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ON THE ANTI-INFLAMMATORY ACTIVITY OF PROTAMINE SULPHATE AND OF HEXADIMETHRINE BROMIDE, INHIBITORS OF PLASMA KININ FORMATION

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It has been suggested that plasma kinins may be responsible for some of the tissue responses to local injury (Lewis, 1958). Bradykinin, the first plasma kinin to be characterized and prepared synthetically, produces the four cardinal signs of inflammation: vasodilation (Holton & Holton, 1952), increased capillary permeability (Holdstock, Mathias & Schachter, 1957; Herxheimer & Schachter, 1959), accumulation of leucocytes (Lewis, communication to Joint Meeting of British and Scandinavian Pharmacological Societies, July 1960), and pain (Armstrong, Jepson, Keele & Stewart, 1957).

Armstrong & Stewart (1962) reported that the antiheparin agents, protamine sulphate and hexadimethrine bromide, are potent *in vitro* inhibitors of the activation of plasma kinins induced by procedures such as the dilution of plasma or serum, or contact with glass. These drugs have, therefore, been tested against various types of inflammatory reaction.

METHODS

Rat paw test. Male Sprague-Dawley rats of from 130 to 170 g were used and were within a 20-g weight range for each experiment. Inflammation was evoked by injecting 0.1 ml. of one of the following solutions or suspensions beneath the plantar aponeurosis of the left hind-paw: the supernatant fluid from a 20% suspension of brewers' yeast in saline; histamine, 60 μg/ml.; 5-hydroxytryptamine, 5 μg/ml.; silver nitrate, 5 mg/ml.; formaldehyde, 30 mg/ml.; kaolin, 100 mg/ml. suspension; and dextran (molecular weight 135,000), 60 μg/ml. Groups of six rats were used. The volume of the left hind-foot of each rat was measured before and at intervals after the inflammatory injection using Kellett's (1958) modification of the method described by Buttle, D'Arcy, Howard & Kellett (1957). The compound to be tested was given 1 hr before inducing the inflammation.

Cotton pellet test. The test was basically as described by Meier, Schuler & Desaulles (1950). Four weighed cotton pellets, each of about 8 mg, were implanted subcutaneously in the pectoral and groin regions of male Sprague-Dawley rats of about 120 g. The rats, in groups of five, were given hexadimethrine intraperitoneally, or cortisone acetate intramuscularly, twice daily for 5 days and were killed at the end of the 5th day. The pellets and granulation tissue were then dissected free from the surrounding tissue, dried at 60° C for 24 hr and weighed. The increase over the original pellet weight gave the weight of granulation tissue formed.

Ultraviolet erythema. Previously depilated male albino guinea-pigs weighing about 250 g were exposed to ultraviolet irradiation, on both sides, for 35 sec from a Hanovia Kromayer lamp (Model 10), the standard

applicator having been replaced by a flat metal disc with three circular apertures of 0.5 cm diameter. There were five animals in each group. They were given hexadimethrine or phenylbutazone, intraperitoneally. Half the dose was given 30 min before, and the rest 1 min before, irradiation. The intensity of the reaction was assessed 90 min later by a trained observer who was unaware of the drugs and doses given. The scoring system was as suggested by Adams (1960), the intensity of each erythema being estimated on a 0 to 4 scale. An " Effective Dose " is the dose needed to reduce the maximum possible score for a group by one-half.

Capillary permeability. Male albino guinea-pigs of 250 to 350 g were given 60 mg/kg of Evan's Blue in saline, intravenously. After 15 min, intradermal injections of 0.1 ml. of 10 μg/ml. of histamine or bradykinin, or 0.1 ml. of saline, were made into the previously depilated sides of each animal. In some experiments the hexadimethrine was given intraperitoneally 45 min before the intradermal injections: in others it was incorporated into the intradermally injected solution as 0.05 ml. of 1 mg/ml. solution, giving a total injection volume of 0.15 ml. After 25 min the animals were killed and the skin over the injection sites was carefully removed. Two diameters of each lesion, at right angles to each other, were measured from the inner skin surface, and the mean result was recorded.

