Objective Method for Evaluation of Analgesic/Anti-Inflammatory Activity

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Abstract □ A method for evaluating analgesic and anti-inflammatory activity in mice was developed. The method is based on the reduction in mobility elicited by painful yeast paw edema; restoration of mobility toward normal is used as an estimation of analgesia. Anti-inflammatory activity is evaluated through changes in paw weight. Aspirin and phenylbutazone produced significant analgesic and anti-inflammatory effects. Acetaminophen and codeine had no marked effect on mobility or inflammation. Chlorpheniramine maleate and dextroamphetamine sulfate produced a significant improvement in mobility but had only a modest effect on the inflammatory state, less than that of aspirin or phenylbutazone. Hydrocortisone caused a significant reduction in inflammation but did not greatly improve mobility. These results indicate that compounds with anti-inflammatory action and a centrally acting component are most effective in producing analgesia in this system.

Keyphrases □ Anti-inflammatory/analgesic activity, evaluation—yeast paw edema method, comparison with aspirin □ Analgesic/anti-inflammatory activity, evaluation—yeast paw edema method, comparison with aspirin

A rapid, relatively simple method of evaluating analgesic and anti-inflammatory activity was developed in contrast to the more subjective Randall–Selitto method (1, 2) and the time-consuming adjuvant arthritis procedure (3). The present method is based on the reduction in mobility resulting from painful yeast paw edema; restoration of mobility toward normal and reduction in paw edema serve as indicators of analgesic/anti-inflammatory activity. The testing of different classes of compounds helped to delineate the limits of the system. It proved to be useful as a means of comparing unknowns with standard drugs such as aspirin and phenylbutazone.

EXPERIMENTAL

Albino female mice[1], weighing 20–24 g., were used for all experiments. The animals had access to food and water at all times.

Effect of Yeast-Induced Paw Edema on Mobility—Paw edema was induced by the subcutaneous injection of 0.05 ml. of a 5% yeast suspension[2] into the plantar surface of both hind feet. Control animals were subjected to sham injection with a 27-gauge needle, as was used in the yeast injections. All animals were lightly anesthetized with ether before injection for ease in handling.

At different times after yeast injection, the groups of animals (control and yeast treated) were placed in activity cages[3] which monitor spontaneous locomotor movements. Activity was measured for 20 min. The change in mobility of yeast-treated animals as compared to controls was expressed as the percent decrease in mobility.

Following locomotor measurements, the animals were sacrificed by cervical dislocation and both hind paws were amputated at the tarsocrural joint (4). Each paw was weighed and the percent inflammatory response was calculated from the increase in paw weight in yeast-treated animals as compared to controls.

[1] Manor Farms M1 strain.
[2] Thompson NBC-600 Nutritional Yeast in 0.9% saline.
[3] Woodard Research Corp., Herndon, Va.

Effect of Compounds on Normal Mice—Compounds were first tested on mice that were not subjected to yeast edema induction. Drugs were suspended in 0.25% gum tragacanth and administered orally by means of an 18-gauge feeding tube. Control mice received no treatment. At 30 min. after drug administration, the mobility of both groups of mice was monitored as already described.

Estimation of Analgesic/Anti-Inflammatory Activity—Three groups of five mice were used for each test. The groups were as follows:

Group I	yeast paw inflammation plus drug
Group II	yeast paw inflammation
Group III	control; sham injection

Yeast paw inflammation was induced as previously described. The drugs to be tested for activity were administered orally as described.

At 30 min. after drug administration (2 hr. following yeast or sham injection), the three groups of animals were placed in the activity cages. In one experiment with hydrocortisone, activity was monitored 1 hr. after drug administration rather than after the usual 30-min. period. In all instances, activity was measured for 20 min.; the total number of counts generated by each of the three groups of animals was used for calculation of drug effect.

After activity measurements, the animals were sacrificed and paw amputations were carried out. This entire procedure was repeated at least four times for each dose level of drug being tested.

The drug effect was estimated according to the procedures presented below. In all instances, the activity of the test compounds was compared to that of aspirin, the standard.

