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TOXICOKINETICS IN FISH: ACCUMULATION AND ELIMINATION OF SIX CHLOROBENZENES BY GUPPIES

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SUMMARY

The kinetics of six chlorobenzenes in guppies has been studied in an accumulation and elimination experiment. Uptake and elimination rate constants and bio-accumulation have been determined and correlated with log P_{oct}. A parabolic curve, with an optimum at log $P_{oct} \approx 5.4$, appears to be a better description of the relation of the uptake rate constant with log P_{oct}, than a straight line. The bio-accumulation follows this non-linear behaviour: an optimum has been found at log $P_{oct} \approx 6.5$.

INTRODUCTION

The importance of the bio-accumulation (b.a., the ratio between the concentration in an organism and in the water, air or food) for the potential hazard of chemicals is generally accepted. In recent proposals for a systematic hazard evaluation b.a. plays an important role (1,2). In the early sixties most attention was paid to b.a. of chemicals in animals, including fish, via the food-chain. Moriarty, however, concluded in 1972 (3) that for aquatic animals, particularly fish, concentration from water was a more important source of contamination by pollutants than was concentration from food. Recent studies (4,5,6,7) lead to the same conclusion. Therefore, the present study was limited to b.a. of chemicals from water. Neely et al. (8) showed that bio-accumulation from water by fish is correlated with hydrophobicity, expressed as P_{oct}, the partition coefficient of a substance between n-octanol and water. This relationship has been confirmed by Chiou et al. (9) and by others. The b.a. was calculated by Neely using an accelerated procedure, based upon the kinetics of a one compartment system, as proposed by Branson et al. (10). In this study a two compartiment model was used to describe the kinetics of six chlorobenzenes (CB's) in guppies. We determined two rate constants of the kinetics of these chlorobenzenes and the bio-accumulation. These parameters have been

correlated with log P_{oct}. The b.a. was compared with data calculated by Branson's method. No anatomical meaning can be ascribed to the compartments in this model without more extensive studies. The character of the compartments may vary with fish species, with individuals, with the type of chemicals tested, etc. An attempt has been made to reduce these and other variables by using a series of chemicals which have closely related structures and by exposing all fishes to a mixture of the chlorobenzenes. Therefore, the b.a. and the kinetic parameters for each compound refer to the same fishes.

METHODS

120 female guppies (Poecilia reticulata), with an average weight of 0.62 g, were exposed to a standardized mixture of 6 chlorobenzenes which had a final calculated aqueous concentration as mentioned in table 1. The guppies were fed daily with a commercial fish food. The chlorobenzenes were dosed in an acetone solution. In addition to the calculated values, the concentrations actually determined are given in table 1. After 19 days the exposure was stopped. The elimination of the chlorobenzenes from the fish was then studied for 9 weeks. During the accumulation and elimination phase three guppies were periodically taken out of the aquarium and individually analyzed to determine the six chlorobenzenes.

Constant flow system

A constant dosing apparatus was used as described by Mount and Brungs (11), with slight modifications. Utrecht tap-water was used and aerated before the acetone solution was added at a concentration of 0.023 ml/l water. The water flow through the closed 175 l aquarium was 27 l/hr. The water temperature was kept constant at $(21.0 \pm 0.5)^{o}$ by a thermostat placed in a buffer vessel before the aquarium. Water hardness was 5° DH and oxygen content 5 ppm. An initial dose of 4.0 ml of stock solution (the mixture of the six chlorobenzenes, mentioned above) was added approximately half an hour before the experiment started. During the accumulation period, a slimy cover formed on the walls of the buffer vessel and, to a lesser extent, in the aquarium. This was caused by the presence of acetone and it consisted mainly of bacteria (Siderocapsa treubii). Therefore this vessel and the aquarium were cleaned regularly.

After 19 days the aquarium was cleaned again and the contaminated water replaced by clean tap-water. The water flow of 27 $l.hr^{-1}$ was maintained, without adding stock solution.

Chemical analysis

Water samples were analyzed every three days by extracting 5 ml of water with an equal volume of n-hexane and analyzing the hexane phase gas-chromatographically. The individual fish were killed in liquid nitrogen and homogenized in a mortar, mixed with three times its weight of dry Na_2SO_4 and then extracted in a soxhlet apparatus with 20 ml of n-hexane. The extract was cleaned up with an Al_2O_3-column, as described by Holden and Marsden (12). All hexane samples were diluted or concentrated (by evaporating with a N_2-stream) when necessary, before analyzing on a TRACOR 550 gas-chromatograph, equipped with a ^{63}Ni electron capture detector. The glass column (2m x 2.5 mm I.D.) was filled with Chromosorb W HP (80-100 mesh) coated with 3 wt. % OV-1.

