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INHIBITORY EFFECTS OF THIOL COMPOUNDS ON KALLIKREIN AND ON EXPERIMENTAL BURNS IN GUINEA-PIGS

BY

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Fractionation of guinea-pig serum by chromatography on diethylaminoethyl cellulose revealed that the 7 S γ-globulin fraction was capable of inducing an increase in capillary permeability when injected into guinea-pig skin. The inhibition of these permeability effects by Soya Bean Trypsin Inhibitor and Dyflos (diisopropylfluorophosphate) suggested two possibilities: either the γ-globulin containing an enzyme which met its substrate in the skin, or the γ-globulin was a substrate which was acted upon by an enzyme in the skin (Davies & Lowe, 1961). Subsequently it was shown that the active γ-globulin fractions contained an enzyme with the properties of serum kallikrein, suggesting that the actual mediator of increased permeability under these circumstances was a kinin (Davies & Lowe, 1963). However, during an investigation of the alternative hypothesis (that the γ-globulin was a substrate), guinea-pig γ-globulin was treated with various enzymes in attempts to identify active products of the reaction. None was found, but in one of these experiments γ-globulin was treated with papain in the presence of a thiol-containing reagent. Two control tubes were included, one containing the thiol compound with γ-globulin and the other, γ-globulin alone. When these mixtures were injected into the skin of the guinea-pig it was found that this treatment with thiol had markedly reduced the permeability activity of the γ-globulin. We have therefore investigated this inhibitory effect of thiols on the kallikrein activity of guinea-pig γ-globulin and also their effect on the oedema resulting from thermal injury in the guinea-pig.

METHODS

Isolation of γ-globulins

The 7 S γ-globulin fraction was isolated from fresh guinea-pig serum by chromatography on diethylaminoethyl cellulose as described previously (Davies & Lowe, 1961). This material was used as the source of kallikrein.

Determination of esterase activity

The modification of the Hestrin procedure described by Roberts (1958) was used to measure esterase activity. *p*-Tosyl-L-arginine methyl ester (1 ml. 0.06 M) was used as substrate and all determinations were made at *p*H 8.6 in 0.2 M tris-(hydroxymethyl)-amino-methane buffer.

Preparation of crude guinea-pig kininogen

Fresh guinea-pig serum was heated at 56° for three hours to destroy peptidase activity. The heated serum was then centrifuged and the supernatant dialysed against barbiturate buffer (*p*H 8.6; $\mu=0.03$). The heated, dialysed serum was separated by zone electrophoresis on ethanolysed cellulose in an apparatus based on that of Porath (1956) as modified by Lowe (1964). The fractions were identified by paper electrophoresis and the individual α-globulin fractions were assayed for kininogen content by the addition of 1 ml. kallikrein solution (1 mg/ml.) to 1 ml. each fraction followed by incubation for 30 min at 37°. Aliquots were added to two volumes of boiling ethanol, boiled for 2 min, cooled, centrifuged and the supernatant evaporated to dryness in a Büchi Rotovapor. The residue was dissolved in water and assayed on the isolated rat uterus preparation. Fractions containing kininogen were mixed, dialysed and freeze-dried.

Determination of increased capillary permeability

Effects on capillary permeability were determined by the injection of 0.1 ml. amounts into the skin of guinea-pigs previously "blued" by intravenous injection of Pontamine Sky Blue, as described previously (Davies & Lowe, 1961, 1963).

Buffer

The buffer used for diluting material for skin tests was prepared by dissolving 5.7 g of barbitone in 500 ml. of hot distilled water, adding 85 g of NaCl and 3.75 g of sodium barbitone and making up to 2 l. with distilled water. The buffer was diluted 1:5 with distilled water on the day of use.

Determination of kinin activity

Kinin activity was assayed on the isolated rat uterus suspended in oxygenated de Jalon solution of the following composition: NaCl 45 g; KCl 2.1 g; $CaCl_2$ 0.1 g; $NaHCO_3$ 2.5 g; glucose 2.5 g distilled water to 5 l.

