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INTERACTIONS OF ORGANIC POLLUTANTS WITH GILLS OF THE BIVALVE MOLLUSCS *ANODONTA CALIFORNIENSIS* AND *MYTILUS CALIFORNIANUS*: UPTAKE AND EFFECT ON MEMBRANE FLUXES. II

JAMES H. SWINEHART and MARCOS A. CHENEY
Department of Chemistry, University of California, Davis, California 95616, USA

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Abstract—1. The uptakes of 2,4,5-T, glyphosate, parathion, paranitrophenol, naphthalene, glycine, and inulin by gills of the bivalve molluscs *Anodonta californiensis* (freshwater) and *Mytilus californianus* (marine) show non-polar compounds are taken up to a greater extent than polar compounds except where active transport occurs.

2. The uptake of glycine by *M. californianus* is reduced by pollutants containing complexing functional groups but not by non-polar compounds.

3. The uptake of parathion alters the polyphosphate–inorganic phosphate balance in *M. californianus*.

4. The uptakes of pollutants parallel their toxicities toward rats.

INTRODUCTION

Organisms can remove metals and organic chemicals from water in both soluble and particulate forms (Martin, 1967). Particulate forms include condensed heavy metals (e.g. iron), organics (humic materials), and chemicals adsorbed on inert materials. Soluble forms include a complete spectrum of organics (pesticides, amino acids, saccharides, lipids, humic materials, etc.) and metal ions from natural and pollutant sources. Complexation or association between metal ions and organics is important because the resulting complexes can be the dominant form of the soluble materials available to an organism for uptake. The introduction of heavy metals and/or organics from sewage water, mine seepage, agricultural sources or areas of high natural biological activity can dramatically alter the complexation equilibria present and thus the bioavailability of chemicals to an organism.

The intracellular pool of free amino acids is an example of how changes in the external medium can affect a component of a biological system. Alteration of such pools in bivalve molluscs occurs when the organism is subjected to stresses such as variations in the osmolality (Pierce, 1971) or pollutants in the medium (Sansone *et al.*, 1978). The flux of amino acids depends on the external divalent cations (Ca^{2+} and Mg^{2+}) concentrations, osmolality of the medium, membrane permeability and ATP (Pierce and Greenberg, 1973, 1976; Watts and Pierce, 1978).

This research involves an investigation of the uptake of potential organic pollutants and glycine moderated by pollutants into gills of the marine bivalve mollusc *Mytilus californianus* and the freshwater bivalve mollusc *Anodonta californiensis*.

The chemicals examined in the course of this study include the amino acid, glycine, and organic pollutants such as: 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), *N*-phosphonomethylglycine (glyphosate), phosphorothioic acid *O,O*-diethyl-*O*-(4-nitrophenyl) ester (parathion) and its breakdown product paranitrophenol, *N,N*-bis(phosphonomethyl)glycine (glyphosine, a ripening agent), and naphthalene (a potential petroleum-based pollutant). The uptake of inulin, a high molecular wt sugar assumed to occupy only extracellular space, was determined to ascertain if some pollutants also occupy only extracellular space.

A study of the uptake of pollutants and how pollutants affect the uptake of actively transported glycine in *M. californianus* can give information about the molecular interactions involved in membrane transport processes and the mode of toxicity of pollutants.

MATERIALS AND METHODS

Molluscs

Specimens of the marine mollusc *M. californianus* were collected from the open coast at the Bodega Marine Laboratory, University of California, Bodega Bay, California. Animals were maintained at 13°C in aerated seawater and were used as soon as possible after collection. Specimens of the freshwater mollusc *A. californiensis* were collected in irrigation canals approximately 5 miles north of Knights Landing, California. Specimens were used within a week of collection and were maintained in an aquarium having water and temperature conditions of the natural environment.

Uptake experiments

All experiments with *M. californianus* were carried out in artificial seawater (ASW) prepared with reagent grade salts and distilled water according to Cavanaugh (1956). The medium used for experiments on gills of *A. californiensis* was distilled water or distilled water containing Ca^{2+} and Mg^{2+}. The divalent cations were added as the chloride hydrates and the concentrations of the divalent cations were determined by atomic absorption spectroscopy. To these solu-

tions organic compounds of known purity, with or without their radiotracers, were added. In the case of glyphosate uptake the results were independent of whether the source was pure glyphosate or the commercial products Roundup or Monsanto 75%.

