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Contribution of Haemoglobin and Membrane Constituents Modification to Human Erythrocyte Damage Promoted by Peroxyl Radicals of Different Charge and Hydrophobicity

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We have investigated the influence of the free radical initiator characteristics on red blood cell lipid peroxidation, membrane protein modification, and haemoglobin oxidation. 2,2'-Azobis(2-amidinopropane) (AAPH) and 4,4'-azobis(4-cyanovaleric acid) (ACV) were employed as free radical sources. Both azo-compounds are water-soluble, although ACV presents a lowed hydrophilicity, as evaluated from octanol/water partition constants. At physiological pH, they are a di-cation and a di-anion, respectively.

AAPH and ACV readily oxidise purified oxyhemoglobin in a very efficient free radical-mediated process, particularly for ACV-derived radicals, where nearly one heme moiety was modified per radical introduced into the system, suggesting that negatively charged radicals react preferentially at the heme group. The radicals derived from both azo-compounds lead to different oxidation products. Methemoglobin, hemichromes and choleglobin were produced in AAPH-promoted hemoglobin oxidation, while ACV-derived radicals predominantly form hemichromes, with very low production of choleglobin.

Red cell damage was evaluated at the level of hemoglobin and membrane constituents modification, and was expressed in terms of free radical doses. Before the onset of the lytic process, ACV leads to more lipid peroxidation than AAPH, and induces a moderate oxidation of intracellular Hb. This intracellular oxidation is markedly increased if ACV hydrophilicity is decreased by lowering the pH. On the other hand, AAPH-derived radicals are considerable more efficient in promoting protein band 3 modification and cell lysis, without significant intracellular hemoglobin oxidation. These results show that the lytic process is not triggered by lipid peroxidation or hemichrome formation, and suggest that membrane protein modification is the relevant factor leading to red blood cell lysis.

Keywords: 2,2'-azobis(2-amidinopropane), 4,4'-azobis(4-cyanovaleric acid), lipid peroxidation, band-3, haemoglobin oxidation

INTRODUCTION

Erythrocyte lipids, membrane proteins and haemoglobin are damaged by free radicals. These processes are of particular interest since the oxi-

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dation and denaturation of haemoglobin, together with membrane components oxidation, are known to be important factors in the removal of senescent[1] and abnormal erythrocytes of several hemolytic conditions, such as thalassemia[2], malarial anemia[3], sickle cell anemia[4], favism in G6PDase defficiency[5], and congenital Heinz body hemolytic anemia[6]. Furthermore, an understanding of the sequence of processes associated to the free radical promoted modification of erythrocytes can help to understand the mechanism of the damage induced by drugs leading to Hb oxidation, such as the antimalaria drug primaquine[7] and bacterial hemolysins[8].

The type of damage and its temporal sequence will be determined by the characteristics (reactivity, selectivity and hydrophobicity) of the initial radical and the locus of its generation. Particularly suitable sources of free radicals for these types of studies are azo-compounds.

Azo-compounds have been widely employed as free radical sources in relation to the damaging role of active oxygen species[9–20]. These compounds have the advantage of producing radicals at controlled rates that are relatively insensitive to the characteristics of the media[21], thus allowing an evaluation of the free radical dose and temporal course of the oxidation process. In particular, it can be assumed that the rate of radical production is insensitive to the presence of metals and/or metal chelators[19]. Furthermore, a variety of azo-compounds of widely different hydrophobicity are commercially available[22]. The hydrophobic characteristics, as well as the net charge of the parent molecule, can widely change the local concentration of the initiator in microheterogenous systems[21], the reactivity of the produced radicals, and the relative importance of the different reaction channels.

The influence of the azo-compound hydrophobicity upon its interactions with red blood cells constituents has been partially discussed by Niki et al.[13] employing 2,2′-azobis(2-amidinopropane) (AAPH) and 2,2′-azobis(2,4-dimethylvalero nitrile) (ACVN) as free radical sources. These authors have reported that both azo-compounds are able to induce significant hemolysis, particularly in the presence of oxygen, and that the damage can be efficiently prevented by chain-breaking antioxidants. These results were interpreted as suggesting that free radical-mediated lipid peroxidation and/or its products play an important role in the hemolytic process. However, the contribution of intracellular haemoglobin oxidation to the lytic event was not evaluated. Azo-compounds derived radicals lead to haemoglobin oxidation and hemichrome formation[15], whose polymerisation leads to Heinz body accumulation[23]. Association of these aggregates to band-3 protein could contribute to the red cell disruption[6].

AAPH, a positively charged azo-compound, is the free radical source most extensively employed in the study of red cell damage inflicted by hydrophilic free radicals[9–11,13,14,19,24–26]. ACV is a negatively charged azo-compound whose hydrophobicity, at physiological pH, can be considered to be between that of the positively charged AAPH and that of the neutral ACVN. Pekiner and Pennock[9,20] have described the lytic effect of ACV on outdated erythrocytes. However, no studies of oxidative damage to fresh erythrocytes have been reported that could allow a comparison with other free radical sources. In the present work, we have carried out a comparative study of the temporal profiles of the induced lysis, lipid peroxidation, membrane protein damage, and haemoglobin transformation promoted by AAPH and ACV. Studies were carried out over a wide range of experimental conditions, employing intact human red cells, lysates and purified Hb in order to assess the role of the azo-compound characteristics on the rate of intracellular and extracellular Hb oxidation. In particular, we have evaluated the effect of the initiator (and derived radicals) charge by conducting experiments over a wide pH range. Changes in pH could modify both the course of the lytic

