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# Kinins and anti-inflammatory steroids

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1. This investigation was designed to re-examine the possibility that anti-inflammatory steroids interfere with the kinin-forming system.

2. We conclude that the anti-inflammatory action of corticosteroids cannot be explained by the inhibition of kinin formation. This view is based on the following findings.

3. Neither hydrocortisone nor prednisolone nor dexamethasone inhibited the activation or activity of intrinsic plasma kinin forming enzymes resulting from dilution, incubation with kininase inhibitors, or exposure to glass, monosodium urate microcrystals or to precipitated complexes of rheumatoid factor and aggregated human $\gamma$-globulin.

4. Hydrocortisone did not inhibit the action of the active kinin-forming enzymes, human salivary or urinary kallikrein, or plasmin on purified kininogen.

5. Hydrocortisone, prednisolone and dexamethasone did not inhibit the hydrolysis of benzoyl-arginine-ethyl ester by human plasmin, plasma kallikrein or hog pancreatic kallikrein.

6. Kinin formation occurred normally in plasma taken from two patients receiving betamethasone and one receiving prednisone.

The role of kinins in inflammatory reactions has been the subject of extensive discussion. Although the evidence is still incomplete, such a role is strongly suggested by the principal biological actions of kinins and by the potency and efficiency of the factors involved in the formation and inactivation of kinins. This has stimulated several studies on the possible effects of known anti-inflammatory compounds on the action of the peptides themselves and their formation. The actions of kinins themselves are not antagonized by non-steroidal anti-inflammatory drugs, (Lewis, 1963). Claims that aspirin and other anti-pyretics inhibit the activities of kinin-forming enzymes (Northover & Subramanian, 1961 ; Spector & Willoughby, 1963), were not confirmed by Lewis (1963) or by Hebborn & Shaw (1963). Moreover, these enzymes were also not inhibited by paramethasone (Davies, Holman, Johnston & Lowe, 1966).

There still remained the possibility that anti-inflammatory agents inhibited the activation rather than the activities of the endogenous plasma kinin-forming system. Cline & Melmon (1966) and Melmon & Cline (1967) have recently claimed that

hydrocortisone in a concentration as low as $10^{-6}$M suppresses the release of kinins by inhibiting both the activation and the action of the kinin-forming enzymes in plasma. In the present investigation the action of three gluco-corticoids (hydrocortisone, dexamethasone and prednisolone) was re-examined to determine whether these steroids can inhibit the activation of the kinin-forming system in plasma by glass, dilution, urate crystals and precipitated complexes of rheumatoid factor and aggregated γ-globulin. The effect of steroids on already active kinin-forming enzymes, such as kallikrein and plasmin, was also examined. The results demonstrate that even at high concentrations steroids had little or no effect on kinin activity or kinin formation.

## Methods

### *Steroid preparations*

Hydrocortisone sodium succinate (Solu-Cortef, Upjohn), and as pure salt: hydrocortisone alcohol (Sigma); prednisolone 21-phosphate (Codelsole, Merck, Sharpe & Dohme) and dexamethasone 21-phosphate (Decadron, Merck, Sharpe & Dohme).

### *Enzyme preparations*

Hog pancreatic kallikrein (210 Frey units/mg) kindly given by Bayer A. G., Leverkusen, Germany; salivary kallikrein (human saliva diluted 1:10 with saline); urinary kallikrein (5.7 Frey units/mg), kindly supplied by Dr. M. Webster; glycerol activated human plasmin 10.4 caseinolytic units/ml. (kindly given by the Michigan Department of Health).

### *Endogenous kinin-forming enzymes in plasma*

These were activated by five different procedures as follows:

(1) *Acid.* Non-contact human plasma (that is, not previously exposed to glass) 0.5 ml. was acidified to pH 2 by addition of N-HCl; after incubation for 10 or 20 min at 37° C it was neutralized with N-NaOH. Some samples were acidified immediately after collection and remained at pH 2 until tested. In some experiments, the re-neutralized plasma was incubated in 0.1 M-phosphate buffer at pH 7.4 with purified kininogen (see below) (0.5–1.0 mg) in the presence of the kininase inhibitor phenanthroline ($10^{-4}$ g/ml.). The total volume of this mixture was 2 ml. and at various time intervals samples of between 0.05 and 0.2 ml. were taken and assayed on the isolated rat uterus against a standard of synthetic bradykinin.

(2) *Dilution.* Human plasma was diluted with 4–15 parts of physiological saline. In some experiments *o*-phenanthroline $10^{-4}$M, disodium versenate $2 \times 10^{-3}$M or ε-amino caproic acid $10^{-3}$M were added to inhibit plasma kininase.

