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# INHIBITION OF SOME POLYMORPHONUCLEAR LEUKOCYTE FUNCTIONS BY ETHACRYNIC ACID*

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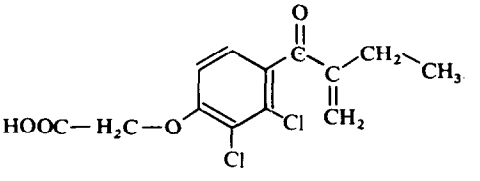
**Abstract**—Ethacrynic acid ($10^{-4}$ M) inhibits exocytosis, phagocytosis and superoxide release in rabbit polymorphonuclear leukocytes (PMN's). Dihydroethacrynic acid is a much weaker inhibitor of these PMN functions. Though ethacrynic acid inhibits ATPase activity in the PMN, this occurs at much higher concentrations than required for inhibition of exocytosis and superoxide release, thus a causal relationship seems unlikely. The same applies to inhibition of ATP generation by ethacrynic acid: the concentration required to decrease ATP level in PMN's is much higher than required for the inhibitory effect on exocytosis. Inhibition of exocytosis by ethacrynic acid can be prevented by dithiothreitol. It is concluded that vulnerable sulfhydryl groups are involved in the inhibition by ethacrynic acid.

The interaction of appropriate stimuli with the plasma membrane of polymorphonuclear leukocytes (PMN's) results in the activation of various cell functions, such as phagocytosis, exocytosis and superoxide production [1–3]. The mechanisms of these processes are largely unknown, as well as their interrelations; they may have a number of steps in common, but under some conditions these processes can proceed independently from one another [4, 5].

To elucidate the mechanism of exocytosis and its relation to other processes, we tested a number of more or less specific inhibitors. In this way the separate steps which underly exocytosis may be identified. We studied the action of ethacrynic acid, a known ATPase inhibitor, on exocytosis, phagocytosis and $O_2^-$ release by polymorphonuclear leukocytes induced by the ionophore A23187 or the chemotactic peptide, formyl methionyl leucyl phenylalanine. Studying the effect of ethacrynic acid on mast cells, Chakravarty *et al.* [6, 7], and Magro [8, 9] found experimental support for the view that a plasma membrane ATPase was involved in the secretion of histamine from these cells. For the adrenal medulla a correlation was established between inhibition of $Mg^{2+}$-activated ATPase of chromaffin granules and inhibition of catecholamine release [10]. In platelets the ATPase of the actomyosin of the platelet membrane appears to play an important role in the release reaction [11]. The PMN too possesses some ATPases. We compared the inhibitory effect of ethacrynic acid on these ATPases with the effect on the PMN functions mentioned.

Ethacrynic acid may also interfere with other cellular processes such as mitochondrial respiration [12], glycolysis [13], anion [14] and cation transport [15]. The question whether ethacrynic acid causes inhibition of exocytosis and related functions via these other inhibitions has been considered.




Ethacrynic acid =
[2,3-dichloro-4-(2-methylenebutyryl)-phenoxy]-acetic acid

## MATERIALS AND METHODS

*PMN's.* PMN's were harvested from the peritoneal cavity of rabbits, as described earlier [17]. The medium used consisted of: 140 mM NaCl, 20 mM Tris–HCl (pH 7.2), 5 mM KCl and 10 mM glucose.

*Reagents.* Ionophore A23187 was obtained as a gift from Eli Lilly Labs. Zymosan was opsonized as described before [17] and used in a final concentration of 0.5 mg/ml. Ethacrynic acid, cytochalasin A, FMLP, zymosan, cytochrome *c* type III, superoxide dismutase, and phorbol myristate acetate were obtained from Sigma Chemical Co.

Reagents insoluble in water were prepared as a stock solution in ethanol. The final ethanol concentration in the mixture with PMN's was kept below 0.5%.

Dihydroethacrynic acid was a gift of Dr. Le Quoc, Besançon.

