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STABILIZATION OF SERUM ALBUMIN BY ANTI-INFLAMMATORY DRUGS

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Abstract—Nonsteroidal anti-inflammatory agents inhibited the heat denaturation of serum albumin at drug levels below 1 mM, as measured by turbidity and viscosity changes. The immunosuppressive, antihistamine, anticholinergic and antimalarial drugs tested did not show this property. Active drugs did not block denaturation induced by alkali, detergents or guanidine, nor did they generally inhibit the denaturation of ovalbumin, fibrinogen, γ-globulin or collagen.

STEROIDAL and nonsteroidal anti-inflammatory agents share a large number of different pharmacological actions. This multiplicity may result from independent interactions with various cellular sites or it may represent multiple secondary indications of a fundamental biochemical effect. The inherent attractiveness of the second possibility calls for care in viewing an effect as fundamental; nevertheless, the possibility of reconciling some of the numerous actions of anti-inflammatory drugs requires that promising leads be examined.

One such lead is the inhibition of protein denaturation by anti-inflammatory drugs reported by Mizushima *et al.*[1-3] and by Browne and Mackey.[4] This effect seems to be related to the reversal of enhanced plasma protein coagulability in drug-treated animals.[5, 6]

Denaturation may be broadly defined as any modification of the secondary, tertiary or quaternary structure of the protein molecules which does not involve the breaking of covalent bonds.[7] Nearly all modifications of a protein's environment can be shown to produce some measurable alteration of its native structure and, since such structural changes may be reversible and quite subtle (for example, the behavior of allosteric enzymes), it is not necessary to envisage drastic pathological counterparts to changes imposed *in vitro*.

Denaturation affects nearly all physico-chemical properties of protein molecules. To approach an understanding of the behavior of anti-inflammatory agents, several kinds of denaturing conditions should be imposed and several different parameters measured. In the present study we examined the influence of anti-inflammatory drugs on the denaturation of bovine serum albumin induced by heat, guanidine and sodium dodecyl sulfate, as measured by the changes in aggregation and configuration shown by turbidity, viscosity and optical rotation.

MATERIALS AND METHODS

Materials

Crystalline and Fraction V grades of bovine serum albumin (BSA) were obtained

from Pentex, Inc., with stated electrophoretic purities of 100 and 98 per cent and protein contents of 98 and 96 per cent, respectively. Lyophilized outdated human plasma, lyophilized fresh human serum, bovine fibrinogen (Fraction I) and bovine γ-globulins (Fraction II) were also obtained from Pentex, Inc. Ovalbumin, three times crystallized, was obtained from Sigma Chemical Co. Rat tail collagen solutions were prepared as described previously.[8] Crystalline BSA was the starting material for defatting procedures; these consisted of the low pH–mixed bed resin method of Foster *et al.*[9] (which did not lead, in the present case, to an initial phase separation), the acetic acid–heptane method of Goodman,[10] and the charcoal treatment of Sogami and Foster.[11]

Several acyl derivatives of albumin were made for the purpose of clarifying the role of free amino groups in the inhibition of denaturation. Succinlylation of albumin was carried out by treating 5 g of Fraction V, in 100 ml of 5% $NaHCO_3$, with 2 g of the anhydride at 4° over a 2-hr period, with the pH maintained above 7·0 by addition of solid $NaHCO_3$. Four acetylated preparations were made similarly, except that 2, 0·75, 0·5 and 0·25 g anhydride were used. The solutions were dialyzed against two 12-l. volumes of distilled water and lyophilized.

The following drugs were obtained through the courtesy of the indicated manufacturers: phenylbutazone and oxyphenbutazone (Geigy); indomethacin (Merck, Sharp & Dohme); 6-mercaptopurine and allopurinol (Burroughs Wellcome); cyclophosphamide (Mead Johnson); methotrexate (Lederle); chlorpheniramine (Schering); guanethidine (Ciba); propanolol (Ayerst); 2-phenyl-4-*p*-chlorophenylthiazol-5-ylacetic acid (PCTA)[12] and β-(4,5-diphenylolazol-2-yl)propionic acid (DPOP)[12, 13] (Wyeth).

