STUDY OF THE REACTION OF NITROSOBENZENE WITH HAEMOGLOBINE

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During the reaction of haemoglobine with nitrosobenzene, the latter was determined polarographically. Nitrosobenzene bound by coordination to the divalent Fe atom of haemoglobine was polarographically inactive; it was set free from this complex by oxidation of the iron with ferricyanide and then determined. At a molar ratio of nitrosobenzene to haemoglobine of 4 : 1, at which the molar ratio of iron to nitrosobenzene is 1 : 1, 0·9 mol of nitrosobenzene is bound by coordination to the iron while 0·1 mol is reduced by SH groups to azo- and azoxybenzene. After the SH groups of haemoglobine had been blocked by cyanoethylation or oxidation, nitrosobenzene was bound to iron in a stoichiometric ratio. Accordingly, the conditions for the polarographic determination of nitrosobenzene after oxidative splitting of the complex with haemoglobine are defined.

The interaction of nitrosobenzene (PhNO) with haemoglobine or oxyhaemoglobine (Hb, HbO_2) was studied because the redox system phenylhydroxylamine/nitrosobenzene (PhNHOH/PhNO) is probably the most efficient haemiglobine-forming agent applied in poisoning with aromatic amino and nitro derivatives[1-9]. It was proved especially on the basis of the extensive work of Kiese's school that haemiglobine (ferrihaemoglobine, Hi) is formed by a conjugate oxidation of HbO_2 and PhNHOH. The second product of this oxidation, PhNO, is reduced enzymatically back to PhNHOH, which is thus returned into the original haemiglobine-forming system. The formation cycle of Hi is interrupted by transformation of PhNHOH to *p*-aminophenol, which is a weak haemiglobine-forming agent and is easily excreted by urine in the form of glucuronide.

We attempted to elucidate the loss of PhNO during its quantitative determination in blood[4,10] and the quantitative splitting off of PhNO from HbPhNO during oxidation with ferricyanide. The aim of the present work was to find what part of PhNO is bound to the haemoglobine iron and what part is reduced with SH groups of the globine component. Further it was necessary to define the conditions of the polarographic determination of PhNO after oxidative splitting of HbPhNO. It was necessary to find out basic polarographic data[11] and to choose the optimum pH value for the determination of PhNO in the presence of ferricyanide, to solve the problem of loss of PhNO by evaporation, and to investigate the losses of PhNO by interaction with proteins.

EXPERIMENTAL

Apparatus and Materials

A polarograph of the type LP 55 was used. Erythrocytes were obtained from human blood, either fresh one or conserved. Nitrosobenzene was prepared in our laboratory by oxidation of phenylhydroxylamine. Chemicals of the highest analytical purity grade were used.

Methods

The solutions were polarographed at the laboratory temperature (22 °C) in a vessel with a separated saturated calomel electrode. The height of the reservoir was 75 cm, drop time in 0·1M-KCl 0·76 s, rate of flow of mercury 3·82 mg/s. The volatility of the free PhNO was even in the presence of 50 vol.% ethanol considerable and therefore the solutions were deoxygenated by bubbling nitrogen prior to the addition of PhNO. In measuring the diffusion current, PhNO was added as a 0·1M solution in ethanol by a micropipette inserted close to the bottom of the polarographic vessel, which was to this purpose provided with a separate closeable inlet. The volume of the added solution was not larger than 1/200 of the polarographed solution. During the oxidative splitting off of PhNO from HbPhNO, a 0·4M aqueous ferricyanide solution was added analogously. PhNO evaporated also into the space of the polarographic vessel, therefore the volume of the solution in it was kept constant, namely 35% of the operating volume of the vessel. At this condition the loss of PhNO was negligible during an hour. The solution was after adding PhNO shortly agitated by a magnetic stirrer coated with Teflon. The half-wave potential of PhNO changed linearly in the pH range from 4 to 10 from +0·08 to −0·29 V in accord with the literature[11]. The calibration curve was linear in the concentration range from $1 . 10^{-4}$ to $2{\cdot}5 . 10^{-3}$M-PhNO. All reactions were followed at pH 9·2, at which the wave of PhNO is sufficiently shifted to negative potentials and separated from the wave of excessive ferricyanide. At this pH value the formed Hi is not yet denaturated, but its SH groups are already oxidised with ferricyanide[12]. The solutions were buffered with 0·012M-$Na_2B_4O_7$ either in the aqueous or 50 vol.% ethanol medium. With respect to the much higher affinity of the haemoglobine iron[12] to PhNO than to oxygen it is not important for the formation of HbPhNO whether the reaction of PhNO proceeds with Hb or with HbO_2. We therefore used mainly better accessible solutions of HbO_2 which were obtained by haemolysis of erythrocytes washed three times with a physiological solution. The haemolysates were centrifuged and the concentration of HbO_2 was then determined spectrophotometrically.