Experimental burns

Guinea-pigs were anaesthetized with ether and one hind-foot was immersed for 20 sec in water at 55°. The development of oedema was followed by measuring with a micrometer across a sagittal section before immersion and at various intervals thereafter.

The percentage inhibition at given times after immersion was calculated as follows:

$$\%\ \text{inhibition at time } t = \frac{\text{Mean increase in size of controls} - \text{Mean increase in size of treated} \times 100}{\text{Mean increase in size of controls}}$$

Groups of four animals were used. The 95% confidence limits for an inhibitory effect were roughly ±30%.

RESULTS

Permeability effects of kallikrein

In general, only the results obtained with cysteine are quoted, but similar effects were found with β-mercaptoethanol, β-mercaptoethylamine, penicillamine and glutathione No effects were shown by cystine, ascorbic acid or hydrazine.

When a freshly prepared mixture of cysteine (1 mg/ml.) and kallikrein (1 mg/ml.) was injected into guinea-pig skin, the permeability-increasing activity of kallikrein was unimpaired but the activity decreased as the period of incubation of cysteine with kallikrein increased (Table 1). Table 2 shows the effects with various concentrations of cysteine.

TABLE 1

INHIBITION BY CYSTEINE OF THE EFFECT OF KALLIKREIN ON CAPILLARY PERMEABILITY (VARIATION IN PERIOD OF INCUBATION)

Period of incubation of cysteine with kallikrein (min)	Mean lesion diameter (mm)	S.E.±
0	11·5	0·2
5	11·5	0·2
20	5·2	0·1
30	3·0	0·06
Kallikrein (0·5 mg/ml.) (no cysteine)	11·4	0·2

Cysteine (1 mg/ml.) incubated with kallikrein (1 mg/ml.): 0·1 ml. injected. 8 animals per group.

TABLE 2

INHIBITION BY CYSTEINE OF THE EFFECT OF KALLIKREIN ON CAPILLARY PERMEABILITY (VARIATION IN CONCENTRATION OF CYSTEINE)

Concentration of cysteine (mg/ml.) incubated with Kallikrein (1 mg/ml.) for 30 min.	Mean lesion diameter (mm)	S.E.±
1·0	7·0	0·1
0·5	5·0	0·05
0·1	8·9	0·5
0	10·2	0·8

8 animals per group

Neither cysteine, nor any of the other thiol compounds, had any effect on increased capillary permeability produced by the injection of histamine, bradykinin, compound 48/80 or crystalline trypsin, either when mixed with the injected material or given to the animals orally or parenterally.

Kinin-releasing effects of kallikrein

Solutions of kallikrein (1 mg/ml.) were incubated for 30 min with equal volumes of either cysteine or de Jalon solution. Aliquots were then incubated for 30 min with crude kininogen (5 mg/ml.); the kinin was extracted with boiling ethanol, the ethanol removed and the residue dissolved in water. The kinin activity was assayed on the isolated rat uterus. Treatment of kallikrein with cysteine almost completely abolished its ability to liberate kinin from kininogen (Table 3). Cysteine, at the concentrations used in the experiments, did not itself alter the response of the rat uterus to kinin.

TABLE 3

INHIBITION BY CYSTEINE OF THE KININ-RELEASING EFFECT OF KALLIKREIN

Incubation mixture	Height of contraction of rat uterus produced by 0·1 ml. alcoholic extract (mm)
Control kallikrein+ kininogen	34
Cysteine-treated kallikrein+kininogen	7
Kallikrein only	0
Kininogen only	0

1 ml. kallikrein solution (1 mg/ml.) incubated for 30 min with 1 ml. kininogen solution (5 mg/ml.) and 1 ml. either cysteine solution (1 mg/ml.) or de Jalon's solution. Mixtures then poured into boiling ethanol and centrifuged. Ethanol extract evaporated and residue dissolved in water (1 ml.).

Esterase activity of kallikrein

A solution of kallikrein (1 mg/ml.) was mixed with an equal volume of cysteine (1 mg/ml.) and allowed to stand at room temperature for 30 min. Both solutions were prepared in 0.2 M tris buffer. A control mixture contained kallikrein with an equal volume of buffer. The presence of cysteine caused no diminution in the ability of kallikrein to hydrolyse *p*-tosyl-L-arginine methyl ester (58.4 μmole TAMe/hr/mg in the absence of cysteine and 57.4 μmole TAMe/hr/mg in its presence).

