With many compliments, H. Gysels

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ELECTROPHORETICAL AND HISTOCHEMICAL INVESTIGATIONS ON SOME NOXIOUS LEPIDOPTERA AND ON THE IMPACT OF PESTICIDES UPON *EPHEMERA DANICA*, A WATER-DWELLING, INNOCUOUS MAYFLY

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It is a well known fact that besides heavy metals, hydrocarbons and other waste products from industrial and „welfare" societies, pesticides play an important role in damaging natural ecosystems. It is also well known that several harmful, often also poisonous compounds reach man by different food chains from water and land. Still relatively little research is being carried out on the influence of pesticides upon the lower animals, that are not directly considered enemies of man. It is necessary to have some basic information on the normal structure of the organisms investigated, of normal characteristics, but also of aetiological and pathological features. This paper deals with histochemical data and agar electrophoretic patterns obtained from both normal, operated and treated insects, familiar to agricultural investigators and to nature conservancy people as well.

Methods used for histological preparations will be described briefly in the course of the paper while specific staining methodes will be mentioned as well ; agar electrophoresis was carried out according to Wieme and Rabaey (1957). To compare relative mobilities the test substances dextran ($m_r = 0$) and human serum albumin ($m_r = 100$) were run at the same plate (indications d-a).

1. A first series of electrophoretic slides deals with the variability of haemolymph patterns of *Stilpnotia salicis* (Lymantriidae), a moth often causing pests in poplar plantations.

Among imagines, a remarkable difference has to be noted with regard to sex : the electrophoretic component nearest to the cathode is considerably stronger pronounced in males (fig. I, 7-8), while fractions of medium mobility appear less clear than in females (fig. I, 6-9). Anodal components are almost absent.

In chrysalids, however, two anodal components are the more pronounced ; one fast migrating is broad and highly concentrated, the second one lies very close to it, but is less concentrated and shows a narrower appearance. A lot of protein fractions with medium mobility is visible, though in a pretty low concentration, while the cathodal component, also recorded in male and female imagines, is fairly pronounced in almost equal concentrations (fig. I, 1-5).

Larval haemolymph patterns show more variations. At first full grown cater-

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pillars have both imaginal and chrysalid proteins, but several components with medium mobility are much more concentrated than in chrysalids. It is a striking feature that the haemolymph patterns of two caterpillars, identical in dimensions, colour etc. are exactly alike when they are fed on *Populus,* but differ slightly from a pattern obtained from an animal fed on *Salix*. In fact, there is only one fraction the mobilitv of which is increased slightly, but unmistakably, towards the anode (fig. II, 1-2-3).

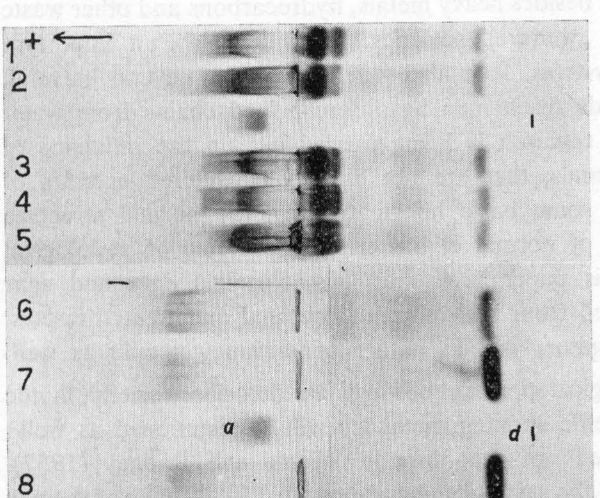

Fig. I. — Haemolymph electropherograms of *Stilpnotia salicis* (1-5 : chrysalids, 6-9 : imagines). Further explanation see text.

