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Inhibition of Protein Aggregation by Vitamin E and Selenium (37742)

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Studies by Mizushima (1, 2), Mizushima and Suzuki (3), and Mizushima and Kobayashi (4) demonstrated that nonsteroidal anti-inflammatory drugs stabilize bovine serum albumin (BSA) to heat-induced aggregation at a concentration of 10^{-3} *M, in vitro*; lower concentrations were not studied. Although phenylbutazone, indomethacin and flufenamic acid were found to stabilize human gamma globulin (HGG) to a slight degree at a concentration of 10^{-3} *M,* oxyphenbutazone and acetylsalicylic acid were found to be ineffective (2, 3). Brown and Mackey (5) have shown further that indomethacin, phenylbutazone and flufenamic acid stabilize BSA at concentrations *in vitro* as low as 5×10^{-5} *M*.

Pollock and Brown (6) recently demonstrated that steroidal anti-inflammatory drugs (at dosages used clinically in humans) and vitamin E, when administered (ip) to rats for 3 days, stabilized hepatic lysosomes isolated from these animals. In a similar study (7), selenium, vitamin E, or simultaneous administration of both at various dosages, was found to stabilize isolated rat liver lysosomes. Since anti-inflammatory drugs and vitamin E also stabilize erythrocyte and leucocyte membranes (8–12), it has been suggested that this stabilizing effect on cell membranes may be due to the interaction of these agents with certain proteins in the membranes (12). These data, together with preliminary clinical evidence[2] that vitamin E may be an effective adjunct to steroid therapy in rheumatoid arthritis, suggest that vitamin E may possess activity similar to that of steroidal or nonsteroidal anti-inflammatory drugs.

The purpose of this study was, therefore, twofold: first to determine whether selenium and/or vitamin E can inhibit the *in vitro* aggregation of various serum proteins and to compare their effect or degree of effect with that of steroidal and nonsteroidal anti-inflammatory agents; and second, to investigate the possible anti-inflammatory activity of selenium and/or vitamin E in adjuvant-induced polyarthritis in rats.

Materials and Methods. Heat-induced aggregation of serum proteins. Drug solutions were prepared immediately before each experiment by dissolving the drugs in isotonic saline. A dilute sodium hydroxide solution was employed to dissolve the acidic drugs and to neutralize the drug solutions to pH 6.5 to 7.0. Appropriate dilutions were then made for 2×10^{-3} through 2×10^{-9} *M* solutions. For vitamin E drug solutions *d-alpha*-tocopherol polyethylene glycol 1000 succinate (TPGS, Eastman Chemical Products, Inc.) was used; sodium selenite (Chromalloy American Corp.) was used as the source of selenium. Other drugs used included hydrocortisone-21-phosphate (Sigma Chemical Co.), acetylsalicylic acid (Aspirin, USP), phenylbutazone (Ciba-Geigy), and indomethacin (Merck, Sharp and Dohme).

The following serum proteins were used: HGG (Cohn fraction II, Nutritional Biochemicals Co.), rat gamma globulin (RGG,

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Cohn fraction II, Miles Laboratories, Inc.) and BSA (crystallized and lyophilized, Sigma Chemical Co.).

Slight modifications of previously described methods (1–4) were used to study aggregation of serum proteins. Two milliliters of RGG or HGG (0.2% dissolved in 0.067 *M* sodium phosphate buffered isotonic saline, pH 6.4) and 2 ml of drug solution were mixed and kept for 20 min at room temperature (20°). The mixture was then heated for 20 min (RGG) or 40 min (HGG) at 70° in a water bath and immediately cooled in an ice bath (0–2°). Two milliliters of BSA (1.0% dissolved in 0.067 *M* sodium phosphate buffered isotonic saline, pH 5.3) and 2 ml of drug solution were mixed and kept for 20 min at room temperature (20°). The mixture was then heated for 2 min at 67° in a water bath and immediately cooled in an ice bath. The degree of turbidity of the mixture was determined by measuring absorbance at 660 nm in a Bausch and Lomb Spectronic 20 colorimeter using 0.067 *M* sodium phosphate buffered isotonic saline as a blank, and protein solutions containing isotonic saline (no drugs) as controls.

Percentage inhibition of denaturation was calculated by the following expression:

$$100 - \left[\frac{\text{Absorbance (with drug) at 660 nm}}{\text{Absorbance (protein alone) at 660 nm}} \times 100\right] = \%\ \text{inhibition.}$$

Control values for heated BSA ($n = 17$), HGG ($n = 58$), and RGG ($n = 49$) were, respectively, 1.06 ± 0.08 (SEM), 0.33 ± 0.04, and 0.32 ± 0.04.