RESULTS AND DISCUSSION

In aqueous tetraborate solutions of all the studied proteins, the diffusion current of PhNO was lowered by adsorption of the protein on the dropping mercury electrode (Fig. 1). The decrease depended on the kind of protein. The wave of PhNO decreased further with time owing both to the reduction of PhNO with SH groups and to nonspecific adsorption on the colloidal protein particles.

The formation of HbPhNO from Hb and PhNO was followed polarographically at a concentration of $1{\cdot}25 . 10^{-4}$M-Hb and pH 9·2 at a potential of −0·8 V. The main part of the equimolar quantity of PhNO with respect to the haemoglobine iron reacted during 30 s and the reaction was finished within 5 min. The reaction rate was not influenced by the presence of 50 vol.% ethanol.

The determination of PhNO in HbPhNO was based on the fact that the PhNO molecules bound by coordination are set free by the oxidation of the divalent iron and cannot be bound by the simultaneously formed Hi. At an excess of potassium ferricyanide at pH 9·2, the SH groups of the globine are simultaneously oxidised[12] so that no PhNO is lost by reduction. The quantity of PhNO set free corresponds to the degree of utilization of iron during the reaction of PhNO with HbO_2. The difference between the added and split off PhNO represents the fraction which underwent side reactions and did not participate in the formation of HbPhNO. We found that PhNO is quantitatively set free from the bond to iron in HbPhNO only by four equivalents of ferricyanide per mol of iron (*i.e.*, 16 mol ferricyanide to 1 mol HbPhNO), and this after 17–21 min (Fig. 2). This excess of ferricyanide was considered as standard. The found quantity of PhNO was extrapolated to zero time, *i.e.*, the instant when ferricyanide was added. The extrapolation was done graphically according to the course of adsorption of PhNO on colloidal Hi (Fig. 2), which meant that the found content of PhNO in the maximum had to be increased by 3%. In this way nearly 90% of added PhNO was found (Table I). It was proved by thin-layer chromatography (Silufol) that the remaining 10% PhNO underwent reduction during the reaction with HbO_2, mainly to azo- and azoxybenzene. These compounds were also found as products of the reduction of PhNO with cysteine, which was

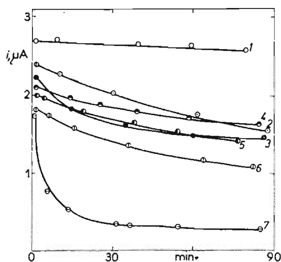

FIG. 1

Time Drop of Diffusion Current of $5 . 10^{-4}$M-PhNO in Aqueous Medium in the Presence of Proteins

1 Pure 0·012M-$Na_2B_4O_7$; *2* 1% gelatin; *3* 5 times diluted human plasma; *4* 10 times diluted white of egg; *5* $1·25 . 10^{-4}$M haemiglobine; *6* 0·5% horse serumalbumine; *7* 4 times diluted white of egg.

