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FRAGMENTATION OF HUMAN HEMOGLOBIN BY OXIDATIVE STRESS PRODUCED BY PHENYLHYDRAZINE

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Exposure of purified human hemoglobin to phenylhydrazine induces the oxidation of hemoglobin and the generation of acid soluble peptides. The extent of protein fragmentation depends on the concentration of phenylhydrazine, incubation time and temperature. The fragments, excluded by gel filtration chromatography on Sephadex G-15, are partially degraded by leucine aminopeptidase and are totally converted to amino acids by acid hydrolysis. The addition of inhibitors for serine proteinases (phenylmethylsulfonylfluoride), cysteine proteinases (leupeptin), aspartic proteinases (pepstatin A) and metalloproteinases (EDTA) does not alter the formation of acid soluble peptides, thus excluding the involvement of erythrocyte proteinases in the generation of peptides. It is suggested that oxygen and phenylhydrazine free radicals produced in the course of hemoglobin oxidation might be responsible for protein fragmentation. We also discuss a possible relationship between the fragmentation of oxidized hemoglobin and the ATP-independent proteolysis stimulated by oxidative agents.

KEY WORDS: Hemoglobin, Fragmentation, Phenylhydrazine, Oxidation, Proteolysis, Free radicals.

INTRODUCTION

The hydrazines are a class of compounds widely used in the laboratory, in industry and in human therapy such as antihypertensive (hydralazine) or antineoplastic agents (procarbazine). The metabolic activation of hydrazines seems to generate radical agents responsible for several toxic effects, including carcinogenesis.[1]

Among different hydrazine derivatives phenylhydrazine is one of the most effective haemolytic drugs[2] used to produce experimental reticulocytosis in rabbits.[3] The biochemical mechanism that mediates the oxidative haemolysis produced by phenylhydrazine is still poorly understood, in spite of several efforts made in this area. A complete elucidation is difficult owing to the large number of different effects produced by phenylhydrazine in the red cells. Treatment of erythrocytes with phenylhydrazine results in a variety of hemoglobin denatured products (methemoglobin, reversible and irreversible hemichromes, sulphohemoglobin, Heinz bodies),[4] a methemoglobin-phenyldiazene complex[5] and a complex of reactive intermediates of phenylhydrazine and heme.[6] Also observed have been changes in erythrocyte membranes such as spectrin degradation,[7] protein cross-linking,[8] band 3 clustering[9] and

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lipid peroxidation,[10] as well as alteration of cellular thiol status by glutathione oxidation,[11] depletion of ATP[12] and inactivation of enzymes.[13]

In addition to these effects, phenylhydrazine is a powerful agent capable of stimulating proteolysis in the red cells.[14] It has been proven that free amino acids are mainly generated from hemoglobin,[15] but the mechanism by which oxidized hemoglobin is rapidly hydrolysed remains unclear to date.

In this paper we report the observation that human purified hemoglobin, devoid of endogenous proteinase contamination, can be cleaved into acid soluble peptides by direct interaction with phenylhydrazine and we believe that this event might be related to previously described enhanced proteolysis.

