Biochemical Pharmacology, Vol. 23, pp. 3107–3120. Pergamon Press, 1974. Printed in Great Britain.

ANTI-COMPLEMENT EFFECTS OF TWO ANTI-INFLAMMATORY AGENTS
NIFLUMIC AND FLUFENAMIC ACIDS*

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(*Received* 16 *November* 1973; *accepted* 15 *March* 1974)

Abstract—Two anthranilate-type anti-inflammatory agents, niflumic acid and its benzene analog, flufenamic acid, as the sodium salts (niflumate and flufenamate) were tested *in vitro* in a number of hemolytic assays to determine their inhibitory effects on guinea-pig complement activity. Phlorizin, a known anti-complementary (anti-C) agent, was evaluated for comparison. Flufenamate was consistently more inhibitory than was niflumate. EAC$\overline{14}$ formation from C$\overline{1}$ and EAC4 was partially inhibited by flufenamate, but not by niflumate at $1{\cdot}8 \times 10^{-3}$ M. Both C2 cleavage and EAC$\overline{142}$ formation were substantially decreased by flufenamate at 9×10^{-4} M, while niflumate was considerably less effective and phlorizin was ineffective. This effect of flufenamate on C2 was apparently not due to chelation of Mg^{2+} or Ca^{2+}, nor to an effect on EAC$\overline{142}$ stability. EAC$\overline{1423}$ formation from C3 and EAC$\overline{142}$ was partially inhibited by flufenamate and strongly inhibited by phlorizin, but niflumate was, at best, weakly inhibitory. None of these agents affected C3 cleavage or EAC$\overline{1423}$ reactivity. EAC$\overline{14235}$ formation from C5 and EAC$\overline{1423}$ was decreased by all three agents at 9×10^{-4} M, with flufenamate the most inhibitory. C5 cleavage was not affected by any of the agents. However, all three agents increased the lability of EAC$\overline{14235}$ at 25°, flufenamate being the most effective agent. The significance of these observations with reference to the anti-inflammatory activities of niflumic and flufenamic acids and the relevance of anti-C activity to drug design are discussed.

THE ROLE of complement (C) as a mediator of inflammation has been studied and reviewed extensively over the past few years.[1,2] The complement system consists of a series of nine components, C1 through C9.† Sequential activation of these components leads to the generation of phlogistic agents, such as anaphylatoxins and leukocyte chemotactic factors and, in addition, C enhances phagocytosis of particles and promotes cell lysis.[3–5]

* The present studies were published, in part, in an abstract for the Fifth International Complement Workshop, Coronado, Calif., February 23–25, 1973. The abstract appeared in *J. Immun.* **111,** 306 (1973).

† The nomenclature in this paper conforms to that described in *Bull. Wld Hlth Org.* **39,** 935 (1968). The symbols E, A and C represent, respectively, a sheep erythrocyte, antibody to erythrocytes (hemolysin) and complement molecules, the individual complement components being designated by number, e.g. C1, C2. The subscripts "a" and "b" are used to designate fragments of the complement molecules. The bar over certain complement components indicates activated enzymes generated by the interaction of native C components with previously activated C components having enzymic activity. For example, EAC$\overline{1\ 4_b,2_a,3_b,5_b}$ would indicate that cleavage products or fragments of C4, C2, C3 and C5 were fixed on the erythrocyte complex by enzymic activation. Throughout this paper, the erythrocyte–complement complexes are indicated without reference to the fixed cleavage products, although the presence of these fragments is implied. The symbol "S" in place of "E" is used to designate a site on an erythrocyte that has bound A and one or more C components.

The mode of inhibition of C by a number of synthetic organic compounds has been studied. These compounds are of diverse structure: e.g. hydroxybenzene derivatives;[6–8] aromatic amino acid derivatives;[9] benzamidines, guanidines and phenoxyacetamides;[10–14] phosphonate esters[15] and others.[16–19] These compounds have been shown to inhibit various steps in the C sequence.

Of considerable interest to us are the mechanisms by which currently available and potential anti-inflammatory agents exert their effects. Knowledge of these mechanisms might be an aid in the development of assays *in vitro* for anti-inflammatory agents.

Recently, data have been presented on the anti-complementary (anti-C) effects of a number of commercially available anti-inflammatory compounds.[20,21] There are also earlier reports on the effects of such agents as hydrocortisone and chloroquine on C function.[22,23]

The present paper deals, in part, with an analysis in isolated systems of the anti-C effects of two structurally related anti-inflammatory compounds, niflumic acid and flufenamic acid, and of phlorizin, a known anti-C agent[6,7] (Fig. 1). The data obtained indicate that the two anti-inflammatory drugs show at least quantitative differences in their anti-C effects *in vitro*. The salt of flufenamic acid and, to a lesser extent, that of niflumic acid, affect the early C reactions involving C1, C4 and C2, as well as the C5 reaction. High concentrations of either of the anti-inflammatory agents in whole serum (human and guinea-pig) caused a decrease in levels of hemolytic C1, C2 and C5. Flufenamate also caused a decrease in C3 levels in human serum.