Analgesic Effect as Indicated by Changes in Mobility—The counts recorded for each group in each test run were averaged. Activity was determined by the following equation:

$$\frac{Y - T}{Y} \times 10^2 = \% \text{ analgesia} \qquad \text{(Eq. 1)}$$

where Y = (mean counts of Group III − mean counts of Group II), and T = (mean counts of Group III − mean counts of Group I).

Anti-Inflammatory Effect as Indicated by Changes in Paw Weight—Paw weights recorded for each group in each test run were averaged. Activity was calculated as follows:

$$\frac{U - D}{D} \times 10^2 = \% \text{ anti-inflammatory response} \qquad \text{(Eq. 2)}$$

where U = (mean paw weights of Group II − mean paw weights of Group III), and D = (mean paw weights of Group I − mean paw weights of Group III).

The drugs studied were aspirin, phenylbutazone, dextropropoxyphene hydrochloride, acetaminophen, codeine sulfate, hydrocortisone acetate, chlorpheniramine maleate, diphenhydramine hydrochloride, pyrilamine maleate, phenyltoloxamine dihydrogen citrate, chlorpromazine hydrochloride, and dextroamphetamine sulfate.

RESULTS

Prior to drug testing, the effect of yeast inflammation on mobility and paw size was investigated. As the results in Table I show, the 2–4-hr. period after yeast injection provided a marked decrease in mobility and a significant inflammatory response, as indicated by the increase in paw weight.

Before the study with the yeast-treated animals, each compound of interest was tested for its effect on the mobility of normal mice. Doses were selected initially on the basis of the known activity of

Table I—Changes in Mobility and Paw Weight during Development of Yeast-Paw Edema in Mice

Hours after Yeast Injection	Decrease in Mobility, %[a]	Increase in Paw Weight, %
1	19.2 ± 7.1	44.2 ± 2.7
2	44.3 ± 14.6	49.4 ± 5.3
3	59.7 ± 18.4	65.3 ± 9.1
4	67.1 ± 12.4	60.3 ± 8.9

[a] Mean ± *SD* of four determinations.

the compounds as determined by various pharmacological tests [mouse phenylquinone writhing test (5), guinea pig histamine aerosol (6), and mouse hot plate test (7)]. If these initial doses had a marked effect on mobility (increase or decrease), they were modified until minimum changes in activity were achieved. The results are presented in Table II. With the exception of chlorpromazine hydrochloride, none of the compounds had any significant effect at the doses selected, all of which were pharmacologically active. Chlorpromazine hydrochloride did cause a significant depression of mobility. However, since this dose was the lowest (0.25 mg./kg.) that had been found previously to be pharmacologically active [in the mouse hot plate test (7) as determined in this laboratory], it was not reduced further.

Following these experiments, the compounds were tested at the same doses in yeast-treated animals as described in the *Experimental* section. Table III shows the results of these studies. Compounds which possess analgesic *and* anti-inflammatory activity (aspirin and phenylbutazone, for example) appear to be very active in this system. A significant analgesic and anti-inflammatory effect was also produced by dextropropoxyphene hydrochloride. The anti-inflammatory activity of dextropropoxyphene hydrochloride was unexpected, since it was not reported previously.

Compounds which are thought to be "pure" analgesics such as acetaminophen and codeine sulfate had little effect, as evidenced by the fact that changes in mobility (analgesic effect) were small and quite variable; as expected, there was no significant anti-inflammatory activity. Also, the tranquilizer chlorpromazine hydrochloride had no significant effect on mobility or on the inflammatory state.

The experiments with hydrocortisone acetate were of interest since they indicated that anti-inflammatory activity alone did not provide a marked analgesic effect (as measured by restoration of mobility). As can be seen in Table III, hydrocortisone acetate produced a significant anti-inflammatory effect at 30 min. after drug administration but did not markedly affect mobility. In another study, the same dose of hydrocortisone acetate was used, but analgesic and anti-inflammatory activities were monitored 1 hr. after dosing. This experiment was performed to ensure sufficient time for hydrocortisone acetate to exert its effect. In this instance, a slightly better anti-inflammatory response was obtained and there was some restoration of mobility. However, the analgesic effect obtained was not as marked as that reached with either aspirin or phenylbutazone, even though the degree of anti-inflammatory effect was comparable with all three compounds.

