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REDUCTION OF 2,6-DICHLOROPHENOL INDOPHENOL BY BOVINE METHEMOGLOBIN REDUCTASE IN THE PRESENCE OF A NUMBER OF NON-STEROIDAL ANTI-INFLAMMATORY COMPOUNDS

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Anti-inflammatory drugs have been reported to inhibit cellular oxidation-reduction reactions [1]. In particular sodium salicylate inhibits a number of enzymes, all of which require NAD^+-NADH as cofactors [2,3].

Within the framework of a study on the metabolism of 2-aryl-1,3-indandiones [4] we are investigating the distribution of 2-phenyl-1,3-indandione (PID) - a well-known anticoagulant with anti-inflammatory activity - over the blood components. The erythrocyte contains a number of enzymes which reduce methemoglobin to hemoglobin, the methemoglobin reductase system (a diaphorase) [5-10]. Some of these enzymes require NADH as a cofactor, while others require NADPH [6,7].

In this context it was interesting to know whether PID would also inhibit methemoglobin reductase.

In this communication we describe the reduction of 2,6-dichlorophenol indophenol (DCIP) by bovine methemoglobin reductase isolated according to Splittgerber [9], in the presence of NADH, sodium salicylate, 2-phenyl-1,3-indandione, phenylbutazone or indometacin.

The assay of enzyme activity was carried out at 20°C with a Cary model 1605 recording spectrophotometer in 1-cm cuvettes at 600 nm, the wavelength of maximum absorption of DCIP. The reaction mixture contained 40 mM Tris-HCl buffer (pH 7.5), 1 mM EDTA and 9-59 μM DCIP (determined in the cuvette by using milli-

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molar extinction coëfficient of 20.1 [9]. When NADH was used, its concentration was 133 µM. The protein concentration was 100 µg/ml. The anti-inflammatory compounds were added in 0.05 ml DMSO. The reactions were started by the addition of the cofactor. In the inhibition studies the inhibitor was added just before the reaction was started.

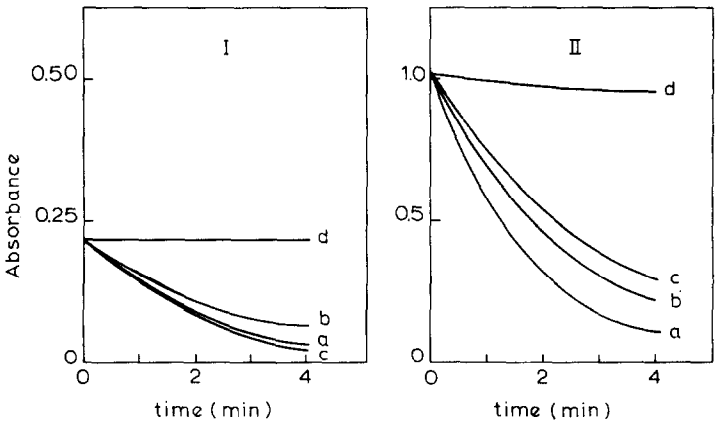

Fig. 1. Reduction of DCIP - 11 µM (I) and 51 µM (II) - by methemoglobin reductase in the presence of (a) 1 mM PID, (b) 1 mM PID + 133 µM NADH and (c) 133 µM NADH and nonenzymatic reduction of DCIP by 1 mM PID (d).

The data presented in Fig. 1 show first of all the effect of addition of PID on the reduction of DCIP by methemoglobin reductase. At 11 µM DCIP (Fig.1, curve Ib) PID produced a decrease of the rate of reduction and at 51 µM DCIP (Fig.1, curve IIb) an increase.

