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The assay of anti-pyretic drugs in mice, using intracerebral injection of pyretogenins

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1. A simple, cheap, and reliable method is described for the screening of new compounds for anti-pyretic activity. It involves the intracerebral injection of a suitable pyretogenin in conscious mice.

2. The pyretic response is related to the logarithm of the dose in a substantially linear manner when "E" Pyrogen is used.

3. A selection of established anti-pyretic drugs were effective in oral doses approaching those used clinically, which shows that the method is unusually sensitive.

4. The response to intracerebral pyretogenin is not unduly influenced by drugs known to cause hypothermia.

5. It is suggested that "E" Pyrogen injected intracerebrally has a direct effect on the central nervous system, rather than an indirect action through release of endogenous pyrogen.

The non-narcotic analgesic drugs frequently possess anti-inflammatory or anti-pyretic properties or both. It is therefore necessary to test all drugs of potential use as analgesics for possible anti-pyretic activity.

Existing tests generally involve a febrile state following the injection of a pyretogenin by the intravenous, subcutaneous or intraperitoneal routes. Potential anti-pyretic drugs are administered immediately before or during the ensuing pyrexia. The present method was developed after Brittain (1966) had reported the effects of noradrenaline and other drugs on body temperature in the conscious mouse after intracerebral injection, using the method of Haley & McCormick (1957). It seemed likely to us that the inconsistency of pyretic reactions in rodents could be due to failure of much of the pyrogen to reach the brain. We therefore studied the effect of intracerebral injection of pyretogenins on body temperature in the conscious mouse and determined the anti-pyretic action of a number of non-narcotic analgesics. Several drugs known to produce hypothermia in mice were also tested in order to establish whether these would mask the pyrexia induced by the intracerebrally injected pyretogenin. For comparative purposes we tested paracetamol against pyrexia induced by intravenous injection of "E" Pyrogen in rabbits.

Methods

Albino mice (*CFW* or *TO* strain) weighing 19–21 g were used throughout. Normally females were used but pilot experiments had shown there was no significant difference between strains or between sexes. All animals were kept overnight in the test room which was thermostatically maintained between 70° and 72°F and were allowed free access to food and water. Two hours before the test, each mouse was placed individually in a solid cage (10 cm^3) and food and water were withdrawn until the test was completed. The cages were fitted with perforated Perspex lids, which enabled the mice to be observed throughout the experimental period. Rectal temperatures were recorded by means of a thermistor, (Standard Telephones and Cables Ltd., Type F15) fitted with a stop so that insertion was to a depth of 1 cm. The thermistor was dipped into olive oil for lubrication immediately before each measurement.

Intracerebroventricular injections were carried out as described by Haley & McCormick, except that a Hamilton 100 μl. syringe with a 25 S.W.G. × $\frac{3}{8}$in. needle (Gillette) was used for injections and the injection volume was constant at 20 μl. The depth of injection was limited to $\frac{1}{4}$in. by shortening the effective length of the needle with a $\frac{1}{8}$in. rubber stop, and in order to facilitate perpendicular injection, the syringe was clamped in a rack-work "X" block (C. F. Palmer), fixed to a retort stand. Penetration was then achieved by rotating the racking screw which lowered the syringe. The location of the injection site was checked by injecting 20 μl. of a 1 in 5 dilution of Indian ink in 0.9% NaCl solution. Histological examination showed that injection was into the third ventricle and ink particles were also distributed throughout the aqueducts and the fourth and lateral ventricles.

