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THE USE OF CHANGES IN CAPILLARY PERMEABILITY IN MICE TO DISTINGUISH BETWEEN NARCOTIC AND NONNARCOTIC ANALGESICS

BY

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An extension of the "squirming test" is described which makes the method specific for nonnarcotic analgesics. The intraperitoneal injection of acetic acid causes squirming and an increase in capillary permeability that is measured by direct estimation of plasma-bound dye (Pontamine Sky Blue) which has leaked into the peritoneal cavity. Nonnarcotic analgesics inhibit squirming and leakage of dye. Values for the oral ED50s for both effects are given for a number of typical compounds. Narcotic analgesics, in doses that produce analgesia, inhibit squirming but do not significantly affect leakage of dye. Drugs that stimulate the central nervous system and also inhibit squirming have no significant effect on leakage of dye over the range of doses which inhibit squirming. Corticosteroids do not significantly inhibit either squirming or leakage of dye.

The use of the squirming syndrome in mice and rats is now well established as a method of testing analgesic compounds. Squirming is here used to denote a syndrome which appears after the injection of mildly irritant substances into the peritoneal cavity. It is variously described as "stretching" by Koster, Anderson & de Beer (1959), "cramping" by Murray & Miller (1960) and "writhing" by Hendershot & Forsaith (1959). Squirming is understood to indicate one or more of the following responses: inward rotation of one or more feet, twisting or turning of the trunk, drawing-in of the abdominal wall, lordosis, and arching of the back.

Although this test is sufficiently sensitive to detect the effect of analgesics which are clinically less active than acetylsalicylic acid, it suffers from the disadvantage of not being specific. The test does not distinguish between narcotic and nonnarcotic drugs and it may even give a positive result with compounds which are not regarded as clinically useful analgesics (Seigmund, Cadmus & Lu, 1957; Eckhardt, Cheplovitz, Lipo & Govier, 1958; Hendershot & Forsaith, 1959). Various authors have discussed the conditions which influence the efficiency of the test (Murray & Miller, 1960; Brown & Hughes, 1962; Okun, Liddon & Lasagna, 1963).

The object of the present work was to gain more information from the test by measuring the concurrent changes in the peritoneal capillary permeability during squirming. By extending the test in this way it was possible to increase the specificity

of the test and so to distinguish between the effects of narcotic and nonnarcotic analgesics.

METHODS

Male and female specific pathogen-free mice of the Alderley Park strain I, weighing 20±1 g, were given test-compounds by stomach tube. After 20 to 25 min each animal was given an intravenous injection of 0.1 ml. of a 4% solution of Pontamine Sky Blue. At 30 min after administration of the analgesic, 1 mg of acetic acid was injected intraperitoneally as 0.4 ml. of a 0.25% (v/v) solution. Each dose group consisted of twelve mice. These were placed in a twelve-compartment Perspex observation box, and the number of squirms for each animal was recorded on a twelve-unit tally counter. After 20 min the mice were killed by dislocation of the neck and the viscera were exposed, after allowing 1 min for blood to drain away from the abdominal wall. The animal was held by a flap of abdominal wall and the viscera were irrigated with distilled water over a Petri dish. The combined washings were filtered through glass wool and made up to 10 ml. in a graduated test tube. 0.1 ml. of 0.1 N-sodium hydroxide solution was added to each tube in order to clear any turbidity due to protein, and the absorption was read at 590 mμ. The amount of dye was expressed as μg/20 g of mouse. Control animals were treated similarly except that they received an oral dose of vehicle alone. The effects in terms of permeability and analgesia are expressed as percentage reductions of control values. Comparisons of potency were made at doses which gave a 50% reduction.

A log dose/effect curve was obtained for each of the compounds. The slope and position of the regression line were calculated by the method of least squares, and the ED50 and fiducial limits by standard statistical methods.

The tests were performed at room temperature (21±1° C). Animals were primed by injecting 0.05 mg of stilboestrol intramuscularly 48 hr before the test. The stilboestrol was administered as a solution in ethyl oleate containing 1 mg/ml.