When the uptake of a particular organic compound was to be studied, a solution 10^{-4} M in that compound or 10^{-5} M in the case of naphthalene, containing a small concentration of the ^{14}carbon labelled radiotracer was prepared. Typically the concentration of the radiotracer was about 10^{-8} M. In some experiments another organic compound was added to this solution in order to determine the effect of the compound on the uptake of the radiolabelled material.

Prior to the experiments, excised gills were treated in the following manner. Gills of the freshwater species *A. californiensis* and the marine species *M. californianus* were incubated in unchlorinated natural freshwater and ASW, respectively, for periods of up to 30 min. The gills were then allowed to drain, dried lightly on a paper towel and placed in the solution used in the experiment. The beakers containing the experimental media and gills were placed on a shaker table. At pre-determined times, gill samples were removed from the solution, washed for several seconds in the experimental solution devoid of the organic compounds, and blotted on filter paper. The gills were placed in tared scintillation vials, dried to constant weight at 50–70°C and weighed to the nearest 0.1 milligram. One millilitre of 0.1 M nitric acid was added to the dried gills, the mixture was allowed to stand for 6 hr, 10 ml of scintillation fluid was added, the mixture was incubated for at least 12 hr, and the radioactivity of the vials were determined with a Beckman Scintillation model 6800 counter. The data were computed as μmoles of organic uptake per gram dry wt of sample using the activity of a known volume of the experimental solution, and thus a known amount of organic material before the gills were added. All data points are an average of two experiments with each point in duplicate.

^{31}*Phosphorus nuclear magnetic resonance (NMR) experiments*

^{31}Phosphorus NMR experiments using gills of *M. californianus* were carried out on a Nicolet 200 MHz NMR spectrometer. Gills were treated as described in the previous section. After proper incubation, exposure to parathion and washing to remove external parathion, the gills were placed in a 20 mm tube and the ^{31}phosphorus NMR spectrum was recorded.

RESULTS

Incorporation of 2,4,5-*T, glyphosate, parathion, paranitrophenol (pH variation), naphthalene, glycine and inulin*

All uptake studies were carried out at 10^{-4} M carrier except for naphthalene which was limited to 10^{-5} M due to its solubility.

A. californiensis. Figure 1 shows uptake of 2,4,5-T at 10^{-4} M with no added glycine and with added glycine at 10^{-3} and 10^{-4} M, and glycine itself. Figure 2 shows the uptake of glyphosate at 10^{-3} M with no added glycine and with added glycine at 10^{-3} and 10^{-4} M. Additionally the uptake of inulin is shown assuming a molecular wt of 5000. The uptakes of both 2,4,5-T and glyphosate are independent of the presence of glycine. The uptake of inulin, which occupies only extracellular space, is shown for comparative purposes and will be used in a later discussion of whether some pollutants are actually taken into the gill or occupy extracellular space. Figure 2

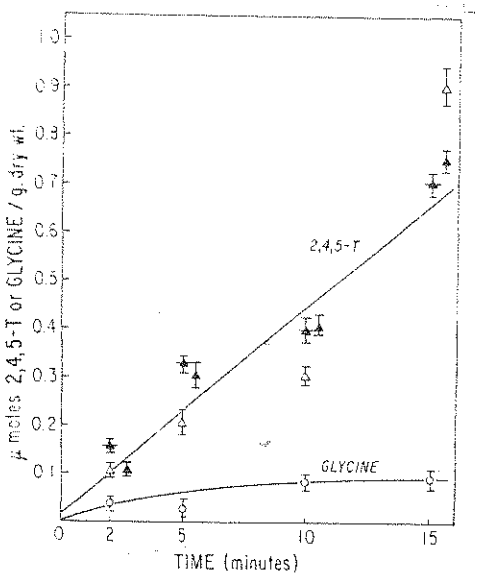


Fig. 1. Uptake of 10^{-4} M 2,4,5-T in distilled water (△) with 10^{-4} M (▲) and 10^{-3} M (▲) glycine, and 10^{-4} M glycine itself (○) by gills of *A. californiensis*.

also shows the uptake of 10^{-5} M naphthalene, a non-polar molecule potentially present in oil spills, into gills of *A. californiensis* both in the presence and absence of glycine at 10^{-5} M. At these concentrations glycine has no effect on the uptake of naphthalene.

