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ANALGESIC-ANTIPYRETIC DRUGS AS INHIBITORS OF KALLIKREIN

BY

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The production of kallidin from guinea-pig and ox serum kallidinogen by the action of guinea-pig serum-kallikrein or human salivary kallikrein was inhibited by various analgesic-antipyretic drugs. This effect was obtained *in vitro* with concentrations of inhibitors which do not inhibit the smooth-muscle-stimulating action of the various polypeptides, but similar to the concentrations needed *in vivo* to obtain an anti-inflammatory action. The vasodepressor action of intravenously administered human salivary kallikrein in the anaesthetized dog was very markedly inhibited by the intravenous administration of doses of various analgesic-antipyretic drugs which only partially antagonized the responses to kallidin and bradykinin and which left the vasodepressor responses to histamine, acetylcholine and 5-hydroxytryptamine unaffected. In rabbits the accumulation of protein-bound dye at the site of intradermal injection of human salivary kallikrein and guinea-pig serum-kallikrein, but not of bradykinin and kallidin, was inhibited very markedly by the systemic administration of various analgesic-antipyretic drugs.

Analgesic-antipyretic drugs have been shown by Collier & Shorley (1960) to antagonize the bronchoconstrictor action of bradykinin in the anaesthetized guinea-pig, and Lecomte (1960) has demonstrated that in the rabbit phenylbutazone will prevent the fall in blood pressure and the local increase in capillary permeability in response to bradykinin.

It is widely recognized that analgesic-antipyretic drugs possess anti-inflammatory properties, both in rheumatoid diseases of man and also in experimentally induced inflammatory reactions in other species, but their mechanism of action is not clear (Smith, 1953). Bradykinin has been proposed by Hilton & Lewis (1955) as a possible mediator in certain inflammatory reactions, and their hypothesis is further substantiated by the fact that a chemically heterogeneous group of substances share the ability to inhibit both bradykinin and inflammatory reactions.

Several enzyme systems which are present in the mammalian body are reported to produce polypeptides either very closely related to, or identical with, bradykinin (Lewis, 1960a). If the liberation of bradykinin is an important mechanism in the production of an inflammatory reaction, then it seems likely that inhibitors of the enzymes which liberate bradykinin *in vivo* should act as anti-inflammatory agents. The experiments described in this paper are intended to test this possibility.

METHODS

Smooth-muscle stimulation. Isolated segments of the terminal ileum of guinea-pigs were suspended in a 20 ml. bath of oxygenated Tyrode solution at 33 to 34° C, and contractions recorded isotonically.

Vasodepressor activity. Dogs were anaesthetized with chloralose and the blood pressure recorded from the central end of the carotid artery by means of a cannula connected to a mercury manometer with a floating writing point. Drugs were administered by the femoral vein.

Accumulation of protein-bound dye. Bradykinin, kallidin or kallikrein was injected intradermally in the depilated flank skin of rabbits which had previously been injected with Evans blue dye intravenously, as described by Bhoola, Calle & Schachter (1960). By measuring the diameter of the region of blue dye accumulation at the injection site on the flank in both untreated control animals and in animals pretreated with various drugs, the degree of inhibition of the response afforded by the drug was determined.

Materials. Bradykinin was a synthetic nonapeptide (Nicolaides & DeWald, 1961) kindly supplied by Dr Collier in the form of a crystalline solid of approximately 80% purity. This peptide is now known to be identical with natural bradykinin (Shorley & Collier, 1960; Lewis, 1960b; Konzett & Sturmer, 1960b).

Kallidin was prepared from an incubated mixture of ox serum, which had been heated to 56° C for 3 hr, and human saliva, according to the method of Holdstock, Mathias & Schachter (1957).

The urinary kinin was prepared from a pooled sample of human urine by *n*-butanol extraction as described by Gaddum & Horton (1959).

Substance P was prepared by extraction of acetone-dried and *n*-butanol-washed ox brain with aqueous acid according to the general method of Amin, Crawford & Gaddum (1954) as subsequently modified by Leach (1959).

Salivary kallikrein for these experiments was obtained from human saliva, diluted at the time of use with 0.9% saline solution.

Serum kallikrein was obtained from guinea-pig or ox serum, which had been dialysed in cellophane tubing against distilled water for 48 hr at 4° C, and stored at this temperature until it was diluted for use with Tyrode solution at 37° C. In the case of both guinea-pig and ox serum maximum activation of the enzyme was obtained by a 1:15 dilution.

