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BRADYKININ AND OEDEMA FORMATION IN HEATED PAWS OF RATS

BY

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Histamine was considered to be the chief mediator in inflammatory reactions until it was shown that specific antihistamine drugs did not always inhibit these reactions. The idea of the polypeptide nature of some of the unidentified permeability factors in inflammatory exudates was introduced by Menkin (1936). Armstrong, Jepson, Keele & Stewart (1957) noted the similarity of the actions of these factors with those of bradykinin, a nonapeptide. This compound or others related to it have since been found in many oedema fluids and were detected in the perfusates of rat paws after the application of heat (Rocha e Silva & Antonio, 1960).

It was considered important to investigate in more detail the extent to which kinins participate in this so-called thermic oedema by following changes in four kinin parameters in the perfusates. As rats which are resistant to the anaphylactoid reaction produced by dextran (the so-called non-reactors) also showed greater resistance to thermic oedema than did the reactors giving the anaphylactoid reaction (Gecse, Karady, Starr & West, 1965), a comparison of the activity of the kinin systems in the perfusates collected from the heated paws of both types of rat was also made. In addition, a study of the effects of drugs on both the development of thermic oedema and the actions of bradykinin has been included.

METHODS

Adult Wistar albino rats were obtained from Fison's Ltd. (Holmes Chapel) and from the Agricultural Research Council's Field Station at Compton. They were tested for their reactivity to dextran (240 mg/kg, intraperitoneally) on three occasions, oedema developing in the extremities over a 2-hr period only in reactor rats (Harris & West, 1961). Both reactor and non-reactor rats were anaesthetized with pentobarbitone sodium (45 mg/kg, intraperitoneally) and their hind paws were perfused according to the method of Rocha e Silva & Antonio (1960). Before collecting the perfusates, the paw space was washed free of blood. The flow of perfusing fluid (Tyrode solution) was regulated to 5–10 drops/min, and then the paw was immersed in a water-bath maintained at the desired temperature for 30 min. Perfusates were collected for three consecutive periods of 10 min during the heating and for further periods after the heating. Samples were also collected from unheated paws to serve as controls.

In other experiments, paws were heated without perfusion after the rats had been anaesthetized and given azovan blue dye intravenously (18 mg/kg, Bonaccorsi & West, 1963). The paws were then examined for the presence of oedema using the plethysmographic method of Buttle, D'Arcy,

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Howard & Kellett (1957), and for blueing and petechial formation (both being measured visually on an arbitary scale from 0 to + + +). Other rats were pretreated with inhibitory drugs at various times before heating (usually at 46.5° C); the inhibitors were injected either subcutaneously into the paw which was to be heated or intraperitoneally.

Assay and characterization of kinins

The methods have been described in detail elsewhere (Dawson, Starr & West, 1966). The free kinin content in the perfusates was determined by direct addition to the isolated test organs, contractions of the rat uterus and guinea-pig ileum and relaxation of the rat duodenum being compared with those produced by different doses of synthetic bradykinin. Further characterization of the kinin-like nature of the activity in the perfusates was indicated by incubating them with chymotrypsin (which destroys bradykinin) or with trypsin (which does not).

Kininogen content of perfusates

The kininogen concentrations were measured by the method of Diniz, Carvalho, Ryan & Rocha e Silva (1961), as modified by Dawson, Starr & West (1966). Samples of 0.5 ml. perfusate were incubated with trypsin under standard conditions and the bradykinin formed was assayed.

Kinin-forming enzyme activity of perfusates

A modification of the method of Amundsen, Nustad & Waaler (1963) was used. Samples of 1 ml. perfusate were added to polythene tubes containing 1 ml. rat plasma substrate which had been previously heated at 61° C for 60 min (Eisen, 1963). Then phenanthroline (100 μg) was added to inhibit specifically the kininase. After incubation at 37° C for 30 min, the reaction was stopped by placing the tubes in ice, and the bradykinin in the incubate was assayed. Kinin-forming activity of the original perfusate has been expressed as the amount of bradykinin formed by the whole of the 10-min sample.

Kininase activity of perfusates

The method used was similar to that of Edery & Lewis (1962) for blood plasma, except that samples of 1 ml. perfusate were incubated with 0.5 μg synthetic bradykinin in 3 ml. 0.9% saline at 37° C.

