THE EFFECTS OF RETINOL, α-TOCOPHEROL, AND ASCORBIC ACID ON THE PLASMA MEMBRANES OF ERYTHROCYTE GHOSTS

by

Anne Marlene Popma

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NAME: Anne Marlene Popma

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EXAMINING COMMITTEE:

Chairman: Dr. T.W. Calvert

Dr. A.J. Davison
Senior Supervisor

Dr. F. W. Banister

Dr. N.M.G. Bhakthan

Dr. K. Colbow

Dr. J. Bryant External Examiner
Associate Professor, Department of Chemistry
University of Puget Sound

Date Approved: 11th Dec 1974

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Author: _____

(signature)

Anne Popma

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ABSTRACT

Erythrocytes have been shown to be damaged by excessive quantities of retinol (vitamin A) and to be protected against this damage by α-tocopherol (vitamin E). Ascorbic acid (vitamin C) has been shown to protect red blood cells against light-induced and free radical-mediated destruction. All three of these vitamins possess oxidative and reductive capacities and are therefore potentially capable of partaking in free radical reactions. This study was undertaken to resolve, in part, their effects in high concentrations on the plasma membranes of living cells and to investigate in a preliminary way the interactions between them.

Membranes were isolated from human erythrocytes in the form of "ghost" cells, and were resealed so that permeability changes could be determined as a measure of the intactness of the membrane. Damage to protein and lipid constituents of the membrane were separately assessed by determining the activity of a membrane-bound protein, glyceraldehyde-3-phosphate dehydrogenase (GAPD), and by following autoxidation of the membrane lipids as measured by the accumulation of lipid peroxides.

Activity of GAPD was destroyed by all concentrations of retinol and ascorbate which were tested. Initial damage caused by ascorbate occurred much more rapidly than an equimolar concentration of retinol, even though the effective damage was considerably less following the first half hour of incubation at 37°C. The inclusion of concentrations greater than 0.5 mM ascorbate with various concentrations of retinol resulted in a loss of GAPD activity which was much more rapid than with either vitamin alone. Smaller amounts of ascorbate were found to exert a slight protective effect on damage induced by retinol. Although incubation under 100% oxygen resulted in only a marginal increase in GAPD destruction in the presence of ascorbate, it doubled the damage caused by retinol by the end of 6 hours of incubation. Cooling the incubation medium to 0°C suppressed retinol-induced damage by 50% but did not slow the destruction caused by ascorbate. The effects of both retinol and ascorbate were reversed by the sulfhydryl compound dithiothreitol (DTT), indicating that the decrease in enzyme activity was associated with a reversible oxidation of sulfhydryl groups. Inclusion of tocopherol, but not tocopherol acetate, in the incubating medium resulted in a 30% inhibition of enzyme damage caused by both retinol and ascorbate.

Membrane permeabilty was measured by the accessibility of GAPD to substrates located outside the cell membrane. Retinol and α-tocopherol both caused an increase in membrane permeability and also an increase in mean cell volume as determined by an electronic Coulter counter. Both of these effects were additive when retinol and tocopherol were present simultaneously. Ascorbate caused no increase in membrane permeabilty and no change in mean cell volume. It did, however, increase turbidity in ghost suspensions and caused a color change from pink to brownish-red and finally to green, indicating destruction of residual hemoglobin as confirmed by visualization of Heinz bodies under phase contrast microscopy.

The formation of lipid peroxides was measured by the thiobarbituric acid (TBA) method. Incubation of ghost cells with retinol resulted in a substantial increase in lipid "peroxidation". This effect was greatly enhanced by the presence of 100% oxygen and was decreased by 66% in the presence of α-tocopherol. The level of absorptivity at 532 nm in the presence of TBA was increased by ascorbate and the presence of α-tocopherol provided almost complete prctection against this effect.

It is concluded, in general, that biological membranes are subject to damage by excessive quantities of vitamins A and C , that physiological quantities of ascorbate and tocopherol can protect to some extent against the damage caused by retinol, and that the contents of living cells normally provide protection against such damage.

To Mom and Dad,

and to Dave

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INTRODUCTION

Vitamins are substances that are essential for the maintenance of normal metabolic function, but which are not synthesized in the body and therefore must be furnished from exogenous sources. A healthy individual ingesting a well balanced diet normally receives adequate amounts of vitamins from his food. However, there are many situations in which the concentration of one or more vitamins may be either excessive or suboptimal. A knowledge of the mode of action of the vitamins is therefore essential to an understanding of human metabolism, both in the healthy and diseased state.

