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A STUDY OF POSSIBLE MEDIATORS OF INFLAMMATORY REACTIONS IN THE MOUSE FOOT

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A number of compounds have been studied for their ability to antagonize the inflammatory reaction produced by injections of formaldehyde and 5-hydroxytryptamine in the mouse foot. An attempt has been made to elucidate the ways in which certain hydroxybenzoates, pyrazolones, sympathomimetic amines, flavone and flavanone glycosides, local anaesthetics, antihistamines and anti-5-hydroxytryptamine substances produce their anti-inflammatory effect.

Several substances occur in damaged tissues which have the ability to increase capillary permeability, the subject having been reviewed by Spector (1958). The presence of such substances does not prove their participation in the mediation of the symptoms observed, and it is difficult to differentiate between the causes and the effects of the process in many types of inflammation. Since Lewis (1927) demonstrated that part of the response of tissues to trauma is mediated by an axon-reflex there has been little work done to discover to what extent this process operates in different types of inflammation. Chambers & Zweifach (1947) have emphasized that little is known about the effect which local changes in blood flow have on the overall inflammatory reaction, and so we have attempted, in the experiments described here, to analyse the anti-inflammatory action of certain drugs which are known to alter blood flow in the skin.

METHODS

Formaldehyde-induced inflammation in the mouse foot. The details of the method have been described by Northover & Subramanian (1961a). Adult mice of either sex weighing between 25 and 35 g were used in groups of 6 animals. The animals were pretreated with the test substance, given subcutaneously, one injection 24 hr and another 30 min before the injection of formaldehyde. Where variations of this procedure were used they are indicated in the text. All premedicant injections were made in a dose-volume of 0.1 ml., and all compounds were given either as freshly prepared solutions or suspensions, adjusted, where required, to *p*H 7 with either sodium hydroxide or hydrochloric acid. Each mouse received 0.1 ml. of an aqueous solution containing 0.5% azovan blue and 0.9% sodium chloride through a lateral tail vein, and then immediately afterwards 0.05 ml. of an aqueous solution containing 4% formaldehyde and 0.9% sodium chloride subcutaneously in the dorsum of the right hind foot. The left hind foot received the same volume of saline alone and acted as a control for the formaldehyde-treated foot. The blue-dye accumulation and the swelling in the test foot compared with the control foot were given subjectively assigned scores of from 0 to 3 by

two observers, and, unless otherwise stated in the text, these scores were given 60 min after the formaldehyde was injected. The mean response of each group was expressed as a % of the response of the control group, which was pretreated with saline in place of the test drug.

5-Hydroxtryptamine-induced inflammation in the mouse foot. These experiments were performed in a similar way to those described above, using a solution containing 0.01% 5-hydroxytryptamine creatinine sulphate and 0.9% sodium chloride in place of the formaldehyde solution. The inflammatory response was recorded 30 min after the injection of 5-hydroxytryptamine.

RESULTS

Hydroxybenzoates and related compounds. Numerous reports have been published on the anti-inflammatory action of salicylates, and the subject was reviewed by Smith (1953) and Smith (1960). Previously we have found (Northover & Subramanian, 1961a) that formaldehyde-induced inflammation in the mouse foot is reduced by both acetylsalicylic acid and sodium salicylate, and it seemed of interest to investigate the effect of other hydroxybenzoates. The results of these experiments are given in Table 1.

TABLE 1

HYDROXYBENZOATES AND RELATED COMPOUNDS AS INHIBITORS OF FORMALDEHYDE-INDUCED INFLAMMATION

* Denotes that the difference between the response of a group and that of the control group is statistically significant ($P<0.05$)

	Dose mg/kg	Mean inflammatory response as a % of the control: Blue colour	Mean inflammatory response as a % of the control: Swelling
Normal saline	—	100	100
Salicylic acid	160	75*	72*
Acetylsalicylic acid	160	59*	52*
Diphenylacetylsalicylic acid	160	100	86
2,5-Dihydroxybenzoic acid (gentisic acid)	160	79	80
2,6-Dihydroxybenzoic acid (γ-resorcylic acid)	320	81	111
3,5-Dihydroxybenzoic acid	160	89	111
3,4-Dihydroxybenzoic acid (protocatechuic acid)	160	54*	69*
3,4-Dihydroxycinnamic acid (caffeic acid)	160	58*	64*
β-(3,4-Dihydroxyphenyl)propionic acid (hydrocaffeic acid)	160	88	83
4-Hydroxy-3-methoxybenzoic acid (vanillic acid)	160	80	83
3,4-Dimethoxybenzoic acid (veratric acid)	160	92	95

Diphenylacetylsalicylic acid has been reported by Weaver, Richards & Martin (1961) to have a greater analgesic action than acetylsalicylic acid in the mouse, but it was inactive in our test. Several dihydroxybenzoic acids were tested, the 2,5 and 3,5 derivatives being virtually inactive, whereas 3,4-dihydroxybenzoic acid was moderately active. The 2,6 derivative was inactive, and this agrees with the reported inability of this compound to affect ultra-violet radiation-induced inflammation in guinea-pig skin (Adams & Cobb, 1958).