The pyretogenins used were "E" Pyrogen (Organon) and T.A.B. vaccine, B.P. (Burroughs Wellcome), diluted with pyrogen-free 0.9% NaCl solution. The drugs reported to have anti-pyretic properties investigated were acetylsalicylic acid (B.D.H.), paracetamol (Monsanto), phenylbutazone (Geigy), mefenamic acid (Parke-Davis), flufenamic acid (Parke-Davis), ibufenac (Boots), indomethacin (Merck) and 4-amino-antipyrine (Hopkin and Williams). They were administered orally as suspensions in approximately 0.5% sodium carboxymethyl-cellulose in a constant dose volume of 0.5 ml. The drugs tested which were known to produce hypothermia included perphenazine (Allen & Hanbury), which was prepared and administered as described above, phenobarbitone sodium (Evans) and reserpine (B.D.H.), which were injected intraperitoneally in an 0.5 ml. dose volume. The reserpine was prepared as an injection solution as described by Leyden, Pomerantz & Bouchard (1956).

In most experiments, one group of mice was injected with 0.9% NaCl solution intracerebrally as control. The 0.9% NaCl solutions used had previously been shown to be substantially free of pyrogen, when tested by the "Test for Pyrogens," described in the *British Pharmacopoeia* (1963). Only solutions which gave a summed response on three rabbits of less than 0.55° C were accepted for use.

Anti-pyretic activity in rabbits was tested as follows, using animals and equipment suitable for use in the "Test for Pyrogens" of the *British Pharmacopoeia* (1963). The rectal temperatures of groups of three rabbits (New Zealand White) were recorded initially and at half-hourly intervals for 5 hr, by means of thermocouples inserted into each rectum to a depth of 7.5 cm., readings being taken on a spot

galvanometer (Cambridge Instruments). Fifteen minutes after the first reading, "E" Pyrogen (0.01 μg/kg) was injected intravenously into each rabbit, and 30 min after this injection the rabbits were dosed orally with paracetamol (125 mg/kg) or 0.9% NaCl solution in a dose volume of 1 ml./kg.

Results

Effect of pyretogenin injection

The time courses of the mean rectal temperature changes in groups of five mice after intracerebral injections of "E" Pyrogen (2.5 μg/kg), T.A.B. vaccine (1 in 100 dilution) or pyrogen-free 0.9% NaCl solution are shown in Fig. 1, together with the calculated standard errors. Rectal temperatures were recorded initially, at 15 min intervals for the first hour, and at 2 hr after injection. Both T.A.B. vaccine and "E" Pyrogen produced a hyperthermic response which was maximal at 30 and 45 min after injection, but the variability in the T.A.B. vaccine response appeared to be greater than the "E" Pyrogen response as judged by the standard errors. Pyrogen-free 0.9% NaCl solution produced a transient fall in body temperature which returned towards the initial at 45 min, with a subsequent gradual decline, up to 2 hr after injection. Brittain (1966) reported a similar response after intracerebral injection of 0.9% NaCl solution in mice.

In all subsequent experiments "E" Pyrogen was used as the pyretogenin.

