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THE ACTION OF SODIUM SALICYLATE AND ASPIRIN ON SOME KALLIKREIN SYSTEMS

BY

P. HEBBORN* AND B. SHAW

From the Research Department, Boots Pure Drug Co., Nottingham

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The effects of sodium salicylate and of aspirin on the actions of pancreatic, salivary and serum kallikreins have been investigated. Kinin production by the three enzymes was assessed using a guinea-pig isolated ileum preparation. The esterolytic activity of pancreatic and salivary kallikreins was measured by determining acid release from the synthetic substrate toluene-*p*-sulphonyl-L-arginine methyl ester. Sodium salicylate (up to 20 mM) or aspirin (up to 5 mM) failed to inhibit kinin production by each of the three enzymes. With salivary kallikrein, a concentration of 50 mM of sodium salicylate was required to produce a 50% inhibition of kinin production. No significant inhibition of esterolytic activity was produced by concentrations up to 5 mM of either sodium salicylate or aspirin. Prior incubation of sodium salcylate or aspirin with the enzymes also resulted in no significant effect of either drug on kinin production or esterolytic activity. Prior incubation of pancreatic or salivary kallikrein with the inhibitor from ox parotid gland (Trasylol) reduced both kinin production and esterolytic activity. Toluene-*p*-sulphonyl-L-arginine methyl ester (0.5 mM) inhibited kinin production by each of the three enzymes. It is concluded that sodium salicylate and aspirin are poor inhibitors of kallikrein activity *in vitro*.

It has been suggested that bradykinin may have some role in inflammatory reactions (Hilton & Lewis, 1955), and there is evidence that enzyme systems which form kinins participate in such reactions (Edery & Lewis, 1962). Anti-inflammatory drugs may act against bradykinin either by direct antagonism or by inhibiting the production of bradykinin by the various enzyme systems which are thought to produce bradykinin or polypeptides closely related to it (Lewis, 1960). Collier & Shorley (1960) have demonstrated antagonism by aspirin and phenylbutazone of bronchoconstriction induced by bradykinin in guinea-pigs. However, Lewis (1961) claims that " aspirin does not antagonize those actions of bradykinin which would be involved in inflammatory reactions."

In a recent paper Northover & Subramanian (1961) have claimed that sodium salicylate, aspirin, and phenylbutazone are inhibitors of serum and salivary kallikreins. Work already in progress on pancreatic kallikrein in our laboratories indicated little or no inhibition by these drugs. These studies have now been extended to include serum and salivary kallikreins and are reported here.

* Present address: Department of Medicinal Chemistry, School of Pharmacy, University of Buffalo, Buffalo, N.Y., U.S.A.

METHODS

Pharmacological assay

The amount of kinin produced by the action of kallikrein on serum globulins was assayed with the guinea-pig isolated ileum preparation. A segment of the terminal ileum was suspended in a 10 ml. bath of aerated Tyrode solution at 35° C and isotonic contractions were recorded. A standard bradykinin solution or aliquots of the test systems were added to the bath at 4 min intervals and remained in contact with the ileum for 1 min.

Biochemical assay

The esterolytic activity of kallikrein was followed by measuring the release of free carboxyl groups from the synthetic substrate toluene-*p*-sulphonyl-L-arginine methyl ester using the method of Troll, Sherry & Wachman (1954). The ester (final concentration 0.020 M) and enzyme were incubated at 37° C in buffer [2-amino-2-(hydroxymethyl)propane-1,3-diol hydrochloride (tris), final concentration 0.2 M, *p*H 8.5] and aliquots (1.0 ml.) removed at suitable intervals (0, 15, 30 and 45 min). Each aliquot was added immediately to formaldehyde solution (1 ml. of 37% formaldehyde, w/v) previously adjusted to *p*H 8.0 with 0.05 N NaOH. Carboxyl groups were estimated by titration of this mixture with 0.05 N NaOH using phenol red (0.2%) as indicator. Controls were run either by omitting the enzyme or by using boiled enzyme. Acid release was calculated by difference.

