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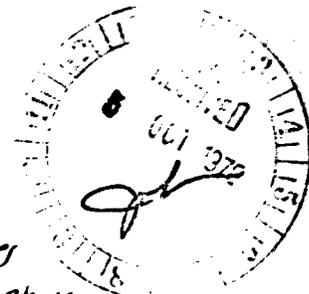
SPECIALTY CHEMICALS DIVISION



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MONSANTO INDUSTRIAL CHEMICALS CO.  
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September 25, 1978



Ms. Joan Urquhart  
EPA-OTS (TS-793)  
Federal Register Section  
401 M Street SW  
Washington, D.C. 20460

Re: Aryl Phosphates  
& Ethyl hexyl diphenyl phosphate  
Ref: OTS 04004

Received from H. Gial 3/3/81

Dear Ms. Urquhart:

The following are Monsanto's comments in reply to the dossier prepared for the Aryl Phosphates for the EPA TSCA Interagency Testing Committee. Our comments consist of submission of additional data not included in the dossier and of suggested revisions of Part I.

1. Enclosed are copies of the following Monsanto papers.

- a. Johannsen, F.R., P. L. Wright, D. E. Gordon, G. J. Levinskas, R. W. Radue, and P. R. Graham, "Evaluation of Delayed Neurotoxicity and Dose Response Relationship of Phosphate Esters in the Adult Hen." Toxicology and Applied Pharmacology, 41:291-304 (1977).

The following conclusions regarding structure-activity relationships may be drawn from this study: (1) Phosphate prepared from o-alkyl-substituted phenols frequently are neurotoxic; however, the o-alkyl group must contain at least one hydrogen atom on the a-carbon for activation. (2) The neurotoxic potency of active o-alkyl-substituted phosphates declines with increased mass and branching of the o-substituent. (3) Neurotoxicity is reduced by further substitution in the ring already containing an o-substituent. (4) A series of unsymmetrical phosphates containing an alkyl group in the p-position of one ring are not neurotoxic even though they contain hydrogen atoms on the a-carbon. (5) p-Tertiary-butylphenyl diphenyl phosphate, with no hydrogen on the a-carbon available for activation, does not produce delayed neurotoxicity.

- b. Saeger, V., "Environmental Fate of Selected Phosphate Esters". Submitted for publication to Environmental Science and Technology, July 1978.

This paper shows that phosphate esters as a class are readily biodegradable.

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Ms. Joan Urquhart  
September 25, 1978  
Page 2

2. The following revisions are recommended in Part I.

General Information

Cresyl Diphenyl Phosphate

- 1.46 Solubility  
2.6 ppm in water at room temperature (1)
- 1.47 Octanol/Water Partition Coefficient and, based on  
octanol/water partition coefficient, probably  
will not bioconcentrate significantly in fish.  
 $3.20 \times 10^{-4}$  (1)
- 1.7 Manufacture by Monsanto was discontinued in 1975.

Triphenyl Phosphate

- 1.45 Vapor Pressure  
1.90 mm (Hg) @ 200°C (2)
- 1.46 Solubility  
1.9 ppm in water at room temperature (1)
- 1.47 Octanol/Water Partition Coefficient  
 $4.25 \times 10^{-4}$  (1)

Tritolyl Phosphate

- 1.45 Vapor Pressure  
0.50 mm (Hg) @ 200°C (2)
- 1.46 Solubility  
0.4 ppm in water at room temperature (1)
- 1.47 Octanol/Water Partition Coefficient  
 $12.8 \times 10^{-4}$  (1)
- 1.7 Manufacture by Monsanto was discontinued in 1975.

- (1) Saeger, V., "Environmental Fate of Selected Phosphate Esters".  
Submitted for publication to Environmental Science and Technology,  
July, 1978.
- (2) Midwest Research Institute, MRI Project No. 4309-C (June 17, 1977)-  
Reference 4, Page II - 33 of the dossier.

Sincerely,



J. Coleman Weber  
Manager  
Product Acceptability

## Evaluation of Delayed Neurotoxicity and Dose-Response Relationships of Phosphate Esters in the Adult Hen<sup>1</sup>

FREDERICK R. JOHANNSEN, PAUL L. WRIGHT, DONOVAN E. GORDON,<sup>2</sup>  
GEORGE J. LEVINSKAS, ROBERT W. RADUE, AND PAUL R. GRAHAM

Monsanto Company, St. Louis, Missouri 63166

Received August 31, 1976, accepted December 28, 1976

Evaluation of Delayed Neurotoxicity and Dose-Response Relationships of Phosphate Esters in the Adult Hen. JOHANNSEN, F. R., WRIGHT, P. L., GORDON, D. E., LEVINSKAS, G. J., RADUE, R. W., AND GRAHAM, P. R. (1977). *Toxicol. Appl. Pharmacol.* 41, 291-304. An oral multiple-dose technique in hens was developed to determine the neurotoxic potential of a series of triaryl-, trialkyl-, and alkyl-aryl phosphates. This potential was estimated by observation of abnormal behavioral signs and always verified by histological examination. This procedure permits dose titration of an active material to quantify dose-response relationships. Initial evaluation of a series of phosphates for neurotoxicity yielded three active materials: tricresyl phosphate, cresyl diphenyl phosphate, and *o*-isopropylphenyl diphenyl phosphate. Subsequent experiments demonstrated that their neurotoxic potential differed markedly. The following conclusions regarding structure-activity relationships may be drawn from this study: (1) Phosphates prepared from *o*-alkyl-substituted phenols frequently are neurotoxic; however, the *o*-alkyl group must contain at least one hydrogen atom on the  $\alpha$ -carbon for activation. (2) The neurotoxic potency of active *o*-alkyl-substituted phosphates declines with increased mass and branching of the *o*-substituent. (3) Neurotoxicity is reduced by further substitution in the ring already containing an *o*-substituent. (4) A series of unsymmetrical phosphates containing an alkyl group in the *p*-position of one ring are not neurotoxic even though they contain hydrogen atoms on the  $\alpha$ -carbon. (5) *p*-Tertiary-butylphenyl diphenyl phosphate, with no hydrogen on the  $\alpha$ -carbon available for activation, does not produce delayed neurotoxicity.

Many organophosphate esters possess physical and chemical properties desirable for general utility as flame retardants, plasticizers, and industrial fluids. They are also used in plasticized automotive upholstery and wall coverings, applications which are of importance to the general populace. The widespread acceptance of phosphate esters for these purposes has been fostered by their relatively low order of toxicity. Yet, one member of this class, tri-*o*-cresyl phosphate, was found to induce delayed neurotoxicity as early as 1930 (Smith and Elvove, 1930). Similar effects have been reported for other chemicals within this class (Heath, 1961; Aldridge and Barnes, 1966; Cavanagh, 1973; Johnson, 1975), and much attention has been given to the evaluation of delayed neurotoxicity by other phosphate esters.

While several animal species show some susceptibility toward a general neurological paralysis, the adult hen generally is accepted as the species exhibiting a degree of

<sup>1</sup> Presented, in part, at the Fifteenth Annual Meeting of the Society of Toxicology in Atlanta, Georgia, March 14-18, 1976.

<sup>2</sup> Industrial BIO-TEST Laboratories, Inc., Northbrook, Illinois.

sensitivity and a clinical syndrome similar to that observed in the human (Aldridge *et al.*, 1969). The following experiments were conducted with industrial phosphate esters to evaluate structure-activity relationships with respect to producing neurological effects in the mature hen. This technique employed a maximum-tolerated multiple-dosing regimen to permit detection of neurotoxicity and to demonstrate dose-response relationships of active phosphate esters.

## METHODS

### *Synthesis and Purity of Test Material*

In general, phosphate samples were prepared by reacting alkyl alcoholic or phenolic precursors and phosphorus oxychloride and subsequently purified. In some cases, the samples were prepared using the Schotten-Baumann reaction and subsequently refined. Mixed alkyl-aryl phosphates were prepared by reaction of alcohol with phosphorus oxychloride followed by reaction with sodium phenate. Trialkyl phosphates were prepared by a catalyzed reaction of excess alcohol with phosphorus oxychloride. In both cases, purification steps followed synthesis.

Gas chromatography (GC) was used to verify purity of the phenolic precursors prior to synthesis and to check the purity of synthesized aryl phosphates. Each aryl phosphate was also subjected to alkaline hydrolysis and the freed phenolics were isolated quantitatively and analyzed by GC.

### *Symmetrical Triaryl Phosphates*

Triphenyl phosphate was prepared from pure phenol. Commercial grade cresylic acid, which is a complex mixture of phenolic derivatives, was used to prepare mixed (*o*-, *m*-, *p*-) isomeric tricresyl phosphate. The total *o*-constituents found in this cresylic acid approached 2.2%. Tri-*o*-cresyl phosphate assayed 98.5% by GC. Additional symmetrical triaryl phosphates, synthesized in the laboratory, were found to be greater than 97% pure by alkaline hydrolysis and subsequent GC analysis: tris-(*o,o*-dimethylphenyl) phosphate, tris-(*o,p*-dimethylphenyl) phosphate, tris-(*o*-isopropylphenyl) phosphate, and tris-(*o*-ethylphenyl) phosphate. Tris-(*m*-ethylphenyl) phosphate was prepared from essentially pure *m*-ethylphenol. It was not analyzed for purity.

### *Unsymmetrical Triaryl Phosphates*

Cresyl diphenyl phosphate was prepared from commercial-grade cresylic acid containing a mixture of phenolic derivatives (approximately 2.2% *o*-constituents). A laboratory sample of cresyl diphenyl phosphate was prepared from pure *m,p*-cresol and pure phenol and yielded greater than 42% *m,p*-cresol and 57% phenol following alkaline hydrolysis and GC analysis. Each of the following unsymmetrical phosphates was prepared by laboratory synthesis. Subsequent hydrolysis of the ester yielded the respective phenolic ratio weight shown in parentheses: *o*-isopropylphenyl diphenyl phosphate (1.1 phenol:1 *o*-isopropylphenol), *p*-isopropylphenyl diphenyl phosphate (1 phenol:1 *p*-isopropyl phenol), and *p*-tertiary-butylphenyl diphenyl phosphate (2 phenols:1 *p*-tertiary-butylphenol). Although prepared in essentially the same manner, from highly purified precursors, no quantitative information is available on *o*-xenyl diphenyl phosphate, *p*-hexylphenyl diphenyl phosphate, or *p*-nonylphenyl, *p*-cumylphenyl diphenyl phosphate.

### *Alkyl and Mixed Alkyl-Aryl Phosphates*

The following phosphates were prepared from essentially pure and/or high quality alkyl alcohols: isodecyl diphenyl phosphate, 2-ethylhexyl diphenyl phosphate, dibutyl phenyl phosphate, and tributyl phosphate.

### *Acute Oral Toxicity*

Single doses of undiluted or corn oil solutions of test materials were administered by gastric intubation to groups of Sprague-Dawley strain male and female rats and White Leghorn adult hens. In general, maximum dosages of 15.8 and 10 g/kg were given to rats and hens, respectively. Triphenyl phosphate is a solid with low bulk density. It was administered in gelatin capsules to hens at 5 g/kg, the maximum dosage it was practical to achieve. If mortalities occurred during the 14-day observation period, additional groups of rats or hens were treated at decreasing fractional log intervals to provide data for calculation of the LD50 value (de Beer, 1945).

### *Acute Dermal Toxicity*

Undiluted test material was applied to intact, clipped skin (dorsal) of New Zealand albino male and female rabbits to determine the minimum lethal dose by the dermal route. Following application, treated areas were covered with plastic to preclude evaporation of the ester. Test material was washed off after the 24-hr exposure period. Animals were held for a 14-day observation period, after which they were sacrificed and subjected to gross autopsy.

### *Neurotoxicologic Evaluation*

Procedures for neurotoxicity testing in hens suggest using a dose at or near the single oral LD50. With many of the phosphates in the present study, hens survived after administration of the largest feasible single oral dose. Consequently, an exaggerated dosing regimen was adopted to afford maximum opportunity for absorption of the ester (Table 1). Undiluted or corn oil solutions of test materials were administered twice daily, for 3 consecutive days, at the maximum feasible dosage. If neither signs of general systemic toxicity nor neurotoxicity were noted, the same dosage schedule was repeated 21 days later. The multiple-dose procedure, at reduced dosages, subsequently was used to quantify dose-response relationships of neurotoxic materials. Groups of adult hens, usually 10 birds per group, were dosed by gastric intubation following a 16-hr fasting period.

TABLE 1

DOSING REGIMEN TO EVALUATE NEUROTOXIC POTENTIAL OF  
PHOSPHATE ESTERS IN CHICKENS

Days on test	Procedure
1-3	Oral dose, twice daily
4-21	Observation for abnormal behavior
21-23	Oral dose, twice daily
24-42	Observation for normal behavior
42	Sacrifice, histopathology

Body weights of individual hens were recorded at 0, 21, and 42 days on test. Birds were observed daily for mortality and possible neurotoxic reactions throughout the total 42-day test period.

Hens were sacrificed on test Day 42 and subjected to a gross pathologic examination. Brain, sciatic nerve, and spinal cord were removed *in situ* from each hen and fixed in 10% buffered formalin. Hematoxylin-eosin-stained sections of these tissues were examined microscopically. Hens which became moribund during the test period were sacrificed. These hens, and those which died, were weighed and subjected to both gross and histopathologic evaluation as described above.

## RESULTS

### Acute Toxicity

Results of acute oral and dermal toxicity studies with a series of aryl and alkyl phosphate esters are presented in Table 2. This series of phosphates exhibits a very low

TABLE 2

ACUTE ORAL AND DERMAL TOXICITY OF PHOSPHATE ESTERS

Phosphate ester	LD50 (g/kg)		
	Rat oral	Hen oral	Rabbit dermal
<b>Symmetrical triaryl</b>			
Triphenyl phosphate	10.8	>5.0	>7.9
Tricresyl phosphate ( <i>o</i> -, <i>m</i> -, <i>p</i> -isomers)	>15.8	>10.0	>7.9
Tri- <i>o</i> -cresyl phosphate	8.4	ND <sup>a</sup>	3.7
Tris-( <i>o</i> , <i>o</i> -dimethylphenyl) phosphate	>15.8	>1.0 <sup>b</sup>	>7.9
Tris-( <i>o</i> , <i>p</i> -dimethylphenyl) phosphate	>15.8	>1.0 <sup>b</sup>	>7.9
Tris-( <i>o</i> -isopropylphenyl) phosphate	>15.8	>1.0 <sup>b</sup>	>7.9
Tris-( <i>m</i> -ethylphenyl) phosphate	>15.8	>10.0	>7.9
Tris-( <i>o</i> -ethylphenyl) phosphate	>15.8	>1.0 <sup>b</sup>	>5.0
<b>Unsymmetrical triaryl</b>			
Cresyl diphenyl phosphate	10.4	>10.0	>5.0
<i>o</i> -Xenyl diphenyl phosphate	>15.8	>10.0	>7.9
<i>o</i> -Isopropylphenyl diphenyl phosphate	10.0	ND	>5.0
<i>p</i> -Isopropylphenyl diphenyl phosphate	ND	>10.0	ND
<i>p</i> -Tertiary-butylphenyl diphenyl phosphate	>15.8	>10.0	>7.9
<i>p</i> -Hexylphenyl diphenyl phosphate	>15.8	>10.0	>7.9
<i>p</i> -Nonylphenyl, <i>p</i> -cumylphenyl diphenyl phosphate	>20.0	>10.0	>7.9
<b>Alkyl-diaryl</b>			
2-Ethylhexyl diphenyl phosphate	>15.8	>10.0	>7.9
Isodecyl diphenyl phosphate	>15.8	>10.0	>7.9
<b>Dialkyl-aryl</b>			
Dibutyl phenyl phosphate	2.4	<2.0	>5.0
<b>Trialkyl</b>			
Tributyl phosphate	1.4	1.8	>3.1

<sup>a</sup> ND, not determined.