The recoveries of the fish analyses of 1,4-di-, 1,2,3-tri, 1,3,5-tri, 1,2,3,5-tetra-, penta- and hexachlorobenzene were 65, 83, 83, 86, 92 and 94% respectively. The recoveries of the determinations of the concentrations of these chlorobenzenes in the aquarium water are: 85, 91, 82, 87, 87 and 91%. The b.a. data presented are corrected for these recoveries.

The concentrations of the chlorobenzenes in the guppies are expressed in µg/g lipid weight, because there appeared to be a significantly better correlation between residue and lipid weight than between residue and total body weight. The lipid content was determined by leading a N_2-stream over the soxhlet hexane extract until constant weight, at room temperature. The weight of the residue was measured. The average fat content of our guppies, as determined in this way, was (with s.d.) 5.4 $\pm$ 2.0%.

Determination of rate constants

We used a two compartment model to describe the kinetics of the CB's:

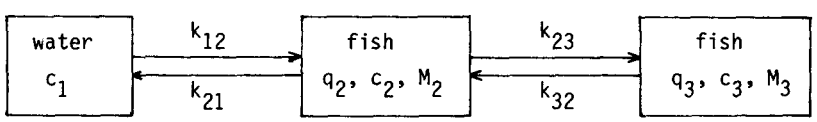

Fig. 1. A two compartment model for the fish.

In this paper the mathematical derivations are given in a condensed form. A more comprehensive treatment has been given in the appendix.

The following symbols and definitions were used:

		dimensions:
c_n	the concentration in compartment n	-- (mass.mass^{-1})
q_n	the quantity in compartment n	mass
M_n	the mass of compartment n, $c_n = q_n/M_n$	mass
r_{nm}	first order rate constant	mass. time^{-1}
nm	as index indicates transport from the nth to the mth compartment	
f.w.	fat weight of a fish	mass
t	time	time
c_{tot}	$(q_2 + q_3)$/f.w.	-- (mass.mass^{-1})
k_{nm}	r_{nm}/M_n except	time^{-1}
k_{12}	r_{12}/f.w.	time^{-1}

The kinetics can be described by three differential equations:

$$\frac{dc_1}{dt} = 0 \text{ (the water concentration being constant)} \qquad \text{(eqn. 1)}$$

$$\frac{dq_2}{dt} = r_{12}c_1 - (r_{21} + r_{23})c_2 + r_{32}c_3 \qquad \text{(eqn. 2)}$$

$$\frac{dq_3}{dt} = r_{23}c_2 - r_{32}c_3 \qquad \text{(eqn. 3)}$$

For the one compartment model $k_{23} = k_{32} = 0$.

From these equations the concentration in the fish at time t can be calculated:

for the one compartment model,
during the accumulation:

$$c_{tot} = \frac{k_{12}}{k_{21}} c_1(1 - e^{-k_{21}t}) \qquad \text{(eqn. 4)}$$

during the elimination:

$$c_{tot} = A\, e^{-k_{21}t}, \text{ where A is the concentration when the dosing stops} \qquad \text{(eqn. 5)}$$

for the two compartment model,
during the accumulation:

$$c_{tot} = A_1(1 - e^{-a_1 t}) + A_2 (1 - e^{-a_2 t}) \qquad \text{(eqn. 6a)}$$

$$\text{where } A_1 + A_2 = \frac{k_{12}}{k_{21}} (1 + \frac{k_{23}}{k_{32}})c_1 \quad \text{is} \qquad \text{(eqn. 6b)}$$

the concentration of c_{tot} in the steady state (c_{totss})

during the elimination:

$$c_{tot} = A_1' \, e^{-a_1 t} + A_2' \, e^{-a_2 t} \qquad \text{(eqn. 7)}$$

when the elimination starts at the steady state, $A_1' = A_1$ and $A_2' = A_2$.

a_1 and a_2 can be solved from the next equation, which is found by solving the second order differential equations in q_2 and q_3, which can be derived from equations 1-3.

