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THE ROLE OF PLASMA IN OXIDATIVE HAEMOLYSIS

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INTRODUCTION

Elucidation of the mechanisms that underlie oxidative haemolysis is a major goal of biomedical research. The reasons for pursuing such a task are multiple and include: *a*) attempts at improving tools of prevention, diagnosis and therapy of some common haemolytic diseases, such as the clinical manifestations of genetically inherited glucose 6-phosphate dehydrogenase deficiency[1]; *b*) to gain adequate insight into the interaction between oxidizing drugs and target cells, using the erythrocytes as an experimental and simplified model for further studies on cytotoxicity mechanisms and for site-directed attacks on vital cell functions, as for instance in cancer therapy[2–4]; *c*) the expectation that unraveling the mechanisms of oxidative haemolysis may improve our knowledge on the properties and on the behaviour of the erythrocyte, when challenged with various xenobiotics. This would disclose several practical applications, e.g. a properly oriented use of the erythrocyte as a vehicle for delivering drugs to the organism and also indicating ways of improving the quality of the erythrocyte for transfusion purposes by means of specific manipulations aimed at prolonging its survival.

These considerations justify the ever increasing body of investigations that are devoted to oxidative haemolysis by specialists in several biological disciplines, mostly biochemistry, genetics, toxicology, physical chemistry. The efforts of these researchers are often coupled with those of clinicians — notably haematologists and paediatricians — faced with haemolytic problems that would be probably overcome by a better knowledge on this topic. In spite of these studies, however, we are still unable to define the sequence of events taking place during the oxidative challenge exerted on a target erythrocyte — for instance predisposed by specific enzyme deficiencies — and also throughout the subsequent steps that mediate progressive fragility of the cell and its eventual destruction once the triggering signal has started.

In our opinion, the main reasons for this unsatisfactory "state of the art" are the following. First, the biochemical properties of erythrocytes, i.e. metabolic pathways,

structural components of the membrane and of the cytosol, the ionic composition, the fine regulation of haemoglobin function, have been so far dissected and analyzed in good detail, yet an integrated and reciprocally coordinated picture of these properties is still missing[5]. A second reason for the confusion in the present research on oxidative haemolysis is the heterogeneity of the numerous model systems that are being developed and investigated, especially "in vitro"[6-18]. It should be kept in mind that these experimental models, each of which is based on a structurally and metabolically different molecule or combination of molecules, may reveal alternative mechanisms of erythrocyte fragility and destruction. Accordingly, the interaction of each of the oxidizing agents with the target erythrocytes can hardly be extrapolated to other xenobiotics affording an oxidative challenge, in spite of the eventual haemolysis that may result in all these experimental systems. In this context, it should be considered that with the wide variety of oxidizing drugs that are currently investigated for their effects on target erythrocytes, at least two major pathways of damage have been identified[19]: 1) peroxidation of membrane lipid components, 2) draining of glutathione and/or of critical sulphydryl groups of membrane proteins, notably spectrin. Other mechanisms, such as disruption of specific enzyme systems essential for maintaining a proper electrolyte balance in the erythrocyte, or extensive haemoglobin degradation, or imbalances in the physiologically coordinated activity of enzymes involved in the detoxication of reactive oxygen species, or possible noxious effects actively triggered by erythrocyte components, such as methaemoglobin, that are usually maintained in low amounts and that could reach "threshold" concentrations during an oxidative stress, can cause further damage.

Owing to these reasons, the currently available picture of oxidative haemolysis is still uncertain, in that it has been drawn with heterogenous model systems, and is mostly descriptive, i.e. it rests on a wide array of biological events for which it is premature to define a precise correlation in terms of mechanisms. In fact, these events are certainly the result of the oxidative stress, but whether and how they are related to each other is still impossible to answer. For some of these oxidation-dependent processes, the link with erythrocyte fragility and lysis is only speculative and not yet proven with certainty. To provide just an example relevant to the latter consideration, it is significant that several genetically determined enzyme deficiencies still affecting metabolic functions currently thought to be crucial to the survival of the erythrocyte are, in reality, non-disease states, i.e. are well tolerated from a clinical **standpoint** and come to observation only fortuitously[20]. These deficiencies affect for instance NADPH diaphorase, glutathione reductase, glutathione peroxidase and glutathione synthetase and their benign nature seems hard to be reconciled with the central role of NADPH and of glutathione in the reductive and oxidative metabolism of the erythrocyte. Since some of these enzyme deficiencies are probably well tolerated because of redundancies in metabolism[20], such redundancy (either involving much more enzyme than is required for performing a given function, or the switching on of auxiliary pathways through the activation of metabolic by-passes) could equally be operating in the experimental models of oxidative haemolysis in which selective enzyme inactivations are commonly observed. This could for instance justify the long survival — at least "in vitro" — of erythrocytes variously depleted of their glutathione (unpublished data) or the unexplained resistance of glucose-6-phosphate dehydrogenase-deficient erythrocytes to haemolysis even when they keep being challenged with some oxidative stimuli[21].

In order to overcome these problems, it seems to be operationally essential to trace

some major requirements of experimental model systems for the study of oxidative haemolysis "in vitro". These requirements include, in our opinion in increasing order of importance: 1) An oxidative challenge, exerted either by xenobiotics or by metabolites thereof, strong enough as to overcome the biochemical defenses of the erythrocyte, mainly represented by the hexose monophosphate shunt activity, glutathione peroxidase, catalase and superoxide dismutase activities. 2) A close similarity with the situation found "in vivo", i.e. a constant "in vitro" destruction of erythrocytes by those external agents that produce haemolysis in a susceptible organism[1]. 3) The choice of suitable parameters to be investigated, i.e. of cellular and molecular phenomena that prove to be related to eventual haemolysis according to a cause-effect correlation: this requirement stems from the consideration that haemolysis *per se* is a multi-composite process and can therefore be the consequence of events unrelated to the specific oxidizing stress or of artefacts such as bacterial growth in the system, or toxic processes, or changes in the osmotic properties of the medium, or modifications of pH, on other processes. 4) The reconstruction of a clean "in vitro" system in which the multi-step chain of events leading to damage and to haemolysis may be reproduced and rationalized in terms of specific molecules rather than of indefinite "factors".

Genetic deficiency of glucose-6-phosphate dehydrogenase, the commonest enzyme abnormality in humans[22], meets requirement no. 1 since it predisposes the affected erythrocyte to metabolic failure toward several types of oxidizing stress. More specifically, its Mediterranean variety seems to be a suitable genetic model for studying oxidative haemolysis, since, *a*) it is characterized by a particularly severe deficiency of activity (approximately 0.1% of the erythrocyte glucose-6-phosphate dehydrogenase activity found in normal subjects[23], *b*) it is associated, although in a characteristically non-constant and unpredictable way, with acute haemolytic attacks that are triggered by oxidizing stimuli like fava beans (favism) and/or a variety of drugs[24], *c*) it is easily available being a polymorphic mutation. However, glucose-6-phosphate dehydrogenase Mediterranean erythrocytes do elude requirement no. 2 since they are not usually haemolyzed "in vitro" by xenobiotics that prove to be haemolytic "in vivo". Such unexpected inadequacy may be inherent to reasons the elucidation of which could determine an essential advancement in our knowledge of oxidative haemolysis. Possible reasons of inadequacy are, *a*) a limited account of the role played by the repeated oxygenation-deoxygenation cycles (Luzzatto, L., personal communication); *b*) the lack of deformation and of the mechanical and osmotic strains that are conversely experienced by the circulating erythrocytes during their transit across capillaries[25,26]; *c*) use of unmodified rather than of metabolically activated drugs or oxidizing agents[27-29]; *d*) use of buffers, most of which may be inadequate especially in respect to isotonicity, conductivity, surface tension and metabolic competence (for instance containing varying and unphysiological amounts of orthophosphate and of other electrolytes), instead of a more natural environment of erythrocytes, i.e. plasma or plasma derivatives.

With reference to the last point, we report here experimental evidence that plasma cannot be considered as an "innocent by-stander" in the process of haemolysis triggered by oxidizing agents. On the contrary, based on the "in vitro" findings obtained with two unrelated experimental systems of oxidative haemolysis, one of which is likely to represent a good model of favism, we suggest the involvement of as yet unidentified, low molecular size plasma components in the final pathway leading to destruction of oxidatively damaged erythrocytes. Both systems have been designed

to work in the test tube, without specific constraints of mechanic and/or osmotic type mimicking of the circulatory condition. Accordingly, we think that the indication we have obtained may be extrapolated to an intravascular type of oxidative haemolysis, although the occurrence of extravascular mechanisms cannot be certainly ruled out in acute haemolytic diseases.