Inhibition of the tuberculin reaction in B.C.G.-sensitized guinea-pigs. Male albino guinea-pigs were sensitized to tuberculin by the intramuscular injection of 0.05 mg (moist weight) of Mycobacterium B.C.G. After 4 to 6 weeks groups of four animals were given hexadimethrine intraperitoneally, or cortisone acetate subcutaneously, 1 hr before the intradermal injection of 10, 30 or 90 U of Old Tuberculin in 0.1 ml. of saline into the previously depilated skin. Both sides of each animal were used, and one injection of each concentration of Old Tuberculin was given on each side. The wheal diameters were measured 24 hr later.

Drugs. These were: hexadimethrine bromide (Abbott), 10 mg/ml. solution with average molecular weight of 6,000; protamine sulphate (Nutritional Biochemicals); phenylbutazone (Geigy), 200 mg/ml. solution; and cortisone acetate (Roussel), 25 mg/ml. suspension.

RESULTS

Rat paw test. The systemic injection of protamine sulphate into rats produced an anaphylactoid reaction which obscured its anti-inflammatory activity. Nevertheless, it significantly inhibited a yeast-induced oedema (Table 1).

TABLE 1

ANTI-INFLAMMATORY ACTIVITY OF PROTAMINE IN THE RAT PAW TEST

Protamine was given 1 hr before the injection of 0·1 ml. of supernatant fluid from a 20% yeast suspension beneath the left hind paw. There were six rats per group. * Denotes that the response shows a statistically significant difference ($P<0·05$) from that for the control group. S.c., subcutaneous; i.m., intramuscular; i.v., intravenous

Dose (mg/kg)	Route	Inhibition of swelling (%) at 1·5 hr	Inhibition of swelling (%) at 6 hr
800	S.c.	47·8*	50·9*
400	S.c.	34·7*	35·6*
400	I.m.	36·6*	48·4*
200	I.m.	35·2*	38·8*
25	I.v.	18·9*	34·6*
12·5	I.v.	13·0	24·9*

Hexadimethrine was tested by different routes against a yeast-induced oedema and the results are shown in Fig. 1. It was inactive orally and most active after intraperitoneal or intravenous injection. A dose/response curve for hexadimethrine against a yeast-induced oedema is shown in Fig. 2.

The effects of hexadimethrine against other experimentally induced inflammations are summarized in Table 2. They fall into two groups: in one of these the inflammatory response failed to develop; in the other the response was only temporarily suppressed.

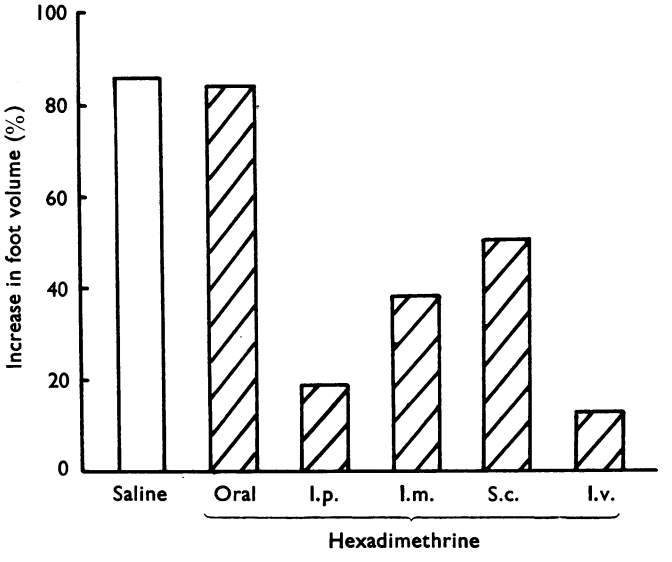

Fig. 1. Anti-inflammatory activity of hexadimethrine against a yeast-induced oedema in the rat's paw, Hexadimethrine (40 mg/kg) or saline was given 1 hr before the injection of 0.1 ml. of the supernatant fluid from a 20% yeast suspension into the paw. Foot volumes were read 1.5 hr after the irritant injection. There were six rats per group. I.p., Intraperitoneal; i.m., intramuscular; s.c., subcutaneous; and i.v., intravenous.