<sup>b</sup> Due to limited sample size, hens were dosed at one level only.

order of oral toxicity to rats and chickens and dermal toxicity to rabbits. Those phosphates containing at least two aryl groups are considered to be practically nontoxic by either route of exposure. Ester substitution to form a dialkyl-aryl or a trialkyl phosphate increased the degree of toxicity. However, these dialkyl-aryl and trialkyl phosphates are still considered to be only slightly toxic following oral or dermal administration.

#### *Neurotoxicologic Evaluations*

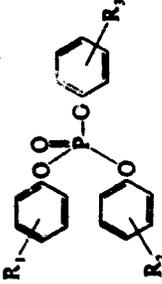
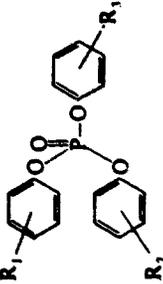
Table 3 presents results of experiments depicting the potential for a series of phosphate esters to produce delayed neurotoxicity. The neurotoxicologic potential of each phosphate was estimated by observation of abnormal behavioral signs in treated hens. Final evaluation of delayed neurotoxicity was based on the results of histologic examination.

Tricresyl phosphate, whether derived from a mixture of *o*-, *m*-, and *p*-isomers of cresol or from a cresol with a high *o*-isomer content, was neurotoxic. Cresyl diphenyl phosphate, made from a mixture containing the three isomers of cresol, was neurotoxic at a cumulative dosage of 1.2 g/kg. In contrast, a 100-fold higher dosage of cresyl diphenyl phosphate, made from cresol containing only *m*- and *p*-isomers, was not neurotoxic. *o*-Isopropylphenyl diphenyl phosphate was neurotoxic at a cumulative dosage of 12 g/kg. A similar 12-g/kg dosage of other esters containing tris-*o*-substituents failed to induce neurotoxicity, i.e., tris-(*o,o*-dimethylphenyl) phosphate, tris-(*o*-ethylphenyl) phosphate, tris-(*o*-isopropylphenyl) phosphate, and tris-(*o,p*-dimethylphenyl) phosphate. A cumulative dosage of 120 g/kg of *o*-xenyl diphenyl phosphate failed to produce signs of neurotoxicity. Two alkyl-diaryl phosphates, isodecyl diphenyl phosphate and 2-ethylhexyl diphenyl phosphate, also were not neurotoxic under our test conditions. Unsymmetrical, *p*-substituted phosphates which produced no delayed neurotoxicity at cumulative dosages of 120 g/kg were *p*-isopropylphenyl diphenyl phosphate, *p*-tertiary-butylphenyl diphenyl phosphate, *p*-hexylphenyl diphenyl phosphate, and mixed *p*-nonylphenyl, *p*-cumylphenyl diphenyl phosphate. An unsubstituted ester, triphenyl phosphate, was not neurotoxic at 60 g/kg, the maximum dosage it was feasible to administer. Dibutylphenyl phosphate and tributyl phosphate were treated at lower dosages because of their acute toxicity to hens. The cumulative dosage for each, however, was greater than their respective single oral LD50 values.

Three neurotoxic esters, tri-*o*-cresyl phosphate, cresyl diphenyl phosphate, and *o*-isopropylphenyl diphenyl phosphate (Table 3), were evaluated further by means of the multiple-dose regimen. This procedure permitted titration of the amount of the ester required to produce neurotoxicity. Results are presented in Fig. 1. A median effective dose (ED50), based on histologic evaluation of nerve tissue, was calculated for each material. The ED50 values were: 0.42 g/kg for tri-*o*-cresyl phosphate, 1.23 g/kg for cresyl diphenyl phosphate, and 10.28 g/kg for *o*-isopropylphenyl diphenyl phosphate. No-effect dose levels for each ester can also be estimated from the dose-response curves.

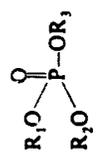
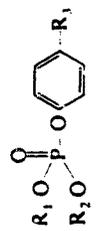
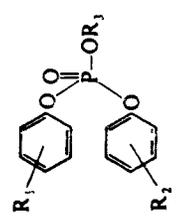
Signs of peripheral polyneuropathy occur on a delayed basis. Gross observation of treated birds revealed treatment-related effects, generally beginning 8-14 days following initial administration of an active phosphate. Overt signs of neurotoxicity exhibited by hens were graded according to the general scheme shown in the legend in Table 4. An

TABLE 3  
NEUROTOXICOLOGICAL RESPONSE OF A SERIES OF PHOSPHATE ESTERS IN THE ADULT CHICKEN

Structure <sup>a</sup>	Phosphate ester	Dose regimen <sup>b</sup>	Cumulative dosage (g/kg)	Behavior	Neurotoxic response (number positive/number of birds)	Histology	
 <p>(I) Symmetrical triaryl</p>	Triphenyl phosphate	5.0 x 2 x 6	60.0	0/9	0/9		
	Tricresyl phosphate	10.0 x 2 x 3	60.0	6/6	6/6		
	Tri- <i>o</i> -cresyl phosphate	0.3 x 1 x 5	1.5	4/4	— <sup>c</sup>		
	Tris-( <i>o,o</i> -dimethylphenyl) phosphate	1.0 x 2 x 6	12.0	0/10	0/10		
	Tris-( <i>o,p</i> -dimethylphenyl) phosphate	1.0 x 2 x 6	12.0	0/10	0/10		
	Tris-( <i>o</i> -ethylphenyl) phosphate	1.0 x 2 x 6	12.0	0/10	0/10		
	Tris-( <i>m</i> -ethylphenyl) phosphate	10.0 x 2 x 6	120.0	0/10	0/10		
	Tris-( <i>o</i> -isopropylphenyl) phosphate	1.0 x 2 x 6	12.0	0/10	0/10		
	 <p>(II) Unsymmetrical triaryl</p>						

H, H, H  
-Me, -Me, -Me  
(mixed *o*-, *m*-, *p*-)  
*o*-Me, *o*-Me, *o*-Me  
*o*-Me, *o*-Me, *o*-Me  
*o*-Me, *o*-Me, *o*-Me  
*o*-Me, *o*-Me, *o*-Me  
*p*-Me, *p*-Me, *p*-Me  
*o*-Et, *o*-Et, *o*-Et  
*m*-Et, *m*-Et, *m*-Et  
*o*-Isop, *o*-Isop, *o*-Isop

H, H, <i>o,m,p</i> -Me	Cresyl diphenyl phosphate (mixed <i>o,m,p</i> -isomers)	0.1 x 2 x 6	1.2	3/10	2/10
H, H, <i>m,p</i> -Me	Cresyl diphenyl phosphate (from <i>m,p</i> -isomers)	10.0 x 2 x 6	120.0	0/8	0/8
H, H, <i>o</i> -Isop	<i>o</i> -Isopropylphenyl diphenyl phosphate	1.0 x 2 x 6	12.0	4/9	5/9
H, H, <i>f</i> -Isop	<i>p</i> -Isopropylphenyl diphenyl phosphate	10.0 x 2 x 6	120.0	0/10	0/10
H, H, <i>o</i> -Phen	<i>o</i> -Xenyl diphenyl phosphate	10.0 x 2 x 6	120.0	0/6	0/6
H, H, <i>p</i> -Tbut	<i>p</i> -Tertiary-butylphenyl diphenyl phosphate	10.0 x 2 x 6	120.0	0/9	0/9
H, H, <i>p</i> -Hex	<i>p</i> -Hexylphenyl diphenyl phosphate	10.0 x 2 x 6	120.0	0/9	0/9
H, H, <i>p</i> -Nonphen, <i>p</i> -Cumphen	<i>p</i> -Nonylphenyl, <i>p</i> -cumylphenyl diphenyl phosphate	10.0 x 2 x 6	120.0	0/6	0/6
(III) Alkyl-diaryl					
H, H, Isod H, H, Ethex	Isodecyl diphenyl phosphate Ethylhexyl diphenyl phosphate	10.0 x 2 x 6 10.0 x 2 x 6	120.0 120.0	0/10 0/10	0/10 0/10
(IV) Dialkyl-aryl					
But, But, H	Dibutyl phenyl phosphate	1.34 x 1 x 2	2.68	0/5	0/5
(V) Trialkyl					
But, But, But	Tributyl phosphate	1.84 x 1 x 2	3.68	0/9	0/9



<sup>a</sup> But, butyl; Cumphen, cumylphenyl; Et, ethyl; Ethex, ethylhexyl; H, hydrogen; Hex, hexyl; Isod, isodecyl; Isop, isopropyl; Me, methyl; Nonphen, nonylphenyl; Phen, phenyl; Tbut, tertiary-butyl; First, second, and third designations, R<sub>1</sub>, R<sub>2</sub>, and R<sub>3</sub>, respectively.  
<sup>b</sup> Dose = (grams per kilograms) x treatment per day x number of days.  
<sup>c</sup> Not determined.

initial generalized weakness of the legs was followed by an unsteadiness of gait (ataxia). These abnormal signs increased in intensity until a partial paralysis of the legs had developed. Finally, there was full immobilization so that affected birds were unable to stand. On occasion, severe cases also showed weakness of the wings and wingdrop was noted.

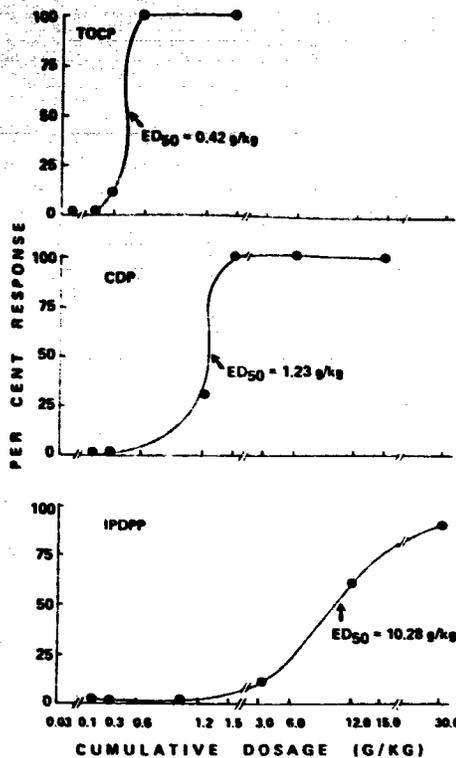


FIG. 1. Dose-response (percentage of hens affected) of three neurotoxic phosphate esters, tri-*o*-cresyl phosphate (TOCP), cresyl diphenyl phosphate (CDP), and *o*-isopropylphenyl diphenyl phosphate (IPDPP) following use of the multiple dose technique in adult hens. Response is based on histologic evaluation of nerve tissue. Ten hens were used at each dose level.

Table 4 presents the gradation and duration of abnormal behavioral signs observed in groups of hens treated at two dosages of tri-*o*-cresyl phosphate. No hens dosed with 0.01 g/kg exhibited abnormal signs, while 6 of 10 hens treated with 0.025 g/kg exhibited characteristic signs of neurotoxicity. Initial signs of neurotoxicity were observed between Days 8 and 14 in four treated hens. However, the appearance of similar signs was delayed until Days 35 and 39 in the other two birds. The severity of abnormal signs exhibited by hens at this dosage level did not exceed a grade of 2. Adverse behavioral signs increased in intensity through day 22 of the test period, following which the intensity decreased. All hens showed an absence of abnormal signs between test Days



26 and 29, even though all birds had been redosed on test Days 21–23. Abnormal signs reappeared in these birds 9–11 days after the second series of doses and continued until termination of the study.

All 10 hens treated with 0.25 g/kg of tri-*o*-cresyl phosphate exhibited abnormal signs 6–13 days after initial treatment. Severity of signs increased rapidly and most hens exhibited a grade of 3 or 4 prior to sacrifice.

Histopathologic evaluation of tissues obtained from treated hens revealed an apparent sequence in the development of neural lesions as seen by light microscopy. The morphological characteristics of lesions produced by all three active phosphates in this study were similar. Figures 2 and 3 show normal sections from sciatic nerve and spinal cord, respectively, of untreated control hens. The development of the neural lesion observed in sciatic nerve and spinal cord sections is shown in Figs. 4–7. Axonal swelling is the first histopathologic lesion observed. This progresses to axonal degeneration during which vacuolation, clumping, and fragmentation of axons are evident (Figs. 4 and 5). Finally, degradation or loss of the myelin sheath occurs at sites of earlier damage on the sciatic nerve (Fig. 6). In severe cases, loss of myelin also occurred in sections of the spinal cord (Fig. 7). In general, the relative severity and degree of tissue involvement observed histopathologically were dose related.

With active phosphates, there was good correlation between gross behavioral signs graded as moderate and more severe and the presence of histopathologic changes in neural tissue. When hens exhibited a slight to mild degree of abnormal behavioral signs, the duration of the signs appeared to be a factor in determining whether neural lesions would be observed microscopically.

#### DISCUSSION

Delayed polyneuropathy, as observed in man and experimentally in the hen, appears to be a graded response and is correlated with both dosage and frequency of application. Therefore, determination of a dose-related relationship for a selected chemical or series of chemicals can be useful in assessing the relative hazard of active materials. Dose-response relationships showing inhibition of selected enzymes, and taken as an indicator of potential neurotoxicity by individual phosphate esters, have been demonstrated (Johnson, 1975). Evaluation of a dose-response relationship of an

FIG. 2. Photomicrograph of longitudinal section of sciatic nerve from an untreated hen showing intact myelinated nerve fibers with occasional Schwann cell nuclei and a few endoneurial fibroblasts. Hematoxylin and eosin stain;  $\times 1500$ .