Histamine and 5-hydroxytryptamine contents of perfusates

Longitudinal muscle strips from guinea-pig ileum and rat fundus were used for assaying histamine and 5-hydroxytryptamine respectively. Each muscle was suspended in aerated Tyrode solution containing atropine (10^{-6} g/ml.) at 30° C, the ileum in the presence of 2-bromolysergic acid diethylamide (10^{-7} g/ml.) and the fundus with mepyramine (10^{-7} g/ml.). All values of the amines are expressed in terms of the base.

Protein content of perfusates

This was measured by determining their absorption in the ultra-violet light at 280 mμ using a Uvispek spectrophotometer.

Intradermal injections

The abdominal areas of both reactor and non-reactor rats were depilated with electric clippers 24 hr before the animals were injected intravenously with azovan blue dye (18 mg/kg). Fifteen minutes later, intradermal injections of bradykinin (0.1 and 1.0 μg), histamine (1 and 10 μg) and 5-hydroxytryptamine (0.1 and 1.0 μg) were made, six injections of 0.1 ml. per rat. The animals were killed 30 min later and the skin over the injection sites was carefully removed and pinned, skin downwards, on to a cork board. The two diameters of each lesion, taken at right angles to each other, were measured and the mean value was calculated. Antagonistic drugs were injected either intraperitoneally 30 min before the intradermal injections of the agonists or intradermally together with the solutions of the agonists.

RESULTS

Oedema formation in heated rat paws

When paws were immersed at 43.5° C for 30 min, slight oedema (with a mean increase in paw volume of about 10%) developed in reactor rats but not in non-reactors; the oedema was accompanied by marked blueing of the paws (a + + reaction). At 45° C, the extent of oedema formation and blueing increased in reactors, while petechiae in the paw and marked salivation also developed. At 46.5° C, oedema in reactors reached a + + + reaction (indicating an increase in paw volume of about 50%), together with intense blueing, marked petechial formation and extensive salivation; at this temperature the paws of non-reactors showed only slight oedema, some blueing and a few petechiae, but salivation did not develop. These results are shown in Table 1. It is apparent that the paws of non-reactor rats have a raised threshold to the heat stimulus. Salivation, which occurred only in reactor rats and was marked at 45° and 46.5° C, ceased as soon as the paw was removed from the heat stimulus, whereas oedema continued to increase for about the next 20 min.

TABLE 1

CHANGES OCCURRING IN REACTOR (R) AND NON-REACTOR (NR) RATS WHEN THE PAWS ARE HEATED AT DIFFERENT TEMPERATURES FOR 30 MIN

Reactions recorded on a relative scale from 0 to + + +

Temperature of water in which paw is immersed (° C)	Intensity of paw oedema		Intensity of paw blueing		Number of petechiae		Degree of salivation	
	R	NR	R	NR	R	NR	R	NR
43·5	+	0	+ +	Trace	0	0	0	0
45·0	+ +	Trace	+ + +	Trace	+	0	+ +	0
46·5	+ + +	+	+ + +	+	+ +	+	+ + +	0

Bradykinin release from heated rat paws

Bradykinin release from the paws of reactor rats increased as the temperature at which the paws were heated was raised above 43.5° C, the temperature at which oedema was first recorded (see Fig. 1). The highest amounts were usually detected during the second 10-min collection period, although the kinin output was usually well maintained throughout (Table 2). The increase in kinin in the perfusates (up to 5-fold at 46.5° C) paralleled

TABLE 2

CHANGES OCCURRING IN REACTOR (R) AND NON-REACTOR (NR) RATS WHEN THE PAWS ARE HEATED AT 46·5° C for 30 MIN AND THEN LEFT AT ROOM TEMPERATURE FOR 60 MIN

Reactions recorded on a relative scale from 0 to + + +. Compare the development of the heat reaction (increase in paw volume) with the output of bradykinin in the paw perfusates

