Identification of intracellular particles of pesticides in ciliate protozoa by Raman microprobe

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Summary. A Raman microprobe technique was used for the identification of intracellular particles of a pesticide added to a culture of the ciliate protozoan *Colpidium campylum.* 2 methods, a wet and a dry, can be used for sample preparation. In both cases it was possible to find characteristic lines of 2 pollutants: 4′,4′-dichlorodiphenyl and β-endosulfan. Some technical problems were encountered: the dry method causes drastic cellular alteration; the cell can move and go out of laser focus in the wet method.

Many ciliate protozoa have a strong endocytic activity. They ingest both nutrient and inert particles and have the ability to concentrate insoluble pollutants if the particles are small enough to be ingested[1]. During experimentation, 2 questions can be asked: Are ingested particles really particles of the pesticide added?; is there a modification of crystalline or chemical configuration in digestive vacuoles of ciliates?

The Raman spectrum can be used as a reference for a substance. It provides information about the kind of chemical structure and crystalline configuration present, even if the reference spectrum has not been theoretically explained. With the Raman microprobe we are able to record Raman lines for microscopic samples. So use is made of the microprobe to give answers to both of the questions asked about the endocytosis of pesticides by ciliate protozoa.

Materials and methods. The Raman microprobe makes it possible to identify a substance by reference to its vibrational frequencies (the motions of atoms in a molecule correspond to the characteristic frequency-stretching vibrations of each kind of molecule) and permits the precise determination of the position of this substance in a microscopic sample. The results of the analysis are recorded on chart paper. The size of the inclusions which can be detected may be, in good cases, as small as 1 micron. Upon radiation by a laser beam the sample scatters light charac-

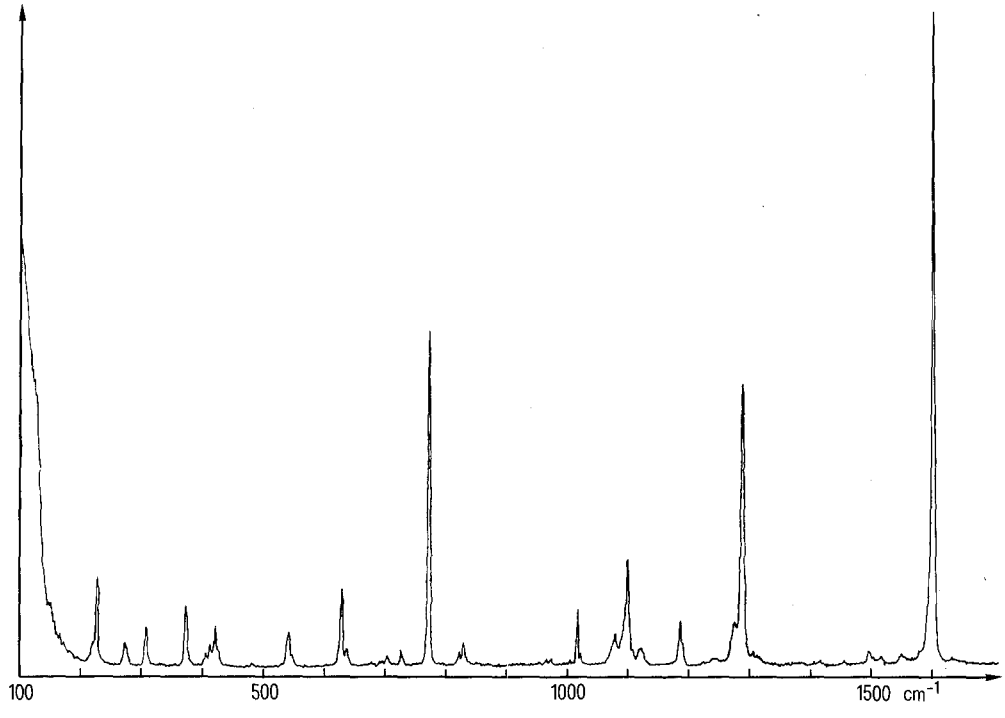

Fig. 1. Reference Raman spectrum of 4′,4′-dichlorodiphenyl.