Pathology resulting from excessive intakes of vitamin A has been well documented in the literature, and although hypervitaminosis A is rare, serious cases of vitamin A poisoning continue to occur. Outbreaks of acute vitamin A intoxication have been noted in Arctic explorers after ingesting large quantities of polar bear liver (Pierce, 1962). Chronic hypervitaminosis A in adults has occurred in patients who received large doses of this vitamin as treatment for dermatological conditions, and who continued subsequent intake without medical supervision (Di Benedetto, 1967; Stimson, 1961). It has also been reported in food faddists who included large doses of vitamin preparations in

their daily dietary regimes (Bergen and Roels, 1965; Russell, Boyer, Bagher and Hruban, 1974). There has been much speculation regarding the possible harmful effect of ingesting heroic doses of vitamin C as recommended by Linus Pauling and others (Pauling, 1970; Stone, 1966; Yew, 1973), but apart from the damage to enzymes and to membrane systems in vitro discussed later, there is little evidence to suggest damage to tissues in the living body. Vitamin E is generally considered to be of low toxicity and, although there are a number of reports of metabolic abnormalities induced in experimental animals by excess of vitamin E (March, Wong, Seier, Sim and Biely, 1973), levels greatly in excess of normal dietary requirements have been administered to human subjects without adverse clinical effects.

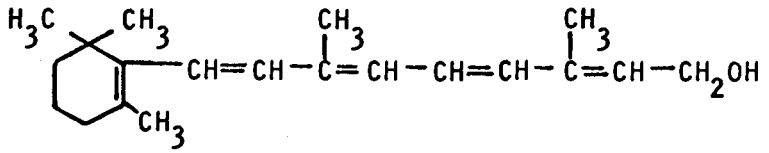
The vitamins A, E and C have beer shown to possess oxidative and reductive capacitites and are therefore potentially capable of causing free radical tissue damage. Vitamin A (retinol), because of the high degree of unsaturation in its isoprenoid chain, is easily oxidized in the presence of oxygen. Being lipid soluble it is often found associated with other hydrophobic substances, e.g. the lipids of biological membranes, and by such an association it may either physically disrupt the ordered structure which existed prior to its implantation, or

initiate a series of damaging chemical reactions as a consequence of autoxidation. Vitamin E (-tocopherol) is also an unstable molecule due to the phenolic group on its aromatic ring. It functions physiologically as an antioxidant and protects molecules such as retinol from oxidative damage. Vitamin C (ascorbic acid) is a strong reducing agent because of its enediol group and is known to have both damaging and protective effects on various tissues as will be discussed later. The molecular structures of retinol, α-tocopherol and ascorbic acid are illustrated in figure 1.

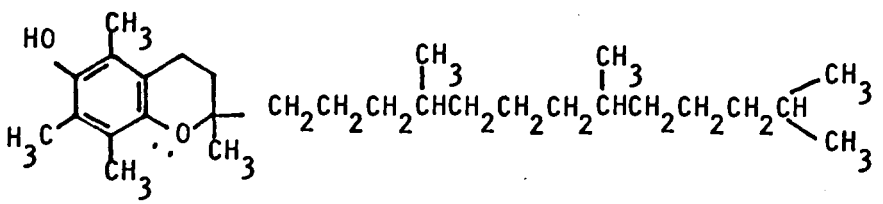
Oxidation is defined as the loss of electrons; the reverse process, reduction, is electron acceptance. Oxidation can proceed through loss of electrons from a substance either in pairs or one at a time. The loss or gain of one electron frequently results in the formation of free radicals, i.e. chemical compounds possessing an odd number of electrons which are highly reactive and potentially damaging in living cells. Once formed, a radical will attempt to stabilize itself, either by combining with another odd-electron species or by generating another radical which propagates a chain reaction. Unless terminated, such phenomena can be extremely destructive and are believed to play a significant role in the degradation of biological systems (Pryor, 1973).