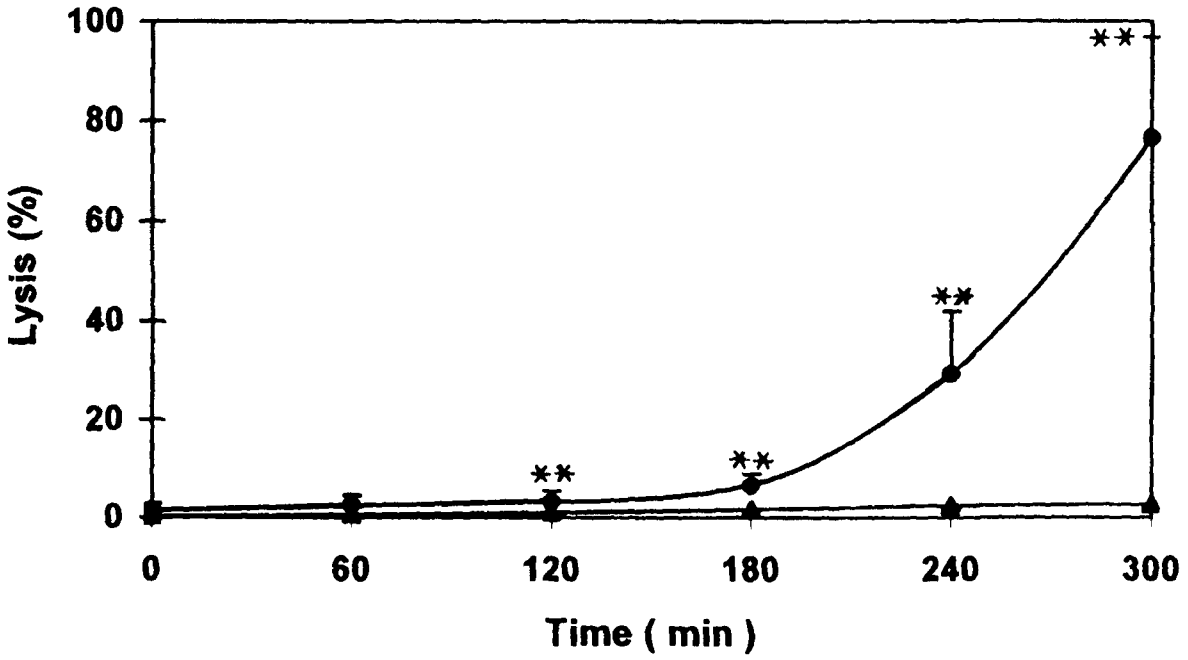

FIGURE 1 Time course of hemolysis of red cells (Hto: 8%) exposed to 75 mM AAPH (•) or 75 mM ACV (▲) at pH 7.4 (n=6). Control values after 300 minutes of incubation were 0.83 ± 0.38 (n=6). * $p < 0.05$ ** $p < 0.01$

process[27] and the production rate and reactivity of water-soluble azo-derived free radicals[28].

MATERIALS AND METHODS

4,4′-Azobis(4-cyanovaleric acid) (ACV) (Sigma) and 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) (Wako) were employed without further purification. Partition constants between the aqueous solution (at fixed pH) and n-octanol were obtained by the hand-shaking method. The concentration of the azo-compound remaining in the aqueous phase was determined spectrophotometrically from its absorbance at 370 nm.

Azo-compounds pKa were obtained from potentiometric titration curves (pH 2.5 to 13). Rates of free radical generation by the azo-compounds were evaluated employing luminol chemiluminescence as a sensor of the steady state free radical concentration[29]. Rates were estimated from induction times following Trolox addition, and considering that each antioxidant molecule is able to trap two free radicals[29,30]. Measurements were carried out at pH 8.3 to increase luminol chemiluminescence intensity. Rates of cleavage, at different pH, were evaluated by measuring, with a Clark-type electrode, the oxygen consumption associated to the process. Measurements were carried out under air at 37°C over a wide pH range.

Blood was collected from human volunteers by venepuncture in the presence of heparine and centrifuged to remove plasma and buffy coats. Erythrocytes were washed three times with ice-cold Krebs-HEPES pH 7.4 (mmoles/L: NaCl, 120; KCl, 4.8; $CaCl_2$, 1.3; $MgSO_4$, 1.2; HEPES, 16) and resuspended in the same buffer to 16%(v/v). Intracellular pH, at a given extracellular pH, was determined in supernatants after rupturing

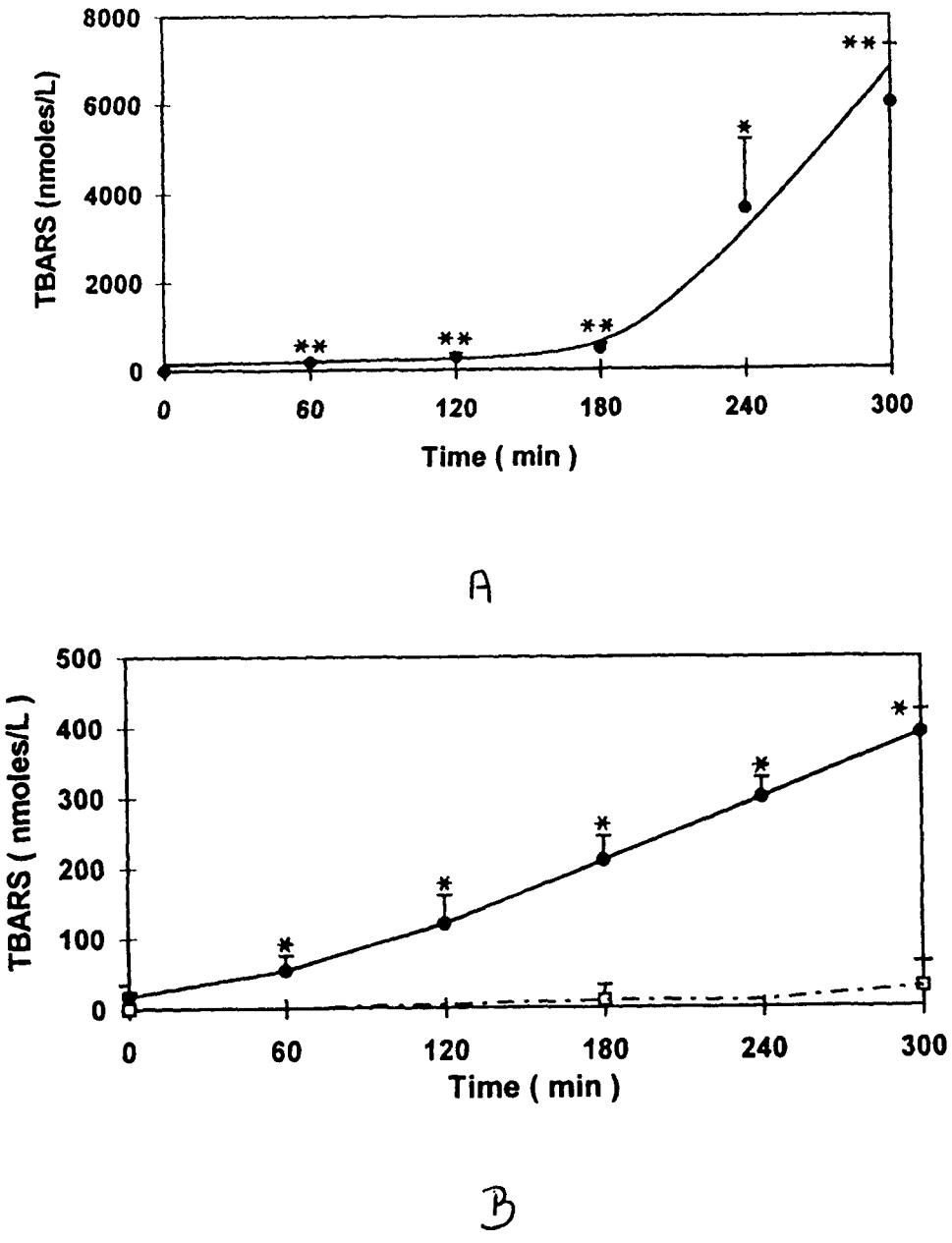

FIGURE 2 Time course of lipid oxidation of red cells (Hto: 8%) exposed to 75 mM AAPH (A) or 75 mM ACV (•, B) or without azo-compounds (□, B) (n=6). * $p < 0.05$ ** $p < 0.01$