$$a^2 - (k_{21} + k_{23} + k_{32})a + k_{21}k_{32} = 0 \qquad \text{(eqn. 8)}$$

so:

$a_1 a_2 = k_{21}k_{32}$ and

$$a_1 + a_2 = k_{21} + k_{23} + k_{32}$$

During the first part of the accumulation, the uptake in the fish can be approximated by

$$\lim_{t \to 0} \frac{dq_{tot}}{dt} = r_{12}c_1$$

for the first six hours, this can be rewritten as $k_{12} = \frac{\Delta c_{tot}}{c_1 \, \Delta t}$ (eqn. 9)

An expression for k_{21} can be derived from eqs. 6 and 8:

$$c_{totss} = (k_{23} + k_{32})\frac{k_{12}}{k_{21}k_{32}} c_1 = \frac{a_1 + a_2 - k_{21}}{a_1 a_2} k_{12}c_1$$

$$k_{21} = a_1 + a_2 - \frac{a_1 a_2}{k_{12}} \times \text{b.a.} \qquad \text{(eqn. 10)}$$

All the variables necessary for the calculation of k_{12} and k_{21}, can be determined in accumulation and elimination experiments. a_2 is the coefficient of the later part of the linear regression of ln c_{tot} with time in days. For this part the period from day 21 of the elimination until the end of the elimination period was used for all cases. For the calculation of a_1 extrapolated values from the a_2 regression equation for the shorter elimination times were substracted from the actual fish concentrations. For these differences we also calculated the linear regression of ln c_t with time in days, The regression coefficient from this equation is a_1.

RESULTS AND DISCUSSION

Table 1 gives the concentrations as calculated from the added stock solution of the chlorobenzene mixture, the average of the determined concentrations from the second to the last day of the accumulation and the approximate concentrations of the CB's in the bacteria-containing material from the buffer vessel which were determined once only. The n-octanol/water partition coefficients (P_{oct}) in table 1 are calculated with Rekker's f-system (13). In our opinion these values are better for rather lipophilic compounds than experimental ones (14).

Table 1.

substance	log P_{oct}	ng/ml added water concentration	ng/ml, average actual water concentration after 2 days*	µg/g wet weight concentration in bacteria
1,4-diCB	3.53	160	116	2.5
1,2,3-triCB	4.20	100	48	10
1,3,5-triCB	4.20	100	43	11
1,2,3,5-tetraCB	4.94	40	12	40
pentaCB	5.69	8	1.2	20
hexaCB	6.44	4	0.3	15

*standard error 10-15%

The high concentrations of chlorobenzenes in the bacteria-containing material may largely explain the great losses in the water concentrations. Other factors, such as bacterial breakdown and vaporization, may also have contributed to these losses.

The accumulation data of the first six hours are represented by k_{12}-values, calculated as described in the methods section. In contrast to all other calculations we used here the water concentrations as calculated from the addition instead of those determined later as the actual concentrations, because the initial concentrations were prepared about half an hour before the start of the experiment by adding the stock solution directly to the aquarium water. All the factors mentioned in an attempt to explain the lower water concentrations actually found from the third day on cannot have influenced, to any great extent, these concentrations during the first six hours. These k_{12} values are given in table 2. The accumulation data for the whole 19 days period are shown in fig. 2. The course of the elimination can be seen in fig. 3. All data points represent the average of three determinations, except for that of day 0 of the elimination, which consists of the average of 15 determinations.

For the calculation of b.a. concentrations in fish were assumed to be constant from the second day for the di- and triCB's and from the 7-th day for tetra-, penta- and hexaCB, and are calculated as the averages of the results after 2 and 7 days respectively. In table 2 k_{12}, b.a., the slopes of the elimination (a_1 and a_2) and k_{21} are summarized for the six CB's.

