT OF EFFECTS ON LOW-MOLECULAR-WEIGHT SUBSTANCES

Foreign substances affect many targets in cells, proteins and nucleic acids as well as low-molecular substances such as lipids, and co-factors (Bridges *et al.*, 1983). Unsaturated fatty acids, which are important components of cellular membranes, are among the targets of xenobiotics. Compounds such as CCl_4, paraquat, ozone, and oxides of nitrogen react with fatty acids to produce free radicals. The result is a change to the structure of the chain of the fatty acid, and hence to the properties of the membrane. The extent of lipid peroxidation depends on various factors such as the contents of unsaturated fatty acids in the membrane, oxygen pressure, contents of β-carotene and α-tocopherol, which act as antioxidants, and on the contents of the enzyme terminating the chain reaction (viz., glutathione reductase). 1,6-Diphenyl-1,3,5-hexatriene built into a phospholipid membrane parallel with chains of fatty acids can be used as a fluorescent probe for the assessment of the effects of xenobiotics on membranes. Any change in the composition of the phospholipid membrane results in reduced fluorescence.

Furthermore, the level of ATP and the transport of ions can be affected by exposure to a foreign substance. The concentration of ATP in cells depends on the rate of synthesis of ATP which may be inhibited at several places in the pathway forming reduced cofactors, by depletion of reduced cofactor levels or by inhibition or uncoupling of oxidative phosphorylation. ATP level may be affected by interference of the chemical with enzymes keeping the concentration of ATP constant.

The levels of cyclic nucleotides can also serve as indicators of cellular damage. The nucleotides have several important functions, especially that of a second messenger in hormonal action, and they participate as mediators in the processes of transportation through the membrane. They are also involved in nerve function, e.g., toxaphene causes an increase in the levels of c-AMP and c-GMP in all tissues of *Leucophaea maderae* (Butler and Crowder, 1977).

It is well known that some inorganic ions, such as Ca^{2+}, K^{+}, Mg^{2+}, Mn^{2+}, and Na^{+}, have major roles in cellular processes. Sufficient concentrations of these ions and their regulation are indispensable for cellular transport, energy production, synthesis of proteins, control of the cell cycle, and other

functions of the cell. If the function of the membrane has been impaired by chemicals, calcium content in the cell may increase by several orders of magnitude (calcium concentration in cells is approximately 10^{-7} M, most of it being bound, while the concentration of calcium in extracellular space is approximately 10^{-3} M). A direct correlation of calcium concentration with the death of the cell has been observed. However, it is quite probable that disorders of an overall balance in the concentrations of several ions enhance the toxic effects of chemicals more than a changed concentration of only one of them.

3.2 DETECTION OF EFFECTS ON NUCLEIC ACIDS

Changes of the contents of nucleic acids caused by exposure to xenobiotics induce changes in genetic material as well as in the proteosynthetic apparatus of the cell. It has been proved that some xenobiotics (e.g., DDT) act on the subcellular level and stimulate transcription. These substances seem to affect either histones or acidic proteins which appear to be involved in the control of genetic expression.

The amount of DNA in cells can be determined by means of a microdensitometer and Feulgen reaction, or by ethidium bromide fluorescence and micrometry. Especially the changes detected by flow cytometry are well reproducible.

However, since the amount of DNA is relatively constant in the majority of insect cells, RNA, and cell proteins must also be measured. The changes of RNA are usually more pronounced after exposure to chemicals than those in DNA. Xenobiotics usually not only enhance RNA synthesis after 30 to 50 minutes of autoradiographic bioassay, but they also conspicuously change the ratio of nucleic RNA/DNA, and enhance the template activity of nucleic RNA. Besides the altered ratio of RNA/DNA, some physiological characteristics (e.g., mitotic index) should also be examined if cell damage is to be detected.

Insect ovary is a most useful tissue to observe changes in the contents of nucleic acids, particularly when exposure causes alterations of fecundity and cell proliferation. The amount of RNA depends on morphological changes during ontogeny, markedly increasing at the onset of vitellogenesis. The amount of DNA usually increases gradually along with the increasing activity of follicular and nutritive cells and with transportation of DNA from other organs.