Time of collection (min)	Mean increase in paw volume (%)		Intensity of blueing		Number of petechiae		Degree of salivation		Mean kinin release (ng)	
	R	NR	R	NR	R	NR	R	NR	R	NR
0–10	0	0	0	0	0	0	0	0	4·6	4·1
10–20	28·0	1·3	+	0	+	0	+ +	0	22·6	7·3
20–30	45·6	8·8	+ +	0	+ +	0	+ + +	0	18·4	4·2
30–40	68·3	14·7	+ + +	+	+ +	+	+ + +	0	17·6	3·1
40–50	71·5	13·6	+ + +	+	+ +	+	0	0	15·4	3·9
80–90	69·4	13·7	+ + +	+	+ +	+	0	0	—	—

the increase in oedema volume, the extent of blueing and petechial formation, and salivation, although the peak of kinin release mostly occurred at an earlier time and often during the heating period (30 min). In contrast, the absence of gross oedema in the non-reactor rats was reflected in the low kinin content of the perfusates from the paws of these animals (Table 2), which only occasionally exceeded the kinin content of the perfusates from unheated paws of reactor animals. In all cases, the increases in the protein levels of the perfusates closely followed those of bradykinin. The release of kininogen and of kinin-forming enzyme in reactor rats followed a similar pattern to that of bradykinin, though the increase was relatively less (Fig. 1); the output of kininase was not affected by the heating procedure.

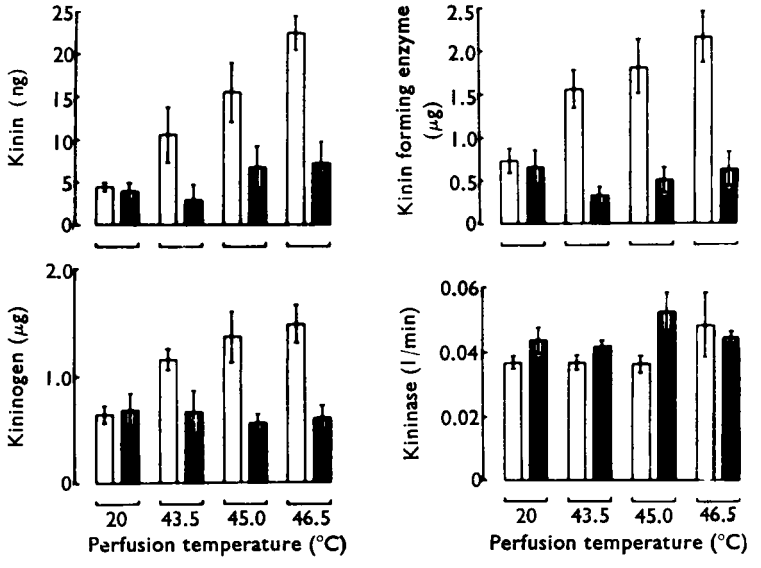

Fig. 1. Effect of subjecting paws of reactor (white column) and non-reactor (black column) rats to different temperatures on the kinin (top, left), kininogen (bottom, left), kinin-forming enzyme (top, right), and kininase (bottom, right) contents of the paw perfusates. The values (±S.E.) obtained during the second 10-min collection period only are shown. Note that non-reactor rats respond feebly to the heat stimulus.

Release of histamine and 5-hydroxytryptamine from heated rat paws

The output of histamine from heat perfused paws of reactor rats remained unchanged (about 20 ng/10 min), even at 46.5° C for 30 min, while that of 5-hydroxytryptamine (10±2 ng/10 min) showed a small but insignificant increase. These findings are in agreement with those of Rocha e Silva & Antonio (1960). No changes occurred in non-reactor rats, and thus histamine and 5-hydroxytryptamine are probably not involved in the reaction of the paw to heat treatment at temperatures up to 46.5° C. However, when heating was carried out at 60° C for 10 min, bradykinin output was reduced in reactor rats almost to control levels but histamine release increased more than 10-fold and 5-hydroxytryptamine release was doubled. Thus, at high temperatures, histamine and 5-hydroxytryptamine probably play more important roles than bradykinin in the heat

reaction. It has already been reported that, in man, histamine and pharmacologically active peptides are released into the circulation as a result of thermal injury (Goodwin, Jones, Richards & Kohn, 1963).

Inhibition of thermic oedema

The effect of intraperitoneal injections of various antagonists on some of the parameters of the heat reaction at 46.5° C in the paws of reactor rats is shown in Table 3. The most effective agents in the doses used were sodium flufenamate, sodium phenylbutazone, calcium acetylsalicylate and dibenzyline. It was usually more difficult to inhibit blueing in the paws than oedema ; for example, pretreatment with dibenzyline or sodium meclofenamate inhibited the oedema by over 60% but had no effect on the intensity of blueing. This difference in the processes of oedema formation and of blueing has been noted previously (Gözsy & Kátó, 1957 ; Wilhelm, 1962). In all cases where the oedema was prevented during the heating period, none developed during the post-heating period, indicating a full inhibition of the response.