Figure 3 shows the uptake of parathion, a neutral molecule, both with and without glycine at 10^{-4} and 10^{-3} M. Glycine has no effect on parathion uptake. Figure 3 also shows data on the acid dependence of

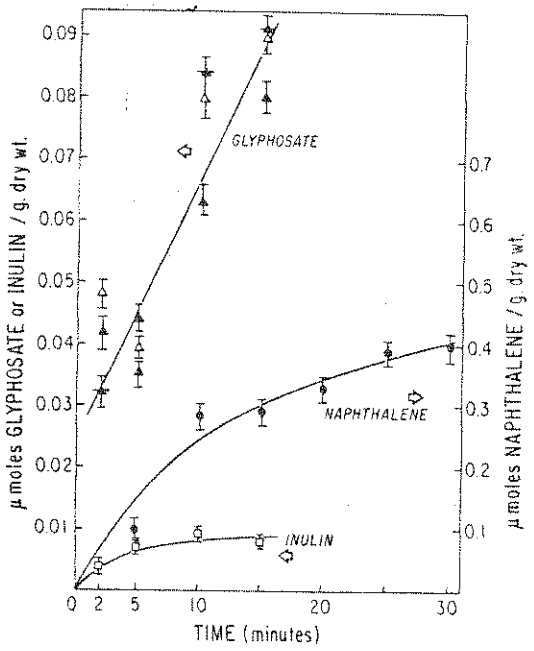
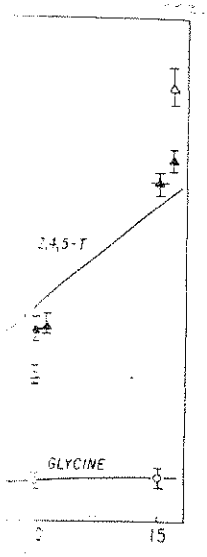


Fig. 2. Uptake of glyphosate 10^{-4} M (△) with 10^{-4} M (▲) and 10^{-3} M (▲) glycine, 10^{-5} M naphthalene (solid line) with 10^{-3} (●) glycine, and inulin (□) at an assumed molecular wt of 5000 by gills of *A. californiensis*.



distilled water (△) with
and 10^{-4} M glycine
californiensis.

M naphthalene, a
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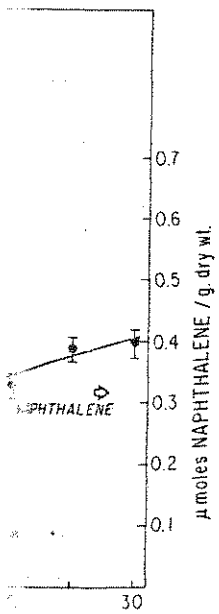


△) with 10^{-4} M (▲)
phthalene (solid line)
□) at an assumed
A. californiensis.

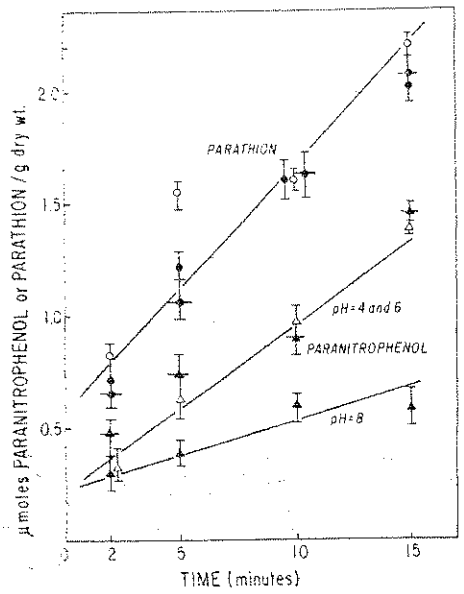


Fig. 3. Uptake of 10^{-4} M paranitrophenol at pH = 8 (▲), pH = 6 (△) and pH = 4 (▲), and the uptake of 10^{-4} M parathion (○) with 10^{-4} M (●) and 10^{-3} (●) glycine by the gills of *A. californiensis*.

the uptake of paranitrophenol, a breakdown product of parathion (Aizawa, 1982). The data on the acid dependence of the uptake of paranitrophenol shows that the neutral form is more readily taken up than the anionic form.

