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INHIBITORS OF HYDROGEN PEROXIDE-INDUCED HAEMOLYSIS OF BOVINE ERYTHROCYTES

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Abstract—The optimal conditions for the haemolysis of bovine erythrocytes by H_2O_2 have been established. The parameters were concentration of erythrocytes, H_2O_2 concentration, time, and influence of the solvent in which the substances tested were dissolved. Some inhibitors of this oxidative haemolysis have been employed to serve as model substances for further antihaemolytic investigations with natural products.

Oxidation is one of several processes which brings about haemolysis of erythrocytes. This can be inhibited by application of antioxidants like vitamin E or selenium. An exhaustive search for effective antihaemolytic–antioxidative natural and related synthetic products has been the subject of our recent investigation [1].

For this purpose an ideal experimental system has been established using H_2O_2 as a haemolysing agent and simple phenols as model inhibitors. Although H_2O_2 has been used in this context by other investigators [2, 3], no standard method has been developed until now for a routine antihaemolytic investigation with bovine blood, which is a more accessible source of erythrocytes.

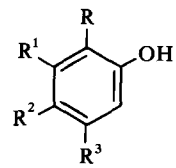
MATERIALS AND METHODS

Citrated bovine blood was used. The blood was stabilized by mixing it with a solution of the following composition (5:1, v/v) [4]: trisodium citrate 13.2 g, citric acid 4.8 g, and dextrose 14.7 g (dissolved in 100 ml doubly-distilled water). This refrigerated blood sample could be used for *ca* 3 weeks.

The erythrocytes were separated by centrifugation and washed with an isotonic buffer suspension 3.95 g, $Na_2HPO_4 \cdot 2H_2O$, 0.76 g KH_2PO_4, and 7.20 g NaCl in 100 ml doubly-distilled water and adjusted to pH 7.4 [5] until the supernatant was colourless. A 0.4% solution of erythrocytes in this buffer was found to be suitable for our test conditions.

Haemolysis tests. Erythrocyte suspension (2 ml) was mixed with H_2O_2 (0.5%), buffer dimethyl sulfoxide (DMSO) and inhibitor dissolved in DMSO as indicated in Table 1. After mixing, the tubes were incubated at 37° for 4 hr, then centrifuged (1000 *g*) for 5 min, and the optical density of the supernatants was measured at 540 nm. The extent of haemolysis and its inhibition were calculated from the measured optical densities.

The haemolysis inhibitors. Keeping in mind the substitution pattern of our natural products which were investigated in our further experiments [1], we selected the following phenols as model substances:



I	R = R^1 = R^2 = R^3 = H	**II**	R = OH, R^1 = R^2 = R^3 = H
III	R^1 = OH, R = R^2 = R^3 = H	**IV**	R^2 = OH, R = R^1 = R^3 = H
V	R = OCH_3, R^1 = R^2 = R^3 = H	**VI**	R^1 = OCH_3, R = R^2 = R^3 = H
VII	R^2 = OCH_3, R = R^1 = R^3 = H	**VIII**	R = tertiary butyl, R^2 = OCH_3, R^1 = R^3 = H
IX	R^1 = R^3 = OH, R = R^2 = H	**X**	R = R^1 = OH, R^2 = R^3 = H
XI	R^1 = R^2 = OH, R = R^3 = H	**XII**	R^1 = OH, R^2 = OCH_3, R = R^3 = H
XIII	R^1 = OCH_3, R^2 = OH, R = R^3 = H	**XIV**	R^1 = R^2 = OCH_3, R = R^3 = H

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Table 1. Composition of mixtures for haemolysis tests (total volume 4 ml)

Components (ml)	No. of tubes I	II	III	IV	V	VI
Erythrocyte suspension	2.0	2.0	2.0	2.0	2.0	2.0
Buffer	2.0	—	—	1.4	1.3	1.3
H_2O	—	2.0	1.9	—	—	—
DMSO	—	—	0.1	—	0.1	—
Inhibitor in DMSO	—	—	—	—	—	0.1
H_2O_2 (0.5%)	—	—	—	0.6	0.6	0.6

The test substances (**I–X**) were obtained from E. Merck (Darmstadt, F.R.G.). Compounds XI–XIV were synthesized in our laboratory by Dakin oxidation [6] of the related aldehydes. The acetates were obtained by acetylation of the phenols by acetic anhydride in pyridine. Before crystallization, the synthesized substances were purified by column chromatography (silica gel; benzene as eluant). The authenticity of the substances was verified by spectroscopic methods. Due to the very low solubility of the tested inhibitors in water, they were dissolved in dimethyl sulfoxide (DMSO). The tolerance limit of the solvent was found to be 0.1 ml/4 ml test combination.