packed erythrocytes by freezing, followed by thawing and centrifugation (20 min at 20000 g, 0 °C). Lysates were obtained by pouring erythrocyte suspensions into a hyposmotic Tris-HCl solution, pH 7.4. Membrane separation was achieved by centrifugation at 20000g during 20 minutes at 4 °C. Haemoglobin was purified from the lysate by column chromatography on

DEAE-Sephadex, according to the procedure described by Winterbourn[31].

In oxidation experiments, an erythrocyte suspension, lysates or purified haemoglobin, was incubated at 37 °C with different concentrations of AAPH or ACV. Blank experiments were carried out without azo-compound addition. Most incubations were performed in Krebs-HEPES buffer, pH 7.4. Because ACV low hydrosolubility, it was first dissolved in concentrated NaOH. After mixing with Krebs buffer, the solution was brought to the desired pH.

Thiobarbituric acid-reactive substances (TBARS) were determined by the method described by Trotta et al.[32]. ACV interferes with TBARS analysis. Reproducible results were obtained by acidification and chloroform extraction prior to the sample treatment. The decrease in band-3 protein was evaluated by PAGE-gel electrophoresis, as previously described[24]. Hb or metHb oxidation was evaluated following haemoglobin absorption spectra changes. Hb, metHb and hemichromes concentrations were determined employing the method and relationships reported by Winterbourn[31]

After oxidation of red cells, Hb transformation was evaluated both in the external medium (corresponding to cells lysed during the process) and in cells that remained intact. The former was achieved by measuring Hb status in the supernatant after centrifugation. Hb status in the intact cell was measured in the supernatant after washing and lysing, by an osmotic shock, the centrifugued cells. Hb status averaged over all the cell population, including intact and lysed cells, was evaluated from the spectra of the supernatant after pouring the erythrocyte suspension in distilled water. Percentage hemolysis was assessed by measurements of Hb released from cells, relative to the total cellular Hb content. Following incubation, a volume of cells suspension was diluted with 40 volumes of ice-cold 0.15M NaCl, or with 40 volumes of ice cold distilled water. After centrifugation, total supernatant Hb concentration was determined spectrophotome-trically[31]. Total Hb was considered as the sum of HbO_2, metHb, and hemicromes.

Statistical analysis

Wilcoxon test was applied to red blood cells oxidation data. Free haemoglobin oxidation data are expresed as mean value ± standard deviation of 3 to 5 independent determinations carried out in duplicates.

RESULTS

Evaluation of pKa and Hydrophobicities

ACV pKa was 4.9 ± 0.1, typical of a carboxylic group in water. For AAPH, pKa was 10.4 ± 0.2, considerably lower than that expected for amidino groups, although higher than that estimated for the AAPH derived radicals[28]. For each compound, a single pKa was observed, corresponding to the titration of two groups per molecule. This implies that the amidino moiety in AAPH and the carboxylic acid in ACV behave as isolated groups.

The octanol/water partition constant K, defined by:

$$K = [Azo]_{octanol}/[Azo]_{water}$$

is a measure of the azo-compound hydrophobicity. The values obtained are shown in Table I.

TABLE I Octanol/water partition constant of ACV and AAPH at different pH

pH	*ACV*	*AAPH*
2.7	2.0 ± 0.2	⩽0.05
7.4	⩽0.05	⩽0.05
7.4[a]	3.1×10^{-5}	5.0×10^{-7}
13.0	---	0.5 ± 0.1

Experimentally determined values are given as averages ± standard deviations of three independent determinations.

a. Partition constant values estimated from the values measured at pH 2.7 (ACV) and 13 (AAPH) and twice the difference between the pKa of the compound and 7.4.

Evaluation of Free Radical Generation Rates

The rate of radical production (R_p) at 37 °C and pH 7.4 can be expressed as

$$R_p(\mu M\ min^{-1}) = 0.15\ [AAPH]$$

and

$$R_p(\mu M\ min^{-1}) = 0.018\ [ACV]$$

when the azo-compound concentration is given in mM units. Rp, for both azo-compounds were very little pH dependent over the 4.2 to 8.6 pH range. The rate of the process changes less than 10 % for AAPH over all the pH range considered, and decreases c.a. 30 % for ACV at the lowest considered pH.

Red blood cell oxidation

Lipid peroxidation, lysis, and Hb oxidation elicited by incubation of a red cell suspension in the presence of azocompounds are relatively slow processes, in spite of a rather high rate of free radical production. Hemolysis extent measured after incubation is shown in Fig. 1. AAPH disrupts nearly 80% of the red cells in 300 minutes, with an induction time of c.a. 180 minutes. On the other hand, after 300 minutes incubation with ACV, only 2.7 ± 0.8 %(n=6) of hemolysis takes place and, even after 75 hours, hemolysis is only 10.8± 0,9%(n=4). These values, although low, are significantly higher than those obtained in control experiments. In order to compare the lytic effect of both azo-compound, data are given in terms of free radical doses (Table II).

The extent of lipid peroxidation, evaluated by TBARS production, is shown in Fig. 2; both azo-compounds produce, at short incubation times, a small but significant oxidation of membrane lipids. An increase in lipid peroxidation rate occurs after 180 minutes exposure to AAPH (Fig 2A), coinciding with an increase in the rate of hemolysis (Fig. 1). TBARS molecules produced per radical introduced into the system have been evaluated for both azo-compounds (Table II).