Use of drugs

The drugs were either dissolved in buffer or adjusted to the appropriate *p*H in aqueous solution and a check made to ensure that addition of a drug caused no *p*H shift in the final incubation mixture. Kinin production in the pharmacological assay and acid release in the biochemical assay were compared in the presence and absence of drug. Controls showed that drugs did not interfere with either assay.

Substrates

For the pharmacological assay, the substrate for salivary and pancreatic kallikrein was prepared from ox serum treated as follows:

(*a*) Glass-contaminated serum, poor in platelets, was obtained by collecting ox blood from a cut jugular vein, allowing the blood to clot at room temperature and centrifuging for 30 min at 2,000 *g* immediately afterwards. The cell-free serum was heated at 56° C for 3 hr, dialysed against distilled water at 4° C for 48 hr, and stored in small volumes in glass vessels at 4° C until used. Each individual sample was used once only. No decrease in activity was noted for serum stored in this manner for 3 weeks, and no serum was used after such a storage period had elapsed.

(*b*) Ox blood was taken from a jugular vein through a siliconed needle and collected in polyethylene vessels to reduce platelet destruction (Kugelmass, 1959). The blood was allowed to clot at 4° C and serum decanted from the contracted clot 3 hr later. To obtain sera which were relatively rich and poor in platelets the differential centrifugation technique of Biggs & Macfarlane (1957) was used. Serum was centrifuged in polypropylene tubes at 80 *g* for 30 min to give serum rich in platelets or at 1,400 *g* for 30 min to give serum poor in platelets. After heating and dialysis as described above, small aliquots were stored in polyethylene vessels at −15° C until required for use. Thawed samples were not re-frozen.

Toluene-*p*-sulphonyl-L-arginine methyl ester was prepared by the method of Troll *et al.* (1954) and recrystallized until pure (confirmed by chemical analysis and by determination of melting point) or obtained commercially (Californian Corporation for Biochemical Research) and used without further purification.

Enzymes

Serum kallikrein was activated by diluting (1:10) unheated, dialysed ox serum with 0.85% saline solution. Salivary kallikrein was obtained by diluting (1:10) fresh human saliva with

0.85% saline solution. Pancreatic kallikrein (Glumorin) was a lyophilized powder obtained commercially (Farbenfabriken Bayer A.G.).

Other materials

Bradykinin was prepared and partially purified (Stage P2) by the method of Elliott, Horton & Lewis (1961) and was 1/400th as potent as synthetic bradykinin. Synthetic bradykinin was kindly supplied by Sandoz Products, London. Sodium salicylate and aspirin (Boots Pure Drug Co.) were of B.P. quality and were not further purified. Kallikrein inhibitor from ox parotid gland (Trasylol) was obtained commercially (Farbenfabriken Bayer A.G.). 2-Amino-2-(hydroxymethyl)propane-1,3-diol hydrochloride (tris) and formaldehyde solution were both commercial preparations (B.D.H., Laboratory Reagent and A.R. grades respectively).

RESULTS

The effect of salicylate on kinin formation

Serum kallikrein

Dialysed ox serum was diluted (1:10) with two volumes of 0.2 M 2-amino-2-(hydroxymethyl)propane-1,3-diol hydrochloride buffer, *p*H 7.8, and seven volumes of 0.85% saline solution and incubated at 37° C for 5 min. Aliquots of 0.5 to 1.0 ml. added to the organ bath produced contractions of the ileum which were submaximal and reproducible. The amount of kinin in the aliquot was assayed against a standard bradykinin solution (using either synthetic bradykinin or natural bradykinin prepared from ox globulin). This was compared with the kinin content of an aliquot of serum diluted with buffer, saline and sodium salicylate solution and incubated under identical conditions. After each comparison between control and test solutions the response of the ileum to the standard bradykinin solution was again determined to demonstrate that the sensitivity of the preparation had not altered during the experiment. Undiluted serum added directly to the bath produced little or no contraction during the 1 min of contact.