RESULTS AND DISCUSSION

Maximum haemolysis of bovine erythrocytes was achieved by 0.5% H_2O_2 solution with an incubation time of 4 hr (Fig. 1). Lower concentrations of H_2O_2 or shorter time intervals did not cause appreciable haemolysis. Higher H_2O_2 concentrations had an adverse effect on haemolysis, apparently due to decomposition of the liberated haemoglobin (Fig.1).

As indicated in chemical formulae, the phenols carrying oxygen functions at appropriate positions were tested for their haemolysis inhibitory activity. The results of the tests are given in Table 2.

The phenols are well known as scavengers of free radicals formed in oxidation processes. Some phenols have been tested for their antihaemolytic activity [2] but as yet no systematic attempt has been undertaken to evaluate the effect of substitution pattern and to study the role of acetylation of the hydroxyl groups.

Apart from the side chains and/or the condensed rings, most of the natural products in our further investigation [1] contained groups in various positions. As such, for a systematic investigation of the antihaemolytic property of these substances, it was advisable to start with the basic phenol (**I**) and then study the influence of additional hydroxyl (or methoxyl) groups in different positions. Phenol **I** showed moderate antihaemolytic activity, which is also corroborated by its mild antioxidative property.* Contrary to the reports of Younkin *et al.* [2], the introduction of a methyl group in the *ortho* position does not affect the activity. Our result is in agreement with its antioxidative property for fats.*

If a second hydroxyl group was in the *ortho* position of the phenol (**II**), an enhancement in activity was observed. This was maintained even if one phenolic group was methylated (**V**). In contrast to this, the presence of a *meta*-hydroxyl group in the phenol (**III**) rendered it completely inactive as an antihaemolytic agent. This can be attributed to the poor oxidizability of this substance.* Partial methylation of **III** to **VI** restored its activity to some extent. Among the dihydroxy products, hydroquinone **IV** was found to be the most active inhibitor of haemolysis. Its susceptibility for fast oxidation to *para*-quinone explains its high activity. Partial methylation of **IV** to **VII** did not result in any loss of activity. Introduction of a bulky group in the *ortho* position (**VIII**) of the hydroxyl group in **VII** produced no untoward effect as regards its activity.

Among the phenols carrying three hydroxyl groups, only pyrogallol (**X**) was a good inhibitor. Hydroxyl groups, as in the case of pholoroglucinol (**IX**), occuring in many natural products, do not seem to be conducive to antihaemolytic activtiy. Under our experimental conditions, phenols **II**, **IV**, **V**, **VIII**, **X**, **XII** and **XIII** had a tendency to show lower antihaemolytic activity at higher concentrations. At such concentrations these substances possessed weak haemolytic activity, as found in independent experiments without the use of H_2O_2 in the test combina-

* The oxidation values were calculated from a stripped lard test for 72 hr at 60°. The results of this investigation will be published separately.

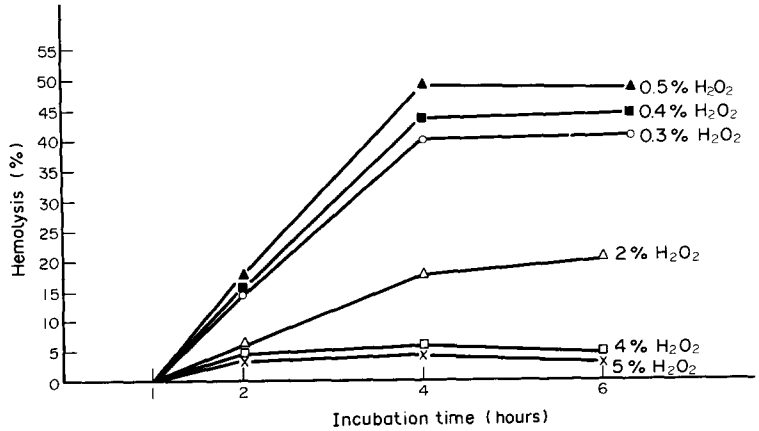

Fig. 1. Time dependence of H_2O_2-induced haemolysis.