M. californianus. Figure 4 shows data on the uptake of 2,4,5-T and glyphosate with no added glycine and with 10^{-4} and 10^{-3} M glycine, naphthalene at 10^{-5} M with and without 10^{-5} glycine, and 10^{-4} M inulin assuming a molecular wt of 5000. Figure 5 shows data for the uptake of parathion with and without 10^{-4} and 10^{-3} M glycine and, for comparison, an average line from five experiments on the uptake of glycine. The incorporations of 2,4,5-T, glyphosate, naphthalene and parathion into gills of *M. californianus* are independent of glycine.

The large uptake of parathion by gills such as those of *M. californianus* and the known effect of parathion on enzymatic processes (Williams and Lansford, 1977) lead to an examination of the ^{31}phosphorus NMR spectrum of such gills with and without exposure to parathion (Fig. 6). The spectra show the appearance of parathion (62.5 ppm) in the gills and the reduction and disappearance of signals attributable to the β-phosphorus in compounds such as ATP and an increase in the signal of inorganic phosphate.

Uptake of glycine by gills of M. californianus *as affected by 2,4,5-T, glyphosate, glyphosine, parathion and naphthalene*

Figure 7 shows the effect of glyphosate at 10^{-4} and 10^{-3} M on the uptake of glycine into gills from *M. californianus*. The effects of glyphosine, which is structurally related to glyphosate, and 2,4,5-T on glycine uptake are similar to that shown for glyphosate in Fig. 7. All of these compounds reduce the uptake of glycine. After 15 min the ratios of glycine uptake at 10^{-4} M and 10^{-3} M added glyphosate, glyphosine and 2,4,5-T vs the uptake with only glycine present are: glyphosate, 0.77 and 0.33; glyphosine, 0.76 and 0.25; and 2,4,5-T, 0.85 and 0.50.

Figure 8 shows that parathion, a compound containing no complexing functional groups, has no effect on the uptake of glycine. Additionally, 10^{-5} M naphthalene does not affect the uptake of 10^{-5} M glycine.

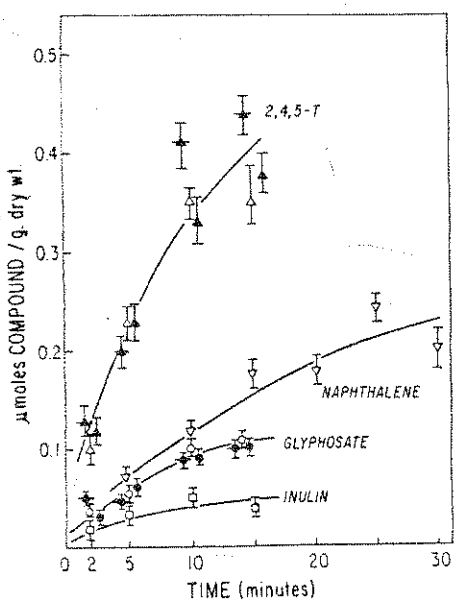


Fig. 4. Uptake of 10^{-4} M glyphosate (○) with 10^{-4} M (●) and 10^{-3} M (●) glycine, 10^{-4} M 2,4,5-T (△) with 10^{-4} M (▲) and 10^{-3} M (▲) glycine, 10^{-5} M naphthalene (solid line) with 10^{-5} M glycine (▽), and 10^{-4} M inulin (□) assuming a molecular wt of 5000 by gills of *M. californianus* in ASW at pH = 7.8.