Table 2*

substance	b.a.**	a_1	a_2	k_{12}	k_{21}
1,4-diCB	$(1.8\pm0.5)\times10^3$	1.00 ± 0.20	-	$(1.8\pm0.4)\times10^3$	1.00 ± 0.20
1,2,3-triCB	$(1.3\pm0.4)\times10^4$	0.45 ± 0.06	0.007 ± 0.012	$(8.3\pm1.3)\times10^3$	0.45 ± 0.06
1,3,5-triCB	$(1.4\pm0.4)\times10^4$	0.40 ± 0.06	0.000 ± 0.014	$(8.0\pm1.2)\times10^3$	0.40 ± 0.06
1,2,3,5-tetraCB	$(7.2\pm1.2)\times10^4$	0.28 ± 0.04	0.064 ± 0.012	$(15\pm2)\times10^3$	0.26 ± 0.04
pentaCB	$(2.6\pm0.5)\times10^5$	0.18 ± 0.04	0.062 ± 0.021	$(22\pm4)\times10^3$	0.11 ± 0.04
hexaCB	$(2.9\pm0.9)\times10^5$	0.062 ± 0.005	-	$(10\pm2)\times10^3$	

* the dimension of a_1, a_2, k_{12} and k_{21} is day^{-1}. For all data the 95% confidence limits are given

** calculated on the basis of fat weight

In table 2 the elimination of hexaCB has been treated as elimination from a one compartment system. This compartment is probably identical to the combined first

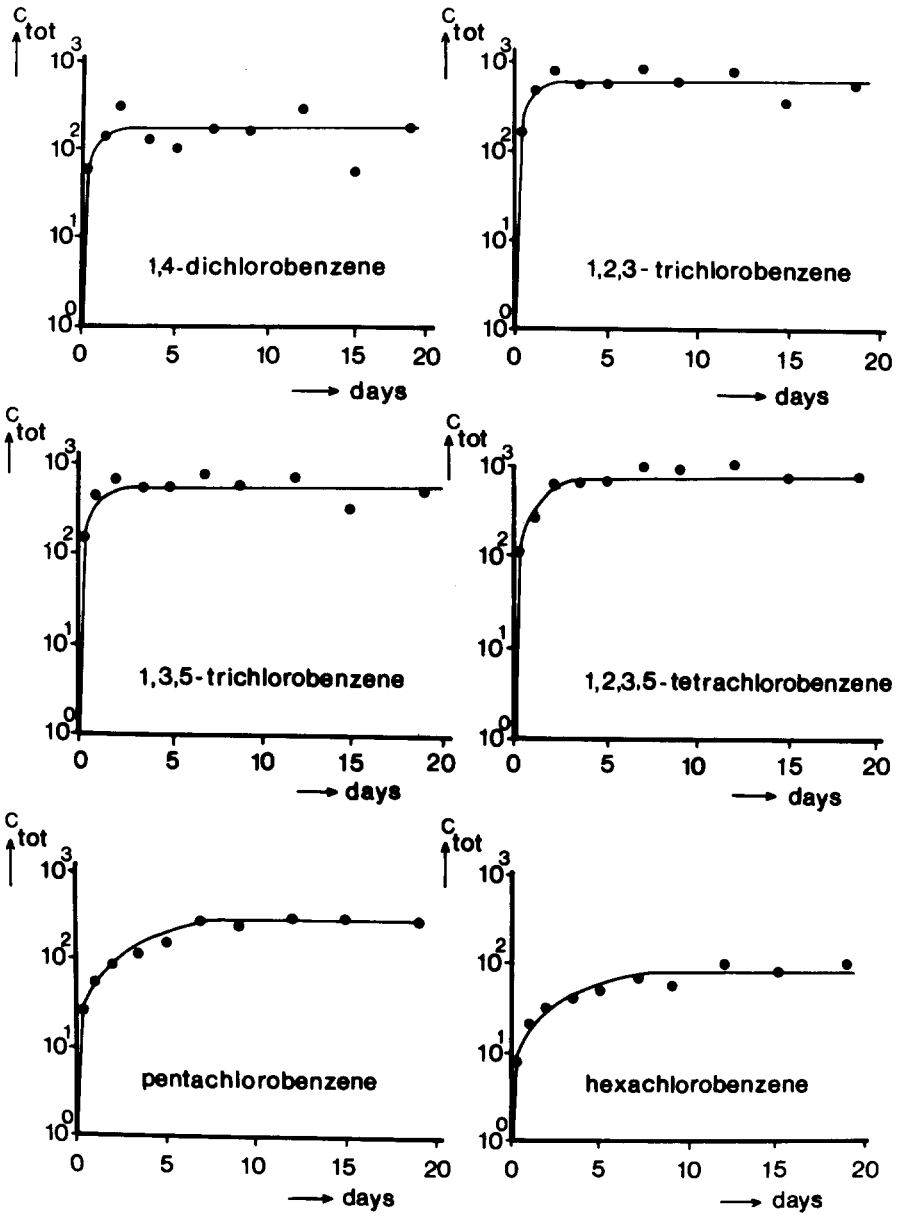

Fig. 2. Residues of chlorobenzenes during the accumulation.
All concentrations (c_{tot}) are in µg (CB)/g (fat weight of the fish).