It is interesting to compare the activity of 3,4-dihydroxybenzoic acid and 3,4-dihydroxycinnamic acid with the inactivity of β-(3,4-dihydroxyphenyl)propionic acid. We conclude that for activity the carboxyl group needs to be either attached to the benzene ring or conjugated with it. 4-Hydroxy-3-methoxybenzoic acid was inactive, which indicates that the activity of 3,4-dihydroxybenzoic acid depends upon the presence of both hydroxyl groups. Not unexpectedly, therefore, 3,4-dimethoxybenzoic acid was inactive.

All compounds which antagonized formaldehyde-induced inflammation were also tested against 5-hydroxytryptamine-induced inflammation in the mouse foot. In the doses which were active against formaldehyde none of these compounds showed any activity against 5-hydroxytryptamine. Keleman (1957) found that salicylate in doses of 600 mg/kg reduces 5-hydroxytryptamine-induced inflammation in the rat foot, but the doses which he used were considerably greater than those used by us.

Pyrazolones. Phenylbutazone is still considered the most potent anti-inflammatory agent among the analgesic–antipyretic drugs at present in use. It has a number of serious side-effects which have stimulated the search for equally effective but less toxic substances. The metabolites of phenylbutazone have been tested for anti-inflammatory action, and of these oxyphenbutazone is the most thoroughly investigated (Burns, Yu, Dayton, Gutman & Brodie, 1960). Domenjoz (1960) has reported that oxyphenbutazone inhibits formaldehyde-induced inflammation in the rat foot and is inactive against ultra-violet radiation-induced inflammation in guinea-pig skin. The results of our experiments are given in Table 2, where it can be

TABLE 2

PYRAZOLONES AS INHIBITORS OF FORMALDEHYDE-INDUCED INFLAMMATION

For meaning of asterisks see Table 1

	Dose mg/kg	Mean inflammatory response as a % of the control	
		Blue colour	Swelling
Normal saline	—	100	100
Phenylbutazone	80	25*	17*
Oxyphenbutazone	80	106	121
Aminopyrine	160	60*	63*
Phenazone	160	104	87

seen that oxyphenbutazone is inactive against formaldehyde-induced inflammation in the mouse foot when administered in a dose of 80 mg/kg. This is in sharp contrast to phenylbutazone, which in the same dose produced an almost complete inhibition. Phenazone was also inactive. None of these compounds was active in the doses quoted in Table 2 against 5-hydroxytryptamine-induced inflammation.

Flavone and flavanone glycosides. These were administered intraperitoneally, as this route offers the maximum chance of absorption for non-irritant water-insoluble compounds. The results are presented in Table 3. Of the 4 compounds tested, only rutin was inactive. Since rutin possesses typical vitamin-P-like properties which are in every respect comparable with those of hesperidin and other

TABLE 3

FLAVONES AND FLAVANONES AS INHIBITORS OF FORMALDEHYDE-INDUCED INFLAMMATION

	Dose mg/kg	Mean inflammatory response as a % of the control: Blue colour	Swelling
Normal saline	—	100	100
Rutin	100	100	100
Hesperidin	100	62*	35*
Neohesperidin	100	41*	42*
Naringin	100	75*	53*

active compounds, to which it is closely related chemically (Levitan, 1949), it was difficult to explain the inactivity of rutin, other than by assuming that it was not absorbed in adequate amounts under the conditions of the experiment. We have no evidence yet to indicate whether the inactivity of rutin is due to lack of absorption or whether, even if it is absorbed, it is inactive.

None of the flavone and flavanone glycosides was active against 5-hydroxytryptamine-induced inflammation.

Local anaesthetics. There are three ways in which local anaesthetics might be expected to reduce formaldehyde-induced inflammation. Firstly, because of their ability to prevent certain actions of 5-hydroxytryptamine, as demonstrated by Sinha & West (1953), but the effect may not be solely dependent upon local anaesthetic properties. Since 5-hydroxytryptamine may be involved in the formaldehyde-induced reaction in the mouse foot, the formaldehyde was given with a local anaesthetic to see if the reaction was reduced. Secondly, local anaesthetics inhibit mono-amine oxidase (Philpot, 1940), and it has been shown by Spector & Willoughby (1960) that mono-amine oxidase inhibitors reduce turpentine-induced pleurisy in rats, so it is possible that local anaesthetics might inhibit inflammation by this mechanism also. Thirdly, it is not certain to what extent the "flare" reaction described by Lewis (1927) operates in the reaction of the mouse foot to formaldehyde, nor the extent to which the flare would contribute to the symptoms of inflammation in the foot.