TABLE 3

EFFECT OF INTRAPERITONEAL INJECTIONS OF ANTAGONISTS ON THE OEDEMA REACTION PRODUCED BY HEATING PAWS OF REACTOR RATS FOR 30 MIN AT 46·5°C

Inhibitions recorded as percentages of the oedema (paw volume) developing without antagonist. Number of petechiae and intensity of blueing recorded on a relative scale from 0 to +++

Antagonist	Dose (mg/kg)	Inhibition of oedema (%)	Number of petechiae in paw	Intensity of blueing in paw
Sodium flufenamate	100	86	0	++
Sodium mefenamate	100	44	+	++
Sodium meclofenamate	100	65	++	+++
Sodium phenylbutazone	200	83	0	+
Calcium acetylsalicylate	500	88	+	+
Dibenzyline	20	73	+	+++

Specific antagonists of acetylcholine, histamine and 5-hydroxytryptamine such as atropine (200 mg/kg), mepyramine (50 mg/kg) and 2-bromolysergic acid diethylamide (10 mg/kg) respectively, as well as metabolic inhibitors of the dextran anaphylactoid reaction such as glucose (3 g/kg) or 2-deoxyglucose (500 mg/kg), had no effect on the thermic oedema. These results support the view that histamine and 5-hydroxytryptamine are not the chief mediators involved in the heat reaction at temperatures up to 46.5° C.

Hexadimethrine bromide, an anti-heparin agent, has been reported to be effective against yeast-induced rat paw oedema when used in doses of 20 mg/kg intraperitoneally (Kellett, 1965) but it was ineffective against thermic oedema. Similarly, Trasylol (Bayer A.G., 5,000 kallikrein inactivator units/ml.) reported by Kaller, Hoffmeister & Kroneberg (1966) to be effective against yeast did not alter the course of thermic oedema in rats, even when used in doses of 10,000 I.U./kg intraperitoneally. Soya Bean Trypsin Inhibitor (Koch-Light & Co., Colnbrook, Bucks) likewise was ineffective, although previous workers had shown it to suppress kaolin-induced rat paw oedema when used in doses of 20 mg/kg intraperitoneally (Hladovec, Mansfeld & Horáková, 1958) and to inhibit plasma kallikrein-mediated kinin release *in vitro* (Werle & Maier, 1952). Cysteine, which was reported to be an efficient inhibitor of thermic oedema (55° C for 20 sec) in guinea-pigs at doses of 200 mg/kg intraperitoneally (Davies & Lowe, 1966), was ineffective against thermic oedema (46.5° C for 30 min) in rat paws.

The inhibitors which were effective when injected intraperitoneally were next tested for their action after local subcutaneous injection into the paws, again when used 30 min before heating (Table 4). Sodium flufenamate and sodium meclofenamate markedly inhibited oedema formation and petechial production, and reduced the intensity of blueing in the paw. Sodium mefenamate again was the least active of the three fenemates tested. Sodium phenylbutazone and calcium acetylsalicylate reduced the thermic oedema by about 50% but had no effect either on the number of petechiae developing or on the intensity of blueing in the paw. Dibenzyline by the subcutaneous route was completely ineffective against the oedema reaction.

TABLE 4

EFFECT OF SUBCUTANEOUS INJECTIONS OF ANTAGONISTS ON THE OEDEMA REACTION PRODUCED BY HEATING PAWS OF REACTOR RATS FOR 30 MIN AT 46·5° C

Inhibitions recorded as percentages of the oedema (paw volume) developing without antagonist. Number of petechiae and intensity of blueing recorded on a relative scale from 0 to +++

Antagonist	Dose (mg)	Inhibition of oedema (%)	Number of petechiae in paw	Intensity of blueing in paw
Sodium flufenamate	4	77	0	+
Sodium mefenamate	4	36	+	+++
Sodium meclofenamate	4	67	0	++
Sodium phenylbutazone	8	53	++	+++
Calcium acetylsalicylate	20	44	++	+++
Dibenzyline	0·8	9	++	+++

Adrenaline in doses of 5 μg was also found to be a potent inhibitor of the thermic oedema when injected into the paw before heating, and petechiae formation and blueing were markedly suppressed. Noradrenaline was some ten times less active (Fearn, Karady & West, 1965), even when tested at 46.5° C for 30 min. This result suggests that the pressor action of these amines is not of major importance, as noradrenaline is the more active pressor agent.