*Exocytosis.* Exocytosis was measured as the release of granule-associated enzymes in the absence

* Part of this work was presented at the Annual Meeting of the Dutch Society of Cell Biology, abstract in *Cell Biol. Int. Reports*, **5**, 461 (1981).

of significant release of cytoplasmic lactate dehydrogenase (LDH). Two secretagogues were used in order to induce exocytosis in the absence of phagocytosis: ionophore A23187 with 1 mM $Ca^{2+}$; and the chemotactic peptide formyl methionyl leucyl phenylalanine (FMLP) combined with cytochalasin A and 1 mM $Ca^{2+}$ [16]. In the former case cells were preincubated in the presence of $Ca^{2+}$, with or without ethacrynic acid, and A23187 was used to initiate exocytosis. In the latter case cells were preincubated with $Ca^{2+}$ and cytochalasin A, and then FMLP was added to induce exocytosis. $3 \times 10^6$ PMN's, in a final volume of 1 ml, were preincubated with (or without) ethacrynic acid for 20 min at 37° in a medium containing 1 mM $Ca^{2+}$. To obtain FMLP-induced exocytosis, $5 \times 10^{-7}$ M cytochalasin A was present during preincubation. After preincubation exocytosis was initiated by addition of A23817 ($5 \times 10^{-7}$ M), or $10^{-8}$ M FMLP, and incubation was carried out for 30 min at 37°.

The cells were centrifuged at 500 *g* and the supernatant was analyzed. Lysozyme was assayed by measuring the rate of lysis of *Micrococcus lysodeikticus* at pH 6.2, according to the method of Shugar [18]. β-Glucuronidase was assayed by measuring the release of *p*-nitrophenol from *p*-nitrophenyl-β-D-glucuronide. Lactate dehydrogenase (LDH) was assayed by measuring the conversion of NADH into $NAD^+$ during the conversion of pyruvate into lactate.

*Phagocytosis.* $3 \times 10^6$ PMN's were preincubated with ethacrynic acid, with 1 mM $Ca^{2+}$ and 1 mM $Mg^{2+}$ present, for 20 min at 37°. Then zymosan was added and the mixture was incubated for 30 min at 37°. The process was stopped with $5 \times 10^{-3}$ M EDTA. Subsequently the zymosan particles taken up were counted by means of oil immersion microscopy. Cells that contained two or more zymosan particles were counted as phagocytic. The concomitant release of lysozyme was measured as described for the exocytosis experiments. During the phagocytosis experiments the leakage of LDH remained below 5 per cent.

*Superoxide ($O_2^-$) production.* Superoxide dismutase-inhibitable superoxide production was induced by phorbolmyristate acetate and determined according to the method of Babior *et al.* [19], with minor modifications. $4 \times 10^6$ PMN's in a final volume of 1.0 ml, were preincubated for 20 min at 37° with medium, 1 mM EDTA, and ethacrynic acid. Then 0.1 mM ferricytochrome *c* and phorbol myristate acetate (100 ng/ml) were added and incubation was carried out for 15 min at 37°. After centrifugation the supernatant was assayed spectrophotometrically at 550 nm. As a comparison, a mixture of the same composition, but with 20 μg superoxide dismutase per ml was treated in the same way. The absorbance values were used to calculate nanomoles of $O_2^-$ produced by $5 \times 10^6$ PMN's in 15 min.

*Assay of ATPase activity.* PMN's ($6 \times 10^7$/ml) were suspended in sucrose (0.34 M sucrose, 20 mM Tris–maleate) and ruptured in a Potter–Elvehjem homogenizer with a motor-driven Teflon pestle (10 min, ice-cooling). A soluble fraction was obtained by centrifuging a portion of the homogenate for 30 min at 40,000 *g*. A particulate fraction, which could be homogeneously resuspended, was obtained by centrifuging another portion of the homogenate at 500 *g*, followed by centrifugation of the supernatant at 40,000 *g*; the two pellets were added together. 0.3 ml cell material, corresponding to $1.5 \times 10^7$ cell PMN's for the supernatant, and $0.4 \times 10^7$ PMN's (or $1 \times 10^7$ PMN's for $Na^+$, $K^+$-ATPase) for the membranes, was used to measure $Ca^{2+}$, $Mg^{2+}$-stimulated ATPase or $Na^+$, $K^+$-stimulated ATPase activity.

The medium for $Ca^{2+}$, $Mg^{2+}$-ATPase consisted of 15 mM Tris–maleate (pH 7.4), 2 mM $Mg^{2+}$, 0.02 mM $Ca^{2+}$; the medium for $K^+$, $Na^+$-ATPase consisted of 100 mM KCl, 50 mM NaCl, 15 mM Tris–maleate (pH 7.4) and 1 mM EDTA. The reaction, in a final volume of 1 ml, was started by addition of 1 mM ATP. After 45 min incubation at 37°, 0.5 ml ice-cold 10% trichloroacetic acid was added. The mixture was centrifuged and, in the supernatant, $P_i$ was determined according to Ames [20], with a modification as described by Sha'afi [21].

