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EFFECT OF WATER AND ETHANOL RADICALS  
ON THE PROTEIN PART OF HUMAN HEMOGLOBIN

PART II. DAMAGE TO AMINO ACID RESIDUES

This work is devoted to the effect of interactions of water and ethanol radicals with human hemoglobin (Hb) amino acid residues. The  $\cdot\text{OH}$  radicals caused the greatest destruction of human hemoglobin amino acid residues. Among them the most sensitive to irradiation were cysteine tryptophan and histidine residues.

1. INTRODUCTION

This work deals with the damage to hemoglobin amino acid residues induced by the action of primary products of water radiolysis and of secondary ethanol radicals.

The effect of the reaction of water and ethanol radicals with the amino acid residues was estimated on the basis of the loss in their content measured on an automatic amino acid analyzer after protein hydrolysis. The obtained results were discussed together with the fluorescence changes in part I [11].

2. MATERIALS AND METHODS

Hemoglobin was obtained and purified according to procedure described in the first part of our study [11]. Globin was isolated from irradiated hemoglobin by removal of the heme moiety at acid pH. The method described by Rossi Fanelli [7] employs acid-acetone at low temperature to split the heme group from the globin, which precipitates and is subsequently redissolved in water.

### 3. IRRADIATION CONDITIONS

Employed irradiation conditions were also analogous as previously described [11]. The preparations were irradiated with 2.5 kGy and 5 kGy under the atmosphere of  $N_2O$  and argon or in both variants with ethanol. Deoxyhemoglobin concentration was  $5 \text{ g} \times \text{dm}^{-3}$ .

### 4. AMINO ACID ANALYSIS

Amino acid composition of hemoglobin was determined following protein hydrolysis in three variants:

- in hydrochloric acid at the concentration  $5.7 \text{ mol} \times \text{dm}^{-3}$ ,
- in p-toluenesulfonic acid (2 × crystallized) at the concentration  $3 \text{ mol} \times \text{dm}^{-3}$  with 0.2% 3(2-aminoethyl)indol from Pierce [5],
- in methanesulfonic acid at the concentration  $4 \text{ mol} \times \text{dm}^{-3}$  with 0.2% tryptamine from Sigma [8].

The amino acid composition of irradiated Hb and globin obtained from irradiated Hb were analysed. The samples containing from 1 to 1.5 mg of protein were hydrolysed in the atmosphere of argon in sealed ampules at  $110^\circ\text{C}$  for 24 h. Amino acid analysis was performed using the automatic analyzer JLC-6AH (JEOL Japan).

### 5. THE -SH GROUPS DETERMINATION

The -SH groups content was estimated by spectrophotometric titration of protein with p-chloromercuribenzoic acid (PCMB) [1]. The PCMB preparation from Chemapol was purified before use. Titration was performed using PCMB of the concentration  $10^{-4} \text{ mol} \times \text{dm}^{-3}$  prepared in the acetate buffer, pH 4.6. Samples containing  $0.5 \times 10^{-4} \text{ mol} \times \text{dm}^{-3}$  of protein were titrated. The protein with PCMB was incubated for 2.5 h in buffer pH 4.6 and then absorption increase was monitored at 255 nm, and the content of free -SH groups was calculated. The -SH groups were determined in the hemoglobin solutions except the preparations irradiated with 5 kGy in the atmosphere of  $N_2O$ . In this case the -SH groups were recorded in solutions of globin obtained from irradiated hemoglobin.