(3) *Glass contact.* Plasma was left in contact with 1 in. specimen tubes or with ballotini 1 g/ml. for 3–15 min as specified. Some experiments were performed in the presence of *o*-phenanthroline $10^{-4}$ g/ml. and of additional kininogen. Glass ballotini, (0.1 mm in diameter) were obtained from English Glass Co.

(4) *Monosodium urate microcrystals.* Equal parts of non-contact plasma and of phosphate buffer 0.1 M, pH 7.4, were agitated with urate crystals, 5 mg/ml. for 5–15 min. Monosodium urate crystals were prepared according to the method of Seegmiller, Malawista, & Howell (1962).

(5) *Rheumatoid factor/aggregated γ-globulin complexes (RF/aggr. γG).* Suspensions of complexes were incubated with 10 times diluted non-contact plasma or synovial exudate, so that the final concentration of complex protein was 50–300 μg/ml. Complexes of rheumatoid factor and aggregated γ-globulins were prepared by the method of Astorga & Bollet (1965), avoiding exposure to glass.

### *Kininogen*

The following kininogen preparations were used as substrates for the enzymes.

(1) Heated human plasma ; non-contact human plasma was heated at 56° C for 1 or 3 hr. Any kinin present was then removed by dialysis and denatured protein by centrifugation.

(2) Heated dog plasma ; non-contact dog plasma was heated at 56° C for 3 hr. This substrate showed little free kinin activity and negligible kininase activity. It was stored at − 10° C.

(3) Purified kininogen ; kininogen was purified from freshly collected human plasma according to the method of Brocklehurst & Mawer (1966). The method involved fractionation of plasma using a Sephadex G-50 column, and followed directly by a DEAE Sephadex column. The protein fraction eluted with 0.02 M-phosphate buffer and 0.16 M-NaCl was passed through a further Sephadex G-50 column. Incubation of 0.5 ml. of this kininogen for 30 min with trypsin 200 μg/ml. in 0.1 M-phosphate buffer pH 7.4 (total volume 2 ml.) produced 300–400 ng of bradykinin/mg of protein. Incubation of the kininogen in phosphate buffer without the trypsin failed to form bradykinin. Incubation of the kininogen with synthetic bradykinin in phosphate buffer caused no destruction of the bradykinin. In some preparations free kinins were detected but in such small amounts (less than 1 ng/ml.) that they did not interfere with the assays.

### *Esterolytic activity*

The hydrolysis of benzoyl-argine ethyl ester (BAEe) was measured at 37° C and pH 8.0 and at a wavelength of 253 mμ according to Schwert & Takenaka (1955). Corticosteroids display a strong absorbance at this wavelength so the experiments were performed in such a way that the light of the double beam spectrophotometer (Unicam, S.P.800) passed through two cells in the sample position. One cell contained kinin-forming enzyme, BAEe and buffer, the other only buffer. When the inhibition by a corticosteroid was investigated, the corticosteroid was present in the cell which contained enzyme BAEe and buffer. In the control experiments the corticosteroid was placed in the cell containing buffer only. The total optical density observed was similar in both experiments and the apparent rate of its change expressing the rate of enzymic activity was strictly comparable.

### *Assay of kinins*

Kinins were directly assayed either on the isolated rat uterus suspended in de Jalon's solution or on the isolated guinea-pig ileum suspended in Tyrode solution. Assays were carried against a standard of synthetic bradykinin triacetate (Sigma).

## Results

### *Kinin formation*

The kinin formation induced in human plasma by various procedures in the presence of hydrocortisone sodium succinate, prednisolone 21-phosphate or dexamethasone 21-phosphate is shown as a percentage of the kinin formation in absence of these steroids in Table 1. Each of these experiments was carried out on a different plasma. It is clear that the formation of plasma kinins resulting from dilution, incubation with kininase inhibitors, or from exposure to glass, monosodium urate microcrystals, or to RF/aggr γG-precipitates was not reduced by any of the steroids. Figure 1A illustrates the formation of kinin in human plasma induced by glass contact alone and in the presence of dexamethasone 0.2, 2 and $4 \times 10^{-4}$M. Kinin formation by plasmin or pancreatic kallikrein was also not diminished by the steroids. The experiment of Fig. 1B illustrates this for pancreatic kallikrein and dexamethasone. Corticosteroids appeared to inhibit kinin formation in six out of sixty-four experiments but this was probably due to errors inherent in these methods of activating the intrinsic plasma kinin-forming enzymes.