TABLE II Comparison of AAPH and ACV derived radical efficiency in promoting lipid peroxidation, band-3 modification and lysis. AAPH and ACV: 75 mM; RBC number: 9×10^8 RBC/mL

Time (min)	*Dose (μM)*	*AAPH*	*ACV*
Lipid peroxidation		*10^3 TBARS/Radical*	
60	80		0.67 ± 0.26(6)
180	240		0.86 ± 0.14(6)
30	340	0.33 ± 0.23(6)[a]	
300	400		0.96 ± 0.08(6)
60	680	0.26 ± 0.07(6)	
180	2020	0.23 ± 0.07(6)	
300	3400	1.78 ± 0.39(6)	
Band-3 modification		*% of decrease*	
30	340	22 ± 1.1(4)[a]	
240	340		3.0 ± 1.4(6)
Lysis		*% of lysis*	
300	3400	76.6 ± 22.5(8)[a]	
2400	3400		5.9 ± 0.5(4)

Values given as averages ± standard deviations. The number of independent experiments are given between parenthesis.

a. $p < 0.01$ with regard to values obtained employing ACV under similar free radical doses.

Although hemolysis promoted by ACV is minimal (Fig. 1), a small but significant ($p<0.05$) amount of intracellular hemichrome is produced (Fig. 3). In spite of the high lytic effect of AAPH after 300 minutes incubation, nearly 98% of the remaining intracellular hemoprotein is oxyHb. However, an extensive oxidation to metHb is observed in the red cell suspension which, after 300 minutes exposure to AAPH, represents nearly 20% of the total Hb concentration. This value is significantly different from control values ($p<0.05$) (Fig. 4).

To gather some insight on the effect of the radical precursor hydrophobicity, experiments were carried out at different extracellular pH values. Intracellular pH follows the external values

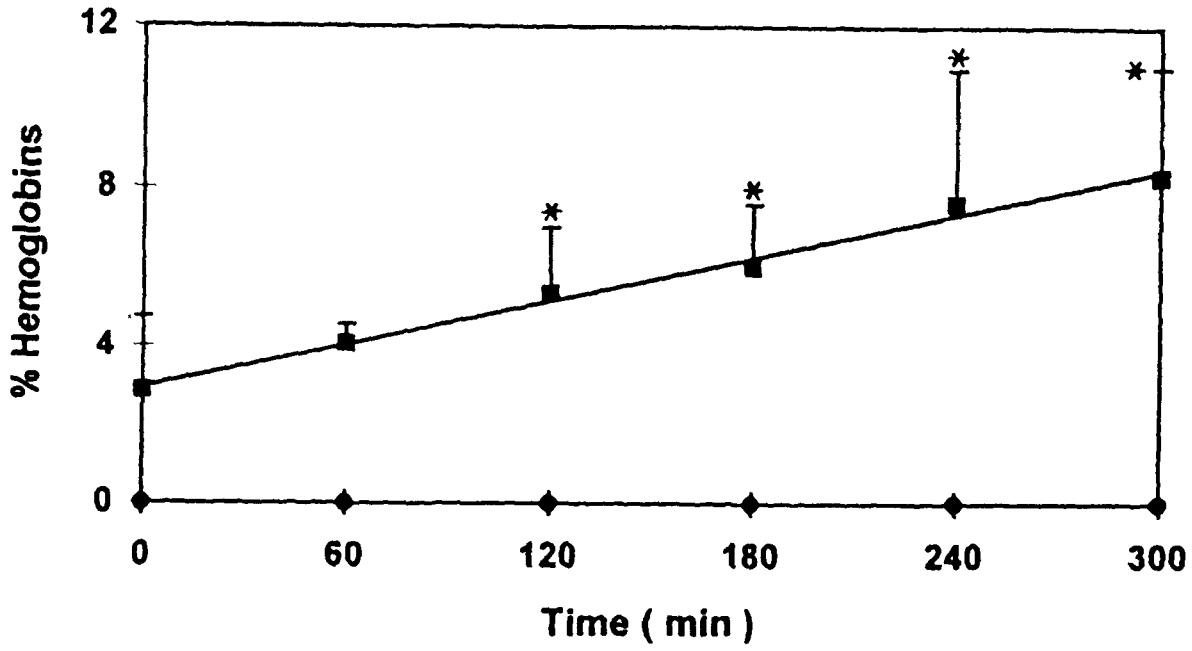

FIGURE 3 Intracellular increase of hemichromes (■) and metHb (•) in red cells (Hto: 8%) exposed to 75mM ACV (n=6). No changes in hemichromes or metHb were observed without ACV. * $p < 0.05$

(intracellular pH was 7.28, 6.52, or 5.48 when the extracellular pH was 7.40, 5.12, or 4.35, respectively) and was unaffected by the presence of the azo-compounds. The percentage of lysis elicited by ACV is shown in Fig. 5. A noticeable increase in hemolysis is observed when the pH decreases. This is an effect due to ACV since, in control experiments, lysis remained below 7 % at all pH tested. Concomitant to the increase in lysis, there is an increase in the rate of intracellular Hb oxidation at low pH (Fig. 6).

A different pH effect is observed when AAPH (75 mM) is employed as free radical source. For this compound, the onset of lysis is delayed to c.a. 240 min instead of 180 min (Fig. 1) when pH is lowered from 7.4 to 5.12 (data not shown).

Free haemoglobin oxidation

Hb in lysates and purified Hb are readily oxidised, both by ACV and AAPH. Purified Hb oxidation rate depends both on the azo-compound and hemoprotein concentration (Fig. 7). Oxyhemoglobin decay profiles are similar when lysates (data not shown) or purified Hb are employed, suggesting that intracellular defences are rather ineffective (at least after dilution) in preventing Hb damage elicited by the azocompounds. However, a quantitative comparison of both sets of data is precluded by the faster autoxidation of purified Hb.

Oxidation rates (expressed as % of reaction/time) increase when the Hb concentration decreases, implying that the reaction order is smaller than one. This indicates an almost quantitative trapping of the original radicals by the hemoprotein. Table III shows heme groups oxidation per radical produced under different experimental conditions.