FIG. 3. Photomicrograph of longitudinal section of spinal cord from an untreated control bird showing myelinated nerve fibers. Hematoxylin and eosin stain;  $\times 1500$ .

FIG. 4. Photomicrograph of longitudinal section of sciatic nerve showing degenerate nerve fibers with ovoid structures (necrotic and fragmented axons), swollen axons, distended neurolemmal sheaths, and degradation of myelin. Hematoxylin and eosin stain;  $\times 1500$ .

FIG. 5. Photomicrograph of longitudinal sections of spinal cord showing fragmentation of swollen, degenerate, and necrotic axons. Hematoxylin and eosin stain;  $\times 1500$ .

FIG. 6. Photomicrograph of longitudinal section of sciatic nerve showing swollen, degenerative, and necrotic axons. Hematoxylin and eosin stain;  $\times 1500$ .

FIG. 7. Photomicrograph of longitudinal section of spinal cord showing degenerative to necrotic axons. Hematoxylin and eosin stain;  $\times 1500$ .

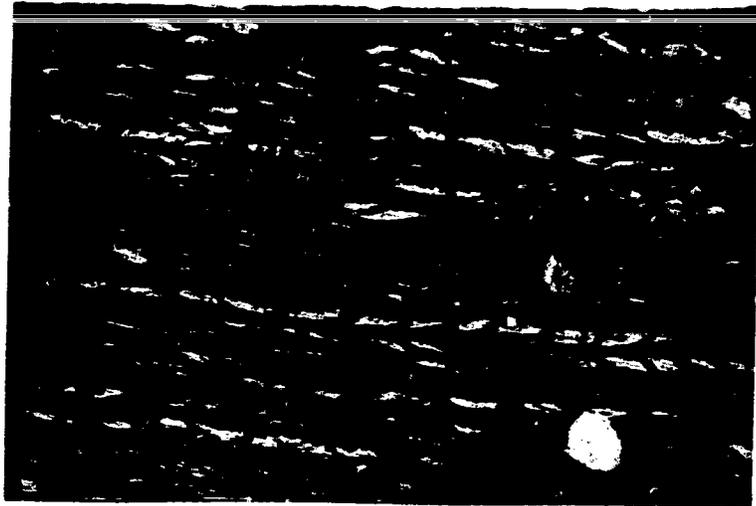


FIG. 2.

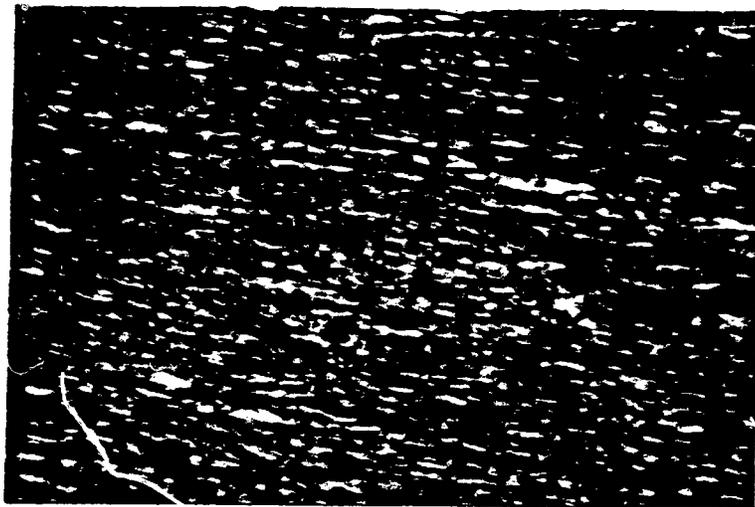


FIG. 3.

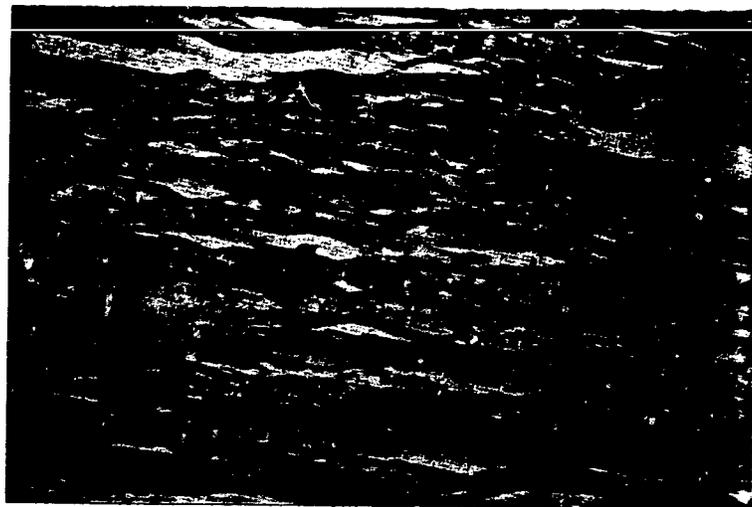


FIG. 4.

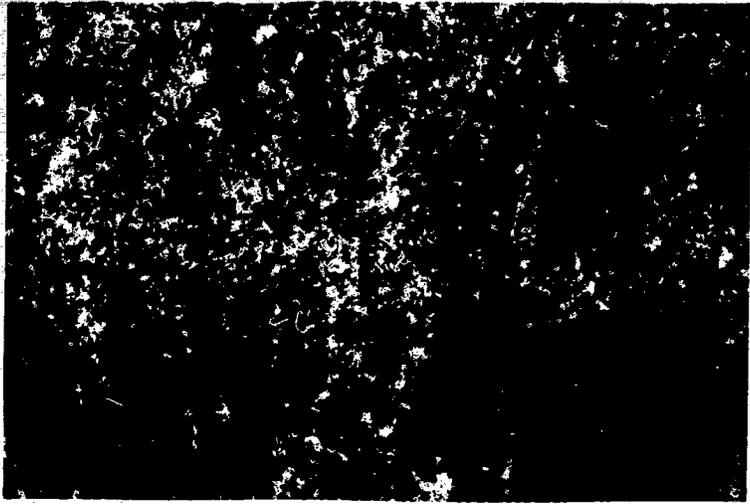


FIG. 5.



FIG. 6.

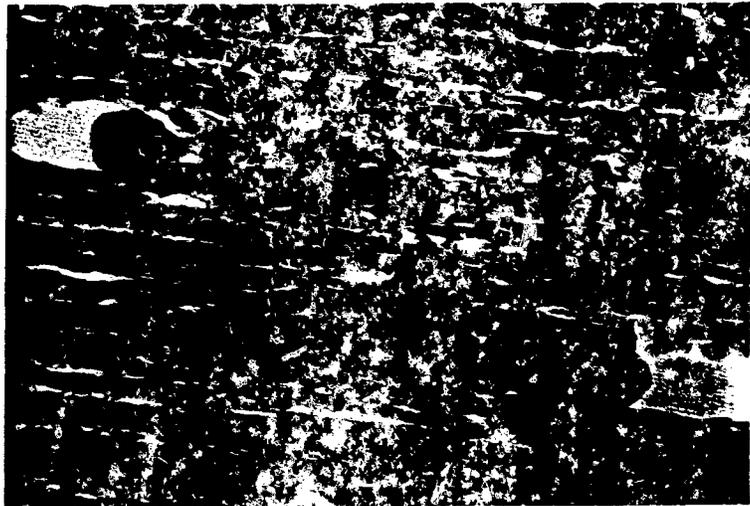


FIG. 7.

active chemical through use of an *in vivo* system has inherent advantages. The multiple-dose technique reported herein is capable of relating the neurotoxic response to use of selected active phosphates following administration to intact test animals.

In the present studies, all triaryl phosphates producing neurotoxicity had alkyl substituents in the *o*-position of one or more of their aryl groups. These results are consistent with the conclusion that *o*-substituted triaryl phosphates produced a high degree of neurotoxicity (Aldridge and Barnes, 1961, 1966; Casida *et al.*, 1961). However, not all *o*-substituted phosphates evaluated here produced neuropathy. The effect of substitution only within a single aryl group (unsymmetrical phosphate) as well as identical substitutions in all three aryl groups (symmetrical phosphate) must be considered for evaluation of structure-activity relationships.

Johnson (1975) has concluded that a substituted aryl phosphate will induce delayed neurotoxicity if the *o*-alkyl group has at least one hydrogen on the  $\alpha$ -carbon atom, thus allowing cyclization to occur. This hypothesis explains the reported neurotoxicity of cresyl diphenyl phosphate derived from *o*-cresol (Aldridge and Barnes, 1966). Positive results reported in our study with a batch of cresyl diphenyl phosphate prepared from a mixture of *o*-, *m*-, and *p*-cresol isomers indicate that small amounts of an active *o*-substituent within a phosphate mixture are capable of producing neurotoxicity. Mixed esters, made from raw materials containing *o*-ethyl and *o*-normal-propyl moieties, reportedly are highly neurotoxic (Bondy *et al.*, 1960). The present study adds *o*-isopropylphenyl diphenyl phosphate to this series of active *o*-substituted phosphates. According to Johnson's hypothesis, the corresponding normal-butyl, but not the *tertiary*-butylphenyl, ester should be neurotoxic. The negative results obtained with triphenyl phosphate and *o*-xenyl diphenyl phosphate are consistent with Johnson's hypothesis since neither material contains a hydrogen atom on the  $\alpha$ -carbon.

Johnson (1975) indicated that the potency for neurotoxicity of active *o*-alkyl-substituted esters declined with increasing mass and branching of the alkyl substituents. The relatively lower neurotoxicity shown by *o*-isopropylphenyl diphenyl phosphate in Fig. 1 is consistent with this view.

It has been suggested (Johnson, 1975) that neurotoxicity can be reduced by additional substitution of an aryl moiety already containing an *o*-alkyl substituent. As a result, symmetrical tri-*o*-esters should be less active than their corresponding unsymmetrical mono-*o*-analogs. While tri-*o*-cresyl phosphate was shown to be highly neurotoxic to the chicken with an ED<sub>100</sub> of 0.6 g/kg, symmetrical triesters prepared from *o,o*-dimethyl- or *o,p*-dimethyl phenols were not neurotoxic at cumulative dosages of 12 g/kg. Additionally, Bondy *et al.* (1960) reported that tris(*o,m*-dimethylphenyl) phosphate was not neurotoxic at a cumulative dosage of 2.5 g/kg. We have shown that tris-*o*-esters prepared from *o*-ethyl phenol and *o*-isopropyl phenol also were negative at cumulative dosages of 12 g/kg, while the mono-*o*-isopropyl phenol ester produced neurotoxicity at this level. Bondy *et al.* (1960) have shown that the mono-*o*-ethyl phenol ester is highly neurotoxic.

Aldridge (1954) and Hine *et al.* (1956) reported that phosphate esters prepared from pure *m*- or *p*-cresol or mixtures of both were not neurotoxic. In contrast to the highly neurotoxic cresyl diphenyl phosphate which was prepared from an isomeric mixture of *o*-, *m*-, and *p*-precursors, cresyl diphenyl phosphate, derived from *m,p*-substituted cresol, was not neurotoxic in our study, even at high dosages. The lack of neurotoxicity of

another *m*-substituted phosphate, tris(*m*-ethylphenyl) phosphate, was reconfirmed at a higher dosage in this study.

Bondy *et al.* (1960) found that tri-*p*-ethylphenyl phosphate was highly neurotoxic. However, these authors also suggested that mixed alkyl-aryl esters, containing only one or two *p*-ethylphenyl groups, were not neurotoxic. Data obtained in the present study indicate that mixed phosphate esters containing one or more of the following *p*-substituted alkyl groups are not neurotoxic: isopropyl, *tertiary*-butyl, normal-hexyl, or nonyl-cumyl. While it has been suggested (Johnson, 1975) that triaryl esters, substituted at the *p*-position, need two hydrogen atoms on the  $\alpha$ -carbon for activation, this assumption does not appear to hold for *p*-substituted unsymmetrical triaryl phosphates.

#### ACKNOWLEDGMENTS

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**RESEARCH & DEVELOPMENT**

ENVIRONMENTAL SCIENCES SECTION

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ENVIRONMENTAL FATE TESTING OF PHOSPHATE ESTERS

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### ABSTRACT

Environmental fate studies encompassing water solubility, water-octanol partition coefficient, and biodegradability were carried out for eleven trialkyl, alkyl aryl, and triaryl phosphate esters. These studies include that phosphate esters as a group exhibit low aqueous solubility, have a moderate potential for bioconcentration, and readily undergo primary and ultimate biodegradation by naturally occurring mixed-microbial populations present in activated sludge and river water.

## INTRODUCTION

Phosphate esters have found wide application as plasticizers and hydraulic fluids where flame retardancy is a desired property. Although such uses may lead inadvertently to introduction into the environment there are relatively few reports in the technical literature of the presence of phosphate esters in the environment or of their environmental fate (1,2,3).

Among the more environmentally important properties of a synthetic chemical are its partitioning characteristics and biodegradability. The n-octanol/water partition coefficient helps predict whether a chemical will tend to bioconcentrate (4). For hydrophobic compounds this partition coefficient may be estimated from aqueous solubility data (5,6). Biodegradability is an important determinant of environmental residence time, and thus affects the availability of a chemical for bioconcentration.

This paper reports on studies of the partitioning characteristics and biodegradability of eleven phosphate esters - triphenyl phosphate (TPP), tricresyl phosphate (TCP), trixylenyl phosphate (TXP), cresyl diphenyl phosphate (CDP), isopropylphenyl diphenyl phosphate (IPDP), t-butylphenyl diphenyl phosphate (tBPDP), isodecyl diphenyl phosphate (IDDP), 2-ethylhexyl diphenyl phosphate (EHDP), dibutyl phenyl phosphate (DBPP), tris-2-ethylhexyl phosphate (TEHP), and tributyl phosphate (TBP).

## EXPERIMENTAL

### Materials

The eleven phosphate esters studied were commercial-grade materials. TPP, TCP, CDP (Santicizer<sup>®</sup> 140), IDDP (Santicizer<sup>®</sup> 148), EHDP (Santicizer<sup>®</sup> 141), tBPDP, and DBPP were manufactured by Monsanto Co. Three of the remaining esters - IPDP (Kronitex<sup>®</sup> 100), TXP (Kronitex<sup>®</sup> TXP), and TEHP -

were obtained from FMC Corporation, and the fourth - TBP (Phosflex<sup>®</sup> 4) from Stauffer Chemical Co.

The chemical name specified for each ester indicates the major component. TPP, TBP, EHDP, and TEHP were relatively pure (>90%) single-component materials as indicated by gas chromatography. TCP and TXP were mixtures of their respective isomers. DBPP contained TBP and butyl diphenyl phosphate as minor components, while the four remaining esters also contained TPP and the appropriate di(alkyl) phenyl or di(alkylphenyl) phenyl phosphate esters. All data in the report pertain to these mixtures as a whole and not to their individual components.