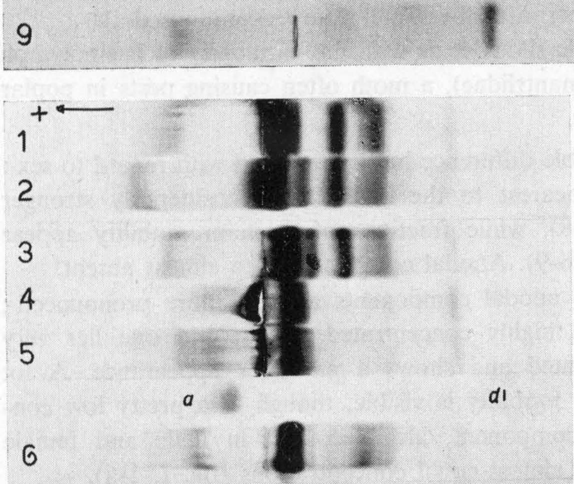

Fig. II. — *Stilpnotia salicis* : larval haemolymph patterns (1-3 : fully grown, 4-5 : young, 6-7 : starving).

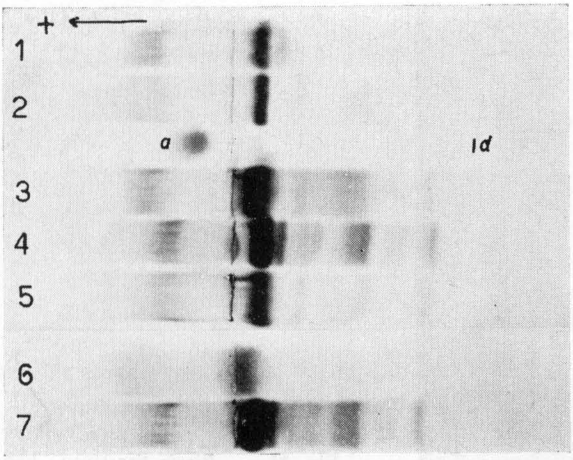

Fig. III : *Stilpnotia salicis :* haemolymph patterns from parasitized larvae. Further explanation see text.

In young caterpillars, on the other hand, all fractions, though clearly visible, are less concentrated except the fastest migrating, broad spotted anodal one (fig. II, 4-5), which is also striking in full grown caterpillars and pupae. This protein remains present also when all other components grow faint and even disappear by shorter or longer periods of starvation (fig. II, 6-7).

Finally haemolymph patterns from young *Stilpnotia* caterpillars can be altered considerably by the activity of parasites, e.g. *Meteorus versicolor* and *Apanteles solitarius* (fig. III, 1-2). In different stages of parasitized larvae one can state that again the anodal component remains intact, even when all othe fractions ar fading, while they are, of course, consumed by the parasite (fig. III, 3-4-5). Externe exhaustion causes fading of the anodal component also ; this corresponds with the stage in which the small larva is motionless, very close to

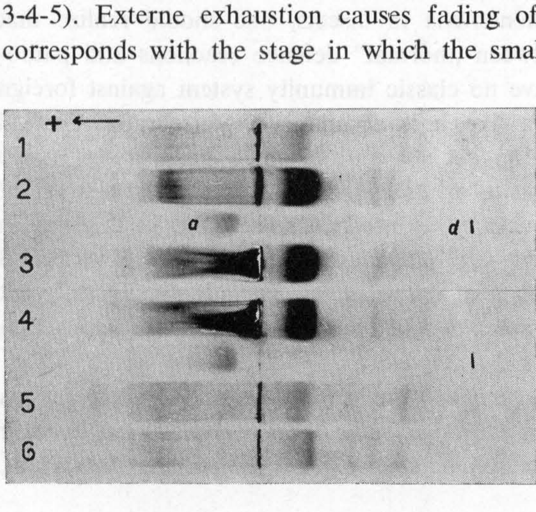

Fig. IV. — *Pieris brassicae :* larval haemolymph patterns (1 : young, 2 : before moulting, 3-4 : fully grown, 5-6 : parasitized).