Inhibition of intradermal bradykinin

Sodium phenylbutazone was one of the most active compounds tested in antagonizing the increase in capillary permeability produced by intradermal injections of bradykinin, and, in contrast to the other effective antagonists, it was active when given locally mixed with the bradykinin (Table 5). The sodium salts of the three fenamic acids were only

TABLE 5

EFFECT OF ANTAGONISTS ON THE INCREASE IN CAPILLARY PERMEABILITY PRODUCED IN THE SKIN OF RATS BY THE INTRADERMAL INJECTIONS OF BRADYKININ (0·1 AND 1·0 μg)

Inhibitions recorded as percentages of the reaction developing without antagonist

Antagonist	Intraperitoneal dose (mg/kg)	Inhibition (%) 0·1 μg	Inhibition (%) 1·0 μg	Intradermal dose (mg)	Inhibition (%) 0·1 μg	Inhibition (%) 1·0 μg
Sodium flufenamate	100	55	45	0·5	0	0
Sodium mefenamate	100	47	24	0·5	0	0
Sodium meclofenamate	100	63	37	0·5	0	0
Sodium phenylbutazone	200	68	53	1·0	82	56
Calcium acetylsalicylate	500	38	25	2·5	17	11
Dibenzyline	20	0	0	0·1	0	0

effective when given intraperitoneally 30 min before the kinin, whereas dibenzyline was completely inactive in this test. Both reactor and non-reactor rats were equally sensitive to intradermal bradykinin, and further experiments showed that their vascular systems were equally sensitive to the kinin (0.1–3 μg), and isolated smooth muscle preparations from both types of rat responded similarly.

It was confirmed that adrenaline, in intradermal doses of 0.1–1.0 μg, is a potent inhibitor of bradykinin, and noradrenaline was found to be about ten times less active (Brown & West, 1965).

All three fenamates, sodium phenylbutazone and calcium acetylsalicylate showed anti-5-hydroxytryptamine and antihistamine activity, particularly when used intraperitoneally against the lower dose level of agonist. As with bradykinin, sodium phenylbutazone was also the only antagonist to be active when given intradermally together with the histamine or 5-hydroxytryptamine. Mepyramine and 2-bromolysergic acid diethylamide, when injected intraperitoneally or intradermally, completely suppressed the lesions produced by histamine and 5-hydroxytryptamine respectively but were without effect against bradykinin.

DISCUSSION

The results obtained from the experiments where the paws were perfused with Tyrode solution indicate that bradykinin is involved in thermal injury in rats. The oedema developing in the paws of animals sensitive to dextran is accompanied by petechial formation, spread of blue dye into the tissue spaces of the paw when such a dye is in the circulation, and systemically-induced salivation. The degree of oedema parallels the release of free bradykinin, bradykininogen and kinin-forming enzyme into the perfusate of the paw and is temperature-dependent up to 46.5° C; on the other hand, the kininase activity is not altered by the heating procedure. Experiments *in vitro* indicate that this heat stimulus itself is not strong enough to activate directly the plasma kinin system so that there probably is an endogenous initiator of the process. The local extravasation of kinin precursors, and their subsequent activation by dilution in the oedema fluid, or by contact with foreign tissue surfaces, also appears unlikely as the peak of kinin release into the paw perfusates always occurs before the oedema is fully developed. It has been found that the protein content of the perfusates parallels that of bradykinin and it thus appears that during the heating process protein migrates to the tissue spaces first and this is then followed by water to give the oedmea reaction. It may be that the pH value of the interstitial fluid in the paws subjected to thermal injury reaches a sufficiently low value to activate the kinin-releasing enzyme, for this enzyme is easily activated in rat plasma (Jacobsen & Waaler, 1966).

The poor response to thermal injury shown by rats resistant to dextran (the non-reactors) was reflected in the small increase in bradykinin in the perfusates from the paws of such animals and the absence of systemically-induced salivation. This suggests that non-reactor rats either have a low content of the initiating substance or possess an increased amount of inhibitor. It has already been shown (Dawson, Starr & West, 1966) that the blood levels of bradykinin, bradykininogen, kinin-forming enzyme (kallikreinogen) and kininase in the twe types of rat are similar. Recent studies show that the plasma from the rats is activated equally by dilution or acidification or by contact with

glass—in each case the time course of the development of bradykinin and the amounts of bradykinin formed are similar.