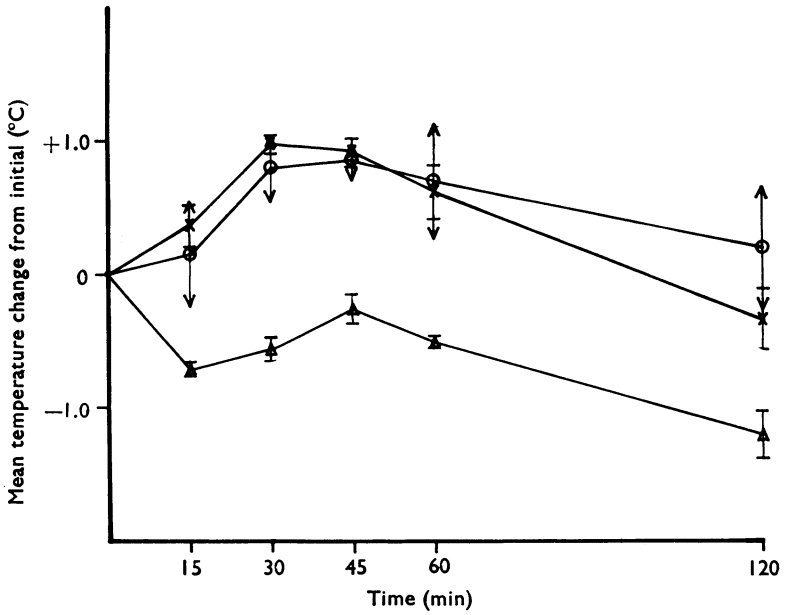

FIG. 1. Effect of intracerebral injections of 0.9% NaCl solution, "E" Pyrogen (2.5 μg/kg) and T.A.B. vaccine (1/100) on the mean rectal temperature changes in groups of five mice during a 120 min period. The crosses (X) represent the temperature response to "E" Pyrogen (mean ± S.E.), the open circles (○) the response to T.A.B. vaccine (mean ± S.E.), and the open triangles (△) the response to 0.9% NaCl solution. The standard errors relating to T.A.B. vaccine are terminated in an arrow head. All substances were injected in a volume of 20 μl.

Responses to graded doses of "E" Pyrogen

It would be impractical to use the entire temperature response curves, as shown in Fig. 1 for assay of either pyrogen or anti-pyretic drugs. In order to simplify the assay procedures, we have adopted the assessment of "temperature index," described by Winter & Nuss (1963), as a means of recording results. Taking as a base the mean initial temperature, the mean temperature changes from the initial at 15, 30, 45 and 60 min were summed and termed the temperature index. The 2 hr reading was ignored because the temperature changes had passed their peak at 60 min, and uniform intervals between temperature measurements are inherent in the conversion of the response curve to a digital form. In our experiments the temperature index for 0.9% NaCl solution was invariably negative and in this respect our results varied from those reported by Winter & Nuss (1963) in their experiments in rats and rabbits.

The values for temperature index relative to log dose of intracerebral "E" Pyrogen in groups of five mice are shown in Fig. 2. There is no significant deviation from linearity over the dose range used but in pilot experiments doses greater than 2.5 μg/kg did not proportionally increase the pyretic response.

Assay of anti-pyretic drugs in mice

All anti-pyretic drugs tested were administered orally to groups of five mice in increasing doses, 15 min before the intracerebral injection of "E" Pyrogen (2.5 μg/kg) into each mouse and rectal temperatures were recorded as described

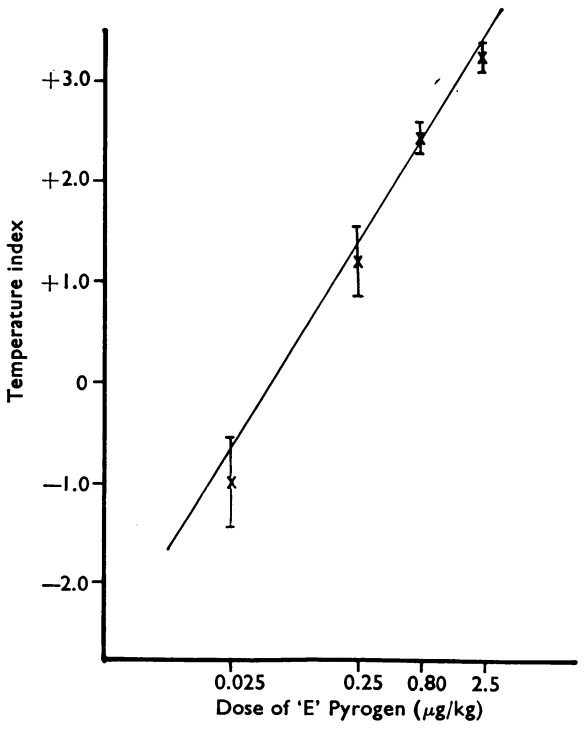

FIG. 2. Effect of increasing doses of "E" Pyrogen injected intracerebrally into groups of five mice. The crosses (X) represent the temperature index (mean ± S.E.) determined for each dose of "E" Pyrogen, the doses being plotted on a log scale.