Methods

Analytical

Gas chromatographic (GC) analyses for phosphate esters were carried out using a Hewlett-Packard 5711A chromatograph equipped with dual-flame ionization detectors. Samples were injected on-column into either 1 or 2 m x 3 mm i.d. glass columns packed with 3% OV-17 on 80-100 mesh Chromosorb W HP. Column temperature varied from 180 to 300°C depending on the ester. Injection port and detector temperatures were 250 and 350°C, respectively, and the helium flow rate was 45 cc/min.

Solubility

Our procedure for determining solubilities consisted of adding 25 ml of the phosphate ester and 500 ml of purified water (Milli-Q water purification system, Millipore Corp.) to a 1 liter glass bottle with an aluminum-foil lined cap. The bottle was agitated on a mechanical shaker for 48 hr and allowed to stand quiescent for 1 week to permit phase separation. The water equilibration was performed in the dark to preclude photodegradation. The aqueous phase was centrifuged at 14,000 rpm for one hour to remove

suspended droplets and then extracted twice with methylene chloride. The extracts were combined and concentrated in a Kuderna-Danish evaporative concentrator and analyzed for phosphate esters.

#### Partition Coefficient

The n-octanol/water partition coefficients were measured by preparing solutions (100 ppm to 1%) of the test material in 100 ml of n-octanol. The octanol solution and 500 ml of purified water were added to a 1 liter glass bottle with a foil-lined cap and shaken for 48 hrs. The mixture was then transferred to a 1 liter separatory funnel and allowed to stand for one week. This equilibration was also performed in the dark. Aliquots of the aqueous phase were drained from the funnel and extracted twice with methylene chloride. The extracts were combined, concentrated, and analyzed for phosphate esters.

The partition coefficient,  $P$ , was calculated using the equation:

$$P = C_o/C_w$$

where  $C_o$  and  $C_w$  are the phosphate ester concentrations in octanol and water, respectively. The original ester concentration in octanol was used in the calculations because in all cases only a negligible amount of the ester partitioned into the aqueous phase. Partition coefficients were used to calculate bioconcentration factors, applying the equation of Neely, et al. (4).

#### Primary Biodegradation

The river die-away method is similar to that employed in a study of phthalic acid esters (7). Settled 200 ml portions of Mississippi River water (St. Louis waterfront) were added to a series of 16-ounce narrow mouth screw-cap bottles. Four microliters of a solution containing 50  $\mu$ g of

phosphate ester per  $\mu$ l of ethanol were injected into each bottle. Each bottle was sealed with a foil-lined cap, mixed, and stored in the dark at room temperature. Heat-sterilized water controls were included to confirm that any decrease in the initial 1 mg/l phosphate ester level was due to biodegradation and not some other physical or chemical phenomenon. A set of positive controls was also prepared with river water and linear alkylbenzene-sulfonate (LAS). For each product the active and control samples were analyzed periodically for residual ester. Analyses were carried out by extracting a bottle and its contents with hexane (3 x 25 ml). The extracts were concentrated and analyzed using the previously described gas-chromatographic procedure.

The activated-sludge method is based on the Soap and Detergent Association's semi-continuous procedure (8) using modified feed (9). Domestic activated sludge from a local treatment plant was used in magnetically-stirred glass vessels of 1.5 liter operating volume.

The phosphate esters were tested at addition rates of 3 and 13 mg/l 24-hr cycle. For measuring primary degradation, 50-ml samples of mixed liquor were withdrawn a few minutes after feeding and at the end of the 24-hr cycle. These samples were extracted with hexane (3 x 25 ml). Concentration and analysis of the extracts were performed as for the river water samples. Sampling was carried out on a one-cycle-per-week basis for each ester for periods ranging from 8 to 39 weeks.

The efficiency of the analytical methods was determined by analyzing mixed liquor containing known phosphate ester concentrations. Each ester was added in duplicate at three levels (2, 4, and 6 mg/l) to activated sludge mixed liquor from a blank semi-continuous activated sludge (SCAS) unit. These samples were analyzed as previously indicated and the concentrations

found were compared to the added concentrations. The average recoveries were: TPP,  $91 \pm 6\%$ ; TCP,  $91 \pm 6\%$ ; TXP,  $80 \pm 4\%$ ; CDP,  $92 \pm 5\%$ , IPDP,  $97 \pm 8\%$ ; tBPDP,  $76 \pm 2\%$ ; IDDP,  $79 \pm 7\%$ , EHDP,  $96 \pm 6\%$ ; DBPP,  $80 \pm 11\%$ ; TEHP,  $74 \pm 4\%$ ; and tBP,  $91 \pm 3\%$ . To verify that disappearance of a phosphate ester was not due to volatilization, the off-gases from each unit were passed through a series of three hexane scrubbers during a complete cycle. No significant ( $<0.5\%/cycle$ ) volatility losses were observed for any of the phosphate esters.

#### Ultimate Biodegradation

The ultimate biodegradability of the phosphate esters was measured using the apparatus and procedure developed by Thompson and Duthie (10) and modified by Sturm (11). Acclimated bacterial seed was prepared using a 14-day Bunch-Chambers (12) die-away with no transfer. In this procedure a 2 liter flask containing 20 mg of appropriate ester, 50 mg of yeast extract, 100 ml of settled SCAS supernatant, and 900 ml of standard BOD water (13) was incubated in the dark for 14 days at ambient temperature under quiescent conditions. At the end of the incubation, equal aliquots from each phosphate ester flask were mixed to form a composite seed. A 9 liter bottle containing 500 ml of composite seed and 5500 ml of BOD water was prepared for each ester and a control. To each of the phosphate ester bottles, a weighed quantity (approximately 120 mg) of the appropriate ester was added. The control bottles received no test material. During the test, CO<sub>2</sub>-free air was bubbled through the test bottles, and the effluent air passed through a set of three CO<sub>2</sub> scrubbers, each containing 100 ml of 0.05N Ba(OH)<sub>2</sub>. The evolved CO<sub>2</sub> was trapped as barium carbonate and quantitated by back-titrating the remaining Ba(OH)<sub>2</sub> with 0.1N HCl. Carbon dioxide production from the

control bottles was in the range of 10 to 15% of the total CO<sub>2</sub> evolved from the phosphate ester bottles. Final values were corrected for CO<sub>2</sub> production from the control.

## RESULTS AND DISCUSSION

The data in Table I indicate that phosphate esters generally exhibit room temperature aqueous solubilities in the low ppm range. The most soluble esters are those with smaller, alkyl groups. The true solubility of tris-2-ethylhexyl phosphate is probably less than 1000 ppm. While shaking with water, the aqueous phase turned cloudy, suggesting the formation of an emulsion or a decomposition product. The turbidity was not removed by centrifugation or extraction.

The octanol/water partition coefficients and calculated bioconcentration factors are listed in Table II. The range of bioconcentration factors covers almost an order of magnitude. Based on these octanol/water partition coefficients, it appears that the esters have moderate potential to concentrate in aquatic organisms. In general, the bioconcentration factors are one to two orders of magnitude less than those reported for some pesticides and PCB's (14, 15).

Currently we are aware of experimental bioconcentration data for only one of the compounds covered in this report. In 1977 Lombardo and Egry reported a bioconcentration factor of 260 for rainbow trout exposed to triphenyl phosphate at approximately the 10 ppb level (2). This is the same range as our value of 420 calculated from the octanol/water partition coefficient.

Several workers have proposed a relationship between aqueous solubility and the octanol/water partition coefficient of hydrophobic compounds in the form:

$$\text{LOG } P = m (\log S) + b.$$

In this equation P is the partition coefficient and S is the solubility in  $\mu\text{moles/l}$ . The linear regression data obtained in this work and in previous investigations are given in Table III.

The data of Hansch and coworkers (5) were generated from a diverse group of 156 common organic compounds. Chiou, et al., (6) studied 34 organic compounds including hydrocarbons, acids, and pesticides. The relatively good agreement between those data and this study on phosphate esters lends support to the concept that solubility may be used to estimate partition coefficients.

River die-away studies on 9 phosphate esters demonstrated that the esters, exposed to the natural microbial population of the river, underwent primary biodegradation at moderate to rapid rates. TPP was employed as a common test material in 3 sets of experiments. The river water in each set was taken from the same source but at different times. The die-away curves for TPP in each set are compared to a positive LAS control in Figure 1. Figures 2, 3, and 4 show die-away curves for the remaining esters. Of the esters tested, only IPDP showed residual ester at the end of the test period. Five of the esters (TPP, TCP, CDP, DBP and TBP) showed complete primary degradation in less than seven days, while tBPDP, EHDP, and IDDP degraded in 10 to 21 days. Sterile-water controls showed no significant evidence of non-biological degradation or loss.

Primary biodegradation rates from our semi-continuous activated sludge (SCAS) studies are summarized in Table IV. The SCAS test, which simulates secondary sewage treatment, shows generally the same trend in degradation rate as river die-away studies. At the 3 mg/l feed level, TPP, TCP, CDP, tBPDP, EHDP, DBPP, and TBP showed rapid primary degradation. TXP, IPDP, and IDDP were in the intermediate range, and TEHP degraded more slowly. In the 3 to 13 mg/l range, the effect of feed level was variable. Apparent inhibiting effects were observed for TXP, IPDP, DBP, and TBP at the 13 mg/l level. Both IPDP and IDDP caused a significant decrease in the biomass as monitored by the suspended solids concentration.

No attempt to isolate intermediates was made in either the river die-away or SCAS studies. To establish that the primary biodegradation observed in these studies represented more than a slight modification of the parent molecule, CO<sub>2</sub> evolution studies were undertaken. By measuring the CO<sub>2</sub> produced and comparing it to the theoretical yield based on the carbon content and weight of the ester, an indication of ultimate biodegradability was obtained.

In Table V the CO<sub>2</sub> produced from each ester is expressed as a percentage of the theoretical yield at three time intervals during the test. Typical curves are shown in Figure 5. It is apparent that the esters tested essentially break down completely to carbon dioxide, water, and inorganic phosphate. The same rate trends observed in the river die-away and activated-sludge tests are apparent in the CO<sub>2</sub> evolution data. TEHP, because of its slow primary degradation rate, would not be expected to produce significant CO<sub>2</sub> in this test.

The degradation pathway for the phosphate esters most likely involves a step-wise enzymatic hydrolysis to orthophosphate and the phenolic or alcohol moieties (16). The alcohol and/or phenol would then be expected to undergo

further degradation. It is obvious from all the data presented that the phenol and/or alcohol moieties have a significant effect on the biodegradability. Although the data are too limited to make any highly significant structural correlations a few tentative conjectures can be made. For trialkyl and alkyl aryl esters, the shorter the alkyl chain the more biodegradable the ester. For alkyl aryl and triaryl esters, increasing the number and size of substituent groups on the phenyl ring leads to decreasing biodegradability. A comparison of the solubility and biodegradation data shows no obvious correlation.

The biodegradation data presented in this study suggest that mixed microbial populations in the environment will degrade phosphate esters. The significance of the results obtained by Westlake, et al., (1) on several triaryl esters (triphenyl, tri-o-cresyl, and trixylenyl) are uncertain. Using mixed bacterial populations isolated from soil they obtained growth on all three esters as sole carbon sources, but were unable to grow the dominant organism in pure culture on the esters. It appears likely that very high ester concentrations of 100 and 1000 mg/l inhibited growth.

Data on non-biological degradation routes for the phosphate esters are limited. A half-life for hydrolysis of TPP under neutral conditions has been estimated at 1.3 years from kinetic data (3). This suggests that biodegradation is the dominant breakdown route in the environment. For aryl and alkyl aryl esters, photolysis remains a possible degradation mechanism.

We have shown that commercial phosphate esters can be effectively treated by activated sludge from domestic sewage treatment plants and are readily susceptible to biodegradation in rivers by naturally occurring microbial populations. Judging from these tests, we feel that phosphate ester contamination

is not likely to become a wide spread environmental problem unless their introduction rate exceeds the degradation capacity of the ecosystem. Because of their low aqueous solubility and moderate potential to bioaccumulate in aquatic organisms, discharge of high levels to the environment should be avoided.

TABLE I

SOLUBILITY IN WATER AT ROOM TEMPERATURE

<u>Phosphate Ester</u>	<u>Solubility<sup>a</sup> (ppm)</u>
tris-2-ethylhexyl	1000 <sup>b</sup>
tributyl	280
dibutyl phenyl	96
t-butylphenyl diphenyl	3.2
cresyl diphenyl	2.6
isopropylphenyl diphenyl	2.2
2-ethylhexyl diphenyl	1.9
triphenyl	1.9
trixylenyl	0.89
isodecyl diphenyl	0.75
tricresyl	0.36

a) Total for all components in commercial mixtures.

b) True solubility is probably lower.

**TABLE II**  
**OCTANOL/WATER PARTITION COEFFICIENTS AND BIOCONCENTRATION STUDIES**

<u>Phosphate Ester</u>	<u>Octanol/Water Partition Coefficient<sup>a</sup></u>	<u>Calculated Bioconcentration Factor</u>
tris-2-ethylhexyl	16,800	250
tributyl	10,100	190
dibutyl phenyl	18,800	270
t-butylphenyl diphenyl	133,000	770
cresyl diphenyl	32,000	360
isopropylphenyl diphenyl	202,000	970
2-ethylhexyl diphenyl	534,000	1600
triphenyl	42,500	420
trixylenyl	427,000	1400
isodecyl diphenyl	273,000	1100
tricresyl	128,000	750

a) Total for all components in commercial mixtures.

TABLE III  
LINEAR REGRESSION DATA FOR PARTITION  
COEFFICIENT - SOLUBILITY RELATIONSHIP

<u>Investigators</u>	<u>m</u>	<u>b</u>	<u>Correlation Coefficient</u>
Hansch, et al. (5) <sup>a</sup>	-0.75	5.23	0.935
Chiou, et al. (6)	-0.67	5.00	0.98
This Work	-0.42	5.42	-0.81

a) Published values converted to express solubility in  $\mu\text{moles/l}$ .