3.3 CHANGES IN PEPTIDES AND PROTEINS AS MARKERS OF EXPOSURE

The effects of exogenous substances on proteins and peptides can be divided into three main groups: inhibition of protein synthesis, inhibition of enzymes,

and induction of enzymes. Other possible effects may be found, such as effects on the release of some hormones, reaction of a xenobiotic with receptors, protein binding, etc.

Protein synthesis can be inhibited at several stages: blocking either the synthesis of aminoacyl t-RNA or the binding of aminoacyl t-RNA to ribosomes or the binding of peptides catalysed by peptidyl transferase, or by inhibiting RNA synthesis directly.

Enzyme inhibition by xenobiotics can be investigated relatively easily. Enzymatic activity can be reliably measured by spectrophotometric methods, or by the even more sensitive fluorimetry, and the difference between controls (or individuals of the same population sampled prior to exposure) and individuals exposed to xenobiotics can be evaluated. ATPase (Mg^{2+} dependent), catalase, acetylcholinesterase, and choline acetyltransferase, are examples of enzymes affected by pesticides.

The induction of enzymes by many chemicals has received much attention as one of the important adaptations of insects to their environment (Terriere, 1984). This induction is not limited to insects, since the mechanisms of action are largely the same as in higher animals. The mechanism of induction is presumed to include the following basic steps:

(1) Exogenous inducer penetrates a cell;
(2) Inducer is bound to the molecule of receptor (protein in cytosol);
(3) Inducer/receptor complex is transferred into the nucleus; and
(4) Transcription and translation result in the production of a respective enzyme.

The most prevalent detoxication systems in insects are microsomal oxidases, glutathione S-transferases and carboxylesterases. There are several kinds of P-450 cytochrome, which is an active enzyme site of the microsomal oxidases system. Some forms of cytochrome P-450 are permanently present in insects, whereas others are induced by a chemical inducers and act against exogenous substrates. The induced cytochrome P-450 is not designated only for metabolism of the inducer but has a more general target. Microsomal oxidases control many reactions by which an atom of oxygen is introduced into a substrate molecule and alkyl groups are removed from methoxy and ethoxy groups, or hydroxyl groups are introduced into aromatic portions of molecules, and substrates are either epoxidated or deaminated. Since so many reactions are possible, almost any pesticide or other toxicant is a suitable substrate for these enzymes.

Glutathione S-transferases have a major role in the metabolism of organophosphorus compounds. There are several types, distinguished by the reactions they catalyse (alkyl-, aryl-, and epoxy-transferases are the most important). They are catalysers of the reaction between substrate and reduced glutathione, producing mercapturic acid derivatives.

Carboyxlesterases are a third group of detoxication enzymes. They hydrolyze mainly carboxyl esters, such as pyrethroids and malathion, but

they can also hydrolyse phosphate or carbamate esters. There are many forms of carboxylesterases in one animal, but only some of them participate in the hydrolyses of xenobiotics (this applies at least to pesticides).

There are many methods for determining the actions and forms of detoxication enzymes induced by xenobiotics. Every sample must be compared with a sample in which no induction has occurred. The activity of microsomal oxidases can be determined by measuring, e.g., *p*-nitroanisole demethylation, 7-ethoxyresorufin *O*-deethylase activity using a spectrophotometer, or various fluorimetric substrates can be used (e.g., ethoxycoumarin, *p*-nitrophenetol).

A method using 3,4-dichloronitrobenzene as a substrate is applicable to spectrophotometric assessment of the activity of glutathione S-transferases. The activity of esterases can be determined in various ways; 1- and 2-naphthylacetate or *p*-nitrophenyl acetate are commonly used as substrates for spectrophotometry, and methylumbelliferone esters for fluorimetry.