previously. Temperature indices with standard errors were calculated for each drug and are recorded, together with the mean temperature changes for each dose of each drug, in Table 1. All the anti-pyretic drugs tested were effective in the test. In most cases the temperature indices calculated for the highest dose of each drug approached that of intracerebral 0.9% NaCl solution. It should be noted that in previous experiments this dose had been shown to have no significant effect on body temperature in normal mice, and it was also without effect on the response to intracerebral 0.9% NaCl solution. The mean temperature index for all tests carried out on 0.9% NaCl solution was −2.15 and, for "E" Pyrogen treated controls, +3.10. In all cases, the relationship between temperature index and log

TABLE 1. *Effect of anti-pyretic drugs on temperature changes induced in mice by intracerebral injections of "E" Pyrogen*

Drug	Dose mg/kg (orally)	Mean temperature change (°C) after 15 min	30 min	45 min	60 min	Temperature index	Anti-pyretic potency mg/kg (orally)
Paracetamol	25·0	−0·93	−0·55	−0·55	−0·32	−2·35±0·45	6·8
	12·5	−0·50	+0·40	+0·11	−0·22	−0·21±0·17	
	6·25	−0·41	+0·50	+0·88	+0·13	+1·10±0·67	
	0	+0·65	+1·05	+1·00	+0·65	+3·35±0·87	
Aspirin	50·0	−1·10	−1·00	−0·40	0·00	−2·50±0·78	15·8
	25·0	−0·35	0·00	+0·31	+0·20	+0·16±0·65	
	12·5	+0·25	+0·70	+0·85	0·00	+1·80±0·69	
	0	+1·05	+0·75	+0·85	+0·65	+3·30±0·55	
Phenylbutazone	25·0	−0·92	−0·75	−0·45	−0·45	−2·57±0·70	8·9
	12·5	−0·40	+0·15	+0·45	+0·35	+0·55±1·04	
	6·25	+0·30	+0·75	+0·65	+0·50	+2·20±0·77	
	0	+0·45	+1·00	+0·70	+0·60	+2·75±0·39	
Ibufenac	25·0	−1·10	−0·55	−0·65	−0·95	−3·25±0·96	10·5
	12·5	+0·25	+0·60	+0·60	+0·15	+1·60±0·31	
	6·25	+0·10	+1·10	+1·00	+0·90	+3·10±0·54	
	0	+0·30	+1·00	+1·30	+1·10	+3·70±0·60	
Flufenamic acid	12·5	−1·10	−0·90	−0·75	−0·05	−2·80±0·99	4·8
	6·25	−0·40	+0·65	+0·30	+0·45	+1·00±1·00	
	3·125	+0·75	+0·85	+0·50	+0·40	+2·50±0·86	
	0	+0·35	+0·65	+0·90	+0·25	+2·15±0·30	
Mefenamic acid	12·5	−0·55	−0·70	−0·15	−0·20	−1·60±0·78	4·0
	6·25	−0·20	+0·55	+0·25	−0·30	+0·30±0·67	
	3·125	+0·25	+0·90	+0·10	+0·40	+1·65±0·45	
	0	+0·44	+0·97	+0·91	+0·72	+3·04±0·61	
4-amino-antipyrine	12·5	−0·60	0·00	−0·40	−0·55	−1·55±0·70	3·9
	6·25	−0·60	−0·05	+0·30	0·00	−0·35±1·10	
	3·125	+0·55	+0·90	+0·60	−0·05	+2·00±0·59	
	0	+0·05	+1·20	+0·80	+0·65	+2·70±0·74	
Indomethacin	12·5	−0·25	+0·70	+0·25	0·00	+0·70±0·20	6·3
	6·25	+0·20	+0·40	+0·35	+0·35	+1·30±0·04	
	3·125	+0·25	+0·63	+0·69	+0·35	+1·92±0·14	
	0	+0·13	+0·96	+1·17	+1·11	+3·37±0·24	
None	Mean saline response	−0·63	−0·45	−0·28	−0·79	−2·15	