TABLE IV  
ACTIVATED SLUDGE PRIMARY BIODEGRADATION

<u>Phosphate Ester</u>	<u>Addition Rate (mg/l/24 hr)</u>	<u>Biodegradation %</u>	<u>Test Duration (Weeks)</u>
triphenyl	3	96 ± 2	12
	13	93 ± 11	7
tricresyl	3	97+	4
	13	99+	4
trixylenyl	3	65 ± 18	14
	13	13 ± 9	25
cresyl diphenyl	3	82 ± 12	22
isopropylphenyl diphenyl	3	49 ± 8	24
	13	35 ± 11	15
t-butylphenyl diphenyl	3	93+	9
	13	84 ± 3	8
isodecyl diphenyl	3	54 ± 6	24
	13	20 ± 9	15
2-ethylhexyl diphenyl	3	74 ± 9	22
dibutyl phenyl	3	95+	4
	13	52 ± 11	21
tris-2-ethylhexyl	3	20 ± 8	34
tributyl	3	96+	13
	13	56 ± 21	21

TABLE V  
CARBON DIOXIDE EVOLUTION OF PHOSPHATE ESTERS

<u>Phosphate Ester</u>	<u>Ester Concentration (mg/l)</u>	<u>% OF THEORY</u>		
		<u>Elapsed Days of Test</u>		
		<u>7</u>	<u>28</u>	<u>48</u>
triphenyl	18.3	61.9	81.8	-
tricresyl	26.4	78.6	82.1	86.3
trixylenyl	20.2	4.7	43.8	65.2
cresyl diphenyl	23.1	53.2	84.5	91.3
isopropylphenyl diphenyl	21.5	9.4	48.8	61.8
t-butylphenyl diphenyl	19.8	43.4	89.8	92.3
isodecyl diphenyl	19.0	13.5	63.3	68.4
2-ethylhexyl diphenyl	21.6	37.2	82.3	-
dibutyl phenyl	19.7	61.5	84.4	-
	20.6	11.1	75.6	84.5
tributyl	20.0	0.9	3.3	-
	19.4	30.4	90.8	-

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Figure 1. TPP biodegradation in river water  
Symbols: ●, Set 1; ■, Set 2; ▲, Set 3; ○, LAS (Set 2)

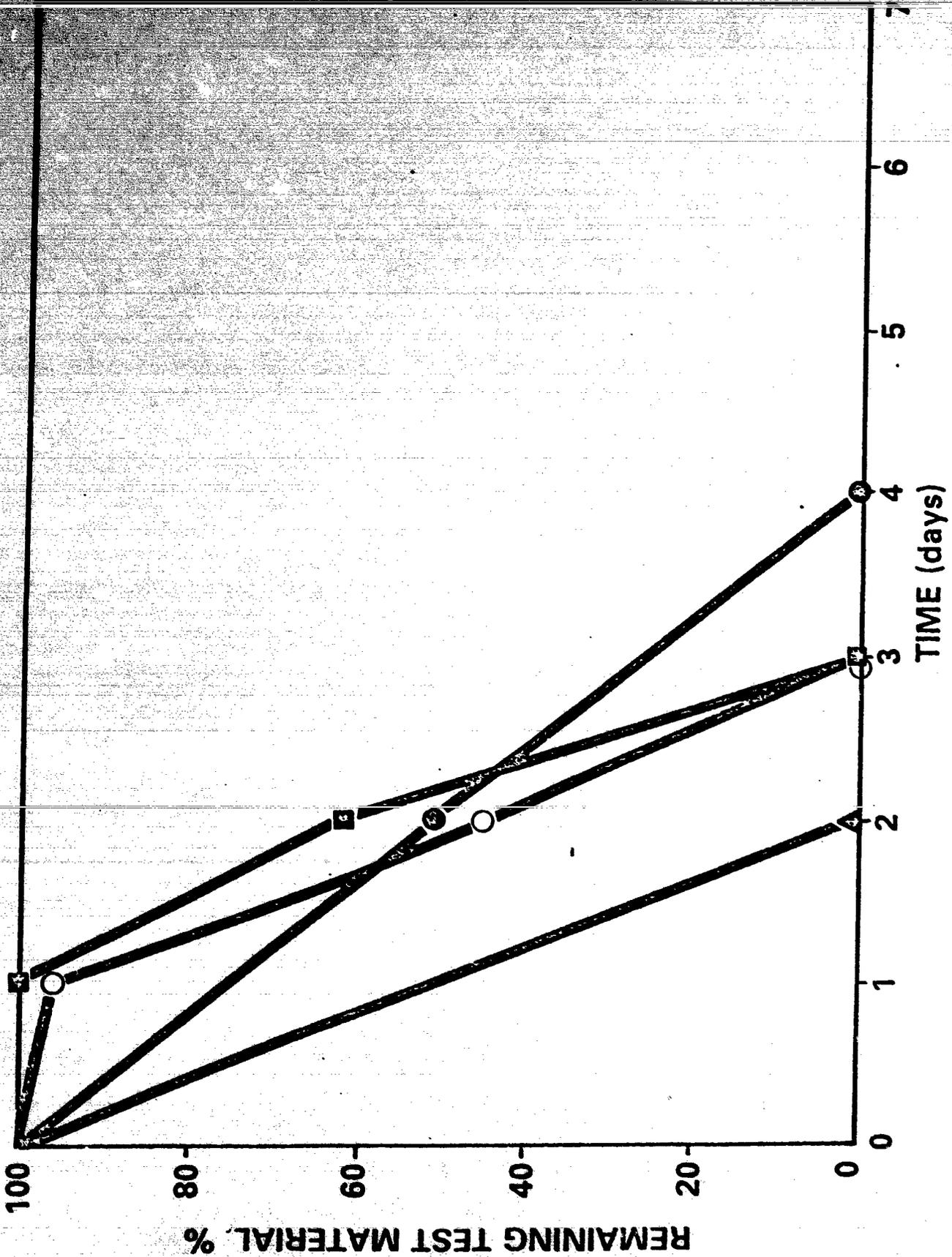


Figure 2. Biodegradation in river water - set 1  
Symbols: ●, TPP; □, tBPDP; △, IDDP

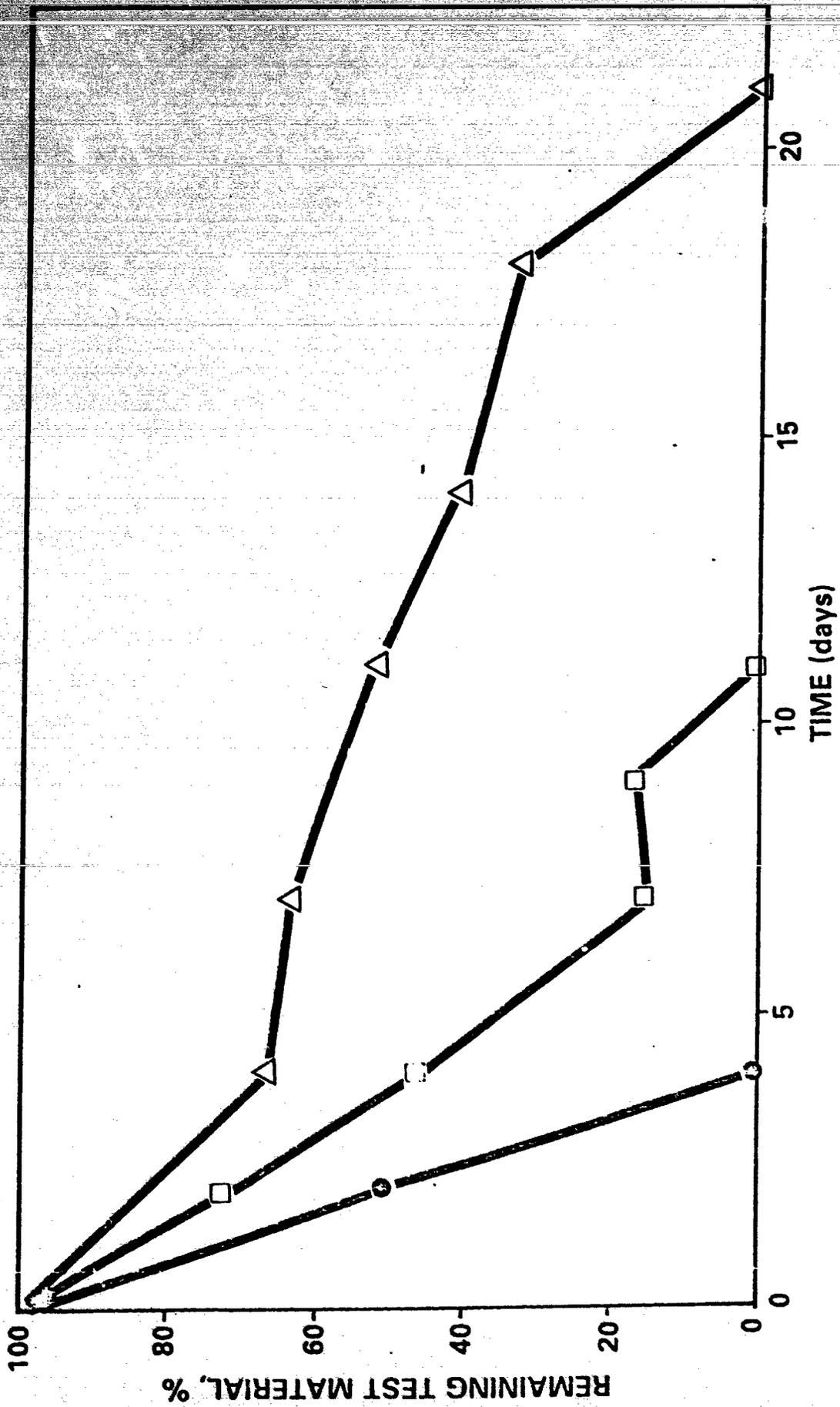


Figure 3. Biodegradation in river water - set 2  
Symbols: ■, TPP; ○, CDP; □, TCP; △, DBPP; ◇, TBP.

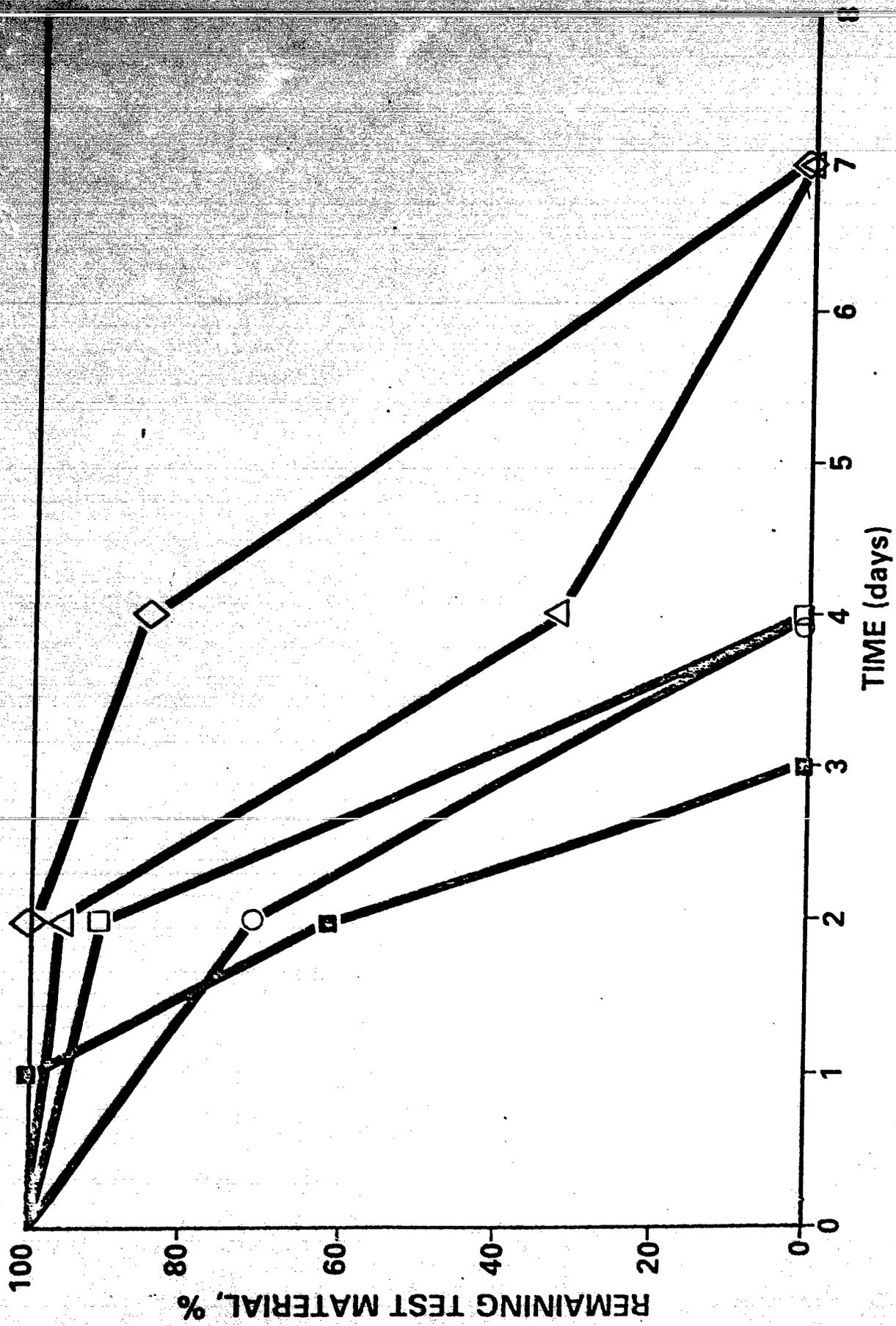


Figure 4. Biodegradation in river water - set 3  
Symbols: ▲, TPP; ○, EHDP; □, IPDP.