Polyarthritis in rats. Methods used to induce and measure drug effects on adjuvant-induced polyarthritis in rats have been previously described (6). In this study 0.9% saline was used as the vehicle for drug solutions.

Results and Discussion. Heat-induced aggregation of serum proteins. Phenylbutazone, indomethacin and hydrocortisone-21-phosphate significantly inhibited aggregation of BSA (Table I) to about the same degree at concentrations of 10^{-5} or 10^{-4} *M, viz,* 20 to 29% inhibition at 10^{-5} *M* and 44 to 58% inhibition at 10^{-4} *M*. Acetylsalicylic acid produced less inhibition at concentrations of 10^{-4} or 10^{-3} *M*. Results obtained with indomethacin and phenylbutazone compare favorably with those previously reported (5). Sodium selenite was inactive at all concentrations tested on BSA, *viz,* 10^{-8} through 10^{-3} *M*.

In contrast to results reported with BSA, those observed with HGG (Table II) and RGG (Table III) indicate that concentrations of anti-inflammatory drugs as low as 10^{-8} to 10^{-6} *M* are effective in attenuating aggregation of these proteins; maximal inhibition was obtained at 10^{-4} *M*. Acetylsalicylic acid was less effective at the concentration of 10^{-6} *M* using HGG or RGG, and was maximally effective at 10^{-3} *M*. Such results indicate lesser potency for acetylsalicylic acid than either phenylbutazone or indomethacin. Sodium selenite produced inhibition of similar *magnitude* using either HGG or RGG, *but* a lower concentration (10^{-8} *M*) was required for this inhibition with HGG; concentrations of 10^{-5} *M* sodium selenite were needed to effect a significant inhibition of aggregation of RGG. Vitamin E produced significant, but very slight inhibition (18%) of aggregation using HGG. With RGG, greater inhibition

TABLE I. Effects of Drugs on Heat-Induced Aggregation of BSA.

Drug	% Inhibition (± SE)[a] at (*M*):			
	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Phenylbutazone		99 ± 0	54 ± 7	29 ± 4
Indomethacin		98 ± 1	58 ± 4	20 ± 5
Acetylsalicylic acid	63 ± 3	34 ± 3	26 ± 6	
Hydrocortisone-21-phosphate			44 ± 7	21 ± 5

[a] Standard error was based on 12 replicate experiments carried out on different days and on different samples of BSA obtained from the source listed under Materials and Methods.

TABLE II. Effects of Drugs on Heat-Induced Aggregation of HGG.

Drug	% Inhibition[a] (± SE)[b] at (*M*):							
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Phenylbutazone		+21 ± 5 (12)	8 ± 7 (12)	30 ± 3 (12)	56 ± 3 (16)	31 ± 6 (20)	24 ± 4 (11)	
Indomethacin		+23 ± 7 (12)	34 ± 4 (12)	35 ± 5 (16)	35 ± 4 (15)	25 ± 8 (11)		
Acetylsalicylic acid	14 ± 3 (12)	40 ± 7 (12)	29 ± 5 (16)	28 ± 6 (11)	17 ± 4 (8)			
Hydrocortisone-21-phosphate			32 ± 8 (12)	30 ± 4 (20)	28 ± 7 (15)	10 ± 9 (12)		
Sodium selenite		7 ± 2 (12)	14 ± 5 (12)	42 ± 6 (12)	44 ± 9 (12)	49 ± 8 (12)	38 ± 4 (27)	+9 ± 3 (18)
Vitamin E			16 ± 3 (9)	18 ± 3 (9)	18 ± 3 (16)	3 ± 2 (12)	+2 ± 3 (12)	+3 ± 3 (12)

[a] Plus signs indicate increased aggregation.

[b] Standard error based on the number of replicates shown in parentheses. Experiments were carried out on different days and on different samples of HGG obtained from the source listed under Materials and Methods.

TABLE III. Effects of Drugs on Heat-Induced Aggregation of RGG.

Drug	% Inhibition[a] (± SE)[b] at (*M*):						
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Phenylbutazone		+4 ± 9 (6)	32 ± 6 (24)	39 ± 4 (22)	40 ± 5 (28)	22 ± 5 (11)	
Indomethacin		+9 ± 5 (6)	38 ± 3 (12)	41 ± 5 (16)	44 ± 3 (14)	42 ± 2 (10)	8 ± 2 (10)
Acetylsalicylic acid	31 ± 2 (12)	60 ± 4 (12)	54 ± 4 (16)	36 ± 3 (12)	22 ± 3 (10)		
Sodium selenite		50 ± 7 (16)	57 ± 7 (20)	27 ± 7 (17)	14 ±10 (17)	0 ±13 (12)	
Vitamin E		+2 ± 4 (6)	54 ± 5 (21)	59 ± 5 (18)	53 ± 6 (18)	2 ± 7 (8)	

[a] Plus signs indicate increased aggregation.