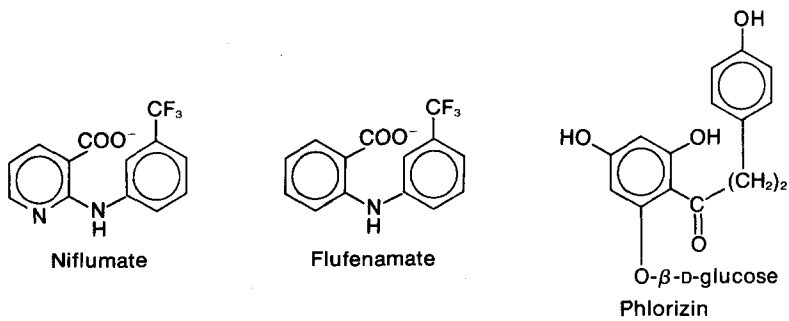

FIG. 1. Compounds studied for anti-complement effects.

MATERIALS AND METHODS

Compounds and chemicals

Disodium ethylenedinitrilotetraacetate (ethylenediaminetetraacetate) dihydrate (EDTA) and phloretin (lot A5133) were purchased from J. T. Baker Chemical Co. Sodium barbital (sodium 5,5′-diethylbarbiturate) was purchased from Merck & Co., Inc. Niflumic acid (lots UPSA-540, NM010 and N^{O} II) was obtained from Squibb England in batch form (non-formulated). Flufenamic acid (lot rx9422) was a gift of Parke, Davis & Co., Detroit, Mich. Phlorizin (lot A-5133) was purchased from Mann Research. Epsilon aminocaproic acid was obtained from Nutritional Biochemicals Co. All other chemicals used were of Fisher, Baker or Mallinckrodt analytic grade.

Preparation of compounds

EDTA was dissolved in water and neutralized with NaOH to pH 7·3 to give a final stock solution of 0·1 M. All other compounds were dissolved, with stirring, in 0·1 N or 0·15 N NaOH at room temperature at a concentration of 0·1 M or less. The solutions were diluted 10-fold in the appropriate buffer and the pH was adjusted to 7·2 to 7·4 by the addition of 0·1 N or 0·01 N HCl. Flufenamic and niflumic acids were tested as their sodium salts, flufenamate and niflumate.

Complement and isolated components

Pooled guinea-pig serum was purchased from Suburban Serum Labs., Silver Spring, Md. Serum was fractionated by various procedures. $C\bar{1}$ euglobulin fraction was prepared by the method described by Nelson *et al.*[24] Partially purified C2 was obtained by the method of Borsos *et al.*[25] Procedures for the isolation and purification of C3, C5, C6, C7, C8 and C9 have been described by Nelson *et al.*[24] All sera and component reagents were stored at −75°. Human serum, as a source of C, was obtained from an individual donor by venipuncture. The blood was allowed to clot at room temperature for 2 hr, then was centrifuged at 4°; the serum was recovered and frozen at −75°. C-EDTA, as a source of C3 and C5–C9, was made by diluting 1 ml of whole guinea-pig serum with 36·5 ml of 0·01 M EDTA in buffer A (see below).

Buffers

(A) Isotonic Veronal–NaCl containing 0·1% gelatin, pH 7·3, ionic strength 0·147, was prepared as described by Kabat and Mayer.[26] Buffer A with 0·01 M EDTA was made by adding one part of 0·1 M EDTA, pH 7·3, to nine parts of buffer A. (B) Equal parts of buffer A and of 5% aqueous glucose solution containing 0·1% gelatin were mixed. (C) One part of buffer A and two parts of buffer B were mixed. When necessary, Ca^{2+} and Mg^{2+} (as chloride salts), at a final concentration of 0·15 and 1·0 mM, respectively, were added to buffers A, B or C, and were then designated A^{++}, B^{++}, C^{++}. The conductivities of buffers and solutions were determined on a conductivity bridge (Yellow Springs Instrument Co., model 31) at 0°. The ionic strengths of solutions were calculated by comparison with conductivities of standard NaCl solutions. The changes in conductivities of buffers due to compounds were negligible.

Preparation of sensitized erythrocyte–complement intermediate complexes (EAC's)

Sheep erythrocytes (E) and rabbit hemolytic antibody (A) were obtained as described by Kabat and Mayer.[26] The procedures used for sensitizing E and for preparing $EAC\bar{1}4$ were those of Mayer *et al.*[26,27] The reactivity of $EAC\bar{1}4$ (T_{max}) was determined by the method of Borsos *et al.*[28] EAC4 was prepared by a modification of Becker's method.[29] $EAC\bar{1}4\bar{2}$ was prepared by the procedure of Shin and Mayer,[30] $EAC\bar{1}4\bar{2}3$ by the procedure of Shin *et al.*[31] and $EAC\bar{1}4\bar{2}35$ by the method of Shin *et al.*[32]

All EAC's, with the exception of $EAC\bar{1}4$ and EAC4, were prepared and used on the same day. A stock suspension of $EAC\bar{1}4$ (1×10^9 cells/ml) was kept between 0 and 4° for 1–3 weeks in buffer B^{++}; aliquots were removed, washed once, then utilized for a particular day's experiment(s).

Complement assays

Assay of individual C components. All assays were based on the lysis of erythrocytes, measured spectrophotometrically at 412 nm. $C\bar{1}$ was assayed by the method of Borsos and Rapp.[29] C2 was determined by the method of Borsos *et al.*,[28] as modified by Mayer and Miller.[27] C3 activity was analyzed by the method of Shin and Mayer,[30] and C5 by the method of Shin *et al.*[31]

A unit of C component activity was defined as the dilution of the component that yielded 63 per cent lysis in the above assay systems. A 63 per cent hemolysis level, using the analysis of Borsos *et al.*,[28] is equivalent to $-\ln(1-y) \doteq 1{\cdot}0$, where y is the fraction of cells lysed. It is also equivalent to the average number of sites (S) per red cell bearing the particular C components under investigation (average SAC.../cell), as indicated on the ordinates of figures included in this paper. In addition, calculations of EAC reactivity in all studies were based on values of $-\ln(1-y)$.

