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Thiophosphoric Acid Derivatives of Ethylamine, DL-Methionine, and L-Proline Ethyl Esters II

Biological Activities in Guppies

By FLORENCE C. KLEE† and ERNST R. KIRCH

Observed biological responses of guppies in 0.01% w/v aqueous solutions of the thiophosphoric acid derivatives indicate that the compounds are acetylcholinesterase inhibitors. The 0.001% solutions furnished additional information concerning the biological activity of these compounds. Structure-toxicity relationship is discussed.

THE SYNTHESIS of thiophosphoric acid derivatives of ethylamine, DL-methionine, and L-proline ethyl esters has recently been published (1).

The thiophosphoric acid derivatives used in this investigation have the general formula

—N—P(X)$_2$, where (X)$_2$ = (O-alkyl)$_2$, (O-C$_6$H$_5$)$_2$, (C$_2$H$_5$O)Cl, or (Cl)$_2$, and N represents the amine or amino acid ester moiety. These structures are similar to those of known anticholinesterases, thus the thiophosphoric acid derivatives may be acetylcholinesterase inhibitors. If so, then the following types of biological responses may be visualized in guppies (*Lebistes Reticulatus*): difficulty in breathing, dizziness, evacuation of rectum and bladder, convulsions, and total paralysis (death).

The purpose of this investigation was to observe the biological responses of baby guppies which have been immersed for 24 hours in 0.01 and 0.001% w/v aqueous solutions of each compound.

EXPERIMENTAL

Table I lists the compounds used in this study. Stock aqueous solutions or aqueous suspensions (0.1% w/v) were prepared from which 0.01 and 0.001% w/v solutions were obtained after making a tenfold dilution of the stock and 0.01% w/v solution.

An alcoholic stock solution and alcoholic dilutions were made of diphenoxyethylamidothiophosphate, since it was not possible to obtain a uniform 0.1% w/v aqueous suspension. Aliquots of the alcoholic solutions were allowed to evaporate to dryness and a volume of distilled water equal to the volume of alcohol was introduced before testing the biological activity of the compound.

Ten milliliters of 0.01 and 0.001% w/v aqueous solutions of each compound were placed in separate vials (2.4 cm. diameter and 5.5 cm. in height) and 10 young guppies were placed in each vial. For control purposes, distilled water was used instead of an aqueous solution of a compound under test and it was observed that all of the guppies survived after 24 hours. The O,O-diethyl phosphorochloridothioate (Ethyl PCT-Monsanto), C$_2$H$_5$O—P(S)—Cl,

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TABLE I.—PER CENT EXPIRED OF GUPPIES

No.	Compound	Moles/L. × 10^{-4} 0.01% w/v	% Expired 24 hr., 0.01% w/v	% Expired 24 hr., 0.001% w/v
1	$C_2H_5NHP(S)(OC_2H_5)_2$	5.07	100	0
2	$C_2H_5NHP(S)(OCH_2CH_2CH_3)_2$	4.44	100	10
3	$C_2H_5NHP(S)(OCH(CH_3)_2)_2$	4.44	100	0
4	$C_2H_5NHP(S)(OCH_2CH_2CH_2CH_3)_2$	3.95	100	40
5	$C_2H_5NHP(S)(OCH(CH_3)CH_2CH_3)_2$	3.95	100	0
6	$C_2H_5NHP(S)(OC(CH_3)_3)_2$	3.95	100	20
7	$C_2H_5NHP(S)(OCH_2CH(CH_3)_2)_2$	3.95	100	20
8	$C_2H_5NHP(S)(OC_6H_5)_2$	3.41	100	100
9	$C_2H_5NHP(S)(OC_2H_5)Cl$	5.33	100	10
10	$C_2H_5NHP(S)Cl_2$	5.62	100	90
11	$CH_3SCH_2CH_2CH(CO_2C_2H_5)NHP(S)(OC_2H_5)_2$	3.04	100	20
12	$CH_3SCH_2CH_2CH(CO_2C_2H_5)NHP(S)(OC_2H_5)Cl$	3.13	100	0
13	$CH_3SCH_2CH_2CH(CO_2C_2H_5)NHP(S)Cl_2$	3.22	100	0
14	$CH_2CH_2CH_2CH(CO_2C_2H_5)N—P(S)(OC_2H_5)_2$	3.39	100	50
15	$CH_2CH_2CH_2CH(CO_2C_2H_5)N—P(S)(OC_2H_5)Cl$	3.50	70	0
16	$CH_2CH_2CH_2CH(CO_2C_2H_5)N—P(S)Cl_2$	3.62	100	0
17	$(C_2H_5O)_2P(S)Cl$ Reference standard	5.31	100	0

an anticholinesterase (2), was chosen as the reference standard for making biological response comparisons.