Figure 1. Molecular structures of vitamin A (retinol), vitamin E (α-tocopherol) and vitamin C (ascorbic acid)

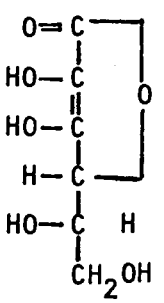
FIGURE 1



Vitamin A (retinol)



Vitamin E (α-tocopherol)



Vitamin C (ascorbic acid)

Radical-induced tissue damage frequently occurs as a result of the interaction of pro-oxidant substances with biological membranes. Membranes are, essentially, complex mixtures of lipids and proteins held together by ionic and hydrophobic forces (Singer and Nicholson, 1972). The lipid component invariably contains unsaturated fatty acids which are highly susceptible to free radical attack. Since the presence of a carbon-carbon double bond weakens the carbon-hydrogen bond of the carbon atom next to it, less energy is required to abstract the hydrogen atom, leaving a radical center on the α-methylene carbon. This highly reactive radical may, in the presence of oxygen, acquire a hydroperoxy group. Such a series of reactions is termed lipid "peroxidation" and profoundly affects membrane structure and function (Dahle, Hill and Holman, 1962; Tappel, 1973).

The erythrocyte, because of its lack of intracellular structures and relative simplicity, provides a unique subject for the study of cellular membranes. Its membrane is composed of 52% protein, 40% lipid and 8% carbohydrate (Steck, 1974). Because of the high content of polyunsaturated fatty acids (Dodge and Phillips, 1967) and direct exposure to molecular oxygen, the erythrocyte is extremely susceptible to peroxidative damage. There is

evidence to suggest, however, that there are two sites of action of oxidant compounds on the red blood cell: one involving the membrane directly and the other involving a cooperative, potentiating action of cellular contents in the damaging process (Ginn, Hochstein and Trump, 1969; Miller and Smith, 1970). In this study, in order to differentiate between these two processes, the membrane has been separated from intracellular constituents in the form of erythrocyte ghosts.

Dodge, Mitchell and Hanahan (1963) define a ghost as being the delicate, discoid body obtained after the removal of hemoglobin. The properties displayed by ghost systems have generally been found to represent properties characteristic of the plasma membrane of intact cells (Hoffman, 1958; Bramley and Coleman, 1972), thus many observations made using ghosts may be applied to in vivo conditions. There are essentially two types of ghost cells: "white ghosts" and "resealed ghosts" (Schwoch and Passow, 1973). White ghosts are free from visible contamination with hemoglobin and are largely devoid of intracellular contaminants. They are widely used for biochemical work, such as the investigation of the enzymological properties of the cell membrane, however their diffusion barrier is completely destroyed and they are therefore unsuitable for

permeability studies (Jung, Carlson and Balzer, 1973). In resealed ghosts emphasis is placed not on extreme purity of the membrane, but on restoration of the original membrane structure and function after hemolysis. As a result they have been used extensively in studies of transport and permeability, and were used in the present work.

RELATED LITERATURE

Dingle and Lucy (1962) have shown that isolated erythrocytes of rabbit, pig, ox, rat and man are rapidly lysed by 10-20 micrograms of retinol per ml and that hemolytic activity is restricted to those retinol derivatives posessing biological activity and capable of preventing the symptoms of vitamin A deficiency. They hypothesized that the site of action of vitamin A is the lipoprotein membrane of cells and their organelles, and that its physiological role is the control of membrane permeability.

Many of the changes seen in hypervitaminosis A are explicable on the hypothesis that vitamin A penetrates lipoprotein membranes and that membranes containing an excess of the vitamin exhibit increased permeabilty or decreased stability. The addition of an excess of retinol to a suspension of mammalian fibroblasts causes local distensions in the plasma membrane, damage to the nuclear membrane, and mitochondrial swelling (Dingle, Glauert, Daniel and Lucy, 1962; Daniel, Dingle, Glauert and Lucy, 1966). Lucy, Luscombe and Dingle (1963) similarly report swelling in the mitochondria of rat liver following the addition of retinol. Dingle, Lucy and Fell (1961) found

that the alterations in mitochondrial structure caused by excess vitamin A resulted in depressed respiratory activity in embryonic chick limb-bone rudiments and similar metabolic disturbances have been reported in liver mitochondria by Seward, Vaughan and Hove (1966) and Pokrovsky, Kon and Natanson (1972). Lucy, Dingle and Fell (1961) reported degradation of embryonic chick cartilage under the influence of hypervitaminosis A and suggested that it might be due to an enhanced proteolytic activity of the chondroblasts.

