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Biological effectiveness, in goldfish, of some *p*-substituted acetanilides alone and in the presence of poloxamers

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The death times of goldfish have been measured in aqueous solutions containing different concentrations of *p*-substituted acetanilides alone or in the presence of poloxamers. Plots of reciprocal death time versus concentration were linear with a positive concentration intercept, the minimum effective concentration (MEC). The MEC values were directly related to the π value (hydrophobic-lipophilic constant) of the functional group on the acetanilide indicating that activity is directly related to lipophilicity. Slopes of reciprocal death time versus drug concentration were linearly related to π values but there was no direct dependence. The presence of poloxamers in aqueous acetanilide solutions reduced the goldfish death time. The effect of the poloxamers is believed to be one of rendering the goldfish membrane more permeable to drugs.

We have previously described the solubilization of some *p*-substituted acetanilides in aqueous solutions of structurally related poloxamers (Collett & Tobin 1978). The extent of solubilization was discussed in terms of relationships between solubilizer and solubilizate structure. This report describes the biological activities of the same acetanilides alone and in the presence of poloxamers. We have attempted to relate biological activity to substituent parameters of the functional group on the acetanilide. Goldfish were used as a model biological system in the light of reports of their suitability for use in studies such as relating structure and toxicity of phenothiazine derivatives (Nightingale et al 1972) and the effect of alkyl chain length on the narcotic properties of alkyl *p*-aminobenzoates (Yalkowsky et al 1973).

MATERIALS AND METHODS

Materials

The *p*-substituted acetanilides and poloxamers used have been described previously (Collett & Tobin 1978). The goldfish, common variety (*Carassius auratus*) 7–8·5 g, 6·5–7·5 cm in length were purchased locally. Before use the fish were kept at 25 to 30 °C. All solutions were made up in Krebs bicarbonate buffer pH 7·4 (Krebs & Henseleit 1932).

Methods

Single goldfish were placed in 150 ml of test solution maintained at 30 ± 0·1 °C. At least four fish were used for each test solution. Time of death was measured as the time taken for complete cessation of gill and mouth movements.

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RESULTS AND DISCUSSION

Preliminary experiments were carried out in which goldfish were placed in Krebs bicarbonate buffer and in buffer solutions containing poloxamers F68 1% and 2% and P65 1% and 2%. The fish were alive after 6 h immersion in buffer and after 1 h in poloxamer solution.

(i) *Effect of drug structure on activity*

Table 1 shows the mean death times for fish placed in various concentrations of the 10 *p*-substituted acetanilides in Krebs bicarbonate buffer.

The kinetics of drug absorption in the goldfish have been studied by several workers (Levy & Gucinski 1964; Disanto & Wagner 1969; Nightingale & Gibaldi 1971). Levy and Gucinski predicted that a plot of reciprocal time of occurrence of pharmacological effect (time of death or overturn time) against drug concentration in the solution in which the fish are immersed will be linear and pass through the origin.

Although this model is successful for several drugs, some exceptions have been reported. Hall & Hayton (1967) found that using dilute ethanol solutions a positive concentration intercept was seen. This was verified by Gibaldi & Nightingale (1968). Nightingale & Gibaldi (1971) subsequently showed using a two-compartment reversible model that at low drug concentrations the reciprocal time-drug concentration plots will be hyperbolic rather than linear and that an intercept will occur. This intercept represents the minimum effective concentration (MEC) necessary for pharmacological effect. Feldman et al (1975) reported MEC values

Table 1. Mean death times produced in goldfish by various concentrations of *p*-substituted acetanilides in Krebs bicarbonate buffer pH 7·4 at 30 °C.