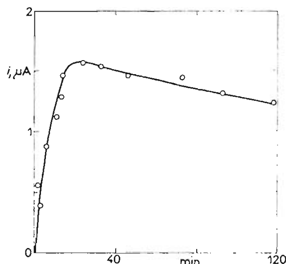

FIG. 2

Time Dependence of Diffusion Current of PhNO set free from $1·25 . 10^{-4}$M-HbPhNO with Potassium Ferricyanide in Aqueous Medium

Volume of solution 30 ml. At time zero, 0·15 ml of 0·4M-$K_3Fe(CN)_6$ was added.

oxidised to cystine (proved polarographically). That the oxidation splitting off of PhNO is quantitative was proved with the use of HbPhNO samples prepared in the stoichiometric composition. These were prepared either by the reaction of HbO_2 with a large excess of PhNO (the excess of PhNO acted as an oxidant of the SH groups), or by the reaction of PhNO with HbO_2 whose SH groups had been blocked by cyanoethylation with an excess of acrylonitrile. In these cases the expected quantity of PhNO was set free (100–108%). From the analytical point of view it was important that the oxidative splitting off of PhNO was in the aqueous ethanol medium instantaneous, the solutions of Hi remained clear and the height of the polarographic wave of PhNO was constant. The diffusion current of PhNO was against the aqueous medium by 24% lower, however it was not lowered by the formed Hi, hence the sensitivity was not decreased by the presence of ethanol. Moreover, the scatter of the diffusion current values of PhNO was in the presence of ethanol appreciably smaller, resulting in a more accurate determination (Table I). The half-wave potential of PhNO was shifted by 70 mV to more negative values, so that the wave of PhNO was better distinguished from the ferricyanide wave. The determination in an aqueous ethanol medium brings therefore a simplification of the analysis and more reproducible results, which are nevertheless in accord with the mean of a larger number of measurements in an aqueous medium (Table I).

We conclude that PhNO can be determined polarographically in prepared samples of HbPhNO. The splitting off of PhNO with ferricyanide could not be proved polarographically at a ratio of PhNO to HbO_2 1 : 10 or less. The spectrophotometric

TABLE I

Quantitative Results

Concentration of Hi or HbPhNO was always $1{\cdot}25 . 10^{-4}$M. Aqueous medium was 0·012M-$Na_2B_4O_7$; aqueous–ethanolic medium was a mixture of equal parts of 0·025M-$Na_2B_4O_7$ and 96% ethanol.

Medium	Presence of Hi	5.10^{-4}M-PhNO i, μA	i, %	±s, %	No of measurements
Aqueous	—	2·67 ± 0·043	100·0	1·7	10
	Hi	1·97 ± 0·27	73·5	13·7	11
Aq.–eth.	—	2·03 ± 0·031	76·0	1·6	10
	Hi	2·02 ± 0·041	75·6	2·0	8
	quantity of PhNO set free from HbPhNO				
Aqueous	Hi	1·76 ± 0·14	89·8	8·2	6
Aq.–eth.	Hi	1·75 ± 0·12	86·7	6·7	6

determination of PhNO according to Kiese[4] (based on splitting off of PhNO from HbPhNO by oxidation with ferricyanide) was also unsuccessful if HbO_2 was in excess against PhNO. These methods are hence unsuitable for the determination of small concentrations of PhNO in blood, which are likely to occur after intoxication with aniline or nitrobenzene. According to our results, even occurrence of traces of free PhNO in blood must be preceded by the reaction of a much larger quantity of PhNO with molecules of the biophase (*e.g.*, with SH groups mainly under formation of azo- and azoxybenzene). This circumstance must be considered in studying the mechanism of the haemiglobine-forming effect of aniline, nitrobenzene, and their derivatives. The present work suggests that the haemiglobine-forming redox system PhNHOH : : PhNO can be blocked also by the reactions of PhNO with SH groups of the globine, and these reactions can then compete with the transformation of PhNHOH to *p*-aminophenol and its excretion by urine in the form of glucuronide[1-9].

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