Changes in the potency of aspirin in the presence of protein-bound dye

Whilst using the combined analgesic-capillary permeability test of Whittle (1964) we found the potency of aspirin in inhibiting acetic acid-induced writhing in mice to be less than we had obtained with the acetic acid model of Koster, Anderson & de Beer (1959) for analgesia estimation only. The most obvious difference between the two tests was the presence of intravenously administered vital dye in the Whittle test. This suggested some kind of interaction between aspirin and the protein-bound dye. In view of the use that has been and continues to be made of intravenous dyes as markers for plasma proteins in a variety of experimental inflammatory situations (Ankier & Whiteside, 1969 and references cited therein: Harrison & O'Donnell, 1970; Vargaftig, Coignet & others, 1971; Zoni, Molinari & Banfi, 1971), we sought confirmation of this interaction.

Groups of 10 male Swiss albino mice (18–22 g) were administered aspirin by stomach tube immediately after the intravenous injection of saline or of a saline solution of pontamine sky blue (diphenyl brilliant blue, colour index no. 24410, Geigy). 1 h later, acetic acid (0·5% v/v) was injected intraperitoneally (0·3 ml/mouse). After 5 min, the number of writhing movements made by each animal was counted for 10 min. The percentage inhibition of writhing in each group was calculated with reference to control groups of 20 mice receiving appropriately saline or dye but with vehicle (0·5% carboxymethylcellulose) in place of aspirin.

That the dye really was influencing the analgesic potency of aspirin in the writhing test was readily confirmed by the demonstration that in the presence of the dye (4% as used by Whittle, 1964) the log/dose-response curve of aspirin was markedly modified (Fig. 1). Thus, doses of aspirin (25–300 mg/kg) that in normal mice produced dose-related inhibitions of the writhing syndrome were virtually without effect in mice injected with the dye, although, at higher doses of aspirin (600 mg/kg), this antagonistic effect of the dye appeared to be reversible. Some animals treated with the dye showed obvious signs of acute toxicity after doses of aspirin above 300 mg/kg, but with 2% dye antagonism of the anti-writhing effects of aspirin was related to the amount of dye solution administered (Table 1).

As the minimal acute lethal oral dose of aspirin in normal mice is about 1 g/kg the observed toxicity of much lower doses of aspirin in the presence of the dye also required confirmation which is found in Table 2. The number of animals dying after any one dose of aspirin increased as the amount of dye administered increased but the higher doses of dye were themselves toxic and this precluded a conclusion as to which substance was potentiating which.

Table 1. *Mean counts* (± s.e.) *of acetic acid-induced writhing in mice treated with aspirin and pontamine sky blue.*

Dose of aspirin mg/kg	No. of animals	Volume of 2% pontamine sky blue (ml) 0·1	0·2	0·3	0·4
0	20	18·6 ± 1·5	21·6 ± 1·7	21·7 ± 1·1	34·3 ± 3·5
12·5	10	20·4 ± 1·2	31·4 ± 4·3*	33·8 ± 3·2**	30·5 ± 3·9
25	10	18·1 ± 2·5	32·7 ± 3·7*	29·8 ± 3·3*	37·8 ± 3·6
50	10	17·0 ± 3·8	23·7 ± 4·1	28·1 ± 4·0	28·0 ± 2·3
100	10	2·5 ± 0·7**	16·3 ± 3·0	24·5 ± 3·8	23·2 ± 3·9*
200	10	1·1 ± 0·6**	10·0 ± 1·6**	22·9 ± 3·0	5·6 ± 1·5**

* $P < 0.05$ ** $P < 0.001$ for differences from control counts by Student's *t*-test. Mice treated with 200 mg/kg aspirin and 0·4 ml 2% dye showed signs of acute toxicity and were possibly incapable of writhing.

These preliminary findings indicate a need for caution in the interpretation of results from experiments using vital dyes as plasma protein markers. This is especially true in considering the probable site of interaction being at the level of protein-binding since, although admittedly in an artificial *in vitro* system, it is evident that aspirin and pontamine sky blue compete for binding sites on plasma proteins (Table 3). Thus the amount of blue dye appearing in the protein-free supernatant fraction increases, for any one concentration of dye, with increasing concentration of aspirin and, for any one concentration of aspirin, with increasing concentration of dye. Certainly drug interaction at this level, particularly with the acidic anti-inflammatory analgesics, is well established (Solomon & Schrogie, 1967; Meyer & Guttman, 1968; Whitehouse, Bluestone & others, 1970).