*ATP.* For the determination of ATP, all cellular processes were stopped by adding an equal volume ice-cold trichloroacetic acid (10%). After neutralization with sodium acetate (1.5 M) the ATP content was determined by the bioluminescence technique, using luciferin–luciferase from fire fly tails, as described by Strehler [22]. In the experiments where the ATP level was measured, glucose was omitted from the medium.

## RESULTS

The effect of ethacrynic acid on exocytosis in the presence of cytochalasin A and chemotactic peptide is depicted in Fig. 1. In the absence of ethacrynic acid there is a strong release of the granule associated enzymes lysozyme and β-glucuronidase. At an ethacrynic acid concentration of $10^{-4}$ M this release is markedly inhibited. In the concentration range considered ethacrynic acid does not induce LDH release, which means that cell lysis does not occur. Dihydroethacrynic acid is a much weaker inhibitor of exocytosis than ethacrynic acid (Fig. 1). Inhibition

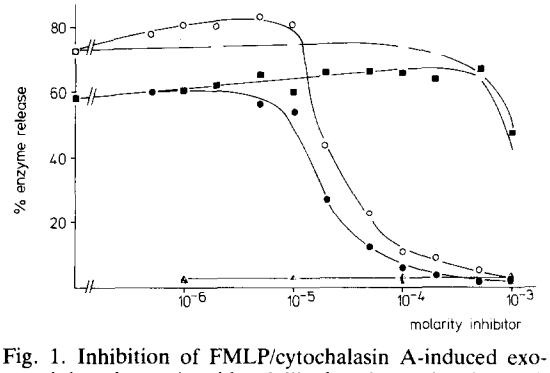



Fig. 1. Inhibition of FMLP/cytochalasin A-induced exocytosis by ethacrynic acid and dihydroethacrynic acid. Each point represents the mean value of four experiments. —○— lysozyme release (ethacrynic acid); —●— glucuronidase release (ethacrynic acid); —▲— LDH release (ethacrynic acid); —□— lysozyme release (dihydroethacrynic acid); —■— glucuronidase release (dihydroethacrynic acid); —▲— LDH release (dihydroethacrynic acid).

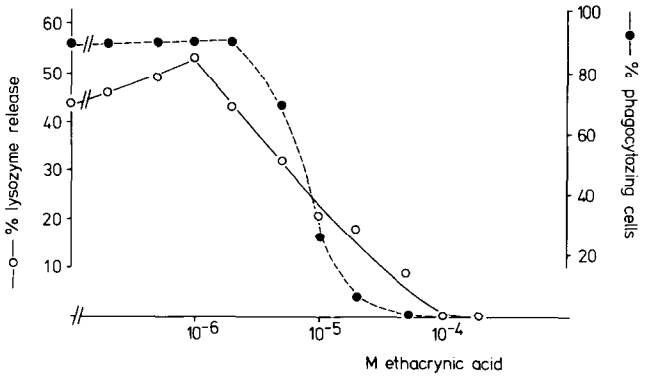



Fig. 2. Inhibition of phagocytosis and concomitant release of lysozyme. —○— lysozyme release; —●— phagocytosis. Phagocytosis was estimated microscopically; cells with two or more zymosan particles were counted as phagocytozing.

by the dihydro-compound occurs only at concentrations higher than 1 mM.

Besides inhibiting exocytosis, ethacrynic acid also inhibits phagocytosis of opsonized zymosan and the concomitant release of lysosomal enzymes (Fig. 2). Both phagocytosis and enzyme release are inhibited at about the same concentration of ethacrynic acid as is chemotactic peptide-induced exocytosis. Superoxide dismutase inhibitable superoxide release is inhibited by the same concentration of ethacrynic acid (Fig. 3), whereas dihydroethacrynic acid is a much weaker inhibitor. With both inhibitors a slight potentiation of lysosomal enzyme and superoxide release was sometimes observed at sub-inhibitory concentrations. This effect, however, was poorly reproducible.

