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DUAL EFFECTS OF PROSTAGLANDINS ON HEAT DENATURATION OF SERUM ALBUMIN

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Abstract—Prostaglandins E_1, E_2, $F_1\alpha$ and dihydro E_1 all inhibit albumin aggregation during heat denaturation. However, in the concentration range 1–17 $\times$ 10^{-5} M, the prostaglandins increase the viscosity at the lower levels, while decreasing it at the higher levels. These agents augment the actions of anti-inflammatory agents in inhibiting turbidity formation while opposing their inhibition of protein unfolding. Modification of the amino, carboxyl or disulfide groups of the albumin resulted in loss of the viscosity-enhancing effect.

THE PHARMACOLOGICAL actions of prostaglandins include smooth muscle stimulation, blood pressure reduction, relaxation and contraction of bronchial muscle, antagonism of hormonal-induced lipolysis, inhibition of gastric secretion and inhibition of platelet adhesiveness.[1–4] A few of the apparently conflicting pharmacological observations may be reconcilable by the findings that members of the prostaglandin group have been shown to inhibit cyclic adenosine monophosphate (AMP) formation in some tissues and enhance it in others, and that direction of the change can vary with dosage.

Since little is known of prostaglandin behavior at the level of molecular interaction, our interests in protein stabilization by anti-inflammatory agents[5] and in the bronchodilator activity of prostaglandin PGE_2[6] led us to study the actions of several prostaglandins on solutions of bovine serum albumin (BSA) undergoing heat denaturation. This paper reports the unexpected findings.

METHODS

Prostaglandin E_2 was prepared enzymatically.[7] 4-Hydroxy-2,3-dimethylcyclopentanone and racemic dihydroprostaglandin E_1 [3-hydroxy-2(3-hydroxyoctyl)-5-oxocyclopentaneheptanoic acid][8] were synthesized by Dr. D. Strike of these Laboratories. We are indebted to Dr. John Pike of the Upjohn Company for samples of PGE_1 (lot 8111-JEP-76A) and $PGF_1\alpha$ (lot 8370-JMB-17D), to Geigy Pharmaceuticals for phenylbutazone, and to Merck, Sharpe & Dohme for indomethacin.

Prostaglandins were assayed for purity by thin-layer chromatography and by alkaline conversion to the dienone with an absorption maximum at 278 nm.[7] In the binding experiments, ultraviolet absorption measured both prostaglandin and protein, and biological activity supplemented prostaglandin assays. For the latter, comparisons were made at various dilutions on the drug-induced contractions of isolated guinea pig ileum. Prostaglandin concentrations of about 10^{-6} M could be detected by the

chemical assay with an error of about 10 per cent, and concentrations of 10^{-9} M could be detected by the biological assay, with an error of about 40 per cent. Both PGE_1 and PGE_2 possessed about the same biological potency as acetylcholine.

BSA was obtained from Pentex, Inc. Crystalline material was used except where albumin derivatives or their Fraction V starting materials were used. Succinylation was carried out as described earlier.[5] Penicilloylation was carried out by reacting 10 g Fraction V BSA with 16·1 g benzylpenicillin in 0·1 M potassium phosphate, pH 7·4, at 37° for 5 days, followed by extensive dialysis and lyophilization. Dinitrophenylation was performed by reacting 2·76 g Fraction V with 2·24 g 2,4-dinitrofluorobenzene in 100 ml of 0·1 N Na_2CO_3; after stirring for 3 hr at room temperature, the mixture was exhaustively dialyzed at 4° against both tap water and distilled water, and the retentate was lyophilized. Sodium borohydride reduction was carried out by the method of Brown,[9] using 2-octanol to suppress foaming. *S*-sulfonation was carried out by reacting 2·76 g Fraction V with 1·51 g Na_2SO_3 in 0·1 M Tris, pH 8·4, containing 8 M urea, at 25° for 3 hr; the product was dialyzed extensively and the retentate was lyophilized. Carboxylic acid group modification was carried out by the carbodiimide–glycine ester procedure of Hoare and Koshland.[10]