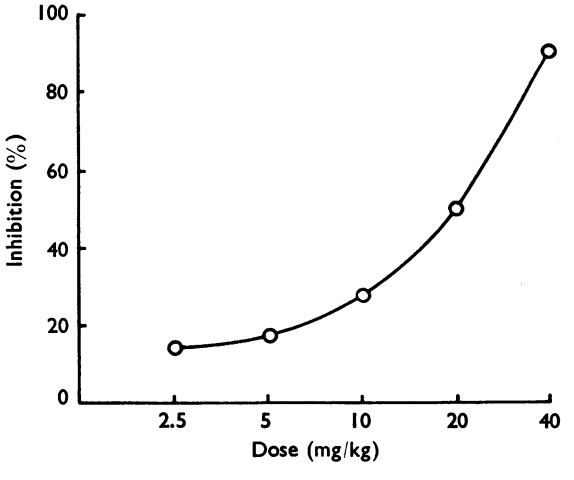

Fig. 2. Dose/response curve for hexadimethrine against a yeast-induced oedema in the rat's paw. Hexadimethrine was given intraperitoneally 1 hr before the injection of 0.1 ml. of the supernatant fluid from a 20% yeast suspension into the paw. Foot volumes were read 6 hr after the irritant injection. There were six rats per group.

TABLE 2

ANTI-INFLAMMATORY ACTIVITY OF HEXADIMETHRINE AGAINST VARIOUS INFLAMMATORY STIMULI IN THE RAT PAW TEST

Hexadimethrine, 40 mg/kg intraperitoneally, was given 1 hr before the injection of 0·1 ml. of irritant beneath the left hind-paw and foot volumes were recorded 1·5, 6 and 24 hr afterwards. There were six rats per group. * Denotes that the response shows a statistically significant difference ($P<0·05$) from that of the control group. N=No inflammation remaining in control group

	Inflammatory stimulus	Inhibition of swelling (%) at 1·5 hr	6 hr	24 hr
I	Kaolin (100 mg/ml. suspension)	81·6*	93·4*	N
I	Dextran (60 μg/ml.)	85·9*	80·8*	N
I	5-Hydroxytryptamine (5 μg/ml.)	86·2*	N	N
I	Histamine (60 μg/ml.)	78·4*	N	N
II	Formaldehyde (30 mg/ml.)	79·4*	50·8*	2·1
II	Silver nitrate (5 mg/ml.)	80·9*	62·6*	5·2

The inflammatory stimuli in the first group (kaolin, dextran, 5-hydroxytryptamine and histamine) must have been rather transient, whereas those in the second (formaldehyde and silver nitrate) were more prolonged and persisted after the inhibitory effects of hexadimethrine have worn off. The foot volumes of hexadimethrine-treated and control groups 24 hr after injection of irritant were very similar for silver nitrate and formaldehyde-injected animals. A second dose of hexadimethrine 3 hr after the formaldehyde injection further delayed the onset of inflammation; but it developed a few hours later, and after 24 hr there was no difference between test and control groups.

Hexadimethrine reduced an established inflammation in the rat's paw. A dose of 40 mg/kg given 45 min after the development of a yeast-induced oedema caused a significant reduction in foot volume, as compared with saline-treated controls, during the subsequent course of the reaction (Fig. 3).