MATERIALS AND METHODS

Chemicals The following chemicals were obtained from commercial sources: phenylhydrazine and fluorescamine from Sigma (USA), leucine aminopeptidase, alanine dehydrogenase, NAD^+ and protease inhibitors from Biochemia-Boehringer (Milan, Italy), DEAE-Cellulose (DE-52) and CM-Cellulose (Cm-52) from Whatman, Ultragel AcA34 from LKB, Sephadex G-15 from Pharmacia. All other chemicals of analytical grade were purchased from Carlo Erba (Milan, Italy). *Preparation of purified human hemoglobin* Heparinized blood (25 ml) was withdrawn from adult healthy donors. Erythrocytes were isolated according to Beutler *et al.*[16] Hemoglobin was purified according to Di Cola *et al.*[17] with slight modifications. Briefly, hemolysate, devoid of membranes, was buffered to pH 7.0 with sodium phosphate (5 mM) and percolated by a DE-52. Hemoglobin (not absorbed) was further purified by gel filtration on Ultragel AcA34 (2.6 × 80 cm). Tubes containing hemoglobin (at least 5 mg/ml) were pooled. Purified hemoglobin (80 mg/ml) was stored at -80°C and used within a month. A prolonged storage rendered the protein more susceptible to fragmentation. Purity of the product was checked, immediately after preparation, by polyacrylamide gel electrophoresis[18] which at all concentrations of protein tested gave a single band in the presence or in the absence of SDS. Alternatively, hemoglobin was isolated according to Lynch[19] *et al.* and further purified by gel filtration as described above. *Assay of hemoglobin breakdown* Hemoglobin (16 mg) was incubated in a volume of 1 ml containing 50 mM sodium phosphate buffer (pH 7.5) in the presence of suitable concentrations of phenylhydrazine. In control samples phenylhydrazine was replaced with buffer. At different times the mixture was deproteinized by cold trichloroacetic acid (10% final concentration) and the release of the acid-soluble amino groups was measured fluorimetrically with fluorescamine using leucine as a standard.[20] All values were corrected for the fluorescence background occurring at zero time incubation. *Measurements of free alanine and amino acids analysis* The concentration of free L-alanine was estimated according to Williamson[21] by fluorimetric assay of NADH as previously described.[17] Amino acids analysis was performed in a LKB 4150 ALPHA amino acids analyzer by cation-exchange resin (Ultropac 11) developed with lithium buffers and standardized with amino acids occurring in physiological fluids. *Preparation of acid soluble material* Hemoglobin (80 mg) was incubated in 5 ml of potassium phosphate buffer (pH 7.4) with 8 mM phenylhydrazine for 3 h at 37°C. Then the solution was deproteinized by the addition of 5 ml of perchloric acid (1 M). After the removal of precipitate, the supernatant was neutralized with KOH (5 M). Insoluble perchlorate was eliminated and the acid soluble fraction used for analysis.

RESULTS

Hemoglobin (16 mg/ml), incubated at 37°C in the presence of 8 mM phenylhydrazine, underwent a rapid oxidation. The colour of the solution turned immediately from a bright red to a dark brown. Within a few minutes the solution became turbid and an insoluble precipitate accumulated at the bottom of the tube. These changes are related to the formation of methemoglobin, hemichromes and Heinz bodies, produced by the oxidative reaction of phenylhydrazine, as revealed by spectral analysis (Figure 1). The appearance of visible turbidity was greatly reduced when the reaction was performed at 0°C, though a slight elevation of base line at 700 nm (indicative of molecular aggregation) occurred also at low temperature (Figure 1). In addition to such well-known spectral changes, we observed that an increasing amount of fluorescamine-reactive acid soluble material was released in the course of incubation at 37°C. The formation of acid soluble material was dependent on the time of incubation, the concentration of phenylhydrazine (Figure 2) and temperature (Figure 3). When the incubation was carried out at 0°C, a very small amount of peptides was released by hemoglobin even at high concentration of phenylhydrazine in spite of the complete oxidation of oxyhemoglobin to methemoglobin and hemichrome. Since fluorescamine is a specific reagent for primary amino groups, it was conceivable that fluorescamine-sensitive material recovered in the acid soluble fraction might be made up by amino acids or peptides generated by hemoglobin fragmentation.

No free alanine could be detected by a very sensitive fluorimetric method capable of revealing even a few nmoles of alanine (data not shown). Similarly, the chromatographic profile obtained by the amino acid analyzer did not show the presence of