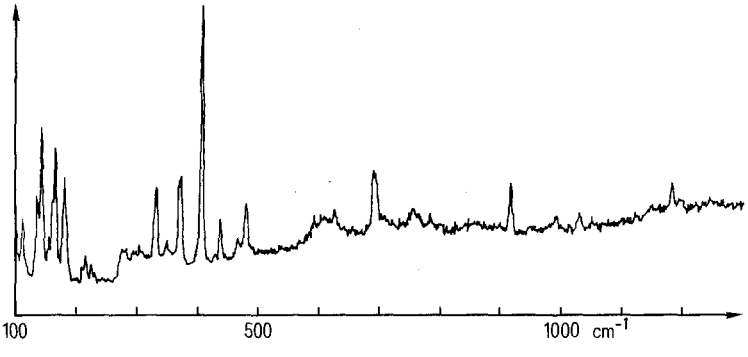

Fig. 2. Reference Raman spectrum of β-endosulfan.

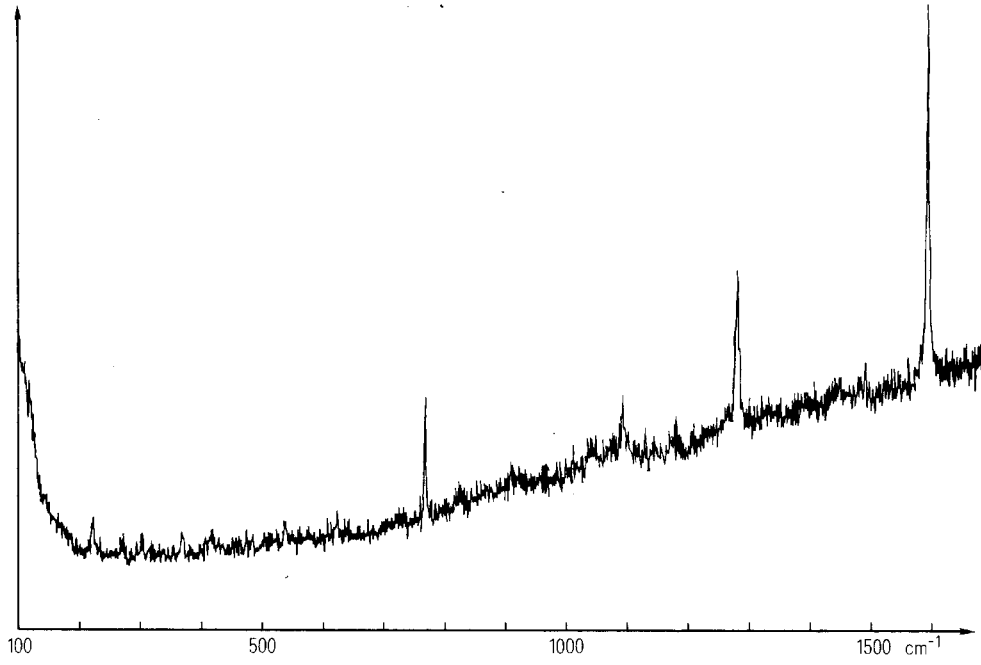

Fig. 3. Raman spectrum of 4′,4′-dichlorodiphenyl in a digestive vacuole of *Colpidium campylum*.