Among the ATPases of the rabbit PMN the $Ca^{2+}$, $Mg^{2+}$-stimulated ATPase, associated with the particulate fraction, represents the bulk of activity. This enzyme activity probably corresponds to the $Mg^{2+}$-ATPase of the plasma membrane of the PMN, as found by Harlan *et al.* [22] and Smolen *et al.* [24]. The effect of ethacrynic acid on exocytosis (measured as lysozyme release) and on ATPase activities is represented in Fig. 4. The $Ca^{2+}$, $Mg^{2+}$-stimulated ATPase of the particulate fraction is more sensitive to ethacrynic acid. However, at concentrations of ethacrynic acid where exocytosis is blocked completely, there is only a relatively small inhibition of ATPase activity (Fig. 4). Dihydroethacrynic acid had only a small inhibiting effect on $Ca^{2+}$-$Mg^{2+}$-stimulated ATPase activity at the highest concentration used.

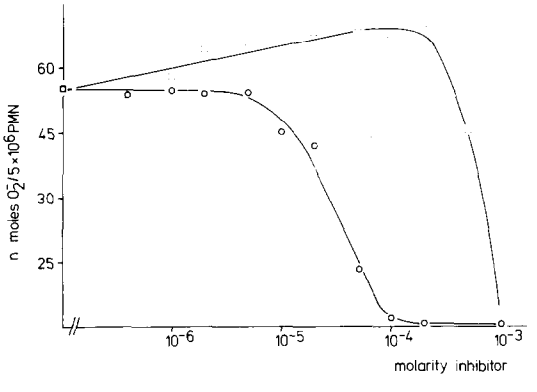



Fig. 3. Effect of ethacrynic acid and dihydroethacrynic acid on superoxide dismutase-inhibitable superoxide release by PMN's. —○— ethacrynic acid; —□— dihydroethacrynic acid. Each point represents the mean value of three experiments.

ATP, mainly derived from glycolysis, is required for PMN functions, and inhibitors of glycolysis inhibit exocytosis, phagocytosis and superoxide production. Therefore a possible interference with ATP production by ethacrynic acid was considered. The effect of ethacrynic acid on the intracellular ATP level of PMN's was measured. Concentrations of ethacrynic acid which suppress exocytosis produce only a small decrease in ATP content with time in resting cells, as compared with the effect of the usual glycolysis inhibitors such as iodoacetate and 2-deoxyglucose (Fig. 5). High concentrations of ethacrynic acid interfere with ATP production, as can be seen in Fig. 6. These concentrations are, however, much higher than those required for inhibition of exocytosis.

In order to locate the effect of ethacrynic acid we used the sulfhydryl compound dithiothreitol. Because cytochalasin A is inactivated by sulfhydryl compounds, we used the divalent cation ionophore A23187 as inducer of exocytosis. Dithiothreitol itself does not interfere with exocytosis; sometimes a potentiating effect was found. It appears that dithiothreitol prevents, wholly or partially, the inhibiting effect of ethacrynic acid on exocytosis when ethacrynic acid and dithiothreitol are simultaneously present in the medium. When, however, dithiothreitol is added after preincubation of PMN's with ethacrynic acid, the inhibiting effect of ethacrynic acid on exocytosis is the same as without dithiothreitol (Table 1).