Electrophoresis is another method for at least semiquantitative determination of the activity of detoxication enzymes, particularly transferases and esterases, and the number and properties of various forms of enzymes. The most common of these methods is electrophoresis on polyacrylamide gel, using gel rods or slabs. A number of substrates is available for visualisation of esterases; individual zones can be characterised by means of various inhibitors. Glutathione S-transferases can be observed on gel, using chloro-2,4-dinitrobenzene or methyl iodide or 1,3-dinitro-4-iodobenzene as substrates.

Besides electrophoresis, enzymes on polyacrylamide gel can be separated by the method of isoelectric focusing which often enables an even more detailed separation. Substrates are the same as for electrophoresis.

Another way of registering and proving the activity of detoxication enzymes is to analyse the products of metabolism of xenobiotics in the insect body. The xenobiotics have toxic effects on proteins, nucleic acids, and other components of cells and tissues, but many detoxication enzymes act against them. Besides the enzyme systems inducible by pesticides and other compounds, which have been mentioned above, these are glucosyltransferases catalysing the conjugation of glucose with pesticides altered by microsomal oxidases, sulphotransferases, or, e.g., formaminidases detoxicating formamidine pesticides.

Dialkylphosphoric acids and dialkylthiophosphoric acids are products of the detoxication of organophosphorus pesticides (Daughton *et al.*, 1976). Various conjugates can be found in excrements (products of the metabolism of xenobiotics by microsomal oxidases and transferases).

Methods used for determining the metabolites of the degradation of pesticides and other xenobiotics are gas chromatography and thin layer chromatography. Substances passing unchanged through the insect alimentary canal can also be detected by these methods.

Spectrophotometry can also be used for determining whether the resistance of a population sample is due to a low sensitivity of the target enzyme. This applies to acetylcholinesterase which is inhibited by organophosphorus and carbamate pesticides. In some insect populations this enzyme occurs in a somewhat modified form which is several times (up to 1000 times in some cases) less sensitive to inhibition. Naturally, kinetic constants for the reaction of the enzyme with a given inhibitor must be ascertained (Nishioka *et al.*, 1976).

Insects whose resistance is based on a high activity of esterases can be detected by electrophoretic methods, quickly and with relatively simple equipment. Well-known examples are *Myzus persicae* and *Culex* mosquitoes. Analyses of populations samples of these insects always bring out a fraction whose esterase activity is considerably higher than in susceptible insects. Preliminary biochemical tests have been based on this fact, showing within a few minutes whether the examined sample is susceptible or resistant. An example is a filter paper test for detection of phenotypes of a high esterase activity in mosquitoes resistant to organophosphates. The test is based on enzymatic hydrolysis of 1-naphthyl acetate as substrate, and subsequent azo-coupling of 1-naphthol with diazonium salt Fast Garnet GBC (Pasteur and Georghiou, 1981).

3.4 EFFECTS OF EXPOSURE ON INSECT METABOLISM AND METABOLITES

In contrast to most of the other organisms, insects undergo substantial metabolic changes during their development. Metabolism (usually measured as oxygen uptake, calorific quotient, or respiration quotient) increases in parallel with the course of development only in embryogenesis, but sometimes it reaches maximum values at maximal cell multiplication. In pupae, oxygen uptake makes a characteristic U-shaped curve, being the highest immediately after pupation, and later rapidly increasing again immediately before adult emergence. Metabolism is also greatly affected by diapause, quiescence, and starvation. The state of the organism at the moment of exposure should, therefore, always be taken into account when its effects are investigated. In most insects, there are no general rules concerning correlation between the body size and intensity of metabolism; in most cases, the larger the insects the lower the metabolism in relation to body weight.

Proteins are the basic structural elements of insect muscles, glands, and other tissues, making up about 20 percent of some tissues such as flight muscles. The synthesis of proteins from free amino acids substantially increases in the course of growth, especially during metamorphosis. At the same time proteins are transferred from organs undergoing atrophy to functional ones.

Blood sugars (in particular disaccharide trehalose and glucose) are the

most readily available sources of energy. Their content in haemolymph is normally 2.6 to 4.4 percent, but it may amount to as much as 11.5 percent. Exposure reduces the content of blood sugars in some cases and, similarly to starvation, glycogen content in the body fat. In contrast, the content of trehalose in blood may increase after a direct injury to cuticle by abrasive chemicals.