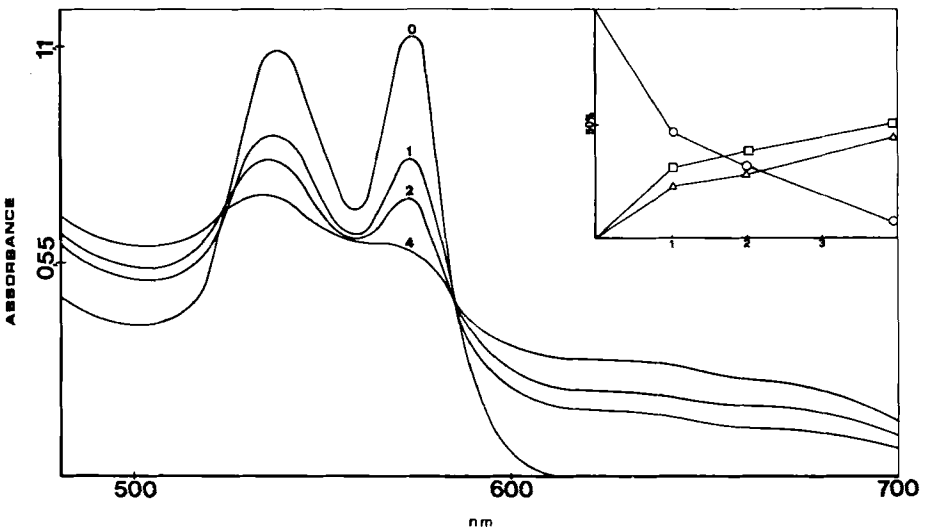

FIGURE 1 Spectrophotometric changes of oxyhemoglobin produced by different amount of phenylhydrazine. The protein (16 mg/ml) incubated at 0°C with phenylhydrazine for 30 min was diluted (18.7 μM) in potassium phosphate buffer 0.1 M (pH 7.0) and the optical spectra were promptly recorded at 6°C. The figures reported on the spectral curves indicate phenylhydrazine/heme ratio. The insert shows the relative changes (%) of oxyhemoglobin (○), methemoglobin (△) and hemichromes (□) against phenylhydrazine/heme ratio, computed by spectral analysis according to French *et al.*[26].

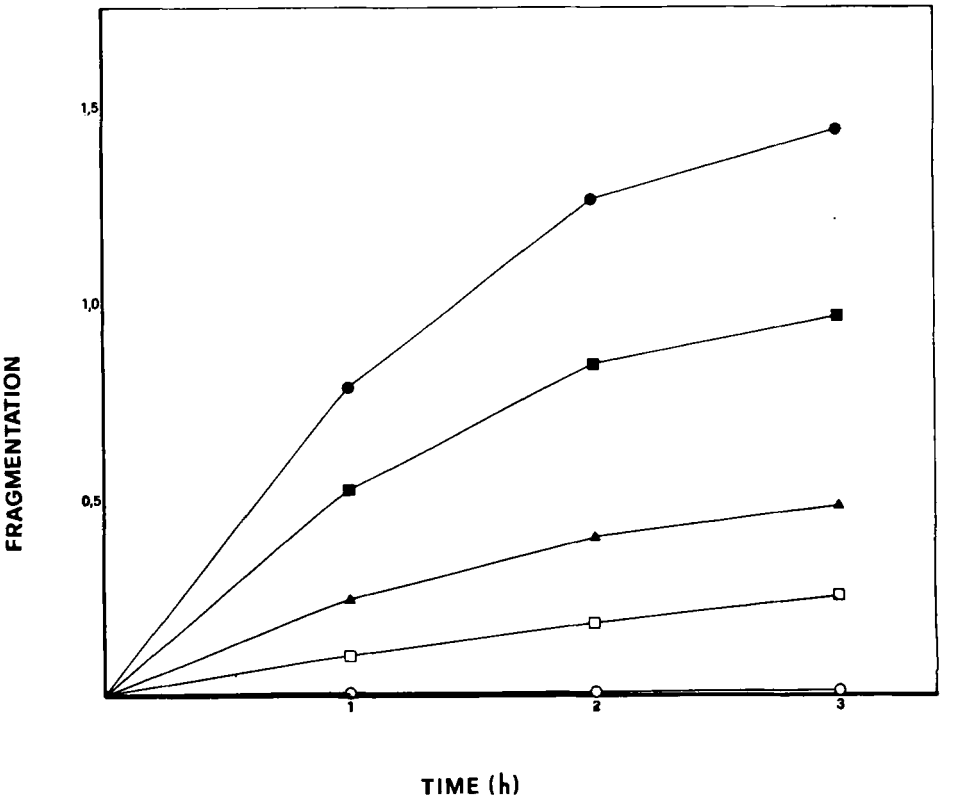

FIGURE 2 Effect of phenylhydrazine on hemoglobin fragmentation. The protein (16 mg ml) was incubated at 37°C with different concentrations of phenylhydrazine. (○) none: (□) 1 mM: (▲) 2 mM: (■) 4 mM: (●) 8 mM. At the times indicated aliquots were removed and acid-soluble amino groups were estimated as described in Methods section and expressed as moles of amino groups mole of heme.

typical amino acid peaks (data not shown). When an aliquot (2 ml) of acid soluble material was gel-filtered, after neutralization, through a Sephadex G-15 column, a single peak of fluorescamine-reactive material appeared in the void volume of the column, thereby excluding that phenylhydrazine itself is involved in the reaction with fluorescamine, but suggesting the presence of peptide(s) greater than 1500 kDa (Figure 3). The profile elution was changed after the incubation of acid soluble material with leucine aminopeptidase. While the void volume peak decreased, a second peak appeared in the phenylalanine elution volume (Figure 3). After acid hydrolysis the former peak was abolished and the latter was greatly increased (Figure 3).

