J. Pharm. Pharmac., 1970, **22**, 456

LETTERS TO THE EDITOR

Investigation of the mechanism of gelatin-induced *in vitro* red cell aggregation

We reported (Görög & Kovács, 1970) that *in vitro* non-steroid anti-inflammatory compounds were highly specific inhibitors of gelatin-induced aggregation of red cells of the rat. The present report is concerned with the mechanism responsible for the inhibitory effect on aggregation and its investigation using compounds with known biochemical mechanisms of action.

The applied method corresponds to that described earlier (Görög & Kovács, 1970). The buffered saline solution of the compounds to be tested was added to the saline suspension of the red cells of the rat and after incubation for 5 min at 20° (or 16 h at 4°) gelatin, 0·6% final concentration, was added to the cell suspension. Aggregation was estimated by the sedimentation rate of the column of cells (ESR).

The potency of the inhibitory effect is compared with phenylbutazone in Table 1. This shows that cell-aggregation was inhibited by fluoride at a higher concentration than that inhibiting red cell glycolysis (2–8 mM). When the red cells were left standing for 16 h in a medium containing fluoride before the addition of gelatin, the aggregation-inhibitory effect of the compound was significantly increased. It is known that after such a time lapse the adenosine triphosphate (ATP) content of the cells is reduced because of inhibited glycolysis. On the other hand, in high concentration, fluoride strongly inhibits the ATPase activity of red cells (Straub, 1952). Our results have therefore been explained by the assumption that the direct effect of fluoride is produced by the ATPase inhibitory action, while responsibility for the enhanced inhibition of cell-aggregation seen after prolonged incubation is ascribed to the reduced energy level of the cells. The red cell aggregating effect of gelatin was strongly inhibited by the two sulphydryl binding compounds, *p*-chloromercuribenzoate (PCMB) and Salyrgan. After incubation of the cells for 16 h the effect of PCMB was

Table 1. *Effect of various metabolic inhibitors on gelatin-induced* in vitro *aggregation of red cells of the rat*

Compound	IC50 (mM)* after incubation for		Relative effectiveness† (Phenylbutazone = 1·0) after incubation for	
	5 min	16 h	5 min	16 h
Fluoride	90	3·0	9782	319
PCMB	1	0·08	108	8·5
Salyrgan	0·49	0·12	53	12·7
DNP	0·24	0·19	26	20·2
PCP	0·045	0·042	4·8	4·4
Na-Arsenate	>30			
Na-Azide	>20			
KCN	>10			
Na–EDTA‡	35			
Na–EGTA§	16	8·0		
Phenylbutazone	0·0092	0·0094	1·0	1·0

* Concentrations producing 50% inhibition on red cell aggregation.
† The smaller the value the greater the inhibitory effect.
‡ 2 mM EDTA or EGTA produced 20 and 23% inhibition respectively.
§ In the presence of 20 mM Mg ion ($MgCl_2$).

strongly increased, while that of Salyrgan showed a moderate increase. According to Weber & Portzehl (1952), these mercuric agents inhibit the energy transfer from ATP to the actomyosin system, which explains their potent inhibitory effect on actomyosin ATPase. Of the compounds investigated, the strongest inhibitor of red cell aggregation was the pentachlorophenol (PCP), a potent uncoupling and actomyosin ATPase inhibitory compound. The inhibitory effect of 2,4-dinitrophenol (DNP) on cell aggregation was also significant. Aggregation of red cells was not inhibited by arsenate, cyanide and azide.

Our results suggest that gelatin-induced aggregation of red cells is a Ca ion dependent process. Aggregation was inhibited slightly by EDTA, but ethylene glycol bis(2-amino-ethyl) tetra-acetic acid (EGTA; Chel-De, Geigy) produced inhibition of aggregation in the presence of excess Mg ion. EGTA is a chelate-forming agent which has practically no binding to Mg ion at neutral pH and forms a specific Ca-chelate in the presence of excess Mg ion (Ebashi, Ebashi & Fujite, 1960), hence its effectiveness emphasizes the significance of the Ca ion in the process. Presumably the relatively high concentration was needed because the gelatin preparation contained fairly large amounts of Ca ion; moreover, in the red cell membrane the Ca ion is bonded at a site which is hardly accessible to the chelate-forming agents.

The results obtained point to a close similarity in the mechanism between the gelatin-induced aggregation of red cells and the aggregation of blood platelets under various influences (Mason & Saba, 1969). On the evidence of our findings the aggregation of red cells—like that of the platelets—is strongly inhibited by compounds which bind sulphydryl groups, inhibit actomyosin ATPase or have an uncoupling effect on oxidative phosphorylation. In the case of platelets, a decisive role in bringing about aggregation is ascribed to the contractile protein with ecto-ATPase activity, situated on the outer surface of the cell-membrane. Much evidence indicates that the physical and enzymatic properties of this superficial contractile protein are altered by the compounds inhibiting the aggregation of platelets (Mason & Saba, 1969). Our results indicate that gelatin-induced aggregation of red cells is an energy-requiring process in which a decisive role is played by a platelet-like Ca sensitive ecto-ATPase (Schatzmann, 1966), most probably the site of action of inhibitory compounds. Our results partly explain the resemblance to be noted between the relative inhibitory effect of non-steroid anti-inflammatory agents on red cell and platelet aggregation.

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March 4, 1970

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