[b] Standard error based on the number of replicates shown in parentheses. Experiments were carried out on different days and on different samples of RGG obtained from the source listed under Materials and Methods.

TABLE IV. Effects of Mixtures of Vitamin E and Selenium on Heat-Induced Aggregation of RGG or HGG.

Drug	Concn (M)	Protein	% Inhibition (± SE)[a]
Vitamin E + sodium selenite	5×10^{-7}[b] 1×10^{-7}	RGG	17 ± 6 (13)
Vitamin E + sodium selenite	5×10^{-7} 1×10^{-6}	RGG	15 ±15 (9)
Vitamin E + sodium selenite	1×10^{-6} 1×10^{-8}	HGG	31 ± 6 (12)

[a] Standard error based on the number of replicates shown in parentheses. Experiments were carried out on different days and on different samples of RGG obtained from the source listed under Materials and Methods.

[b] Percentage inhibition of 11 replicates of this concentration alone in RGG was 30 ± 9 (mean ± SE).

was observed (59%) with vitamin E at concentrations (10^{-6} to 10^{-4} M) similar to those used with HGG. Hydrocortisone-21-phosphate was tested with HGG only, producing maximal inhibition of about 30% at concentrations as low as 10^{-6} M.

Although a synergistic effect of vitamin E and sodium selenite was demonstrated *in vivo* on rat liver lysosomes (7), no synergism was found when these compounds were admixed and studied using RGG or HGG, Table IV.

Polyarthritis. The effects of the following dose levels of vitamin E were tested by daily injections (ip): 0.039 and 0.39 IU/kg. Dose levels of selenium (in the form of sodium selenite) were: 0.0005, 0.002, and 0.005 mg/kg. Combinations of these agents were also tested by daily ip injections at the dose levels indicated in parentheses: vitamin E (0.039 IU/kg) plus selenium (0.005 mg/kg); vitamin E (0.039 IU/kg) plus selenium (0.002 mg/kg); and, vitamin E (0.34 IU/kg) plus selenium (0.005 mg/kg). Vitamin E and/or selenium when administered alone or simultaneously had no effect on adjuvant-induced arthritis on Days 2, 4, or 21 (after inoculation with heat-killed mycobacteria), with one exception. The only significant inhibitory effect observed was that with vitamin E (0.039 IU/kg) plus selenium (0.002 mg/kg) on the arthogram score on Day 21; this was 28%. Hind paw swelling was unaltered. Mean swelling (ml ± SE) on Days 2 and 4, respectively, in the arthritic control animals was 1.04 ± 0.09 ($n = 8$) and 0.93 ± 0.08 ($n = 8$). Mean hind paw volumes (ml ± SE) on Day 21 in the right and left arthritic control hind paws, respectively, was 3.28 ± 0.20 and 2.83 ± 0.29; negative control values were 1.37 ± 0.02 for either paw. The arthogram score (±SE) on Day 21 in the arthritic controls was 15.9 ± 1.6. These data are consistent with those reported previously (6). In those studies it was shown that although vitamin E stabilized lysosomes it had no effect on adjuvant arthritis. Experiments were extended in this study to include mixtures of vitamin E and selenium (as sodium selenite) that were found to stabilize lysosomes *in vivo* (6). Again, however, it was found that neither vitamin E, selenium, nor various dosages of these substances given simultaneously inhibited arthritis.

We can only conclude that although certain plasma proteins or cellular or organelle membranes are stabilized by these compounds, such effects are not pertinent to the onset and development of polyarthritis in rats. This conclusion is based, however, on the assumption that the membranes of lysosomes derived from cells involved in inflammation are identical to or at least similar to the membranes of lysosomes of rat liver cells. Nevertheless, results resported herein on plasma proteins may indicate a useful effect of vitamin E and/or selenium nutritionally, or in certain inflammatory diseases which may not have been truly represented by adjuvant arthritis.

Summary. Nonsteroidal anti-inflammatory drugs were found to prevent heat-induced aggregation of bovine serum albumin (BSA), human gamma globulin (HGG), or rat gamma globulin (RGG) *in vitro*. Vitamin E and selenium, in the form of sodium selenite, also stabilized HGG or RGG. Neither vitamin E, sodium selenite, nor various combinations of these agents, when administered daily (ip) to polyarthritis rats, prevented the onset or development of the arthritis. Results are discussed briefly.

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