Kinetic assays of EAC formation. These assays are referred to as site assays, since they measure the relative numbers of sites (S) on the cells that have reacted with the particular C component. The kinetic uptake of $C\bar{1}$ by EAC4 was measured by the method of Borsos and Rapp.[29] C2 site formation was assayed by the method of Borsos *et al.*[28] The kinetic assays for C3 site formation have been described by Shin and Mayer,[30] and those for C5 site formation by Shin *et al.*[31] The general protocol for these assays was as follows: A suspension of the particular EAC was prepared at appropriate concentrations and kept at 0°. In a separate tube, the C component under investigation was diluted at 0° in the appropriate buffer, in the presence or absence of the compound being studied. All tubes were then placed at 25° for 2–5 min for equilibration. Kinetic studies were started by adding an equal volume of C component (with or without compound) to the cells that were maintained in suspension by frequent shaking. The time at which half the volume of component was added to cells was designated zero time. Aliquots were removed and the cell samples were washed and assayed by addition of the remaining C components required for hemolysis. (See Results for details.) The initial reaction rates of component uptake (site formation) were determined by inspection of the slopes of the initial, relatively linear portions of the plotted kinetic curves (usually up to about 2 min). The initial rate is equivalent to the number of sites formed/cell/min, and the ratios of the slopes of the reactions in the presence and absence of compounds were used to calculate the extent of any inhibitory effects.

RESULTS

Effects of niflumate and flufenamate on C reactions utilizing isolated components

The anti-C effects of niflumate and flufenamate were first observed in an assay for C-dependent immune hemolysis, utilizing guinea-pig C and the compounds at 7×10^{-4} M (unpublished observations). Investigation of the anti-C effects of these agents on individual C reactions, using isolated guinea-pig components, was undertaken in an effort to delineate more clearly the mechanism(s) of these effects.

Interaction of EAC4 and $C\bar{1}$. At 25°, partially purified guinea-pig $C\bar{1}$ (final concentration, 1 unit/ml) was incubated with EAC4 ($3{\cdot}75 \times 10^7$/ml) in the presence of $1{\cdot}8 \times 10^{-3}$ M niflumate or flufenamate or buffer C^{++}. Two ml of incubation mixture was sampled at various times, diluted in 8 ml of cold buffer C^{++}, and centrifuged

at 5°. The cells were washed twice with 5 ml of cold buffer C^{++}, resuspended with 1 ml of this buffer, and assayed for $EAC\overline{1}4$. Only flufenamate decreased the reaction rate of $EAC\overline{1}4$ formation; a decrease of 45 per cent in reaction rate was observed (Fig. 2). No irreversible effect on hemolytic $C\overline{1}$ was observed when niflumate or flufenamate (at 1·8 or 3·6 × 10^{-3} M) was incubated with $C\overline{1}$ alone in buffer C^{++} for 15 min, dialyzed against buffer B^{++}, and assayed.

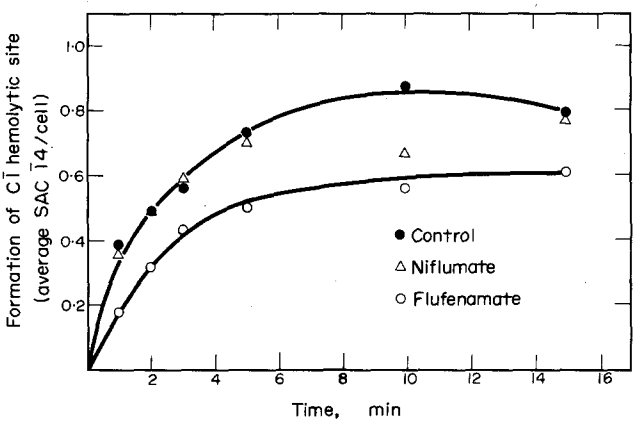

FIG. 2. $EAC\overline{1}4$ formation from $C\overline{1}$ and EAC4 at 25° and μ = 0·11, in the presence of 1·8 × 10^{-3} M niflumate, flufenamate or buffer control.

A possible reason for the apparent decrease in rate of $EAC\overline{1}4$ formation could be a compound-related decrease in stability of $EAC\overline{1}4$ or EAC4. Separate aliquots of these cells were incubated with each of the compounds at 1·8 × 10^{-3} M, under conditions identical to those described for the kinetic studies (Fig. 2). After 15 min at 25°, the cells were washed, resuspended and assayed. A small decrease (8 per cent) was noted in the reactivity of EAC4 incubated with compounds as compared with that of cells incubated with buffer. However, significant decreases in reactivity of $EAC\overline{1}4$, 25 and 17 per cent, were caused by flufenamate and niflumate, respectively, in an identical experiment. The observation that niflumate decreased the reactivity of $EAC\overline{1}4$ was unexpected, based on the kinetic data previously obtained (Fig. 2) which indicated no effect of this compound on $EAC\overline{1}4$ formation.