Table II—Effect of Test Compounds on Mobility of Normal Mice

Compound	Activity[a] Control	Activity[a] Treated
Aspirin, 150 mg./kg.	3228 ± 28	2914 ± 206
Phenylbutazone, 150 mg./kg.	2519 ± 389	2812 ± 410
Dextropropoxyphene hydrochloride, 25 mg./kg.	3012 ± 159	2671 ± 734
Acetaminophen, 300 mg./kg.	2829 ± 273	2271 ± 738
Codeine sulfate, 10 mg./kg.	3040 ± 159	3096 ± 128
Hydrocortisone acetate, 15 mg./kg.	2867 ± 285	2329 ± 454
Chlorpheniramine maleate, 30 mg./kg.	3025 ± 59	2951 ± 348
Diphenhydramine hydrochloride, 20 mg./kg.	2551 ± 277	2818 ± 119
Pyrilamine maleate, 50 mg./kg.	3115 ± 852	2830 ± 948
Phenyltoloxamine dihydrogen citrate, 15 mg./kg.	2551 ± 277	2504 ± 523
Chlorpromazine hydrochloride, 0.25 mg./kg.	2833 ± 107	2395 ± 157[b]
Dextroamphetamine sulfate, 1.5 mg./kg.	2943 ± 382	2608 ± 259

[a] Mean ± *SD* of three determinations. The three values obtained for determination of the mean were the total number of counts registered by each of three groups of five mice/20-min. period. [b] Significantly different from control at $p = 0.02$ (Student's t test).

Table III—Restoration of Mobility and Reduction of Inflammation during Yeast-Paw Edema

Compound	Percent Analgesia[a]	Percent Anti-Inflammatory[a]
Aspirin, 150 mg./kg.	68.4 ± 7.7[b]	31.6 ± 9.6[b]
Phenylbutazone, 150 mg./kg.	59.1 ± 23.8[b]	33.2 ± 9.8[b]
Dextropropoxyphene hydrochloride, 25 mg./kg.	35.7 ± 23.1[b]	23.5 ± 8.1[b]
Acetaminophen, 300 mg./kg.	8.0 ± 10.3	3.7 ± 8.2
Codeine sulfate, 10 mg./kg.	18.5 ± 15.8[c]	7.5 ± 8.3
Hydrocortisone acetate, 15 mg./kg. (A)[d]	11.3 ± 14.0	21.3 ± 12.2[b]
Hydrocortisone acetate, 15 mg./kg. (B)[d]	18.6 ± 14.6[c]	27.9 ± 17.1[b]
Chlorpheniramine maleate, 30 mg./kg.	93.8 ± 15.6[b]	20.1 ± 11.9[e]
Chlorpromazine hydrochloride, 0.25 mg./kg.	16.5 ± 22.0	4.5 ± 5.4
Dextroamphetamine sulfate, 1.5 mg./kg.	50.3 ± 36.9[b]	19.1 ± 11.9[e]
Control (0.25% tragacanth)	3.6 ± 5.4[f]	5.5 ± 8.0[f]

[a] Mean ± *SD* of six experiments with exception of control values. [b] Significantly different from control at $p = 0.01$. [c] Significantly different from control at $p = 0.05$. [d] Experiment A = 30 min. after drug administration and Experiment B = 1 hr. after drug administration. [e] Significantly different from control at $p = 0.02$. [f] Mean ± *SD* of 15 experiments.

Dextroamphetamine sulfate, a known CNS stimulant stated to have analgesic activity (8), produced a marked analgesic effect in the present system. Dextroamphetamine sulfate also had an anti-inflammatory effect, which is in agreement with previously reported findings that dextroamphetamine sulfate was moderately active against formaldehyde-induced inflammation in the mouse foot (9). The degree of anti-inflammatory activity obtained in the present studies with dextroamphetamine sulfate was not marked and did not coincide with the degree of analgesia obtained.