Glass test tubes (18 × 155 mm) containing 5 ml of buffered solutions (2·5 ml of 2% BSA in 0·05 M Tris-acetate plus 2·5 ml prostaglandin in Tris-acetate, pH 6·0, containing 5% dimethyl formamide) were placed in a water bath whose temperature was controlled to 0·01° by a "micro-set" thermoregulator probe and relay. After exactly 10 min, the tubes were cooled in ice water and then allowed to equilibrate in a second bath at 25° (23° in the human serum experiment). Samples were rejected if flocculation occurred. Turbidity, if any, was read at 660 nm. Viscosities were determined as described earlier,[5] and results were expressed as reduced viscosity:

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

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.

RESULTS

Heating of albumin solutions causes molecular unfolding, resulting in an increase in viscosity, and molecular aggregation, resulting in an increase in turbidity. Nonsteroidal anti-inflammatory agents inhibit heat denaturation and both of the accompanying changes in the physical properties of the solution.[5] Prostaglandins also inhibit the aggregation, but their influence on viscosity sets them apart from the anti-inflammatory compounds.

Variation with denaturing conditions

As the denaturation temperature rises, unfolding and aggregation increase, as illustrated in both parts of Fig. 1 at zero prostaglandin concentration. However, with increasing prostaglandin concentrations, the turbidity curves drop, while the viscosity curves remain level or rise. Opposition between the effect of prostaglandin and the effect of temperature should result in the steepest drops occurring at the lowest temperatures, and this is what one finds in the upper part (turbidity) of Fig. 1. Ob-

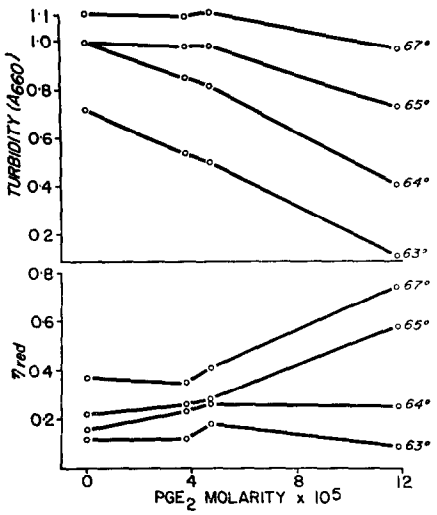

FIG. 1. Effect of PGE_2 concentration on turbidimetric and viscosimetric changes at various denaturation temperatures.

versely, reinforcement of the heating effect by the chemical should produce the greatest rise with increasing prostaglandin concentration at the highest temperature, as we found in the viscosity studies shown in the lower part of Fig. 1.

Using guanidine (2 M and 4 M) as the denaturant, no enhanced change in optical rotation[5] was found with PGE_2 at $5{\cdot}7 \times 10^{-5}$ M, $1{\cdot}1 \times 10^{-4}$ M or $5{\cdot}7 \times 10^{-4}$ M.

Variation with structure of prostaglandin and related compounds

PGE_1, $PGF_1\alpha$, dihydro PGE_1 and PGE_2 all decreased the turbidity of heated BSA solutions throughout most of the prostaglandin concentration range studied, 1–17 $\times 10^{-5}$ M (Figs. 2 and 4). In contrast, their effects on viscosity varied with their concentrations. In general, at the lower levels they increased viscosity, while at the higher levels they decreased it. In seven experiments in which crystalline BSA was warmed at 65° for 10 min and PGE_2 concentrations were varied over a range of 1×10^{-6}–$1{\cdot}7 \times 10^{-4}$ M, maximal viscosity appeared at about 6×10^{-5} M ($6{\cdot}2 \pm 2{\cdot}8 \times 10^{-5}$ M; range $2{\cdot}3$–$11{\cdot}4 \times 10^{-5}$ M).