Hb oxidation products are markedly different when the hemoprotein oxidation is promoted by ACV or AAPH (Fig. 8 and 9). Similar results were obtained with purified Hb (data not shown). AAPH promotes both hemichromes and

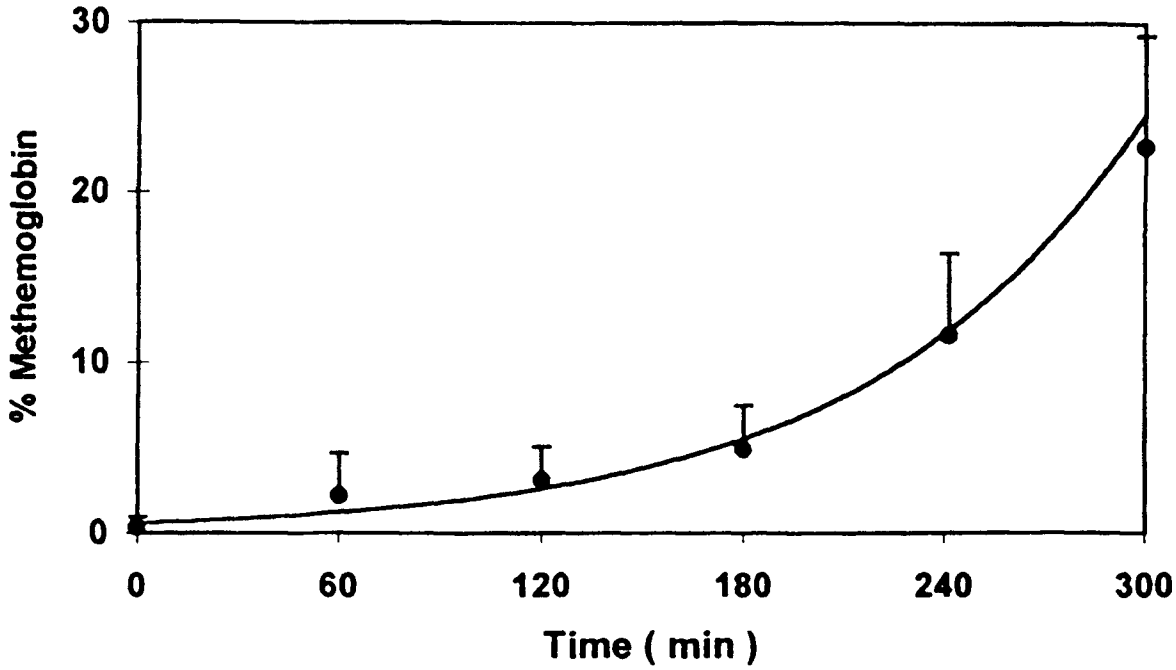

FIGURE 4 Time course of metHb increase in a red cells suspension of erythrocytes (Hto: 8%) exposed to 75 mM AAPH (n=6)

metHb formation (Fig 8), whereas only hemichromes are produced in ACV mediated oxidation (Fig. 9). Interestingly, some metHb is formed with ACV at short incubation times, which later disappears. This suggests that, at least partially, hemichromes could be formed from initially produced metHb. This is supported by results obtained with samples containing metHb, where a clear diminution in its concentration is observed (data not shown). Choleglobin, evaluated by the difference between the initial Hb concentration and the sum of Hb, metHb and hemichromes, was always below 10% for ACV. Higher values (up to 20% of the transformed Hb) were obtained employing AAPH. Hb oxidation rates were also evaluated at pH 4.3. Both azo-compounds are more efficient at this pH (data not shown). However, it is interesting to note that incubation with AAPH for times longer than 40 minutes at low pH leads to extensive hemoprotein precipitation previous to the total oxyHb modification. This would indicate that AAPH derived radicals could be preferentially interacting with the globin moiety, leading to an irreversible modification of the hemoprotein.

TABLE III Initial rate of heme groups modification and number (n) of heme groups modified per radical introduced into the system

Azocompound	*Hb (μM)*	*Initial Rate (%/min)*	*n*
AAPH	7.0	3.00 ± 0.08(4)	0.140 ± 0.005(4)
	13.8	2.20 ± 0.07(10)	0.193 ± 0.006(10)
	41.3	0.70 ± 0.02(4)	0.193 ± 0.005(4)
ACV	7.0	2.00 ± 0.04(4)	0.778 ± 0.016(4)
	13.8	1.04 ± 0.06(10)	0.783 ± 0.046(10)
	41.3	0.25 ± 0.01(4)	0.579 ± 0.009(4)

Values given are averages ± standard deviations. Values between parenthesis correspond to the number of independent determinations.

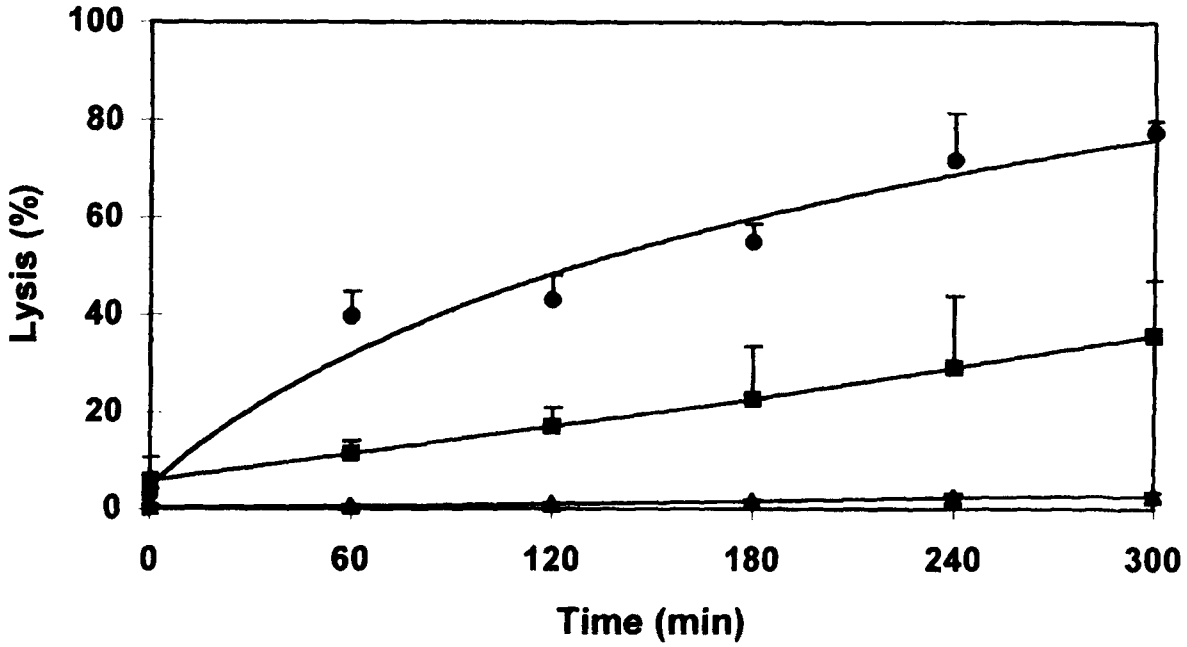

FIGURE 5 Effect of pH on red cells lysis promoted by ACV. (▲) 75 mM ACV, pH 7.4 (n=6); (■) 6,6 mM ACV, pH 5.12 (n=3); (•) 6.6 mM ACV, pH 4.3 (n=3)

Oxygen saturation produces a noticeable decrease in Hb oxidation rate only if ACV is the free radical source. Hb consumption after 60 minutes of incubation were 68 ± 1.7 % (n=10) and 33 ± 6.2% (n=4) in air and oxygen saturated atmospheres, respectively. On the other hand, AAPH (10mM) decreased oxyhemoglobin in 78 ± 3%, both in air or oxygen saturated atmospheres. Control experiments (without azo-compound) showed an hemoglobin decrease of 29 ± 4.1 % and 25 ± 3.8% in air and oxygen saturated atmospheres, respectively.