TABLE 4

LOCAL ANAESTHETICS AS ANTAGONISTS OF FORMALDEHYDE-INDUCED AND 5-HYDROXYTRYPTAMINE-INDUCED INFLAMMATION

The local anaesthetics were injected with the formaldehyde or 5-hydroxytryptamine in a volume of 0·05 ml. at a final concentration which is indicated below

	Concentration in mg/ml.	Mean inflammatory response as a % of the control: Formaldehyde: Blue colour	Formaldehyde: Swelling	5-Hydroxytryptamine: Blue colour	5-Hydroxytryptamine: Swelling
Normal saline	—	100	100	100	100
Procaine hydrochloride	10	46*	40*	61*	43*
Amethocaine hydrochloride	2	49*	39*	102	73*
Cinchocaine hydrochloride	1	71*	47*	110	64*

We have tested the ability of three local anaesthetics to prevent formaldehyde-induced and 5-hydroxytryptamine-induced inflammation in the mouse foot, and the results are given in Table 4. The local anaesthetics were dissolved in the solution of formaldehyde or 5-hydroxytryptamine, and the values for the concentration of local anaesthetic which are quoted in Table 4 represent the final concentration in the solution injected. All three local anaesthetics reduced the response to both formaldehyde and 5-hydroxytryptamine, but the response to formaldehyde was reduced more than the response to 5-hydroxytryptamine, which suggests that local anaesthetics are not anti-inflammatory because of their antagonism to 5-hydroxytryptamine but rather by some other mechanism, which is probably related to their ability to prevent the " flare " reaction.

TABLE 5

SYMPATHOMIMETIC AMINES AS ANTAGONISTS OF FORMALDEHYDE-INDUCED INFLAMMATION

* Denotes that the difference between the response of a group and that of the control group is statistically significant ($P<0.05$). † Denotes that the difference between the response of a group treated with a sympathomimetic amine alone and that of the group treated with the same sympathomimetic amine with an antagonist is statistically significant ($P<0.05$). Doses of sympathomimetic amines are calculated as free base. A signifies 10 mg/kg phenoxybenzamine hydrochloride, and B signifies 4 mg/kg dichloroisoprenaline hydrochloride. The antagonists were administered by the subcutaneous route 25 min before the injection of formaldehyde and azovan blue, whilst the sympathomimetic amines were administered subcutaneously in the opposite hind limb 15 min before the injection of formaldehyde and azovan blue

	Dose mg/kg	Antagonist	Mean inflammatory response as a % of the control: Blue colour	Swelling
Normal saline	—	—	100	100
—	—	A	89	93
—	—	B	100	114
(−)-Noradrenaline	0·4	—	28*	46*
(−)-Noradrenaline	0·4	A	74†	62*†
(−)-Noradrenaline	0·4	B	30*	35*
(±)-Isoprenaline	0·4	—	45*	47*
(±)-Isoprenaline	0·4	A	30*	45*
(±)-Isoprenaline	0·4	B	79†	92†
(−)-Phenylephrine	4·0	—	55*	64*
(−)-Phenylephrine	4·0	A	62*	40*
(−)-Phenylephrine	4·0	B	46*	61*
(−)-Noradrenaline + (±)-Isoprenaline	0·4 + 0·4	—	28*	31*
(±)-Amphetamine	20·0	—	60*	38*
Tyramine	4·0	—	97	101

Sympathomimetic amines. In Table 5 are presented the results of some experiments to elucidate the mechanism by which sympathomimetic amines produce an anti-inflammatory action. Noradrenaline and isoprenaline were highly active, phenylephrine and amphetamine were moderately active, whereas tyramine was inactive. Phenoxybenzamine block α-receptors (Ahlquist, 1948), whereas dichloroisoprenaline blocks β-receptors (Powell & Slater, 1958). Phenoxybenzamine reduced the effect obtained with noradrenaline, whereas the responses to isoprenaline and

phenylephrine were unchanged. Similarly, dichloroisoprenaline reduced the inhibition obtained with isoprenaline but did not influence the inhibition with noradrenaline and phenylephrine. It might be expected from these results that stimulation of both α- and β-receptors with a mixture of isoprenaline and noradrenaline would produce a greater effect than either amine alone. There is tentative support for this from the results of one experiment in which isoprenaline and noradrenaline were given together, where the effect was slightly greater in reducing the swelling than that seen with either compound alone.