Retinol was the first compound of physiological importance to be shown to have an action both on the release of lysosomal enzymes in the living cell and also on isolated organelles (Dingle, 1961; Fell and Dingle, 1963). Fell, Dingle and Webb (1962) examined the specificity of the action of vitamin A on lysosomal preparations and found it to be similar to that found for erythrocytes and mitochondria. Roels, Trout and Guha (1965) found the stability of liver lysosomes in vitamin A-deficient rats to be greatly impaired and Roels, Anderson, Lui, Shah and Trout (1969) found the rate of release of hemoglobin from erythrocytes of vitamin A-deficient rats to be considerably faster than that from cells of pair-fed controls. An optimal amount of retinol therefore appears necessary to stabilize biological membranes. Lucy and Dingle (1964) have

found that, unlike the observed hemolytic effect of high concentrations of retinol, treatment of erythrocytes with low concentrations (0.1 to 1.0 micrograms/ml) increased their resistance to hypotonic hemolysis. Raz and Livine (1973) likewise found a protective effect of retinol at low concentrations and compared it to the effect produced by lipid soluble anaesthetics (Seeman, 1969; Roth and Seeman, 1971).

Such phenomena are not resricted to <u>in vitro</u> situations. Morriss (1973a,b) found that a single large injection of vitamin A palmitate administered to a pregnant rat results in malformations and prenatal death in the offspring. Ultrastructural abnormalities included membrane swelling, cytoplasmic vacuoles, "budding", changes in the extra- to intracellular fluid ratio, and condensed and swollen mitochondria.

Observations made by phase-contrast and electron microscopy have shown that erythrocytes assume various bizarre forms within one minute of the addition of an excess of vitamin A (Glauert, Daniel, Lucy and Dingle, 1963; Murphy, 1973). The initial effect is a greatly increased surface area with no accompanying change in volume. Large indentations appear on the surface of the cells and vacuoles

form by a process resembling micropinocytosis. The cells then become spherical and loss of hemoglobin begins as breaks appear in the membranes of some cells. It was proposed that the retinol molecule is incorporated in the membrane, which expands in order to accomodate the additional molecules, and that lysis arises from mechanical weakening or rearrangement of the structure of the membrane. Bangham, Dingle and Lucy (1964) found that retinol readily penetrates and expands monolayers of lecithin and cholesterol at an air-water interface. Reels et al. (1969) performed similar experiments on artificial lipid structures and suggested that the effect of retinol on membranes was due to an interaction between retinol and the polar group of phosphatidylcholine. Lucy and Dingle (1962) had previously reported on the ability of vitamin A to interact with protein, and suggested that retinol acts as a cross-linking agent between lipid and protein.

The appearance of breaks in the cell membrane occurs only at temperatures above 20°C although at lower temperatures other morphological changes are still observable. This was believed to be evidence of a two-step process in the hemolysis of the erythrocyte by retinol: (1) an initial penetration of the membrane which occurs both in the cold and at physiological temperatures, and (2) an

oxidation of the polyene chain of retinol which occurs above 20°C (Dingle and Lucy, 1965). Lucy (1969) found that colloidal dispersions of retinol were easily oxidized by molecular oxygen and Fisher, Lichti and Lucy (1972) found this reaction to have the characteristics of a free radical-catalyzed autoxidation. Krishnamurthy and Kartha (1973), however, found no evidence of "peroxidation" of erythrocyte lipids even when hemolysis was complete with 100 micromoles of retinol per ml.

The rapid hemolysis by retinol at 37°C has been shown to be prevented by an equimolar quantity of α-tocopherol (Lucy and Dingle, 1964). Electron micrographs of erythrocytes treated with retinol and α-tocopherol simultaneously showed an initial sequence of changes similar to that seen with retinol alone. After 15 minutes, however, cells were still intact, though they contained vacuoles which may have formed as a result of initial expansion of the cell surface. Retinol seemed to have penetrated the membrane both in the presence and absence of α-tocopherol, but tocopherol prevented subsequent rupturing which occurred with retinol alone.

Although the exact biological function of vitamin E has been controversial for over 30 years, it is generally

recognized as a naturally occurring, lipid-soluble antioxidant (Tappel, 1972; Molenaar, Vos and Hommes, 1972). One of its sites of action is the erythrocyte membrane and persons deficient in vitamin E are known to exhibit increased susceptibility to hemolytic anemia (Tsen and Collier, 1960; Binder, Herting, Hurst, Finch and Spiro, 1965; Oski and Barness, 1968).