Lipids are the most important reserves of energy in the insect body. Their contents vary, ranging between 2.5 to 45 percent of dry weight and increasing approximately 10 times during ontogeny. A reduction of the fat content, especially in the cells of the body fat, as a result of exposure to chemicals can be proven by biochemical and histological methods as disappearance of fat vacuoles from cells (by selective staining for fats in sections or squashes).

Metabolism of pigments (e.g., melanins, pterines, carotinoids) is usually connected with physiological processes and stimulated by some abiotic and biotic factors. However, pigment metabolism may be affected by food, and in relation to selection (e.g., the selective pressure of predation), they may also be affected by substances polluting the environment (industrial melanism), which thus indirectly help the propagation of dark (melanic) forms. Blood pigments are relatively rare in insects (haemoglobin, haemocyanine) and their function is usually accessory to tracheal respiration, so that their intoxication (e.g., by heavy metals) has limited effects on the insect organism.

Respiratory metabolism varies greatly in insects. Even under physiological conditions, it is affected by many factors, such as temperature, humidity, developmental stage, sex, activity. It is usually measured as oxidative metabolism (cycle of citric acid and cytochromes) or by means of the exchange of gases (oxygen and carbon dioxide).

The content of oxygen in respired air may not be a decisive factor for the insect organism, since oxygen uptake in some species is more or less independent of the oxygen tension in the environment. Other species can live for days and even months under anoxic conditions, switching to anaerobic metabolism. A modest increase in the content of CO_2 is tolerated by insects, high concentrations are narcotic. The larvae of insects living deep in soil are very resistant. However, CO_2 affects the opening of stigmata, thus hastening desiccation of the organism.

In general, xenobiotics are the cause of a higher oxygen uptake (up to three times compared with controls) which is easily detected by the usual methods.

3.5 HAEMOCYTES AND IMMUNE RESPONSES AS POSSIBLE INDICATORS OF TOXICITY

The examination of changes in either the total or part of insect haemocytes is a suitable system for detecting effects of toxic substances. Metabolites

and toxins of entomopathogenic bacteria (*Bacillus thuringiensis*, *B. sphaericus*), fungi and nematodes can also be tested in this way. All these substances induce an irreversible cytopoiesis of the host's haemocytes, at the same time either inactivating or inhibiting the synthesis of bacteriolytic cationic proteins in the haemolymph of immune insects. Unlike with microbial metabolites, there are very few data on the haemograms of insects exposed to xenobiotics, on the role of haemocytes, other cells of the reticuloendothelial-like system, and direct detoxication of herbicides. Many of the available data are contradictory, so that, at the present time, this method cannot be generally employed and should be applied only under specific conditions.

There is probably a relation between exposure to pesticides and changes in haemograms: absorption of labelled insecticides by haemocytes had been proven, but detoxication did not take place. Pericardial cells are presumed to participate in detoxication. Insects possessing fewer free haemocytes are more susceptible to xenobiotics (e.g., parathion), and this susceptibility seems to be the result of lower activity of non-specific haemocyte carboxylesterases. Cytopoiesis has been proven in insects exposed to lethal doses of arsenates, nicotine dichlorodiethyl ether, carbon tetrachloride, and DDT.

Although information on the immune system (or rather its mechanisms in insects) is incomplete, the observed changes in the defence of the organism can be recommended as a suitable system for detection of the effects of pesticides on insects. The effectiveness of defense mechanisms reveals much of the physiological state of the host, so that any changes in the immune response (such as, a reduced proteosynthesis of bacteriolytic proteins, increased parasitisation, etc.) reflect a physiological imbalance of the organism. These changes can be induced among others by pesticides or their residues, whether insects are their targets or they penetrate into insect tissues from the polluted environment.