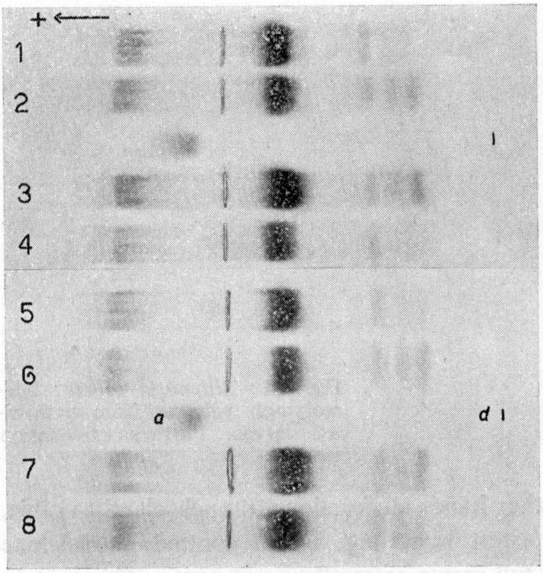

Fig. V. — *Pieris brassicae*: haemolymph patterns from chrysalids (1-2 : normal, 3-8 : after probable cancerigenous treatment. Further explanation see text.

death (fig. III, 6). In fully grown parasitized caterpillars a similar process seems to take place ; the fastest anodal component remains almost untouched by parasite activity, while all other fractions appear in lower concentrations or fade (fig. III, 7).

2. A series of experiments with regard to the characteristics of pathological tissues, features of parasitism and experimental tumours was carried out on *Pieris brassicae*, the Large White butterfly, well known as a cabbage field pest.

While studying pathological phenomena in insects, one should realize that it is very difficult to distinguish between „normal" defence reactions and pathological situations. Indeed, insects have no classic immunity system against foreign objects that may enter their bodies, like it is common in Vertebrates. Instead of antibodies, a typical blood reaction occurs, during which haemocytes try to enclose the object, in general a parasite or its egg. During this process a lot of haemocytes perish and in turn are enclosed by newly arrived ones. In this way, a „tumour" may grow, but it is clear that such an inflammation concretion, identified as a tumour by a considerable number of investigators, is undoubtedly different from a neoplastic tumour that originated from abnormal cell growth, and which is a really pathological phenomenon.

In this light a number of experiments were set up to follow the reactions of insect organs and tissues, after some foreign bodies had carefully been brought into the *Pieris* chrysalids. A small cellophane strip e.g. caused melanin formation, necrosis of the adjacent fat cells and connective tissues, and hypertrophy of the digestive tract epithelium : the single original cell layer became chaotically stratified (fig. VIII). Some authors are inclined to call this a neoplasm (e.g. Matz, 1963), while a number of cells show giant nuclei ; with a greater number, however, the normal nucleus/plasma proportion is reduced. Mitotic figures were almost absent, one of the reasons why Day (1952) preferred to consider this „pseudoepithelium" as a kind of wound tissue and not as a neoplasm. On the other hand a number of investigators would not hesitate to call the whole a „melanotic tumour".

Quite another reaction was obtained by introducing a crystal of benzidin, a substance generally agreed to be cancerigenous for Vertebrate animals. Contrary to the normal situation, the testis from the treated animal showed no mitosis at all, but resulted in an impression of general disintegration and loss of chromatophilia in the generative elements. The strongly atrophic and dysplastic material tended to accumulate in some rounded conglomerations (r.c.) ; moreover, a strong thickening and pigmentation of the testis coat (t.c.) and of the follicle walls (f.w.) were recorded (fig. IX).

Thirdly, a number of chrysalids was interiorly sectioned to cut important nerves and ducts ; to another series a drop of haemolymph obtained from other individuals was added. According to a number of authors these procedures may also start tumour formation in insects (e.g. Hema, 1966 and Sutherland, 1967).

Electrophoresis of the haemolymph of both normal, parasitized and treated *Pieris* caterpillars and chrysalids was carried out in order to state whether these actions would influence the patterns or not.

Young larvae provide haemolymph patterns with poorly concentrated protein fractions (fig. IV, 1), whereas fully grown caterpillars show a broad, highly concentrated protein spot of medium mobility, besides 3-4 lesser concentrated, slower migrating fractions pretty close to the cathode (fig. IV, 3-4). In the course of larval development, perhaps as a consequence of moulting, these slowest fractions may disappear (fig. IV, 2). In parasitized animals they remain fairly visible, while the strongly concentrated, faster migrating broad component is reduced either into a narrow fraction, or into a rather faint, indistinctly limited spot (fig. IV, 5-6).