In examining the molecular specificity of antioxidants in preventing vitamin A-induced hemolysis, Lucy and Dingle (1964) found that, although α-tocopherol and α-tocopherol acetate were effective, α-tocopherol succinate was relatively ineffective and the phosphate ester was itself hemolytic. Hydroquinone was without effect even when 500 micromoles/ml were added simultaneously with 15 micromoles/ml retinol and it was concluded from this that inhibition by the tocopherols was possibly not due to neutralization of free radicals or the breaking of auto-oxidative chain reactions, but rather to a physical effect not unlike that of cholesterol (Lucy, 1972). This conclusion was supported by the fact that the acetate ester of α-tocopherol, which was particularly effective in inhibiting lysis, does not contain the hydroxyl group that can yield hydrogen and thereby scavenge free radicals (Tappel, 1962). Compounds of the vitamin K series were also

tested by Lucy and Dingle (1964). Vitamin K1 was found to inhibit retinol-induced lysis effectively, while menadione (vitamin K3), which has the same structure as vitamin K1, but no isoprenoid chain, was inactive. Ubiquinone-30, which has a long side chain, was very effective in inhibiting lysis despite the fact that its antioxidant activity is much less than that of α-tocopherol (Green, Diplock, Bunyan, Edwin and McHale, 1961). Two other long chain compounds, squalene and phytol, which have no activities as vitamins, were also capable of inhibiting lysis by retinol.

Krishnamurthy and Kartha (1973) found both vitamins E and D to inhibit hemolysis induced by vitamin A. The sulfhydryl compounds glutathione and cysteine were also effective in arresting hemolysis, but synthetic antioxidants such as diphenyl-p-phenylenediamine (DPPD), butylated hydroxytoluene (BHT) and ethoxyquin were ineffective. Ascorbic acid at low concentrations prevented hemolysis, but at higher levels it increased the damage.

Dolbeare and Martiage (1972) also found small amounts of ascorbate to exert a protective effect in that it decreased β-glucuronidase activity when incubated with lysosomal suspensions. Higher concentrations of ascorbate not only failed to inhibit the enzyme, but enhanced activity

above that of controls. Schothorst, van Steveninck, Went and Suurmond (1970) reported that 5 mM ascorbate inhibited the *in vitro* photohemolysis of erythropoietic protoporphyria (EPP) erythrocytes. A similar concentration of ascorbate slowed the loss of 2,3-diphosphoglycerate (2,3-DPG) from stored blood (Wood and Beutler, 1973), decreased the formation of lipid peroxides in vitamin E-deficient rat liver homogenates (Chen, 1973), and partially restored glycolysis in rat heart homogenates following treatment with 100% oxygen (Horn and Haugaard, 1966). Exposure to hyperbaric oxygen for one hour created a 40% decrease in the levels of ascorbate in rat lungs and Wills and Kratzing (1972) suggested that this was due to the *in vivo* utilization of ascorbic acid as an antioxidant.

It has been demonstrated that low, but not high, concentrations of ascorbic acid produce swelling or lytic changes in isolated liver mitochondria (Hunter, Scott, Hoffstein, Guerra, Weinstein, Schneider, Schutz, Fink, Ford, Smith, 1964). Wills (1969) reported that low concentrations (0.5 mM) of ascorbate enhanced lipic "peroxidation" in rat liver microsomes, and Comolli (1971) found that this effect increased with increasing age of the animal. Wills and Wilkinson (1966) found that ascorbate increased the formation of lipid peroxides and release of enzymes caused

by irradition of mitochondria and lysosomal membranes, and Wills (1966) found that ascorbic acid increased the rate of "peroxidation" of unsaturated fatty acids catalyzed by whole homogenates of liver, heart, kidney and spleen.

Ascorbic acid is known to undergo autoxidation to dehydroascorbate with the production of H_2O_2, a process catalyzed by metals and metalloproteins (Blaug and Hajratwala, 1972). This autoxidation is capable of inhibiting the activity of solutions of catalase (Orr, 1966) and has also been reported to increase lytic sensitivity of erythrocytes to H_2O_2 (Serrill, Jefferson, Quick and Mengel, 1971). Dehydroascorbic acid by itself does not have the same effect (Orr, 1966; Hunter *et al.*, 1964) and damage may therefore be due to intermediate radical species such as the monodehydroascorbate radical (Green and O'Brien, 1973; Haase and Dunkley, 1969a,b) or oxygen radicals such as O_2^-, .OOH or .OH (Orr, 1967a,b).