Drugs were administered orally 15 min before the intracerebral injection of "E" Pyrogen (2·5 μg/kg) and the mean temperature changes from the initial in groups of five mice were calculated. Temperature indices were determined, with standard errors, from the sum of the mean temperature changes at 15, 30, 45 and 60 min after injection, and were used to compare the anti-pyretic potencies of the drugs. The anti-pyretic potency is the dose of drug necessary to reduce the response of "E" Pyrogen (2·5 μg/kg) to that of 0·25 μg/kg in the absence of the drug.

dose of anti-pyretic drug was substantially linear and this is illustrated for paracetamol in Fig. 3. For comparative purposes the time course of the mean temperature changes for the doses of paracetamol used for constructing the temperature index-log dose curve for paracetamol are illustrated in Fig. 4.

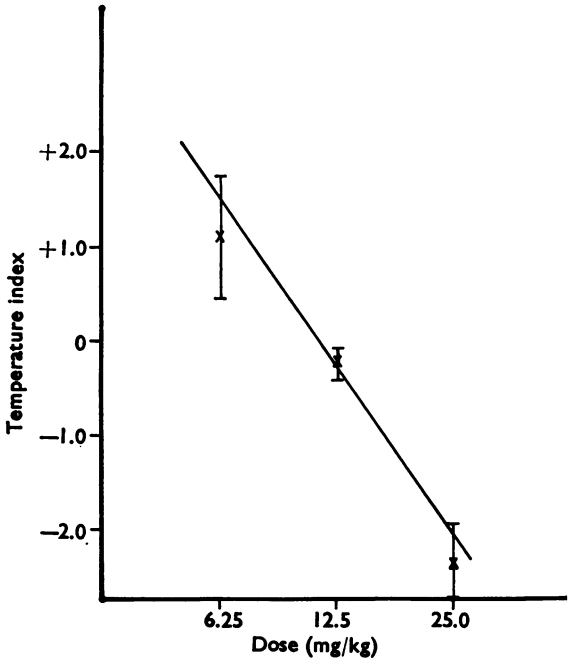

FIG. 3. Effect of paracetamol on the temperature response to "E" Pyrogen (2.5 μg/kg) injected intracerebrally into groups of five mice. The crosses (X) represent the temperature index (mean ± S.E.) determined for each dose of paracetamol, the doses being plotted on a log scale.

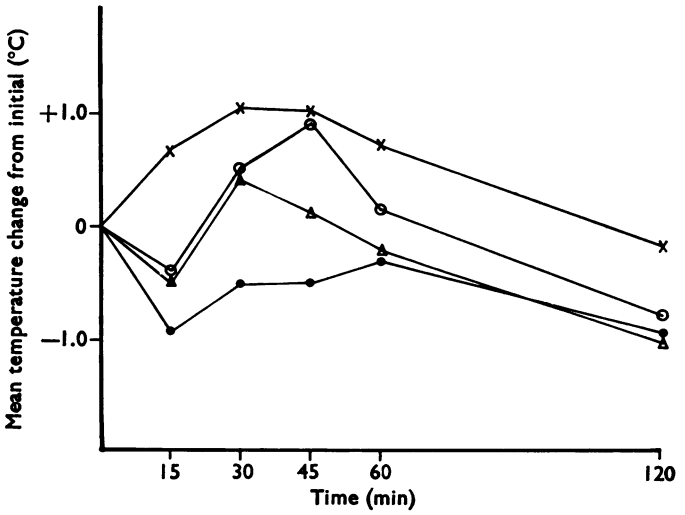

FIG. 4. Effect of paracetamol on the mean temperature changes induced in groups of five mice by the intracerebral injection of "E" Pyrogen (2.5 μg/kg), over a 120 min period. The crosses (X) represent the response to untreated mice, the open circles (○) the response to paracetamol 6.25 mg/kg, the open triangles (△) the response to paracetamol 12.5 mg/kg and the closed circles (●) the response to paracetamol 25 mg/kg. The paracetamol was administered orally 15 min before the "E" Pyrogen injection.