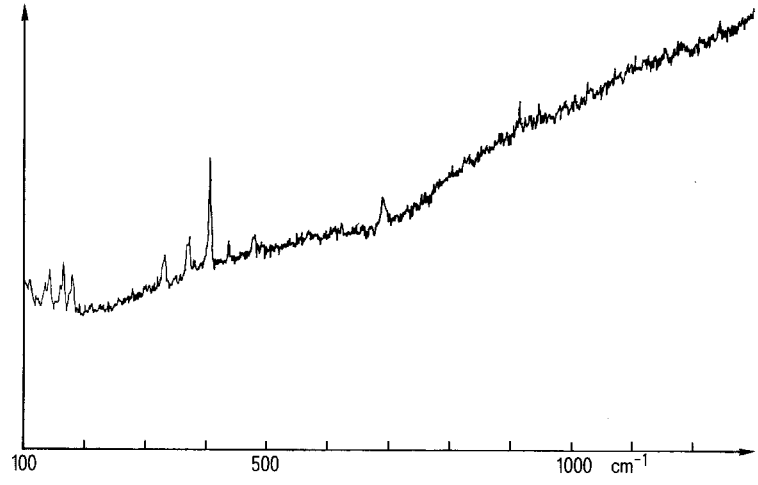

Fig. 4. Raman spectrum of β-endosulfan in a digestive vacuole of *Colpidium campylum*.

Wavelength and characteristics of lines of 4′,4′-dichlorodiphenyl and endosulfan in reference spectra and in a digestive vacuole of *Colpidium campylum*

Endosulfan	Endosulfan in *Colpidium*	4′,4′-Dichlorodiphenyl	4′,4′-Dichlorodiphenyl in *Colpidium*
112 W	112	227 M	227
135 W	135	272 VW	272
145 M	145	307 W	307
160 M	160	372 W	372
167 M	167	420 W	420
182 M	182	540 W	540
332 W	332	628 M	628
375 W	375	770 S	770
407 S	407	1017 W	1017
437 W	437	1098 M	1098
480 W	480	1185 W	1185
692 W	692	1285 S	1285
917 W	917	1600 VS	1600

Intensities: VW, very weak; W, weak; M, medium; S, strong; VS, very strong.

teristic for the substance which constitutes the inclusion. The Raman spectrum provides a good 'fingerprint' of the product.

The pesticides, 4′,4′-dichlorodiphenyl and β-endosulfan, were used in acetone solution in the concentration range 2.5 to 25 mg/l. 20 µl of acetone solution was added to 1 ml of 10 mM Tris-HCl buffer (pH 7.2) and the mixture was treated for 1 min in an ultrasonic glassware cleaning apparatus (Kerry Ultrasonic Inc.). The pesticide was thus dispersed as very small particles which could be ingested by ciliates.

24-h *Colpidium* cultures were centrifuged (1000 rpm/3 mn); the supernatant was discarded and cells were resuspended in 10 mM Tris-HCl buffer. The volume of the ciliate suspension was adjusted to 4 ml for a cell concentration within 50,000 to 100,000 cells/ml. Then pesticide suspension was added to the protozoa. At various time intervals aliquots were fixed with an equal volume of 4% glutaraldehyde and the cells were decanted.

For Raman examination, 2 methods were used: 1. 1 drop of treated sample was examined under a cover glass with a

microscope; 2. 1 drop was placed on a cleaned glass slide and dried.

Under a 800-1000-times magnification, the laser was focused (1-2 µm) on digestive vacuoles containing apparently insoluble material. The major diffusion lines were surveyed, then a Raman spectrum could be studied between 100 to 3500 cm^{-1}; the more interesting diffusion lines occur between 100 and 2000 cm^{-1}.

Results and discussion. It has been possible to obtain spectra for intravacuolar pesticides with both methods: figures 1 and 2 give reference spectra, and figures 3 and 4 give the spectrum response of digestive vacuoles of ciliates. The table gives the wavelength and characteristics of the lines found in the control and the samples for 4′,4′-dichlorodiphenyl and β-endosulfan respectively. In both cases, the most important lines of reference of the sample are the same as those of the pesticide added. No essential modification in chemical structure occurs, because both the reference and sample spectra are the same.

Both wet and dry methods can give good results in analysis but each method has its own advantages. With the wet method, research is quite easy. But currents can occur in the preparation due to the local thermic effect of the laser beam, so that the cell can move and no longer remain in focus. With a very thin preparation, the phenomenon is reduced. With the dry method, many cells are drastically altered morphologically, and collapsed, so it is very difficult to see precisely the structures that are interesting for analysis. The advantage is that the cells do not move under laser focus.