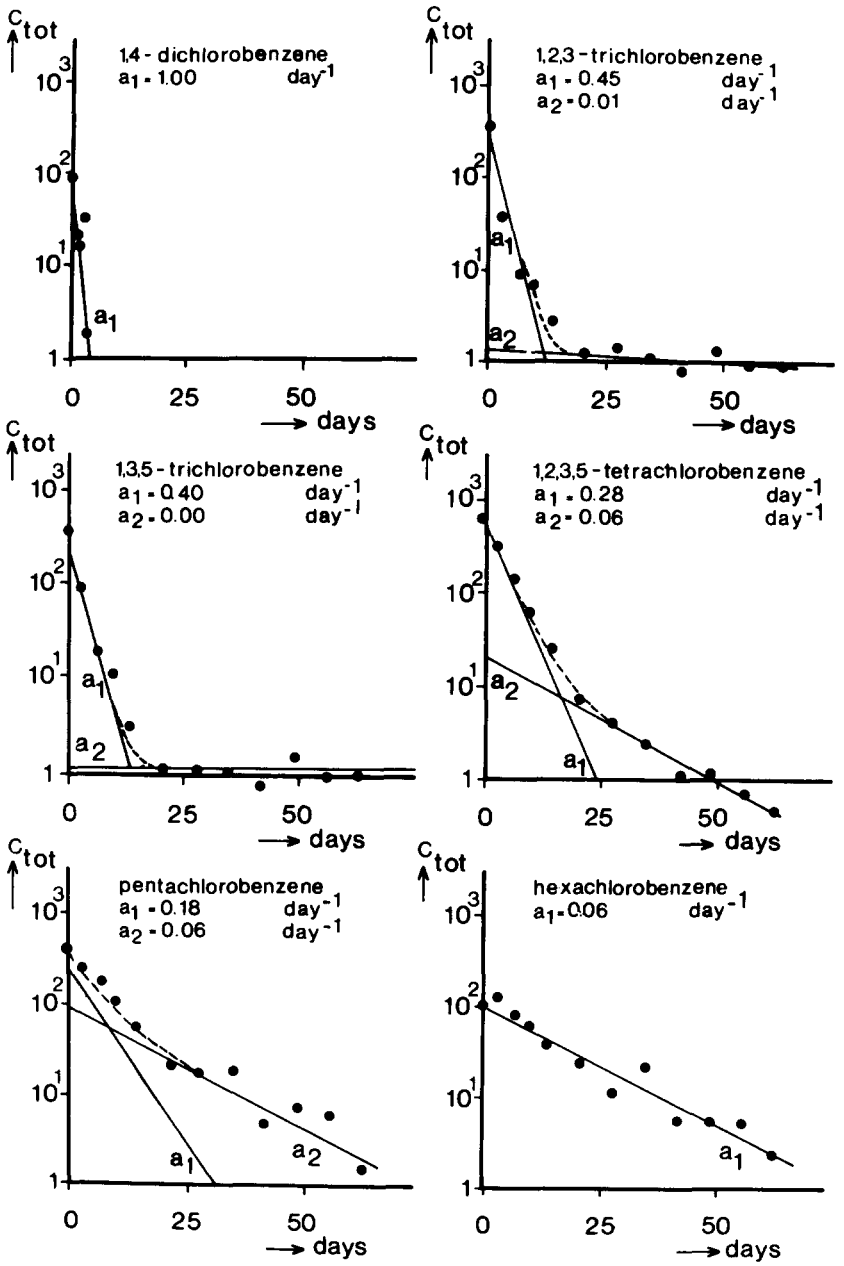

Fig. 3. Residues of chlorobenzenes during the elimination.
All concentrations (c_{tot}) are in µg (CB)/g (fat weight of the fish).