## DISCUSSION

According to our experiments, ethacrynic acid

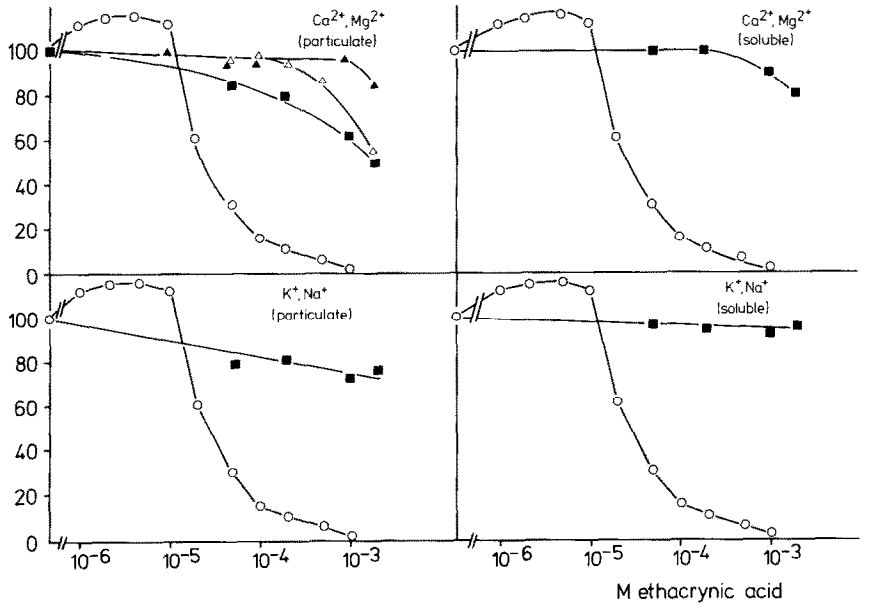



Fig. 4. Effect of ethacrynic acid on ATPase activities as compared to the effect on exocytosis. —■— ATPase activity; —○— lysozyme release. ATPase activities and lysozyme release (exocytosis) are relative to the activity and release in the absence of inhibitor (=100). In the absence of inhibitor, the amounts of $P_i$, liberated in 45 min, at 37° by the equivalent of $10^7$ cells, were as follows: Ca,Mg-ATPase, particulate fraction: $34.5 \times 10^{-8}$ moles $P_i$; Ca,Mg-ATPase soluble fraction: $2.9 \times 10^{-8}$ moles $P_i$; K,Na-ATPase, particulate fraction: $3.2 \times 10^{-8}$ moles $P_i$; K,Na-ATPase, soluble fraction: $1.8 \times 10^{-8}$ moles $P_i$. Each point represents the mean value of four experiments. For the Ca,Mg-stimulated ATPase the effect of dihydroethacrynic acid (—▲—) is included and as a comparison the effect on lysozyme release (—△—).

inhibits exocytosis, phagocytosis and superoxide release in rabbit polymorphonuclear leukocytes. Complete inhibition of all three processes occurs at about the same concentration. It is known from the literature that ethacrynic acid interferes with ATPases [6, 9], inhibits energy metabolism [12] and inhibits ion transport through membranes [14, 15]. Our results are in good agreement with observations of Dunham *et al.* [25], who found that ethacrynic acid inhibited lysosomal enzyme release in dogfish phagocytes.

Ethacrynic acid has been described as an inhibitor of several ATPases [6–9, 26]. We found that this applies to the PMN ATPases as well; the $Ca^{2+}$, $Mg^{2+}$-stimulated ATPase of the particulate fraction is especially sensitive to ethacrynic acid. In several types of secretory cells a $Ca^{2+}$, $Mg^{2+}$-stimulated ATPase has been implicated in exocytosis [7, 8, 10, 11]. Studying the effect of ethacrynic acid on histamine release from mast cells, and on $Ca^{2+}$, $Mg^{2+}$-stimulated ATPase of the plasma membrane of these cells, Magro and Chakravarty *et al.*, found a correlation between inhibition of histamine release by exocytosis, and the inhibition of the $Ca^{2+}$,

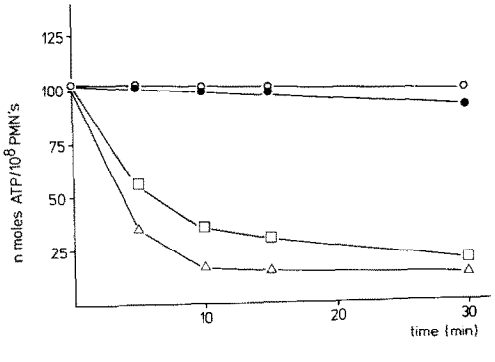



Fig. 5. Time dependence of the effect of ethacrynic acid, 2-deoxyglucose and iodoacetate on ATP content of resting rabbit PMN's. —○— ethacrynic acid, 0.05 mM; —●— ethacrynic acid, 0.2 mM; —□— iodoacetate, 0.5 mM; —△— 2-deoxyglucose, 2.0 mM.

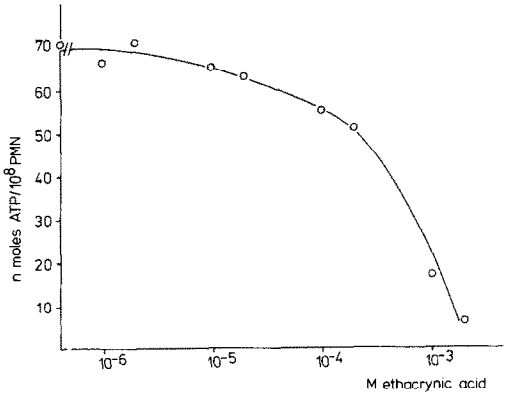



Fig. 6. Concentration dependence of the effect of ethacrynic acid on ATP content of resting PMN's.