Fig. 1 shows that no inhibition of kinin production was apparent in the presence of sodium salicylate at 5 mM final concentration in the incubation mixture. Similar results were obtained with 1 mM sodium salicylate and with 1 mM and 5 mM aspirin. The latter concentration is ten times that claimed by Northover & Subramanian (1961) to cause 50% inhibition of kinin production and is approximately twice the blood concentration reached during anti-rheumatic therapy.

The effect of sodium salicylate on the inactive kallikrein precursor and on serum kininogen was determined by incubating one volume of ox serum with an equal volume of sodium salicylate solution for 30 min at 37° C, adding eight volumes of saline and incubating for a further 5 min at 37° C. Ox serum diluted with an equal volume of saline instead of salicylate solution was treated similarly. Prior incubation in the presence of an equal volume of a 50 mM solution of sodium salicylate did not affect the amount of kinin produced on subsequent activation of the kallikreinogen by dilution (Fig. 2).

A similar lack of inhibition by sodium salicylate was obtained whether kallikrein was derived from ox serum or from citrated plasma. Variation of platelet concentration and storage in plastic or glass did not affect this result.

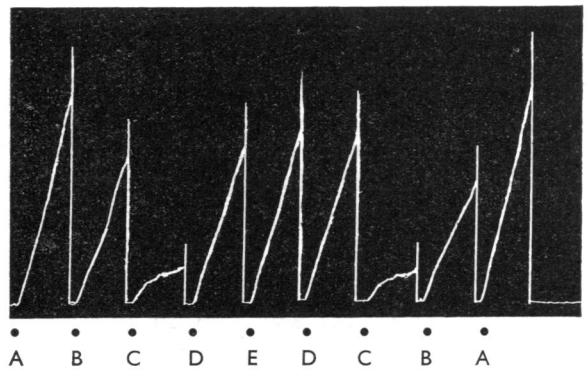

Fig. 1. Effect of sodium salicylate on kinin production by serum kallikrein. Contractions of guinea-pig isolated ileum to standard bradykinin solution (prepared from partially-purified bradykinin isolated from ox globulins): 15 μg at A, 10 μg at B and 5 μg at C. Contractions to: D, 0.5 ml. of solution of guinea-pig serum diluted 1 : 10 with buffered saline and incubated at 37° C for 5 min; E, the same with sodium salicylate, 5 mM final concentration. Contact time, 60 sec.

Salivary kallikrein

Heated, dialysed, ox serum (0.1 to 0.2 ml.) and saliva were incubated together in phosphate buffer, *p*H 7.0, or in 2-amino-2-(hydroxymethyl)propane-1,3-diol buffer, *p*H 7.8. Water or sodium salicylate solution was added to give a final volume of 1 ml. In the incubation mixture, the final buffer concentration was 40 mM and the saliva dilution was 1 : 50. After incubation at 37° C for 5 min, 0.5 ml. of the mixture was removed and assayed for kinin content. Neither serum nor saliva incubated separately under similar conditions contracted the gut.

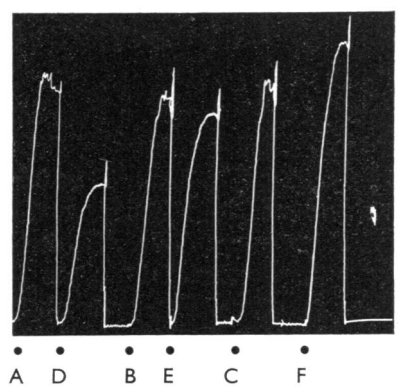

Fig. 2. Effect of prior incubation of serum kallikreinogen and kininogen with sodium salicylate. Contractions of guinea-pig ileum to: A, 0.5 ml. of a solution of guinea-pig serum diluted 1 : 10 with saline and incubated at 37° C for 5 min; B, serum diluted 1 : 2 with saline, incubated at 37° C for 30 min and then treated as A; C, serum diluted 1 : 2 with sodium salicylate solution (final concentration 25 mM), incubated at 37° C for 30 min and then treated as A; D, E and F, standard bradykinin solution (prepared from partially-purified natural bradykinin) containing 5 μg, 10 μg and 15 μg respectively. Contact time, 60 sec.