Although the two systems under study have already been in part investigated from the standpoint of the cellular damage they elicit, especially in the glucose-6-phosphate dehydrogenase-deficient erythrocytes[19,30-38], they will be considered here mainly with respect to their dependence on plasma components. On the basis of this specific feature, namely the requirement of so far unidentified plasma components, both systems, i.e. t-butylhydroperoxide and divicine in combination with ascorbate, do differ from a variety of other oxidant or peroxidant drugs including primaquine, α-naphthol, diamide, naphthoquinone and a mixture of hydrogen peroxide and sodium azide[20]. Major interest for the divicine and ascorbate combination stems from the fact that it appears to be a satisfactory model of favism[19,33,39], the haemolytic manifestations of which, as already mentioned, are quite bizarre and clearly related to so far undefined "factors of risk". A better elucidation of the way and of the mechanisms by which plasma interferes with the glucose-6-phosphate dehydrogenase-deficient erythrocytes challenged with divicine and ascorbate, is expected to identify such factors of haemolytic risk, with obvious advancements in the prevention of favism.

MATERIALS AND METHODS

A) *Blood Samples and Enzyme Assays*

Blood samples were drawn from normal and glucose-6-phosphate dehydrogenase-deficient subjects. All glucose-6-phosphate dehydrogenase-deficient subjects were hemizygous males of Sardinian ancestry and had abnormally low levels of erythrocyte glucose-6-phosphate dehydrogenase activity, ranging from 0.5 mIU/g Hb to 9 mIU/g Hb[23,40]. These can therefore be classified as having the Mediterranean variety of glucose-6-phosphate dehydrogenase deficiency[41].

All blood samples were drawn using heparin as anticoagulant or, alternatively, by defibrinating with glass beads, and processed immediately or within 24 hrs. In the latter cases samples were taken at 2°C and usually shipped from Sassari (Sardinia) overnight. In all cases the buffy coat was carefully discarded after each of three washings with isotonic solution and, in the defibrinated samples, complete removal of leukocytes was obtained as reported by Beutler *et al.*[42]

Assays of glucose-6-phosphate dehydrogenase activity were carried out at 25°C using the buffer recommended by the World Health Organisation, as described elsewhere[23,40,43]. Ca^{2+} – ATPase and Na^+/K^+ – ATPase activities were assayed in frozen-thawed haemolysates following the same conditions as described by Hanahan and Ekholm[44], with the exception that the incubations were continued for 30 min and at 37°C rather than at 44°C. Intracellular hexose monophosphate shunt and glycolytic activities were estimated as reported previously[45].

B) *Oxidative Haemolysis Induced by t-Butylhydroperoxide*

Washed erythrocytes were incubated at a final 10% haematocrit with 1 mM t-butyl-

hydroperoxide (previously titrated with $Na_2S_2O_3$) in phosphate buffered saline (20 mM Na phosphate, pH 7.4, containing 146 mM NaCl and 5 mM glucose). After 90 min of gentle stirring at 25°C, aliquots were removed and analysed for malonyl-dialdehyde, hydroxyl radical formation, methaemoglobin and cellular potassium. Erythrocytes were washed three times with phosphate buffered satine (PBS) and divided in two parts. A first aliquot was re-incubated at a 10% haematocrit in one of the following reference buffers (which were adjusted to the same values of osmolarity and pH as those found in the corresponding plasma): PBS, iso-osmotic TES (0.04 M N-tris (hydroxymethyl)methyl-2-aminoethane sulfonic acid, pH 7.4, containing sodium chloride up to the proper osmolarity), "plasma-like buffer" (having the same ionic composition as measured in a standard plasma). The second aliquot of t-butylhydroperoxide-treated erythrocytes was re-incubated in parallel in native plasma or in fractions obtained from plasma as reported in a following section. After 30 min at 37°C in glass vials, the extent of haemolysis taking place in the suspensions was estimated by measuring the absorbance of the supernatant at 540 nm. One hundred percent haemolysis was obtained by addition of 1.8 ml of deionized water to 0.2 ml of the erythrocyte suspension, followed by centrifugation and estimation of the absorbance of the supernatants at 540 nm. Experiments with carbon monoxide-saturated erythrocytes were carried out in tightly capped glass vials under a CO/O_2 (20/80, vol/vol) atmosphere both in the first step with t-butylhydroperoxide and in the re-incubation step.

C) *Oxidative Haemolysis Induced by Divicine and Ascorbate*

Washed erythrocytes were incubated under sterile conditions and in the presence of gentamycin (50 μg/ml). Unless stated otherwise, erythrocytes were incubated at a final 10% haematocrit in iso-osmotics TES, pH 7.4, containing divicine and ascorbate at the concentration indicated, i.e. ranging from 1.5 to 5.0 mM divicine and from 0.15 to 0.5 mM ascorbate, and 5 mM glucose. Divicine was freshly prepared before each experiment by hydrolyzing commercial vicine (Serva, Feinbiochemica GMBH & Co., Heidelberg, F.R.G.) with 1 N HCl for 20 min at 100°C, followed by immediate cooling at 2°C and adjustment of pH to 7.4. After incubating for 210 min at 37°C with continuous stirring in glass flasks, the erythrocytes were washed three times with iso-osmotic TES and then divided in two parts which were re-incubated at 37°C at a final 10% haematocrit under gentle stirring in plastic containers. A first aliquot was incubated in the same reference buffer as used in the primary incubation step (usually iso-osmotic TES, containing glucose and gentamycin). The second aliquot of pre-treated erythrocytes was incubated in parallel in native plasma or in fractions therefrom (see below). At various time intervals during re-incubation, aliquots of erythrocytes were taken and haemolysis was estimated as with the t-butylhydroperoxide.

D) *Fractionation of Plasma Increased-haemolyzing Activity*

The procedures followed were slightly different for fractionation of the extra-haemolyzing activity as measured with the t-butylhydroperoxide system and with the divicine + ascorbate combination, respectively.

t-butylhydroperoxide system Pooled plasma from four different subjects (300 ml) was heated for 10 min at 100°C, cooled at 0°C and the supernatant recovered by centrifugation at 12,000 g. The clear supernatant was lipid-depleted[46] with a methanol/chloroform/H_2O mixture (1/2/6, v/v) and the aqueous phase recovered and lyophilized. The lyophilized extract, dissolved in 20 ml water, was brought to an osmolarity of 310 mOsm/l H_2O. The heat-treated and delipidated plasma derivative was then submitted to filtration on a Diaflo YM 5 membrane (Amicon Ltd., Stonehouse, England) and the filtrate was finally passed on a Diaflo YM 2 membrane and recovered.

The Diaflo YM 2 membrane filtrate was submitted to proteolysis with trypsin and/or with pronase. Two mg of TPCK-trypsin (Worthington Biochemical Co., New Jersey, U.S.A.) were added to 1 ml of the filtrate. After incubation for 24 hrs at 37°C, the mixture was heated for 10 min at 100°C or alternatively treated with 1.3 mg of soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, Mo., U.S.A.), then cleared by centrifugation. Incubation of the Diaflo YM 2 membrane filtrate (1 ml) with 0.1 mg of pronase E (Sigma Chemical Co., St. Louis, Mo., U.S.A.) was carried out for 24 hrs at 37°C, then the mixture was heated for 10 min at 100°C and cleared by centrifugation.

Before assaying the increased-haemolyzing activity present in the plasma fractions obtained as described above, these fractions were constantly taken to the same values of osmolarity and pH as measured in the starting plasma.

Divicine + Ascorbate The procedure was simpler than with the t-butylhydroperoxide system and involved heat treatment of pooled plasma, followed by filtration on a Diaflo YM 5 membrane, as described above.