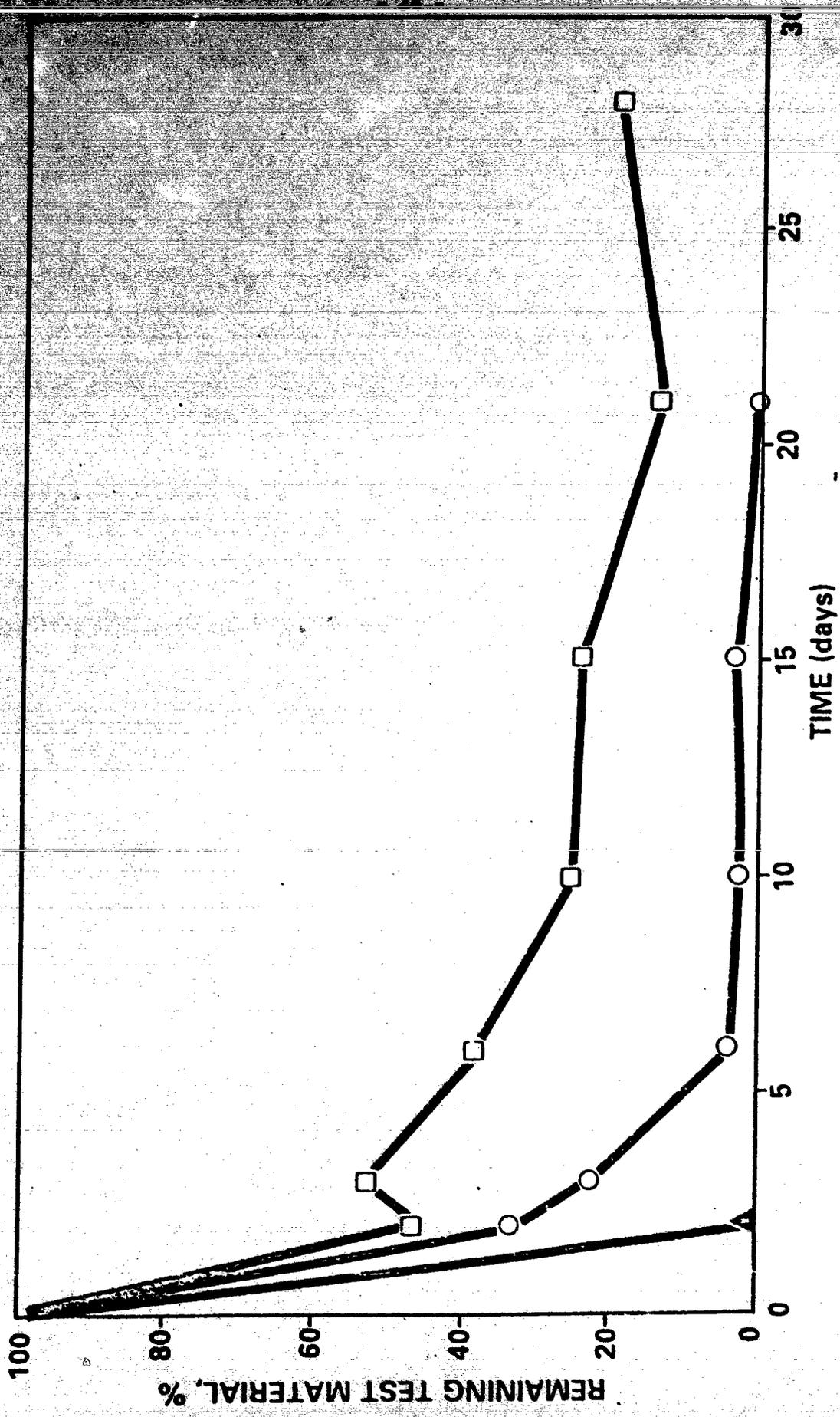
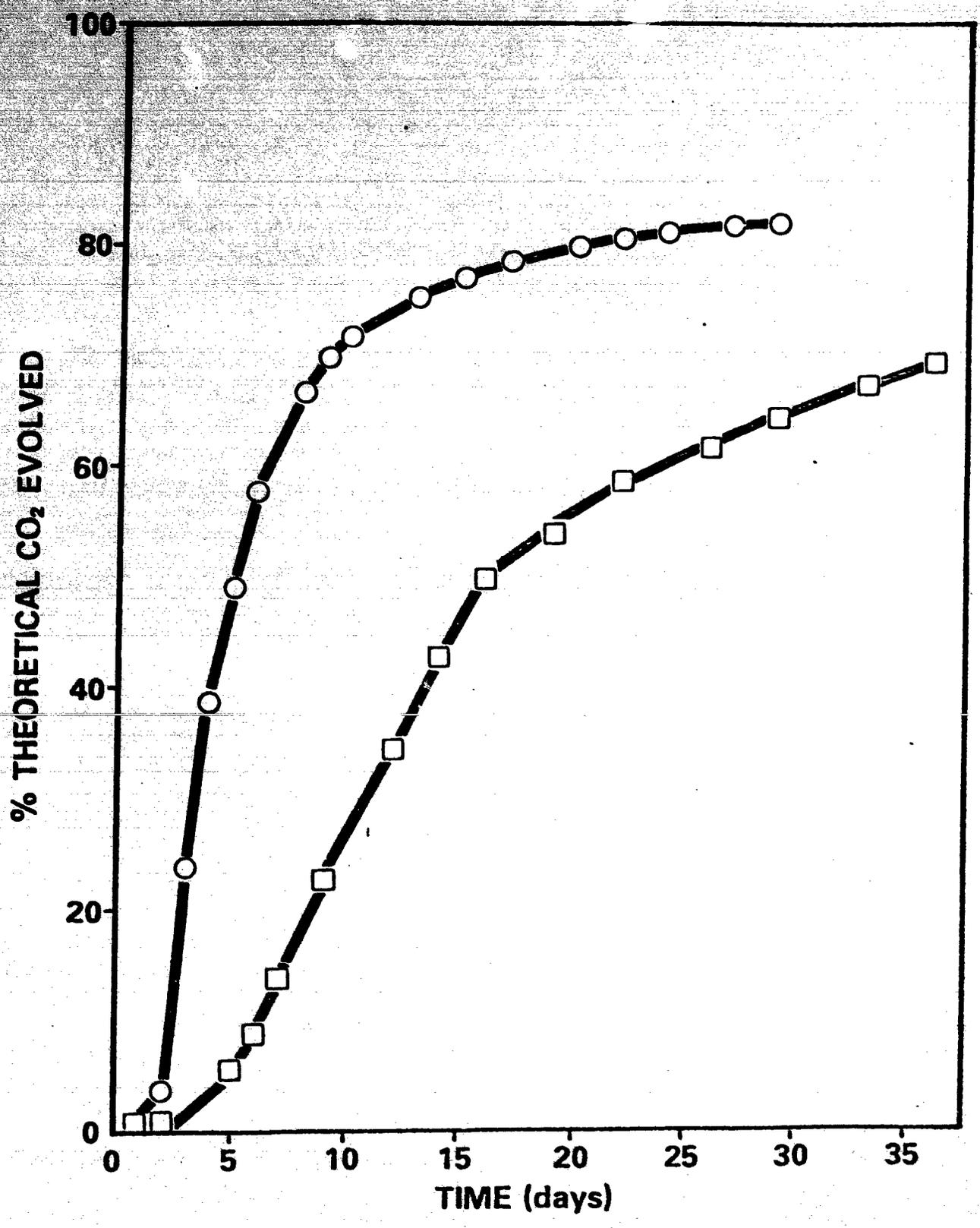


Figure 5. Carbon dioxide evolution for TPP and IDDP  
Symbols: ○, TPP; □, IDDP



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**RESEARCH & DEVELOPMENT**

ENVIRONMENTAL SCIENCES SECTION

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ENVIRONMENTAL FATE TESTING OF PHOSPHATE ESTERS

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### ABSTRACT

Environmental fate studies encompassing water solubility, water-octanol partition coefficient, and biodegradability were carried out for eleven trialkyl, alkyl aryl, and triaryl phosphate esters. These studies include that phosphate esters as a group exhibit low aqueous solubility, have a moderate potential for bioconcentration, and readily undergo primary and ultimate biodegradation by naturally occurring mixed-microbial populations present in activated sludge and river water.

## INTRODUCTION

Phosphate esters have found wide application as plasticizers and hydraulic fluids where flame retardancy is a desired property. Although such uses may lead inadvertently to introduction into the environment there are relatively few reports in the technical literature of the presence of phosphate esters in the environment or of their environmental fate (1,2,3).

Among the more environmentally important properties of a synthetic chemical are its partitioning characteristics and biodegradability. The n-octanol/water partition coefficient helps predict whether a chemical will tend to bioconcentrate (4). For hydrophobic compounds this partition coefficient may be estimated from aqueous solubility data (5,6). Biodegradability is an important determinant of environmental residence time, and thus affects the availability of a chemical for bioconcentration.

This paper reports on studies of the partitioning characteristics and biodegradability of eleven phosphate esters - triphenyl phosphate (TPP), tricresyl phosphate (TCP), trixylenyl phosphate (TXP), cresyl diphenyl phosphate (CDP), isopropylphenyl diphenyl phosphate (IPDP), t-butylphenyl diphenyl phosphate (tBPDP), isodecyl diphenyl phosphate (IDDP), 2-ethylhexyl diphenyl phosphate (EHDP), dibutyl phenyl phosphate (DBPP), tris-2-ethylhexyl phosphate (TEHP), and tributyl phosphate (TBP).

## EXPERIMENTAL

### Materials

The eleven phosphate esters studied were commercial-grade materials. TPP, TCP, CDP (Santicizer<sup>®</sup> 140), IDDP (Santicizer<sup>®</sup> 148), EHDP (Santicizer<sup>®</sup> 141), tBPDP, and DBPP were manufactured by Monsanto Co. Three of the remaining esters - IPDP (Kronitex<sup>®</sup> 100), TXP (Kronitex<sup>®</sup> TXP), and TEHP -

were obtained from FMC Corporation, and the fourth - TBP (Phosflex<sup>®</sup> 4) from Stauffer Chemical Co.

The chemical name specified for each ester indicates the major component. TPP, TBP, ENDP, and TEHP were relatively pure (>90%) single-component materials as indicated by gas chromatography. TCP and TXP were mixtures of their respective isomers. DBPP contained TBP and butyl diphenyl phosphate as minor components, while the four remaining esters also contained TPP and the appropriate di(alkyl) phenyl or di(alkylphenyl) phenyl phosphate esters. All data in the report pertain to these mixtures as a whole and not to their individual components.

#### Methods

##### Analytical

Gas chromatographic (GC) analyses for phosphate esters were carried out using a Hewlett-Packard 5711A chromatograph equipped with dual-flame ionization detectors. Samples were injected on-column into either 1 or 2 m x 3 mm i.d. glass columns packed with 3% OV-17 on 80-100 mesh Chromosorb W HP. Column temperature varied from 180 to 300°C depending on the ester. Injection port and detector temperatures were 250 and 350°C, respectively, and the helium flow rate was 45 cc/min.

##### Solubility

Our procedure for determining solubilities consisted of adding 25 ml of the phosphate ester and 500 ml of purified water (Milli-Q water purification system, Millipore Corp.) to a 1 liter glass bottle with an aluminum-foil lined cap. The bottle was agitated on a mechanical shaker for 48 hr and allowed to stand quiescent for 1 week to permit phase separation. The water equilibration was performed in the dark to preclude photodegradation. The aqueous phase was centrifuged at 14,000 rpm for one hour to remove

suspended droplets and then extracted twice with methylene chloride. The extracts were combined and concentrated in a Kuderna-Danish evaporative concentrator and analyzed for phosphate esters.

#### Partition Coefficient

The n-octanol/water partition coefficients were measured by preparing solutions (100 ppm to 1%) of the test material in 100 ml of n-octanol. The octanol solution and 500 ml of purified water were added to a 1 liter glass bottle with a foil-lined cap and shaken for 48 hrs. The mixture was then transferred to a 1 liter separatory funnel and allowed to stand for one week. This equilibration was also performed in the dark. Aliquots of the aqueous phase were drained from the funnel and extracted twice with methylene chloride. The extracts were combined, concentrated, and analyzed for phosphate esters.

The partition coefficient, P, was calculated using the equation:

$$P = C_o/C_w$$

where  $C_o$  and  $C_w$  are the phosphate ester concentrations in octanol and water, respectively. The original ester concentration in octanol was used in the calculations because in all cases only a negligible amount of the ester partitioned into the aqueous phase. Partition coefficients were used to calculate bioconcentration factors, applying the equation of Neely, et al. (4).

#### Primary Biodegradation

The river die-away method is similar to that employed in a study of phthalic acid esters (7). Settled 200 ml portions of Mississippi River water (St. Louis waterfront) were added to a series of 16-ounce narrow mouth screw-cap bottles. Four microliters of a solution containing 50  $\mu$ g of

phosphate ester per ml of ethanol were injected into each bottle. Each bottle was sealed with a foil-lined cap, mixed, and stored in the dark at room temperature. Heat-sterilized water controls were included to confirm that any decrease in the initial 1 mg/l phosphate ester level was due to biodegradation and not some other physical or chemical phenomenon. A set of positive controls was also prepared with river water and linear alkylbenzene-sulfonate (LAS). For each product the active and control samples were analyzed periodically for residual ester. Analyses were carried out by extracting a bottle and its contents with hexane (3 x 25 ml). The extracts were concentrated and analyzed using the previously described gas-chromatographic procedure.

The activated-sludge method is based on the Soap and Detergent Association's semi-continuous procedure (8) using modified feed (9). Domestic activated sludge from a local treatment plant was used in magnetically-stirred glass vessels of 1.5 liter operating volume.

The phosphate esters were tested at addition rates of 3 and 13 mg/l 24-hr cycle. For measuring primary degradation, 50-ml samples of mixed liquor were withdrawn a few minutes after feeding and at the end of the 24-hr cycle. These samples were extracted with hexane (3 x 25 ml). Concentration and analysis of the extracts were performed as for the river water samples. Sampling was carried out on a one-cycle-per-week basis for each ester for periods ranging from 8 to 39 weeks.

The efficiency of the analytical methods was determined by analyzing mixed liquor containing known phosphate ester concentrations. Each ester was added in duplicate at three levels (2,4, and 6 mg/l) to activated sludge mixed liquor from a blank semi-continuous activated sludge (SCAS) unit. These samples were analyzed as previously indicated and the concentrations

found were compared to the added concentrations. The average recoveries were: TPP,  $91 \pm 6\%$ ; TCP,  $91 \pm 6\%$ ; TXP,  $80 \pm 4\%$ ; CDP,  $92 \pm 5\%$ , IPDP,  $97 \pm 8\%$ ; tBPDP,  $76 \pm 2\%$ ; ICDP,  $79 \pm 7\%$ , EHDP,  $96 \pm 6\%$ ; DBPP,  $80 \pm 11\%$ ; TEHP,  $74 \pm 4\%$ ; and tBP,  $91 \pm 3\%$ . To verify that disappearance of a phosphate ester was not due to volatilization, the off-gases from each unit were passed through a series of three hexane scrubbers during a complete cycle. No significant ( $<0.5\%$ /cycle) volatility losses were observed for any of the phosphate esters.

#### Ultimate Biodegradation

The ultimate biodegradability of the phosphate esters was measured using the apparatus and procedure developed by Thompson and Duthie (10) and modified by Sturm (11). Acclimated bacterial seed was prepared using a 14-day Bunch-Chambers (12) die-away with no transfer. In this procedure a 2 liter flask containing 20 mg of appropriate ester, 50 mg of yeast extract, 100 ml of settled SCAS supernatant, and 900 ml of standard BOD water (13) was incubated in the dark for 14 days at ambient temperature under quiescent conditions. At the end of the incubation, equal aliquots from each phosphate ester flask were mixed to form a composite seed. A 9 liter bottle containing 500 ml of composite seed and 5500 ml of BOD water was prepared for each ester and a control. To each of the phosphate ester bottles, a weighed quantity (approximately 120 mg) of the appropriate ester was added. The control bottles received no test material. During the test, CO<sub>2</sub>-free air was bubbled through the test bottles, and the effluent air passed through a set of three CO<sub>2</sub> scrubbers, each containing 100 ml of 0.05N Ba(OH)<sub>2</sub>. The evolved CO<sub>2</sub> was trapped as barium carbonate and quantitated by back-titrating the remaining Ba(OH)<sub>2</sub> with 0.1N HCl. Carbon dioxide production from the

control bottles was in the range of 10 to 15% of the total CO<sub>2</sub> evolved from the phosphate ester bottles. Final values were corrected for CO<sub>2</sub> production from the control.