Drugs. The analgesic drugs used were of B.P. quality and were administered as solutions or ball-milled suspensions in a dispersing agent containing (per l.): Lissapol NX 1 ml., Lissapol C 1 g, and Dispersol OG 30% 3.3 ml. (each from I.C.I.), adjusted to *p*H 7. Acetylsalicylic acid was administered as a solution of the sodium salt prepared immediately before use by neutralization of the acid with a slight excess of sodium bicarbonate. Doses of amphetamine sulphate, ephedrine hydrochloride, and mebanazine oxalate (α-methyl benzyl hydrazine oxalate, Actomol, I.C.I.) refer to the salts. Pontamine Sky Blue 6 BX, batch number 14530, was obtained from G. T. Gurr Ltd. Variations in the absorption maxima and concentration of dye were noted in different batches of this material. The present work was carried out using a 4% solution (w/v) in distilled water which was stored at 4° C until required for use. Phenylquinone (2-phenyl-1,4-benzoquinone) was obtained from Kodak Ltd.

RESULTS

Difference in sensitivity of different strains of mice

Brown & Hughes (1962) have examined the sensitivity of mice of various strains to squirming produced by phenylquinone. The mice used in the present study gave mean numbers of squirms of 36.2 and 49.5 after 20 and 30 min respectively, in response to an intraperitoneal dose of 1 mg/kg of phenylquinone. This rate of squirming compares with that of the more active strains of Brown & Hughes (1962) who used an intraperitoneal dose of 2 mg/kg. Comparative studies using other agents have not so far been reported, but similar variations in sensitivity between strains are likely to exist. Changes in permeability after phenylquinone are similar in time course to those produced by acetic acid, but they are less conspicuous and hence less useful as the basis for an assay method.

Effects of environmental temperature

Parkes & Pickens (personal communication, 1960) have shown that the rate of squirming increases with environmental temperature, and a useful increase in sensitivity can be obtained by carrying out the test at 35° C. The changes in permeability are also sensitive to temperature, but the optimum temperature appears to be about 20° C. Any increased permeability at higher temperatures is presumably offset by more rapid reabsorption of dye. Miles & Miles (1952) found that an environmental temperature of 37° C reduced the leakage of circulating dye induced by histamine in guinea-pigs.

Effects of priming with stilboestrol

Fig. 1 shows the effect of priming with stilboestrol on male and female mice. The increase in sensitivity was seen in each sex over a range of doses of acetic acid. At the dose used in the method described above it amounted to a twofold increase

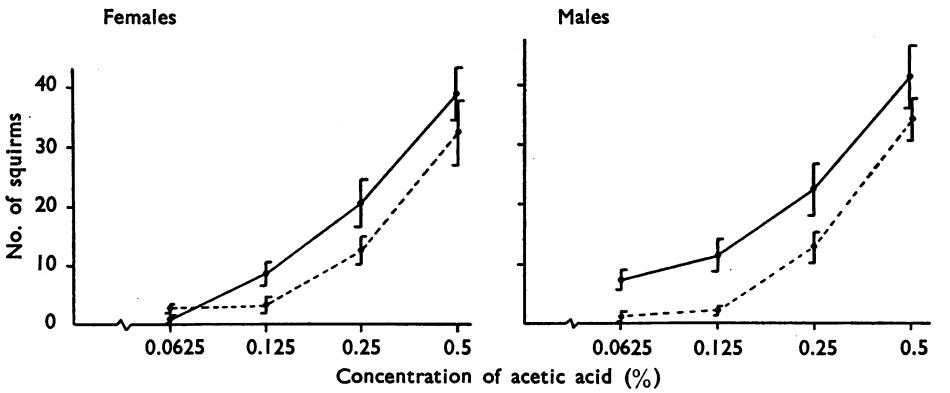

Fig. 1. The effect of priming on the number of squirms of male and female mice induced by intraperitoneal injection of acetic acid. The primed animals received 0.05 mg of stilboestrol in ethyl oleate 48 hr before testing. Abscissa: the concentration of acetic acid injected; ordinate: the number of squirms per animal in 20 min. Each point is the mean for twelve mice with standard error. The continuous line represents primed animals and the broken line unprimed animals.

in sensitivity. The effect did not appear to be directly related to the stage of oestrus. Priming does not materially affect the estimates of potency, but provides a useful means of increasing sensitivity.