Histamine diphosphate, acetylcholine bromide and 5-hydroxytryptamine creatinine sulphate were used, and the doses refer to free base in each case. Sodium salicylate, acetylsalicylic acid, 2:6-dihydroxybenzoic acid, sodium α-4-*sec*-butylphenoxypropionate and phenylbutazone were used as freshly prepared solutions adjusted to neutrality (*p*H 7) with sodium hydroxide.

RESULTS

The inhibition of kinin formation by analgesic-antipyretic drugs. When guinea-pig or ox serum was diluted 1:15 with Tyrode solution and incubated for 5 min at 37° C, a smooth-muscle-stimulating kinin was produced. When diluted serum was added to an organ bath containing a strip of isolated guinea-pig ileum it caused a slightly delayed slow contraction. As little as 0.4 ml. of the diluted serum usually produced a maximal contraction. The undiluted serum, when added to the bath directly, produced no contraction for at least 2 min. If the contact time was extended beyond this, a very slow contraction occurs which reaches a maximum in about 6 min. The incubation of the diluted serum was then performed in the presence of 100 μg/ml. 2:6-dihydroxybenzoic acid, and when the incubated mixture was added to the organ bath the response was reduced to 15% of that obtained in the control. When the incubation of the diluted serum was performed in the absence of 2:6-dihydroxybenzoic acid, but an equivalent amount was added to the organ bath 1 min prior to the addition of the incubated and diluted serum, however, the response was the same as in the control, as shown in Fig. 1. This indicates that the inhibitor does not alter the responsiveness of the gut to the kinin when it is formed, but rather it inhibits its formation from kallidinogen by the enzyme kallikrein, which is activated by the dilution of the serum.

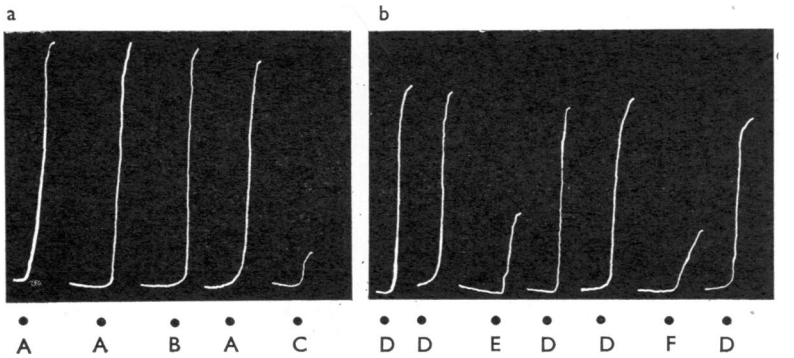

Fig. 1. Effect of analgesic-antipyretic drugs on formation of kallidin. Contractions of isolated guinea-pig ileum to 0.2 ml. incubated 1:15 dilution of guinea-pig serum at (A); to 20 μg 2:6-dihydroxybenzoic acid at (B); to 0.2 ml. 1:15 dilution guinea-pig serum incubated in presence of 100 μg/ml. 2:6-dihydroxybenzoic acid at (C); to 0.1 ml. incubated mixture of heated ox serum and salivary kallikrein at (D); to 0.1 ml. mixture of heated ox serum and salivary kallikrein incubated in the presence of 10 μg sodium salicylate at (E); to 0.1 ml. mixture of heated ox serum and salivary kallikrein incubated in presence of 10 μg phenylbutazone at (F). Contact time—60 sec.