and second compartment in the other cases. Therefore it is not possible to calculate from a_1 a k_{21} value for hexaCB which has the same meaning as the other k_{21} values. Also it is not possible to split up the elimination graph into two straight lines. Probably a_1 and a_2 will not differ greatly and will be both in the order of 0.06. But even if the values of a_1 and a_2 were known more precisely, k_{21} could not be calculated with reasonable accuracy from these data, mainly due to the errors in b.a. and k_{12}. The values of a_2 are difficult to interpret. It is possible that the second compartment from which the triCB's are eliminated with a rate a_2, differs from that to which the a_2 values of the tetra- and pentaCB's refer. In figs. 4,5 and 6 the logarithms of the b.a., k_{12} and k_{21} are plotted against log P_{oct}. When linear regression equations are calculated, it appears that the most satisfactory equation is found for log k_{21} vs. log P_{oct} (i.e. without hexaCB):

$$\log k_{21} = -0.419 \log P_{oct} + 1.435 \qquad r = -0.988 \qquad s = 0.048 \qquad \text{(eqn. 11)}$$

The other two regression equations give poor results, as appears from the much larger standard deviations (both over 0.2). When hexaCB is excluded from the calculations, the result is improved:

$$\log \text{b.a.} = 0.980 \log P_{oct} - 0.063 \qquad r = 0.991 \qquad s = 0.099 \qquad \text{(eqn. 12)}$$

$$\log k_{12} = 0.462 \log P_{oct} + 1.837 \qquad r = 0.925 \qquad s = 0.140 \qquad \text{(eqn. 13)}$$

In both cases the extrapolated value for hexaCB deviates greatly from the experimental one. This problem can be completely eliminated by introducing a quadratic log P_{oct} term into the equations, which now include hexaCB:

$$\log \text{b.a.} = 3.411 \log P_{oct} - 0.264 (\log P_{oct})^2 - 5.513 \qquad s = 0.039 \qquad \text{(eqn. 14)}$$

$$\log k_{12} = 3.174 \log P_{oct} - 0.293 (\log P_{oct})^2 - 4.277 \qquad s = 0.039 \qquad \text{(eqn. 15)}$$

The remaining standard deviations can be completely attributed to the errors in the data. From eqn. 14 it follows that the b.a. of chlorobenzenes is optimal at log $P_{oct} \approx 6.4$. Equations 11, 14 and 15 may also be valid for other hydrophobic compounds, which share with the chlorobenzenes a low rate of metabolism by fish. Because the equations above are based on only six compounds, which for quadratic equations is a particularly small number, these results should be confirmed using more chemicals. The occurrence of non-linear relationships between biological