Effects of p*H and concentration of acetic acid*

Squirming and increased permeability were produced by all of the acids tested (acetic, citric, hydrochloric, lactic and phosphoric acids) and the response appeared to be related to *p*H and to concentration (Fig. 2). In this experiment solutions of acetic acid were neutralized with sodium hydroxide solution to give solutions containing 0.25 and 0.5% of acetate, with a range of *p*H's from 3.1 to 5.4 Squirming and leakage of dye were maximal at a *p*H of less than 3.8, and the change in both parameters was greatest in the range 4.0 to 5.0. This is the range of *p*H where the

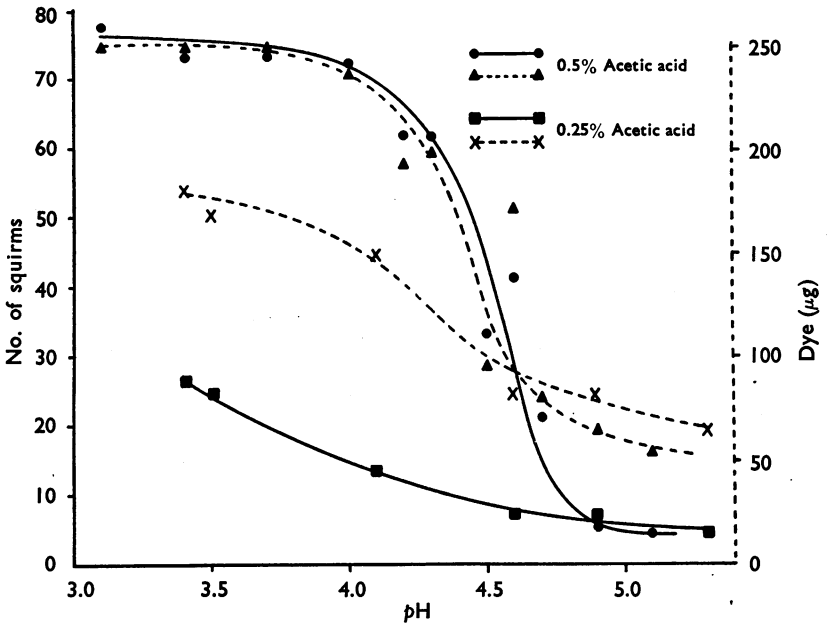

Fig. 2. The effect of *p*H and concentration of acetic acid/acetate solution on squirming and capillary permeability in mice. Ordinate: the number of squirms per animal in 20 min (solid line) and the amount of dye recovered in μg (broken line); abscissa: *p*H of injected solution. Solutions of acetic acid were partially neutralized with the calculated amount of sodium hydroxide solution and were diluted to give final concentrations of 0.25% (■ and ×) and 0.5% (● and ▲) of acetic acid.

concentration of un-ionized acid is changing most rapidly (the pK_a of acetic acid is 4.75).

Other acids were tested but found to be less satisfactory than acetic acid over the same range of concentrations. Citric acid gave a flat dose/response curve and at higher concentrations produced convulsions. Lactic acid and hydrochloric acid gave irregular dose/response curves.

Effects of narcotic and nonnarcotic analgesics

Narcotic analgesics did not cause a significant effect on the permeability response, when the criterion of activity was reduction of the response in treated animals to 50% of the value in control animals. Table 1 records values for the ED50's of

TABLE 1

COMPARISON OF ANALGESIC ACTIVITIES OF NARCOTIC ANALGESICS AGAINST SQUIRMING INDUCED BY ACETIC ACID

The analgesics were given orally 30 min before the squirming test

Compound	ED50 (mg/kg)	95% confidence limits
Morphine	1·3	0·9–1·8
Methadone	4·1	2·7–6·0
Pethidine	6·6	5·0–9·0
Codeine	7·2	5·5–9·3