E) *Analytical Procedures*

Elution of the erythrocyte-bound plasma proteins After re-incubation of t-butylhydroperoxide-sensitized erythrocytes in plasma, complete haemolysis was achieved by addition of 9 ml of cold deionized water to 1 ml of packed erythrocytes and erythrocyte ghosts. The pellet obtained by centrifugation at 12,000 g was suspended and then incubated for 30 min at 25°C in 6 M urea (final concentration). After centrifugation at 12,000 g the supernatant was collected and the precipitate was carefully suspended in 2.5 ml of glycine-HCl, pH 2.3, and left for 30 min at 25°C. The supernatant was then combined with the urea supernatant and the resulting mixture, containing the eluted proteins, was dialyzed with two changes against 100 volumes of PBS at 4°C. The same elution procedure was followed for comparison with both native and t-butylhydroperoxide-sensitized erythrocytes re-incubated in PBS.

Radioiodine labelling Radioiodination of proteins eluted from erythrocyte ghosts was carried out by using sodium iodide, according to the chloramine-T procedure[47].

Immunoelectrophoresis This was carried out by conventional procedures.

Consumption of complement Activation of serum complement was triggered by means of antibody-coated sheep erythrocyte ghosts, which were prepared as follows: ten ml of 3% (v/v) suspension of washed sheep erythrocytes were incubated 15 min at 37°C with 1 ml of a rabbit anti-sheep erythrocyte antiserum (a gift from Dr. G. Corte)

in barbitone saline buffer[48]. After 3 washings, erythrocytes were haemolyzed with 9 vols of H_2O and the resulting ghosts washed 3 times. Activation of serum complement was achieved by incubating 10 ml of fresh human serum with 3 ml of the antibody-sensitized sheep erythrocyte ghosts for 15 min at 37°C, followed by centrifugation for 30 min at 12,000 g. Although the supernatant proved to be completely depleted of complement haemolytic activity, it was also incubated 30 min at 56°C.

Haemolytic assays of complement These were performed using antibody-coated sheep erythrocytes as target cells[48].

Production of hydroxyl ($OH^{\cdot}$) radicals Mannitol-inhibitable production of ethylene from methional, as an indicator of $OH^{\cdot}$ radicals formation, was measured by means of gas liquid chromatography[49], as reported previously[30].

Lipid peroxidation Formation of malonyldialdehyde (MDA), as a measure of lipid peroxidation, was estimated as described by Stock and Dormandy[50].

Intraerythrocytic potassium This was estimated in supernatants from a mixture of 0.5 ml of washed erythrocytes with 1.0 ml of 10% TCA, by atomic absorption spectrophotometry with an air/acetylene flame[51].

RESULTS

A) *t-Butylhydroperoxide*

1) **Cellular mechanisms of erythrocyte sensitization and damage** Figure 1 shows the time course of haemolysis of t-butylhydroperoxide-pretreated normal erythrocytes upon their re-incubation in an iso-osmotic buffer (PBS) and in native and heat-treated plasma, respectively. While haemolysis progresses slowly in the reference buffer (and in other iso-osmotic buffers as well — data not shown), it is consistently more rapid and extensive either in native or deproteinized plasma. Exposure of erythrocytes to carbon monoxide prior to and throughout treatment with t-butylhydroperoxide abolishes haemolysis almost completely (Table I). This indicates a critical requirement for the unliganded iron of haemoglobin in the process of haemolysis and confirms the specific role of methaemoglobin[30]. Once formed as a result of the oxidative stress applied on the erythrocyte, methaemoglobin is likely to trigger the production of $OH^{\cdot}$ radicals through a homolytic rupture of the oxygen-oxygen bond in t-butylhydroperoxide, thereby starting the sequence of events that terminate in erythrocyte destruction[30]. This conclusion is suggested also by the fact that the CO-treated erythrocytes fail to undergo the following other events that are conversely observed upon treatment of the native erythrocytes with t-butylhydroperoxide and that are apparently related to haemolysis[30]: a) formation of methaemoglobin, b) production of $OH^{\cdot}$ radicals or of oxygen derivatives of comparable reactivity, c) lipid peroxidation, d) a dramatic loss of cellular potassium (Table I).

Exposure of erythrocytes to t-butylhydroperoxide under conditions identical to those reported in Figure 1 results in the impairment of two enzyme systems of the membrane that are devoted to maintaining a proper electrolyte balance between the intracellular and the extracellular environment. As shown in Table II, there is in fact a substantial inactivation of both Ca^{2+} – ATPase and of Na^+/K^+ – ATPase (ouabain-

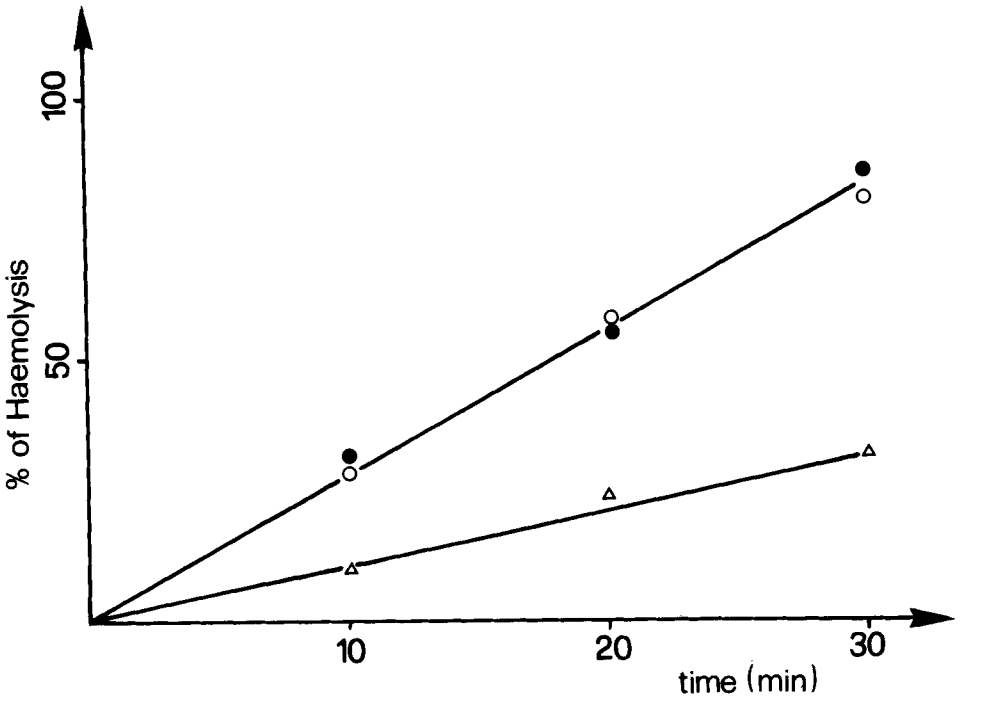

FIGURE 1 Haemolysis of t-butylhydroperoxide-sensitized erythrocytes during re-incubation in different media. Following incubation with 1 mM t-butylhydroperoxide as reported under "Methods", erythrocytes were washed and re-incubated at 37°C at a final 10% haematocrit in PBS (△—△), in native (O—O) and in heated plasma (●—●), respectively.

inhibited) activities. It may be significant and perhaps relevant to the mechanism of haemolysis that inactivation of both membrane systems progresses even following removal of t-butylhydroperoxide and during re-incubation of the pre-treated erythrocytes in iso-osmotic buffer or in plasma. However, the electrolyte imbalances resulting from such inactivation are not the only mechanism responsible for eventual haemolysis. This is clearly ruled out by the identical extents of inactivation observed in the three different media that still elicit a comparably distinct degree of haemolysis (see Fig. 1).

2) **Partial characterization of the plasma components involved in the haemolysis by t-butylhydroperoxide** A major feature of the t-butylhydroperoxide-induced effects on both normal and glucose-6-phosphate dehydrogenase-deficient erythrocytes is the greater extent of haemolysis that occurs in plasma over a variety of iso-osmotic buffers (Fig. 1). Such feature ("increased-haemolysis") was constantly observed in blood samples from all subjects examined in this respect. Cross incubations with homologous plasma showed increased-haemolysis without exception, suggesting the presence of ubiquitous plasma components that in some way interact with the

TABLE I
Influence of O_2 and CO on both pre-haemolytic events and haemolysis of t-butylhydroperoxide sensitized erythrocytes

Incubation in	MetHb* %	MDA* nmoles/g Hb	Ethylene produced* nmoles/g Hb	Potassium* (nmoles/liter RBC)	Haemolysis** %
Air	85	143.5	273	3	63
Carbon Monoxide	—	17.1	45	104	2

* Estimated after incubation of washed erythrocytes with 1 mM t-butylhydroperoxide for 90 min at 25°C as described previously[30].
** Estimated after re-incubation of t-butylhydroperoxide-pretreated erythrocytes in autologous plasma for 30 min at 37°C.

damaged erythrocytes and determine their destruction. Replacement of plasma with serum did not modify the extent of increased-haemolysis at all. Conversely, the type of anticoagulant used was found to influence increased-haemolysis significantly: thus, this effect was largely abolished by use of EDTA or acidified citrate dextrose as anticoagulants. However, in spite of these findings and of the impairment of Ca^{2+} – ATPase activity by treatment of the erythrocytes with t-butylhydroperoxide, no obvious evidence emerged for a role for calcium or for any of a number of divalent cations tested. In fact, addition of salts of calcium, iron, zinc, manganese, alone or in various combinations, to the reference iso-osmotic buffer (0.04 M TES containing sodium chloride up to an osmolarity of 310 mOsm/l H_2O) did not enhance the extent of haemolysis at all (data not shown).