TABLE 2. *Effect of hypothermic drugs on mouse temperature and on temperature changes induced in mice by intracerebral injections of "E" Pyrogen*

Drug	Dose (mg/kg)	Route	Dose of "E" Pyrogen (μg/kg)	Mean initial temperature (°C)	Mean temperature change (°C) after 15 min	30 min	45 min	60 min	Temperature index
Phenobarbitone sodium	100	I.P.	Nil	35·90	−0·90	−1·10	−1·15	−1·45	−4·60±0·89
	100	I.P.	2·5	35·55	+0·35	+0·05	−0·85	−0·95	−1·40±0·40
	25	I.P.	Nil	37·15	−1·05	−1·00	−1·05	−1·80	−4·90±0·83
	25	I.P.	2·5	37·50	−0·75	−0·10	−0·55	−0·75	−2·15±0·94
Reserpine	2·5	I.P.	Nil	26·70	−0·05	−0·25	−0·19	−0·20	−0·69±0·50
	2·5	I.P.	2·5	28·30	+0·50	+0·50	+0·40	+0·05	+1·45±0·39
Perphenazine	10	Oral	Nil	37·70	−0·70	−1·65	−1·55	−1·70	−5·60±0·66
	10	Oral	2·5	37·35	−0·15	−0·10	−0·55	−0·80	−1·60±0·92

Phenobarbitone sodium and perphenazine were administered 15 min and reserpine was administered 4 hr before the rectal temperatures of groups of five mice being recorded at 15 min intervals for 60 min. The mean temperature changes from the initial were calculated. Temperature indices, with standard errors, were determined from the sum of the mean temperature changes at 15, 30, 45 and 60 min.
In a similar series of experiments, "E" Pyrogen (2·5 μg/kg) was injected intracerebrally into each mouse immediately after the initial temperature had been recorded.

The relative potencies of the drugs are listed in Table 1 and are reported in terms of the dose of drug necessary to reduce the response (temperature index) of an intracerebral dose of 2.5 μg/kg of "E" Pyrogen to that of 0.25 μg/kg in the absence of the drug. This parameter has been arbitrarily chosen and is calculated from the log dose/temperature index curves for each drug, and from the log dose/temperature index curve for graded doses of "E" Pyrogen (Fig. 2). Thus the temperature index for a dose of "E" Pyrogen of 0.25 μg/kg intracerebrally is +1.3 and the dose of paracetamol estimated to be necessary to give this temperature index, after 2.5 μg/kg of "E" Pyrogen (Fig. 3), is 6.8 mg/kg orally.

Effect of hypothermic drugs

Table 2 shows the results we obtained with phenobarbitone sodium, perphenazine and reserpine administered alone and with intracerebral injections of "E" Pyrogen (2.5 μg/kg), which was administered 15 min after treatment with phenobarbitone sodium and perphenazine and 4 hr after treatment with reserpine. Groups of five mice were used throughout. All three drugs produced a hypothermic effect, as judged by the temperature indices. The hypothermic effect of the reserpine was nearing maximal at the time of the experiment and consequently the fall during the experiment was smaller than with the other drugs. In each case the effect of intracerebral "E" Pyrogen injection was pyrexia, indicated by a reduction in the degree of hypothermia produced by the drugs.