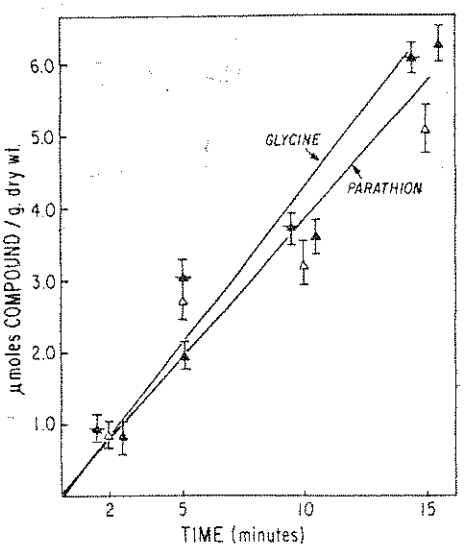


Fig. 5. Uptake of 10^{-4} M parathion (△) with 10^{-4} M (▲) and 10^{-3} M (▲) glycine, and 10^{-4} M glycine itself by gills of *M. californianus* in ASW at pH = 7.8.

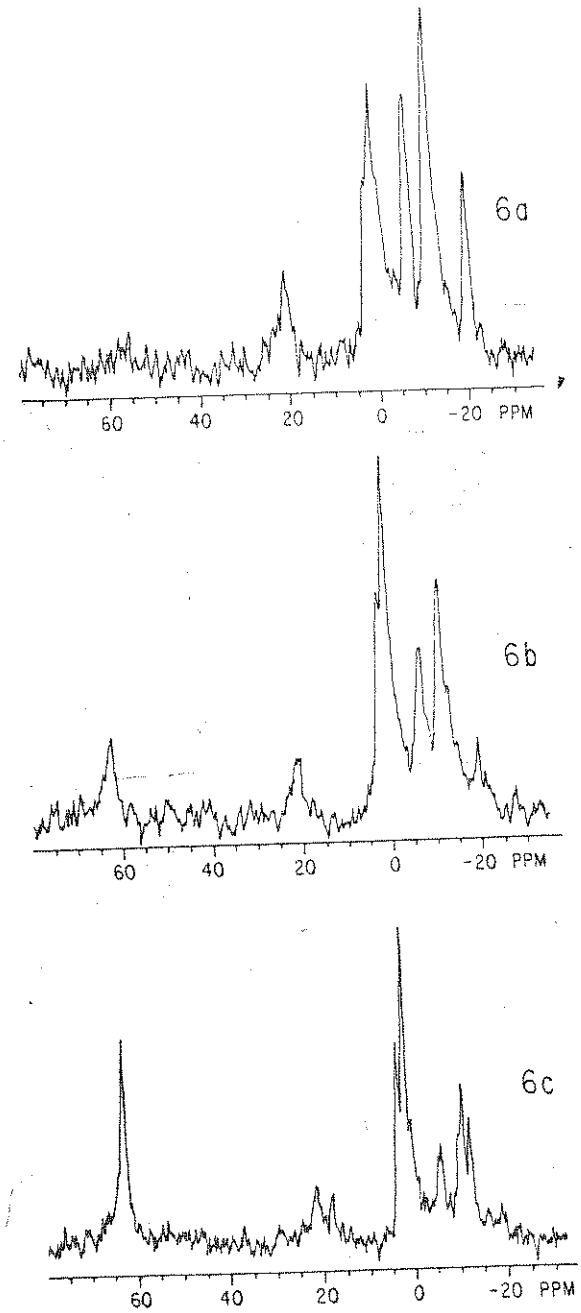


Fig. 6. (a) ^{31}P NMR of gills of *M. californianus*, (b) ^{31}P NMR of gills of *M. californianus* after 30 min incubation in 10^{-3} M parathion, and (c) ^{31}P NMR of gills of *M. californianus* after 4 hr incubation in 10^{-3} M parathion.

DISCUSSION

Uptake experiments

In the case of the gills of *A. californiensis* the data in Figs 1–3 and from a previous study on 2,4-D (Swinehart and Cheney, 1983) show that the order of uptake of the compounds investigated is inulin < glycine ≈ glyphosate < 2,4-D < 2,4,5-T ≈ PNP^- < PNPH < parathion. The pK of paranitrophenol is 7.1, and PNPH and PNP^- are the neutral and anionic