DISCUSSION

Partition constant values obtained for both compounds at different pHs can be explained in terms of protonation-deprotonation equilibrium. pKa values obtained in the present work shows that ACV is a neutral molecule at pH 2.7, and a di-anion at pH 7.4. On the other hand, AAPH is a neutral molecule at pH 13, and a di-cation at pH 7.4 and 2.7. Table I shows that at pH 7.4, AAPH is considerably more hydrophilic than ACV. If it is considered that only uncharged molecules can penetrate lipid domains, this increased hydrophilicity can be related to an intrinsic factor (the difference in K for the neutral species) and to the larger difference between its pKa and the working pH.

The present study shows that radical production for both azo-compounds is almost pH independent over the pH range considered. Hanlon and Seybert[28] have reported a moderate increase (40 %) in the rate of AAPH consumption when pH changes from 7.5 to 5.5. However, in Hanlon and Seybert's work[28] only the loss of the parent azo-compound was measured, and the results could have been influenced by a change in pri-

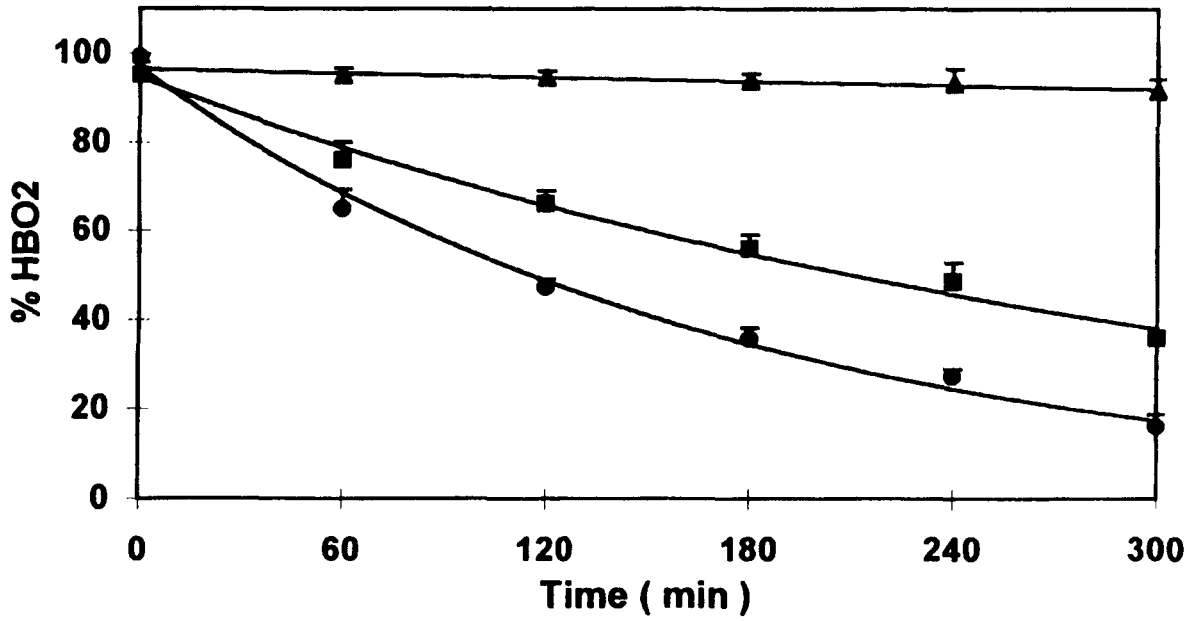

FIGURE 6 Effect of pH on the oxidation of intracellular Hb in red cells exposed to ACV. (▲) 75 mM ACV, pH 7.4 (n=6); (■) 6.6 mM ACV, pH 5.12 (n=3); (•) 6.6 mM ACV, pH 4.3 (n=3)

mary recombination efficiency with pH. Neverteless, we can conclude that pH has a minimum effect in the rate of the parent compound consumption, the rate of free radical production, and the oxygenation of the primary radicals.

Intact erythrocytes[24], as well as erythrocyte ghost membranes[33], are oxidised by a free radical chain mechanism. The kinetic chain length when AAPH is employed as initiator, is rather short (between 3.3 and 7.5), at least at the earliest stages of the process[34]. It has been reported that in AAPH promoted red blood cells oxidation lipid peroxidation takes place at early stages of the damaging process[11,34]. The data presented in Fig. 2A show that, although significant lipid peroxidation is observed prior to hemolysis, there is an increase in the rate of the process after the onset of the lytic process. This behaviour is opposite to that reported by Sato et al.[10], who found that TBARS accumulate linearly with time from the beginning of the incubation till complete hemolysis. Nevertheless, there seems to be consensus regarding the fact that lipid peroxidation is not the main process leading to red cell lysis[10,11,24,34].

Table II shows that, when applied in similar doses, AAPH derived radicals are considerably more lytic than ACV derived radicals. A similar conclusion is reached if the effect of both free radical sources are evaluated at similar free radical fluxes(1.5 μ M/min). Under these conditions, after 75 hours of incubation, the percentage of lysis elicited by ACV and AAPH was 10,8 ± 0,9% and 38,3 ± 11,3% respectively (n=4) (data not shown). As ACV derived radicals, due to their reduced hydrophilicity (Table I), are more efficient in promoting lipid peroxidation (Table II) and intracellular Hb modification, these two processes (Hb oxidation and lipid peroxidation) are not the main factors determining the extent of the lytic process. It is interesting to note that, regarding band-3 protein modification, AAPH radicals are more damaging that ACV derived