Substances which modify the action of histamine. Both histamine and 5-hydroxytryptamine occur in the skin of the mouse, and both have the ability to increase capillary permeability under some circumstances (Spector, 1958). It was of interest to see what effect the depletion of skin histamine with polymyxin B sulphate (Bushby & Green, 1955 ; Parratt & West, 1957) would have upon the reaction of the mouse foot to formaldehyde. If histamine is involved in the production of the symptoms of inflammation, then the depletion of the stores of skin histamine would be expected to reduce the response. From Table 6 it can be seen that the pre-

TABLE 6

SUBSTANCES WHICH ALTER THE HISTAMINE CONTENT OF THE TISSUES OR MODIFY ITS ACTION, AS INHIBITORS OF FORMALDEHYDE-INDUCED INFLAMMATION

No group showed a response which was significantly different ($P<0{\cdot}05$) from the control group. Polymyxin B sulphate was administered intraperitoneally, and contained 6,970 units per mg

Name of drug	Daily dose in mg/kg	No. of days of treatment	Mean inflammatory response as a % of the control	
			Blue colour	Swelling
Normal saline	—	—	100	100
Mepyramine maleate	4·0	2	80	85
Polymyxin B sulphate	4·0	3	115	92
Aminoguanidine bicarbonate	20·0	2	93	95
L-Thyroxine sodium	1·0	5	94	79

treatment of the mouse with polymyxin B sulphate had no effect on the response to formaldehyde, indicating that histamine is not an important mediator of the response. This conclusion is supported by the observation that the antihistamine compound, mepyramine maleate, was not active in reducing the response to formaldehyde, neither was a histaminase inhibitor, aminoguanidine bicarbonate, nor thyroxine, which has been shown to increase the sensitivity of the tissues to histamine (Spencer & West, 1961).

Substances which modify the action of 5-hydroxytryptamine. In Table 7 are presented the results obtained with a number of substances which in one way or another interfere with 5-hydroxytryptamine metabolism. Reserpine depletes the skin of much of its stores of 5-hydroxytryptamine (Parratt & West, 1957), and we found that reserpine was capable of reducing the response to formaldehyde very considerably. Reserpine has a number of other actions in the body, notably its ability to deplete the tissues of their stores of catechol amines (Burn & Rand, 1958), and so the evidence obtained from depletion studies with reserpine must

be treated with some reserve, but it suggests that 5-hydroxytryptamine may play some part in the response of the mouse foot to formaldehyde. This suggestion is supported by the fact that several antagonists of 5-hydroxytryptamine (shown in Table 7) were moderately active antagonists of formaldehyde-induced inflammation.

TABLE 7

SUBSTANCES WHICH ALTER THE 5-HYDROXYTRYPTAMINE CONTENT OF THE TISSUES OR MODIFY ITS ACTION, AS INHIBITORS OF FORMALDEHYDE-INDUCED AND 5-HYDROXYTRYPTAMINE-INDUCED INFLAMMATION

* Denotes that the response of a group shows a statistically significant difference ($P<0.05$) from the control group. Reserpine, as a stabilized aqueous solution, was administered intraperitoneally

	Dose mg/kg	No. of days of treatment	Mean inflammatory response as a % of the control: Formaldehyde Blue colour	Formaldehyde Swelling	5-Hydroxytryptamine Blue colour	5-Hydroxytryptamine Swelling
Normal saline	—	—	100	100	100	100
Reserpine	0·4	10	66*	24*	—	—
Cyproheptadine hydrochloride	0·2	2	72*	76*	—	—
	0·4	2	66*	68*	—	—
	0·8	2	56*	62*	13*	19*
Yohimbine hydrochloride	4·0	2	50*	65*	27*	49*
Phentolamine methane sulphonate	8·0	2	50*	63*	13*	19*
Thenaldine tartrate	40·0	2	62*	62*	40*	46*

Cyproheptadine is a potent inhibitor of 5-hydroxytryptamine (Stone, Wenger, Ludden, Stavorski & Ross, 1961), and the other antagonists have been reviewed by Gyermek (1961). It should be noted that for all these compounds the inhibition of the inflammatory response to injected 5-hydroxytryptamine was more complete than with formaldehyde-induced inflammation. It is not unusual, however, to find that the ability of an antagonist to prevent the action of injected agonist is greater than its ability to prevent the action of the agonist liberated endogenously. The evidence seems to indicate that in the mouse foot the injection of formaldehyde leads to the release of 5-hydroxytryptamine which is partly responsible for the capillary hyperpermeability.