Drug conc (mol $l^{-1} \times 10^3$)	No. of fish	Mean death time, T(min)	1/T (min^{-1})	s.d.
Acetanilide				
3·255	5	4·75	0·211	0·72
2·959	6	6·17	0·162	0·68
2·663	4	8·19	0·122	1·82
2·071	4	33·00	0·030	6·98
4-Hydroxyacetanilide				
86·00	5	19·00	0·053	1·06
68·80	5	24·70	0·040	1·94
51·60	6	34·00	0·029	5·25
43·00	5	40·90	0·024	2·88
4-Methoxyacetanilide				
7·264	6	4·00	0·250	1·41
5·448	5	7·40	0·135	1·78
4·722	5	10·50	0·095	2·44
3·632	4	45·00	0·022	7·26
4-Ethoxyacetanilide				
2·009	8	4·25	0·235	0·27
1·786	6	6·83	0·146	0·98
1·674	4	7·88	0·127	1·75
1·339	5	24·10	0·041	7·97
4-Nitroacetanilide				
1·138	6	6·79	0·147	0·83
1·027	5	10·40	0·096	2·72
0·910	5	18·10	0·055	1·02
0·855	6	27·92	0·036	3·76
4-Acetamidobenzoic acid				
9·805	6	4·29	0·233	0·43
7·844	6	5·67	0·176	1·47
5·883	6	9·50	0·105	0·60
3·922	5	23·10	0·043	3·93
4-Fluoroacetanilide				
2·612	4	2·00	0·500	0·54
2·350	5	2·70	0·370	0·63
2·089	4	4·13	0·242	1·02
1·828	4	7·50	0·133	0·45
4-Chloroacetanilide				
0·590	6	6·04	0·166	0·63
0·472	6	7·88	0·127	1·44
0·354	4	14·88	0·067	3·51
0·295	6	21·13	0·047	4·49
4-Bromoacetanilide				
0·374	5	18·50	0·054	1·65
0·336	5	26·50	0·038	8·11
0·318	4	40·00	0·025	7·53
0·299	4	52·75	0·019	14·71
4-Iodoacetanilide				
0·192	5	19·40	0·052	1·47
0·172	4	24·40	0·041	3·71
0·153	5	32·90	0·030	2·07
0·134	5	50·20	0·020	2·75

for four local anaesthetics in goldfish. In order to calculate MEC's they assumed a linear relation between reciprocal time and drug concentration. Such a linear relationship was found for each *p*-substituted acetanilide. Fig. 1 shows a typical example, 4-hydroxyacetanilide.

Table 2 gives the MEC values for each substituted acetanilide with the slope (K) and correlation for

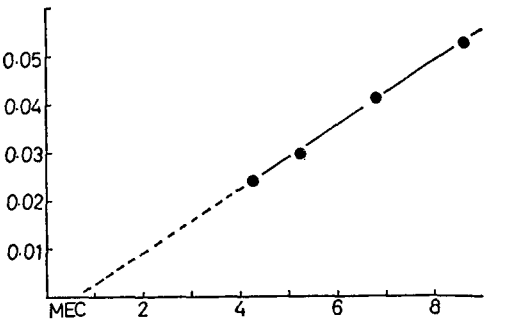

FIG. 1. Plot of reciprocal death time versus concentration of 4-hydroxyacetanilide in Krebs bicarbonate buffer at 30 °C. Ordinate: 1/T (min^{-1}). Abscissa: drug concentration (mol $litre^{-1} \times 10^2$).

each plot. Feldman et al (1975) showed a relation between log MEC and log of the partition coefficient of four local anaesthetics, demonstrating that the activity of the local anaesthetics in producing overturn in goldfish was due in part to the lipophilicity of the drug molecule.

The lipophilicity of a compound can be expressed in terms of π, the hydrophobic-lipophilic constant (Fujita et al 1964). π is related to partition coefficient by equation 1.

$$\log \frac{P_X}{P_H} = \pi \qquad (1)$$

where P_X and P_H refer to the partition coefficients of the substituted and parent compounds respec-

Table 2. The slopes (K) and intercepts (MEC) for plots of reciprocal death time against drug concentration for *p*-substituted acetanilides in Krebs bicarbonate buffer pH 7·4 at 30 °C.

Drug	π[a]	Intercept (MEC) (mol $litre^{-1} \times 10^3$)	Slope (K) (litre mol^{-1} $min^{-1} \times 10^{-3}$)	R[c]
Acetanilide	0	1·872	1·517	0·999
4-Hydroxyacetanilide	−0·36	6·410	0·007	0·999
4-Methoxyacetanilide	−0·133	3·251	0·624	0·999
4-Ethoxyacetanilide	0·367[b]	1·216	2·823	0·990
4-Nitroacetanilide	0·499	0·766	3·904	0·998
4-Acetoamidobenzoic acid	0·091	2·604	0·326	0·999
4-Fluoracetanilide	0·309	1·560	4·707	0·999
4-Chloracetanilide	0·714	0·183	4·166	0·996
4-Bromoacetanilide	1·130	0·262	4·832	0·994
4-Iodoacetanilide	1·303	0·098	5·506	0·999