Similar conditions had resulted in complete inhibition of the permeability activity of kallikrein (Table 1), therefore attempts were made to explain this discrepancy. The conditions of the two experiments were not quite the same, for example "tris" buffer had been used as diluent in the esterase determination and barbiturate buffer in the permeability test.

The experiment was therefore repeated with both cysteine and β-mercaptoethanol diluted in barbiturate buffer. They were mixed with a solution of kallikrein in saline and allowed to stand at room temperature for 30 min. Aliquots (1 ml.) were then added to tris buffer (1 ml.) containing *p*-tosyl-L-arginine methyl ester (0.06 M; 1 ml.) while other portions were used in the skin test.

Cysteine again completely inhibited permeability activity but had no effect on esterase activity. β-mercaptoethanol also completely inhibited permeability activity but had a weak inhibitory effect on esterase activity (Table 4).

TABLE 4

THE ACTION OF CYSTEINE AND β-MERCAPTOETHANOL ON THE ESTERASE AND CAPILLARY PERMEABILITY-INCREASING ACTIVITY OF γ-GLOBULINS

Mixture	Esterase activity (μmoles TAMe/hr/mg)	% inhibition of esterase activity	Mean lesion diameter (mm)	% inhibition of lesion diameter
γ-globulin	58·0	—	10·1	—
γ-globulin + cysteine	58·3	0	5·2	100
γ-globulin + β-mercaptoethanol	49·2	14·9	4·1	100
Buffer alone	—	—	4·5	—

β-mercaptoethanol solution was 5·0 mg/ml. in barbiturate buffer. Cysteine solution was 1 mg/ml. in barbiturate buffer. γ-globulin was 0·8 mg/ml. in saline.

For the assays, γ-globulin (1 ml.) was added to each inhibitor (1 ml.) and allowed to stand 30 min at room temperature before assaying.

The cysteine and β-mercaptoethanol has been used at different concentrations, in fact, on a molar basis, the β-mercaptoethanol was about twice as concentrated as the cysteine. A further experiment was performed using cysteine at 5 mg/ml. final concentration. Once again, no loss of esterase activity resulted. When the cysteine concentration was raised to 50 mg/ml. about 20% inhibition resulted but this is not considered reliable since this higher concentration of cysteine caused some interference in the assay procedure.

When penicillamine was used in an equivalent molar amount to the β-mercaptoethanol 28% inhibition of the esterase activity occurred but when β-mercaptoethylamine was used no inhibition was produced.

TABLE 5
EFFECT OF CYSTEINE ON BURNS IN GUINEA-PIGS

Exp.	Dose (mg/kg)	Route	Time of dosing	% inhibition of oedema 1 hr	2 hr	5 hr
13563	200	iv.	10 min before	70	80	51
9563	200	iv.	10 min before	55	71	44
14563	100	iv.	10 min before	95	96	95
21563	25	iv.	10 min before	83	68	41
23563	5	iv.	10 min before	34	17	8
6663	25	iv.	10 min after	28	39	15
	25	iv.	30 min after	18	29	14
	25	iv.	60 min after	—	14	9
	25	iv.	10 min before	99	94	100

TABLE 6
INHIBITORY EFFECT OF ORALLY ADMINISTERED CYSTEINE ON BURNS IN THE FEET OF GUINEA-PIGS

Exp.	Dose (mg/kg)	% inhibition of oedema 1 hr	2 hr	5 hr
17763	200	98	88	98
23763	200	74	74	0
25763	200	97	83	34
23763	100	70	45	11
25763	100	72	46	13
23763	50	45	33	21
25763	50	31	29	28
23763	25	38	24	0

Dose given 30 min before burn.

Experimental burns

In the initial series of experiments, penicillamine, cysteinamine, β-mercaptoethanol and cysteine were given intravenously in a range of doses, ten min before immersion of the foot into hot water. Cysteine was the most active and the detailed results with this compound are shown in Table 5. Marked and long-lasting inhibition of the oedema was caused by doses as low as 25 mg/kg. Some inhibitory effect was evident even when the compound was given ten minutes after burning. Oral administration was also effective (Table 6).