OBJECTIVES OF THE STUDY

Since the vitamins A, E and C are all known to be involved in lipid "peroxidation" and free radical tissue damage it is important to understand the mechanisms involved, not only separately, but in combination as well. All too often the integrated description of a system is obscured in attempts to isolate a part. In nutritional studies, interrelationships between the vitamins are especially important since an organism functions not as a result of isolated chemical reactions, but as a highly complex and interdependent unit.

Effects of retinol, α-tocopherol and ascorbic acid on the erythrocyte membrane, both by themselves and in conjunction with each other, have been studied in the present research. An attempt has been made to provide answers to the following questions:

1. What is the effect of retinol on the membrane of the erythrocyte ghost and how does it compare with other reports of membrane damage induced by an excess of vitamin A?

2. How do the membrane effects of vitamin A compare with and relate to those created by different concentrations of ascorbic acid?

3. What substances are effective in counteracting the damaging effects of retinol and/or ascorbate?

4. How do the antioxidant effects of α-tocopherol compare with and relate to the reported antioxidant effects of ascorbic acid?

MATERIALS AND METHODS

Materials

Retinol, ascorbic acid, DL-α-tocopherol acetate, nicotinamide adenine dinucleotide (NAD), dithiothreitol(DTT) and DL-glyceraldehyde-3-phosphoric acid were purchased from the Sigma Chemical Company. DL-α-tocopherol was obtained from Calbiochem. These materials were of the highest grade commercially available, and were used without further purification.

Buffer Mixtures

Solutions were prepared from reagent-grade chemicals. Osmolarities were calculated by totaling the concentrations of all ionizable components in the solution, neglecting deviation of individual salts from ideal behavior. These values were checked for accuracy with a Precision osmometer model 2007. Final pH of each solution was measured on a Radiometer pH meter type PHM28.

Erythrocytes

Stored human blood was obtained from the Red Cross Blood Bank in Vancouver and was used within five weeks of collection after confirmation that no measurable loss of activity of glyceraldehyde-3-phosphate dehydrogenase or of the integrity of the cell membranes had occurred during storage. Citrate phosphate dextrose was the anticoagulant used. Packed erythrocytes were obtained by centrifuging in a Beckman J21 centrifuge at 3000 rpm for 15 minutes at 4° C. Both the plasma and buffy coat were carefully removed by aspiration, and the cells were washed twice with at least five times their volume isotonic saline buffer (0.155 M NaCl per liter of 5 mM PO4 buffer, pH 7.4).

Ghost Preparation

Ghosts were prepared according to the methods of Bodeman and Passow (1972) and Lepke and Passow (1972) with slight modification. General procedure for the preparation of ghost suspensions was as follows: (1) hemolysis, (2) reversion, (3) resealing (Schwoch and Passow, 1973). Hemolysis involves the release of hemoglobin and other intracellular constituents and is accomplished by pipetting a concentrated red blood cell suspension into a hypotonic

solution. Osmolarity of the hemolyzing medium was 40 millicsmolar (mOsm) since ghosts prepared at lower osmolarities tend to fragment into smaller vesicles (Duchon and Collier, 1971), contain significantly less protein, and are more susceptible to damage than are ghosts prepared at higher osmolarities (Bramley, Coleman and Finean, 1971). In detailed electron microscopic studies, Seeman (1967) and Seeman, Cheng and Iles (1973) have shown that hemolysis is associated with the formation of 100-1000 A° holes which are concentrated in a circular zone of about 1 μ diameter. In most cells the holes appear 10-15 seconds after immersion in the hemolysing medium and close up 5-10 seconds thereafter. Hoffman (1958) showed that at the end of the hemolytic event the hemoglobin distribution between cells and medium is at complete equilibrium. Immediately after establishing this equilibrium distribution the ghosts regain their impermeability to hemoglobin (reversion). Nevertheless, the membranes of many ghosts continue to be highly permeable to smaller molecules and ions. Reconstitution of the low permeability to alkali ions characteristic of the intact red blood cell membrane can be accomplished by restoration of isotonicity (310 mOsm) and "resealing" at 37°C (Rodeman and Passow, 1972).