Although each of the biologically active prostaglandins so far tested shows the divergent unfolding–aggregation effect, the structural requirements are nevertheless comparatively narrow. When the side chains in the PGE series were replaced by simple methyl groups, the resulting prototype of the cyclic moiety, 4-hydroxy-2,3-dimethylcyclopentanone, was incapable of altering either turbidity or viscosity in the range of 1–17 $\times 10^{-5}$ M. Owing to the close relation between prostaglandins and open long chain fatty acids, we also examined the actions of a saturated fatty acid, stearic acid, and the unsaturated precursor of PGE_2 arachidonic acid. In the concentration range 3×10^{-5} M to 3×10^{-4} M, each inhibited the heat-induced turbidity and viscosity increases; they produced no enhancement of the viscosity increase characteristic of the prostaglandins.

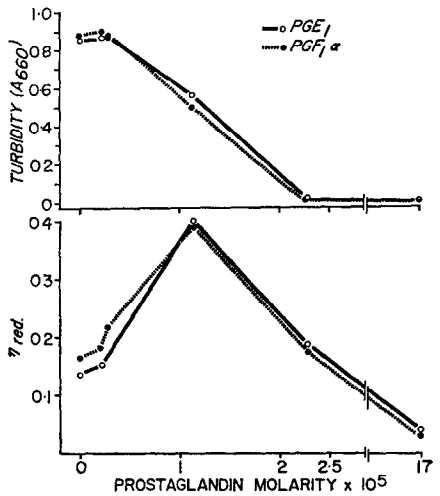

FIG. 2. Effects of PGE_1 and $PGF_{1\alpha}$ on turbidimetric and viscosimetric changes in heated BSA solutions. Determinations were made at 25° after heating for 10 min at 65°.

Anti-inflammatory agents plus prostaglandin

Since the nonsteroidal anti-inflammatory agents and the prostaglandins similarly affect the aggregating stage of heat denaturation of albumin but dissimilarly affect its unfolding, it was of interest to examine the net effect when the two species were present together. The activity of exogenous anti-inflammatory drugs might possibly modify or be modified by the activity of locally high levels of exogenous or endogenous prostaglandins. Moreover, in rheumatoid arthritis or acute allergy, the altered temperature and pH of inflamed tissues can be assumed to favor configurational changes in the proteins.

Figure 3 shows that increasing amounts of indomethacin diminish both the turbidity and viscosity of BSA and that, as anticipated, PGE_2 (3×10^{-5} M) lowers the turbidity curve while raising the viscosity curve. This can be viewed as a modification of the action of the anti-inflammatory drug by prostaglandin or, as indicated in Fig. 4, a modification of the action of prostaglandin by an anti-inflammatory drug. In Fig. 4, the curve of viscosity versus PGE_2 concentration retains its typical shape in the presence of 10^{-4} M phenylbutazone, but it is markedly lowered.

While it is known that albumin can bind phenylbutazone[11] and that prostaglandin-like materials have been identified in plasma,[12] there have been no reports of prostaglandin binding to albumin. We tested the possibility of such binding by means of a Sephadex G-25 equilibrium procedure;[13] protein and prostaglandin bound to protein would be excluded by the gel and remain in the external phase; unbound prostaglandin would pass freely between the internal and external phases. Equilibrium mixtures contained 1·5 g Sephadex, the prostaglandin and albumin, each at 2×10^{-5} M, and 0·05 M potassium phosphate buffer, pH 7·2, total volume 10·0 ml. PGE_1 and PGE_2 were tested, as well as BSA Fraction V (both undenatured and denatured by heating the buffered solution at 65° for 10 min) and defatted crystalline BSA; for controls, prostaglandin, albumin or Sephadex was omitted. After 20 hr of shaking at 25°, the excluded solution contained 93 per cent of the theoretical albumin, based upon