Anti-pyretic effect of paracetamol in rabbits

The time course of the mean temperature changes of groups of three rabbits treated orally with either 0.9% NaCl solution or paracetamol (125 mg/kg) 30 min after the intravenous injection of "E" Pyrogen (0.01 μg/kg) is shown in Fig. 5. The "E" Pyrogen has produced pyrexia in both groups, but this effect has been antagonized in the paracetamol-treated group for a period of approximately 3 hr.

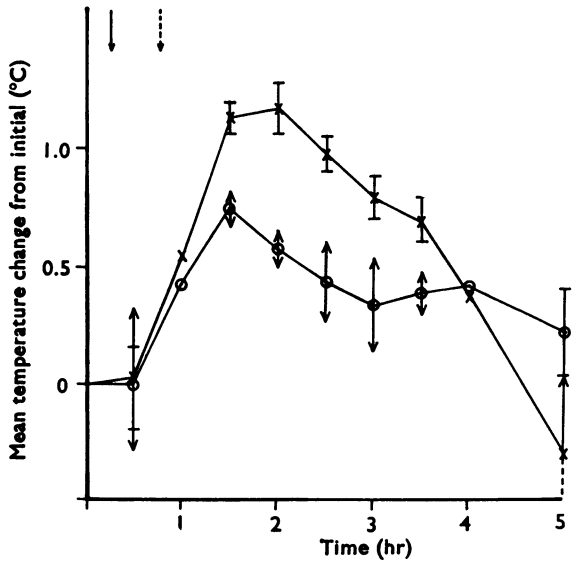

FIG. 5. Effect of paracetamol on the temperature changes (mean ±S.E.) induced in groups of three rabbits by "E" Pyrogen 0.01 μg/kg injected intravenously. The crosses (X) represent the mean responses in saline-treated rabbits and the open circles (○) the mean response in paracetamol-treated rabbits. At ↓, 0.01 μg of "E" Pyrogen was injected intravenously into each rabbit. At ↓, one group was treated orally with 0.9% NaCl solution 2 ml./kg and the other group with paracetamol 125 mg/kg orally, in the same dose volume.

Discussion

These results demonstrate that the method described produces reasonably consistent pyrexia which is sensitive to clinically useful anti-pyretic drugs. The techniques used are easily carried out after a little practice, and the duration of the test is short compared with other anti-pyretic tests described in the literature. A major advantage of the method as a screening test lies in its sensitivity to anti-pyretic drugs given orally in doses equivalent to those used clinically. Because of this sensitivity and the small size of the test animal, only small amounts of compound are required. The response to intracerebrally injected "E" Pyrogen was not affected unduly by drugs which produce hypothermia, in the limited series of experiments we have carried out.

It is obvious that in any test involving measurement of body temperature, environmental control preceding and during the test is vital if consistent results are to be obtained. This is especially true of this method in which mice are the test animals.

The method is unsuitable for assessing the effectiveness of drugs in an already established pyrexia because the pyretic effect is of short duration. The anti-pyretic test in rabbits which we used for testing paracetamol is better suited for this type of assessment.

The test does not always permit comparisons of the relative potencies of the drugs since some log dose/response curves for the drugs tested deviate from the parallel. This was especially true of indomethacin. Modification of the experimental details may, however, make specific comparisons possible. For instance, we have made no attempt to ensure that the peak activity time of each drug coincided with the maximal pyretic effect of the "E" Pyrogen, the present aim being the development of the method as a screening test. Similarly, we have routinely allowed 45 to 60 min between drug administration and maximal pyretic response, which will not necessarily be the optimal time for peak effect with the different doses and the various drugs tested.