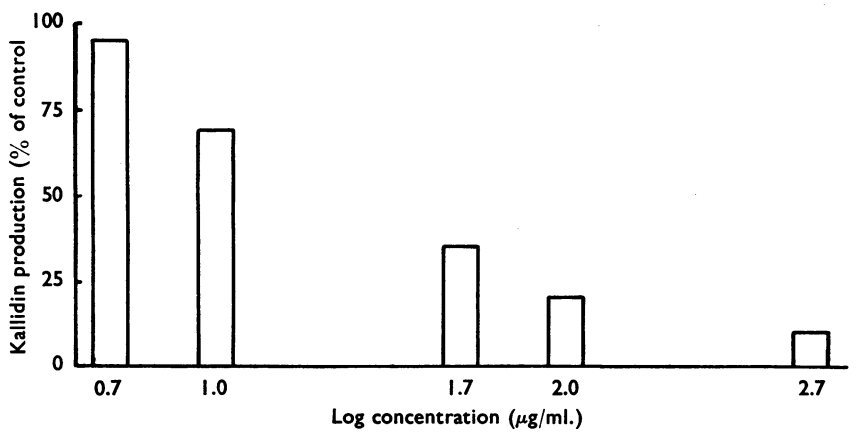

Fig. 2. The inhibition of kallidin formation by serum kallikrein, using fresh dialysed ox serum, in the presence of various concentrations of 2:6-dihydroxybenzoic acid, diluted 1:15 with Tyrode solution, and incubated for 5 min at 37° C. The production of kallidin will be approximately proportional to the antilogarithm$_{10}$ of the height of contraction of the gut.

Fig. 2 gives the results of experiments to determine the degree of inhibition of ox serum kallikrein, using various concentrations of 2:6-dihydroxybenzoic acid. Table 1 gives the concentrations of various inhibitors which were needed to reduce the kinin formation by 50% in the case of ox serum and human salivary kallikrein. Essentially similar results were obtained with guinea-pig serum.

Ox serum which had been heated to 56° C for 3 hr was incubated for 5 min at 37° C with 10 times its volume of human salivary kallikrein solution (final concentration equivalent to saliva diluted 1:100). A smooth-muscle-stimulating kinin was formed which caused an isolated segment of guinea-pig ileum to contract slowly,

TABLE 1

INHIBITION OF KALLIDIN FORMATION IN THE PRESENCE OF ANALGESIC-ANTIPYRETIC DRUGS

The inhibitors were incubated with the enzyme-substrate mixture in the final concentration shown. The incubated mixtures were added to a segment of guinea-pig ileum in an isolated organ bath and the heights of contraction recorded. The amount of kallidin formed is proportional to the antilogarithm$_{10}$ of the height of contraction, in the range of doses used in this experiment

Inhibitor	Concentration required to inhibit kinin formation by 50%, in μg/ml.	
	Ox serum kallikrein Mean$\pm$s.e.	Human salivary kallikrein Mean$\pm$s.e.
Salicylate	116$\pm$13	121$\pm$15
Aspirin	87$\pm$9	89$\pm$8
2: 6-Dihydroxybenzoic acid	39$\pm$7	47$\pm$8
Sodium α-4-*sec*-butylphenoxy-propionate	74$\pm$10	82$\pm$11
Phenylbutazone	29$\pm$6	34$\pm$5

after a slight delay. Approximately 0.2 ml. of the incubated mixture usually produced a maximal contraction. The kallikrein solution and the heated serum were without significant effect when added to the bath separately. When the incubation was performed in the presence of various analgesic-antipyretic drugs the kinin was not formed so rapidly, and the concentrations of these inhibitors required were very similar to those given in Table 1. The possibility that the responsiveness of the gut to the kinin was altered was excluded by adding an equivalent amount of each inhibitor to the organ bath 1 min prior to the addition of the kinin containing mixture ; the response was not affected.

In the concentrations needed to inhibit kinin formation by 50%, the analgesic-antipyretic drugs tested in these experiments did not affect the response of the isolated guinea-pig ileum to synthetic bradykinin, substance P, urinary kinin, histamine, acetylcholine or 5-hydroxytryptamine.

The effect of these inhibitors on other kinin-forming enzyme systems is being investigated.

Antagonism between analgesic-antipyretic drugs and the vasodepressor effect of various substances in the dog. Fig. 3 shows the results of a typical experiment, where several substances which produce a fall in blood pressure were administered intravenously to an anaesthetized dog before and after an infusion of a moderately high dose of one of the analgesic-antipyretic drugs. The fall in blood pressure produced by bradykinin, kallidin, histamine, acetylcholine and 5-hydroxytryptamine was either unaffected or very slightly reduced, whereas that produced by human salivary kallikrein was abolished. In some experiments the response to salivary kallikrein was a sharp fall in blood pressure, a partial return to the baseline, and then a very protracted and sometimes incomplete return to the original value. In this case the antagonists would not only reduce the main fall in blood pressure but also hasten the return to the baseline blood pressure. This confirms a preliminary communication of Guth (1960), who has shown that salicylates inhibit the vasodepressor effect of salivary kallikrein. Of the five antagonists tested, salicylate, acetylsalicylate, α-4-*sec*-butylphenoxypropionate, 2:6-dihydroxybenzoic acid and