The behavior of the fish was closely observed for 2 hours, and at the end of 24 hours the number of fish which survived was recorded. There were no survivors in the 0.01% w/v aqueous solutions of the compounds with the exception of N-ethoxychlorophosphinothioyl-L-proline ethyl ester (Table I). The three fish (survivors) matured in 1 month and the male and a female expired shortly thereafter.

The 0.01% w/v (3.04 to 5.62 × 10^{-4} *M*) aqueous solutions of the compounds produced biological responses similar to those of known acetylcholinesterase inhibitors. Before total paralysis occurred, the guppies exhibited difficulty in breathing and intermittent convulsions (head and tail flutter) followed by coma. Many of the fish lost their sense of balance and were seen in abnormal positions.

An unusual spinal effect was seen when the fish were subjected to the diphenoxyethylamidothiophosphate (0.01% w/v). This compound caused the bending of the tail of several fish to such an extent that the tip of the caudal fin was adjacent to the head of the fish.

The fish which survived after 24-hour chemical treatment with aqueous 0.001% w/v solutions were transferred to fresh water and observed periodically for several months to determine the sex, the months required for the fish to mature, and any abnormal development.

Behavior Toward Food.—An interesting observation concerning the behavior of the fish in fresh water toward food was made immediately after the 24-hour chemical treatment with 0.001% solutions of the compounds (Table II). Most of the fish consumed the food, but the fish which had been in solutions of the dihalo and four carbon chain derivatives, ignored the food. Fish previously in contact with the *sec*-butyl ester, actually leaped out of the water in an attempt to get away from the food which had touched them.

Sex Differentiation(?)—Unfortunately, the female/male ratio cannot be established at the start of the experiment since it is impossible to determine the sex of a baby guppy. However, after the guppies matured, it was found that the female/male survival ratio was greater than two to one, since there were more female survivors than males when both sexes survived (Table II). The larger number of female survivors may be due to the possibility that the compounds are more toxic to the male guppies or else most of the baby guppies (sex unknown) are females. The first reason seems more probable, since experimentation involving mature fruit flies shows that in general, the compounds are more toxic to the males than to females.

It is interesting to note that with the exception of the ethoxychloro derivative in the proline series, there were no male survivors in the ethyl and ethoxychloro derivatives in the three series of compounds, the reference standard, the normal, and isobutyl esters.

It is noteworthy that only the male fish survived in the aqueous solution of the normal propyl ester.

Effect on Growth.—Normal guppies mature in about 3 months after birth as evidenced by full

TABLE II.—SOME BIOLOGICAL RESPONSES OF GUPPIES

Compound[a]	% Expired 24 hr.	Behavior[b] Toward Food	Sex Survived[c] and Growth[d]
$X(OC_6H_5)_2$	100	..	None
XCl_2	90	B	None[e]
$X(OCH_2CH_2CH_2CH_3)_2$	40	B	F(n)
$X(OC(CH_3)_3)_2$	20	B	M(n) F(n)[f]
$X(OCH_2CH(CH_3)_2)_2$	20	B	F(n)
$X(OC_2H_5)Cl$	10	A	F(R)
$X(OCH_2CH_2CH_3)_2$	10	A	M(n)
$X(OCH(CH_3)_2)_2$	0	A	M(n) F(n)
$X(OCH(CH_3)CH_2CH_3)_2$	0	C	M(n) F(n)
$X(OCH_2CH_3)_2$	0	A	F(n)
$Y(OCH_2CH_3)_2$	20	A	F(n)
$Y(OC_2H_5)Cl$	0	A	F(R) + I_2
YCl_2	0	A	M(R) F(R)
$Z(OCH_2CH_3)_2$	50	A	F(R)
$Z(OC_2H_5)Cl$	0	A	M(R) F(R)
ZCl_2	0	A	M(R) F(R)
$(C_2H_5O)_2P(S)Cl$	0	A	F(R) + I_1