### 6. RESULTS

The amino acid residues content was measured in irradiated Hb and globin obtained from irradiated Hb solutions. Similar results in both cases indicated the absence of detectable amounts of free amino acids in the examined

Table 1

Amino acid composition in control and irradiated human hemoglobin with the dose 5 kGy

AA-aminoacid	Control		N <sub>2</sub> O		N <sub>2</sub> O + Et		Argon		Argon + Et	
	mol AA/mol Hb	%AA	mol AA/mol Hb	% AA	mol AA/mol Hb	% AA	mol AA/mol Hb	% AA	mol AA/mol Hb	% AA
Lysine	42.6 ± 2.9 <sup>b</sup>	100	39.5 ± 0.64 <sup>b</sup>	92.7	41.6 ± 1.6 <sup>b</sup>	97.7	41.9 ± 1.93 <sup>b</sup>	98.4	42.4 ± 0.84 <sup>b</sup>	99.5
Histidine	35.8 ± 1.39	100	29.3 ± 0.64	81.8	39.3 ± 5.4	93.0	34.4 ± 3.87	96.1	34.8 ± 1.29	97.2
Arginine	11.2 ± 0.26	100	10.57 ± 0.13	94.3	11.08 ± 0.39	98.9	11.2 ± 2.58	100.0	11.3 ± 0.26	100.8
Aspartic acid + asparagine	47.9 ± 1.99	100	46.4 ± 1.09	96.9	49.8 ± 3.87	103.9	48.3 ± 1.3	100.8	49.2 ± 1.9	102.7
Threonine	28.4 ± 0.13	100	24.8 ± 1.16	87.3	28.1 ± 1.9	98.9	27.5 ± 1.9	96.8	28.2 ± 1.9	99.3
Serine	28.35 ± 2.7	100	23.6 ± 2.58	83.2	27.4 ± 1.55	96.6	26.2 ± 3.2	92.4	25.3 ± 2.6	89.2
Glutamic acid + glutamine	31.3 ± 1.99	100	29.1 ± 0.58	92.9	31.6 ± 2.55	100.9	31.0 ± 2.6	99.0	31.9 ± 0.77	101.9
Proline	26.9 ± 1.3	100	23.1 ± 0.26	85.6	26.3 ± 1.87	97.8	27.0 ± 0.45	100.3	27.0 ± 1.9	100.3
Glycine	38.7 ± 4.5	100	35.9 ± 0.90	92.8	38.7 ± 0.77	100.0	38.8 ± 0.90	100.2	38.2 ± 1.9	98.7
Alanine	69.6 ± 1.93	100	63.7 ± 1.93	91.5	69.6 ± 0.84	100.0	67.7 ± 1.9	97.2	67.7 ± 2.6	97.2
Valine	59.7 ± 2.77	100	53.9 ± 1.93	90.3	57.6 ± 2.6	96.5	59.0 ± 2.6	98.8	57.7 ± 0.3	96.6
Methionine	5.4 ± 0.64	100	4.77 ± 0.64	88.3	5.05 ±	93.1	5.3 ± 1.3	98.1	5.6 ± 0.45	103.7
Leucine	69.6 ± 3.9	100	63.7 ± 1.29	91.5	70.25 ± 3.5	100.9	68.3 ± 1.3	98.1	68.3 ± 2.6	98.1
Tyrosine	10.8 ± 0.77	100	10.1 ± 0.25	93.5	10.4	96.3	10.6 ± 0.5	98.1	11.1 ± 0.6	102.8
Phenylalanine	28.9 ± 1.54	100	26.03 ± 0.51	90.1	28.4 ± 3.9	98.3	27.6 ± 3.9	95.5	28.9 ± 0.9	100.0
Tryptophan	6.0	100	-	-	-	-	6.0	100.0	-	-
Cysteine	5.99 ± 0.29	100	4.52	75.2	6.3 ± 0.29	104.8	5.05 ± 0.25	84.0	6.01	100.0

Number of measurements n = 3 - 10

<sup>a</sup> Data according to G. Fermi and M. F. Perutz, *Atlas of Molecular Structures in Biology 2. Hemoglobin and Myoglobin*, Clarendon Press, Oxford 1981, pp. 4-5.<sup>b</sup> Average standard deviation calculated at measurements number not smaller than 5. Statistically significant differences in relation to control were estimated by t-Student test. The underlined values show statistically significant differences at  $\alpha = 0.05$ . The content of amino acid residues was determined following protein hydrolysis in hydrochloric acid and p-toluenesulfonic acid; tryptophan in methanesulfonic acid, and cysteine by Boyer's method without hydrolysis.