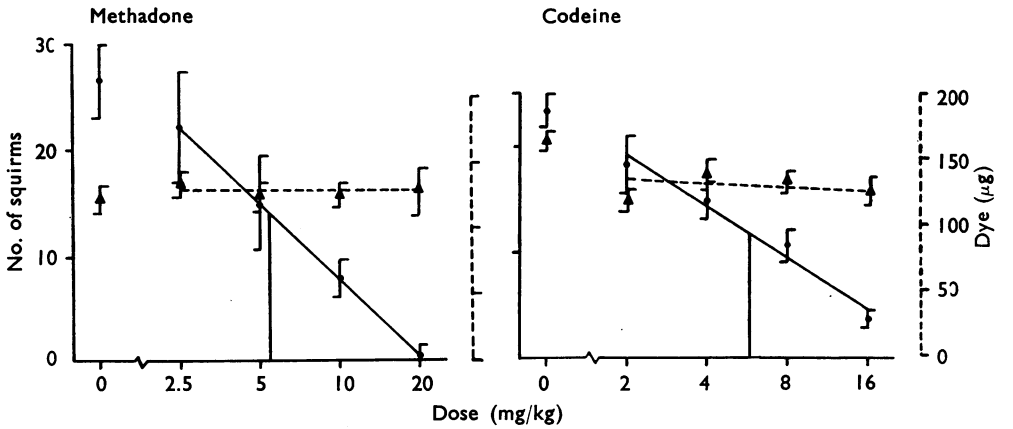

Fig. 3. Analgesic effects of methadone and codeine in mice. Ordinate: the number of squirms per animal in 20 min (solid line) and the amount of dye recovered in μg (broken line); abscissa: oral dose of drug in mg/kg. Treatment time before testing was 30 min. Vertical lines give standard errors of the means.

some typical narcotic analgesics, but since reduction of the permeability response did not approach 50% over the range of doses which inhibited squirming, there are no corresponding ED50's for the permeability effect. The compounds were given orally 30 min before injecting acetic acid. Typical dose/response curves for codeine and methadone are shown in Fig. 3.

Nonnarcotic analgesics antagonized squirming and also reduced the permeability response. Values of the ED50's for both effects are given in Table 2, and the dose/effect curves for sodium acetylsalicylate and cinchophen are shown in Fig. 4.

TABLE 2

COMPARISON OF THE ACTIVITIES OF NONNARCOTIC ANALGESICS AGAINST SQUIRMING AND INCREASE IN PERITONEAL PERMEABILITY

	Inhibition of squirming		Inhibition of permeability response	
Compound	Oral ED50	95% confidence limits	ED50	95% confidence limits
Given 0·5 hr before tests				
Amidopyrine	25·1	18·5–34	200	
Cinchophen	46·4	32–66	130	83–205
Sodium acetylsalicylate	48·8	35–67	104	61–176
Phenazone	78·5	59–105	445	
Paracetamol	137·0	102–182	415	
Sodium salicylate	190·0	134–268	270	155–470
Salicylamide	211·0	150–298	519	298–930
Given 1 hr before tests				
Phenylbutazone	61·5		200	

Effects of nonanalgesic compounds

Central nervous system stimulants give a large proportion of the " false positives " which are detected by the conventional squirming test. Although the characteristics

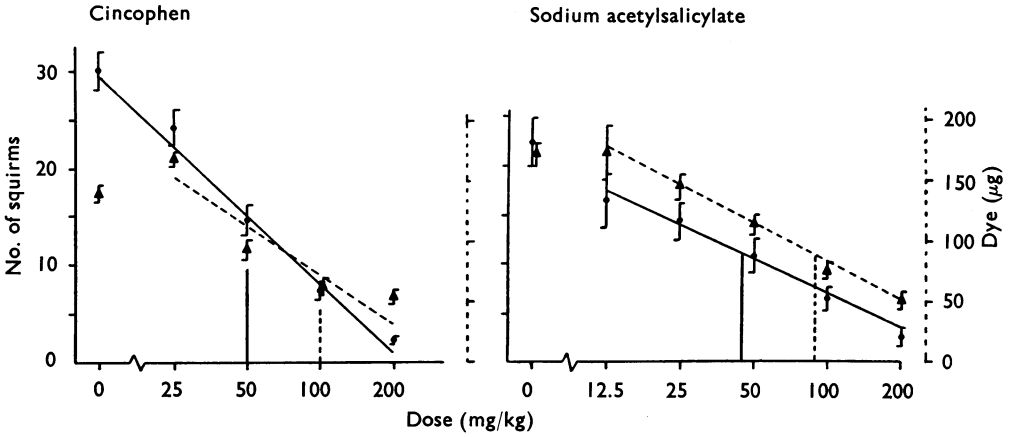

Fig. 4. The effects of cinchophen and of sodium acetylsalicylate on squirming and capillary permeability. Ordinate: the number of squirms per animal in 20 min (solid line) and the amount of dye recovered in μg (broken line). Treatment time before testing was 30 min. Vertical lines give standard errors of the means.