Washed and packed erythrocytes were hemolyzed by suspending them in thirty volumes of cold 40 mOsm PO4 buffer containing 4 mM MgCl2 and 0.01 mM CaCl2, pH 7.4. Ca 2+ and Mg 2+ were included to reduce the permeability of the finished membrane preparations (Bramley and Coleman, 1972). The suspension was swirled gently and left to stand on ice for ten minutes. Isotonicity was restored by adding an appropriate volume of 2.7 M NaCl. The medium was kept on ice for a further ten minutes and then incubated at 37°C for one hour. The resulting ghosts were sedimented by centrifugation at 12,000 rpm for 10 minutes at 4°C and washed three times with ten times their volume isotonic buffer (0.125 M NaCl per liter of 20 mM PO4 buffer, pH 7.4) containing 2mM MgCl2. Washed ghosts were kept in an ice bath and used within 12 hours. Permeability and glyceraldehyde-3-phosphate dehydrogenase activity changed at a rate of less than 1.5 % per hour while standing under these conditions.

Hemoglobin Determination

To determine the percentage of hemoglobin removed from the ghost cells, the concentration of hemoglobin inside the intact cell was first calculated according to the method of Robin and Harley (1964). 0.05 ml red cells

were hemolyzed with 6 ml of 0.2% Triton X-100 and centrifuged at 3000 rpm for 10 minutes at 4 C. Absorbance of the supernatant was read on a Beckman DBGT spectrophotometer at 620 nm, once prior to and once following the addition of a drop of 0.15 M $K_3Fe(CN)_6$. $K_3Fe(CN)_6$ oxidizes the Fe 2+ of hemoglobin to Fe 3+ (methemoglobin) and the increment in absorbance at 620 nm (absorption peak of methemoglobin) is a measure of the hemoglobin originally present. Concentration within the red cell in millimoles per litre was calculated from the following formula:

$$\text{Hemoglobin Concentration} = \frac{\Delta A \text{ 620 nm on addition } K_3Fe(CN)_6}{\Delta \epsilon}$$

where ΔA 620 = change in absorbance at 620 nm
$\Delta \epsilon$ = difference between extinction coefficients of hemoglobin and methemoglobin (3.09)

A sample of ghosts was similarly treated with 0.2% Triton X-100 and after centrifugation at 12,000 rpm for 10 minutes, the hemoglobin was determined as described above. From these two values the mean hemoglobin concentration in 20 separate ghost preparations was calculated to be 10% plus or minus 5% of that in whole red cells.

Protein Determination

Protein was determined by the method of Lowry, Rosebrough, Farr and Randall (1951). Ghost suspensions (10 µl) in 1 ml of water were mixed with 5 ml of freshly made alkaline copper solution (1 ml of 0.5% $CuSO_4.5H_2O$ in 1% potassium sodium tartrate per 50 ml 2% Na_2CO_3 in 0.1 N NaOH) and allowed to stand for ten minutes at room temperature. The solution was mixed after addition of 0.5 ml of Folin phenol reagent (diluted with an equal volume of water) and allowed to stand for 30 minutes. The blue color produced by the Folin reagent and phenol groups of tyrosine and tryptophan residues in the protein sample has an absorption peak at 750 nm. Absorbance was read at this wavelength and protein concentration was determined, using bovine serum albumin as the usual arbitrary standard, according to the linear range of the Beer Lambert Law. Ghost cells were found to contain approximately 13% of the protein in whole erythrocytes.

Cell Permeability

Glyceraldehyde-3-phosphate dehydrogenase (GAPD) is a glycolytic enzyme known to reside on the cytoplasmic face

of the erythrocyte membrane (Schrier, 1963; Duchon and Collier, 1971). Approximately 60-80% of this enzyme remains associated with the membrane during hypotonic hemolysis, representing 5% of total ghost protein (McDaniel and Kertley, 1974; Shin and Carraway, 1973). It catalyzes the following reaction:

3-phosphoglyceraldehyde + NAD+ + inorganic phosphate $\rightleftharpoons$ 1,3-diphosphoglycerate + NADH + H+ (1)

The absorption peak of NADH (reduced nicotinamide adenine dinucleotide) is at 340 nm and rate of the reaction can therefore be determined by observing the increase in absorbance at this wavelength. Preliminary observations indicated that the reaction is approximately first order with respect to time. If substrates of reaction (1) are excluded from inside the cell, membrane permeability can be determined by the extent to which the reaction is accelerated by the addition of substrates to the suspending medium. If this value is compared with the reaction rate with 100% permeability, as induced by treatment with the nonionic detergent Triton X-100, the percent accessibility of the enzyme can be calculated as follows:

$$\text{Percent Accessibility} = \frac{\text{rate of reaction minus detergent}}{\text{rate of reaction plus detergent}} \times 100$$

