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Interaction of anti-inflammatory drugs with serum proteins, especially with some biologically active proteins

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The interaction of clinically established anti-inflammatory drugs with some proteins has shown these drugs to strongly inhibit heat coagulation of whole serum at a concentration attainable in the sera of patients. Phenylbutazone and sodium salicylate do not inhibit the biological activity of three biologically active and labile serum proteins, namely, necrotizing factor, heterogenous serum and complement. However, they do influence the effect of heat on these proteins. The relation between this drug action *in vitro* and the possible mode of action of the proteins *in vivo* is discussed.

As reported by Mizushima & Suzuki (1965) and Mizushima (1966) many non-steroidal anti-inflammatory drugs interact with plasma proteins and especially with Cohn's fraction IV and V. All the acidic anti-inflammatory drugs stabilize serum albumin and some other fractions against heat coagulation, but paradoxically, accelerate the heat precipitation of the crude globulin fraction (fraction IV-4). It was postulated that this property of drugs may have some connection with their mode of anti-inflammatory action. It is important in discussing possible modes of drug action to know if the drug effect *in vitro* is observable in a medium similar to a body fluid and at a drug concentration which can be attained in the serum of patients. We have examined the interaction of anti-inflammatory drugs with proteins, especially with biologically active and heat labile proteins, using nearly undiluted serum and at therapeutic (serum) drug concentrations (Hollander, 1962; Rechenberg, 1962; Mizushima, unpublished observations).

Experimental

DRUGS

Salicylic acid (commercial), phenylbutazone (Geigy), flufenamic acid (synthesized), and ibufenac (Boots) were used. All compounds were dissolved in isotonic saline and neutralized with sodium hydroxide to give the appropriate concentrations for each experiment.

BIOLOGICALLY ACTIVE AND HEAT LABILE PROTEINS

Necrotizing factor. Fresh sera from active rheumatoid patients were obtained from the clotted blood, stored below −20° and used within 2 days. The serum was mixed with the drug solution and 0·1 ml of the mixture was injected intradermally into the backs of male guinea-pigs weighing 300–400 g. Necrotizing activity was recorded 4 hr after the injection as described by Mizushima, Kasukawa & Oshima (1962). To

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compare the activity of two sera, injections were made at the same site on the left and the right side of the one animal.

Heterogenous serum. Fresh goat serum obtained from clotted blood was used. The storage of the serum, method of injection into guinea-pigs and recording of the biological activity were similar to procedures used in the experiments with necrotizing factor.

Complement. Pooled fresh sera from 10 guinea-pigs (stored below −20°) was used. Complement activity of the sera was 230 units measured by Mayer's method (Kabat & Mayer, 1961). Serum, 2·5 ml, was diluted 300 fold and the percentage of cells suffering haemolysis was recorded.

Results

Effect of drugs on the denaturation of some serum proteins, using whole serum. Normal sera were obtained from two healthy adults. The protein concentration of a mixture of the two sera was 77 mg/ml. Paper electrophoresis of the serum showed Alb. (64%), α-gl. (12%), β-gl. (11%) and γ-gl. (13%). The pH of the serum was adjusted to 6·3 by adding a small amount of N hydrochloric acid. To 0·45 ml of the serum was added 0·05 ml of a drug solution and the mixture was incubated at room temperature for 20 min. (0·05 ml of saline was added in place of the drug solution in the controls). The mixture was heated at 57° for exactly 3 min to denature (coagulate) heat-sensitive proteins. After cooling the mixture, 2·5 ml of phosphate-buffered saline (0·07 M sodium phosphate—0·15 M saline) pH 6·3 was added. The turbidity of the solution was measured spectrophotometrically at 660 mμ.

The average percentage inhibition of the heat coagulation of serum proteins by the added drugs is shown in Table 1. The results showed that

TABLE 1. INHIBITORY ACTION OF ANTI-INFLAMMATORY DRUGS ON HEAT COAGULATION OF WHOLE HUMAN SERUM*

Drugs†	Final concentration mg/ml‡	Percentage inhibition of coagulation
Salicylic acid	0·18	81
Phenylbutazone	0·12	72
Flufenamic acid	0 03	43
Ibufenac	0·04	59

* Whole human serum was heated at 57° C for 3 min.
† Drugs (neutralized with NaOH) were added *in vitro* to the serum.
‡ These concentrations of the drugs can usually be attained in the sera of patients after oral medication

all four anti-inflammatory drugs stabilized some serum protein(s) against heat coagulation when present at concentrations attainable *in vivo* and in nearly undiluted serum.

Interaction of drugs with heterogenous serum. In experiment A, 0·1 ml of a solution of phenylbutazone or 0·1 ml of saline was added to 0·9 ml of goat serum and the mixture allowed to stand at room temperature for at least 20 min. The mixture was then heated at 47° for 60 min. The purpose of this experiment was to see whether or not phenylbutazone influenced heat inactivation of the biologic activity of heterogenous

serum. In experiment B, the goat serum was first heated at 47° for 60 min and then 0·1 ml of the drug solution or saline was added to 0·9 ml of the heated serum.