Inhibition of the reaction of $EAC\overline{1}4$ with C2. The effects of niflumate and flufenamate on the interaction of $EAC\overline{1}4$ with C2 was investigated. During this reaction, C2 is activated by the enzymatic action of $C\overline{1}$ present on $EAC\overline{1}4$. $EAC\overline{1}4$ (7·5 × 10^7/ml) and C2 (1 unit/ml) were incubated at 25° in the presence of 9 × 10^{-4} M niflumate, flufenamate or phlorizin in buffer A^{++}. At intervals, 1-ml samples were taken and diluted in 4 ml of cold buffer A^{++}. The cells were centrifuged, washed once with 4 ml buffer, and resuspended with 1 ml buffer. A total of 1·5 ml C-EDTA (1/37·5) at 0° was added and the reaction mixtures were incubated at 37°. As can be seen in Fig. 3, the initial rate of $EAC\overline{142}$ formation was markedly decreased (86 per cent) by flufenamate, but was only slightly affected (18 per cent) by niflumate. Phlorizin, by comparison, caused no inhibition.

The effects of niflumate and flufenamate on the fluid-phase consumption of C2 by $EAC\overline{1}4$ were also investigated in an experiment that was similar in design to that

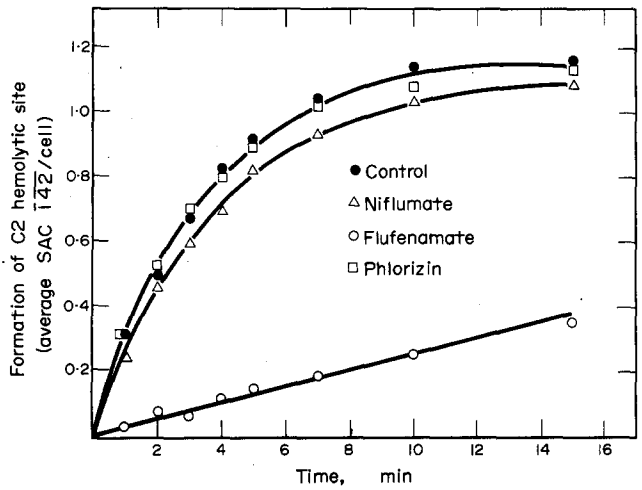

FIG. 3. EAC$\overline{142}$ formation from C2 and EAC$\overline{14}$ at 25° and $\mu = 0{\cdot}147$, in the presence of 9×10^{-4} M niflumate, flufenamate, phlorizin or buffer alone.

shown in Fig. 3. The fluid phase was obtained after incubation of EAC$\overline{14}$, C2 and compound for 10 min at 25°. Dialysis of the undiluted supernatant against buffer A^{++} overnight was followed by measurement of C2 consumption. Flufenamate showed a 68 per cent inhibition of C2 consumption by EAC$\overline{14}$, and niflumate caused a small decrease (9 per cent) (Table 1).

TABLE 1. EFFECTS OF COMPOUNDS ON UPTAKE AND FLUID-PHASE CONSUMPTION OF C COMPONENTS BY SENSITIZED ERYTHROCYTE–C COMPLEXES

	% Inhibition					
	C2*†		C3‡		C5*	
Compound	S.F.§	Fluid‖	S.F.	Fluid	S.F.	Fluid
Niflumate	18	9	1	0	35	3
Flufenamate	86	68	23	4	73	5
Phlorizin	0	ND¶	92	0	22	0

* EAC$\overline{14}$ or EAC$\overline{1423}$ was incubated with C2 or C5, respectively, for 10 min at 25° in the presence of 9×10^{-4} M compound.

† Data on S.F. and fluid consumption (see footnotes §,‖) were obtained from separate experiments.

‡ EAC$\overline{142}$ was incubated with C3 for 10 min at 25° in the presence of $1{\cdot}8 \times 10^{-3}$ M compound.

§ S.F. indicates the uptake of a C component by the appropriate EAC complex. Inhibition was calculated from the ratio of the average number of C sites formed per cell $[-\ln(1 - y)]$ in compound-treated and control incubations.

‖ Fluid indicates the decrease (consumption) in C component reactivity in the fluid phase of incubation mixtures. Inhibition was calculated from the ratios of the C titers of the dialyzed supernatants of compound-treated and control reactions.

¶ Not determined.

Since flufenamate and, to a lesser extent, niflumate could be inhibiting EAC$\overline{142}$ formation by affecting C2 directly or by affecting the stability of EAC$\overline{14}$ or EAC$\overline{142}$, the following studies were performed. The stability of C2 in the presence of niflumate

or flufenamate was determined. C2 (1 unit/ml) in buffer A^{++} was incubated with compound ($1{\cdot}8 \times 10^{-3}$ M) for 20 min at 25° and then dialyzed in the cold overnight. C2 hemolytic activity was decreased 11 per cent by flufenamate. Niflumate had no effect.