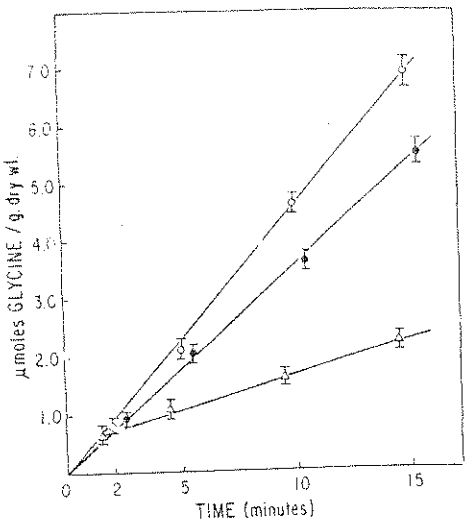


Fig. 7. Uptake of 10^{-4} M glycine (○) with 10^{-4} M (●) and 10^{-3} M (△) glyphosate by gills of *M. californianus*.

forms respectively. Data in Figs 4–6 and from a study of 2,4-D and paranitrophenol show that for the gills of *M. californianus* the order of uptake of the compounds studied is inulin < glyphosate < 2,4-D ≈ 2,4,5-T ≈ paranitrophenol ≪ parathion ≈ glycine. In this case glycine is known to be actively transported. At the pHs of the studies of *A. californiensis* glycine is a dipolar ion (zwitterion) and glyphosate, 2,4-D and 2,4,5-T are anions. In studies involving *M. californianus* these ionic compounds will be partially complexed with the Mg^{2+} and Ca^{2+} present in ASW.

Naphthalene uptake by gills of *A. californiensis* (Fig. 2) and *M. californianus* (Fig. 4) are 0.4 and 0.2 μmoles/g dry wt at 15 min respectively. The concentration of carrier in this experiment is 10^{-5} M and

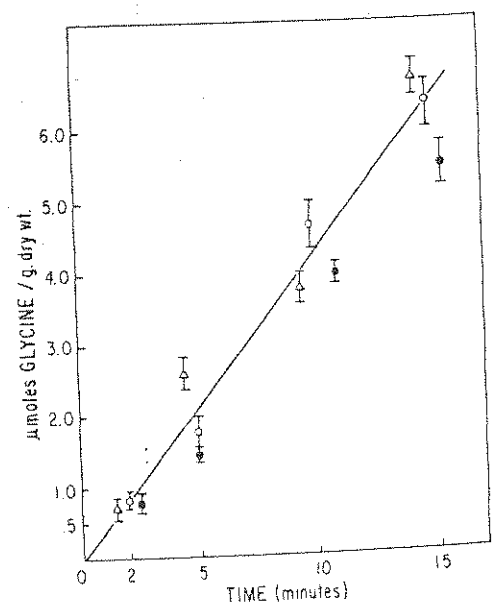


Fig. 8. Uptake of 10^{-4} M glycine (○) with 10^{-4} M (●) and 10^{-3} M parathion (△) by gills of *M. californianus*.

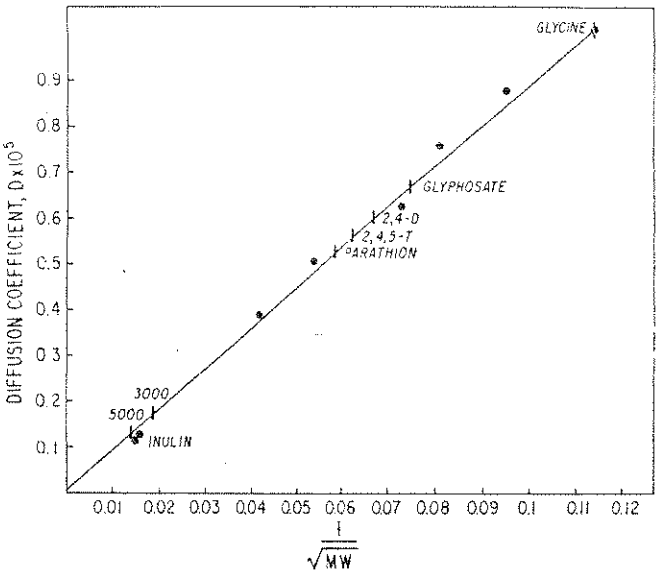
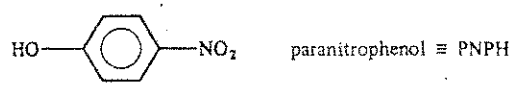


Fig. 9. Plot of values of diffusion coefficients (D) of various compounds vs the inverse of the square root of their molecular wts.