[a] The compounds in each series are arranged according to decreasing toxicity as determined by the per cent expired after 24 hours in the 0.001% w/v aqueous solutions. $(C_2H_5O)_2P(S)Cl$ is the reference standard; X = $C_2H_5NHP(S)$; Y = DL-$CH_3SCH_2CH_2CH(CO_2C_2H_5)NHP(S)$; Z = L-$CH_2CH_2CH_2CH(CO_2C_2H_5)N{-}P(S)$. [b] A, Consumed food; B, ignored food; C, darted away from food. [c] M, Male; F, female. [d] n, Normal, fish matured in 3 months; R, rapid, fish matured in two months; I_1 inhibited or retarded, fish were immature after 3 months; I_2, inhibited or retarded, fish were immature after 6 months. [e] The remaining fish expired 3 hours after transfer to fresh water. [f] Gave birth to young.

development of the body. Caudal fin pigmentation and modification of the pelvic fins in the male as claspers are secondary sexual characteristics which can be easily observed.

Growth Stimulation.—It was noted that both male and female fish matured in 2 months when they had been in aqueous solutions of the dihalo derivatives in the methionine and proline series, and the ethoxychloro derivative in the latter series (Table II). Female guppies, the only survivors, matured in 2 months after being in contact with aqueous solutions of the ethoxychloro derivatives in the ethylamine and methionine series, reference standard, and the ethyl derivative in the proline series.

The greatest stimulation of growth was produced by the aqueous solution of N-(dichlorophosphinothioyl)-L-proline ethyl ester. Gigantism, or abnormal overgrowth, of the female and male fish, which are slightly more than twice the size of normal fish of the same age, was observed.

Growth Inhibition or Retardation.—After 3 months there is no apparent change in size of some of the fish which survived in aqueous solutions of the ethoxychloro derivative in the methionine series and reference standard. The fish which had been exposed to the methionine derivative are the same size and immature even at 6 months of age (Table II). It is possible that these fish are males, since the only fish which did mature are females.

Abnormal Body Development.—The female fish which survived in the aqueous solution of the ethoxychloro ester (methionine series) do not have well-proportioned bodies. Their short bodies and unusually long caudal fins give the fish a "stretched out" appearance. The ventral side is not curved like that of normal fish, but appears to be rather straight. This peculiar shape was noted in other female fish but, unfortunately, the previous chemical treatment is unknown since all the female survivors were placed into the same vessel with all the male survivors for the purpose of observing whether reproduction can occur. The female fish which had matured rapidly and were not well-proportioned expired at the age of $4^1/_2$ months.

In contrast, the giant female fish resulting from the 24-hour contact with the dichloro derivative in the proline series are beautifully proportioned and appear to be in perfect health at 6 months of age.

Reproduction.—The guppies which had undergone 24-hour contact with the same compound were placed into 2 L. of fresh water and observed periodically until they matured. Only the fish which had been in the aqueous solution of the tertiary butyl ester produced progeny (Table II).

The male and female survivors were introduced into the same container and after 6 months, it is interesting to note, only one of the many females had given birth to young. There is a possibility that the synthetic thiophosphoric acid derivatives cause sterility in guppies in view of the fact that alkylating agents are known to produce transient sterility in male rats (3).

Tail Pigment.—Normal female guppies do not have highly pigmented tails. The tip of the caudal fin is a very pale yellow and practically unnoticeable. The giant female fish which resulted after 24-hour contact with an aqueous solution of the N-(dichlorophosphinothioyl)-L-proline ethyl ester have large tails and the bright yellow pigment near the end of the fin can be easily seen in daylight. The ends of the caudal fins of the females which had been exposed to other compounds are green and black.

The bodies and tail of normal male guppies are orange, green, blue, and black. The pigment of the male survivors is essentially the same as that of fish which have not been in 24-hour contact with thiophosphoric acid derivatives, however, there appears to be a predominance of brilliant red color and almost complete absence of orange pigment.

DISCUSSION

The structures of the synthesized compounds are similar to those of compounds known to be acetylcholinesterase inhibitors. Since very frequently the anticholinesterase activity *in vitro* of a given organophosphorus compound is not related to its toxicity *in vivo*, guppies were chosen to investigate the biological activity of the thiophosphoric acid derivatives of ethylamine, DL-methionine, and L-proline ethyl esters. Fish possess acetylcholinesterase in addition to other enzymes, and their biological responses can be readily observed when enzyme inhibition occurs.