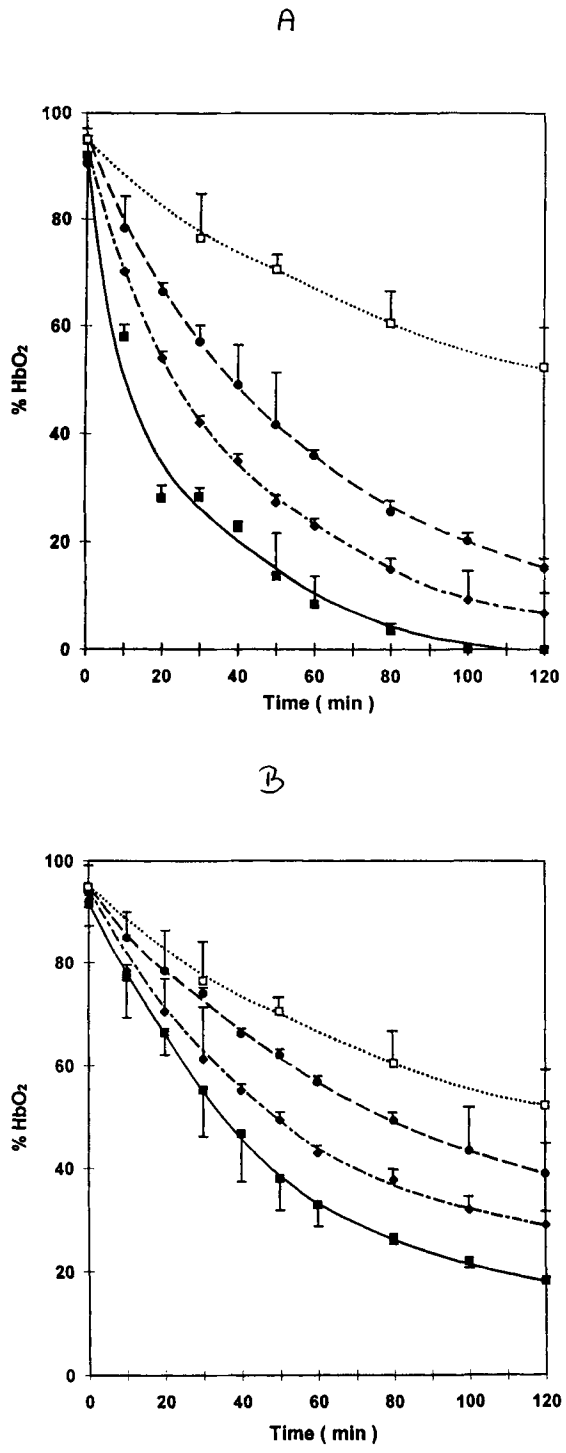

FIGURE 7 Purified Hb (13.8 μM) transformation as a function azo-compound concentration. (A) AAPH concentrations: (□) control; (•)5 mM; (◊) 10 mM; (■)20 mM. (B) ACV concentrations: (□) control; (•) 5 mM; (◊)10 mM; (■)20 mM

radicals. While a dose of 340 μM of AAPH derived radicals produces a 22 % decrease in band-3, only a 3 % decrease is observed when ACV radicals are considered (Table II). This higher capacity of AAPH derived radicals in modifying membrane proteins could explain its higher lytic capacity.

Hb oxidation is low prior to hemolysis and, particularly for AAPH, negligible in the intact erythrocytes. If the fast oxidation of purified Hb by the azo-derived radicals is considered (Fig. 7), the low oxidation rate in intact erythrocytes implies that azo-derived radicals are excluded from the cytosolyc Hb pool and/or that a large concentration of intracellular antioxidants protect the hemoprotein. The strong effect of pH observed when ACV is employed (Fig 6), and the lack of pH effect on AAPH induced Hb oxidation in the erythrocytes, strongly supports exclusion of the parent azo-compounds and/or the derived radicals from the cytosol. This is due to the incapacity of the radical sources to cross the barrier imposed by the membrane at a pH where they are charged compounds, as it is expected for AAPH over all the pH range considered. The larger amount of intracellular Hb oxidation observed with ACV is compatible with its smaller hydrophilicity and faster reaction with the hemoprotein.

Hb oxidation induced by AAPH produces a mixture of compounds, with predominance of metHb and hemicromes (Fig. 8), being the ratio between them almost independent of the heme group modification extent. This implies that AAPH derived radicals are highly inefficient in a further transformation of metHb into hemichromes. These behaviour contrasts with that observed when ACV radicals are considered (see following discussion).

ACV mediated haemoglobin modification presents similar characteristics to that of AAPH, but also noticeable differences. Particularly remarkable are the differences in product distribution and its dependence with oxygen pressure and pH. These differences must be related to differences in the charge of the free radicals and/or the hydrophobicity of the parent azo-compounds and their derived radicals.

The data included in Table III show a negligible dependence of Hb oxidation rate with the substrate concentration, implying nearly total capture of the azo-derived radicals by the hemoprotein. The n value of nearly one, obtained when ACV is employed, would indicate that most of the radicals produced are reacting with the heme moiety. The smaller values of n obtained with AAPH indicate that an important fraction of radicals trapped by the hemoprotein is reacting with the globins, without significant changes in the heme-group status. This is compatible with the higher proportion of coleglobin obtained when AAPH is employed[15]. The lack of metHb observed at long incubation times when ACV is employed as radical source (Fig. 9) implies that metHb is readily transformed to hemichromes. On the other hand, this process is considerably less efficient (or absent) when AAPH is employed as free radical source. Hemichromes production implies an attack to the porphyrin ring and, when ACV is employed, the process could be favoured by the negative charge of the parent azocompound and the produced radicals, that could direct the damage towards the positively charged heme moiety.

The very high efficiency of Hb transformation when ACV is employed (Table III) is also compatible with specific interactions favoured by coulombic and/or hydrophobic forces. Interestingly, large proportions of hemichromes were also measured employing ACVN, a result that was interpreted in terms of favourable hydrophobic interactions[15].