of this type of compound can be distinguished in other tests, the inhibition of squirming by these nonanalgesic compounds can be an embarrassment in a screening programme. Table 3 shows the effect of amphetamine, ephedrine and mebanazine, a monoamine oxidase inhibitor: all inhibited squirming but did not affect the permeability response significantly and hence can be distinguished from nonnarcotic analgesics.

TABLE 3

EFFECT OF CENTRAL NERVOUS SYSTEM STIMULANTS ON SQUIRMING AND CAPILLARY PERMEABILITY

Percentages are calculated from the means of groups of mice compared with controls (12 to 24 mice)

Compound	Oral dose (mg/kg)	No. of mice	Reduction of squirming (%)	Change in permeability (%)
Amphetamine	4	10	46·5	+14
	8	10	92·1	+16
Ephedrine	5	12	16·2	+16
	10	24	53·1	+ 1·3
	20	12	88·2	+16
Mebanazine	10	10	5·9	−21·4
	20	10	38·3	−13·5
	40	10	64·5	−23·8

Corticosteroids, such as cortisone and paramethasone acetate (Metilar, Syntex Corp.), had little effect when given parenterally, 2 hr before challenging with acetic acid (Table 4). Animals treated with cortisone 24 and 2 hr before challenging with acetic acid showed a small decrease in the number of squirms and an increase in permeability, neither of which is significant ($P=0.05$). Similar results were obtained with paramethasone when animals were treated with 2 mg/kg, administered orally 18 and 1 hr before the injection of acetic acid.

TABLE 4

EFFECT OF ANTI-INFLAMMATORY STEROIDS ON SQUIRMING AND CAPILLARY PERMEABILITY

Percentages are calculated from the means of groups of mice compared with controls (12 to 24 mice). I.v. = intravenous; s.c. = subcutaneous

Compound	Dose (mg/kg)	Time before tests (hr)	Route	No. of mice	Change in squirming (%)	Change in permeability (%)
Paramethasone	2	2	I.v.	6	+12	−1
acetate	2	18 and 1	Oral	12	−14·5	+12
Cortisone	250	2	S.c.	6	−19·2	+19·6
	100	24 and 2	S.c.	12	−1·5	+25·1

DISCUSSION

Methods based on the squirming response in rodents are amongst the most sensitive methods of detecting analgesic activity. The conventional squirming test is sensitive but not specific, and attempts to improve specificity by the control of experimental conditions have met with little success.

The measurement of concurrent changes in peritoneal capillary permeability therefore appears to offer a relatively simple way of testing nonnarcotic analgesics. The effect on capillary permeability and squirming have similar time-courses, and it has been proposed that both these effects are consequences of the activation of a protease system (Whittle, 1963). Nonnarcotic analgesics could inhibit squirming by interfering with the local reaction to peritoneal irritation and so reduce the intensity of afferent nervous stimulation. Squirming could also be inhibited by narcotic analgesics which act by blocking central nervous synaptic transmission in the pathway for pain.

The sensitivity of the test depends on the conditions under which the test is performed and may therefore vary from one laboratory to another. For this reason preliminary experiments may be needed to find the dose of acetic acid which will produce a satisfactory response under the conditions of the test. In general, satisfactory assays have been obtained when control animals have exhibited more than fifteen squirms per mouse in 20 min. At this level of response it is usually possible to recover at least 140 μg of dye from the peritoneal cavity. When the values for rate of squirming and dye concentration are lower, the dose/response curves for nonnarcotic analgesics become flat and so the test gives erratic results.

In testing for analgesia it is desirable, though rarely possible, to estimate the tissue response to the applied stimulus. When the method is based on the use of thermal, electrical or mechanical stimuli it is usually only possible to measure the amount of energy applied. The present method makes it possible to measure the local response to peritoneal irritation as an increase in capillary permeability, and to correlate this response with that arising from the simultaneous stimulation of sensory nerve endings.

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