If these derivatives are acetylcholinesterase inhibitors then certain biological responses such as difficulty in breathing, dizziness, and convulsions should have been observed. These responses were observed but other responses such as growth stimulation and retardation were also noted. It appears to the authors that the compounds probably have anticholinesterase activity.

The compounds of the three series are arranged in Table III according to decreasing toxicity as determined by the expiration time of the fish which represents time, in minutes, when the last fish of each group of 10 ceased to breath. For making biological response comparisons, $(C_2H_5O)_2P(S)Cl$, an anticholinesterase, was chosen as the reference standard.

Structure-Toxicity Relationship.—The compounds as 0.01% solutions are toxic to guppies. Based on the maximum time for expiration (Table III), in general, the ethylamine derivatives are faster acting than the methionine derivatives, and the latter are faster acting than the proline derivatives. As the expiration time decreases, most probably the toxicity increases, and with this assumption, the following observations concerning toxicity of the compounds are made.

Varying X[1] Groups of the Amine[2]—P—Nucleus:

Ethylamine Series, $C_2H_5N—P(X)_2$.—Observations of the results obtained with the 0.01% solutions indicate that halogen derivatives are the most toxic to fish (Table III). Branching of the three carbon chain produces an ester which is one-third as toxic as the straight chain ester because the expiration time is almost tripled. The four carbon esters are about one-half as toxic as the normal propyl ester. The two carbon ester is the least toxic since the biological responses of the guppies were observed for almost 2 hours.

Comparison of the results observed with the 0.001% solutions indicate that the dichloro and phenyl esters are the most toxic to guppies because there were no survivors (Table II). Both sexes survived in the isopropyl, secondary, and tertiary butyl esters; however, it is interesting to note that there were more female survivors than males. The fish which had been exposed to the tertiary butyl ester produced offspring. Only females survived in the normal butyl, isobutyl, ethyl, and ethoxychloro esters. The latter ester stimulated growth since the females matured in 2 months. There were exclusively male survivors in the normal propyl ester which suggests that females are more susceptible than males to this compound.

Varying X[3] Groups of the Amine[2]—P—Nucleus: DL-

Methionine Series, DL-$CH_3SCH_2CH_2CH$—NH—P

$(X)_2$; **L-Proline Series,** L-$CH_2CH_2CH_2CHN$—$P(X)_2$

—In the two amino acid series, the 0.01% solutions of the diethyl esters are the most toxic to guppies. The diethoxy and dichloro derivatives are more toxic (quicker acting) than the ethoxychloro ester (Table III). However, the proline derivatives are less toxic (slower acting) than the corresponding methionine derivatives. The ethoxychloro ester in the proline series is the least toxic of the amino acid derivatives because there are survivors.

The 0.001% solutions furnished additional information in regards to the biological activity of these compounds in guppies.

When the X groups attached to the methionine nucleus[4] are ethoxy and chlorine, the ester has the unusual ability to retard or inhibit the growth of some of the guppies (possibly males) and stimulated the growth of the females, since the only fish which matured are females (Table II). Replacement of the ethoxy group by chlorine produced the dihalo derivative which caused rapid growth of both females and males. The latter matured in only 2 months. When X groups are OC_2H_5, only females survived which matured in about 3 months.

As 0.001% solutions, the proline derivatives obtained when diethoxy, ethoxychloro, and dichloro groups are attached to the proline nucleus[5] produced rapid growth in female guppies. The latter two compounds also caused rapid growth in the males that survived. The dichloro derivative stimulated growth to such an extent that both male and female fish are more than twice the size of normal fish of the same age.

Varying the Amine[2] of the —P—OC_2H_5 **Nucleus.**

—It was found that the methionine derivative is more toxic than the ethylamine and proline derivatives (Table III). Female guppies are the only survivors in the 0.001% solutions of these compounds (Table II). The proline derivative stimulated growth as evidenced by the females maturing in a relatively short time.