DISCUSSION

The effect of kallikrein as a mediator of inflammation has not yet been demonstrated unequivocally, but that it plays such a role can not be doubted seriously (Davies & Lowe, 1963 ; Rocha e Silva & Rosenthal, 1961 ; Spector, Westall & Willoughby, 1962).

The present results lend further weight to this argument. Cysteine and other thiol compounds inhibited the permeability-increasing effects of kallikrein and also the prolonged oedema resulting from burning. There are several reports in the literature on the anti-inflammatory effects of thiol compounds. Lecomte & Bohrenstayn (1953) found the cysteinamine and cystinamine, injected locally, diminished the tuberculin reaction in man, and, given systemically, reduced the increased vascular permeability in rabbit skin which follows the application of chloroform. Coulon, Charlier & Vander-

missen (1954) showed that cysteinamine when given to rats injected peri-articularly with kaolin inhibited the later phases of the reaction but were without effect on the early, oedematous phase. Cession-Fossion, Lecomte & Franchimont (1962) attributed these anti-inflammatory effects to the hypotensive effects of cysteinamine. We feel that the effects on burns we have described are not due to hypotension, because of the specificity of the reaction cysteine inhibited the effects of kallikrein but not those of 48/80, bradykinin, histamine or trypsin. Several workers have shown that thiol compounds potentiate the effects of bradykinin both *in vitro* and *in vivo* (Picarelli, Henriques & Oliveira, 1962; Ferreira & Rocha e Silva, 1962; Erdös & Wohler, 1963). This potentiation appears to be caused by the binding of cobalt, which is a co-factor necessary for the activity of a kinin-destroying carboxypeptidase. There is some evidence which indicates that kinins are released during thermal injury (Rocha e Silva & Rosenthal, 1961). The inhibitory effect of cysteine on thermal oedema in guinea-pigs is consistent with the view that kallikrein is inhibited and hence no kinin is produced.

To postulate *in vivo* mechanisms solely from the effect of inhibitors is, of course, an unsure line of argument. Final proof will depend on the demonstration of liberation of kallikrein at the site of a burn and this has not yet been achieved. The *in vivo* effect of cysteine might well be due to some action other than an inhibition of kallikrein, we have not, for example, studied the effects of thiols on the liberation of corticosteroids in the guinea-pig but Van Cauwenberge (1956) found that, although cysteinamine and cystine depleted adrenal ascorbic acid in the rat, cysteine was much less effective.

The failure of thiols to inhibit the *in vitro* esterase activity of kallikrein is puzzling because we have prevously presented evidence relating the esterase and permeability activities of kallikrein (Davies & Lowe, 1963). Although this relationship may be fortuitous, other explanations for the apparent discrepancy can be put forward. Thiol containing compounds are known to reduce a critical disulphide linkage in the γ-globulin molecule (Palmer & Nisonoff, 1963). It is possible that destruction of this disulphide bond renders the kallikrein molecule incapable of binding to its natural substrate (kininogen). The change in the kallikrein molecule may, however, be insufficient to affect its binding to small molecular substrates such as *p*-tosyl-L-arginine methyl esters and hence these substrates are hydrolysed. There is a big difference in the molecular size of kininogen (molecular weight of purified bovine kininogen, according to Habermann 1963), is 48,000) and that of *p*-tosyl-L-arginine methyl ester (M. W. 342.4). This difference in molecular size supports the hypothesis that it is the change in the spatial arrangement of the kallikrein molecule brought about by the thiol compounds which accounts for the differential inhibition.

SUMMARY

1. Cysteine and other thiol-containing compounds inhibited the permeability-increasing and kinin-releasing activities of guinea-pig serum kallikren. Inhibition increased as the cysteine and kallikrein were incubated together, becoming maximal after 30 min. The esterase activity of kallikrein was not inhibited.

2. Administration of cysteine to guinea-pigs markedly reduced the oedema resulting from the immersion of one hind-foot in hot water.

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