hemoglobin solutions which might have occurred as the possible products of polypeptide bonds degradation. The decrease of amino acid residues content was interpreted as the damage. The analysis of the amino acid composition of control Hb and irradiated with the dose of 2.5 and 5 kGy were carried out. At the dose of 2.5 kGy the measurements were performed following protein hydrolysis in p-toluenesulfonic and methanesulfonic acid. In the latter case the hydrolysis enables to determine the tryptophan content. Cysteine was estimated by titrating the -SH groups with PCMB.

The level of -SH groups decreased by 25% (Table 2) and histidine by 11% in Hb irradiated with 2.5 kGy under the atmosphere of N<sub>2</sub>O without ethanol. In the preparations irradiated with the same dose under the atmosphere of argon without ethanol, and argon and N<sub>2</sub>O with ethanol there were no differences in the level of amino acids between irradiated and control Hb.

Table 1 presents the content of amino acid residues in Hb irradiated with 5 kGy in relation to control and literature data. These results clearly indicate that losses of Hb amino acid residues content mainly occur in the preparations irradiated in the atmosphere of N<sub>2</sub>O and do not exceed 25%. Cysteine (25%) and histidine (18%) display the largest decrease of content while proline, serine and threonine only about 15%. The loss of lysine, arginine, glutamic acid + glutamine, alanine, valine, tyrosine and phenylalanine is from 5% to 10%. Cysteine residues content also decreased in Hb irradiated under the atmosphere of argon (16%). The content of the remaining amino acid residues does not show statistically significant differences in relation to the control. The presence of ethanol in the preparations irradiated in N<sub>2</sub>O and argon totally reduces the losses of amino acid residues content.

Table 2

The -SH groups content in control and irradiated  
in N<sub>2</sub>O atmosphere human hemoglobin

Dose [kGy]	0.0	0.83	1.25	2.5	5.0
-SH [mol/mol Hb]	5.99±0.29	5.83±0.27	4.85±0.13	4.49±0.34	4.52

The -SH groups content of Hb irradiated in N<sub>2</sub>O were determined in a wider dose range (Table 2). The results indicate that at the dose of 1.25 kGy about 20% of the -SH groups do not react with PCMB. The maximum decrease of the -SH groups is about 25% at 2.5 kGy and remains unchanged up to the dose of 5 kGy. Within the examined dose range at the greatest about 1.5 mol -SH/mol Hb is modified as the result of the interaction of Hb with the ·OH radicals.

## 7. DISCUSSION

The modifications of amino acid residues leading to their loss during the analysis by means of the analyzer are relatively insignificant (irradiation conditions given) and initiated mainly by the  $\cdot\text{OH}$  radicals ( $\text{N}_2\text{O}$  atmosphere). At the dose 2.5 kGy in  $\text{N}_2\text{O}$  only histidine content decreases while tryptophan remains the same in spite of the data from part I that tryptophan fluorescence in guanidine hydrochloride ( $6 \text{ mol} \times \text{dm}^{-3}$ ) decreased by about 23% [11]. Though insignificant the fluorescence decrease still suggests modifications within indol ring which may not have any influence on tryptophan behaviour during amino acid analysis. The differences can derive from the use of different methods. In this case fluorescence measurement is a more sensitive indicator of tryptophan residues destruction than the analysis employing the amino acid analyzer.

The measurements of the free -SH groups content in the irradiated Hb show their limited reactivity with water radiolysis products. The Hb molecule contains six cysteine residues: two in the  $\beta$  chain at the position  $\beta$  F9 and  $\beta$  G14 and one in the  $\alpha$  chain  $\alpha$  G11 [6]. The  $\beta$  F9 cysteine residues are close to the molecule surface reacting with the reagents to the -SH groups in native Hb. The  $\alpha$  G11 and  $\beta$  G14 designated as „masked” react with the reagents to the -SH groups after protein denaturation.