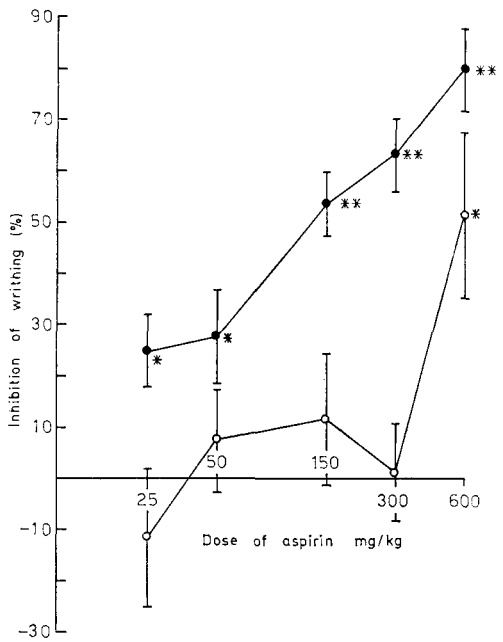

FIG 1. The percentage inhibition of acetic acid-induced writhing produced by aspirin in the absence of (closed circles) and in the presence of (open circles) pontamine sky blue (4%, 0·1 ml/mouse). Each point represents the mean inhibition and standard error (vertical line) in 10 mice, except for open circles with 600, 300, 25 (9 mice) and 150 (8 mice) mg/kg aspirin, expressed as a percentage of the writhing in the respective control groups ($*P < 0{\cdot}05$, $**P < 0{\cdot}001$). There was no significant difference between the control groups but with 150 and 300 mg/kg of aspirin the percentage inhibitions obtained in the presence of the dye were significantly different ($P < 0{\cdot}05$ and $P < 0{\cdot}001$ respectively) from those found in the absence of the dye (Student's *t*-test).

Table 2. *Percentage of animals dead (groups of* 10) *2h after the administration of aspirin and/or pontamine sky blue.*

Dose of aspirin	Volume of 4% pontamine sky blue (ml)				
mg/kg	0	0·1	0·2	0·3	0·4
0			0	40	80
30			0	50	100
100		0	10	100	
300	0	10	70	100	
600	0	20	100		
1000	0	50	100		
2000	30	80	100		
4000	50	80	100		

Table 3. *Displacement of pontamine sky blue from human plasma protein in the presence of increasing amounts of aspirin.*

Amount of aspirin added (mg) in 1 ml $NaHCO_3$ solution	Concentration of pontamine sky blue added in 1 ml distilled water					
	0%	1%	2%	3%	4%	5%
0	0	0	1·5	4	11·5	47·5
12·5	0	1	2	4·5	15	53·5
25	0	2	3·5	7	23	73
50	0	2	4	13·5	58	94
100	0	3	7	22	70	100

The figures (obtained by subtracting spectrophotometric transmittance readings from 100; λ = 605 nm) represent the amount of blue dye present in 1 in 5 dilutions of the protein free supernatants obtained by centrifuging (3000 rev/min for 10 min) the contents of tubes containing human plasma (1 ml) previously incubated (30 min at 37°) with pontamine sky blue (1 ml) and aspirin (1 ml) followed by precipitation of the proteins with 10% trichloroacetic acid (2 ml). Mean values from two experiments using fresh plasma; similar results were obtained with old plasma except that more blue appeared in each supernatant.

Since current opinion is that plasma protein-bound drug is pharmacologically inactive (Goldstein, 1949; Brodie, 1965) then competition between drug and dye for plasma binding sites would adequately explain the apparent increase in toxicity, but not the reduction in anti-writhing potency, of aspirin in the presence of vital dye. However, binding to plasma protein may provide a transport mechanism for aspirin to the site of inflammation and pain, where it would then be released in its active form; in the presence of the dye less drug is bound and transported. Certainly such a transport mechanism has already been proposed for phenylbutazone (Wallenfels & Sund, 1959).

An alternative interpretation is that aspirin displaces some of the dye from its binding sites on plasma protein and that the dye in its free form is responsible for the increased toxicity and the apparent reduction in the analgesic activity of aspirin.

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