Ficoll Density Gradient Separation of Extracellular DDT from *Chlorella*

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ABSTRACT

DDT, when used at concentrations exceeding its water solubility, forms heterogeneous particles which are not separable from phytoplankton by normal centrifugation or by filtration. Accurate and complete kinetic studies of DDT-phytoplankton interactions therefore, have not been possible. We have found that extracellular DDT particles are separable from Chlorella cells by centrifugation in a discontinuous Ficoll density gradient. The DDT is retained by an 11.5 percent Ficoll layer while the Chlorella cells sediment on a 30 percent layer. By the use of this technique, approximately 99 percent of extracellular DDT particles can be removed from a suspension of Chlorella.

INTRODUCTION

The contamination of the environment by DDT (1,1,1-Trichloro-2,2-bis(p-chlorophenyl)ethane) and its adverse effects on living systems is a prime example of the possible consequences of the uncontrolled use of toxic pesticides (1,2,3). Reports that DDT enters the aquatic food chain primarily at the first trophic level and is concentrated upward have focused attention on DDT-phytoplankton interactions (1,2,3). Kinetic studies are basic to the understanding of such interactions. However, accurate and complete kinetic experiments on the uptake of DDT by phytoplankton populations have been unsuccessful due to problems directly related to the hydrophobic nature of DDT (4,5).

It has been shown that when using concentrations of DDT within its reported water-solubility limits ($3.4X10^{-7}$ to $1X10^{-4}$ g/100 ml) (6), heterogeneous DDT particles ranging in size from 1 to 7 microns form and sediment from the aqueous phase on standing (7,8). An attempt was made to study the kinetics of ^{14}C-DDT uptake by Chlorella pyrenoidosa at concentrations near the upper solubility limit of DDT (9). We observed that when using low-speed centrifugation for the harvesting and washing of the cells, 90 percent of the

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DDT sedimented from the test suspension and from the control, indicating that the DDT is co-sedimenting with the cells. Further attempts to separate labeled cells from extracellular DDT by centrifugation at lower speeds and by filtration were unsuccessful. It was therefore impossible to differentiate DDT associated with cells from extracellular DDT. The use of concentrations of ^{14}C-DDT below the aqueous solubility limit was rejected due to the substantial loss of DDT from the aqueous suspension by co-evaporation with water (10) and the low specific activity of the ^{14}C-DDT[1].

In this paper, we report a simple and rapid technique for the separation of extracellular DDT from *Chlorella* cells using Ficoll density gradient centrifugation.

MATERIALS AND METHODS

Ficoll (Pharmacia Fine Chemicals Inc., New Jersey), a synthetic high molecular weight polymer prepared by the polymerization of sucrose with epichlorohydrin, was selected as the gradient material because of its (i) high water solubility, (ii) low viscosity which permits rapid sedimentation and easy pipetting, and (iii) osmotic neutrality (11).

In a preliminary experiment, a series of stock Ficoll solutions were prepared which ranged in concentration from 10 to 30 percent (w/v) in 5 percent increments and maintained at 4°C.

A discontinuous gradient was prepared by introducing 1.0 ml of each stock solution, beginning with 30 percent, into a 15 ml conical centrifuge tube. The test solution of ^{14}C-DDT or a suspension of *Chlorella* cells (see below) was layered on top of the upper (10 percent) Ficoll layer and the gradient was centrifuged at 800 g for 5 minutes at 0-4°C. Each layer of the gradient was carefully aspirated using a square-cut blunt needle attached to a 5 ml disposable syringe, and extracted with 2.0 ml anhydrous ether in the cold (12). Triplicate 100 µl aliquots of the ether extract were evaporated to dryness (13), dissolved in 10 ml Omnifluor (98% PPO and 2% BIS-MSB in toluene, New England Nuclear, Boston, Mass.) and counted in a Nuclear-Chicago 725 Liquid Scintillation spectrometer.

^{14}C-DDT was dissolved in ethanol and diluted with doubly distilled, deionized, dust-free water to a final concentration of 1.5 µg/ml (2.23×10^5 dpm/ml). The

[1]Dichlorodiphenyl(*ring*-Cl4(U))trichloroethane, 99 percent *p,p'*-DDT (specific activity 23.9 mC/ml) (Amershan/Searle Corp., Arlington Heights, Ill.).

flask containing this standard solution was foil wrapped to prevent possible photodegradation and stored at 4°C.

Although this concentration exceeded the solubility of DDT in water (3), no precipitate was initially observed. However, after two days a fine white precipitate was clearly seen at the bottom of the flask. DDT sedimented from this solution and from solutions containing higher concentrations of ^{14}C-DDT within an hour after preparation when centrifuged at 800 g for 5 minutes. These data corroborate the observations of Bowman et al. and Wilson et al. (7,8).