Guanidine hydrochloride was purchased from Matheson, Coleman & Bell and sodium dodecyl (or lauryl) sulfate (SDS) from Nutritional Biochemicals. Other chemicals were of the highest commercially available quality and were purchased from Aldrich, Eastman, Mann, Fisher, and Merck.

Turbidimetric assay

A modification of Mizushima's method was used in the albumin experiments. The test system contained 2·5 ml of buffered 1% BSA and 2·5 ml of buffer or test solution. (Unless otherwise specified, BSA was Fraction V.) The buffer was 0·05 M Tris acetate, pH 6·0; dimethylformamide (DMF) was added to a maximum of 2·5% (v/v) if necessary for solubilization of the test compound. Control and test systems were heated at 69° for exactly 4 min, cooled and their turbidities read at 660 mμ. Agents which decreased turbidities by less than 20 per cent at 1 mM were tested a second time at the same level; those which decreased turbidities by more than 20 per cent were judged active and were retested at 0·8, 0·6, 0·4, 0·2 and 0·1 mM. Compounds retaining activity at 0·1 mM were run at still lower levels in a third series.

Collagen precipitation was followed kinetically as described earlier.[8]

Viscosimetry

Two types of viscosity measurements were made, one for simple comparisons of drug effects and the second for kinetic studies. In both cases, we used Ostwald–Fenski–Cannon viscosimeters (no. 100) giving buffer flow times of 60–63 sec at 25°. Details of the kinetic experiments are given with the results. In the more routine procedure, test solutions consisted of 2·5 ml of freshly prepared 2% (w/v) BSA

(crystalline) in 0·05 M Tris acetate buffer, pH 6·0, plus 2·5 ml of drug dissolved in 5% DMF-Tris acetate. After incubating at 65° for 10 min, tubes were cooled in ice water and then held at room temperature for 15 min. Flow times were determined at 25° and results were expressed as reduced viscosity ($\eta_{red.}$):

$$\eta_{red.} = 1/c \left(\frac{\eta}{\eta_0} - 1\right)$$

where c is the concentration of albumin in grams per 100 ml and η/η_0 is the ratio of the time of flow of the protein solution to that of the solvent. Calculations of percentage inhibition are based upon normalization of the rise in $\eta_{red.}$ associated with the heating. Dilution and testing schedules were similar to those used for turbidimetry.

Optical rotation

A Carl Zeiss polarimeter, with accuracy of ±0·005°, was used. The light source was a sodium lamp and measurements were made at 24–25°. Reagents were dissolved in water, giving final pH's between 7·5 and 7·8.

RESULTS

Quantitative responses to heat-induced denaturation varied with the purity and concentration of the albumin and concentration of DMF. Relatively stable systems, such as serum or plasma, required either higher protein concentrations or higher temperatures to show changes similar to those of albumin solutions. Fraction V BSA was less stable than serum, but more stable than crystalline BSA. DMF levels between

TABLE 1. EFFECTS OF VARIOUS DRUGS ON HEAT PRECIPITATION OF BOVINE SERUM ALBUMIN

Compound	% Inhibition at various concentrations (mM)*					
	0·01	0·04	0·08	0·1	0·3	0·6
Phenylbutazone	0	3	10	32	76	100
Oxyphenbutazone	2	5	5	8	20	42
Indomethacin	0	5	11	24	56	95
Flufenamic acid	0	8	27	39	91	100
DPOP†	0	10	18	24	59	94
PCTA	4	15	28	41	84	100

* DMF was present at 2·5%. In eight separate experiments, values for net absorbancies of controls were 0·734 ± 0·032. The following showed less than 20 per cent inhibition when run in duplicate at 1 mM: acetylsalicylic acid, sodium salicylate, *m*-hydroxybenzoic acid, phenacetin, anthranilic acid, serotonin, histamine, acetylcholine, 6-mercaptopurine, 1-aminocyclopentane carboxylic acid (ACPC), chloroquine, promethazine, allopurinol, atropine and dimethyl sulfoxide.

† See text for abbreviations.

0 and 5 per cent did not affect the turbidity of control systems, but 8 and 10 per cent DMF increased it by about 25 per cent, while at the same time lowering the sensitivity to drugs.

