release of lysosomal enzymes from PMN leukocytes[12], the effect has been considered of no physiological relevance, since these cells were thought to be poor PGs productors. Our data demonstrate that the statement is not correct and that PMN leukocytes are able to synthetize substantial amounts of PGE_2 when subjected to phagocytic stimuli. In our model, hydrocortisone, at a concentration proved to be effective in other biological systems[13-16], failed to inhibt release of prostaglandins and lysosomal enzyme β-glucuronidase. This finding is consistent with other reports[17-20] and casts doubts on whether corticosteroids may act as anti-inflammatory agents by interfering with PG-synthetase activity in cells involved in the inflammatory response.

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Evidence that superoxide radicals are involved in the hemolytic mechanism of phenylhydrazine[1]

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Summary. Phenylhydrazine produces in the red blood cell the same effect as the enzymic system xanthine oxidase-xanthine, a superoxide radical generator system. Both effects are inhibited by the enzyme superoxide dismutase.

Molecular oxygen is fundamental for life of aerobic organisms; however when it acts through the formation of highly active free radicals, it can turn toxic[3-6] and even mortal[7]. One of these free radicals is the superoxide radical (SR), which is generated by the reduction of molecular oxygen when it interacts with a bivalent metal[8]. Much evidence exists relating SR with the initiation of unsaturated fatty acids oxidation[9-11].

There is scarce information as regards the hemolytic mechanism of phenylhydrazine (PH), although it is well known that many of its effects are oxygen dependent. It was demonstrated[12] that PH in presence of hemoglobin produces hydrogen peroxide and therefore SR, since the former is an end product of the dismutation of this free radical. These SR could initiate the peroxidation of the lipidic components of the erythrocyte membrane producing its destruction and liberation of hemoglobin.

Using hemolytic and peroxidative measurements as a damage criterion, we found that the system xanthine oxidase (XO)-xanthine (X), which generates SR, produces a high cellular lysis level which is narrowly related to a high peroxidation grade. Both phenomena are inhibited by the enzyme superoxide dismutase (SOD). In addition, PH produces a similar hemolytic and peroxidative effect as the XO-X system, these processes being also inhibited by SOD. From this, we postulated that the hemolytic action of PH is closely related with that of SR.

Materials and methods. Chemicals: PH-HCl and Folin Ciocalteu's phenol reagent were obtained from E. Merck A. G., Darmstadt, Federal Republic of Germany. Sephadex G-100, epinephrine, xanthine, xanthine oxidase and 2-thiobarbituric acid were obtained from Sigma Chemical Co., St. Louis, U.S.A. All other chemicals used were of analytical grade.

Methods: SOD was prepared from bovine erythrocytes according to the method of McCord and Fridovich[13] with the following modification: the final extract was purified in a Sephadex G-100 (100 cm × 3 cm) column equilibrated and eluted with NaCl 0.15 M. The enzymic activity of SOD was assayed according to the method of Misra and Fridovich[14], obtaining an extract with an activity of 3500 units/mg protein. The denatured enzyme was obteined by heating at 100 °C for 20 min.

The hemolytic and peroxidation asays were carried out in rat's blood using oxalate as anticoagulant. The red blood cells were washed and suspended in an equal volume of NaCl 0.15 M and the assays were performed in several test tubes which contained: 1 ml of the cell suspension; 0.1 ml of 2×10^{-3} M PH; 0.1 ml of 2×10^{-3} M xanthine; enzymic extracts of SOD and xanthine oxidase. The final incubation volume was always 1.4 ml. These test tubes batteries were incubated at 37 °C with gentle agitation. At intervals of 30 min 1.5 ml of NaCl 0.15 M was added to the corresponding test tube, then after shaking, an aliquot of 1.5 ml was extracted for the hemolysis test and another of 0.8 ml for the peroxidation assay.

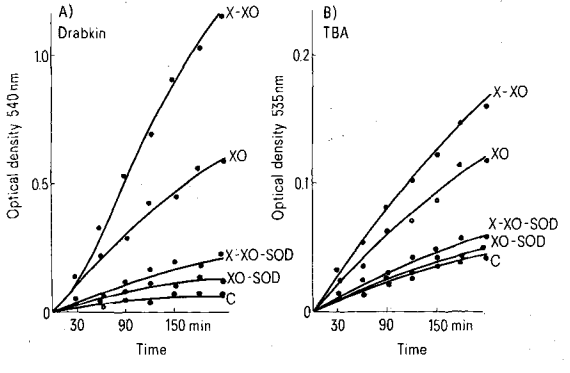

Fig. 1. Effect of the xanthine oxidase (XO)-xanthine (X) system on the hemolysis (A) and peroxidation (B) of red blood cells and its inhibition by SOD. The experimental conditions are described in the text. XO: 0.6 units (Sigma units); SOD: 1.2 units; C: control.