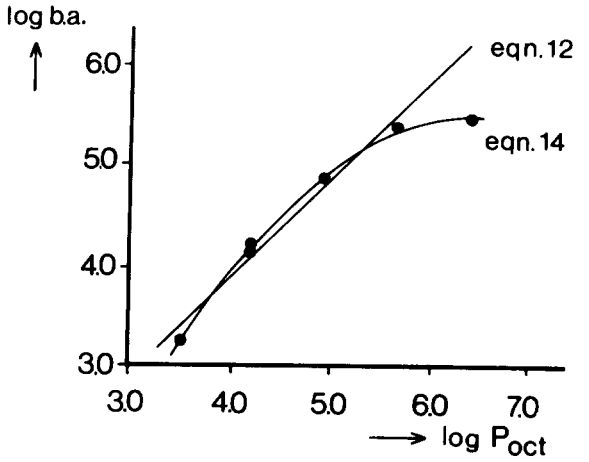

Fig. 4. Relation between log b.a. and log P_{oct} for six CB's

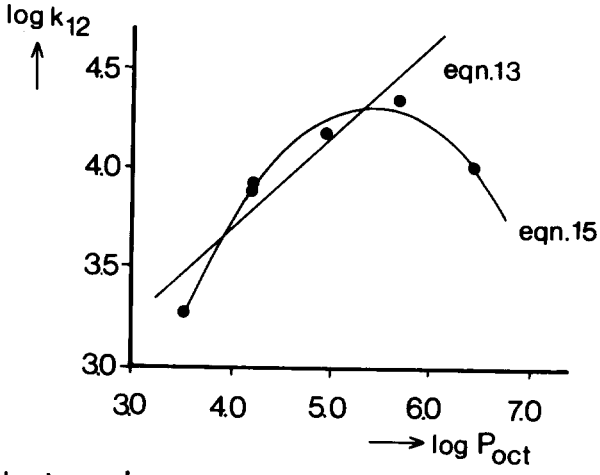

Fig. 5. Relation between log k_{12} and log P_{oct} for six CB's

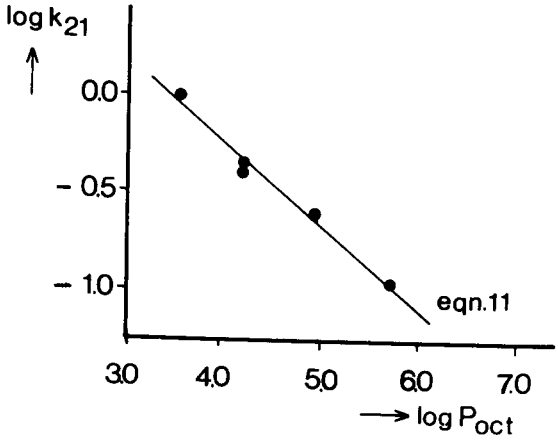

Fig. 6. Relation between log k_{21} and log P_{oct} for five CB's

activity or permeability and log P_{oct} is well known (Hansch in Drug Design, p. 297 (15)). Limitations in the linear increase of log b.a. with log P_{oct} have been reported by Sugiura (16) and Zitko (17). In both cases, however, the experimental conditions differ much from those of this study. The parabolic model used in eqns. 14 and 15 is not the only possible description for this kind of data. Kubinyi (18) pointed out that in many cases a bilinear curve fitted his data better than a parabolic one. But six figures are already the minimum needed to calculate a parabolic curve and are insufficient for calculation of a bilinear one. This model, therefore, cannot be tested on our data. If b.a. is calculated from the accelerated procedure as proposed by Branson et al. (10) an acceptable agreement with our experimental b.a. data is obtained. The calculated b.a.'s of the chlorobenzenes, in the order of table 2, are 1.8×10^3, 1.8×10^4, 2.0×10^4, 5.4×10^4, 1.2×10^5 and 1.6×10^5. PentaCB has the greatest deviation the value found being more than twice as high as the calculated one.

CONCLUSIONS

The bio-accumulation of chlorobenzenes increases with log P_{oct}, until reaching an optimum value at log $P_{oct} \approx 6.5$. For even more lipophilic compounds a decrease in bio-accumulation is expected. This reduction is caused by a sharp decrease in the magnitude of the uptake rate constant, k_{12}, beyond the optimum value at log $P_{oct} \approx 5.4$. There is no indication of a non-linear dependance of log k_{21} (the elimination rate constant) from log P_{oct} in the investigated lipophility range (log P_{oct} 3.5 - 5.7). Therefore a possible deviation from this linear behaviour should occur at higher log P_{oct} values than for log k_{12} (the uptake rate constant).

In spite of its simplicity, the accelerated test of Branson et al. leads to reasonably good estimations of the b.a. factors, also for those chlorobenzenes which show a two compartment accumulation and elimination.

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APPENDIX to Toxicokinetics in fish: accumulation and elimination of six chlorobenzenes by guppies

For symbols and definitions, see Toxicokinetics in fish

$$\frac{dc_1}{dt} = 0 \tag{1}$$

$$\frac{dq_2}{dt} = r_{12}c_1 - (r_{21} + r_{23})c_2 + r_{32}c_3 \tag{2}$$

$$\frac{dq_3}{dt} = r_{23}c_2 - r_{32}c_3 \tag{3}$$

$$(2): \frac{dq_2}{dt} = r_{12}c_1 - \frac{(r_{21} + r_{23})}{M_2} q_2 + \frac{r_{32}}{M_3} q_3$$

$$q_2' = r_{12}c_1 - (k_{21} + k_{23})q_2 + k_{32}q_3 \tag{4}$$

$$(3): \frac{dq_3}{dt} = \frac{r_{23}}{M_2} q_2 - \frac{r_{32}}{M_3} q_3$$

$$q_3' = k_{23}q_2 - k_{32}q_3 \tag{5}$$

eqn. 4 can be rewritten as $k_{32}q_3 = q_2' + (k_{21} + k_{23})q_2 - r_{12}c_1$ (6)

and after differentiation $k_{32}q_3' = q_2'' + (k_{21} + k_{23})q_2'$ (7)