It is well known that body fat of immunised caterpillars of *Galleria mellonella* (De Verno *et al.*, 1984) and diapausing pupae of *Hyalophora cecropia* (Locke, 1980) produce bacteriolytic proteins if transferred into cultivation medium. However, the presence of haemocytes is necessary for induction of proteosynthesis *in vitro*. Haemocytes of *G. mellonella* caterpillars treated with sublethal doses of an insecticide or exposed to the effects of sterile homogenates of some insect pathogens show that either bacteriolytic proteins are not produced at all, or the amount of synthesised proteins is much lower than in controls. Our preliminary results seem to indicate that the ability of haemocytes to induce synthesis of bacteriolytic proteins could be a reliable test of the function of damaged haemocytes of insects from heavily polluted areas.

4 GENETIC CHANGES AS INDICATORS OF CHEMICAL EXPOSURE

Various assay systems have been developed for detecting the mutagenic actions of chemicals. Microbial tests are the most sensitive and quickest of them all, but their results are insufficient for two reasons:

(1) The ability of higher organisms to limit and repair the damage done by genetically active substances has not been delineated; and

(2) Tests are incapable of detecting indirect-acting mutagens and carcinogens activated by enzymes present only in higher animals.

The use of insects as test organisms is, therefore, preferable, enabling a more accurate assessment of the danger to humans.

4.1 MUTAGENICITY TESTING

Tests in *Drosophila melanogaster* seem to be the most desirable (Wurgler *et al.,* 1984), as the species has several useful qualities: a short generation time (10–12 days), numerous progeny, few chromosomes, and many diverse markers. These organisms are grown easily, research with this species is relatively inexpensive, and the organisation of genes and chromosomes is comparable. It is a relatively long-lived organism to which chemicals can be applied in different ways, and their effects on individual stages of the development of gametes can be studied. Furthermore, indirect mutagens are activated by the enzymatic system the same as they are in mammals. *Drosophila* can be used to measure a wide range of genetic changes: recessive lethal (or visible) mutations, deletions, translocations, chromosome loss, dominant lethal, to non-disjunction, and recombination.

The sex-linked recessive lethal test, the so-called Basc (Muller-5) test is the best known and used most commonly. Recessive lethal mutations are the products of molecular changes inside genes, or of slight deletions. The substances inducing them may be dangerous to the gametes of reproductively competent people. The test is simple and objective and its results are easily interpreted. Kale and Baum (1982) have demonstrated that cigarette smoke caused sex-linked recessive lethal changes in spermatocytes of larvae. With the number of smokers as high as it is, even a slight increase in the mutagenic rate may substantially affect the gene pool of a large part of the human population.

Chemical mutagens may also induce chromosomal aberrations, or changes resulting from chromosome breakage or from disruption of chromosome behaviour during meiosis. Some of them may be detected in *Drosophila* by a translocation assay or an aneuploidy test (Valencia *et al.,* 1984). One of the heritable (reciprocal) translocation tests uses 2 markers, bw (brown eye colour) and e (ebony body colour). The former is localised on the second chromosome, the latter on the third. Reciprocal translocations between the

2nd and 3rd chromosomes, between the 2nd and Y, or 3rd and Y chromosomes can be detected through these characters. Aneuploidy tests serve for detecting chromosome loss and non-disjunction. Most of them include sex chromosomes, but changes in autosomes can also be assessed. The sex chromosomes of both parents are labelled by visible mutations, so that the loss (or gain) of a whole chromosome or its part can be identified in the F_1 generation of flies.

Zimmering (1983) used repair-deficient mutants of *Drosophila* for detection of chromosome aberrations. This system is much more sensitive than the others, enabling tests of chemicals at low concentrations. Conventional tests require much higher concentrations, which may be the cause of "false positives" that result from aberrant metabolic products.

The sex-linked recessive lethal test is the most commonly used for practical testing of chemicals. Although it means substantial economy of time, results are known only two generations later. Quicker tests have been worked out in recent years, based on the induction of mitotic recombination. Breakage of chromatids induced by mutagens leads to their exchange, i.e., mitotic recombination, which is realised as a mosaic in the dividing somatic or germ cells. The mosaic can be detected if the chromatids bear suitable genetic markers. A test system enabling comparison of the effects of mutagens in somatic and germ cells is based on changes in the pigmentation of eyes and in the egg phenotype.