No inhibition of kinin formation was observed with sodium salicylate in concentrations up to 20 mM. A 50% inhibition of kinin formation was obtained at 50 mM sodium salicylate, whilst 25 mM produced a 10% inhibition. Aspirin (1 and 5 mM) caused no inhibition.

Incubating the enzyme or substrate for 30 min at 37° C in the presence of 5 mM sodium salicylate followed by 5 min incubation of the complete system did not inhibit enzyme activity.

Pancreatic kallikrein

Pancreatic kallikrein (0.1 unit) was added to an incubation mixture similar to that described for salivary kallikrein and incubated for 5 min at 37° C with and without added sodium salicylate solution. No reduction in the amount of kinin produced could be demonstrated at concentrations of sodium salicylate up to 20 mM and of aspirin up to 5 mM. A similar result was obtained after first incubating the enzyme with sodium salicylate for 30 min at 37° C.

The effect of the substrate preparation

In order to see if the method of preparation of serum had any influence on the sensitivity of the system to the effect of salicylate, three different preparations of serum were used as substrates for salivary and pancreatic kallikreins (see " Methods " for details). Some serum was prepared in glass vessels and could have had constituents activated in the course of preparation. Therefore serum was also prepared in contact only with plastic surfaces. This serum was divided into two preparations, one containing platelets and the other relatively free of them. The differences in methods of preparation had no effect on the final results. Sodium salicylate (up to 10 mM) never inhibited kinin production.

Inhibition of kinin production

*Toluene-*p*-sulphonyl-*L*-arginine methyl ester*

The addition of this ester to the incubation mixture containing either pancreatic or salivary kallikrein caused an inhibition of kinin production which was dependent on the final concentration of the ester (Fig. 3, a). Thus 0.5 mM toluene-*p*-sulphonyl-L-arginine methyl ester produced an 80% inhibition and 0.1 mM a 10% inhibition in systems containing 0.1 ml. of ox serum. This inhibition is competitive (Chapman, Goodell & Wolff, 1959) and the degree of inhibition observed varied with the amount of ox serum used as substrate. Similar results were obtained for serum kallikrein.

Kallikrein inhibitor (Trasylol)

The kallikrein inhibitor from ox parotid gland (Trasylol, 1 unit), added to the incubation mixture containing pancreatic kallikrein (0.1 unit) or an equivalent amount of salivary kallikrein and incubated for 5 min, produced little or no inhibition of kinin production. Incubation of enzyme and inhibitor for 30 min before adding the substrate completely inhibited kinin formation. The kallikrein inhibitor did

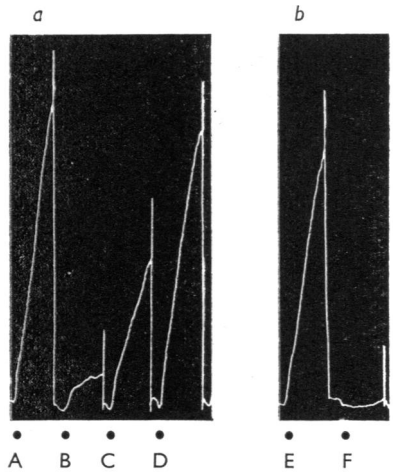

Fig. 3. Effect of inhibitors of kinin formation. *a*, Inhibitory effect of toluene-*p*-sulphonyl-L-arginine methyl ester on kinin formation by pancreatic kallikrein. Contractions of guinea-pig ileum to pancreatic kallikrein incubated with serum substrate at 37° C for 5 min. Final concentrations of the arginine ester in the incubation mixture: nil at A, 0.5 mM at B, 0.25 mM at C and 0.1 mM at D. *b*, Inhibitory effect of kallikrein inhibitor (Trasylol) on salivary kallikrein. Contractions of guinea-pig ileum to: E, saliva incubated for 30 min at 37° C followed by 5 min incubation with buffer and serum substrate; F, as E, but saliva first incubated with 1 unit of kallikrein inhibitor. Contact time, 60 sec.