Chlorpheniramine maleate was chosen as an example of the antihistamines and proved to be extremely active, as can be seen in Table III. The anti-inflammatory effect was modest, but almost 100% restoration of mobility was achieved. This marked response of an antihistamine was somewhat unexpected, since histamine is not thought to be an important mediator of the inflammatory response in the mouse (4, 9, 10). This effect of chlorpheniramine maleate, like that of aspirin, was dose dependent (Figs. 1 and 2). The two dose-response lines were tested for parallelism, and an estimate of relative potency was made according to the method of Litchfield and Wilcoxon (11). Chlorpheniramine maleate was significantly more potent than aspirin; its relative activity was 23 times that of aspirin (relative activity lay between 8.7 and 59.7 times that of aspirin; 95% confidence limits).

Because of the marked activity of chlorpheniramine maleate, several other antihistamines were studied in this system. As shown in Table IV, diphenhydramine hydrochloride, pyrilamine maleate, and phenyltoloxamine dihydrogen citrate (all tested at doses that did not affect normal mobility, Table II) were much less effective than chlorpheniramine maleate, suggesting that chlorpheniramine maleate exerted its effect in this system through other than antihistaminic activity.

DISCUSSION

The method described here detected compounds which have analgesic and/or anti-inflammatory activity, as judged by the results obtained with classical drugs such as aspirin and phenylbuta-

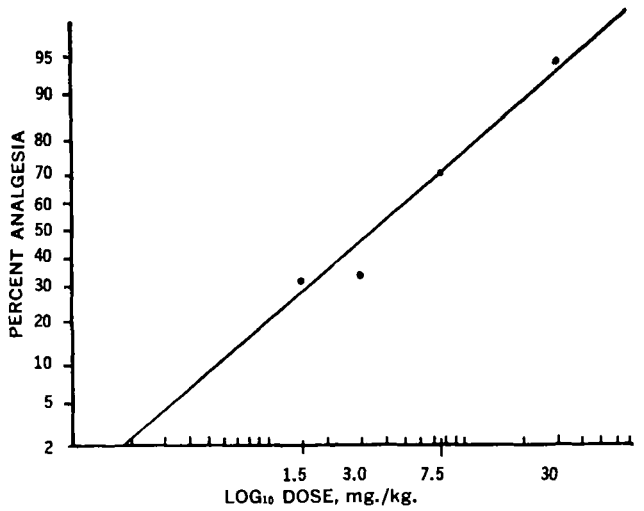

Figure 1—*Analgesic effect of chlorpheniramine maleate. The ED_{50} = 3.6 mg./kg. (1.5–8.6) [determined according to the method of Litchfield and Wilcoxon (11)].*

zone. Aspirin proved to be very effective in this system (ED_{50} = 82 mg./kg.) and was established as the standard against which other drugs are tested in routine screening. Because of its simplicity and the objective nature of the measurements involved, the method proved to be valuable for the rapid detection of analgesic/anti-inflammatory activity.

Experiments with different classes of compounds helped to define those entities most active in the system and also provided some information as to possible mechanisms of drug action. The most obvious point to be made is that anti-inflammatory activity alone did not produce the highest degree of analgesia (as measured by improvement in mobility). The results with hydrocortisone acetate, aspirin, and phenylbutazone led to this conclusion. All three compounds had a comparable anti-inflammatory effect (28–33% reduction in edema), but the analgesic effect of hydrocortisone acetate was much less (19% analgesia) than that of aspirin and phenylbutazone (68 and 59% analgesia, respectively). Yet, the necessity of anti-inflammatory activity was demonstrated by the low and variable percent analgesia and negligible anti-inflammatory effect obtained with the "pure" analgesics, codeine sulfate and acetaminophen, and by the tranquilizer, chlorpromazine hydrochloride. From these data, it seems probable that other parameters were involved in drug action in this system.

Chlorpheniramine maleate demonstrated highly significant analgesia and significant, but not correspondingly, marked anti-inflammatory activity. Since other antihistamines tested were much less effective and since histamine is not an important mediator in the mouse (4, 9, 10), it was thought that the activity of chlorpheniramine maleate was not due to its antihistaminic action. This conclusion was supported by reports that chlorpheniramine maleate and several other antihistamines are active against granuloma pouch edema and formalin-induced inflammation in rats (12, 13). As anti-inflammatory activity did not agree well with antihistaminic activity, the authors (12) of one paper suggested, for the antihistamines, "the possibility of a centrally mediated inhibition" against formalin edema. Similarly, dextroamphetamine sulfate, a known CNS stimulant and reported analgesic (8), was effective in restoring mobility and reducing inflammation.