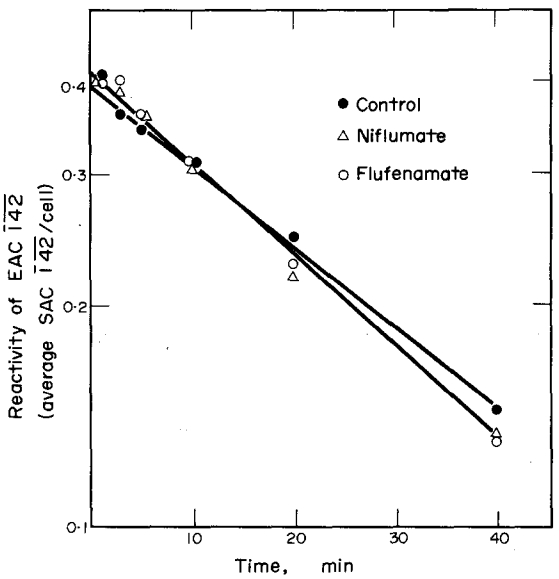

FIG. 4. Effect of 9×10^{-4} M niflumate or flufenamate on the stability of EAC$\overline{142}$ at 30° and $\mu = 0{\cdot}147$. The stability of EAC$\overline{142}$ in buffer alone is also indicated.

The decay of EAC$\overline{142}$ hemolytic reactivity at 30° was not significantly affected by flufenamate or niflumate at 9×10^{-4} M in buffer A^{++} (Fig. 4). In addition, niflumate at a concentration of $3{\cdot}6 \times 10^{-3}$ M did not affect the decay rate. EAC$\overline{1}$4 reactivity was not decreased by niflumate or flufenamate at 9×10^{-4} M when incubated in buffer A^{++} followed by cold centrifugation and washing with buffer B^{++}. This latter result was contrary to that found previously for EAC$\overline{1}$4 stability (see above) under $C\overline{1}$ kinetic assay conditions that employed a 2-fold greater concentration of compounds.

The possibility that flufenamate was interfering with C2 activation by chelating Mg^{2+} or Ca^{2+} was investigated. One volume of EAC$\overline{1}$4 ($1{\cdot}5 \times 10^{8}$/ml) was added to one volume of a mixture containing C2 (1 unit/ml) and flufenamate ($1{\cdot}8 \times 10^{-3}$ M). Reactions were set up in buffer A containing 2×10^{-3} M Mg^{2+} and the normal concentration of Ca^{2+} ($1{\cdot}5 \times 10^{-4}$ M); with excess Ca^{2+} ($1{\cdot}5 \times 10^{-3}$ M) and normal Mg^{2+} concentration (1×10^{-3} M); or with normal concentrations of both metals (buffer A^{++}). Appropriate controls were included. The mixtures were incubated for 10 min at 25°, cooled, centrifuged and washed twice in buffer A^{++}; the cells were then resuspended and assayed for C2 uptake. As indicated in Table 2, neither Mg^{2+} nor Ca^{2+} in molar excess over flufenamate significantly reversed the inhibition of C2 uptake by EAC$\overline{1}$4.

The results indicated in this section suggested that flufenamate and niflumate were interfering with the interaction of $\overline{C1}$ and C2 in incubations containing EAC$\overline{14}$ and C2.

TABLE 2. EFFECT OF Ca^{2+} AND Mg^{2+} ON FLUFENAMATE INHIBITION OF UPTAKE OF C2 BY EAC$\overline{14}$

% Inhibition		
Excess Ca^{2+}*	Excess Mg^{2+}*	Buffer A^{++}
79	75	82

* Molar excess of Ca^{2+} or Mg^{2+} over flufenamate (at $1{\cdot}8 \times 10^{-3}$ M) in incubation mixture.

Reaction of EAC$\overline{142}$ and C3. The next step in the complement sequence, involving C3, was then investigated. Each of the compounds at 9×10^{-4} M was incubated with C3 (5 units/ml) and EAC$\overline{142}$ ($7{\cdot}5 \times 10^{7}$/ml) in buffer B^{++} at 25°. Samples of 0·5 ml were taken at intervals and diluted in 4·0 ml of cold buffer B^{++}. The cells were centrifuged, washed with 4 ml buffer, and resuspended with 0·25 ml buffer B^{++} for assay of EAC$\overline{1423}$ reactivity. As indicated in Fig. 5, niflumate decreased the initial reaction rate by 21 per cent, flufenamate caused a 30 per cent decrease, and phlorizin caused an 84 per cent decrease.

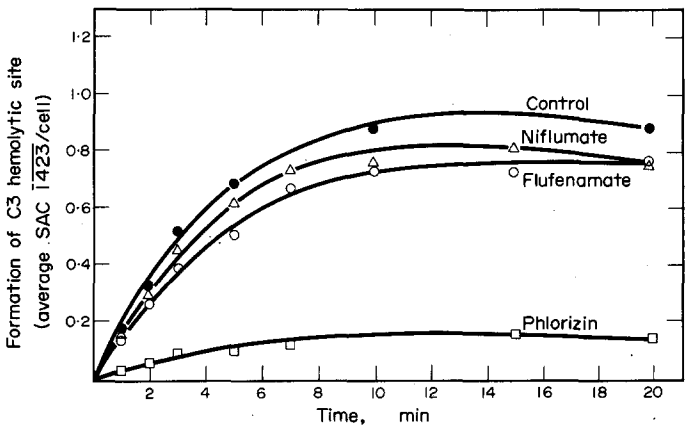

FIG. 5. EAC$\overline{1423}$ formation from C3 and EAC$\overline{142}$ at 25° and $\mu = 0{\cdot}072$, in the presence of 9×10^{-4} M niflumate, flufenamate, phlorizin or buffer.