It is worth noting that approximately 4.5 -SH groups/tetramer Hb were detected in the preparations irradiated with 5 kGy under the atmosphere of  $\text{N}_2\text{O}$ . At this dose Hb was an insoluble precipitate and the -SH groups determination was carried out following globin precipitation. Such globin is well dissolved in acetate buffer, pH 4.6. The presence of about 4.5 -SH groups per Hb molecule reacting with PCMB after globin unfolding shows that they do not react with  $\cdot\text{OH}$  radicals under the irradiation conditions assumed by us. Cysteine is highly sensitive to water radicals, and the -SH groups usually constitute the active sites of proteins. The presence of 4.5 -SH groups in the Hb molecule indicates that under irradiation conditions used the Hb molecules are not dissociated into subunits to a great extent after their interaction with  $\cdot\text{OH}$  radicals and the protein core of the molecule practically stays beyond the direct radical action. Taking into account the results of previous paper [9] one can suggest Hb unfolding in the molecule surface regions containing  $\alpha$  helices with the tryptophan residues A12.

Apart from tryptophan residues destruction and the -SH groups destruction at the dose 2.5 kGy in  $\text{N}_2\text{O}$  also histidine content was found to decrease. Hb contains 38 residues among which 20 are exposed to the reactions with hydrogen ions in the pH range 6–8 [2] and hence they are very likely to react with the  $\cdot\text{OH}$  radicals as well, yielding modifications which affect histidine

content decrease. Losses in the content of the remaining amino acid residues, not exceeding 15%, were stated at the dose of 5 kGy.

Kumta and Tappel [3, 4] analysed the amino acid compositions of Hb solution irradiated at the concentration  $1 \text{ g} \times \text{dm}^{-3}$  under the atmosphere of air and  $\text{N}_2$ . They applied paper chromatography for amino acid separation and regarded the amino acids containing sulphur next histidine, phenylalanine, serine and threonine as most labile. The decrease of amino acid residues content was observed at 10 kGy.

Keeping in mind slight differences irradiation conditions in both studies it can still be concluded that amino acid residues modifications detectable by the applied chromatography methods occur at relatively high doses. The changes of fluorescence and ability to bind oxygen, on the other hand, happen at significantly lower doses [9, 11].

Besides our previous studies showed that  $\cdot\text{OH}$  radicals initiated aggregation of hemoglobin [10]. The bonds induced by the action of  $\cdot\text{OH}$  radicals are covalent. Taking into account changes in the amino acid composition induced by the  $\cdot\text{OH}$  radicals indicate modifications of cysteine, tryptophan and histidine residues. These may be targets of  $\cdot\text{OH}$  radicals with Hb and may be involved in the process of aggregation.

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## WPLYW RODNIKOWYCH PRODUKTÓW RADIOLIZY WODY I RODNIKÓW ETANOŁOWYCH NA BIAŁKOWĄ CZĘŚĆ HEMOGLOBINY CZŁOWIEKA

### CZ. II. USZKODZENIA RESZT AMINOKWASOWYCH

W pracy zajmowano się uszkodzeniami reszt aminokwasowych hemoglobiny indukowanymi działaniem rodników hydroksylowych, hydratowanych elektronów i rodników etanołowych. Jako kryterium uszkodzenia przyjęto obniżenie (ubytek) w zawartości reszt aminokwasowych w napromieniowanych preparatach hemoglobiny. Zawartość reszt aminokwasowych oznaczano po hydrolizie białka za pomocą analizatora aminokwasów. Poziom grup -SH określano metodą miareczkowania p-chlorortęciobenzoesanem.

Najbardziej efektywne w uszkodzeniu reszt aminokwasowych Hb były rodniki  $\cdot\text{OH}$ , natomiast jako najbardziej promienioczułe w badanych warunkach napromieniania można wymienić reszty cysteiny, tryptofanu i histydyny.