In *Pieris,* haemolymph runs from pupae hardly differ from runs obtained from fully grown caterpillars (fig. V, 1-2). Moreover, none of the treated specimen runs seem to have undergone any remarkable influence, neither by introducing cellophane or benzidin (fig. V, 3-4), nor by adding foreign but specific haemolymph (fig. V, 5-6) or by sectioning nerves and ducts (fig. V, 7-8).

3. From earlier research, from literature and from previous experiments men-

tioned above, one may easily learn that insect haemolymph electrophoretic patterns are specific and often reflect successive stages in development. They do not quickly undergo profound changes by pathological situations caused by external influences, although there may be minor alterations in mobilities.

This does not apply to the impact of pesticides commonly used in agriculture. With *Hyponomeuta evonymella,* a Microlepidopterid moth mostly feeding on fruit trees, it could be recorded that the haemolymph pattern was changed completely 12 hours after pesticide treatment of the trees infested with caterpillars.

Since it is well known that pesticides of many types remain persistent in the environment and may severely threaten natural life in waters and soil, by poisoning plants or animals in different food chains, several authors have tried to trace the reputation to be one of the best indicators for different kinds of pollution. (cf. Muirhead Thompson, 1971).

Among all water-dwelling insect larvae, Mayfly larvae (Ephemeroptera) have the reputation to be one of the best indicators for different kinds of pollution. From recent zoogeographical data it was learned that most Mayfly species,

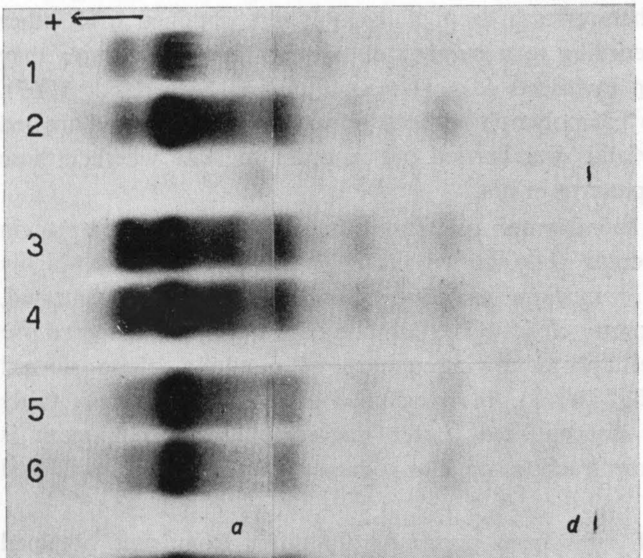

Fig. VI. — *Ephemera danica:* variability in normal larval haemolymph patterns (1-7) and influence of detergents (8) and pesticides (9).

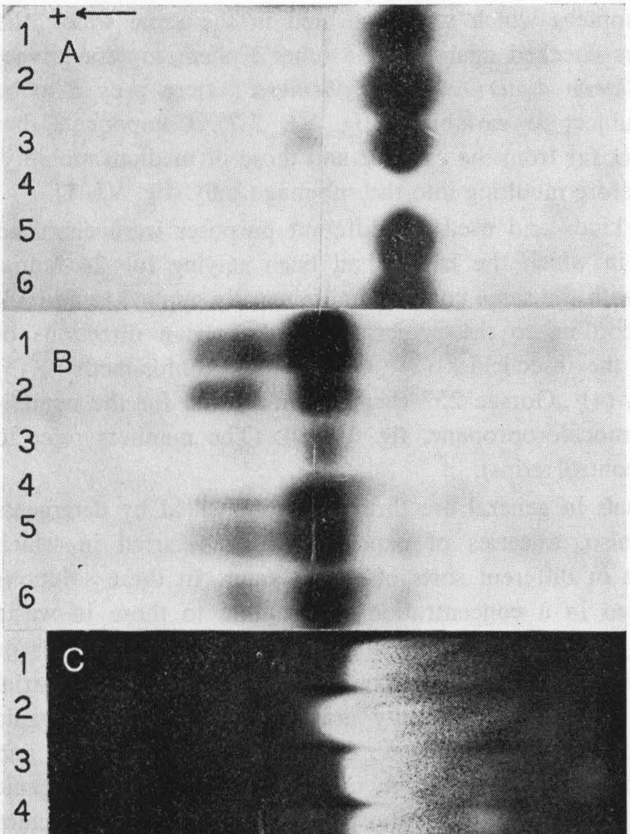

Fig. VII. — *Ephemera danica:* larval haemolymph patterns after treatment with pesticides (2-3-4) and detergents (5-6) Control strips: 1. Specific staining for A. Alcalic phosphatases, B Esterases, C. Amylases. Further explanation see text.