The reaction of EAC$\overline{142}$ with C3 was then examined by analyzing both fluid-phase C3 consumption and EAC$\overline{1423}$ formation in the same incubation mixture. EAC$\overline{142}$ and C3 were, however, incubated at 25° with niflumate or flufenamate at $1{\cdot}8 \times 10^{-3}$ M. One sample was taken after 10 min, cooled immediately without dilution, and centrifuged at 0°. The supernatant fluids were removed and dialyzed against two changes of cold buffer B^{++} overnight prior to the determination of C3 activity. The cells were washed twice in buffer B^{++}, then were resuspended and assayed for the presence of C3 sites. The results are shown in Table 1. Phlorizin decreased EAC$\overline{1423}$ formation by 92 per cent and flufenamate by 23 per cent, but niflumate had no effect.

Analyses of supernatant fluids showed essentially no interference with C3 consumption by any of the compounds (Table 1). The results obtained with phlorizin agree with those of Müller-Eberhard *et al.*[33] Control incubations of C3 with compounds at $1{\cdot}8 \times 10^{-3}$ M and 25° for 15 min, followed by dialysis, indicated a decrease of 18 per cent in C3 hemolytic activity caused by flufenamate. The other agents were without effect.

The effects of niflumate, flufenamate or phlorizin on the hemolytic reactivity of $EAC\overline{1423}$ were tested. $EAC\overline{1423}$ ($7{\cdot}5 \times 10^7$/ml) was incubated in the presence of compound (9×10^{-4} M) at 25° for 15 min in buffer B^{++}. The cells were added to nine parts of cold buffer B^{++}, centrifuged, washed once with eight parts of buffer, resuspended and then assayed. Essentially no change in $EAC\overline{1423}$ stability was noted.

The inhibitory effect of flufenamate on $EAC\overline{1423}$ formation, therefore, could probably be accounted for by its effect on C3 in the fluid phase. Niflumate is considered to have, at best, a minimal effect on C3 uptake, at the concentrations tested.

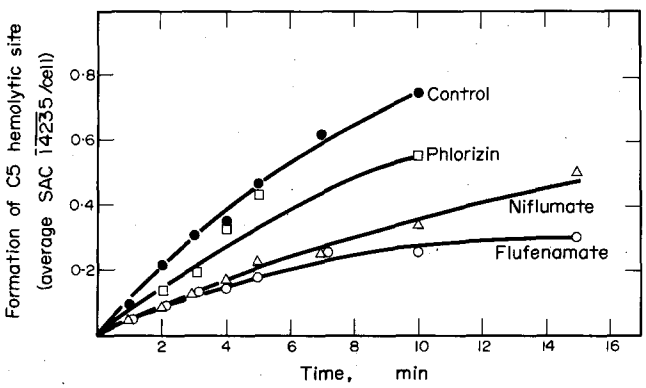

FIG. 6. $EAC\overline{14235}$ formation from C5 and $EAC\overline{1423}$ at 25° and $\mu = 0{\cdot}072$, in the presence of 9×10^{-4} M niflumate, flufenamate, phlorizin or buffer.

Reaction of $EAC\overline{1423}$ *with C5.* The effect of niflumate and flufenamate on $EAC\overline{14235}$ formation was investigated. C5 (2 units/ml) was incubated with $EAC\overline{1423}$ ($7{\cdot}5 \times 10^7$/ml) and niflumate, flufenamate or phlorizin at 9×10^{-4} M in buffer B at 25°. At intervals, 0·5-ml samples were taken, diluted in 4·5 ml cold buffer B, resuspended with 0·25 ml buffer B and assayed for $EAC\overline{14235}$. In Fig. 6, it can be seen that flufenamate, niflumate and phlorizin decreased the initial velocity of $EAC\overline{14235}$ formation by 57, 51 and 21 per cent respectively.

Both supernatant C5 and $EAC\overline{14235}$ formation were analyzed in a subsequent experiment, using a protocol similar to that indicated above (also Fig. 6). Each of the three agents was incubated at 25° at a final concentration of 9×10^{-4} M. Samples were taken after 10 min and handled as for the C3 analyses above. The reactivity of the $EAC\overline{14235}$ formed was measured and, after dialysis against cold buffer B^{++}, the supernatant fluids were assayed for C5. In this experiment (Table 1), 35, 73 and 22 per cent inhibition of C5 uptake was observed for niflumate, flufenamate and phlorizin respectively. Analysis of the supernatant fluids indicated that none of these

compounds affected significantly the fluid-phase consumption of C5 by $EAC\overline{1423}$ (Table 1).

The stability of $EAC\overline{14235}$ in the presence of niflumate, flufenamate or phlorizin was next investigated. $EAC\overline{14235}$ ($7{\cdot}5 \times 10^7$/ml) was incubated at 25° with 9×10^{-4} or $1{\cdot}8 \times 10^{-3}$ M compound for 15 min. The results, indicated in Table 3, show that at a concentration of $1{\cdot}8 \times 10^{-3}$ M, 91, 41 and 33 per cent of $EAC\overline{14235}$ hemolytic reactivity was lost in the presence of flufenamate, niflumate and phlorizin respectively. This effect of phlorizin substantiates that found by Shin *et al.*[32] However, at the concentration of compound utilized in the kinetic experiments (Fig. 6), only flufenamate caused a decrease of $EAC\overline{14235}$ stability.