Turbidimetric and viscosimetric procedures both demonstrate that clinically useful nonsteroidal anti-inflammatory agents inhibit heat denaturation of bovine serum albumin and that inhibition varies directly with the drug concentration (Tables 1–3). Other types of drugs in general do not inhibit denaturation; the ineffective drugs included those used primarily for their immunosuppressive, antitumor, antihistamine,

TABLE 2. DRUG-INDUCED INHIBITION OF VISCOSITY RISE ASSOCIATED WITH ALBUMIN DENATURATION

Compound	% Inhibition at various concentrations (mM)*					
	0·1	0·2	0·4	0·6	0·8	1·0
Phenylbutazone	6	43	55	55	60	72
Oxyphenbutazone	14	32	57	53	75	76
Indomethacin	2	29	53	66	81	98
Flufenamic acid	35	56	68	80	83	80
Mefenamic acid	19	24	46	58	67	82
Ibufenac	10	31	60	60	70	83
DPOP†	0	30	46	79	85	94
PCTA	37	47	50	70	83	96
Salicylic acid		7	34			38
Histamine	13	12	20	16	22	23

* In twenty experiments, values of $\eta_{red.}$ for heated and unheated controls were 0·272 ± 0·052 and 0·025 ± 0·021 l. The following showed less than 20 per cent inhibition when run at 1 mM only: allopurinol, antipyrine, phenacetin, chloroquine, Imuran, 6-mercaptopurine and ACPC. The following were run at two levels, giving inhibitions greater than 20 per cent at 1 mM, but less than 20 per cent at 0·4 mM: benzoic acid, phenylacetic acid, anthranilic acid and methotrexate.
† See text for abbreviations.

TABLE 3. EFFECTS OF DEFATTING PROCEDURES ON ALBUMIN DENATURATION AND STABILIZATION*

Additive†	Turbidity (net absorbance)			
	Crystalline albumin	Albumin defatted by		
		Acid-resin	Acid-solvent	Charcoal
None	0·934	0·986	1·008	0·990
Arachidonic acid	0·862	0·960	0·946	0·902
Palmitic acid	0·876	0·970	0·960	0·940
Linoleic acid	0·694	0·838	0·862	0·816
Myristic acid	0·772	0·868	0·906	0·838
Phenylbutazone	0·530	0·750	0·805	0·741
Indomethacin	0·430	0·658	0·776	0·738

* All samples were heated at 69° for 4 min in the presence of 2·5% DMF.
† Triplicate assays; concentrations: arachidonic and palmitic acids, 5×10^{-5} M; linoleic and myristic acids, 1×10^{-4} M; phenylbutazone and indomethacin, 5×10^{-4} M.

anticholinergic, antimalarial and bronchoconstrictor activities. Salicylate was less effective than other nonsteroidal anti-inflammatory agents, while related hydroxybenzoates acted comparably to salicylate in the viscosity studies and more weakly than salicylate in the turbidity experiments.

When viscosity changes were followed at the denaturation temperature as a function of time, we obtained rate plots such as those shown in Figs. 1–3. At 52°, indomethacin, oxyphenbutazone, mefenamic acid and flufenamic acid (all at 5×10^{-4} M) decreased the rate of denaturation of BSA by 44–65 per cent (Fig. 1). In a similar experiment with 6·5% BSA carried out at 57°, inhibition varied directly with the phenylbutazone concentration over the range 5×10^{-5} M to 1×10^{-3} M (Fig. 2). At 65°, phenylbutazone, indomethacin and sodium salicylate (5×10^{-4} M) all decreased the rate at which 7·2% reconstituted human plasma increased in viscosity. Similarly,

phenylbutazone and sodium salicylate decreased the rate of viscosity increase for 4·5% reconstituted human serum at 65° (Fig. 3).