Varying the Amine[2] of the —P—OC_2H_5 **Nucleus.**

—The ethylamine derivative is much more toxic than the methionine derivative which, in turn, is more potent than the proline derivative (Table III). All of these esters (0.001%) promoted rapid growth of the females and the proline ester, in addi-

[1] $(X)_2$ = $(OC_2H_5)_2$, $(OC_2H_5)Cl$, and $(Cl)_2$.

[2] Ethylamine or the DL-methionine and L-proline ethyl esters.

[4] DL-Methionine nucleus, DL-$CH_3SCH_2CH_2CH$—NH—P—

[5] L-Proline nucleus, L-$CH_2CH_2CH_2CH$—N—P—

TABLE III.—EXPIRATION TIME OF GUPPIES IN 0.01% w/v AQUEOUS SOLUTION OF THE COMPOUND[a]

Minutes	Compound	Minutes	Compound
	Ethylamine Series[b]		DL-Methionine Series[c]
12	$X(OC_2H_5)Cl$	16	$Y(OC_2H_5)_2$
	XCl_2	30	YCl_2
20	$X(OCH_2CH_2CH_3)_2$	257	$Y(OC_2H_5)Cl$
	$X(OC_6H_5)_2$		L-Proline Series[d]
37	$X(OCH_2CH(CH_3)_2)_2$	120	$Z(OC_2H_5)_2$
	$X(OCH_2CH_2CH_2CH_3)_2$	180	ZCl_2
46	$X(OCH(CH_3)CH_2CH_3)_2$	1440	$Z(OC_2H_5)Cl$[e]
	$X(OC(CH_3)_3)_2$		
56	$X(OCH(CH_3)_2)_2$		
112	$X(OC_2H_5)_2$		

[a] Reference standard, $(C_2H_5O)_2P(S)Cl$, expiration time, 11 minutes; for control purposes, distilled water was used instead of the 0.01% solution of the compound under test, and it was observed that all of the guppies were alive after 24 hours. [b] X = $C_2H_5NHP(S)$. [c] Y = DL-$CH_3SCH_2CH_2CH(CO_2C_2H_5)NHP(S)$. [d] Z = L-$CH_2CH_2CH_2CH(CO_2C_2H_5)N—P(S)$. [e] Survival of 30% after 24 hours.

tion, had the same effect on males (Table II). The methionine ester is outstanding in that it retarded or inhibited the growth of some of the guppies (possibly the males).

Varying the Amine[2] of the —P(S)Cl Nucleus.—

The order of decreasing toxicity is ethylamine, methionine, followed by proline (Table III). There were no survivors in the 0.001% solution of the ethylamine derivative (Table II). The amino acid derivatives actually stimulated the growth of both sexes. The proline derivative excelled in this ability in that it caused gigantism.

SUMMARY AND CONCLUSIONS

The observed biological responses of baby guppies which had been immersed in 0.01% w/v aqueous solutions of the thiophosphoric acid derivatives of ethylamine, DL-methionine, and L-proline ethyl esters, indicate that these derivatives probably have anticholinesterase activity. It was noted that the phenyl ester caused extreme bending of the caudal fin of some of the fish.

Other biological responses were observed with the 0.001% solutions. There were very few male survivors, and only one of the many female survivors produced progeny. Behavior toward food, growth stimulation, growth retardation or inhibition, abnormal body development, and enhancement of pigmentation were observed.

Almost all of the amino acid derivatives, the ethoxychloro derivative (ethylamine series), and the reference standard caused rapid growth of both sexes or of the female survivors. Maximum growth stimulation was observed with the dichloro proline derivative. The female fish which had matured rapidly and were not well-proportioned expired at the age of $4\frac{1}{2}$ months.

The ethoxychloro methionine derivative and the reference standard caused growth inhibition of some of the guppies (possibly the males) and stimulated the growth of the females.

There were exclusively male survivors in the normal propyl ester.

The fish which had been in the dichloro and butyl derivatives (ethylamine series) ignored food. The guppies previously in contact with the *sec*-butyl ester actually leaped away from the food which had touched them.

Pigmentation was enhanced in both sexes.

Based on the maximum time for expiration, in general, the ethylamine derivatives are the fastest and the proline derivatives are the slowest acting. Structure-toxicity relationship is discussed. Observations of the results obtained with the 0.01% solutions indicate that halogen derivatives (ethylamine series) and diethoxy derivatives (both amino acid series) are the most toxic to guppies.

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