TABLE 3. STABILITY OF $EAC\overline{14235}$

Compound*	Concn (M)†	% Decrease of C5 sites
Niflumate	9×10^{-4}	8
	$1{\cdot}8 \times 10^{-3}$	41
Flufenamate	9×10^{-4}	68
	$1{\cdot}8 \times 10^{-3}$	91
Phlorizin	9×10^{-4}	0
	$1{\cdot}8 \times 10^{-3}$	33

* Compounds were incubated with $EAC\overline{14235}$ ($7{\cdot}5 \times 10^7$/ml) in buffer B for 15 min at 25°. The cells were centrifuged, washed twice and resuspended for assay.

† The effects of compounds at 9×10^{-4} M were determined in an experiment separate from that with $1{\cdot}8 \times 10^{-3}$ M concentrations.

The increased lability of $EAC\overline{14235}$ in the presence of flufenamate is comparable to the decrease in $EAC\overline{14235}$ formation. This finding suggests that the enhanced lability caused by flufenamate is at least partially responsible for the apparent decrease in $EAC\overline{14235}$ formation observed kinetically (Fig. 6). The lack of an effect of phlorizin or niflumate on $EAC\overline{14235}$ stability under kinetic assay conditions indicated the existence of other undefined mechanisms for inhibition of $EAC\overline{14235}$ formation.

Finally, the direct effect of compounds on C5 was tested. C5 (4 units/ml) was incubated at 25° with $1{\cdot}8 \times 10^{-3}$ M compound in buffer B for 15 min. After dialysis and assay, only an 8 per cent decrease in C5 hemolytic activity due to flufenamate was noted. Niflumate and phlorizin had no effect on C5 activity.

Effect of niflumate and flufenamate on C1, C2, C3 and C5 hemolytic activities in whole serum of human and guinea-pig

The studies of Aoki and Von Kaulla[34] indicated that flufenamate, at 10^{-2} M, caused a decrease in whole C activity in human serum. The following experiments were an extension of these studies.

Guinea-pig serum, 1:10 (one part plus nine parts buffer), was incubated with either niflumate or flufenamate at 9×10^{-3} M in buffer A^{++}. The samples were dialyzed prior to assay of C1, C2, C3 and C5 hemolytic activities (Table 4). The presence of either flufenamate of niflumate caused a marked loss in hemolytic C1, C2 and C5 activities. Flufenamate was more effective than niflumate in decreasing C1 and C2

activities. The effects of niflumate or flufenamate on C3 in a number of similar experiments with whole guinea-pig serum were not clear because of the substantial decrease in hemolytic C3 activity after dialysis of untreated guinea-pig serum samples. When human serum (1:10) was incubated in a similar manner with either niflumate or flufenamate, 94–100 per cent inhibition of C3 and C5 hemolytic activities was observed (Table 4). C1 and C2 levels in treated human serum were not assayed.

TABLE 4. INHIBITION OF C ACTIVITY IN WHOLE SERUM WITH LARGE CONCENTRATIONS OF NIFLUMATE OR FLUFENAMATE

Compound (9×10^{-3} M)*	% Inhibition of C1†	C2	C3	C5
		Guinea-pig serum		
Niflumate	62	45	‡	>99
Flufenamate	94	99	‡	>99
		Human serum		
Niflumate			94	100
Flufenamate			95	>99

* Undiluted serum, 0·5 ml, was added to 4·5 ml of 10^{-2} M compounds neutralized in buffer A^{++} and incubated for 30 min at 37°. The samples were then dialyzed against two changes of cold buffer B^{++} overnight at 5°.

† Data for C2, C3 and C5 were obtained from one experiment; C1 inhibition was measured in a subsequent experiment.

‡ Marked decreases in C3 hemolytic activity occurred on dialysis of guinea-pig serum. The effect of niflumate on C3, therefore, is not easily interpretable. However, flufenamate consistently caused decreases >99 per cent.

Since inhibition of C1, C2, C3 and C5 hemolytic activities was observed, it seemed likely that flufenamate (and possibly niflumate) might be causing the sequential fluid-phase activation and, therefore, destruction of the hemolytic activities of the C components. The experiment indicated in Table 4 was repeated with the compounds and guinea-pig serum in the presence of either 0·02 M EDTA or 0·04 M ε-aminocaproic acid (EACA). EDTA inhibits the early cation-dependent reactions of the C sequence, involving C1, C4 and C2.[26] EACA has been shown to inhibit the conversion of plasminogen to plasmin;[35] the latter is capable of activating C1.[36]

The presence of EDTA or EACA did not prevent niflumate or flufenamate from causing decreased levels of hemolytic C2 and C5 in guinea-pig serum. Once again, inconclusive results were obtained for the effects of compounds on C3.

DISCUSSION

One of the reasons for initiating the studies presented in this paper was to evaluate the use of tests *in vitro* for anti-C effects as a follow-up to the use of C-dependent systems *in vivo* for detecting anti-inflammatory agents. Specifically, from a basic standpoint, we wished to obtain more detailed information on the anti-C properties of two anti-inflammatory agents that are chemical analogs.

The results presented in this paper indicate some of the variations in profiles of C inhibition that have been observed in isolated C component systems (Table 1). A compound may inhibit a step by affecting cleavage of a component (e.g. flufenamate

and C2 activation); it may interfere with site formation without affecting activation (e.g. niflumate, flufenamate or phlorizin on C5); it may decrease the stability of an intermediate C complex (e.g. niflumate, flufenamate or phlorizin on $EAC\overline{14}2\overline{3}5$; Table 3); or it may affect site formation apparently solely by inhibiting transfer of the activated component to its receptor site (e.g. phlorizin inhibition of $EAC\overline{14}\overline{23}$ formation). The inhibitory effect of flufenamate on $C\overline{1}$ interaction with EAC4 (Fig. 2) has not been investigated in detail, but may involve effects on $C\overline{1}$ binding to EAC4 (unpublished observations).