[a] From Dearden, J. C., Tomlinson, E. (1971) J. Pharm. Pharmac. 23: 73S–76S.
[b] Calculated from eqn 16, 17 and 19 of Fujita et al (1964). J. Am. chem. Soc. 86: 5175–5180
[c] Correlation coefficient.

tively. It might be expected, therefore, that a plot of log MEC against π would be linear. Fig. 2 shows the plot of log MEC against π for the *p*-substituted acetanilides. Linear regression analysis yields equation 2.

$$-\log \text{MEC} = 1{\cdot}06\pi\ (\pm 0{\cdot}11) + 2{\cdot}60$$

n	s	F	r			
10	0·17	94·62	0·960	..	..	(2)

If the chloro compound is excluded, the fit is improved:—

$$-\log \text{MEC} = 0{\cdot}99\pi\ (\pm 0{\cdot}07) + 2{\cdot}58$$

9	0·10	235·9	0·985	..	..	(3)

The slope of the line indicates a direct dependence of MEC on partition coefficient. Thus the activity of the *p*-substituted acetanilides in goldfish is directly related to their lipophilicity.

While the intercept is representative of the equilibrium distribution of the *p*-substituted acetanilide i.e. the concentration in the bathing solution necessary to achieve a "killing" concentration in the goldfish, the slope (K) of plots of reciproal death time against concentration represents the rate of achieving this equilibrium distribution. Fig. 3 shows the plot of these slopes (K) against π for the *p*-substituted acetanilides. A linear relation is seen, represented by equation 4.

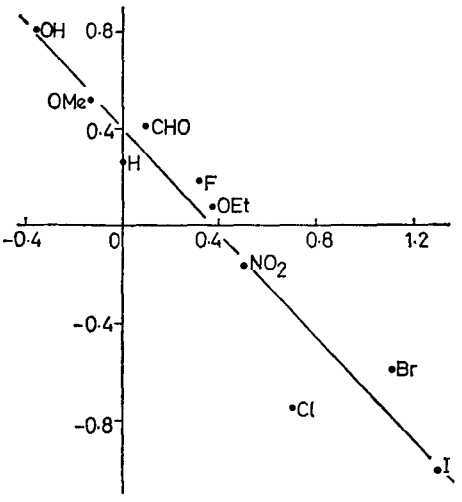

FIG. 2. Plot of log MEC versus π values for *p*-substituted acetanilides in Krebs bicarbonate buffer at 30 °C: Ordinate: log M.E.C. + 3. Abscissa: π.

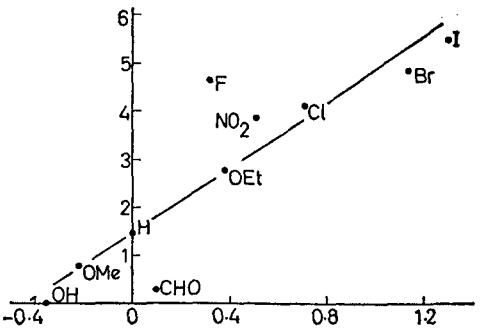

FIG. 3. Slope (K) (ordinate: K × 10^{-2}) for plots of reciprocal death time versus concentration of substituted acetanilide as a function of the π (abscissa) value of the substituent group on the acetanilide molecule.

$$K = 3{\cdot}43\pi\ (\pm 0{\cdot}63) + 1{\cdot}50$$

n	s	F	r			
10	1·01	29·97	0·888	..	..	(4)

In the absence of the fluoro compound

$$K = 3{\cdot}51\pi\ (\pm 0{\cdot}41) - 1{\cdot}23$$

n	s	F	r			
9	0·64	75·08	0·956	..	..	(5)

Although not directly dependent on the partition coefficient, it would appear from equation 4 that the time taken to achieve a "killing" concentration in the goldfish will depend on the lipophilicity of the drug. Since the drug has to penetrate a lipophilic barrier (the goldfish membrane) this observation is to be expected. Thus the lipophilicity of the drug in the bathing solution will determine not only the concentration of the drug necessary to kill the fish but also will be a measure of the time taken to achieve this concentration in the goldfish.