Since one important function of serum albumin is to transport long-chain fatty acids, the binding of these molecules might be expected to influence albumin denaturation and stabilization. Defatting by several techniques results in a small but consistent increase in lability to heat, as shown by the higher turbidity values of Table 3. The addition of long-chain fatty acids provides some stabilization for both the defatted and non-defatted albumins, but the defatted preparations remain less stable. This

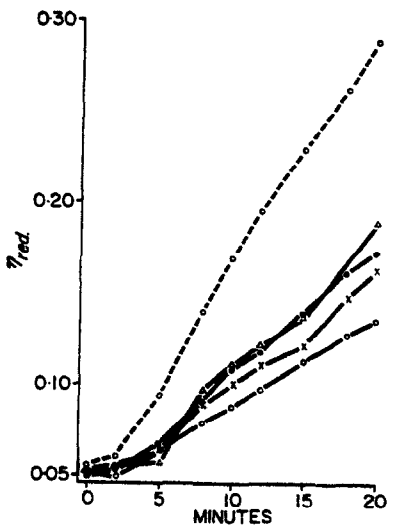

FIG. 1. Effect of anti-inflammatory drugs on the viscosity of albumin at 52°. All drugs at 0·5 mM; BSA at 6·5%. O---O, control; △—△, indomethacin; ●—●, oxyphenbutazone; ×—×, mefenamic acid; O—O, flufenamic acid.

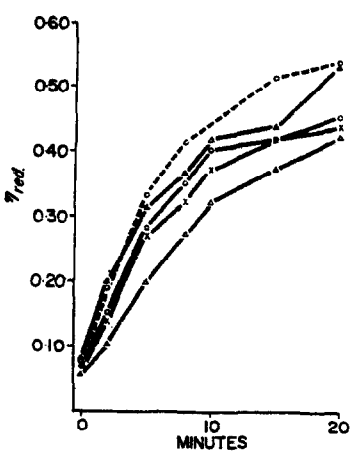

FIG. 2. Effect of various concentrations of phenylbutazone on the viscosity of 6·5% albumin at 57°. O---O, control; ▲—▲, 0·05 mM; O—O, 0·1 mM; ×—×, 0·5 mM; △—△, 1·0 mM.

difference in susceptibility to stabilization appears even more marked in the presence of established anti-inflammatory agents.

Chemical denaturation

Denaturation of BSA occurs rapidly when the pH is raised from 10 to 12.[14] The anti-inflammatory drugs (10^{-3} M) enhanced this denaturation rather than inhibiting it. In the absence of any drug, $\eta_{red.}$ increased from 0·062 to 0·395 on raising the pH, while in the presence of sodium salicylate it increased to 0·730 and with indomethacin to 0·566. The enhancement is probably not relevant to anti-inflammatory mechanisms, since 5×10^{-4} M *p*-hydroxybenzoate gave the same small increase as 5×10^{-4} M sodium salicylate.

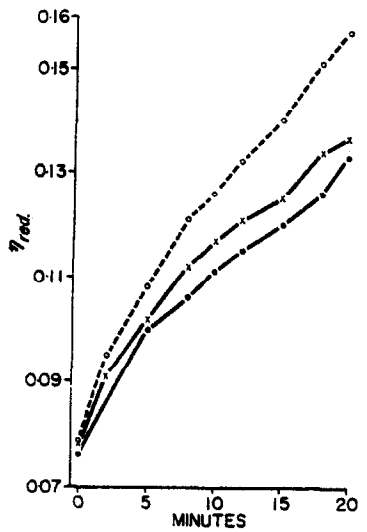

FIG. 3. Effect of anti-inflammatory drugs on the viscosity of human serum at 65°. Drugs at 0·5 mM; serum reconstituted to 4·5%. O—O, control; ×—×, phenylbutazone; ●—●, sodium salicylate.

At a concentration of 5 M, guanidine induces the denaturation of BSA; in the present experiments, it increased the negative specific rotation ($-[\alpha]_D$) of crystalline BSA from 60·0 to 85·6 and that of Fraction V BSA from 59·1 to 95·3. This guanidine effect was increased 10 per cent by sodium salicylate (10^{-3} M). It was slightly increased by phenylbutazone and indomethacin (each at 5×10^{-4} M), even in the presence of 5% DMF (which, alone, slightly decreased it). Viscosimetric analysis of the same systems gave similar results, i.e. no reversal by the drugs of guanidine-induced denaturation.