Tests presently available for screening anti-pyretics vary in the species of animal used, in the pyretogenin necessary to induce fever and in the route of administration. Borison (1964) strongly recommends a method in which purified bacterial lipopolysaccharide, derived from *Salmonella typhosa*, is administered to unrestrained conscious cats by intracerebroventricular injection, body temperature being recorded by means of a thermocouple implanted in the retroperitoneal space. He considers that this method fulfils the two requirements of an anti-pyretic test—namely, that body temperature is predictably raised in a fashion akin to that occurring in disease and that the method of recording body temperature does not influence the response to anti-pyretic treatment. The method we have developed fulfils these requirements. Borison's (1964) method of implanting a thermocouple in the retroperitoneal space is undoubtedly preferable to the recording of rectal temperature we have used, but it is impracticable for screening purposes, because of the relatively large animal used, and because of the work involved in preparing the animals for experiment. It is of interest that in an earlier paper, Sheth & Borison (1960) found that whereas tolerance to repeated intravenous injections of pyrogen developed, it did not develop to intracerebroventricular injections. Bacterial pyretogenins have been used by the intraperitoneal route in rats by Winter & Nuss (1963) and intravenously in

rabbits by Winter, Risley & Nuss (1963). In both cases, rectal temperature was recorded and the pyrogen used was a lipopolysaccharide derived from *Escherichia coli*. It is probable that the pyretogenin used by Winter & Nuss (1963) acted indirectly by causing release of endogenous pyrogen, there being a lag period of some 2 hr before pyrexia become apparent. In similar experiments we have found that "E" Pyrogen (Organon) is satisfactory for the testing of anti-pyretic drugs in rabbits and is active in one hundredth of the dose used by Winter *et al.* (1963). We could not, however, produce consistent pyretic effects in rats.

Yeast induced fever, introduced by Smith & Hambourger (1935) is the most widely used screening test, and has been used for assessing the anti-pyretic activity of non-narcotic analgesic drugs by Boxill, Nash & Wheeler (1958), Domenjoz (1960), Winder, Wax, Scotti, Scherrer, Jones & Short (1962), Adams, Cliffe, Lessel & Nicholson (1963), Rosenthal, Markley & Schubert (1963), Winder, Wax, Serrano, Jones & McPhee (1963) and Winder, Wax & Welford (1965). The test involves injecting a suspension of yeast subcutaneously into rats on the afternoon before the test. On the morning of the test, rats which have developed a suitable pyrexia are selected for the experiment, the remainder being discarded. The relationship between yeast induced pyrexia and clinical fever has not been established but yeast probably causes the release of endogenous pyrogen. Zymosan, prepared from yeast cell walls, activates plasma complement and causes increased phagocytic activity of reticulo-endothelial cells (Fizpatrick & DiCarlo, 1964). Atkins, Bodel & Francis (1967) have shown that when heat-killed staphylococci are phagocytosed by monocytes *in vitro*, pyrogen is released. They also state that endotoxins of Gram-negative bacteria release pyrogen from granulocytes *in vitro*, but fail to cause pyrexia in rabbits made leucopenic by administration of nitrogen mustard. They believe that most microbial agents produce fever indirectly by liberating endogenous pyrogen from the tissues of the host. The production of pyrexia by yeast is both delayed and variable, suggesting a similar indirect mechanism.

We consider that "E" Pyrogen injected intracerebrally acts directly, because the response is rapid and of short duration and because there are less than fifty leucocytes per μl. present in the cerebrospinal fluid. Furthermore, these are mainly lymphocytes which according to Hahn, Char, Postel & Wood (1967) do not release pyrogen. Cooper, Cranston & Honour (1967) have found it necessary to use the pyrogen released from at least 2×10^5 peritoneal exudate cells, given intracerebrally, consistently to produce pyrexia in rabbits.

It seems probable that clinical fever may be induced either directly by micro-organism endotoxin or indirectly via release of endogenous leucocyte pyrogen and consequently tests employing either method of inducing pyrexia must be considered relevant to the clinical condition.

We believe that the method described involves the direct action of bacterial pyrogen on the temperature regulating centre in the hypothalamus. The reproducibility of pyrexia is attributed to this direct action, and to the exclusion of cardiovascular effects, likely to be encountered when endotoxins from micro-organisms are injected parenterally.

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