Table 1. The effect of ethacrynic acid on A23187—or zymosan—induced exocytosis in rabbit PMN's: modification by dithiothreitol

| Additions | % Lysozyme release with A23187 | % Lysozyme release with zymosan | Phagocytosis |
|---|---|---|---|
| — | 89 ± 6 | 51 ± 5 | + |
| dith* | 89 ± 3 | 55 ± 5 | + |
| EA* | 2 ± 2 | 1 ± 4 | − |
| EA + dith | 60 ± 8 | 37 ± 7 | + |
| EA, then dith (after preinc) | 0 ± 3 | 2 ± 5 | − |

Cells were preincubated for 20 min, at 37° with the reagents indicated, without A23187 or zymosan. In the experiment with A23187, 1 mM $Ca^{2+}$ was present in the medium. In the experiment with zymosan 1 mM $Ca^{2+}$ and 1 mM $Mg^{2+}$ were present. After preincubation, A23187 ($5 \times 10^{-7}$ M) or zymosan, and in one case, dithiothreitol were added, and the mixture was incubated for 30 min at 37°.

The values given are the mean values of four experiments ± S.D.

* EA: 0.1 mM ethacrynic acid; dith: 1 mM dithiothreitol; +: more than 90% of the cells with particle uptake. −: less than 10% of the cells with particle uptake.

$Mg^{2+}$-ATPase [6–8]. Though ATPases probably are important for leukocyte function as well, it seems highly unlikely that inhibition of exocytosis and related functions in PMN's by ethacrynic acid is due to inhibition of ATPase activity. Concentrations of ethacrynic acid which cause complete inhibition of exocytosis result in only a relatively small inhibition of ATPase activity.

Exocytosis, phagocytosis and superoxide production are ATP-requiring processes, and accordingly inhibitors of glycolysis inhibit these functions [27, 28]. We have accordingly investigated the possibility that inhibition by ethacrynic acid is due to interference with ATP-generation. Known inhibitors of glycolysis such as iodoacetate and 2-deoxyglucose inhibit ATP production; a rapid decrease of the ATP level is the result. Concentrations of ethacrynic acid which inhibit exocytosis and superoxide release do not have this effect on ATP-levels. Hence it is unlikely that ethacrynic acid interferes significantly with glycolysis in this concentration range.

Another possible target for ethacrynic acid is inhibition of an ion transport system [14, 15]. Korchack *et al.* found that the anion channel blocker 4,4′-diisothiocyano-stilbene-2,2′-disulfonic acid (DIDS) inhibits exocytosis but not superoxide production in human PMN's [29]. They suggested that anion channel blockers specifically inhibit fusion of lysosomes with the plasma membrane.

In the case of ethacrynic acid it seems unlikely that inhibition of exocytosis and $O_2^-$ production is due to interference with ion transport. Dihydroethacrynic acid has been described to have the same potency of inhibiting anion transport as does ethacrynic acid [14]. The addition compound of ethacrynic acid with sulfhydryl compounds (i.e., cysteine) inhibited cation transport even more strongly than ethacrynic acid [30]. In our system dihydroethacrynic acid is a much weaker inhibitor than ethacrynic acid. Moreover, the formation of an addition compound with the sulfhydryl compound dithiothreitol annihilates the inhibitory effect of ethacrynic acid. Thus it must be considered unlikely that the effect of ethacrynic acid on exocytosis and $O_2$-release is due to interaction with the ion transport systems.

Ethacrynic acid reacts with SH-compounds because its structure contains a carbonyl group in conjugation with a double bond. Dihydroethacrynic acid, a compound with similar properties but less reactivity towards SH groups (because the molecular entity for the reaction with this group is missing) has been found to be a much weaker inhibitor of exocytosis and superoxide release than ethacrynic acid. Furthermore, the inhibitory effect of ethacrynic acid is prevented by dithiothreitol. Both results indicate that the inhibition of exocytosis, phagocytosis and superoxide release by ethacrynic acid may be due to the ability of this compound to act as a sulfhydryl reagent.

This is also in agreement with a previous study [31] in which we have presented data which indicate that vulnerable sulfhydryl groups, probably located intracellularly, are involved in the process of exocytosis.

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