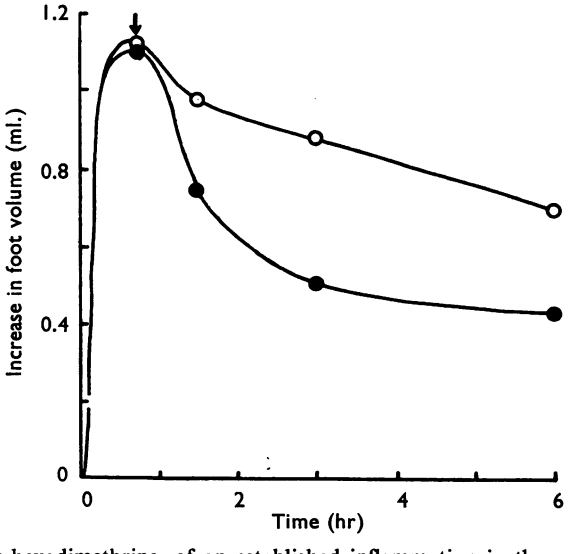

Fig. 3. Inhibition, by hexadimethrine, of an established inflammation in the rat's paw. Inflammation was induced by injecting 0.1 ml. of the supernatant fluid from a 20% yeast suspension into the paw. At the arrow, saline (4 ml./kg) ○——○, or hexadimethrine (40 mg/kg) ●——●, was given intraperitoneally. There were six rats per group.

The dose of hexadimethrine used in most of these tests produced some degree of hypothermia but this did not affect its anti-inflammatory activity. Given to rats kept at 37° C throughout the experiment, hexadimethrine inhibited a yeast-induced oedema to the same extent as in animals maintained under normal laboratory conditions.

The effect of heparin on the anti-inflammatory activity of hexadimethrine in the rat paw test was also examined. Intraperitoneal injection of heparin on a mg/mg basis 15 min after giving hexadimethrine by the same route caused complete loss of activity. Heparin given intravenously or subcutaneously did not appear to affect the response to intraperitoneal hexadimethrine. But the results were unsatisfactory, as injection of the irritant caused bleeding in the paws of some of the rats.

Cotton pellet test. Hexadimethrine, in daily divided doses of 2.5, 5 and 10 mg/kg intraperitoneally, suppressed granulation-tissue formation around subcutaneous cotton

TABLE 3

ANTI-INFLAMMATORY ACTIVITY OF HEXADIMETHRINE IN THE COTTON PELLET TEST

There were five rats per group, with four pellets in each rat. Animals were dosed twice daily and killed at the end of the fifth day. I.m., Intramuscular; i.p., intraperitoneal. Weights of granulation tissue are means ± standard errors

Treatment	Dose (mg/kg/day)	Route	Weight of granulation tissue (mg)	Inhibition (%)	*P*
Saline	—	—	18·85±1·62	—	—
Cortisone acetate	20	I.m.	9·88±0·59	47·6	<0·001
	10	I.m.	14·02±1·40	25·6	<0·05
	5	I.m.	16·91±1·73	10·3	>0·05
Hexadimethrine	10	I.p.	10·68±1·15	43·4	<0·001
	5	I.p.	13·88±1·27	26·4	<0·05
	2·5	I.p.	14·06±1·38	25·4	<0·05

pellets, and was about twice as active as intramuscular cortisone acetate. The hexadimethrine-treated rats lost weight in the early stages of the experiment, but this trend was reversed by the third day.

Ultraviolet erythema. The effect of hexadimethrine was less convincing than in previous tests (Table 4). There was no dose/response relationship, so that it was not possible to calculate an " Effective Dose." However, there was inhibition of the response with doses

TABLE 4

EFFECT OF HEXADIMETHRINE AGAINST AN ULTRAVIOLET ERYTHEMA

Half the dose was given intraperitoneally 30 min before and the remainder 1 min before exposure to ultraviolet light. Intensity of reaction was estimated 90 min later. Maximum score per animal=24. There were four guinea-pigs per group

Treatment	Dose (mg/kg)	Mean erythema response	Approximate " Effective Dose " (mg/kg)
Saline	—	18·9	
Phenylbutazone	16	4·1	
	8	10·2	6
	4	14·0	
Hexadimethrine	16	7·0	
	8	10·5	?
	4	6·5	

as low as 4 mg/kg which is about two-thirds the " Effective Dose " for phenylbutazone in this test. Hexadimethrine, in common with other anti-inflammatory agents, only delayed the development of the erythema.