To exclude that endogenous contaminant proteinase(s) could be responsible for the generation of peptide fragments, hemoglobin was oxidized by phenylhydrazine in the presence of typical inhibitors for each known class of proteases (serine, cysteine, aspartic and metallo-proteinase, respectively).[22] Phenylmethylsulphonylfluoride (0.2–2 mM), leupeptin (0.01–0.2 mg/ml), pepstatin A (0.002–0.02 mg/ml) and EDTA (0.2–2 mM) were totally ineffective in reducing the fragmentation (data not shown).

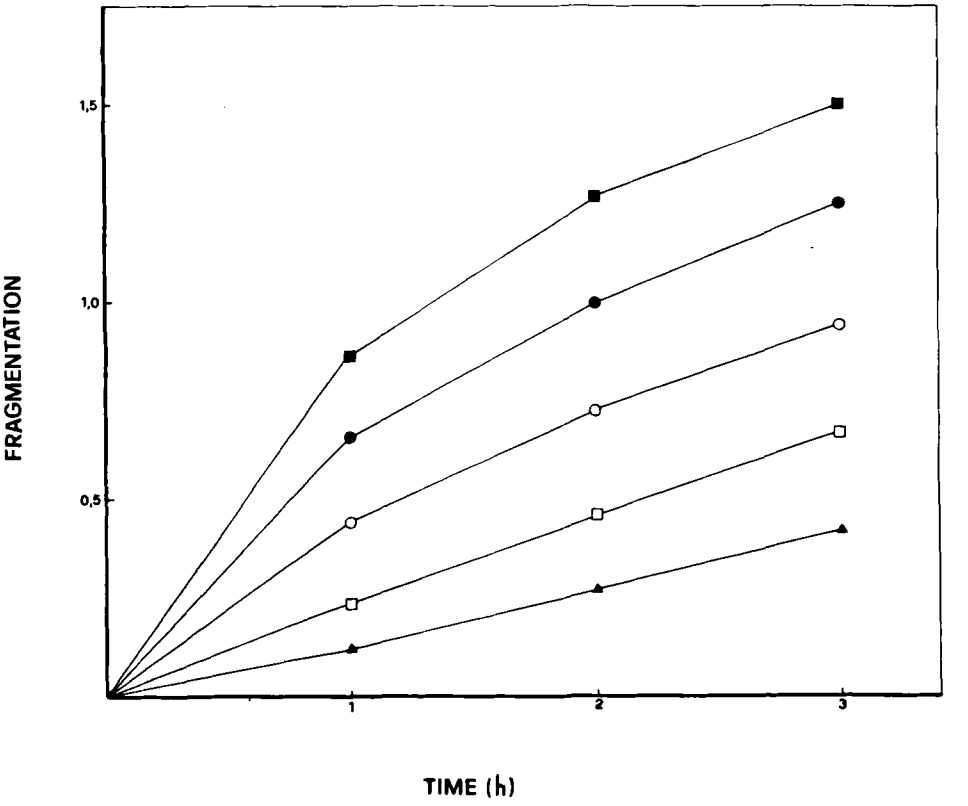

FIGURE 3 Effect of temperature on hemoglobin fragmentation. Hemoglobin (16 mg/ml) was incubated in the presence of phenylhydrazine (8 mM) at 0°C (▲), 10°C (□), 20°C (○), 30°C (●) and 40°C (■). At the times indicated the amount of acid-soluble amino groups was monitored as described in the Methods section and expressed as nmoles of amino groups/mole of heme.

DISCUSSION

The results described herein shows that phenylhydrazine, in addition to a number of effects already known,[4-15] produces, by interaction with hemoglobin, the generation of acid soluble peptides. It has been reported that hemoglobin oxidized by phenylhydrazine is more susceptible to proteolytic degradation by erythrocyte proteinases.[14,15] However, it seems extremely improbable that an endogenous proteolytic contamination could be responsible for the generation of peptides, since similar results are obtained with hemoglobin purified by two different procedures, no other band besides hemoglobin can be detected in electrophoresis and, finally, proteinase inhibitors totally fail to restrain the peptide generation.