Theoretically, it is possible to embed the cells in a solid medium, but many solvents can extract the interesting product so that it is lost during preparation. The research into good preparation conditions continues. The medium has to be hydrosoluble, must not be fluorescent and must give a minimal Raman diffusion spectrum.

With the Raman microprobe, it is not possible to identify particles below a size of 1 or 2 µm. The concentration of the substance must be sufficiently high; this last condition is always fulfilled when we study precipitated or crystalline material in the cell. It is necessary to know the reference spectrum of a substance to identify it in the cell.

So we think that Raman spectrometry can be a very interesting method for biological studies which involve looking for chemical changes in studying their crystalline configuration.

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Neurosecretory system and storage of paraldehyde fuchsin positive neurosecretory material in the dorsal aorta in an earwig, *Anisolabis annulipes* Lucas (Dermaptera: Labiduridae)

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Summary. Each of the 2 groups of medial neurosecretory cells has 10-12 A and 2-3 B-cells. Each pars intercerebralis lateralis has 3-4 B-cells only. The 2 nervi corporis cardiaci I (Ncc I) join with the lateral wall of the aorta and Ncc II terminate in corpora cardiaca (Cc). Only 1 corpus allatum is present. The paraldehyde fuchsin positive neurosecretory material is stored in the dorsal aorta and not in the Cc which indicates the neurohaemal nature of the aorta.

In most of the insect orders the paraldehyde fuchsin positive (PF-positive) neurosecretory material (Nsm) produced by the medial group of brain neurosecretory cells (Nsc) is stored in the corpora cardiaca (Cc), but in Dermaptera divergent views have been given regarding the storage of the Nsm[2-8]. Ozeki[2] and Gabe[3] pointed out that Cc stores the medial as well as lateral neurosecretion in all Dermaptera, whereas Awasthi[5,6], studying 2 species of earwigs, found Nsm in the dorsal aorta only. These variations suggested it might be useful to gather more information on this topic, and the present communication describes the neurosecretory system and storage organ for Nsm in the earwig *A. annulipes*.

Materials and methods. Earwigs were reared in the laboratory on sliced bread. The brain and endocrine aortal complex was taken out by dissecting the insects in Bouin's fixative, and fixed for 18-24 h. The fixed materials (both bulk preparations and sections) were stained in PF (Ewen[9]) and sections were cut 6-8 µm thick.

Observations. The neurosecretory cells. Each protocerebral hemisphere of *A. annulipes* bears a group of median Nsc. Each group consists of 10-12 compactly arranged A-cells and 3-4 B-cells which are somewhat oval in shape. Nsc are lodged very close to the periphery of the brain and are dorsal in position. The pars intercerebralis lateralis has 2-3 B-cells which are smaller in size than the median B-cells. Cyclic secretory activities have been observed in A-cells only. In a freshly moulted earwig these cells have a very small amount of Nsm, which gradually increases as the insect becomes older and is maximal during mating and oviposition.

The neurosecretory pathways. The axons of each group of Nsc in the brain converge and form a medial neurosecretory pathway (Mnp) (figure 1) in each brain lobe. The 2 pathways of the 2 brain lobes cross over. Posterior to the crossing point they run separately on the dorsal side of the protocerebrum and emerge as nervi corporis cardiaci I (Ncc I). Each Ncc I enters the dorsal aorta on its lateral side. Similarly 2 short Ncc II arise from the posterior region of the brain and join with the anterior region of the Cc.

The dorsal aorta. The aorta is located above the Cc and the corpus allatum (Ca) and is full of Nsm. The Cc and Ca lack PF-positive Nsm. The wider anterior part of the aorta receives the neurosecretory axons of the Ncc I from the lateral sides. This region has many more neurosecretory granules than the posterior region. The granules are totally