## RESULTS AND DISCUSSION

The data in Table I indicate that phosphate esters generally exhibit room temperature aqueous solubilities in the low ppm range. The most soluble esters are those with smaller, alkyl groups. The true solubility of tris-2-ethylhexyl phosphate is probably less than 1000 ppm. While shaking with water, the aqueous phase turned cloudy, suggesting the formation of an emulsion or a decomposition product. The turbidity was not removed by centrifugation or extraction.

The octanol/water partition coefficients and calculated bioconcentration factors are listed in Table II. The range of bioconcentration factors covers almost an order of magnitude. Based on these octanol/water partition coefficients, it appears that the esters have moderate potential to concentrate in aquatic organisms. In general, the bioconcentration factors are one to two orders of magnitude less than those reported for some pesticides and PCB's (14, 15).

Currently we are aware of experimental bioconcentration data for only one of the compounds covered in this report. In 1977 Lombardo and Egly reported a bioconcentration factor of 280 for rainbow trout exposed to triphenyl phosphate at approximately the 10 ppb level (2). This is the same range as our value of 420 calculated from the octanol/water partition coefficient.

Several workers have proposed a relationship between aqueous solubility and the octanol/water partition coefficient of hydrophobic compounds in the form:

$$\text{LOG } P = m (\log S) + b.$$

In this equation  $P$  is the partition coefficient and  $S$  is the solubility in  $\mu\text{moles/l}$ . The linear regression data obtained in this work and in previous investigations are given in Table III.

The data of Hansch and coworkers (5) were generated from a diverse group of 156 common organic compounds. Chiou, et al., (6) studied 34 organic compounds including hydrocarbons, acids, and pesticides. The relatively good agreement between those data and this study on phosphate esters lends support to the concept that solubility may be used to estimate partition coefficients.

River die-away studies on 9 phosphate esters demonstrated that the esters, exposed to the natural microbial population of the river, underwent primary biodegradation at moderate to rapid rates. TPP was employed as a common test material in 3 sets of experiments. The river water in each set was taken from the same source but at different times. The die-away curves for TPP in each set are compared to a positive LAS control in Figure 1. Figures 2, 3, and 4 show die-away curves for the remaining esters. Of the esters tested, only IPDP showed residual ester at the end of the test period. Five of the esters (TPP, TCP, CDP, DBP and TBP) showed complete primary degradation in less than seven days, while tBPDP, EHDP, and IDDP degraded in 10 to 21 days. Sterile-water controls showed no significant evidence of non-biological degradation or loss.

Primary biodegradation rates from our semi-continuous activated sludge (SCAS) studies are summarized in Table IV. The SCAS test, which simulates secondary sewage treatment, shows generally the same trend in degradation rate as river die-away studies. At the 3 mg/l feed level, TPP, TCP, CDP, tBPDP, EHDP, DBPP, and TBP showed rapid primary degradation. TXP, IPDP, and IDDP were in the intermediate range, and TEHP degraded more slowly. In the 3 to 13 mg/l range, the effect of feed level was variable. Apparent inhibiting effects were observed for TXP, IPDP, DBP, and TBP at the 13 mg/l level. Both IPDP and IDDP caused a significant decrease in the biomass as monitored by the suspended solids concentration.

No attempt to isolate intermediates was made in either the river die-away or SCAS studies. To establish that the primary biodegradation observed in these studies represented more than a slight modification of the parent molecule, CO<sub>2</sub> evolution studies were undertaken. By measuring the CO<sub>2</sub> produced and comparing it to the theoretical yield based on the carbon content and weight of the ester, an indication of ultimate biodegradability was obtained.

In Table V the CO<sub>2</sub> produced from each ester is expressed as a percentage of the theoretical yield at three time intervals during the test. Typical curves are shown in Figure 5. It is apparent that the esters tested essentially break down completely to carbon dioxide, water, and inorganic phosphate. The same rate trends observed in the river die-away and activated-sludge tests are apparent in the CO<sub>2</sub> evolution data. TEHP, because of its slow primary degradation rate, would not be expected to produce significant CO<sub>2</sub> in this test.

The degradation pathway for the phosphate esters most likely involves a step-wise enzymatic hydrolysis to orthophosphate and the phenolic or alcohol moieties (16). The alcohol and/or phenol would then be expected to undergo

further degradation. It is obvious from all the data presented that the phenol and/or alcohol moieties have a significant effect on the biodegradability. Although the data are too limited to make any highly significant structural correlations a few tentative conjectures can be made. For trialkyl and alkyl aryl esters, the shorter the alkyl chain the more biodegradable the ester. For alkyl aryl and triaryl esters, increasing the number and size of substituent groups on the phenyl ring leads to decreasing biodegradability. A comparison of the solubility and biodegradation data shows no obvious correlation.

The biodegradation data presented in this study suggest that mixed microbial populations in the environment will degrade phosphate esters. The significance of the results obtained by Westlake, et al., (1) on several triaryl esters (triphenyl, tri-o-cresyl, and trixylenyl) are uncertain. Using mixed bacterial populations isolated from soil they obtained growth on all three esters as sole carbon sources, but were unable to grow the dominant organism in pure culture on the esters. It appears likely that very high ester concentrations of 100 and 1000 mg/l inhibited growth.

Data on non-biological degradation routes for the phosphate esters are limited. A half-life for hydrolysis of TPP under neutral conditions has been estimated at 1.3 years from kinetic data (3). This suggests that biodegradation is the dominant breakdown route in the environment. For aryl and alkyl aryl esters, photolysis remains a possible degradation mechanism.

We have shown that commercial phosphate esters can be effectively treated by activated sludge from domestic sewage treatment plants and are readily susceptible to biodegradation in rivers by naturally occurring microbial populations. Judging from these tests, we feel that phosphate ester contamination

is not likely to become a wide spread environmental problem unless their introduction rate exceeds the degradation capacity of the ecosystem. Because of their low aqueous solubility and moderate potential to bioaccumulate in aquatic organisms, discharge of high levels to the environment should be avoided.

TABLE I

SOLUBILITY IN WATER AT ROOM TEMPERATURE

<u>Phosphate Ester</u>	<u>Solubility<sup>a</sup> (ppm)</u>
tris-2-ethylhexyl	1000 <sup>b</sup>
tributyl	280
dibutyl phenyl	96
t-butylphenyl diphenyl	3.2
cresyl diphenyl	2.6
isopropylphenyl diphenyl	2.2
2-ethylhexyl diphenyl	1.9
triphenyl	1.9
trixylenyl	0.89
isodecyl diphenyl	0.75
tricresyl	0.36

a) Total for all components in commercial mixtures.

b) True solubility is probably lower.

TABLE II

HEXANOL/WATER PARTITION COEFFICIENTS AND BIOCONCENTRATION STUDIES

<u>Phosphate Ester</u>	<u>Hexanol/Water Partition Coefficient<sup>a</sup></u>	<u>Calculated Bioconcentration Factor</u>
tri-2-ethylhexyl	16,800	250
tributyl	10,100	190
dibutyl phenyl	18,800	270
t-butylphenyl diphenyl	133,000	770
cresyl diphenyl	32,000	360
isopropylphenyl diphenyl	202,000	970
2-ethylhexyl diphenyl	534,000	1600
triphenyl	42,500	420
triptylenyl	427,000	1400
isodecyl diphenyl	273,000	1100
tricresyl	128,000	750

a) Total for all components in commercial mixtures.

TABLE III  
LINEAR REGRESSION DATA FOR PARTITION  
COEFFICIENT - SOLUBILITY RELATIONSHIP

<u>Investigators</u>	<u>m</u>	<u>b</u>	<u>Correlation Coefficient</u>
Hansch, et al. (5) <sup>a</sup>	-0.75	5.23	0.935
Chiou, et al. (6)	-0.67	5.00	0.98
This Work	-0.42	5.42	-0.81

a) Published values converted to express solubility in  $\mu\text{moles/l}$ .

TABLE IV  
ACTIVATED SLUDGE PRIMARY BIODEGRADATION

<u>Phosphate Ester</u>	<u>Addition Rate (mg/l/24 hr)</u>	<u>Biodegradation %</u>	<u>Test Duration (Weeks)</u>
triphenyl	3	96 ± 2	12
	13	93 ± 11	7
tricresyl	3	97+	4
	13	99+	4
trixylenyl	3	65 ± 18	14
	13	13 ± 9	25
cresyl diphenyl	3	82 ± 12	22
isopropylphenyl diphenyl	3	49 ± 8	24
	13	35 ± 11	15
t-butylphenyl diphenyl	3	93+	9
	13	84 ± 3	8
isodecyl diphenyl	3	54 ± 6	24
	13	20 ± 9	15
2-ethylhexyl diphenyl	3	74 ± 9	22
dibutyl phenyl	3	95+	4
	13	52 ± 11	21
tris-2-ethylhexyl	3	20 ± 8	34
tributyl	3	96+	13
	13	56 ± 21	21

TABLE V  
CARBON DIOXIDE EVOLUTION OF PHOSPHATE ESTERS

<u>Phosphate Ester</u>	<u>Ester Concentration (mg/l)</u>	<u>% OF THEORY</u>		
		<u>Elapsed Days of Test</u>		
		<u>7</u>	<u>28</u>	<u>48</u>
triphenyl	18.3	61.9	81.8	-
tricresyl	26.4	78.6	82.1	86.3
trixylenyl	20.2	4.7	43.8	65.2
cresyl diphenyl	23.1	53.2	84.5	91.3
isopropylphenyl diphenyl	21.5	9.4	48.8	61.8
t-butylphenyl diphenyl	19.8	43.4	89.8	92.3
isodecyl diphenyl	19.0	13.5	63.3	68.4
2-ethylhexyl diphenyl	21.6	37.2	82.3	-
dibutyl phenyl	19.7	61.5	84.4	-
	20.6	11.1	75.6	84.5
tributyl	20.0	0.9	3.3	-
	19.4	30.4	90.8	-

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Figure 1. TPP biodegradation in river water  
Symbols: ○, Set 1; ■, Set 2; ▲, Set 3; ○, LAS (Set 2)

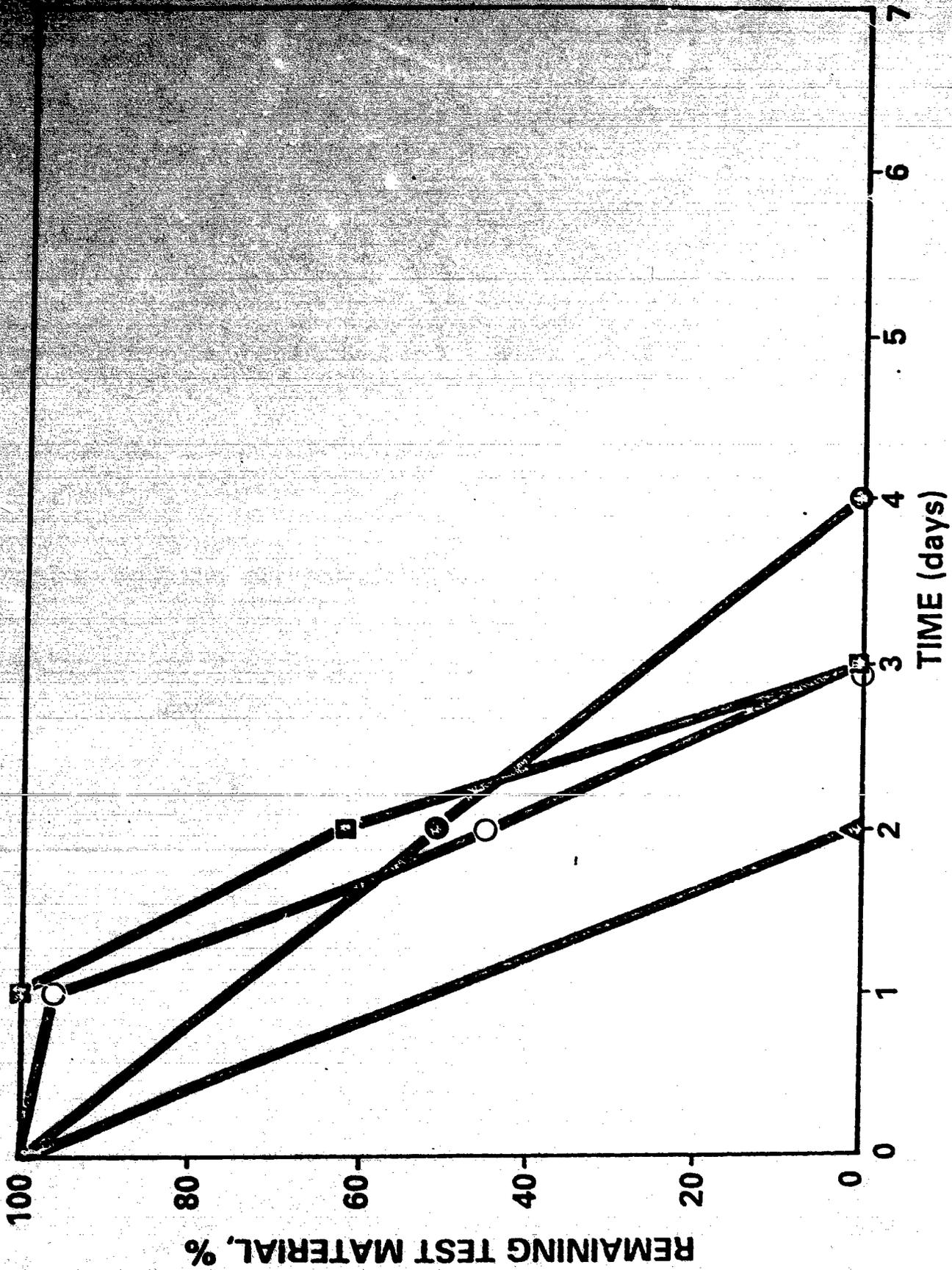


Figure 2. Biodegradation in river water - set 1  
Symbols: ●, TPP; □, tBPDP; △, IODP