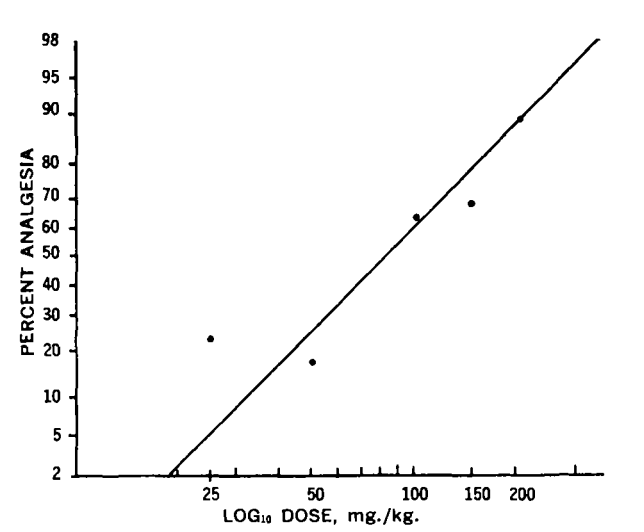

Figure 2—*Analgesic effect of aspirin. The ED_{50} = 82 mg./kg. (54.3–123.8) [determined according to the method of Litchfield and Wilcoxon (11)].*

Table IV—Effect of Antihistamines on Yeast-Paw Edema in Mice

Antihistamine	Percent Analgesia[a]	Percent Anti-Inflammatory[a]
Chlorpheniramine maleate, 30 mg./kg.	93.8 ± 9.7[b]	20.1 ± 11.9[c]
Diphenhydramine hydrochloride, 20 mg./kg.	19.0 ± 23.1	16.1 ± 12.3
Pyrilamine maleate, 50 mg./kg.	20.1 ± 16.1[d]	10.6 ± 10.6
Phenyltoloxamine dihydrogen citrate, 15 mg./kg.	6.9 ± 13.6	7.4 ± 12.5

[a] Mean ± *SD* of six experiments. [b] Significantly different from control at p = 0.01. [c] Significantly different from control at p = 0.02. [d] Significantly different from control at p = 0.05.

Dextropropoxyphene hydrochloride, which is thought to be a "pure" analgesic producing its activity by acting on the CNS (14), also demonstrated significant analgesic and anti-inflammatory activity. The mode of action of this significant anti-inflammatory effect is unclear.

It could be concluded that this method of evaluation of analgesic/anti-inflammatory compounds is based on a complex mechanism involving cellular permeability and perhaps a centrally mediated response, as indicated by the data obtained on the compounds tested. But the degree and extent of influence of these parameters have not yet been fully delineated, and mechanisms of drug action can only be postulated at this time.

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Relationship between MAO Inhibitory and Anticonvulsant Properties of Substituted Cinnamides

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Abstract □ Substituted cinnamides were synthesized to investigate their *in vitro* MAO inhibitory properties and their ability to protect against convulsions produced in mice by subcutaneous injections of pentylenetetrazol. The degree of protection offered by these compounds in no way paralleled their enzyme inhibitory properties.

Keyphrases □ MAO inhibitors, cinnamides—synthesis, correlation to anticonvulsant activity □ Cinnamides, substituted—synthesis, relationship between MAO inhibition and anticonvulsant activity □ Structure–activity relationships—cinnamides, MAO inhibition □ Spectrophotofluorometry—determination of MAO activity, cinnamides □ Manometry (Warburg)—determination of MAO activity, cinnamides