The increased-haemolytic effect afforded by plasma or serum on the t-butylhydroperoxide-treated erythrocytes prompted us to attempt fractionation of the responsible factor(s). A first set of experiments showed that erythrocyte destruction taking place in plasma is paralleled by extensive coating of erythrocytes by several proteins, notably immunoglobulins and components of the complement system. However, such coating proved to be a side phenomenon following the haemolysis by itself and not primarily involved in its mechanism. This conclusion emerged from the following lines of evidence: a) re-adsorption of the eluted plasma proteins to t-butylhydro-

TABLE II
Inactivation of Ca^{2+} – ATPase and of Na^+/K^+ – ATPase activities in normal erythrocytes incubated with t-butylhydroperoxide

Incubation medium	Time* (min)	Ca^{2+} – ATPase (μmoles Pi/g Hb/hr)	Na^+/K^+ – ATPase (μmoles Pi/g Hb/hr)
	zero	40	6.2
t-Butylhydroperoxide in PBS	90	28	4.1
PBS	30	11	1.9
Native plasma	30	12	1.1
Heated plasma	30	13	1.5

* The experimental conditions were as described under the "Methods" section and in the legend to Fig. 1. The times indicated refer to the first incubation with 1 mM t-butylhydroperoxide (90 min) and, following washing of erythrocytes in PBS to remove t-butylhydroperoxide, to re-incubation for 30 min at 37°C in PBS, in native and in heated plasma, respectively.

TABLE III
Properties of the plasma increased-haemolyzing activity measured on t-butylhydroperoxide-sensitized erythrocytes

Fractionation step	Increased-haemolytic activity*
Native plasma	67
Heated plasma	72
Heated, lipid-depleted plasma**	70
Diaflo YM 5 filtrate**	76
Diaflo YM 2 filtrate**	67
Trypsin-treated**	68
Pronase-treated**	66
Trypsin + pronase-treated**	67

* Estimated by subtracting the percent of haemolysis measured upon incubation of the t-butylhydroperoxide-sensitized erythrocytes in PBS for 30 min at 37°C from the percent of haemolysis occurring in paired incubations of the same erythrocyte suspensions in the various plasma derivatives. The values reported are the mean of the three independent experiments.
** Obtained as described under the "Methods" section.

peroxide-treated erythrocytes (estimated after their radioiodination as described under "Methods") was not accompanied by any increased-haemolysis of these cells as compared with those incubated in iso-osmotic buffer; b) attemps at identifying specific receptor sites for the binding of plasma or serum proteins on the surface of the t-butylhydroperoxide-treated erythrocytes were unsuccessful; c) the preliminary consumption of serum complement (see "Methods") did not abolish or even decrease the extent of increased-haemolysis; d) procedures known to destroy proteins (see below) did not affect the extra-haemolytic activity of serum or plasma.

Once established that the increased-haemolytic activity of plasma is unrelated to protein components, more drastic fractionation procedures were followed. It became soon apparent that such activity is totally thermostable, not associated with the lipid components of plasma, and completely resistant to digestion by proteolytic enzymes. Table III shows these and other properties of the plasma factor(s) producing increased-haemolysis of the t-butylhydroperoxide-treated erythrocytes. It is clear that the responsible factor(s) may have a molecular weight below 1,000, although a more precise analysis of the molecular size was consistently hampered by loss of activity during gel chromatography. Also, all attempts at fractionating the increased-haemolytic activity by means of ion chromatography (both anion and cation exchange) were unsuccessful.

In spite of the failure to isolate and characterise the plasma molecule(s) responsible for the increased-haemolytic activity, the possibility that this effect may be due to artefacts can be ruled out because: a) the untreated erythrocytes do survive with a normal metabolic competence as concerns glucose consumption, glycolytic and hexose monophosphate shunt activities and content of intracellular metabolites, when they are incubated in the same media that produce conversely extra-haemolysis of the t-butylhydroperoxide-damaged erythrocytes. Therefore it is clear that the various fractions obtained from native plasma through the procedures listed in Table III are used in the assays for haemolysis under conditions that are not only iso-osmotic but also isotonic with respect to native erythrocytes[52]. b) The increased-haemolytic activity can be recovered from fractionation procedures involving concentration and

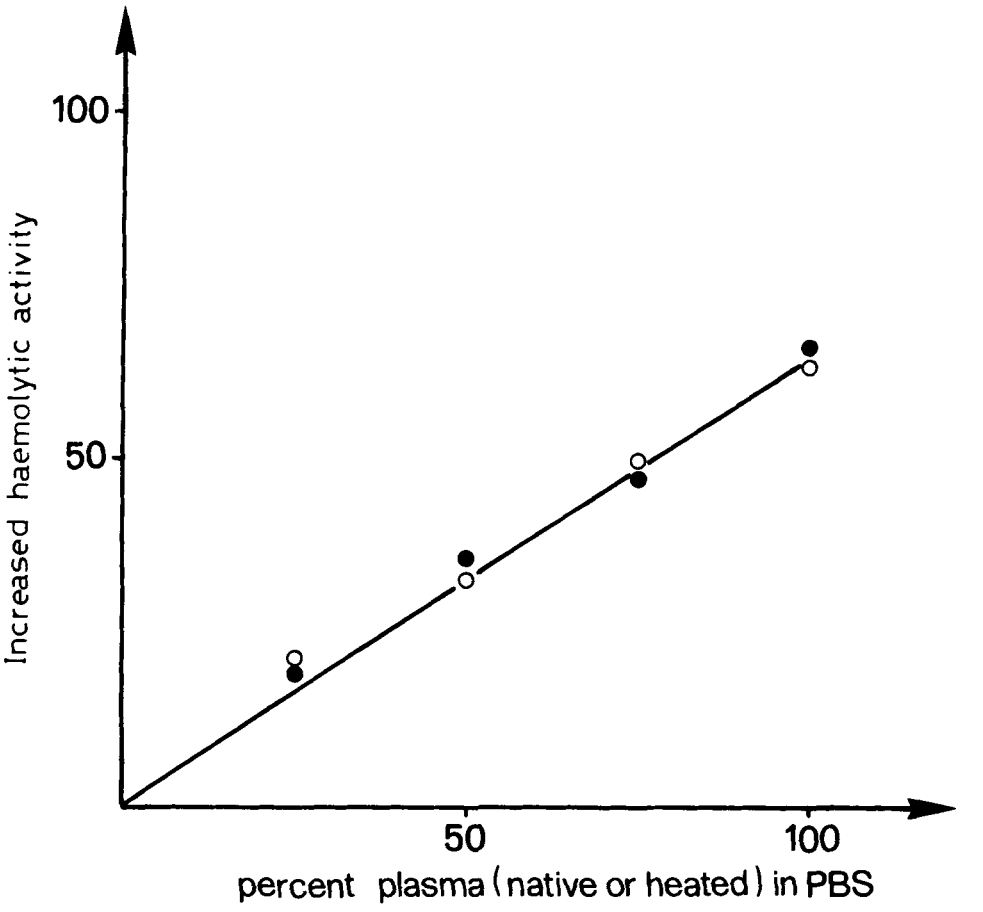

FIGURE 2 Concentration-dependence of the increased-haemolytic activity present in native and heated plasma and measured on t-butylhydroperoxide-sensitized erythrocytes. For experimental conditions see "Methods" and the legends to Fig. 1 and Table III. After exposure to 1 mM t-butylhydroperoxide erythrocytes were washed and re-incubated in either native (O—O) or heated plasma (●—●), variously diluted with PBS as indicated on the abscissa. The osmolarity and the pH were checked before and after re-incubation.

lyophilization steps. When the same procedures were followed starting from inactive fractions to provide adequate controls (e.g. from iso-osmotic buffers), no increased-haemolytic activity was discernible. This excludes artefacts arising from the types of fractionation that were used. c) With all the fractions obtained from plasma the assays of increased-haemolytic activity showed invariably a remarkable concentration-dependence (Fig. 2).