Protein fragmentations have been observed in the course of irradiation producing highly reactive free radicals such as hydroxyl and superoxide radicals.[23] The reaction of phenylhydrazine with oxyhemoglobin is extremely complex, since, in addition to end reaction compounds (nitrogen and benzene,[24]) several intermediate products are

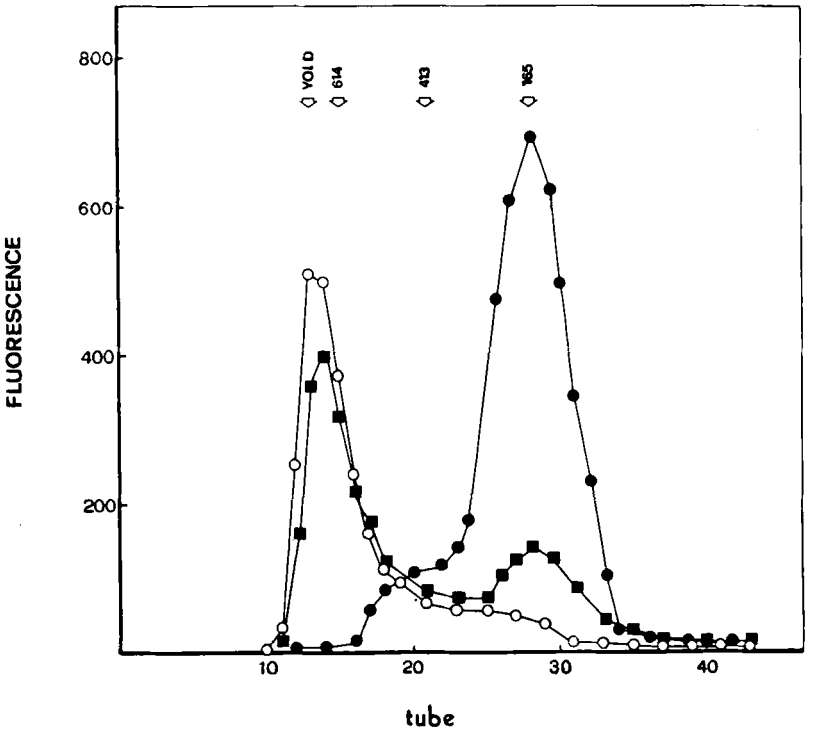

FIGURE 4 Elution profile on Sephadex G-15 of acid soluble fragments. Hemoglobin (16 mg/ml) was incubated with phenylhydrazine (8 mM) for 3 h at 37°C. The mixture was deproteinized and the acid soluble fraction (2 ml) was applied to the column (1.6 × 40 cm) eluted with sodium borate buffer 0.1 M (pH 8.5). Untreated sample (○), sample incubated with leucine aminopeptidase (10 IU) for 16 h at 25°C (■), sample hydrolysed at 110°C for 24 h in HCl (6 M) (●). Amino groups was monitored by fluorescamine reaction and expressed as fluorescence arbitrary units. Arrows and figures indicate the elution volume and molecular weight of standards used for column calibration (Blue Dextran, glutathione disulfide, N-carbobenzoxy-glycyl-glycyl-phenylalanine, phenylalanine)

generated such as phenyldiimide,[25] phenylhydrazyl radical,[26] phenyl radical[27] as well as different by side products such as superoxide,[28] hydrogen peroxide[29] and probably hydroxyl radical.[30] This latter compound has been believed to be involved in the degradation of irradiated proteins[23] and hydrogen peroxide (though at high concentration) has been proven to mediate the fragmentation of albumin.[31] It is possible that the same compounds are involved in the fragmentation of hemoglobin in the course of its oxidation by phenylhydrazine.

The present report can suggest a key for the interpretation of the accelerated release of free amino acids by red cells exposed to phenylhydrazine. It has been proven, by measurements of free alanine generation, that erythrocytes contain an ATP-independent system involved in the proteolytic degradation of oxidant-damaged hemoglobin.[15] It has been proposed that oxidized hemoglobin is more susceptible to a proteolytic attack by specific endoproteinase(s) able to selectively recognize modified hemoglobin, but this enzyme(s) is not so far clearly identified. Although we showed

that the peptide(s) generated by phenylhydrazine-hemoglobin interaction is not completely hydrolysed to amino acids by leucine aminopeptidase, the complementary effect of different mono-di-tri-aminopeptidases occurring in red cells could produce a complete degradation of such a peptide(s). If also in intact red cells, hemoglobin can undergo fragmentation by phenylhydrazine and other oxidants, it is possible that the peptide(s) could be further degraded by erythrocyte peptidases, thus accounting for the elevated rate of amino acid generation.

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