formerly widespread in rivers and rivulets in the Benelux countries, are really extinct now in vast areas polluted by industrial expansion, by waste products of a dense population, or both.

At present, *Ephemera danica* can still be found only in the Ardennes and in some rare lowland brooks that have escaped environmental pollution up to now. While it was easy to obtain haemolymph in quantities sufficient for electrophoresis, the large larvae of this species were chosen for the following experiments.

After a comparison had been made with haemolymph pherograms from some Plecoptera and Trichoptera which were collected in the same water, the specificity of *Ephemera* was checked against some other Ephemeroptera larvae, *Ecdyonurus venosus* and *Cloeon dipterum*. The *Ephemera* pattern proved to be highly specific and little subject to variability (fig. VI, 2-7). Components that in younger larvae migrate not far from the cathode and those of medium mobility, tend to fade in the period before moulting into the subimago only (fig. VI, 1).

Pesticides of different kinds and used for different purposes were dissolved or brought into the water in which the larvae had been staying for 24 hours. Concentrations were made with the same content that is usually applied in agricultural practise, at least, according to the prescriptions of Belgian directions of use. This was the case for the insecticides (2) „Gusathion" (asinfos-methyl), (3) „Perfekthion" (dimethoate), (4) „Gorsac 25" (heptachlorine) and for the nematicide „Nemagon 20" (dibromochloropropane, fig. VI, 9). (The numbers refer to fig. VII ; A1, B1 en C1 are control strips).

While freshwater animals in general are threatened and killed by detergents and washing-preparations also, a series of experiments was started in which the Mayfly larvae were put in different sorts of waste water. In these solutions the pollutants were dissolved in a concentration comparable to those in waste water, in outlets of houses, or in sewages discharging freely in brooks, dykes or rivers. The products examined were a detergent commonly used for both industrial and domestic purposes, a concentrated, slightly acid solution with an organic fraction mainly consisting of the following type : $C_{14}H_{29}$-CH_3-CH-OSO_3Na ; and a product used in dishwashers, in powder form, chiefly containing an anorganic fraction in which considerable amounts of phosphates, sulphates and silicates could be detected (numbers 5 and 6 in fig. VII).

The influence of all products, pesticides and detergents, upon the larvae after 24 hours can be summarized briefly. Most animals were dead ; in some of them, however, a trace of life was still present while antennae or legs were moving convulsively from time to time ; all together, not quite an astonishing result. After haemolymph was taken from the larvae, they were fixed (Bouin and Carnoy fixation) and sectioned in order to perform some histochemical staining.

In the electropherograms the influence of the products can be stated easily already after routine staining by amido-black or naphtol blue black. Most components, both fast migrating and slow migrating ones, have profoundly changed mobilities ; some of them, clearly present in the control run, have disappeared completely with the intoxicated larvae. On the other hand, some new fractions may appear, possibly as a consequence of the dissolution of the original protein fractions. Moreover, the impact of the detergents clearly shows their

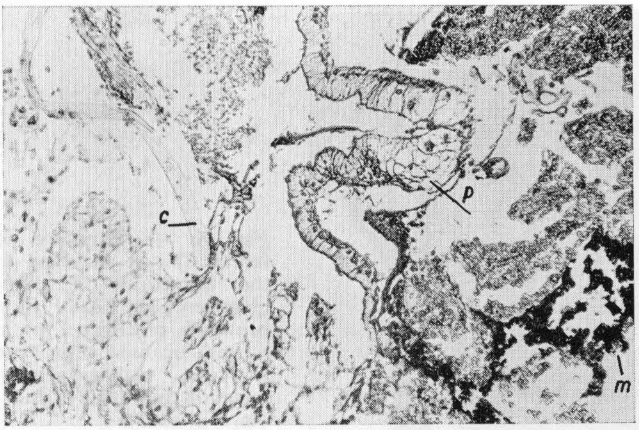

Fig. VIII. — Hypertrophic reaction against a cellophane strip (c), carefully brought into a chrysalid (hemalum-eosin, 7 μ, x100), results in melanin formation (m), necrosis and hypertrophy of the digestive tract epithelium (p).