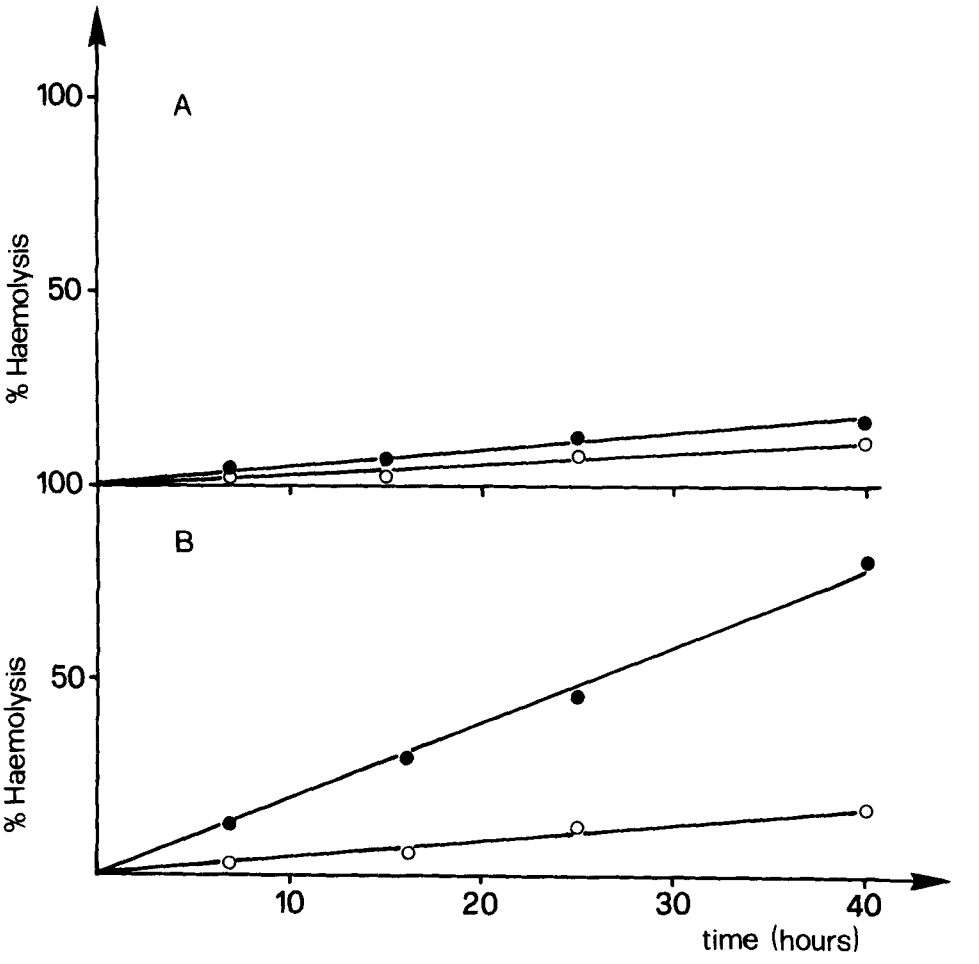

FIGURE 3 Haemolysis of normal and glucose-6-phosphate dehydrogenase-deficient erythrocytes pre-incubated with divicine and ascorbate during re-incubation in different media. Following incubations with 1.5 mM divicine and 1.5 mM ascorbate as reported under "Methods", normal (A) and glucose-6-phosphate dehydrogenase-deficient erythrocytes (B), were washed and re-incubated at 37°C at a final 10% haematocrit in iso-osmotic TES, pH 7.4 (O—O) or in heated plasma (●—●), respectively, for the times indicated.

B) *Divicine – Ascorbate*

1) **Cellular mechanisms of erythrocyte damage** At variance with the t-butylhydroperoxide system which fails to discriminate normal from glucose-6-phosphate dehydrogenase-deficient erythrocytes because of the strength of the oxidizing stress, divicine in combination with ascorbate proved to be selective enough in affording extensive damage to glucose-6-phosphate dehydrogenase-deficient and not to normal erythrocytes having the glucose-6-phosphate dehydrogenase B type. Such selectivity

was obtained at concentrations of divicine lower than reported previously[57,40,53] and by use of acidic rather than of β-glucosidase-catalyzed hydrolysis for preparing divicine.

Moreover, the considerably longer time intervals of incubation required with this system as compared with t-butylhydroperoxide (both of the primary incubation with divicine and ascorbate and, following removal of the oxidizing compounds, of the re-incubation in plasma or in reference buffers) suggested the introduction of modifications which proved to be critical for obtaining the survival of the normal erythrocytes while allowing for fragility and lysis of the glucose-6-phosphate dehydrogenase-deficient cells to take place in the same environment. The most critical condition in this respect was the use of plastic tubes throughout the re-incubation step (see "Methods"), while the glass vials used in previous experiments provided little discrimination between normal and glucose-6-phosphate dehydrogenase-deficient erythrocytes concerning their survival, which was comparably lower.

Fig. 3 shows the differential fragility of normal and glucose-6-phosphate dehydrogenase-deficient erythrocytes toward an oxidizing stress provided by 1.5 mM divicine and 1.5 mM ascorbate. In a number of similar experiments performed on paired samples of normal and glucose-6-phosphate dehydrogenase-deficient erythrocytes the concentrations of divicine that proved to be discriminating between the two cell populations ranged between 1 mM and 2 mM, while ascorbate could be lowered to 0.1–0.5 mM concentrations without significant effects on the extent of haemolysis. In the experiment illustrated in Fig. 3, re-incubation of the erythrocytes (either glucose-6-phosphate dehydrogenase-deficient or normal) following their exposure to divicine plus ascorbate was carried out in heat-treated plasma that affords extra-haemolysis in all glucose-6-phosphate dehydrogenase-deficient erythrocyte samples. In fact, the increased-haemolysis that is observed in native plasma shows conversely wide individual variations in different subjects (see below).

Like with the t-butylhydroperoxide model of oxidative haemolysis, the role of the haemoglobin iron in the process of erythrocyte fragility and lysis was investigated

TABLE IV

Effects of pre-incubation of glucose-6-phosphate dehydrogenase-deficient erythrocytes with CO and nitrite on the extent of haemolysis in the divicine–ascorbate system.

Pre-incubation	Re-incubation medium	Haemolysis % at 18 hrs
—	Iso-osmotic TES	5.7
—	Native plasma	29.4
—	Heated plasma	100.0
CO*	Iso-osmotic TES	4.0
CO	Native plasma	6.6
CO	Heated plasma	12.0
Nitrite**	Iso-osmotic TES	9.5
Nitrite	Native plasma	92.0
Nitrite	Heated plasma	100.0

* Erythrocyte suspensions were pre-treated with CO for 45 min, then submitted to incubation for 3.5 hrs with 5 mM divicine and 0.5 mM ascorbate (see "Methods") under a CO/O_2 (20/80, vol/vol) atmosphere and, after washing, to re-incubation for 18 hrs under the same CO/O_2 atmosphere in the media indicated.

** Erythrocytes were pre-treated at 25°C with 0.15 M $NaNO_2$ in iso-osmotic TES, pH 7.4, for 30 min. After six repeated washes in iso-osmotic TES, pH 7.4, the erythrocytes, containing 100% MetHb, were incubated under air with divicine and ascorbate as the control erythrocytes containing HbO_2 and then re-incubated in the media indicated (see "Methods").

upon prior treatment of cell suspensions with CO or nitrite, respectively. In these experiments, carried out on erythrocytes from glucose-6-phosphate dehydrogenase-deficient subjects, the concentration of divicine was deliberately raised up to 5 mM (with 0.5 mM ascorbate), in order to maximize the extent of haemolysis. It is clear from inspection of Table IV that CO-treated erythrocytes are almost completely protected from the haemolysis induced by treatment with divicine and ascorbate and triggered by re-incubation in either native or heat-treated plasma. As already mentioned, in the control sample incubated under air, the increased-haemolysis in deproteinized plasma is complete, while that observed in untreated plasma is significant but far from being total in the subject investigated in this experiment. This limited extent of increased-haemolysis in native plasma allows one to appreciate the enhancing effect that is determined when the target erythrocytes submitted to the oxidizing stress with divicine and ascorbate initially contained a high amount of methaemoglobin. Accordingly, methaemoglobin seems to predispose the erythrocytes to a greater fragility in the experimental conditions used with the divicine–ascorbate system, although a precise knowledge of how this is happening is still lacking.