Capillary permeability. Intravenous or intraperitoneal hexadimethrine, in doses up to 30 mg/kg, failed to modify the permeability response to intradermal histamine or bradykinin (1 μg) in guinea-pigs previously treated with Evan's Blue. However, when 50 μg of hexadimethrine was incorporated in the histamine or bradykinin solution the blueing response was partially suppressed. But whether this was due to a reduced permeability response or to some other effect, possibly necrosis, produced in the tissues by the hexadimethrine, was not clear.

Tuberculin reaction in B.C.G.-sensitized guinea-pigs. Hexadimethrine showed marked activity in suppressing the tuberculin reaction in sensitized guinea-pigs: a single intraperitoneal dose of 3.75 mg/kg caused about 50% reduction in wheal diameter. With higher doses the wheals often failed to develop, but 48 hr after the Old Tuberculin injection faint pink patches appeared. The doses of cortisone acetate used had only a slight effect (Table 5).

TABLE 5

INHIBITION OF TUBERCULIN REACTION IN B.C.G.-SENSITIZED GUINEA-PIGS

Guinea-pigs were sensitized with 0·05 mg (moist weight) of Mycobacterium B.C.G. 28 days before testing. Drugs were given intraperitoneally 1 hr before the intradermal injection of Old Tuberculin. There were five animals per group. * Denotes that the response shows a statistically significant difference ($P<0·05$) from the control group

Treatment	Dose (mg/kg)	Mean wheal diameter (mm) for units of Old Tuberculin		
		10	30	90
Saline	—	9·5	13·0	16·7
Cortisone acetate	12·5	9·0	12·1	14·4
	6·25	9·0	11·4	13·5*
Hexadimethrine	7·5	1·5*	2·0*	2·6*
	3·75	4·4*	6·8*	8·4*

DISCUSSION

The significance of plasma kinins in inflammatory reactions is uncertain. No conclusive evidence is available that they are present in active form in inflammatory exudates although their precursors are certainly there. Rocha e Silva & Rosenthal (1961) demonstrated plasma-kinin activity in rat tissue after thermal injury, but as Tyrode solution was injected at the site of injury kinin formation may have been due to a dilution effect rather than to the inflammatory stimulus. Recent studies on dogs have shown that lymph draining injured tissues has an increased kinin-forming activity (Edery & Lewis, 1963). Some support for a role of kinins in the inflammatory process is afforded by the fact that some nonspecific inhibitors of bradykinin on the guinea-pig isolated ileum preparation (Rocha e Silva & Leme, 1963) will reduce the oedema after thermal injury in the rat's paw (Rocha e Silva & Antonio, 1960).

There are probably two main intrinsic kinin-forming systems in plasma, one involving the fibrinolytic system and the other dependent upon activation of plasma kallikreins. Proteolytic enzymes are known to be activated after injury, and inhibitors of proteolysis

such as ϵ-aminocaproic acid and soya bean trypsin inhibitor have been tested for anti-inflammatory activity. Bertelli, Proto & Rossano (1962) protected rats subjected to egg white oedema with ϵ-aminocaproic acid (300 mg/kg), and in the granuloma pouch test a 20% decrease in wall thickness was produced in rats treated with 200 mg/kg of the inhibitor for 5 days (Ferluga, Nikulin & Stern, 1963). Soya bean trypsin inhibitor will suppress a kaolin oedema in the rat's paw (Hladovéc, Mansfeld & Horáková, 1958), and also reduces the haemorrhagic necrosis following the intradermal injection of a mixture of bacterial endotoxin and adrenaline (Zweifach, Nagler & Troll, 1961).