The distribution of DDT in the gradient was determined in the following manner: An aliquot of 1-3 day old ^{14}C-DDT standard solution (approximately $3X10^4$ dpm) was layered onto the previously described gradient and centrifuged as above. It was found that 80 percent of the total radioactivity introduced was retained on top of the 10 percent layer, 19.4 percent was in the 10 percent layer and only 0.6 percent was recovered from the combined 15 and 30 percent layers. When a 4-7 day old solution of ^{14}C-DDT was used, the mean total radioactivity within the 10 percent layer increased by 3 to 4 percent. This suggested that larger DDT particles had formed and for this reason, the 10 percent Ficoll layer was increased to 11.5 percent in all subsequent experiments. Tests showed that 80-85 percent of ^{14}C-DDT from 4 to 7 day old solutions was retained above the 11.5 percent Ficoll layer.

The distribution of Chlorella cells in the gradient was determined by gently layering a 1.0 ml aliquot of the cell suspension (approximately $4X10^4$ cells/ml) in late log phase on top of a prepared gradient and centrifuging as above. After centrifugation, each layer was removed, diluted to 10 ml with growth medium and centrifuged. The supernatant was decanted and the pellet suspended in 1 ml of medium. The number of cells per layer was determined using a Spenger-Neubauer Haemocytometer. The Chlorella cells were observed to pass freely through the 11.5 percent Ficoll layer but approximately 5-10 percent were retained by the 15 percent layer.

In subsequent experiments, it was found that the most effective Ficoll gradient was composed of 1.0 ml of 30 percent Ficoll, which functioned as a cushion for the cells, and 2.0 ml of 11.5 percent Ficoll, which effectively retained up to 99 ± 4 percent of the extracellular DDT particles (Table I). The use of a slightly larger volume (3-4 ml) of 11.5 percent Ficoll simplifies the removal of this layer without disturbing the band of cells resting on the 30 percent layer.

Chlorella cells recovered from the gradient did not appear plasmalyzed when viewed with the light microscope. However, few unicellular marine and fresh water algae are as hardy as *Chlorella* and care should be taken to determine possible osmotic damage to the cells.

RESULTS AND DISCUSSION

The technique reported in this paper permits the separation of *Chlorella* cells from extracellular DDT particles which would have otherwise co-sedimented with the cells. Therefore, accurate and complete kinetics of DDT uptake by *Chlorella*, and possibly other algae, can now be determined for the first time. Such studies should lead to better understanding of

TABLE I

DISTRIBUTION OF ^{14}C-DDT RADIOACTIVITY IN FICOLL DENSITY GRADIENTS

	Radioactivity (X 10^{-3} dpm/ml)					Percent radioactivity recovered from				
		Recovered from								
		Ficoll Layers			Cell Band	Ficoll Layers			Cell Band	
Test Solution	Introduced	I	II	III		I	II	III		Total
^{14}C-DDT										
1	5.0	5.0	1.3	0.1	–	100.0	26.0	2.0	–	128.0
2	15.0	12.1	4.6	bkg	–	80.0	30.0	0	–	110.0
3	26.1	21.0	4.6	0.2	–	81.0	17.0	0.7	–	98.7
4	30.0	27.0	2.6	0.1	–	90.0	9.0	0.3	–	99.3
Mean and S.E.						87.8±4.0	20.5±4.0	0.8±0.4		109.1±5.9[a]
Data normalized to 100% (Mean and S.E.)						80.5±3.6	18.8±3.6	0.7±0.4		100.0
^{14}C-DDT and *Chlorella* Cells[b]										
1	12.8	7.0	2.4	–	2.3	55.0	18.8	–	18.8	91.8
2	15.0	9.0	4.0	–	2.4	60.0	26.6	–	16.0	102.6
3	23.4	15.0	3.0	–	3.9	64.0	13.0	–	16.6	93.6
Mean and S.E.						59.6±2.1	19.5±2.3		16.9±0.5	96.0±2.7
Data normalized to 100% (Mean and S.E.)						62.0±2.2	20.3±2.4		17.7±0.5[c]	100.0

[a] A loss in volume due to ether evaporation during the ether extraction procedure produced an apparent increase in ^{14}C-DDT remaining in solution which was not corrected for in the first two experiments. Subsequent experiments were corrected for volume changes.

[b] 2-4 X 10^4 cells were in contact with ^{14}C-DDT for 1 minute in the light at 25° C (5). The recovery of cells was 96-98 percent from the cell band, and 0-1 percent from the 11.5 percent Ficoll layer.

[c] Approximately 17 percent ^{14}C-DDT is bound or incorporated by the cell.

Distribution of ^{14}C-DDT radioactivity in Ficoll density gradients after centrifugation (800 g for 5 minutes at 0-4° C). (I) 1 ml of test solution, (II) 2 ml of 11.5 percent Ficoll, (III) 1 ml of 30 percent Ficoll. I and III were extracted with 1 ml anhydrous ether and II was extracted with 2 ml anhydrous ether. The cell band layering on 30 percent Ficoll was removed, suspended in 10 ml growth medium, centrifuged, and resuspended in 1 ml distilled water. Each value consists of the average from triplicate samples.

DDT-phytoplankton interactions. In addition, this technique may be particularly useful to marine algologists since it could be performed simply and rapidly on board ship. Also, the Ficoll density separation method may be useful in kinetic studies of other highly water-insoluble and ecologically important chemicals, such as the herbicide 2,4,5-T and the polychlorinated biphenyls (PCB's).

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