not reduce the activity of preformed kinin, nor did it affect the response of the ileum to standard bradykinin solutions. The effect on salivary kallikrein is illustrated in Fig. 3, b. With serum kallikrein, prior incubation of the inactive precursor with the kallikrein inhibitor followed by activation of the enzyme resulted in no inhibition of enzyme activity.

The effect of salicylate on esterolytic activity of kallikreins

Pancreatic kallikrein

The spectrophotometric method for following hydrolysis of arginine esters (Werle & Kaufmann-Boetsch, 1960) could not be used in the present series of experiments because of interference due to light absorption by the drugs employed.

The effect of sodium salicylate and aspirin on the esterolytic activity of kallikrein was investigated with a system in which toluene-*p*-sulphonyl-L-arginine methyl ester was hydrolysed by pancreatic kallikrein. Buffer present in the system ensured that the *p*H of the mixture did not change during the experiment. The release of acid from the substrate by the enzyme followed a linear course over the period 0 to 45 min. Linear regression coefficients relating acid release and time were calculated for each pair of experiments in which hydrolysis in the absence and presence of drug was measured. The effect, E, due to the drug was calculated from the equation:

$$E(\%) = \frac{\text{regression coefficient of drug} - \text{regression coefficient of control}}{\text{regression coefficient of control}} \times 100$$

A negative value of E indicated inhibition and a positive value activation. The results are given in Table 1.

TABLE 1

EFFECT OF SODIUM SALICYLATE, ASPIRIN AND KALLIKREIN INHIBITOR (TRASYLOL) ON ESTERASE ACTIVITY OF PANCREATIC KALLIKREIN AGAINST TOLUENE-*p*-SULPHONYL-L-ARGININE METHYL ESTER

Differences due to drugs shown as percentages ± s.e. of means. Numbers of experiments shown in parentheses. +activation, −inhibition

	No prior incubation. Final concentration in mM				Prior incubation at 37° C for 1.25 hr. Final concentration in mM			
	0·1	0·5	1·0	5·0	0·05	0·1	0·5	1·0
Sodium salicylate	+3 ±2 (3)	−13 ±12 (10)	−6 ±10·5 (6)	+21 ±11·4 (5)	—	+19 ±7 (4)	−14 ±10·7 (9)	+4 ±11·4 (5)
Aspirin	—	+21 ±11 (9)	−16 ±5 (7)	+5 ±21 (7)	−11 ±10 (3)	—	+11 ±12·8 (8)	−5 ±14 (7)
Trasylol	−15±21·8 (8)				−62±7·2 (11)			

To show the effect of a known inhibitor of pancreatic kallikrein, the inhibitor from ox parotid gland (Trasylol) was used. As can be seen from Table 1, when equal amounts of enzyme and inhibitor were first incubated at 37° C for 1.25 hr before adding substrate, 62% inhibition occurred. Without prior incubation very little effect on enzymic activity occurred. The effects of both sodium salicylate and aspirin were tested with or without prior incubation, the effects of which were slight.

Technical difficulties inherent in the titration procedure were mainly concerned with the visual assessment of the colour change of the phenol red indicator. Also, the titrations involved the use of small volumes of alkali, which were measured using an "Agla" micrometer syringe. The combination of these factors led to unavoidable variations in titration values and consequently in linear regression coefficients which are reflected in the standard errors given in Table 1. Such variations meant that experimental points did not always lie on a straight line. This type of variation is well illustrated in the typical titration curves in Fig. 4. However, it was clear that with the method of calculation it was possible to detect inhibition of kallikrein activity by this system. The results in Table 1 show that neither sodium salicylate nor aspirin greatly inhibited esterolytic activity when they were used in concentrations up to 5 mM.