DISCUSSION

It is proposed that formaldehyde-induced inflammation in the mouse foot may be conveniently divided into two parts, the first involving 5-hydroxytryptamine as mediator and the second some mediator which is unrelated to 5-hydroxytryptamine. The portion of the total response which is due to the release of 5-hydroxytryptamine can be prevented by either depleting the skin of 5-hydroxytryptamine or by giving the mouse an antagonist of 5-hydroxytryptamine. It also seems probable that the portion of the total response which is due to the " second mediator " can be prevented by treatment with certain analgesic–antipyretic drugs. An explanation of the mechanism of action of a substance against formaldehyde-induced inflammation in the mouse foot is made much easier in those cases where the substance is inactive against 5-hydroxytryptamine-induced inflammation. Under these circumstances it follows that the substance acts on a mediator other than 5-hydroxytryptamine. Thus, in the doses used, the hydroxybenzoates, the pyrazolones, the flavone and

flavanone glycosides were inactive against 5-hydroxytryptamine-induced inflammation, and hence they produce their action against an irritant-induced inflammation by inactivating the second factor.

Another possibility is that an anti-inflammatory agent might operate by releasing or activating some endogenous factor which is anti-inflammatory. It is well known that the salicylates, for example, release both adrenal cortical (Smith, 1953) and adrenal medullary hormones (Smith, 1955), and although the adrenal cortical hormones are inactive against formaldehyde-induced inflammation in the mouse foot (Northover & Subramanian, 1961a) the adrenal medullary amines have been shown in the present experiments to be active.

We do not know by what mechanism the flavanone glycosides are anti-inflammatory. One possibility is that they are metabolized in the body to hydroxybenzoates (Masri, Murray & De Eds, 1960), and it may be that these latter compounds are the active substances.

The analgesic–antipyretic drugs afford the most information concerning their mode of action and hence the nature of the second mediator. One possibility that we have considered is that these compounds inhibit the activity of a plasma kinin-forming enzyme system or act as antagonists of plasma kinin. Collier, Holgate, Schachter & Shorley (1960) have reported that salicylates inhibit the bronchoconstrictor action of bradykinin, and we have shown (Northover & Subramanian, 1961b) that salicylates and other analgesic–antipyretic drugs inhibit plasma kallikrein, which is a plasma kinin-forming enzyme. There are two reasons for thinking, however, that plasma kinin is not an important mediator in the reaction of the mouse foot to formaldehyde. Firstly, the mouse resembles the rat in that its capillaries are extremely insensitive to the permeability-increasing effects of plasma kinin (Miles & Wilhelm, 1960); and, secondly, 2,6-dihydroxybenzoic acid, which is a potent inhibitor of plasma kallikrein (Northover & Subramanian, 1961b), was not active against formaldehyde-induced inflammation in the mouse foot.

Ungar, Damgaard and Hummel (1952) have suggested that the analgesic–antipyretic drugs are anti-inflammatory because they inhibit another plasma kinin-forming enzyme system, namely, fibrinolysin. Quite apart from the fact that plasma kinin has such a weak inflammatory action in the mouse skin, it seems unlikely that this explanation fits all the facts, at any rate in so far as the mouse skin is concerned. Acetylsalicylic acid is inactive *in vitro* against fibrinolysin, whereas salicylic acid is active, and in order to explain the undoubted activity of acetylsalicylic acid as an anti-inflammatory agent these authors proposed that acetylsalicylic acid is active *in vivo* because it is hydrolysed to salicylic acid. It is difficult to account for the greater potency, on a molar basis, of acetylsalicylic acid compared with salicylic acid, as has been shown not only in our experiments (Table 1) but also in those of Adams & Cobb (1958), if the more potent compound is metabolized into the less active one. It is possible that differences in the rate of penetration or of protein-binding might explain this discrepancy.

In view of the evidence at present at our disposal we think the most satisfactory explanation of the anti-inflammatory activity of analgesic–antipyretic drugs, and of the nature of the second mediator, is in terms of the plasma globulin permeability-

enhancing enzymes which have been extensively studied by Miles & Wilhelm (1960) and which are known to be inhibited by salicylates (Spector & Willoughby, 1959). These enzymes behave as though they are esterases, and are inhibited *in vitro* by dyflos, and we know from previous work (Northover & Subramanian, 1961a) that this compound is one of the most active antagonists of formaldehyde-induced inflammation in the mouse foot. Further work is in progress to correlate anti-inflammatory activity with activity against these enzymes.

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