A comparison of the relative inhibitory activities of flufenamate, niflumate and phlorizin in each of the C2, C3 and C5 steps yields striking results. Both anti-inflammatory agents inhibit C2 site formation while phlorizin does not. Flufenamate is 2- to 3-fold more inhibitory at the C5 step (site formation or site stability) than is niflumate or phlorizin. The latter compound, however, is at least 4-fold more effective in preventing C3 site formation than is flufenamate (Table 1).

Data were presented (Fig. 2) that indicated an effect of flufenamate on $C\overline{1}$, since C2 cleavage, as measured by fluid-phase depletion in the presence of $EAC\overline{1}4$, was markedly decreased. This decrease could not be accounted for by a small effect of flufenamate directly on C2. Binding of flufenamate to C2 or other effects of flufenamate that occurred during the kinetic experiment cannot be ruled out by the data presented.

The partial inhibition by flufenamate of $EAC\overline{1}4$ formation from $C\overline{1}$ and EAC4 and the instability of $EAC\overline{1}4$ under these conditions (Fig. 2) could be explained by an effect of flufenamate on $C\overline{1}$ binding, but this does not seem to explain the inhibition of C2 cleavage by $EAC\overline{1}4$.

Inhibitory effects of the two anti-inflammatory agents on the hemolytic activities of C1, C2, C3 and C5 in whole guinea-pig serum and of C3 and C5 in human serum have been demonstrated. Aoki and Von Kaulla[34] concluded that the change in the electrophoretic mobility of β1C protein (representing C3) induced by flufenamate was due to direct interaction of β1C with this compound, since the electrophoretic changes were not prevented by EDTA or EACA, compounds known to affect activation of hemolytic C. Aoki and Von Kaulla[34] also found that large concentrations of EDTA and EACA did not prevent, but perhaps partially reversed, the decrease of whole hemolytic C activity in human serum caused by flufenamate.

During the present studies, it was observed that neither EACA nor EDTA prevented the inactivation of hemolytic C1, C2 or C5 in guinea-pig serum. The results with C3 were ambiguous, due to decreases of this component after dialysis of untreated samples. It appears possible that more than one mechanism may be involved in the inactivation by niflumate or flufenamate of C in whole serum. It seems unlikely that these compounds will bind to four components of the same serum system, namely C, to alter hemolytic activities. Results presented in the present study (Table 4) are consistent with a number of hypotheses, one being that flufenamate in whole serum causes activation of C1 and a change in molecular size to a hemolytically inactive molecule that is still capable of cleaving C2. Additional data on this point, however, are not yet available.

Evidence has accumulated that indicates that the C, kinin, fibrinolytic and clotting systems may interact *in vitro* and *in vivo*.[2,37] Aoki and Von Kaulla[34] have suggested a correlation between the ability of certain compounds to enhance fibrinolysis, to

decrease levels of whole C in human serum, and to change the electrophoretic mobility of β1C serum protein. Flufenamate enhances fibrinolysis *in vitro* about 4-fold better than does niflumate.[38] In our studies, flufenamate was consistently more inhibitory *in vitro* than was niflumate in the C test systems described.

The results presented cause one to question the importance of the anti-C effects reported for niflumate and flufenamate in relation to their anti-inflammatory activities. The levels of these compounds in our tests *in vitro* might not be obtained *in vivo*, although the concentration of niflumic acid in plasma after oral administration to humans has been found to be 69–103 μg/ml (2·5 to 3·7 $\times$ 10^{-4} M).[39] It is possible that a mechanism for concentrating drugs at sites of inflammation (e.g. by permeability or protein–drug equilibrium changes) might be operative *in vivo*. However, in light of other demonstrated effects of niflumate and flufenamate, such as enhancement of fibrinolysis[38] and inhibition of prostaglandin synthesis at smaller concentrations,[40,41] we cannot make a strong case for the importance of anti-C activity in contributing to anti-inflammatory activity of the compounds. Nevertheless, specific C assays *in vitro* have been shown, in the present studies, to be useful in determining mechanisms of anti-C effects.

An additional important question concerning future drug development is whether the chronic administration of an anti-inflammatory agent that is exquisitely active against C *in vitro* could, by virtue of this activity, cause adverse effects in man that might include increased susceptibility to infections. Also, the profile of C inhibition that would be most desirable in an anti-inflammatory (anti-rheumatic) compound, if this activity is important in drug design, must be considered. For example, based on the present studies, increased instability of EAC$\overline{14235}$ would indicate the potential for destroying, *in vivo*, the initial substrate for production of a C($\overline{567}$) trimolecular chemotactic complex.[42] However, in the case of the C5 reaction step, neither niflumate nor flufenamate affected the activation (cleavage) of C5. Presumably, $C5_a$ chemotactic factor was produced.[43] The relative importance of these two chemotactic factors as well as of $C3_a$ chemotactic factor[44] in the inflammatory process is still unknown and may vary depending on the particular inflammatory disease. Similar analyses of other profiles of C inhibition may afford additional information concerning design of an anti-inflammatory drug.

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