In the experiment in Table 2, kinin formation was induced in 1 ml. samples of plasma containing additional kininogen either by dilution, by incubation at pH 2 for 10 min or by salivary kallikrein. The effect of $10^{-6}$–$10^{-4}$M hydrocortisone sodium succinate on each mode of activation was observed and all experiments were repeated six times. The means and standard errors show that this anti-inflammatory steroid did not inhibit the activation of the endogenous plasma enzymes or the action of salivary kallikrein on purified kininogen.

In another series of experiments, one of which is illustrated in Fig. 2, substrate prepared from heated dog plasma was used to examine the effect of steroids on kinin formation by human urinary kallikrein. Fig. 2A shows the formation of kinins from this substrate. Hydrocortisone either dissolved in a little alcohol or

TABLE 1. *Effect of hydrocortisone, prednisolone and dexamethasone on kinin formation in human plasma*

| Mode of kinin formation | Concentration of steroid | | |
|---|---|---|---|
| | $2 \times 10^{-6}$M | $2 \times 10^{-5}$M | 2–4 $\times 10^{-4}$M |
| | Hydrocortisone sodium succinate | | |
| Dilution and kininase inhibitors | | 120 | 100, 100, 120 |
| Glass | | 100, 100, 110 | 115, 100, 50, 65 |
| Pancreatic kallikrein 20 mu./ml. | | | 100, 70 |
| Plasmin 0·05 caseinolytic units/ml. | | | 110 |
| | Prednisolone sodium phosphate | | |
| Dilution | 70 | 100 | |
| Glass | 110 | 50 | |
| | Dexamethasone sodium phosphate | | |
| Dilution and kininase inhibitors | 100, 95, 90, 90, 100, 100 | 80, 100, 70, 50, 95 100, 100, 100, 100 | 100, 100, 100, 70 70, 100, 100, 100 |
| Glass | 90, 100 | 90, 50, 60 | 110, 80, 100, 120 100 |
| Monosodium urate microcrystals | | 100 | 150 |
| RF/aggr γ | | 100 | 100 |
| Pancreatic kallikrein 20 mu./ml. | 180, 130, 100 | 100 | 100, 100 |
| Plasmin 0·1 caseinolytic units/ml. | 100 | 100 | 90 |

Values give kinin-formation as percentage of formation found in absence of steroid.

the water soluble sodium succinate in concentrations up to $10^{-3}$M did not reduce the kinin formation by urinary kallikrein in heated dog plasma substrate, as shown in Fig. 2B. High concentrations of hydrocortisone ($10^{-2}$M) depressed the response of the guinea-pig ileum and it is probable that this rather than true enzymic inhibition was responsible for the smaller responses in Fig. 2B. A further experiment illustrated in Fig. 2C shows that although kinin formation was not affected by hydrocortisone sodium succinate, kallikrein inhibitor Trasylol $10^{-4}$ g/ml.

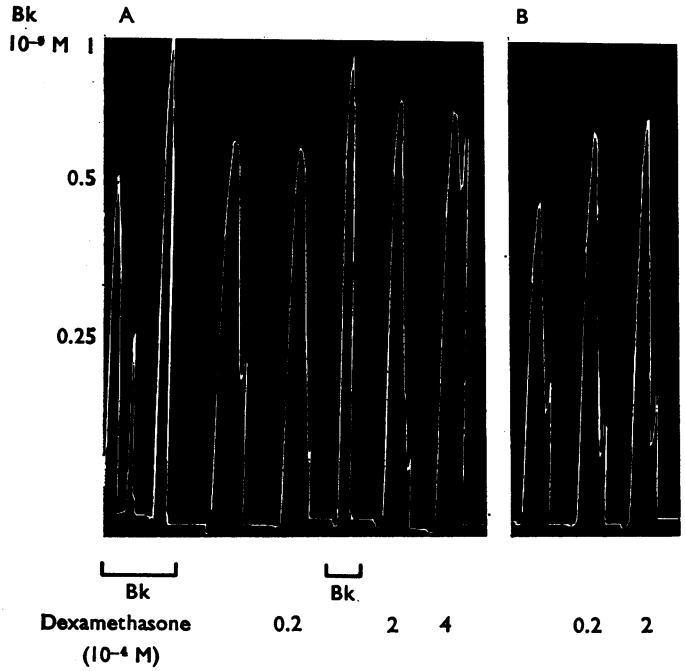


FIG. 1. Dexamethasone does not inhibit kinin formation induced in human plasma by glass contact or by pancreatic kallikrein. Rat uterus; 5 ml. bath. Bk, Bradykinin 0.25, 0.5 and $1 \times 10^{-9}$M. During the responses to bradykinin the drum was running at a slower speed. Dexamethasone in the concentrations specified was incubated at −15 min (A) with the plasma and (B) with pancreatic kallikrein. A: 0.15 ml. of plasma, 10 times diluted, was shaken in specimen tubes of 1 in. diameter from 0–5 min and then 0.5 ml. was tested. B: Heated plasma was incubated with pancreatic kallikrein 0.025 Frey units/ml. at 37° C from 0–5 min and then 0.1 ml. was tested.