The effect of oxygen pressure observed in the present work is similar to that previously reported by Minetti et al.[15]. These authors reported that the effect of changing the oxygen pressure was considerably more important for azo-cyanovaleronitrile (a highly hydrophobic compound) than for AAPH. In fact, the results obtained in the present work show that increas-

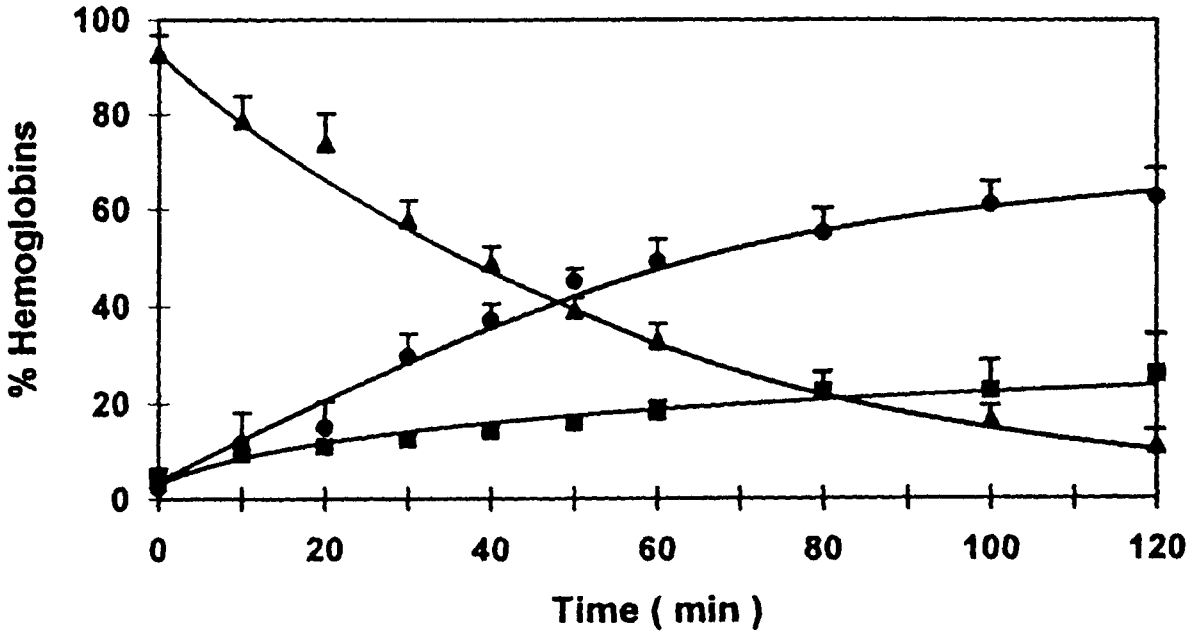

FIGURE 8 metHb (•) and hemicromes (■) production and oxyHb consumption (▲) during Hb (13.8 μM) exposure to AAPH (10 mM). Data obtained employing lysates

ing the oxygen pressure does not modify the rate of Hb oxidation by AAPH but notably reduces it when ACV is employed as free radical source. Considering that the latter compound and/or the produced radicals are negatively charged species, they could be directed towards the heme target, increasing the damaging role of the relatively short-lived carbon centred radicals. The system could then be more sensible to oxygen concentration if carbon centred radicals are more damaging than secondary peroxyl radicals[15]. However, this explanation considers a competition for carbon centred radicals between oxygen and Hb molecules and predicts that increasing Hb concentration should increase the damage associated to carbon-centred radicals. The data given in Table III would argue against this possibility, favouring an explanation based on an enhanced reactivity of non-fully oxygen saturated Hb molecules. In this sense, the origin of the oxygen effect would be similar to that proposed in the autoxidation of Hb[35]. In this system, the enhanced autoxidation observed at low oxygen pressures was explained through a possible role of the distal hystidine as a nucleophile, which could contribute to the electron transfer from Hb to the attacking oxidant. The large proportion of hemichromes, observed when Hb is oxidised by ACV, should be compatible with this mechanism.

In conclusion, our results show that the lytic process is not triggered by lipoperoxidation nor hemichrome formation, whereas membrane proteins modification seems to be relevant on lysis onset. The course of the damage promoted by AAPH and ACV show similarities and noticeable differences. Regarding haemoglobin transformation, both free radicals sources readily oxidise free Hb with high efficiency. However, there are significant differences in the product distribution and in the response of the process to changes in pH and oxygen pressure. Regarding

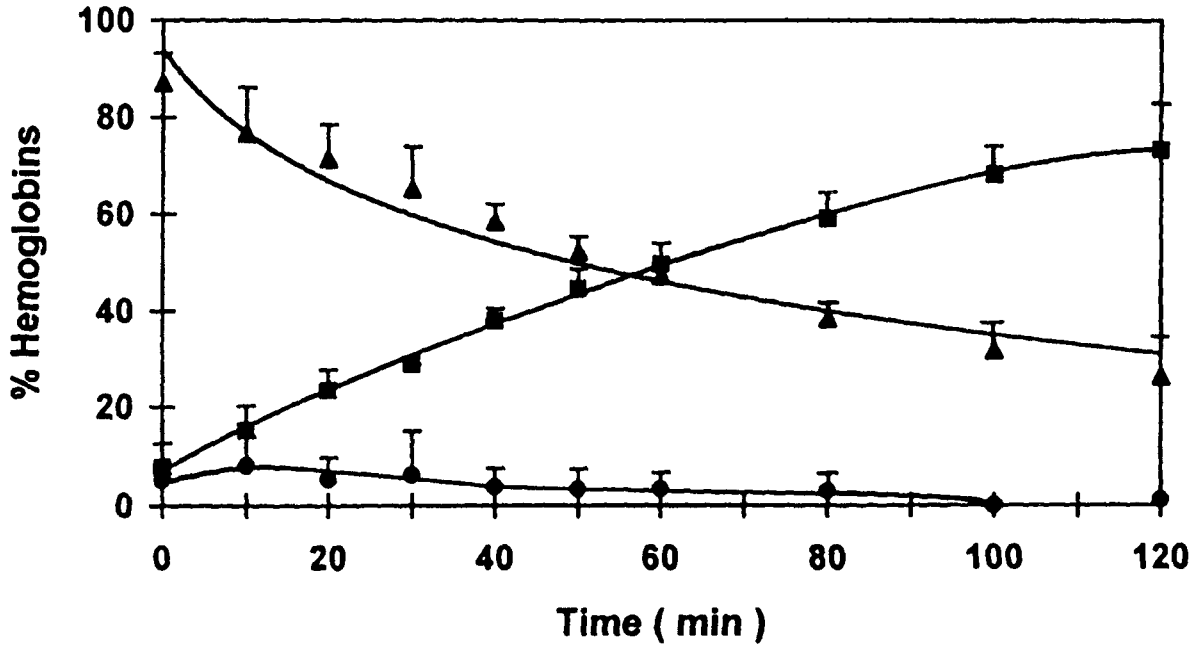

FIGURE 9 metHb (•) and hemicromes (■) production and oxyHb consumption (▲) during Hb (13.8 μM) exposure to ACV (10 mM). Data obtained employing lysates

these parameters, ACV, although hydrosoluble, behaves as a hydrophobic azo-compound, probably due to its charge and reduced hydrophilicity. These differences in oxidation mechanisms, particularly in hemichrome formation, could be of importance in *vivo* processes since Heinz bodies promote red cell recognition and removal by macrophages[4].

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