In view of the complexity of the oxidant damage induced by divicine and ascorbate on the target erythrocytes, we have listed in Table V all known effects elicited by this system on glucose-6-phosphate dehydrogenase-deficient cells and, at higher concentrations of both compounds, also on normal erythrocytes. An obviously distinctive response between these two types of erythrocytes is the limited acceleration of the hexose monophosphate shunt activity of the glucose-6-phosphate dehydrogenase-deficient cells, contrasting with the remarkable enhancement of this pathway in the normal erythrocytes[19,35,37]. A somewhat unexpected feature of the divicine-ascorbate system, in view of its auto-oxidation mechanism[36], is the constant failure to detect any appreciable peroxidation of membrane lipids. at least by means of the thiobarbituric acid reaction[50].

While some of the effects reported in Table V can be safely recognized as early events (e.g., oxidative draining of glutathione and of reduced pyridine nucleotides), other effects require longer incubations in the presence of the oxidizing agents. For most of these late events it is still impossible to establish their precise correlation in terms of mechanisms. For instance, the severe impairment of the erythrocyte calcium pump, that has been observed as a constant feature of favism[54,55], could be the result either of a primary damaging effect of divicine on the membrane Ca^{2+}ATPase or of the extensive draining of cellular reducing equivalents that divicine induces rapidly by way of a redox cycling mechanism[38].

2) **Role of the plasma component(s) responsible for the increased-haemolysis with divicine + ascorbate** We have already mentioned the much greater difficulties encountered when shifting from the t-butylhydroperoxide system to the divicine–ascorbate combination. Basically, these difficulties were introduced by the prolonged times of incubation that increase the possibility of artefacts in an exponential way. For instance, in all experiments, more attention had to be paid to the several variables (e.g., pH, ionic strength, conductivity, osmolarity, presence of crucial metabolites like glucose, sterility, stirring of the suspensions, adequate haematocrits, maintenance of the normal shape and metabolic competence of erythrocytes, etc.) that would affect the parameter under study, i.e. viability or lysis of the erythrocytes, inde-

TABLE V
Intracellular damaging effects induced by divicine and ascorbate on glucose-6-phosphate dehydrogenase-deficient erythrocytes.

Effect	Ref. no.
Depletion of GSH, NADPH and NADH	19, 33, 36, 37
Formation of Methaemoglobin	19, 37
Production of $OH^{\cdot}$ radicals	56, 37
Inactivation of G6PD	40
Inactivation of glutathione peroxidase	39, 57
Inactivation of Ca^{2+} – ATPase	54, 55
Inactivation of Na^{+}/K^{+} – ATPase	58
Inactivation of Ca^{2+} – transglutaminase	58
Accumulation of intracellular calcium	54, 55
Loss of cellular potassium	54
Enhanced degradation of membrane proteins	55
Formation of membrane protein aggregates	19

pendently of the specific oxidative stimulation provided by the divicine–ascorbate combination.

Due to these reasons, we believe that attempts at achieving the chemical characterization and the identification of the plasma component(s) responsible for increased-haemolysis with this system is an unattainable task until some cellular phenomenon, closely related to haemolysis, is recognized as an earlier and adequate target to be experimentally followed instead of measuring the extent of haemolysis itself. Unfortunately, identification of a cellular event having these features is not yet available, this justifying the attempts at elucidating in more detail the sequence of steps that divicine in combination with ascorbate does produce in the target erythrocytes.

In spite of the problems and uncertainties inherent to the assay of the plasma component(s), however, some properties have been consistently found which allow us to rule artefacts under the experimental conditions used in this study. As shown in Table VI, the factor(s) producing increased-haemolysis in the divicine–ascorbate system is

TABLE VI
Properties of the plasma increased-haemolyzing activity measured on glucose-6-phosphate dihydrogenase-deficient erythrocytes pre-treated with divicine and ascorbate.

Fraction no.	Fractionation step	Increased-haemolytic activity*
1	Native plasma	71.0
2	Dialyzed and lyophilized plasma**	2.7
3	Heated plasma	88.0
4	Heated + dialyzed plasma (2 + 3)***	76.4
5	Diaflo YM 5 filtrate****	84.0

* Estimated by subtracting the percent of haemolysis measured in iso-osmotic TES, pH 7.4, from the percent of haemolysis observed in paired incubation of the same glucose-6-phosphate dehydrogenase-deficient erythrocyte suspensions (pre-treated with 2 mM divicine and 0.2 mM ascorbate). The re-incubation time was 18 hrs at 37°C. The values indicated represent the mean of three separate experiments.
** Native plasma was preliminarily dialyzed against 1,000 volumes of deionized H_2O, then lyophilized and taken to the starting values of volume, pH and osmolarity.
*** A 5 ml aliquot of heated plasma (fraction no. 3) was supplemented with an amount of dialyzed and lyophilized plasma (400 mg dry weight, corresponding to 5 ml of native plasma).
**** Heated plasma was directly submitted to filtration on a Diaflo YM 5 membrane.

TABLE VII
Consumption of the plasma increased-haemolytic activity measured on divicine-pre-treated erythrocytes

Heated plasma	Increased-haemolytic activity*
Before haemolysis**	67.5
After oxidative haemolysis***	3.8

* Estimated on glucose-6-phosphate dehydrogenase-deficient erythrocytes pre-incubated with 5 mM divicine and 0.5 mM ascorbate and re-incubated in the media indicated for 12 hrs at 37°C. All values are the mean of four parallel experiments.
** Heated plasma was submitted to filtration on a Diaflo YM 5 membrane.
*** Heated plasma, after filtration on a Diaflo YM 5 membrane, was allowed to react on glucose-6-phosphate-deficient erythrocytes pre-treated with 5 mM divicine and 0.5 mM ascorbate at a final 50% haematocrit. A control mixture of the same erythrocytes in PBS was incubated in parallel, showing no appreciable haemolysis. After 6 hrs of incubation at 37°C, when haemolysis produced by heated plasma had gone to completion, the mixture was centrifuged at 30,000 g and the supernatant filtered on a Diaflo YM 5 membrane. The filtrate was then brought to the same osmolarity and pH as the heated plasma and its increased-haemolytic activity measured as described above.

(are) thermostable and its (their) molecular weight is below 5,000 dalton, although a more detailed characterization was not feasible. The increased-haemolytic activity was invariably found to be concentration-dependent.

An important property apparently excluding artefacts is the complete abolition of the increased-haemolytic activity once the target erythrocytes had been destroyed in the haemolytic system. As shown in Table VII, a sample of heat-treated plasma, filtered on a Diaflo YM 5 membrane, was allowed to produce complete lysis of erythrocytes preliminarily incubated with 5 mM divicine and 0.5 mM ascorbate (these high concentrations were deliberately selected in order to achieve complete haemolysis in a reasonably short time interval). The resulting haemolysate was centrifuged and then filtered on a Diaflo YM 5 membrane in order to remove high molecular weight components and, after correcting the osmolarity and the pH to the normal values, its activity was tested in the same haemolytic system, i.e. using the erythrocytes pre-treated with 5 mM divicine and 0.5 mM ascorbate. No increased-haemolytic activity whatsoever was recovered, this indicating complete consumption of the responsible factor(s) following display of its (their) effect.

In order to distinguish between the two possibilities of an actively occurring consumption and of a passive adhesion of the factor(s) to the erythrocytes, respectively, a sample of heat-treated plasma, preliminarily filtered on a Diaflo YM 5 membrane, was added to erythrocytes at a final 50% haematocrit. After producing complete haemolysis by sonication, the supernatant obtained by centrifugation at 20,000 g was again cleaned by filtration on a Diaflo YM 5 membrane and its increased-haemolytic activity tested. As shown in Table VIII, the preliminary adsorption of heat-treated plasma on native erythrocytes, followed by mechanical disruption of the cells, is sufficient to abolish its increased-haemolytic activity. The plain adsorption on erythrocytes, without any further haemolysis, led to inconclusive results mostly because of a poor reproducibility (data not shown).