surface-active characters : some pherograms have become completely hazy by melting of the different fractions (fig. VI, 8).

More detailed information on the influence of pesticides and detergents can be obtained by staining the electrophoretic runs by methods specific for enzymes (Manwell and Baker, 1967). Among others, alkaline phosphatases seem to suffer no harm form the above mentioned substances, except from heptachlorine which seems to inhibit phosphatase activity completely (fig. VII A, 4). Mobilities of esterase-active components are thoroughly modified, a phenomenon which could be confirmed by two different staining methods for non-specific esterases (fig. VII B, 1-6) : indoxyl acetate) ; cholinesterase activity is influenced and even destroyed by all substances tested (all recipes from Wieme, 1965).

Digestive enzymes are also influenced, but in quite a different way : proteases

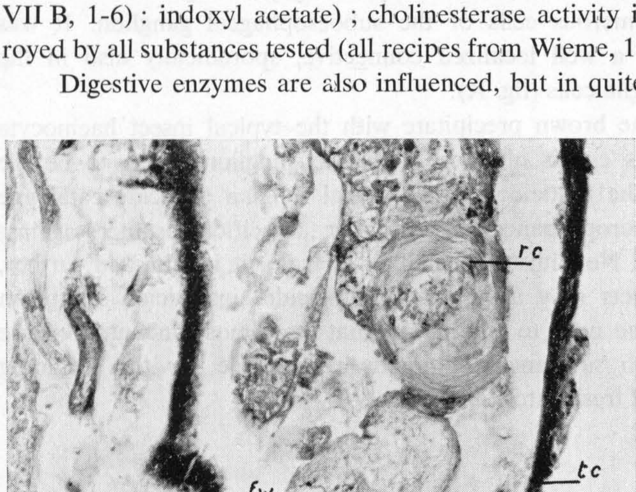

Fig. IX. — Testis from *Pieris* chrysalid treated with benzidin, a product cangerigenous for Vertebrates (hemalum-eosin, 7 μ, x300). Further explanation see text.

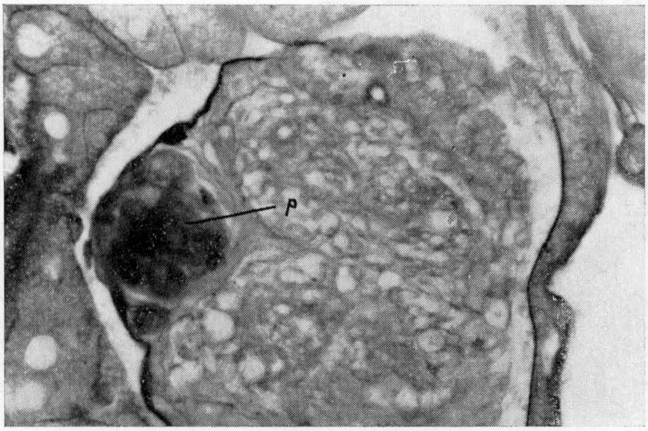

Fig. X. — Exposure of larval *Cloeon* mayflies to the nematicide dibromochloropropane leads to a brownish precipitate (p) in a well localised connective of the suboesophageal ganglion (paraldehydefuchsin, 7 μ, x425).

apparently remain untouched while amylases get modified mobilities, though slightly, and in some cases seem to be activated. By adding dimethoate, even a new component with amylase activity had to be noted (fig. VII C, 3 ; staining methods cf. Uriel, 1960 and Gysels, 1968). In fact this is not surprising at all, if one takes into account that detergents render membranes more permeable and thus increase the extractability of proteins from cells.