Substitution of (6) and (7) in (5) gives

$$q_2'' + (k_{21} + k_{23} + k_{32})q_2' + k_{21}k_{32}q_2 - r_{12}k_{32}c_1 = 0 \tag{8}$$

solutions of q_2 must have the form $me^{at} + n$. Substituting in (8) gives

$$me^{at}(a^2 + (k_{21} + k_{23} + k_{32})a + k_{21}k_{32}) + k_{21}k_{32}n - r_{12}k_{32}c_1 = 0 \tag{9}$$

From this eqn. it is clear that $n = \frac{r_{12}}{k_{21}}$ (10)

and $a^2 + (k_{21} + k_{23} + k_{32})a + k_{21}k_{32} = 0$ (11)

This equation generates two solutions for a, a_1 and a_2, for which

$$a_1 + a_2 = -(k_{21} + k_{23} + k_{32}) \quad (12a)$$

$$a_1 a_2 = k_{21}k_{32} \quad (12b)$$

so a_1 and a_2 are both negative

The general solution is a linear combination of the two a-terms.
When $q_2 = 0$ at $t = 0$ is used as additional information for the accumulation period, the next equation of q_2 is found for this period:

$$q_2 = A_1(1 - e^{a_1 t}) + A_2(1 - e^{a_2 t}) \quad (13)$$

in which $A_1 + A_2 = \frac{r_{12}}{k_{21}} c_1$ is the value of q_2 in the steady state (14)

When the elimination starts at the steady state, the equation of q_2 during the elimination is

$$q_2 = A_1 e^{a_1 t} + A_2 e^{a_2 t} \quad (15)$$

In the same way as for q_2 a differential equation with q_3 can be derived from (4) and (5):

$$q_3'' + (k_{21} + k_{23} + k_{32})q_3' + k_{21}k_{32}q_3 - r_{12}k_{23}c_1 = 0 \quad (16)$$

The solution of q_3 has the same form as q_2.
During the accumulation:

$$q_3 = B_1(1 - e^{a_1 t}) + B_2(1 - e^{a_2 t}) \quad (17)$$

in which $B_1 + B_2 = \frac{r_{12}k_{23}}{k_{21}k_{32}} c_1$ is the value of q_3 in the steady state (18)

During the elimination from the steady state:

$$q_3 = B_1 e^{a_1 t} + B_2 e^{a_2 t} \tag{19}$$

The total concentration in the fish is $c_{tot} = (q_2 + q_3)/\text{f.w.}$, (20)
so in the accumulation period

$$c_{tot} = K_1(1 - e^{a_1 t}) + K_2 (1 - e^{a_2 t}) \tag{21}$$

and when eliminated from the steady state

$$c_{tot} = K_1 e^{a_1 t} + K_2 e^{a_2 t} \tag{22}$$

in which $K_1 + K_2 = (A_1 + A_2 + B_1 + B_2)/\text{f.w.}$ is the concentration in the fish in the steady state (c_{totss}). So

$$c_{totss} = (1 + \frac{k_{23}}{k_{32}})\frac{k_{12}}{k_{21}} c_1 \tag{23}$$

Substituting (12a,b) in (23) gives

$$c_{totss} = -(a_1 + a_2 + k_{21})\frac{k_{12}}{a_1 a_2} c_1 \text{ , or}$$

$$k_{21} = -(a_1 + a_2) - \frac{a_1 a_2}{k_{12}} \times \frac{c_{totss}}{c_1} \tag{24}$$

Differentiating (20) gives $\frac{dc_{tot}}{dt} = (\frac{dq_2}{dt} + \frac{dq_3}{dt})/\text{f.w.}$ (25)

and after substituting (2) and (3) in (25)

$$\frac{dc_{tot}}{dt} = k_{12}c_1 - k_{21}c_2 \tag{26}$$

For the very first part of the accumulation, this can be reduced to

$$\lim_{t \to 0} \frac{dc_{tot}}{dt} = k_{12}c_1 \tag{27}$$

so for this period $k_{12} = \frac{\Delta c_{tot}}{c_1 \, \Delta t}$ (28)

Expressions for k_{23} and k_{32} can be found with (12a,b) and (24)

K_1 and K_2 in (21) and (22) can be calculated from the conditions at the moment when the accumulation starts:
(21) becomes after differentiation, at $t \to 0$

$$\lim_{t \to 0} \frac{dc_{tot}}{dt} = -a_1K_1 - a_2K_2 \tag{29}$$

and combining with (27) $- a_1K_1 - a_2K_2 = k_{12}c_1$ (30)

K_1 and K_2 can be calculated from this equation and (22).

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