TABLE 2. *Effect of hydrocortisone on kinin formation in fresh human plasma using purified human kininogen as substrate*

| Mode of kinin formation | Concentration of hydrocortisone sodium succinate | | | |
|---|---|---|---|---|
| | Nil | $10^{-6}$M | $10^{-5}$M | $10^{-4}$M |
| Dilution | 4·6 ±0·6 | 4·3 ±0·3 | 4·7 ±0·9 | 4·3 ±0·3 |
| Acidification | 2406 ±354 | 2390 ±128 | 2423 ±161 | 2321 ±559 |
| Salivary kallikrein | 390 ±245 | 520 ±312 | 599 ±312 | 535 ±282 |

Each value (ng bradykinin/ml. plasma) is the mean of six experiments (±standard errors) carried out with the same sample of plasma and kininogen.

completely prevented the formation of kinins. The kinin formation catalysed by human urinary kallikrein acting on heated human plasma was also not inhibited by the steroids.

### *Esterolytic activity*

Kinin-forming enzymes hydrolyse synthetic esters of substituted arginine. Pre-incubation of human plasmin, human plasma kallikrein and dog pancreatic

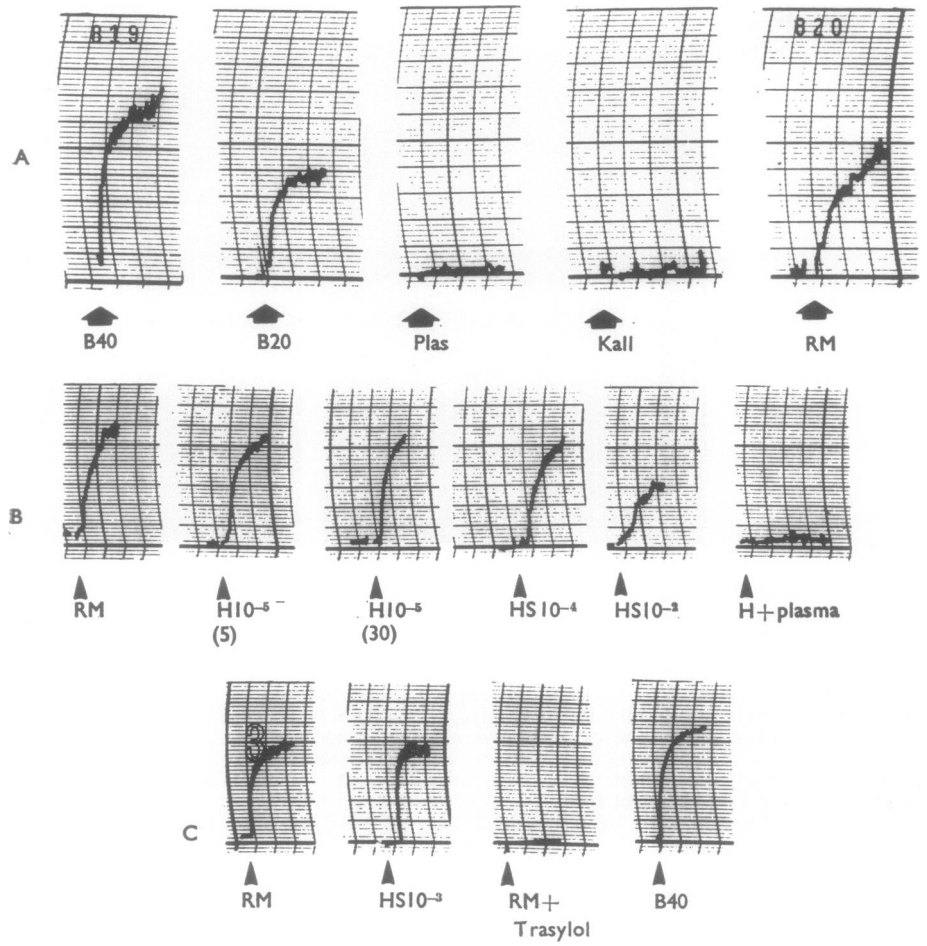


FIG. 2.A: Formation of kinin by human urinary kallikrein and dog plasma $B_{40}$ and $B_{20}$, 40 and 20 ng respectively of bradykinin added to the 5 ml. muscle bath containing the guinea-pig ileum; Plas, 0.4 ml. Tris buffer pH 8.0 and 0.1 ml. plasma incubated for 2 min at 37° C, 0.05 ml. added to the bath (no kallikrein); Kall, 0.4 ml. Tris and 0.05 ml. kallikrein, 0.1 ml. water (no substrate); RM, complete reaction mixture consisting of 0.4 ml. Tris buffer, 0.1 ml. plasma, 0.05 ml. of human urinary kallikrein (0.3 units). After 2 min at 37° C, a sample (0.05 ml.) was removed and added to the muscle bath. B: Failure of hydrocortisone to inhibit kinin formation by urinary kallikrein. RM, Reaction mixture as described above; $H10^{-5}$ (5); hydrocortisone (alcohol) at $10^{-5}$M preincubated for 5 min with substrate before adding the enzyme; $H10^{-5}$ (30), preincubation for 30 min with hydrocortisone before addition of kallikrein; $HS10^{-4}$, $HS10^{-2}$, reaction mixture in the presence of the indicated molarity of hydrocortisone sodium succinate; H+Plasma, hydrocortisone $10^{-4}$M+plasma substrate (no kallikrein). C: The results obtained from a second experiment in which reaction mixture carried out in the presence of 10 kallikrein inhibiting units of Trasylol.

kallikrein with the soluble salts of hydrocortisone, prednisolone or dexamethasone in concentrations up to $10^{-3}$M did not inhibit the hydrolysis of BAEe by these enzymes.

*Kinin formation in patients receiving steroids*