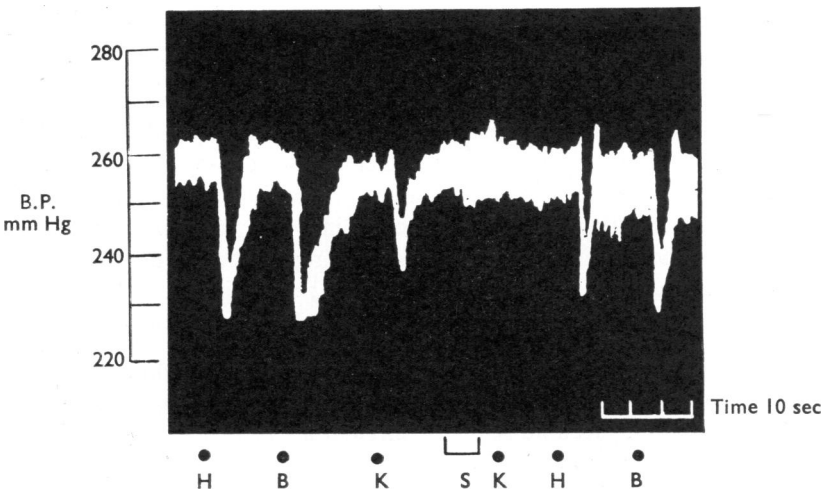

Fig. 3. The antagonism shown by sodium salicylate towards the vasodepressor effect of histamine, bradykinin and human salivary kallikrein. Carotid blood pressure of a 5 kg female dog, anaesthetized with chloralose. H, histamine 4 μg; B, bradykinin 8 μg; K, human salivary kallikrein, equivalent to 0.08 ml. saliva; S, sodium salicylate, 200 mg/kg.

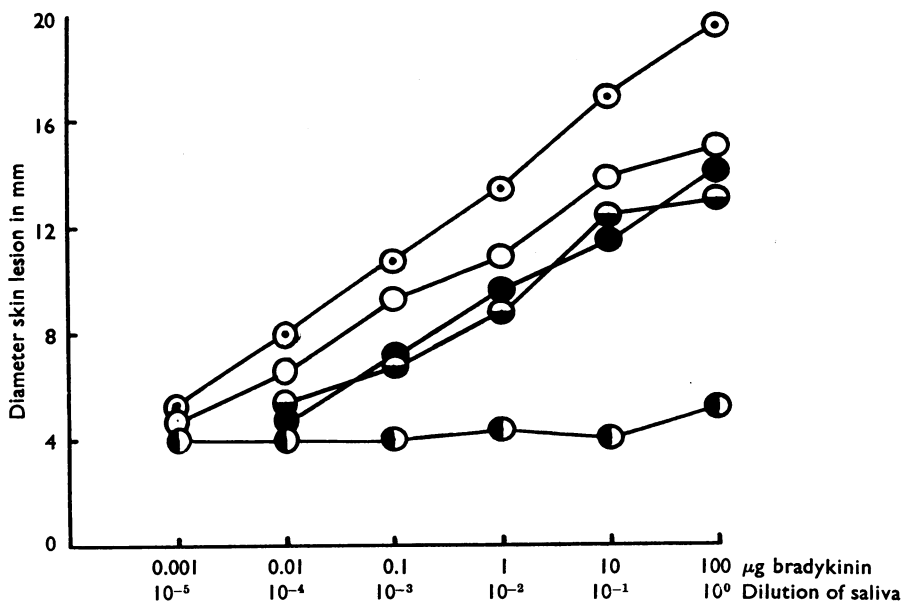

Fig. 4. The antagonism between intravenously administered salicylate and 2 : 6-dihydroxybenzoic acid and the capillary permeability enhancing effect of intradermally injected human salivary kallikrein and bradykinin respectively, in the rabbit. ⊙ —— ⊙, Control rabbits, given normal saline and injected with kallikrein; ○ —— ○, given sodium salicylate 100 mg/kg and injected with kallikrein; ◐ —— ◐, given 2:6-dihydroxybenzoic acid 50 mg/kg and injected with kallikrein; ● —— ●, given 2:6-hydroxybenzoic acid 50 mg/kg and injected with bradykinin; ◒ —— ◒, given normal saline and injected with bradykinin.

phenylbutazone, the last two were the most potent, being effective in doses of 50 mg/kg; the others required at least 150 mg/kg to produce a comparable inhibition.