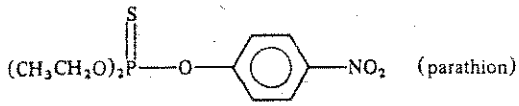
is limited by the solubility of naphthalene. If the experiments could have been carried out at 10^{-4} M carrier, as was the case with other compounds, the uptake could be up to 10 times greater—4 and 2 μmoles/g dry wt—and clearly falls into the range of the uptake of non-polar compounds such as parathion and paranitrophenol, PNPH. The number associated with the uptake of each compound is the μmole/g dry wt taken up at 15 min, and these numbers will be used in comparative calculations concerning the diffusion of compounds.

The order of uptake of the compounds studied for both *A. californiensis* and *M. californianus* shows that parathion is taken up to a greater extent than any other compound. In fact its uptake by gills of *M. californianus* approaches that of glycine, 6 μmoles/g dry wt vs 7 μmoles/g dry wt at 15 min. Whether the non-ionic character of parathion is responsible for its greater uptake is, in part, answered by the uptake of paranitrophenol which has a pK of 7.1 and represents part of the parathion molecule and a known breakdown product of parathion:



paranitrophenol ≡ PNPH

and



(parathion)

respectively. The data in Fig. 3 show that the uptake of paranitrophenol into gills of *A. californiensis* at pH = 4 and 6, is 1.3 μmole/g dry wt, while that at pH = 8 is 0.5 μmol/g dry wt. Thus the neutral form is taken up to greater degree than the anionic form. For comparison the uptake of parathion is 2.2 μmole/g dry wt. Clearly the non-ionic character of parathion and potentially the nitrophenol part of the molecule are important in its enhanced uptake properties.

All of the ionic substances studied except actively-transported glycine in gills of *M. californianus* are taken up to a lesser degree than either paranitrophenol and parathion. Within the limits of these experiments, which of the compounds are actually taken up and which fill only extracellular space? This question can be answered by examining the uptake of inulin, which is known to occupy only extracellular space, vs the uptake of other substances and comparing the uptake with the diffusion coefficients based on molecular wt. Figure 9 shows a plot of literature values of the diffusion coefficients (D) of various compounds vs the increase of the square of the molecular wt. The molecular wt of inulin is reported to be between 3000 and 5000 g/mole, and its diffusion coefficient is then $(0.15 \pm 0.20) \times 10^{-5}$. Comparing ratios of diffusion coefficients with respect to inulin with the ratio of values of uptake at 15 min with respect to inulin will give an indication of which compounds are only occupying extracellular space.

For gills of *A. californiensis* the ratios of the diffusion coefficients of glyphosate and glycine with respect to inulin are 4.5 and 7.1 respectively while the ratios of uptake are 10 and 8 respectively. Thus it appears that the gills of *A. californiensis* do not take up glycine and take up glyphosate only to a small degree. Other substances are taken up to a greater degree and clearly do not only occupy extracellular space.

For gills of *M. californianus* the ratios of the diffusion coefficients of glyphosate, 2,4-D (Swinehart and Cheney, 1983), 2,4,5-T, paranitrophenol, parathion and glycine with respect to inulin are 4.5, 4.0, 3.6, 3.3 and 6.6 respectively while the ratios of uptake are 2.5, 10, 10, 150 and 175 respectively. Thus it

appears that the gills of *M. californianus* take up glycine and parathion to a greater extent and 2,4-D, 2,4,5-T and PNP to a lesser extent. There appears to be no uptake of glyphosate. The diffusion coefficients in Fig. 9 are determined in distilled water and not ASW, but the results are certainly indicative.

The uptakes of parathion for both *M. californianus* and *A. californiensis* exceeds those of other pesticides (Figs 3 and 5). For gills of *M. californianus* parathion uptake is approximately the same as that of glycine (Fig. 5) which is actively transported.