Plasmas from two patients treated with betamethasone 3 mg daily for ulcerative colitis and malignant hypertension respectively and from one patient with nephrotic syndrome receiving prednisone 50 mg daily were studied. On five-fold dilution at 30° C in the presence of phenanthroline 1 mg/ml., 1 ml. of these plasmas formed 3 to $6 \times 10^{-12}$ moles of kinin in 15 min. Shaking in a specimen tube for 5 min generated 0.6 to $1.0 \times 10^{-9}$ moles/ml. of plasma. Plasmas from subjects not receiving corticosteroids form, under these experimental conditions, similar amounts of kinin.

## Discussion

Melmon & Cline (1967) have suggested that steroids may exert their anti-inflammatory action partly by inhibiting the activation and the activity of kinin-forming enzymes. In view of its importance this observation has been re-examined independently in three laboratories. The kinin-forming enzymes of plasma were activated by several procedures but no inhibition by corticosteroids was observed. The enzymes which were already active such as urinary kallikrein (from the same source as that used by Melmon and Cline), human plasmin and hog pancreatic kallikrein were also not inhibited by steroids.

The difference between these findings and those of Melmon and Cline might be partly due to the different ways by which kinin formation was induced and assessed. For example, Melmon and Cline activated plasma by glass contact for 10–30 min. Owing to the presence of enzyme inhibitors and of kininase in plasma, the kinin levels found after 30 min would be well past their peak concentration. This might well account for the finding of these authors that glass contact increased kinin level in plasma only 2 to 3 times above the control values. Moreover, studies by Margolis & Bishop (1963) make it highly unlikely that exposure to glass surfaces, particularly such small surfaces as were used by Melmon and Cline, could consume up to 50% of the total kininogen responsive to trypsin.

The data of Melmon and Cline also showed that there was little correlation between the kinins detected and the levels of residual kininogen (kinin yielding substrate). The latter were estimated by converting all remaining kininogen to kinin with the aid of trypsin. The evaluation of results obtained in this way is complicated by the possibility that different kinin-forming enzymes act on different fractions of plasma kininogens and release different amounts of kinins (Margolis & Bishop, 1963 ; Jacobsen, 1966 ; Vogt, 1966).

Finally it is possible that some enzymes digest plasma kininogens without forming kinins. For example Hochstrasser & Werle (1967) report that carboxy-peptidase B destroys 40% of bovine plasma kininogen without forming kinin. A similar enzyme is present in leucocytes. In view of these uncertainties kinin formation may be more reliably assessed by measuring the kinins themselves rather than the loss of kininogen.

The present work does not support the suggestion that corticosteroids suppress inflammation by inhibiting kinin formation.

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