These findings are also illustrated by the Lineweaver-Burk plots of the enzymatic reduction of DCIP with and without PID (Fig. 2). (The rate of the nonenzymatic reduction (less than 5% of the final rate) of DCIP by NADH, PID or phenylbutazone was measured and deducted from the final velocity). The rather confu-

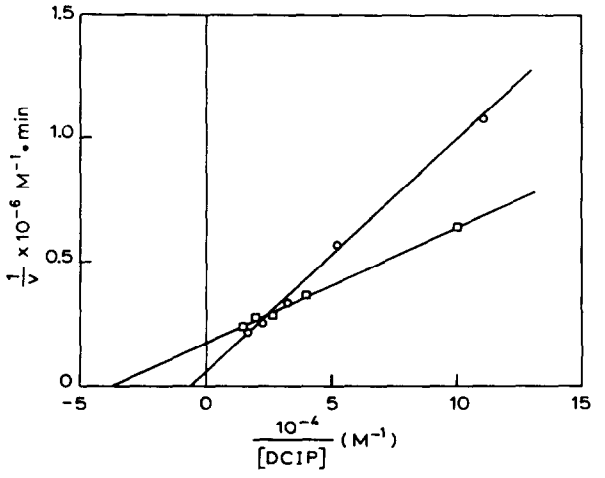

Fig. 2. Lineweaver-Burk plots of the reduction of DCIP by methemoglobin reductase in the presence of 133 µM NADH (□-□) and of 133 µM NADH + 1 mM PID (o-o). Each point represents the mean value of 6 experiments.

sing kinetics could be interpreted as originating from a retardation of the reduction at DCIP concentrations below 42 µM and an acceleration at DCIP concentrations above 42 µM (Fig. 1).

Additionally, Fig. 1 shows that DCIP can be reduced by methemoglobin reductase

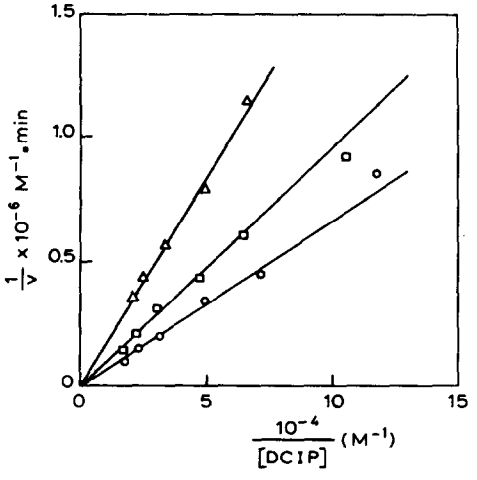

Fig. 3. Lineweaver-Burk plots of the reduction of DCIP by methemoglobin reductase in the presence of 0.35 mM PID (□-□), of 0.5 mM PID (o-o) and of 0.5 mM phenylbutazone (Δ-Δ). Each point represents the mean value of 6 experiments.

in the presence of PID instead of NADH. This reduction was found to be of the first order with respect to PID and DCIP. The double reciprocal plot for this reduction is given by Fig. 3. The rate of reduction increases continuously with the substrate concentration. In other words, the values of K_m and V_{max} are infinitesimally high using PID as a cofactor. Such kinetics are also known of catalase in the reaction [11]:

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$$

The difference in kinetics between the reduction of DCIP with NADH as a cofactor and that in the presence of PID suggests the existence of two active sites, one for NADH and one for PID. This was strengthened by the observation that sodium salicylate - a competitive inhibitor of methemoglobin reductase - had no effect at all on the reduction of DCIP with PID as electron donor. It should be pointed out, however, that PID has been described as an inhibitor of an other diaphorase, rat liver DT-diaphorase [12,13].

Phenylbutazone acted like PID: also replacement of NADH and the same kinetics, perhaps indicating that PID and phenylbutazone interact with the same active site on the enzyme. However, the rate of reduction was lower in this case (Fig. 3).

Indometacin like sodium salicylate inhibited the reduction.

Thus, the tested anti-inflammatory drugs fall in two groups: substitutes for NADH - PID and phenylbutazone - and inhibitors of methemoglobin reductase - sodium salicylate and indometacin - . This point and the possibility that a well-known anticoagulant - PID - and a well-known anti-inflammatory drug - phenylbutazone - may affect the hemoglobin-methemoglobin ratio in the red blood cell - in other words the oxygen transport - are worthy of further investigation.

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