BSA is also denatured by sodium dodecyl sulfate. In the present experiments, 5% and 1% SDS produced nearly the same increase in $-[\alpha]_D$ (to 66·5 and 65·1 respectively), and phenylbutazone (10^{-3} M) had no effect on either change. At 1%, SDS raised $\eta_{red.}$ from 0·037 to 0·210, and neither flufenamic acid nor sodium salicylate (10^{-3} M) inhibited the rise.

Denaturation of other proteins

One major question is whether anti-inflammatory agents inhibit the heat denatura-

tion of all proteins generally or of only a few serum proteins or perhaps of serum albumin alone. We studied this by following viscosity changes in solutions of ovalbumin, bovine fibrinogen and bovine γ-globulin, and turbidity changes in rat tail collagen at the lowest temperatures capable of producing significant denaturation.

With ovalbumin (6·5%) at 52°, indomethacin and phenylbutazone (10^{-3} M) actually accelerated the denaturation, as shown by the viscosity rises. With fibrinogen (5%) at 38°, the results were erratic; over the range 10^{-5} to 10^{-4} M, phenylbutazone inhibited the viscosity rise most at the lower concentrations and least at the higher; indomethacin and mefenamic acid both accelerated the rise. With γ-globulin (4%) at 57°, the results again varied with drug concentration; 5×10^{-4} M levels of indomethacin, aspirin and mefenamic acid accelerated the viscosity increase, while 1×10^{-4} M levels inhibited it. With collagen at 37°, 10^{-4} M levels of phenylbutazone, indomethacin and mefenamic acid produced no effect on either lag time or fibril growth rate.

None of the acylated BSA preparations underwent any measurable heat precipitation, even when heated at temperatures up to 95° for as long as 60 min.

DISCUSSION

This study shows that nonsteroidal anti-inflammatory agents inhibit the heat denaturation of bovine serum albumin. They diminish both the solubility loss and the viscosity rise which accompany structural changes in the protein.

Turbidimetric assays for BSA denaturation are quite sensitive to anti-inflammatory drug concentrations which span the entire clinically useful range (0·01–1·0 mM). The viscosimetric assays are somewhat less sensitive, detecting activity down to only about 0·1 mM. Turbidimetric assays are also more reproducible than viscosimetric, giving control values which agree to within 5–10 per cent in series run under the same experimental conditions. These differences probably stem from the fact that turbidity is highly sensitive to the size of particles and depends very little on their shape or hydration, while viscosity is more a function of shape and hydration than of particle size. These assays therefore convey complementary information.

The mechanism by which anti-inflammatory drugs affect albumin is far from clear, because albumin aggregation probably involves alterations in electrostatic, hydrogen, hydrophobic and disulfide bonding. The drugs act at concentrations at least 100 times lower than the fatty acid stabilizers studied earlier.[15] The difference may be due to a more facile association with the exposed amino groups ($R - NH_3^+$) to prevent lateral alignment of charged bonding regions; failure of acetylated albumin to aggregate supports this idea.

Our findings certainly do not, in themselves, implicate albumin denaturation in the inflammatory process, but there may be a relevant connection to studies made on the antigenicity of albumin aggregates. Maurer[16] has shown that heat denaturation of BSA produces aggregates which are immunologically distinct from the parent protein, and aggregate-like molecular species seem to be present as part of a microheterogeneous population in unheated albumin;[9, 17] such spontaneously occurring albumin aggregates behave as antigens in the production of at least one immuno-inflammatory disease, serum sickness nephritis.[18]

6-Mercaptopurine, cytoxan, methotrexate and 1-aminocyclopentane carboxylic acid (ACPC) do not inhibit denaturation (Tables 1 and 2), indicating that the test is not

selective for drugs whose activity stems from inhibition of cellular proliferation[19] or, in effect, protein synthesis. Thus, at the present time, stabilization of albumin to heat denaturation helps to characterize a drug only at the level of molecular interactions. Such characterizations have a 2-fold value: they provide a simple supplemental screening procedure and they focus on the fairly new and potentially powerful idea that drugs may act through changes in protein conformation.

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