Illustrations of typical individual pairs of determinations are given in Fig. 4. In Fig. 4, A, inhibition by the kallikrein inhibitor (Trasylol) is shown and in Fig. 4, B, the negative effect of sodium salicylate (0.5 mM).

Salivary kallikrein

Fresh saliva was collected and centrifuged at 1,800 *g* for 5 min to separate debris. The supernatant liquid was used as a source of salivary kallikrein. The esterolytic activity of the samples of saliva varied and was always very low compared with the purified pancreatic kallikrein; relatively large volumes had to be used to give enough hydrolysis products to be measured titrimetrically (4 ml. of saliva plus 1 ml. of 0.025 M toluene-*p*-sulphonyl-L-arginine methyl ester). Several samples of saliva did not give a linear rate of acid release and were therefore discarded.

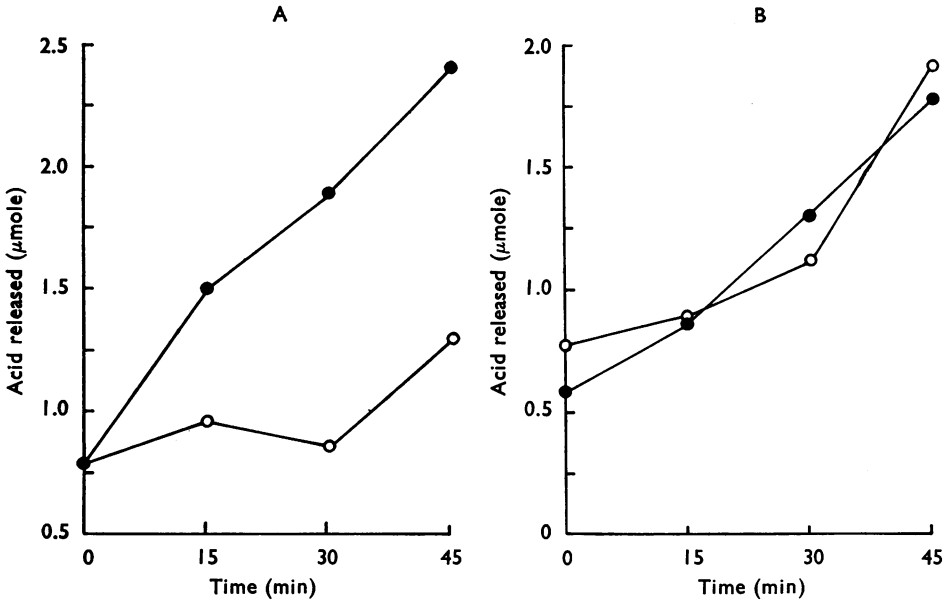

Fig. 4. The effect of kallikrein inhibitor (Trasylol) and of sodium salicylate on the hydrolysis of toluene-*p*-sulphonyl-L-arginine methyl ester by pancreatic kallikrein. Both compounds were first incubated with the enzyme at 37° C for 1.25 hr. A, kallikrein inhibitor; B, sodium salicylate (0.5 mM). ● — ● Enzyme alone, ○ — ○ enzyme plus drug.

The results were as follows: with no prior incubation, 5 mM sodium salicylate gave 9% activation (s.e. of mean = ±19, 7 pairs) and 5 mM aspirin caused 18% inhibition (s.e. of mean = ±27.5, 4 pairs); after 1.25 hr of prior incubation, 5 mM aspirin resulted in 35% inhibition (s.e. of mean = ±12, 3 pairs). These results indicate that at 5 mM concentration neither sodium salicylate nor aspirin greatly inhibited salivary kallikrein activity. Within the limits of the large standard errors these findings are in general agreement with those obtained using the pharmacological assay.