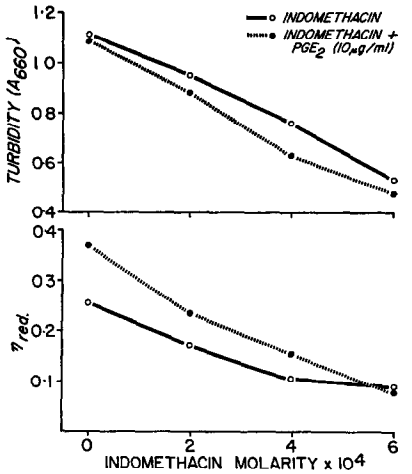

FIG. 3. Effects of indomethacin plus PGE_2 on denaturation of BSA. PGE_2, $2{\cdot}8 \times 10^{-5}$ M; experimental procedure as in Fig. 2.

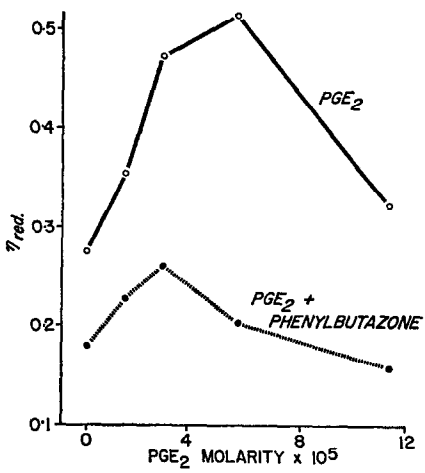

FIG. 4. Effects of phenylbutazone plus PGE_2 on denaturation of BSA. Phenylbutazone 10^{-4} M; experimental procedure as in Fig. 2.

water regain of the dry dextran. For the prostaglandin, chemical assays showed at most 10 per cent enrichment in the external phase and biological assays showed none. The results were the same for the two prostaglandins and the various albumins.

Another experiment was set up to determine whether phenylbutazone could displace trace quantities of prostaglandin which might be bound to BSA Fraction V. The protein (5 g) was incubated with 89 milligrams of phenylbutazone at 4° for 5 days, dialyzed, and the dialysate analyzed for prostaglandins by thin-layer chromatography. No displaced prostaglandin could be detected by the methods used.

Effects on modified and unpurified albumins

Several derivatives of BSA were prepared for the purpose of learning which functional groups were involved in the observed viscosity changes. In a few cases, notably *S*-sulfonation, the method of altering functional groups could be viewed as drastic enough to denature directly, but if this did occur irreversibly it was not reflected in large viscosity changes. Values for η_{red} of unmodified, unheated BSA were 0·030 $\pm$ 0·009, while 10 min of heating at 65° gave values of 0·189 $\pm$ 0·025. Largest initial values of η_{red} for derivatives were 0·113 (succinylated), 0·070 (sulfonated) and 0·066 (carboxyl-modified BSA). The other three derivatives showed initial η_{red} values between 0·028 and 0·042.

The albumins which had undergone *N*-acylation (succinylation, penicilloylation) or carboxyl modification (coupling to glycine ethyl ester) proved to be resistant to heat-induced viscosity and turbidity changes. Nor did PGE_2 at several levels between 1×10^{-5} and 17×10^{-5} M promote any increase in viscosity.

N-dinitrophenylation and modification of the disulfide bonds (sulfonation or reduction to thiol groups) gave derivatives whose viscosity rose on heating. These rises were inhibited by PGE_2 at 2×10^{-5} and 17×10^{-5} M.

Defatted crystalline BSA responded to both PGE_2 and dihydro PGE_1 ($1–17 \times 10^{-5}$ M) similarly to the non-defatted BSA, i.e. enhanced viscosity at the lower levels, decreased viscosity at the higher levels, and decreased turbidity at all levels.