Finally a series of histological preparations was performed. Besides by common Mallory and eosin-haemalum, they were stained by paraldehyde-fuchsin (Gabe, 1968). Treatment of larval *Cloeon dipterum* mayflies by „Nemagon 20" led to a brownish precipitate in numerous cells of the suboesophageal ganglion. It was intensively concentrated in a well localized connective, sporadically also in the adjoining commissures and glia cells (fig. X).

The resemblance of the brown precipitate with the typical insect haemocyte reaction against a parasite's egg is obvious. Thus the ganglion seems to be the probable site of attack of the pesticide. In the offical Belgian pesticide catalogue „Nemagon 20", dibromochloropropane, is listed under „Specific substances against eelworms" (= Nematodes). Notwithstanding this predicate, it is admitted further, that „some of these products may also have a fungicide, insecticide and even herbicide effect". There is no need to emphasize, that the almost uncontrolled use of major quantities of such substances is greatly responsible for the far-going damages in the natural life of freshwaters and soils.

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SUMMARY

A first series of electrophoretic slides deals with the variability of haemolymph patterns of *Stilpnotia salicis* (developmental stages, parasitism...).

Another series of experiments with regard to the characteristics of pathological tissues and experimental tumours was carried out on *Pieris brassicae* (input of foreign substances, nerve sectioning...).

Insect haemolymph patterns are specific and do not quickly undergo profound changes, by pathological situations caused by external influences. They are, however, completely changed by the influence of pesticides and detergents, as was recorded with *Ephemera danica* larvae. By specific staining methods it was demonstrated that especially enzyme patterns are altered seriously.

SAMENVATTING

Met behulp van de elektroforese in agar werd de hemolymfe onderzocht van verschillende insekten, zowel van enkele voor land- en bosbouw schadelijke soorten (o.a. koolwitje en zijdevlinder) als van de voor de mens onschadelijke, stromend water bewonende larven van één van onze grootste Eéndagsvliegen. Naast enkele gegevens over de variabiliteit te wijten aan verschillende ontwikkelings- en andere fysiologische toestanden worden mede aan de hand van specifieke histochemische kleuringen enkele voorbeelden gegeven van de mogelijke invloed die bepaalde landbouwgiften en wasmiddelen kunnen uitoefenen op het metabolisme van beslist onschadelijke en voor het oekologisch evenwicht van de waterlopen onmisbare insekten.

RESUME

L'article présent offre quelques conclusions sur les variations en composition protéique de l'hémolymphe de quelques insectes nuisibles à l'agriculture, ainsi que sur l'influence que peuvent effectuer des pesticides et des détergents sur le métabolisme des insectes non nuisibles habitant l'eau douce courante.

Les experiences furent effectuées avec l'électrophorèse en agargel, combiné avec des méthodes de coloration histochimiques. Ainsi il y a moyen de comparer les changements en composition protéique provoqués par des agents naturels intérieurs et extérieurs, p.e. la situation physiologique et les parasites, avec les perturbations et détériorations causées par les produits chimiques appliqués par l'homme dans sa lutte contre les pestes agricoles.

ZUSAMMENFASSUNG

Mit Hilfe der Elektrophorese in Agar wurde die Haemolymphe verschiedener Insekten untersucht, sowohl die einiger Arten, die für Land- und Forstwirtschaft schädlich sind *(Pieris* und *Stilpnotia*), als auch die der für den Menschen unschädlichen, fliessendes Wasser bewohnenden Larven einer unserer grössten Eintagsfliegen (Agarpherogrammen mit einigen spezifischen histochemischen Färbungen angefült).

Neben einigen Daten über die Variabilität, die durch verschiedene Entwicklungs- und andere physiologische Zustände verursacht wird (auch die spezifischen histochemischen Färbungen spielen dabei eine Rolle), liefert diese Arbeit einige Beispiele des eventuellen Einflusses, den bestimmte Gifte, die in der Landwirtschaft und bei der Herstellung von Waschmitteln gebraucht werden, auf den Metabolismus ausüben können : Metabolismus von wirklich unschädlichen Insekten die für die Oekologie der Wasserläufe unbedingt notwendig sind.

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