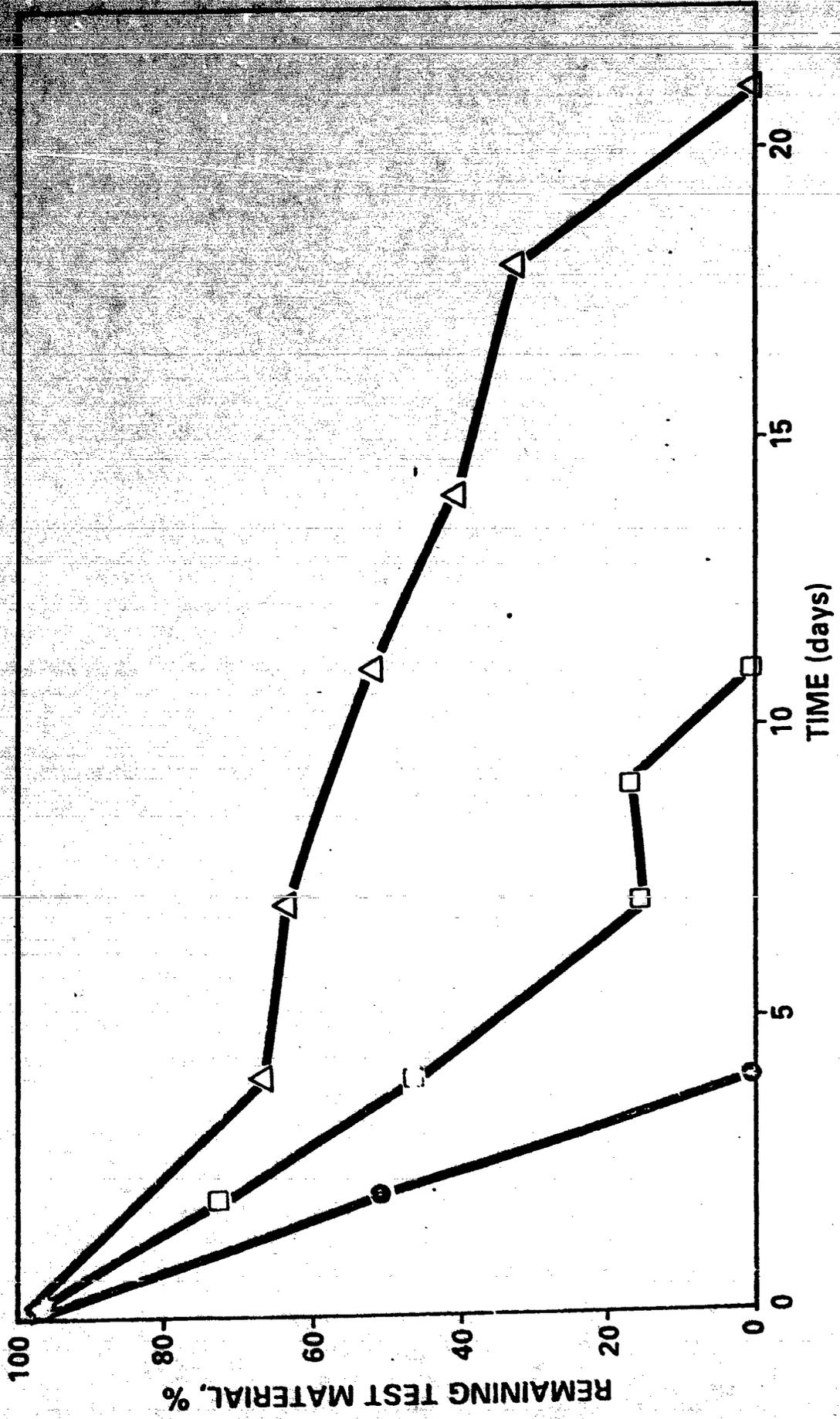


Figure 3. Biodegradation in river water - set 2  
Symbols: ■, TPP; ○, CDP; □, TCP; △, DBPP; ◇, TBP.

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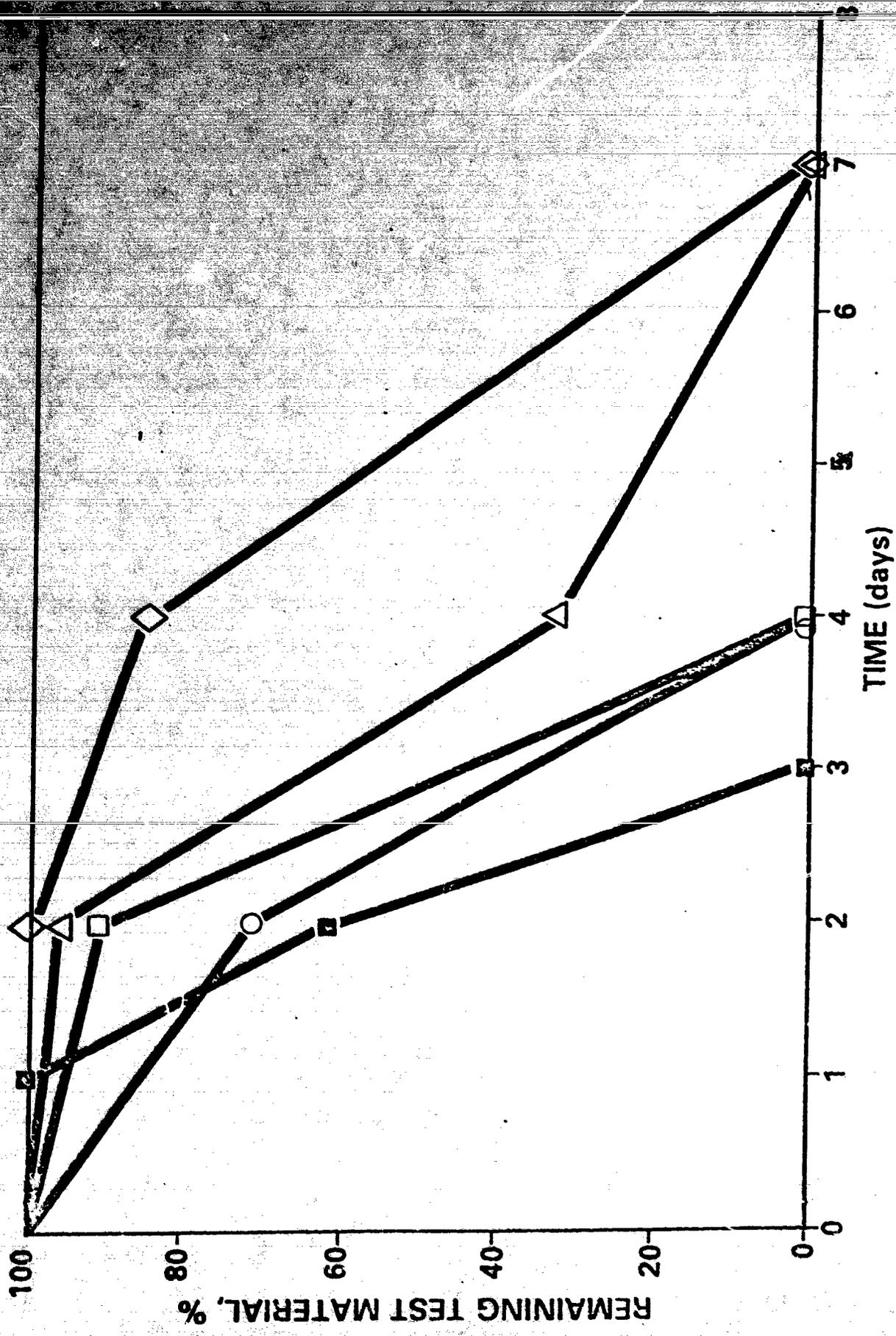


Figure 4. Biodegradation in river water - set 3  
Symbols: ▲, TPP; ○, EHDP; □, IPDP.

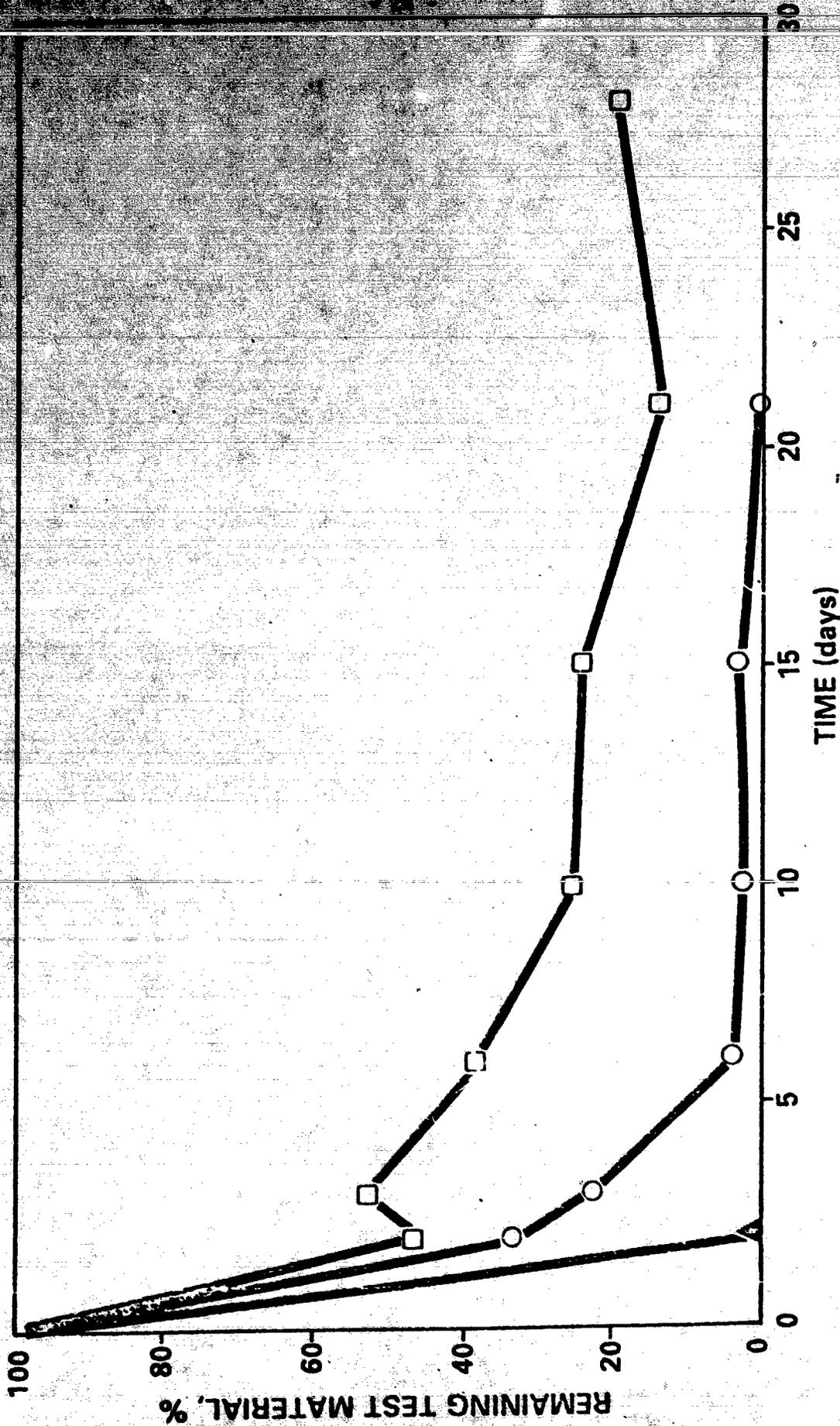
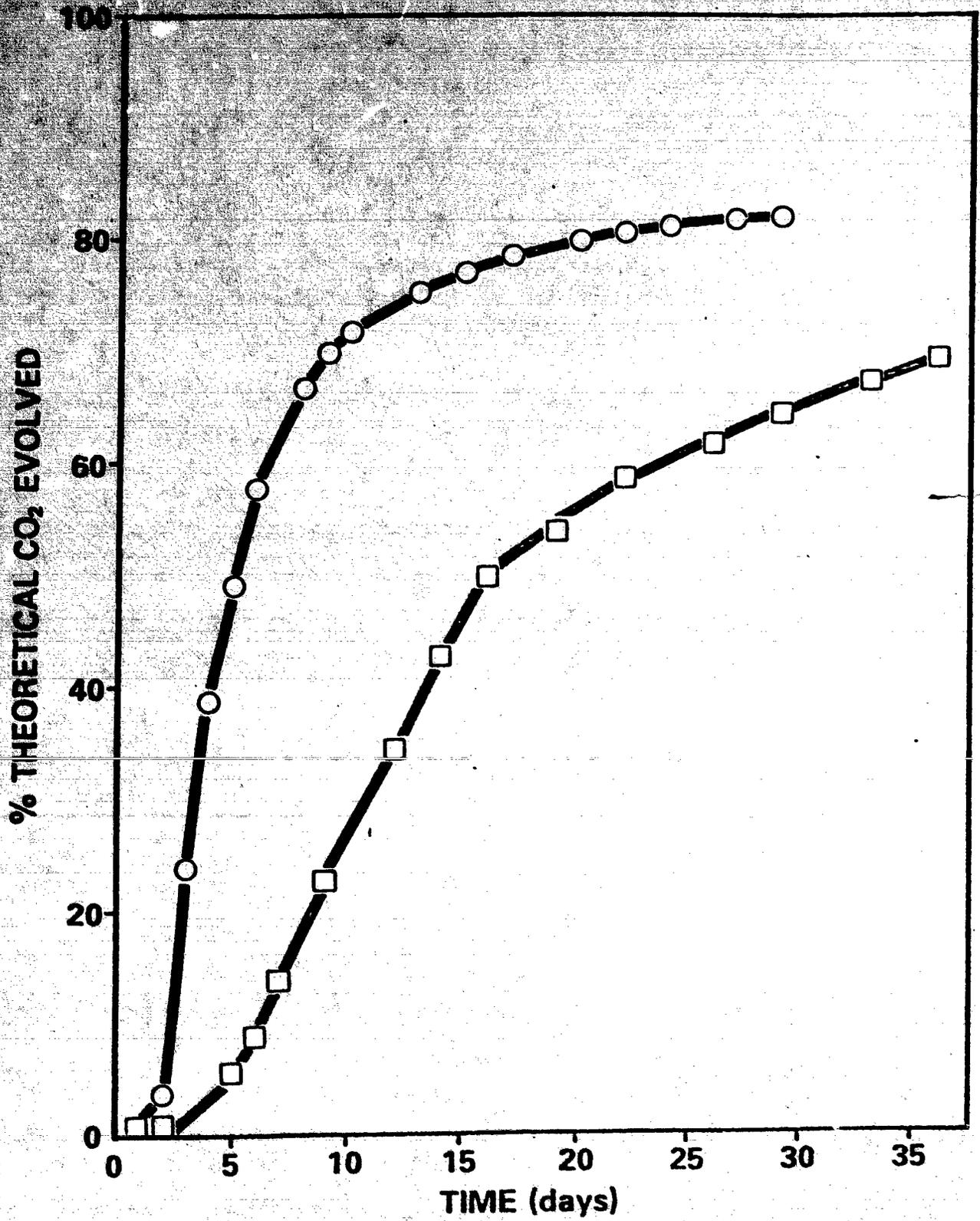


Figure 5. Carbon dioxide evolution for TPP and IDDP  
Symbols: ○, TPP; □, IDDP





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