TABLE VIII
Effect of mechanical haemolysis on the plasma increased-haemolytic activity measured on divicine-pre-treated erythrocytes

Heated plasma	Increased-haemolytic activity*
Before haemolysis**	76.5
After mechanical haemolysis***	zero

* Estimated on glucose-6-phosphate dehydrogenase-deficient erythrocytes as described in Table VII.
** Heated plasma was filtered on a Diaflo YM 5 membrane.
*** Heated plasma, after filtration on a Diaflo 5 membrane, was incubated for 10 min at 37°C with glucose-6-phosphate dehydrogenase-deficient erythrocytes at 50% haematocrit. Following sonication in a MSE mod. "Mullard" PG 100 sonifier, until reaching complete haemolysis, the suspension was stirred for 60 min at 37°C, then centrifuged at 30,000 g and the supernatant filtered on a Diaflo YM 5 membrane. The filtrate was brought to the same osmolarity and pH as the heated plasma and its extra-haemolytic activity measured on the same glucose-6-phosphate dehydrogenase-deficient erythrocytes pre-incubated with 5 mM divicine and 0.5 mM ascorbate.

These findings, on the whole, indicate that the process of haemolysis *per se*, irrespective of the procedures used (either mechanical or following an oxidative stress), leads to a consumption of the plasma increased-haemolytic activity.

If some physiological meaning is assigned to these data, then a massive haemolysis taking place "in vivo" would be expected to produce some refractoriness to haemolysis itself. This situation, although admittedly speculative, is reminiscent of a typical feature of the haemolytic crisis of favic subjects which is, by definition, self-limited in extent[59,60].

3) **Individual susceptibility to the divicine + ascorbate haemolytic system** We have repeatedly mentioned the wide variability in the extent of extra-haemolysis that is observed when the erythrocytes submitted to the oxidative stress with divicine and ascorbate are then re-incubated in autologous plasma. This variability contrasts with the constant susceptibility to extra-haemolysis that is shown by the same pre-treated erythrocytes upon their re-incubation in heat-deproteinized plasma from all subjects examined, irrespective of whether normal or glucose-6-phosphate dehydrogenase-deficient. Table IX shows such behaviour applying to a number of glucose-6-phosphate dehydrogenase-deficient hemizygous subjects. In 3 out of 7 cases investigated in this respect, pre-treatment of plasma with trypsin produced the same effect afforded by heat exposure, i.e. a virtually complete increased-haemolytic activity. Therefore it appears that the increased-haemolytic activity is a constant feature of plasma from all subjects. However, such activity, which seems to be due to ubiquitous component(s), is more or less expressed in native plasma from the individual subjects depending on absence or presence, respectively, of protein molecules which counteract the haemolytic activity itself.

It may well be that these "protecting" plasma proteins are always present in plasma, but rather that their concentrations are in some subjects below a threshold critical for the masking of haemolytic activity. Alternatively, the individual predisposition to the haemolytic system "in vitro" might be related to the extent of binding of the increased-haemolytic, small molecular weight factor(s) to these plasma

TABLE IX

In vitro haemolysis of erythrocytes from various glucose-6-phosphate dehydrogenase-deficient hemizygous subjects following oxidation by divicine and ascorbate and re-incubation in native and deproteinized plasma

Subjects (GD⁻ males)	% Haemolysis*			
	in PBS	in plasma		
		native	heated	trypsinized
A.G.	14	76	93	n.d.
G.M.	20	22	69	67
A.A.	4	9	86	78
C.A.	7	12	79	n.d.
T.M.	29	97	100	n.d.
E.S.	14	89	98	n.d.
A.P.	31	67	81	100

* Estimated after a preliminary incubation of erythrocytes with 5 mM divicine and 2.5 mM ascorbate for 3.5 hrs at 37°C in PBS. The erythrocytes were then washed in PBS and re-incubated in the media indicated for 8 hrs at 37°C. For other details see "Methods". Each experiment was carried out in triplicate.

proteins. This binding in turn could be affected by other properties of plasma, such as for instance small variations of ionic composition, pH, metabolites, etc. While a conclusion on this point is clearly remote and awaits a precise elucidation of both extra-haemolyzing factor(s) and of the plasma proteins as concerns their identification and reciprocal interaction, it is attractive to interpret the well known individual predisposition to haemolytic risk of the glucose-6-phosphate dehydrogenase-deficient subjects[24] in terms of the complex yet reproducible effects observed "in vitro" with the divicine-ascorbate system.

DISCUSSION

The main goal of this investigation was to give proper attention to the role of plasma in the process of oxidative haemolysis. While such a role is clearly an enhancement of erythrocyte lysis with the t-butylhydroperoxide system, the situation seems to be much more complicated with the divicine-ascorbate system in which two opposite phenomena have been observed: a) amplification of haemolysis, which is produced by low molecular weight plasma components, b) significant protection against haemolysis itself, as afforded, yet according to variable patterns in the individual subjects, by thermolabile and trypsin-sensitive plasma factors.

The difference between the two systems under study might be only artefactual, i.e. due to the hardly comparable strength of the two oxidizing solutions. However, attempts at producing lower oxidative damage by decreasing the t-butylhydroperoxide concentrations failed to reveal any difference between the lytic effects of native and of deproteinized plasma, respectively. Therefore the protecting role of plasma proteins seems to be a specific feature of the divicine-ascorbate system only.

Another reason of interest in the divicine-ascorbate combination is its mimicry of favism. Considerable evidence has been accumulating over recent years for a causal role of divicine and of its cogener isouramil in the pathogenesis of this acute hemolytic disease[19,34-39]; the importance of ascorbate seems to be minor and probably related to

maintenance of either pyrimidine aglycone in a reduced state capable of sustaining auto-oxidation.

Attempts at rationalizing in biochemical terms the variegate effects of plasma in the mechanism of oxidative haemoloysis as investigated with the divicine–ascorbate system should develop according to the following schedule:

1) Identifying a specific biochemical event induced by divicine in the glucose-6-phosphate dehydrogenase-deficient erythrocyte at a site not too remote from eventual haemolysis and clearly related to it. This is a primary requirement since, as we have repeatedly mentioned, monitoring of haemolysis is quite simple to be carried out but open to a number of artefacts. If such a divicine-triggered event is identified, then it can be operationally followed in the experimental system under study, thereby avoiding the uncertainties linked to evaluation of haemolysis itself. In our opinion, this is the most critical problem of the overall research and requires a careful scrutiny of the intracellular damaging effects of divicine and ascorbate in terms of time sequence and of integrated mechanisms.
2) After defining a suitable target effect, the fractionation of the plasma molecule(s) responsible for the increased-haemolytic activity should be markedly accelerated and would be expected to lead to isolation and chemical characterization of this (these) molecule(s). A side effect of this crucial achievement would be the reconstruction "in vitro" of the interrelated events that characterize "in vivo" the onset of the acute haemolytic crisis of favic patients.
3) Once the haemolyzing, low molecular weight plasma molecules have been identified, then attention could be paid to the nature and the function of the plasma proteins for which we have postulated a protecting role against haemolysis. Again, the development of a suitable "in vitro" system for measuring increased-haemolysis or any pre-haemolytic state would be the first requirement to be undertaken. Actually, provided that such a system becomes available, the study of the protecting plasma proteins would be mostly based on fractionation and assays of the haemolysis-inhibiting activity. We believe that this still remote phase of the present investigation would be the most rewarding in terms of prevention of favism and of other clinical consequences of glucose-6-phosphate dehydrogenase deficiency.

The requirements of the first of the three above mentioned steps of this research are rather satisfactorily met with the t-butylhydroperoxide system, which seems to represent a simpler and better integrated model of oxidative haemolysis as compared with the divicine–ascorbate combination. Specifically, the sequence of cellular events that take place following the oxidative stress with t-butylhydroperoxide has been elucidated to a satisfactory level of confidence[30–32] and the times required are limited enough as to avoid at least some of the pitfalls mentioned in the "Results" section. However, using the t-butylhydroperoxide rather than the divicine–ascorbate system for attempting fractionation and chemical identification of the plasma increased-haemolysis components (step 2) could be a misleading approach because these components might prove to be different with the two systems of oxidative haemolysis. In this case, then also step 3 would not be practicable and the only result would be the availability of haemolyzing plasma molecules devoid of any physiological significance.