The antagonism between analgesic-antipyretic drugs and the vasodepressor effects of salivary kallikrein were manifest for only a short time, and within 45 min of the injection of 2:6-dihydroxybenzoic acid, for example, the response to kallikrein was already back to control values. Since the effect was so short-lived, it was thought possible that the mode of action involved adrenal medullary stimulation. To test this possibility the experiment was repeated in a dog which had both adrenal glands excluded prior to the experiment; 2:6-dihydroxybenzoic acid still inhibited the fall in blood pressure produced by salivary kallikrein.

Antagonism between analgesic-antipyretic drugs and the accumulation of protein-bound dye at the site of injection of kallikrein. Bradykinin injected intradermally into rabbits causes the accumulation of circulating protein-bound Evans blue at the site of injection. We have used synthetic bradykinin and confirmed the findings of Bhoola, Calle & Schachter (1960) that the slope of the curve relating relative diameter of skin blueing to intradermal dose of bradykinin is flat. With human salivary kallikrein there is also a flat dose-response curve (Fig. 4). Rabbits which had been given intravenous infusions of various analgesic-antipyretic drugs were also tested, and it was found that, whilst the response to injected salivary kallikrein was very nearly abolished, the response to injected bradykinin was only very slightly affected.

Table 2 gives the doses of various analgesic-antipyretic drugs which are required to inhibit the response to injected salivary kallikrein by 50% in the dog and in the rabbit.

TABLE 2

INHIBITORS OF THE VASODEPRESSOR AND CAPILLARY PERMEABILITY ENHANCING ACTION OF HUMAN SALIVARY KALLIKREIN, IN THE DOG AND IN THE RABBIT RESPECTIVELY

	Dose of antagonist (mg/kg intravenously) required to inhibit response by 50%	
Antagonist	Vasodepressor effect Mean±s.e.	Capillary hyperpermeability Mean±s.e.
Salicylate	181±32	192±28
Aspirin	153±22	174±25
2:6-Dihydroxybenzoic acid	87±19	95±14
Sodium α-4-*sec*-butylphenoxy-propionate	119±20	113±18
Phenylbutazone	76±12	79±13

The inhibition of the action of kallikrein in the rabbit was manifest within 5 min of the intravenous injection of the analgesic-antipyretic drug, and lasted for several hours, as shown in Fig. 5. We are not able to explain the longer duration of action of these antagonists in the rabbit than in the dog.

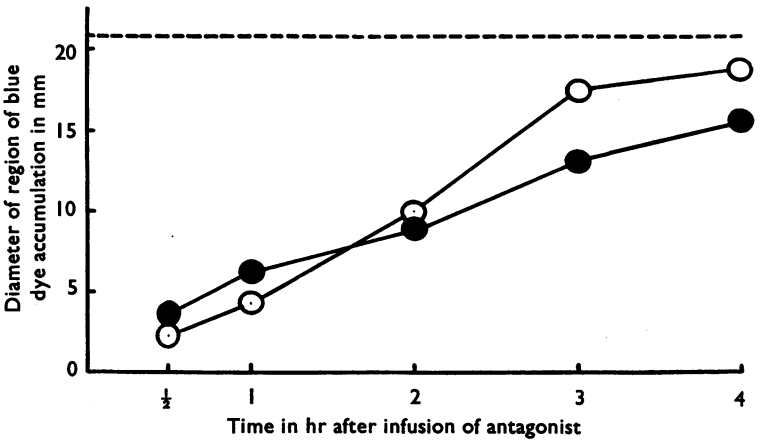

Fig. 5. The inhibition of the capillary permeability enhancing effect of intradermally injected human salivary kallikrein in the rabbit, using 2: 6-dihydroxybenzoic acid and acetylsalicylate, administered as a single dose intravenously. ○ —— ○, 2: 6-dihydroxybenzoic acid, 50 mg/kg; ● —— ●, acetylsalicylate, 200 mg/kg. The horizontal line marked - - - - denotes the mean response of untreated control animals. The dose of salivary kallikrein injected was 0.1 ml. of a 1 : 100 dilution of saliva.