DISCUSSION

The release of kinins in physiological systems may be the result of either proteolytic or esterolytic action (Lewis, 1961). Evidence indicates a close parallel between the esterase activity of kallikreins and the pharmacological activity associated with the release of kinins by these enzymes. Werle & Kaufmann-Boetsch (1960), using salivary and pancreatic kallikrein, compared esterolytic activity against benzoylarginine ethyl ester with pharmacological activity, measured by blood-pressure effects in the anaesthetized dog. A close correlation between these activities was shown. Furthermore the kallikrein inhibitor from ox parotid gland (Trasylol) suppressed to the same degree the hypotensive and the ester-splitting action of the kallikreins. Moreover, benzoylarginine ethyl ester inhibited the release of kinin from serum by salivary kallikrein.

Chapman *et al.* (1959) have also reported similar findings, while Habermann (1961) correlated the pharmacological and enzymic effects of pancreatic kallikrein and several snake venoms and confirmed the general concept of esterase release of

kinin as proposed by Werle & Kaufmann-Boetsch (1960) and, earlier, by Hamburg & Rocha e Silva (1957). Webster & Pierce (1961) have also shown that urinary, pancreatic and plasma kallikreins are all capable of splitting arginine esters, with comparable activities.

In view of such evidence we considered it reasonable to compare the effects of sodium salicylate and of aspirin on the esterolytic activity of pancreatic and salivary kallikreins on an arginine ester (toluene-*p*-sulphonyl-L-arginine methyl ester) with the effect on their kinin-forming activities. The results indicate that this assumption of parallel effects was justified. Neither sodium salicylate nor aspirin inhibited kinin formation at concentrations up to 5 mM, and similarly the hydrolysis of the synthetic ester was little affected by these compounds at concentrations over the range 0.1 to 5 mM. It was not possible to test the esterolytic activity of serum kallikrein against the synthetic substrate, but sodium salicylate at concentrations up to 20 mM did not inhibit kinin formation. This concentration of sodium salicylate was also ineffective on kinin production by pancreatic and salivary kallikreins.

An inhibitor of kallikrein (Trasylol) inhibited both pharmacological and biochemical effects. In the pharmacological system it completely inhibited the production of kinin by pancreatic and salivary kallikrein. In the biochemical system the inhibitor greatly decreased esterase activity after prior incubation with pancreatic kallikrein. In the pharmacological system, 0.5 mM toluene-*p*-sulphonyl-L-arginine methyl ester almost completely inhibited kinin production. This confirmed the work of Werle & Kaufmann-Boetsch (1960) and of Chapman *et al.* (1959). In the same system a concentration of 50 mM sodium salicylate produced only a 50% inhibition.

The results obtained with kallikrein inhibitor and the arginine ester suggest that, by comparison, sodium salicylate and aspirin are poor inhibitors of kinin production. By contrast, Northover & Subramanian (1961) reported that serum and salivary kallikrein activity was 50% inhibited by aspirin or sodium salicylate at concentrations less than 1 mM.

Differences between the results of these authors and of ourselves may have been due to differences in serum preparation. Margolis (1958) has pointed out that activation by glass might be an important factor in the production of kinin in serum. Our results were the same whether serum was collected in plastic or glass. Alexander (1953) has shown that platelets contain a substantial amount of esterase activity. However, variation of the platelet concentration in serum had no effect upon the results, which all suggested little action on kinin production by sodium salicylate or by aspirin up to a concentration of 5 mM. With serum collected in glass a final concentration of sodium salicylate of about 50 mM was required for 50% inhibition. These results differ considerably from those of Northover & Subramanian (1961) and at the moment we can offer no explanation for these differences.

In view of such conflicting evidence we consider that the basis of the anti-inflammatory action of sodium salicylate and aspirin remains still a matter for conjecture.

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