(ii) *Effect of poloxamers P65 and F68 on drug activity*

To test the effects of the two poloxamers on drug activity, two drugs were selected:— 4-hydroxyacetanilide, the least lipophilic of the 10 compounds ($\pi = -0{\cdot}36$) and 4-iodoacetanilide, the most lipophilic ($\pi = +1{\cdot}303$). Solutions were made up each containing $4{\cdot}3 \times 10^{-2}$ mol l^{-1} 4-hydroxyacetanilide with 1% F68, 2% F68, 1% P65 and 2% P65 respectively in Krebs bicarbonate buffer. Similar solutions were made containing $0{\cdot}134 \times 10^{-3}$ mol l^{-1} of 4-iodoacetanilide. Table 3 presents the death times for fish immersed in these various poloxamer solutions It can be seen from the Table that the presence of either poloxamer decreases the death time for each

Table 3. Mean death times for goldfish in 4-hydroxyacetanilide and 4-iodoacetanilide solutions containing various concentrations of poloxamers P65 and F68 in Krebs bicarbonate buffer pH 7·4 at 30 °C.

4-Hydroxyacetanilide			4-Iodoacetanilide		
Concn of poloxamer	No. of fish	Mean death time (min)	Concn of poloxamer	No. of fish	Mean death time (min)
0	5	40·90	0	5	50·20
1% P65	5	14·35	1% P65	6	41·88
2% P65	5	13·50	2% P65	6	39·92
1% F68	6	16·88	1% F68	5	48·85
2% F68	5	15·95	2% F68	5	42·55

compound. There are two possible explanations of this effect: the poloxamer could be forming a complex with the drug which is absorbed more rapidly than the drug alone, or the poloxamer could be affecting the goldfish membrane in some way to make it more permeable to the drug. To decide which theory applied, the fish were pre-treated in poloxamer solutions for 10 min and then transferred to poloxamer-free drug solutions after careful rinsing. Control fish were placed in Krebs bicarbonate buffer for 10 min. Since the drug and poloxamer are never in contact, complexes cannot be formed and any reduction in death time must be due to some effect of the poloxamer on the goldfish membrane. Results for these experiments are given in Table 4.

Table 4 shows that pre-treatment with poloxamer solution leads to a decreased death time, indicating that the poloxamer in some way interacts with the goldfish membrane to increase its permeability to the substituted acetanilides. This is in agreement with the results obtained using polysorbate 80 (Levy & Anello 1968) and STDC (Gibaldi & Nightingale 1968a), both of which act by increasing goldfish membrane permeability.

Table 4. Effect of pre-treatment with poloxamer solutions on the death times for goldfish in solutions of 4-hydroxyacetanilide and 4-iodoacetanilide in Krebs bicarbonate buffer pH 7·4 at 30 °C.

Pre-treatment solution	Test Drug	No. of fish	Mean death time (min)
Krebs buffer	4-Hydroxyacetanilide[a]	5	43·20
1% P65	,,	5	15·10
1% F68	,,	5	19·05
Krebs buffer	4-Iodoacetanilide[b]	5	51·80
1% P65	,,	5	42·00
1% F68	,,	5	49·20

[a] $4{\cdot}3 \times 10^{-2}$ mol litre^{-1}.
[b] $0{\cdot}134 \times 10^{-2}$ mol litre^{-1}.

There is a significant difference between the death times produced by the two poloxamers and between the death times produced by different concentrations of each poloxamer. Levy et al (1966) also found significant differences in the death times produced by secobarbital in 0·01, 1 and 2% solutions of polysorbate 80. This was explained in part by the different amounts of drug binding to the surfactant at different surfactant concentrations, but even when this was corrected for, differences in absorption rate were still seen for the different surfactant concentrations.

The difference between the two substituted acetanilides is marked. Considering 1% F68, the ratio of death times for 4-hydroxyacetanilide with and without poloxamer present is 0·41, for iodoacetanilide it is 0·81. Poloxamer F68 increases the absorption of the more lipophilic compound much less than the hydrophilic compound. The effect of the poloxamer on the goldfish membrane would appear to enhance the passage of the substituted acetanilides by a second mechanism other than passive diffusion.

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