Owing to these reasons we think it largely preferable to explore the divicine–ascorbate system in better detail. In fact, a critical appraisal of the present knowledge on this experimental system reveals several limitations as far as the intracellular

damaging effects are concerned. Some of the effects, listed in Table V, have no apparent correlation with the eventual haemolysis, at least if they are considered individually and not as a summation of events. For instance, the depletion of reducing equivalents is not sufficient to produce erythrocyte destruction *per se*, as shown by the limited extent of haemolysis that is undergone by the pre-treated erythrocytes in the reference buffers even at long incubation times. Accordingly, oxidation of reduced coenzymes cannot replace haemolysis properly as a test of extensive cellular damage.

Other cellular effects induced by divicine and ascorbate in the susceptible, i.e. glucose-6-phosphate dehydrogenase-deficient erythrocytes, seem to deserve more consideration in terms of mechanisms. In this respect, methaemoglobin formation, inactivation of glutathione peroxidase and impairment of the calcium pump might be more or less directly related to the process of haemolysis, although much remains to be clarified. The role of methaemoglobin in the process of haemolysis is still undefined. Specifically, while in the t-butylhydroperoxide system a precise mechanism started by the homolytic cleavage of the oxygen-oxygen bond and liberating $OH^{\cdot}$ radicals could be easily envisaged[30], in the ascorbate–divicine system the picture is much more complicated. Recently, we have shown that the interaction of ascorbate with methaemoglobin results in the production of $OH^{\cdot}$ radicals and the underlying mechanism has been rationalized in terms of H_2O_2 formation, followed by a Fenton-type mechanism in which ascorbate acts to reduce methaemoglobin[61]. Clearly, the addition of divicine introduces further complexity to the system, although the results of the experiment reported in Table IV appear to suggest an active role of methaemoglobin itself in the onset of haemolysis. As a result of these uncertainties on the mechanism, monitoring the extent of methaemoglobin formation as a measure of oxidative haemolysis would be probably an unreliable parameter. Further studies are necessary in order to rule out with certainty the possibility of methaemoglobin formation as a side phenomenon following extensive oxidative damage. The recent findings of a clearcut correlation between high methaemoglobin levels and massive haemoglobinuria in favism[62], in spite of their clinical interest and of practical significance for monitoring the progress of the disease, do not solve the problem of the relationship between methaemoglobin and haemolysis.

The inactivation of glutathione peroxidase by divicine and ascorbate[39,57] seems to contribute to produce significant damage in the normal and even more in the glucose-6-phosphate dehydrogenase-deficient erythrocytes. In fact, in spite of apparently limited inactivation of this important H_2O_2-scavenging erythrocyte enzyme, such effect is counteracted by complete stability and in some experiments by slight activation of superoxide dismutase[39]. Accordingly, the resulting shift in the ratio of glutathione peroxidase to superoxide dismutase activities upon exposure of erythrocytes to divicine and ascorbate is expected to enhance the formation of highly toxic oxygen derivatives, notably H_2O_2 and $OH^{\cdot}$ radicals. Like for methaemoglobin, it may be significant and relevant to haemolysis to find a ratio between glutathione peroxidase and superoxide dismutase activities that is displaced in the same direction, i.e. lower than normal, in favism[39]. On the basis of these considerations, further investigations on this specific mechanism of glutathione peroxidase inactivation could be of help for developing a suitable means of estimation of pre-haemolytic damage.

In view of the well established role of intracellular calcium in cytotoxicity, as investigated in various cell types, the recent observation of an impairment of the calcium pump by divicine and ascorbate seems to be of major importance[54,55]. Although this

cellular effect appears to be nonspecific and related to other oxidizing stimuli like phenylhydrazine[63] and t-butylhydroperoxide (Table II), the elevation of intraerythrocytic calcium levels that follows exposure of glucose-6-phosphate dehydrogenase-deficient erythrocytes to divicine and ascorbate might play some role in the genesis of the haemolytic attack of favic patients. This view is supported by the findings of significantly lowered values of Ca^{2+} – ATPase activity and of dramatically enhanced intracellular calcium content in erythrocytes from acute favic patients[54,55].

The critically disordered calcium homeostasis both in favism and in the "in vitro" experiments with divicine and ascorbate can hardly been ascribed to inactivation of Ca^{2+} – ATPase activity only. In fact such inactivation is not complete and is not reversed by addition of calmodulin[64], suggesting the possibility that other cellular mechanisms and especially an enhanced calcium influx may be important in producing a remarkable accumulation of intracellular calcium. Whatever mechanism is operating, this phenomenon is expected to trigger several side effects including: *a*) loss of cellular potassium ("Gardos" effect[65]; *b*) activation of a calcium-dependent neutral proteinase that has been shown to degrade soluble and membrane proteins as well, through a complicated regulatory interplay with Ca^{2+} itself, a natural protein inhibitor and the availability of protein substrates[66,67]; *c*) formation of γ-glutamyl-ϵ-lysine cross-linked protein polymers *via* the activation of a specific calcium-dependent transglutaminase[68–72].

Of the three effects related to increases in intracellular calcium concentrations, leakage of potassium from erythrocytes and extensive degradation of membrane proteins have been consistently observed in the "in vitro" experiments where erythrocytes were exposed to divicine and ascorbate and re-incubated in calcium-containing media[54]. Conversely, the formation of transglutaminase-dependent polymeric material in these conditions was poorly detectable. Moreover, similar findings, i.e. loss of intracellular potassium and extensive membrane protein degradation were obtained in erythrocytes from 7 favic patients during acute haemolytic crisis. Again, no clear evidence for transglutaminase-catalyzed cross-linked polymers was obtained in these haemolytic patients, not even in those who showed elevations of intraerythrocytic calcium above 0.4 mM, that seems to be the critical threshold for activation of the transglutaminase[68]. Indeed, all membrane protein aggregates observed during the disease proved to be reducible by thiol groups and therefore to be related to disulfide bridge formation as a result of the oxidative stress.

The specific role of abnormal calcium homeostasis in the genesis of haemolysis both "in vitro" and "in vivo" still requires further studies which are in progress in our laboratory. Although there are some reports that loading of calcium into erythrocytes by means of ionophore A 23187 does not affect the survival of these cells[73], it should be mentioned that an elevation of intracellular calcium following exposure to divicine and ascorbate will take place in glucose-6-phosphate dehydrogenase-deficient erythrocytes extensively damaged by the oxidative challenge at several sites. Furthermore, the contribution of plasma to eventual haemolysis should also be considered. Therefore, the specific role of, *a*) metabolic imbalances produced by divicine, *b*) elevation of intracellular calcium and related phenomena, such as the "Gardos" effect, *c*) haemolyzing activity of plasma, must be taken in due account for elucidating the process of oxidative haemolysis in this model system of favism.

The data concerning the perturbation of erythrocyte calcium homeostasis that follows an acute oxidative stress both "in vitro" and "in vivo" are strongly reminiscent of the situation described for other cell types, notably hepatocytes[74,75]. In the

isolated hepatocytes a strong oxidant damage has been reported to produce abnormal subcellular compartmentation of the calcium pool, followed by unequivocal signs of cytotoxicity such as "blebbing" and eventually by extensive lysis[74]. Although the picture for the erythrocyte is obviously simplified and different, it is interesting that divicine induces on the glucose-6-phosphate dehydrogenase-deficient erythrocytes heavy morphological alterations and especially the appearance of "crossbonded" cells that have been also observed as a typical haematological feature of favism[25,26].

Besides bearing a likely relevance to the process of oxidative haemolysis, the raised levels of intraerythrocytic calcium and the related phenomena might prove to adequately probe from an experimental standpoint a pre-haemolytic situation. While other cellular effects of the divicine-ascorbate system (e.g. lipid peroxidation as estimated by malondialdehyde formation) are lacking, intracellular calcium accumulation, or leakage of potassium, or later effects on proteolysis, could be useful for this purpose. Evidence has already been reported that loss of erythrocyte potassium is a pre-lytic phenonemon with an oxidative challenge involving peroxidation and enhanced by thyroxine[76]. Attempts are currently being made in this direction, in order to rationalize the assay of erythrocyte fragilization and to proceed subsequently to a better biochemical characterization of the plasma interactions on the damaged erythrocytes.

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