The results of both experiments (Table 2) indicate that phenylbutazone

TABLE 2. INFLUENCE OF PHENYLBUTAZONE ON HEAT INACTIVATION OF THE BIOLOGICAL ACTIVITY OF GOAT SERUM*

Serum		1	2	3	4	5	6	7	8	9	10	11	12
Unheated serum		++±	++	++	+	++	++	++	++	+	+	++	+
Expt. A (Drug was added before heating)	Saline	++‡	++	++	+	+	+	+	++	+	+	+	±
	Phenylbutazone†	+‡	+	+	+	+	±	+	+	+	+	−	−
Expt. B (Drug was added after heating)	Saline							++	++	+	±	+	+
	Phenylbutazone†							++	++	+	±	+	+

* Whole goat serum was heated at 47° C for 60 min.
† Phenylbutazone at a final concentration of 0·1 mg/ml was added *in vitro* to the serum.
‡ ++, +, ±, − refer to the degree of inflammation of guinea-pig skin caused by goat serum.

did not alter the biologic activity of goat serum but influenced the degree of inactivation of the biologic activity obtained by mild heating.

Interaction of drugs with necrotizing factor. The experimental methods were the same as those used in experiment 2 except that fresh sera from rheumatoid patients were used in place of goat serum and these human sera were heated at 42° for 60 min.

The results (Table 3) show that phenylbutazone did not alter the

TABLE 3. INFLUENCE OF PHENYLBUTAZONE ON HEAT INACTIVATION OF NECROTIZING FACTOR*

Serum		1	2	3	4	5	6	7	8	9	10	11	12
Unheated serum		+‡	++	+++	++	+++	++	++	+++	+++	++	++	
Drug was added before heating	Saline	+‡	±	+	−	++	−	−	+	++	−	−	
	Phenylbutazone†	+‡	±	++	+	+++	+	±	++	++	+	+	
Drug was added after heating	Saline				±	++	+	+	+	++			
	Phenylbutazone†				−	++	+	+	+	+++			

* Whole rheumatoid serum was heated at 42° C for 60 min.
† Phenylbutazone at a final concentration of 0·1 mg/ml was added *in vitro* to the serum.
‡ +++, ++, +, ±, − refer to the degree of inflammation of guinea-pig skin caused by necrotizing factor.

necrotizing activity, while it influenced the degree of inactivation of the necrotizing factor obtained by mild heating.

Interaction of drugs with some component of complement. The method of experiment was the same as in experiment 2 except that fresh guinea-pig

serum and both sodium salicylate and phenylbutazone were used. The serum was heated at 50° for 20 min for heat inactivation.

The results (Table 4) indicated that salicylate and phenylbutazone

TABLE 4. INFLUENCE OF SALICYLATE AND PHENYLBUTAZONE ON HEAT INACTIVATION OF COMPLEMENT*

Drugs†	Final concentration‡	Percentage immune haemolysis§	
		Drugs were added before heating	Drugs were added after heating
Control	—	41a	46d
Salicylic acid	0·20 mg/ml	38b	45e
Phenylbutazone	0·12 mg/ml	38c	46f
Unheated serum	—	73	73

* Whole guinea-pig serum was heated at 50°C for 20 min.
† Drugs were added *in vitro* to the serum.
‡ These concentrations of the drugs can be attained in the sera of patients.
§ 2·5 ml serum diluted 300-fold was used.
b and c to a: significant ($P < 0·01$), e and f to d: not significant.

slightly but definitely ($P < 0·01$) influenced the degree of heat inactivation of complement.

Discussion

The mode of action of non-steroidal anti-inflammatory drugs is still obscure, but their inhibitory effects on oxidative phosphorylation (Whitehouse, 1964), some enzymic reactions (Rechenberg, 1962) and the formation of some inflammatory mediators and factors (Spector & Willoughby, 1963) are considered important. This suggests that non-steroidal anti-inflammatory drugs interact in some way with proteins. We have found that the interaction of these drugs with proteins in nearly undiluted serum takes place using drug concentrations that can be attained clinically in the sera of patients. The drugs could, therefore, interact *in vivo* with and stabilize some protein(s) in the serum or in the tissues of patients adequately treated with them. This *in vitro* property seems to be fairly specific to active non-steroidal anti-inflammatory drugs (Mizushima, 1965).

It is likely, judging from our findings and those on drug effects on enzymic systems (Rechenberg, 1962), that non-steroidal anti-inflammatory drugs at low concentration neither alter strongly the conformation of proteins directly nor inhibit strongly the specific combination of proteins, but do influence the conformational changes suffered by some proteins on heating. It is of interest that there are many reports which indicate the importance of protein denaturation as a cause of inflammation (Opie, 1962, 1963; Ishizaka, 1965).

Necrotizing factor (Lovell, Pryce & Boake, 1954), heterogenous serum and complement were used by us merely as examples of biologically active, heat-labile proteins. There is no evidence that these particular proteins are important as a cause of inflammation, and furthermore, their biological activity was not impaired by the drugs we used.

Regarding the experimental conditions of this study, these biologically active proteins were inactivated by *mild* heating, though the degree of heating was not physiological. Some denaturation of proteins could possibly occur *in vivo*. In these experiments, whole serum was coagulated at pH 6·3, a pH which could be attained in strongly inflamed tissue.

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