Human serum, of which albumin comprises about three-fourths of the solids, also becomes more viscous when heated in the presence of PGE_2. Table 1 shows that at

TABLE 1. EFFECT OF PROSTAGLANDIN E_2 ON VISCOSITY OF HUMAN SERUM*

Additive	η_{red}
None	0·100
PGE_2 ($5·7 \times 10^{-5}$ M)	0·142
Phenylbutazone (4×10^{-4} M)	0·034
Phenylbutazone + PGE_2	0·076
Indomethacin (4×10^{-4} M)	0·038
Indomethacin + PGE_2	0·076

* Lyophilized serum reconstituted to 4% in water. Viscosity assayed at 23° after heating for 10 min at 65°.

$5·7 \times 10^{-5}$ M, the prostaglandin increased η_{red} by 42 per cent in the absence of any anti-inflammatory agent and partially suppressed the inhibitory actions of both phenylbutazone and indomethacin.

DISCUSSION

Alteration by prostaglandins of the secondary, tertiary or quaternary structure of albumin and perhaps other globular proteins may lead to increased susceptibility to proteolytic attack, changed antigenicity, and increased or decreased capacity for binding small molecules.

During denaturation there are changes in individual protein molecules, in the interactions between protein molecules, and in the interactions between protein molecules and other molecules present. The extensive modifications in molecular configuration are deduced indirectly from measurements of sedimentation, viscosity, electrophoresis and light scattering. Configurational changes often expose previously buried functional groups; as a consequence, the state of dispersion may change, producing association, polymerization, aggregation or coagulation. In each of these associative changes, independent molecules unite into fewer particles, and the changes can be measured by sedimentation, viscosity, light scattering and electron microscopy. Although changes in viscosity may reflect either unfolding or molecular aggregation, changes in turbidity usually ensue only from previous changes in the secondary or tertiary structure and occur only if the protein concentration is high enough. So a dichotomy between viscosimetric and turbidimetric changes is understandable, if not expected.

The denaturation of serum albumin has been characterized as follows:[14] hydrogen bonds are first ruptured and rearranged, and the molecules aggregate in the form of a metastable polymer of units linked by hydrogen and hydrophobic bonds; following this, a rapid intermolecular disulfide interchange occurs. If a denaturing substance is present when the albumin is heated, the unfolding may be followed by a second step in which the denaturant molecules displace water and are adsorbed on protein sites which are not available before heating.

The present prostaglandin findings do not conform to this scheme insofar as we adduced no evidence for significant binding with either native or denatured albumin. Moreover, it is difficult to account for the inhibition of turbidity formation and for the reversal of viscosity enhancement at higher prostaglandin levels. Water molecules may be part of the answer. They are capable of forming doubly hydrogen-bonded bridges between carboxyl oxygen atoms of adjacent residues along the chain and thereby stabilizing tightly coiled α-helices. If thermally induced expulsion of water led first to increased unfolding and later to precipitation, then interaction with prostaglandin might alter the properties of the aqueous medium and block the formation of new water–water bonds at the expense of the stabilizing water–protein bonds.

The effects of PGE_1, $PGF_{1\alpha}$ and dihydro PGE_1 on turbidity and viscosity appeared at lower concentrations than did those of PGE_2. PGE_2, PGE_1 and dihydro PGE_1 have one hydroxyl and one carbonyl in their rings; these factors are therefore not responsible for the lesser activity of PGE_2. Dihydro PGE_1 has no chain double bond, PGE_1 and $PGF_{1\alpha}$ have only one, and PGE_2 has two. The extra double bond adds rigidity to the molecule but, if a factor of this kind modifies the interaction, the mechanism is unclear. Moreover, in seeking new insight into the structural determination of function, it is sobering to note that in eight biological test systems which included smooth muscle contraction, vasodilation and depression of blood pressure,[15] PGE_2 was more active than PGE_1 in three, less active in one, and as active in four.

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