Table 2. Inhibition by the test substances (mean ± S.D.; $n = 5$)

Test substances	Concentration in μmole/ml; % inhibition 20×10^{-2}	10×10^{-2}	5×10^{-2}	2×10^{-2}	1×10^{-2}
I	65.2 ±0.5	67.0 ±1.6	66.3 ±1.1	55.0 ±1.0	—
II	60.1 ±1.2	70.0 ±1.4	74.2 ±0.5	89.4 ±2.0	86.6 ±0.7
IIa	(77.9) ±1.3	(71.1) ±0.9	(60.0) ±1.7	—	—
III	0	0	0	—	—
IV	—	84.0 ±0.6	88.7 ±1.5	93.7 ±0.8	73.5 ±1.3
V	67.1 ±0.7	89.2 ±0.9	88.7 ±1.4	89.2 ±1.1	60.1 ±2.1
Va	(90.6) ±1.0	(60.4) ±2.3	(40.7) ±1.7	—	—
VI	69.5 ±0.9	71.2 ±1.4	30.4 ±2.1	—	—
VII	94.0 ±2.1	95.0 ±1.7	95.0 ±1.2	95.4 ±1.5	—
VIIa	(77.4) ±1.0	(66.5) ±0.6	—	—	—
VIII	—	—	82.1 ±2.3	80.8 ±0.7	99.0 ±1.3
VIIIa	(79.8) ±0.9	—	—	—	—
IX	0	0	0	—	—
X	56.0 ±2.4	80.6 ±0.8	70.7 ±1.1	—	—
Xa	(59.0) ±1.3	(43.4) ±0.9	(30.8) ±1.9	—	—
XI	0	0	0	—	—
XII	70.1 ±0.7	85.0 ±0.5	88.3 ±1.4	91.3 ±1.7	—
XIIa	(84.4) ±1.8	(66.1) ±1.1	(53.5) ±1.7	—	—
XIII	78.1 ±0.6	89.5 ±2.1	90.4 ±1.3	85.0 ±1.1	—
XIIIa	(51.2) ±1.7	(50.0) ±0.8	(42.7) ±1.5	—	—
XIV	92.3 ±0.7	98.1 ±1.6	97.6 ±1.9	95.7 ±0.8	—
XIVa	(87.6) ±1.8	(73.4) ±2.4	(60.2) ±1.2	—	—

The values in parentheses are for the corresponding acetates

tion. Compound **XI** was rather haemolytic at all concentrations. However, by selective methylation of the hydroxyl groups of **XI**, very active antihaemolytic compounds (**XII**, **XIII**, **XIV**) were obtained.

Expecting that acetylation would increase the lipophilic properties of the phenols, we also examined the antihaemolytic activity of some phenol acetates. In fact, they were found to be less active than the related phenols. The activity of the acetates seems to depend on their degree of hydrolysis under our experimental conditions. The hydrolysis of acetates was verified by extracting the incubated test substances with ether and comparing them with the corresponding phenols on TLC plates.

In Table 2 the antihaemolytic values of the acetates (**IIa**, **Va**, **XIIa**) at higher concentrations are observed to be of the same order of magnitude as those of the corresponding phenols (**II**, **V**, **XII**) at lower concentrations. This indicates that partial hydrolysis to phenols might be the cause of the activity shown by the acetates. In comparison with the acetates, the nicotinates of the phenols have a lower degree of hydrolysis under our experimental conditions. Accordingly, very low antihaemolytic activity was obtained in the case of the nicotinates. The phenol acetates as such seem to play no structural role in preventing haemolysis in contrast to vitamin E acetate [7].

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