In many types of rat paw oedema the plurality of the mediators involved has been confirmed, but in thermic oedema the participation of histamine and 5-hydroxytryptamine is unlikely as no increases in their release were detected in the paw perfusates at temperatures up to 46.5° C. This finding agrees with that of Rocha e Silva & Antonio (1960) who found only a small output of these amines when paws were heated at 45° C; likewise, Högberg & Uvnäs (1957) showed that the histamine stores in the skin of rats were stabilized and resistant to histamine releasers when the tissue was heated at 45° C for 30 min. Furthermore, large doses of specific inhibitors of histamine and 5-hydroxytryptamine did not in any way modify the thermal injury in rats. The participation of these two amines is greater at 60° C where the reaction is more severe; in fact, their release at this temperature into the oedema fluid was found in the present study, at a time when bradykinin levels were little above the basal values.

The most effective parenteral antagonists of the thermal injury were the fenamates, phenylbutazone, acetylsalicylate and dibenzyline, though sometimes inhibition of blueing (indicating an increase in vascular permeability as the blue dye is attached to blood protein) was much more difficult to achieve than inhibition of oedema. This was particularly so with dibenzyline and sodium meclofenamate. Subcutaneously, all except dibenzyline were effective antagonists of the oedema response, but only two (sodium flufenamate and meclofenamate) markedly reduced the blueing reaction. This suggests that some antagonists may not be able to reduce the early phase of the thermal injury but are effective in the later stages when the oedema reaction becomes prominent. Dibenzyline was completely ineffective against locally injected bradykinin and only sodium phenylbutazone antagonized bradykinin by the two routes tested. The agents that prevented or markedly reduced the thermic injury had no effect on the formation of bradykinin *in vitro* and the results showed they possessed antihistamine and anti-5-hydroxytryptamine properties.

These results show that bradykinin is involved in the thermic oedema reaction, but it is not alone in this respect for the following reasons. Large amounts of exogenous bradykinin are required to be injected subcutaneously into rat paws to produce an oedema of similar intensity; the suppression of thermic oedema but not of blueing by some antagonists suggests that more than one mediator is involved (Brown & Robson, 1964); the inability of kallikrein inhibitors to modify the oedema reaction suggests that kinins do not initiate the reaction; and the intrinsic plasma kinin system of rats resistant to dextran and to heat is similar to that of rats sensitive to heat, and both types of animal react similarly to injected bradykinin. The resistance of non-reactors to thermal injury may be linked with the difficulty by which stored catecholamines (active antagonists of bradykinin) are released (Fearn, Karady & West, 1966) when injected with liberators such as histamine. The anti-oedema activity of parenteral dibenzyline, reported by Rocha e Silva & Antonio (1960) and confirmed in the present study, can be explained on the basis of occupation of α-adrenergic receptors by dibenzyline, so that more freely-circulating catecholamine is available to reduce the bradykinin effects or even its formation and release. On the other hand, the suggested stabilizing action of the other anti-inflammatory agents on blood vessels (Starr & West, 1966) may explain

some of the intradermal results found in the present study and contribute to their efficacy against thermic injury by suppressing permeability changes. The large doses required to show the actions of known potent anti-inflammatory drugs probably precludes the use of rat-paw thermic oedema in testing for anti-inflammatory activity.

SUMMARY

1. Bradykinin is released into the perfusion fluid of rat paws subjected to heat at temperatures up to 46.5° C. At higher temperatures, histamine is released. Rats resistant to the dextran anaphylactoid reaction release less bradykinin at the lower temperatures and are relatively resistant to thermal injury.

2. Sodium flufenamate, sodium mefenamate, sodium meclofenamate, sodium phenylbutazone, calcium acetylsalicylate and dibenzyline are potent inhibitors of the thermic oedema reaction but do not always inhibit petechial formation and blueing of the paw when a blue dye is in the circulation.

3. All the antagonists except dibenzyline are potent inhibitors of the local bradykinin reaction but only when administered intraperitoneally. Sodium phenylbutazone is the exception as it inhibits bradykinin even when injected intradermally together with the kinin.

4. It is concluded that bradykinin is involved in the thermal injury reaction in rats. Histamine and 5-hydroxytryptamine do not play a major part in the reaction when the temperature used is below 46.5° C. It is suggested that other unidentified mechanisms are also concerned in the reaction of the paw to heat.

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