DISCUSSION

There is no conclusive evidence available at the moment to indicate the nature of the mediator of the inflammatory response. It is possible that different types of inflammatory stimuli, in different species and even at different times during an inflammatory reaction, involve different mediators. There is considerable evidence, however, that some of the products of protein degradation are involved in the process, and the subject has been reviewed recently by Lewis (1960a) from the point of view of the part which plasma kinins play in these reactions. Spector (1958) has reviewed the factors which control capillary permeability and the way in which endogenous proteolytic enzymes exert their effect.

The implication of a particular mediator in the inflammatory reaction places upon the investigator the responsibility for demonstrating several crucial facts. For example, it is necessary to show that the mediator actually occurs in the damaged tissues during an inflammatory reaction. The mediator should also be shown not to occur in the tissues, at any rate in an active form, at other times. The administration of exogenous mediator should cause a reaction closely simulating inflammatory reactions elicited in other ways. Antagonists of the inflammatory response to this mediator should also be capable of inhibiting the effects of other inflammatory stimuli. Plasma kinin can fulfil some of these criteria at the moment.

There is no direct evidence that the liberation of bradykinin occurs in inflammatory reactions. However, Elliott, Horton & Lewis (1960) with the pure natural bradykinin which they prepared from ox blood, and Konzett & Sturmer (1960a) with a synthetic peptide of the same structure, indicate that not only is pure bradykinin capable of producing many of the typical symptoms of inflammation but that in some species it is one of the most potent substances known in this respect.

In this paper we have attempted to bring forward some evidence in favour of the theory (Hilton & Lewis, 1955) that the endogenous liberation of plasma kinin is an important step in the mediation of the inflammatory reaction, at any rate under certain conditions. The compounds which we have tested belong to a chemically heterogenous group of substances with the ability to inhibit certain types of inflammatory reaction, as well as certain enzyme systems which are known to produce plasma kinin.

One would expect that in general the more potent anti-inflammatory agent should also be the more potent inhibitor of the kinin-forming enzyme system, if the kinin is in fact involved in the inflammation. This has proved to be the case; the two most potent inhibitors of kallikrein in our experiments, namely, phenylbutazone and 2:6-dihydroxybenzoic acid, have both been shown to be more potent than, for example, salicylates in the treatment of rheumatoid diseases of man. Reid, Watson, Cochran & Sproull (1951) demonstrated the considerably greater potency of 2:6-dihydroxybenzoic acid compared with salicylate in acute rheumatic fever in man. Similarly, Buttle (1951) has shown that 2:6-dihydroxybenzoic acid is more potent than salicylate in preventing formaldehyde-induced foot swelling in mice. Against this evidence, however, is the fact that Adams & Cobb (1958) showed that 2:6-dihydroxybenzoic acid was considerably less potent than salicylate in inhibiting the ultra-violet radiation induced erythema in the skin of guinea-pigs. Phenylbutazone is considered to be among the most potent anti-rheumatoid drugs at present in therapeutic use.

The concentrations of analgesic-antipyretic drugs which we have found necessary to inhibit kallikrein bear a similarity to the blood levels of these substances which Goodman & Gilman (1955) have reported to be necessary for a significant anti-rheumatoid effect in man (approximately 350 μg/ml. in the case of salicylate). The present series of experiments do not exclude the possibility that a significant part of the anti-inflammatory action of the analgesic-antipyretic drugs is caused by the inhibition of plasma kinin rather than by the inhibition of the plasma kinin-forming enzymes. Other workers have previously shown that various pharmacological actions of bradykinin can be inhibited by analgesic-antipyretic drugs. Thus Collier & Shorley (1960) have demonstrated a specific antagonism between the broncho-constrictor action of bradykinin and various salicylates in the anaesthetized guinea-pig, using doses appreciably less than were found to be necessary, for example, in our experiments with dogs and rabbits. It has even been claimed that in very high doses salicylate will inhibit the contraction of the isolated guinea-pig ileum to histamine (Mongar & Schild, 1957). Lecomte (1960) has shown an antagonism between large doses of phenylbutazone and the cardiovascular and capillary permeability enhancing actions of bradykinin in the rabbit. In our experience, however, based upon the use of three independent test preparations, the evidence seems to indicate that the inhibition of the kinin-forming enzyme systems takes place in a lower concentration of analgesic-antipyretic drug than is required to inhibit the actions of the plasma kinin itself, and hence may more probably explain the anti-inflammatory action of this class of compounds.

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