A new test for detection of the mutagenic and recombinational actions of chemicals has been described by Graf *et al.* (1984). The test is applicable only to the detection of somatic mutations, using 2 recessive mutants. It has many advantages: speed (it takes only 1 generation), and the exposure of each disc represents a potential risk for thousands of cells. Detection of mutations by wing mosaic is more precise than by eye mosaic.

The true crossing-over (meiotic recombination) can also be used for the monitoring of genetic effects of chemicals on *Drosophila*. Cross-over is spontaneous in females; in males it is usually blocked but does occur at a very low rate. Numerous chemicals (e.g., formaldehyde) are known to induce meiotic recombination.

These test systems can include various modifications using *Drosophila*. No other species has been studied so extensively. Species whose eggs are easily examined can be used for assessment of the genetic effects of chemicals by a test for dominant lethal mutations (DLM) (LaChance 1967).

The main cause, DLM, seems to be induced chromosome aberrations; it may be expressed at any developmental stage, but death usually occurs during embryogenesis, which is the reason it is based on the evaluation of egg hatchability. The results of this test are not unequivocal, since they may be distorted by sterility.

Parthenogenic species are special cases in the detection of lethal mutations. Recessive lethal mutations (RLM) are realised in the haploid phase after

exposure to a mutagen. *Habrobracon juglandis* is a suitable species for these studies. Haploid males develop from unfertilised eggs; the chorion is so thin that even the stage in which the embryo died can be determined. These properties facilitate the detection of RLM in the F_1 generation by determining the hatchability of eggs laid by individual F_1 virgin females: if a female bears RLM, the specific embryonal lethality of her offspring is about 50 percent.

Test schemes similar to those for *Drosophila* have been developed for other insect species. Inagaki and Oster (1969) studied mutagenic effects of alkylating agents on the spermatozoa of *Bombyx mori* using two recessive genes affecting the colour of serosa and of the eyes of adults: gene pe (pink)—white eggs, and gene re (red)—red eggs (normal eggs are black). Exposed control males were mated to double recessive females, and the colour of their eggs were examined. Most mutations were mosaics. Nothiger and Dubendorfer (1971) investigated somatic crossing-over in *Musca domestica*. Individuals heterozygous for bwb (brown body) and w (white eye) produced clones of mutant cells.

The present survey shows that insects may be valuable for the assessment of genetic risks for people living in a chemical environment. Insects are preferable to other eukaryotic test systems, because of the speed with which results are obtained and because of the large number of offspring. However, the usefulness of insects is primarily by enhancing our understanding of the mechanisms of mutagenesis.

4.2 CYTOGENETIC METHODS

Cytogenetic methods are used to detect changes in chromosome structure (translocations, rings, inversions, bridges), changes in chromosome content (loss or gain of a part of a chromosome), or changes in chromosome number (aneuploidy). These alterations may result from chromosome breakage or from disruption of chromosome behaviour during meiosis. Presently, insect chromosome preparations are obtained almost exclusively from brain, nerve ganglia, salivary glands, testes and ovaries.

One of the simplest methods to stain chromosomes is with acetic orcein or aceto-lactic orcein, although Giemsa is also used often.

Banding techniques have been used more and more lately for the study of insect chromosomes, because the banding pattern of chromosomes facilitates not only discrimination of individual chromosomes but also detection of various chromosomal aberrations. Fluorescence pattern is obtained by Hoechst 33258 or quinacrine staining. Each stain gives an excellent longitudinal differentiation of heterochromatin while euchromatin fluoresces homogeneously. Giemsa staining, commonly used for C-, g- and N-banding of human chromosomes, has been modified for use in insects.
Polytene chromosomes in the salivary glands of *Diptera* are a very good

model for the study of cytogenetic effects of chemicals. The size and banding pattern of the polytene chromosomes enable detection even of only one disc deletion.