Northover & Subramanian (1961) reported that salicylates and phenylbutazone inhibit plasma-kinin formation induced by dilution or treatment with kallikrein *in vitro*, but this was not confirmed by Hebborn & Shaw (1963). However, locally injected sodium salicylate completely suppresses the permeability response to intradermal kallikrein in guinea-pigs (Spector & Willoughby, 1962).

Protamine and hexadimethrine prevent the activation of plasma kinins *in vitro* (Armstrong & Stewart, 1962), and have been investigated for anti-inflammatory activity in several tests. Both were active against a yeast-induced oedema in the rat's paw; hexadimethrine was about fifteen-times more active than protamine. Systemic injection of protamine caused an anaphylactoid reaction and it was considered unsuitable for further testing. The effect of these substances on mast cells has been investigated by Kimura, Young & Ebert (1959), who showed that protamine caused greater mast cell disruption than did hexadimethrine, *in vitro* and *in vivo*.

Hexadimethrine was active against a variety of inflammatory stimuli in the rat paw test and was most active intravenously and intraperitoneally but was inactive orally. With formaldehyde and silver nitrate the oedema was only temporarily suppressed.

In the cotton pellet test hexadimethrine was about twice as active as cortisone acetate in preventing the formation of granulation tissue. The significance of this result is not clear. There is no evidence for the participation of plasma kinins in granulation-tissue formation, and the possibility that some other mechanism is involved must be considered. Hexadimethrine causes a considerable rise in blood sugar levels (Kellett, unpublished observation) and Nagy, Rédei & Karády (1961) have reported on the inhibition of granulation-tissue formation in rats with alloxan diabetes.

No dose/response relationship could be obtained for hexadimethrine against an ultraviolet erythema, but it caused a significant inhibition of the response at 4 mg/kg. The " Effective Dose " for phenylbutazone is about 6 mg/kg.

Hexadimethrine injected intravenously into guinea-pigs did not affect the capillary permeability response to intradermal injection of histamine or bradykinin. Eisen (1964) depressed the permeability response to intradermal kaolin with systemic doses of hexadimethrine. He suggested that the kaolin response may be due to activation of proteolytic enzymes since it could also be prevented with soya bean trypsin inhibitor. The permeability responses to histamine and bradykinin are direct effects, and systemic injection of hexadimethrine would be unlikely to affect them. The results of tests involving locally injected hexadimethrine are not clear.

Hexadimethrine was about three-times as active as cortisone acetate in suppressing the tuberculin reaction in B.C.G.-sensitized guinea-pigs.

Apart from its effect in the cotton-pellet test, which has already been discussed, we have assumed that the anti-inflammatory activity of hexadimethrine is due to inhibition of kinin formation. But the large dose needed to suppress the inflammatory response to injected irritant in the rat paw, compared with the concentration required to inhibit kinin formation in plasma or serum *in vitro*, suggests that some other mechanism may be involved. This is being investigated, and preliminary results suggest the participation of the adrenal medulla. Irritation produced by injected hexadimethrine may also have an effect.

SUMMARY

1. Plasma kinins may be responsible for some of the tissue responses to local injury. Hexadimethrine bromide and protamine sulphate are potent inhibitors of plasma kinin formation *in vitro* and the effects of these drugs on inflammatory reactions have been investigated.

2. Protamine produced an anaphylactoid reaction in rats and was not investigated in detail.

3. Hexadimethrine inhibited oedema formation induced by a variety of irritants and reduced granulation tissue formation in rats. It suppressed the tuberculin reaction in B.C.G.-sensitized guinea-pigs. Given locally, it partially prevented the increase in vascular permeability produced by intradermal injection of histamine and bradykinin.

4. These results support the possibility that kinins play a part in inflammatory reactions. However, the high doses of hexadimethrine needed to suppress oedema formation, and the unexpected finding that granulation tissue production is inhibited, suggest that more than one mechanism may be involved. The drug has an irritant effect and also causes hyperglycaemia and these factors may be responsible for some of its anti-inflammatory activity.

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