Hemolysis determinations were performed according to Drabkin's method[15]. The peroxidation measurements were carried out using the thiobarbituric acid test (TBA)[9] with the following procedure: to the 0.8 ml aliquot was added 1.5 ml of 20% w:v trichloroacetic acid solution. After centrifugation, the upper phase was extracted and heated 15 min at 100 °C with 2.4 ml of 0.67% w:v thiobarbituric acid solution. The coloration obtained after this assay was registered spectrophotometrically at 535 nm. Proteins were assayed according to the method of Lowry et al.[16].

Results. 1. Effect of SR on red blood cells. It is a well known fact that there are many enzymic systems which generate SR[17]. One of the best known is the XO-X system[18–22]. The incubation of red blood cells in a solution which contains XO and X reveals the effect of the SR on the structure of the erythrocyte (figure 1). Notice that, in time, not only a significant increase on the hemolysis grade was produced but also in the peroxidation level. Although the figures are not comparable, there is a direct correlation between both phenomena. Moreover, the addition of SOD produces a clear inhibition on both processes, and this result is an open demonstration that hemolysis and peroxidation are dependent on SR. The XO effect, without X, may be due to the fact that this substrate already exists in the erythrocyte.

2. SOD effect on the hemolysis and peroxidation induced by PH. In figure 2 it may be observed that PH produces a similar effect as the system XO-X. Notice that there is the same correlation between both phenomena; hemolysis and peroxidation. SOD inhibits the PH effect proportionally to the units of the enzyme added. This effect vanishes when an enzymic denatured extract is used. We believe that this result also demonstrated that SR are involved in the mechanism of hemolytic action of PH. SOD, also, inhibits the hemolysis and peroxidation of the controls. These results, not showed here, will be the subject of a further communication.

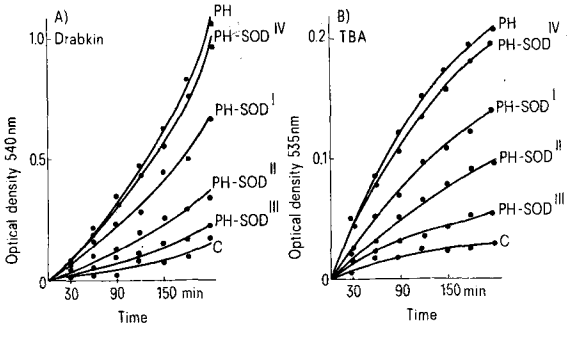

Fig. 2. Phenylhydrazine (PH) effect on the hemolysis (A) and peroxidation (B) of red blood cells and its inhibition by SOD. The experimental conditions are described in the text. SOD^{I}: 0.3 units; SOD^{II}: 0.6 units; SOD^{III}: 1.2 units; SOD^{IV}: denatured extract; C: control.

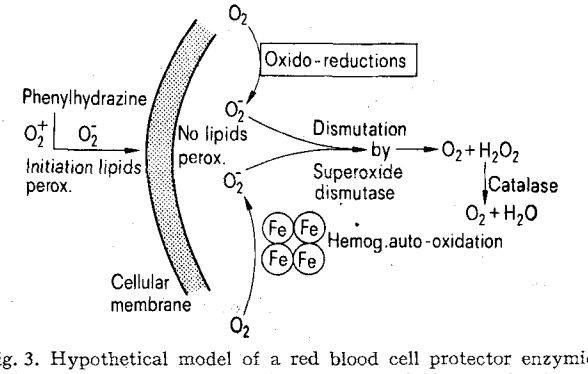

Fig. 3. Hypothetical model of a red blood cell protector enzymic chain. Free radicals generated either by hemoglobin autoxidation or by another oxido-reduction system would be dismutated by SOD to molecular oxygen and hydrogen peroxide which could be destroyed by catalase and converted to non toxic species.

Discussion. From these results it can be inferred that PH produces red blood cell lipid peroxidation which is inhibited by SOD. This fact suggests a direct correlation between SR and the mechanism of action of this chemical. In addition, the erythrocyte susceptibility to a system which generates SR also confirms the relation: phenylhydrazine-superoxide radical-peroxidation.

It has been proposed[9] that the high activity of SOD in the red blood cell is related to a protector function of the inner structure against the deteriorative effect of oxygen. Fundamentally this may be due to the large susceptibility of hemoglobin to autoxidation with the formation of SR[23]. This process explains not only the presence of SOD but its specific role.

The superoxide dismutation does not eliminate at all its toxicity, since this last process generates hydrogen peroxide which is toxic, and moreover it may react with the superoxide to give the hydroxyl radical[24], another free radical of high reactivity.

The great activity of catalase in the erythrocyte would form together with SOD, a simple enzymic chain which would protect the erythrocyte against SR. Figure 3 shows the scheme of this hypothesis.

The blood normal plasma does not present SOD activity[25], and therefore it may be thought that the outer structure of the erythrocyte is unprotected against any SR generator system. Possibly, this is not a genetic error but a regulator system of the erythrocyte mean life. The erythrocyte would suffer successive peroxidation processes along its useful life in the blood stream, which would weaken its membrane and cause its lysis.

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