4.3 RESISTANCE TO PESTICIDES AND ITS DETECTION

Resistance to xenobiotics has been developing in insects as a response to chronic exposure of insect populations to pesticides. Insect genotypes are selected whose makeup ensures survival, even when the insects are treated with doses of pesticides much higher than those killing susceptible insects.

The genetic basis of insecticide resistance has not yet been resolved. Resistance has a single genetic basis in many cases; in some cases, a polygenic basis is more likely. The degree of resistance may be affected by the genetic background.

Population-genetic changes always occur during the selection for resistant populations. They can be detected by common genetic methods (e.g., hybridological analysis, cytogenetic techniques). Some authors have analysed information on hereditary insecticide resistance in some insect species, e.g., individual genes for resistance to some insecticides have been identified in *Musca domestica*, *Drosophila*, and *Lucilia cuprina*. An interesting phenomenon has been observed in *Anopheles* mosquitoes: significantly higher numbers of chromosome inversions have been found in dieldrin-resistant and DDT-tolerant strains.

Several mechanisms of resistance, often occurring in combination, enable exposed insects to survive. Resistance is enhanced several times by the combination of two or more of these mechanisms. Among the most important mechanisms of resistance are an increased amount or activity of detoxication enzymes, slower penetration of xenobiotics through the cuticle into the insect body, and a modified target, most often a modified active site of an inhibited enzyme.

Resistance insects can be detected by conventional toxicity methods. Basic toxicity tests include the spraying of insects in Potter's tower, topical application, dip tests, fumigation, and powdering. Resistant individuals can also be detected through changes in their enzymatic make-up. Some detoxication enzymes (e.g., microsomal oxidases, esterases, and glutathione S-transferases) are almost always highly active in resistant individuals. The higher activity is measured easily either by spectrophotometry or by means of electrophoresis or isoelectricfocusing. Using kinetic constants, enzymes can be characterised by type of inhibition caused by a particular substance.

5 METHODS TO STUDY EFFECTS OF XENOBIOTICS

It is useful to observe populations of insect species and the changes they undergo as indicators of chemically- or environmentally-induced changes. A

single species can be used for bioindication if enough individuals are analysed. For instance, an increase in the production of ammonia by larvae of *Somatochlora cingulata* indicates pollution of water by trichloro- and dichloroacetic acids (Correa *et al.*, 1985).

Another example is detection of parasitisation in populations exposed to xenobiotics. Purrini (1983) investigated parasitisation of soil insects and other soil invertebrates at two localities in West Germany in areas polluted by massive emissions of sulphur dioxide. Compared with control areas in Austria and Spain, parasitism was higher by 25 to 70 percent at the polluted localities. New species of pathogens were found there apart from the increased infestation.

While there are few papers on parasitism resulting from pesticide use or the presence of other toxic substances, investigation into the frequency of parasitism in populations is expected to provide basic preliminary information about the situation at a given locality.

Other systems of bioindication use groups of species, or all species in a biocoenosis. The long-term effects of chemicals on populations have been investigated as a part of integrated plant protection (Sechser *et al.*, 1984); there are several bioindicatory systems using water insects. Some of these are applicable not only to the detection of the effects of xenobiotics but also to the assessment of eutrophication of water resulting in lower oxygen content (Jacob and Walther,
1981).

6 CONCLUSIONS

The present survey shows that there are various methods for detecting xenobiotics and assessing their effects in insects. They are indispensable for the surveillance of substances foreign to the human environment, and for practical analysis of insect populations resistant to insecticides and other substances distributed in the environment. This paper demonstrates gaps in our knowledge about these methods and the effects of chemicals. For instance, many data exist on direct detection of mutations, but information on immune responses in insects is scanty.

Recommendations for improvement of methods for use in insects to detect exposure to chemicals should be aimed at the following:

(1) Improvement of chemical methods for the detection of xenobiotics in tissues and organs;

(2) Development of methods to detect effects at the subcellular level;

(3) Basic research on the effects of xenobiotics on haemocytes and the insect immune system;

(4) Research into new models to detect mutations and resistance to toxicity; and

(5) Improvement in bioindication systems for as many species of a given biocoenosis as possible.

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