The relative uptake of parathion, 2,4-D, 2,4,5-T and glyphosate parallel their known toxicities. The LD_{50} values for the compounds administered orally to rats are: parathion 3.6–13 mg/kg, 2,4-D 320 mg/kg, 2,4,5-T 300 mg/kg, and glyphosate 5000 mg/kg (The Merck Index, 1976). In a related study (Antunes-Madeira and Madeira, 1979) it has been shown that insecticide-induced increases in the permeability of liposome membranes parallel toxicity toward mammals, and that parathion is especially effective. With regard to uptake of a pollutant vs its toxicity, it is important to recognize that the inherent toxicity of a molecule is not necessarily measured by LD_{50} values for orally administered compounds. For example parathion is both taken up more rapidly by gills of bivalve molluscs (and presumably other tissue) and has a smaller LD_{50} value than other pollutants studied. Of the weight of parathion administered to an organism, measured as mg administered per kg of organism, a very large percentage is actually taken up by the organism as compared to glyphosate. However, the inherent molecular toxicity of parathion may not be larger than that of glyphosate. This is important in assessing from a molecular view the reason for the toxicity of a compound.

The ^{31}phosphorus NMR measurements of gills of *M. californianus* with and without parathion chronicle the incorporation of the pesticide by the appearance of a single at 62.5 ppm (Gurley and Ritchley, 1976). The signal at 20 ppm corresponds to phosphonic acid derivatives, in particular aminoethylphosphonic acid (Glonek *et al.*, 1970). The changes observed in the 5 to −25 ppm region reflect the conversion of polyphosphate compounds represented by resonances at −6, −11 and −20 ppm to inorganic phosphate which has a resonance at about 1 ppm. This observation is consistent with the general observation that parathion affects enzymatic processes (Williams and Lansford, 1977) and according to the above observation has an immediate effect on the polyphosphate–inorganic phosphate balance in the organism. Additionally Rao and Rao (1981) have shown that methyl parathion reduces total lipids and phospholipids in tissue; presumably to supply energy because of the perturbation caused by the pollutant. This observation is consistent with our observation of polyphosphate to inorganic phosphate conversion.

Effects of compounds on glycine uptake and glycine on compound uptake

Observations concerning the effect of one organic compound on the uptake of another can give information about the sites at which uptake occurs. Interesting cases are the effects of 2,4-D, paranitrophenol, glyphosate, glyphosine (a ripening agent) and 2,4,5-T on the influx of glycine into the gills of *M. californianus*. Glycine influx, an active transport process, is reduced by the presence of 2,4,5-T, glyphosate (Fig. 7), and glyphosine, and previously published work with 2,4-D and paranitrophenol (Swinehart and Cheney, 1983) shows that these compounds also reduce glycine uptake. However, glycine uptake is not reduced by parathion (Fig. 8). If glycine initially binds to the gill surface in a Mg^{2+} complex in the glycine uptake process (Swinehart *et al.*, 1980), 2,4-D, paranitrophenol, glyphosine, glyphosate and 2,4,5-T, all of which have complexing functional groups, can effectively compete with glycine for Mg^{2+}. Such a competition reduces the amount of glycine available for transport across the membrane (Sillen and Martell, 1971). Interference with this surface complexation process has been proposed to explain the reduction of glycine influx into gills of *M. californianus* by Hg^{2+}, Cu^{2+} and Fe^{2+} (Swinehart, Crowe and Lee, 1983) Parathion and naphthalene, which have no complexing functional groups, can not compete with glycine for Mg^{2+} and thus do not reduce glycine uptake. The fact that glycine does not interfere with the uptake of the pollutants studied indicates that the paths for their uptake do not involve metal complexation in the case of *M. californianus* and are unrelated to the path for glycine uptake.

Summary

This study shows that: (1) non-polar compounds such as parathion are taken up to a greater extent than polar compounds by both types of gills; in fact for *M. californianus*, the uptake of parathion equals that of actively-transported glycine; (2) comparisons of uptakes with that of inulin which occupies only extracellular space show the following compounds are not taken up, glycine for *A. californiensis* and glyphosate for *M. californianus* gills; (3) the uptake of parathion alters the polyphosphate–inorganic phosphate balance in gills of *M. californianus*; (4) the uptake of glycine by *M. californianus* is reduced by pollutants containing complexing functional groups (2,4,5-T, glyphosate and glyphosine) but not non-polar pollutants (parathion and naphthalene) and thus the effects of pollutants on the uptake of glycine by *M. californianus* are consistent with proposals concerning the metal moderated binding of glycine to gill surfaces; and (5) the uptake of various pollutants parallel their toxicities toward rats.

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