A large group of styrylquinoliniums (1), hydrazine derivatives (2), and semicarbazides (3, 4) are known as MAO [EC 1.4.3.4 monoamine:O_2 oxidoreductase (deaminating)] inhibitors. The high degree of inhibition by styrylquinoliniums was proposed to be related to the presence of the styryl group and, more specifically, to its ethylenic moiety (1). This is of interest because of the structural similarity between the styryl group and the phenethyl group of catecholamines and also in view of the mechanism of inhibition of MAO suggested by Belleau and Moran (5). Furthermore, MAO inhibitors were reported to possess antidepressant (6) and pronounced anticonvulsant properties (7, 8), presumably due to an increase in the concentration of brain amines. Certain derivatives of cinnamic acid reported to exhibit anticonvulsant activity (9, 10) and the ability of oleic acid to inhibit the enzyme MAO (11) led the authors to synthesize some cinnamides as MAO inhibitors. Attempts were made to correlate their MAO inhibitory properties with their anticonvulsant activities as a function of their chemical structure.

CHEMISTRY

The various substituted cinnamides, synthesized by the route outlined in Scheme I, are recorded in Table I.

Hippuric acid (I*a*) was synthesized by the benzoylation of glycine which, on treatment with suitable aromatic aldehydes and acetic anhydride in the presence of anhydrous sodium acetate, was converted into corresponding substituted oxazolones (I*b*). Compounds I*b* failed to react with benzocaine to give I–III in the absence of triethylamine. In the present study, it was found that the addition of two to three drops of triethylamine was sufficient to start the reaction, indicating that the presence of the slightly basic medium is essential to open the oxazolone ring. Corresponding hydrazides (IV–VI) were prepared by refluxing I–III with hydrazine hydrate (99–100%) in absolute ethanol. From these hydrazides (IV–VI), substituted semicarbazides (VII–XVIII) were prepared by refluxing them with suitable arylisocyanates in dry benzene. Substituted thiosemicarbazides (XIX–XXX) were obtained by refluxing a mixture of IV–VI and suitable arylisothiocyanates in ethanol.

EXPERIMENTAL

Melting points were taken in open capillary tube and were corrected.

Hippuric Acid (I*a*)—This was synthesized by the benzoylation of glycine according to a method reported earlier (12).

2-Phenyl-4-(*p*-substituted-benzylidene)oxazole-5-ones (I*b*)—The various oxazolones were prepared by heating a mixture of 0.96 mole of aromatic aldehyde, 1.07 moles of powdered dry hippuric acid, 0.98 mole of powdered freshly fused sodium acetate, and 2.9 moles of high grade acetic anhydride on an electric hot plate. As soon as all of the constituents melted out, the mixture was heated on a steam bath for 2 hr. The mixture was allowed to stand overnight, and the solid mass which separated out was filtered and washed with 100-ml. portions of ice-cold ethanol and finally with 100 ml. of boiling water. The various 2-phenyl-4-(*p*-substituted-benzylidene)-oxazol-5-ones were collected by filtration, dried, and used for subsequent reaction without further purification. The melting points of these compounds were found to correspond with values reported in the literature (13, 14).

α-Benzoylamino-*N*-(*p*-ethylbenzoate)-*p*-substituted-cinnamides (I–III)—These compounds were synthesized by refluxing equimolar portions of appropriate 2-phenyl-4-(*p*-substituted-benzylidene)-oxazole-5-one and benzocaine in absolute ethanol in the presence of two to three drops of triethylamine on a steam bath for 6–8 hr. On distilling the excess of ethanol, the cinnamides which separated out were filtered and recrystallized from suitable solvents. The cinnamides were characterized by their sharp melting points and elemental analyses.

α-Benzoylamino-*N*-(*p*-benzhydrazide)-*p*-substituted-cinnamides (IV–VI)—The various α-benzoylamino-*N*-(*p*-ethylbenzoate)-*p*-substituted-cinnamides were refluxed with 99–100% hydrazine hydrate (1:2 molar ratio) in absolute ethanol on a steam bath for 6–8 hr. On cooling, the solid mass which separated out was filtered and recrystallized from the appropriate solvent.

α-Benzoylamino-*N*-[*p*-(4-substituted-phenylsemicarbazides)-benzoyl]-*p*-substituted-cinnamides (VII–XVIII)—Equimolar portions of the suitable α-benzoylamino-*N*-(*p*-benzhydrazide)-*p*-substituted-cinnamide and arylisocyanate were mixed in dry benzene and re-