GAPD was assayed according to the method of Steck and Kant (1974). 0.1 ml of the ghost suspension were preincubated at 25°C for one minute in cuvettes with 0.3 ml of isotonic PO4 buffer, pH 7.4, with and without 0.2% (v/v) Trition X-100. To a cuvette containing 1.24 ml of 30 mM sodium pyrophosphate, pH 8.4 and 0.06 ml 0.4 M sodium arsenate, 0.1 ml 20 mM NAD was added. The reaction was initiated by adding 0.2 ml 15 mM 3-phosphoglyceraldehyde, pH 7.0, the solution mixed, and the reaction was followed spectrophotometrically at 340 nm. If the addition of GAPD is assumed to be the starting point of the reaction, the increase in absorbance between the first and second minute of reaction may be used as a measure of enzyme activity. The rate of the reaction was determined by the formula:

$$R = \frac{\Delta A}{60 \times \epsilon^{\mu M}_{340}}$$

where R = rate of the reaction in μM/sec
ΔA = change in absorbance between the first and second minute of reaction
$\epsilon^{\mu M}_{340}$ = molar extinction coefficient of NADH (0.00622)

Lipid "Peroxidation"

Lipid peroxides were estimated from the production of malonaldehyde as measured by the 2-thiobarbituric acid (TBA) test described by Tsen and Collier (1960). TBA reacts in a ratio of 2:1 with malonaldehyde, a product of the oxidation of unsaturated fatty acids, to form a red pigment which absorbs maximally at 532 nm. Although this reaction does not directly reflect the formation of lipid peroxides (Patton, 1974; Sawicki, Stanley and Johnson, 1963), it parallels direct measurements of lipid "peroxidation" under a wide variety of experimental conditions and has been widely used as a general measure of peroxide formation (Barber and Bernheim, 1967; Dahle et al., 1962). To a centrifuge tube containing 2 ml of ghost suspension, 2 ml of 10% trichloroacetic acid and 4 ml of 0.67% 2-thiobarbituric acid were added. The mixture was placed in a boiling water bath for 10 minutes, cooled, centrifuged, and the supernatant was removed. Absorption spectra of the supernatant was measured between 420 and 600 nm with water as a blank. The height of the peak at 532 nm was expressed in absorbance units, A 532, and indicates the amount of lipid peroxide present.

Cell Volume

Cells were counted and their volume was measured by use of an electronic blood cell counter: the Coulter Counter. Dilution was 1:50,000 in isotonic buffer. Ghost cells prepared as described were found to have an average volume of 47.5 cubic microns.

Phase Contrast Microscopy

The preparations were examined with a Zeiss Photomicroscope. Membrane samples were diluted in the appropriate buffer and examined at a magnification of 1600 x.

Test Systems

For most experiments the test system consisted of a 15 ml volume in a 25 ml Erlenmyer flask. The incubation medium was 0.125 M NaCl in 20 mM PO4 buffer, pH 7.4, and ghost cells were diluted to give a 10% suspension. Temperature was ordinarily 37°C.

Retinol was dissolved in 100% ethanol to give a 50 mg/ml stock solution. This was stored under nitrogen in the

cold and in the dark, and was used within three weeks. Dilutions were made immediately before use by rapid injection into the incubating buffer while stirring with a with a Vortex mixer. In spite of these precautions, effective concentration in the incubation media varied slightly from day to day as the lipid-soluble retinol molecules occasionally adhered to the glass container and/or formed lipid droplets (especially at high concentrations) rather than dispersed evenly in the aqueous medium.

Ascorbate was always freshly prepared in a cold solution. pH was adjusted to neutrality and appropriate volumes were added to the ghost suspensions.

α-tocopherol was prepared fresh each day by dissolving it in a 4:1 ratio with 100% ethanol. Dilutions were made in ethanol and added to test media by rapid injection with a Hamilton syringe. Equal volumes of ethanol alone (0.15 ml in 15 ml) were found to have no effct on cell morphology or permeabilty. In cases where tocopherol was to be used in conjunction with another vitamin, ghosts and tocopherol were pre-incubated for 30 min. at 37°C before addition of the other substances.

The effect of different oxygen concentrations was examined by bubbling either 100% nitrogen or 100% oxygen through the incubation medium for 15 minutes and flushing the surface for another 5 minutes prior to the addition of ghost cells. Flasks were sealed with rubber stoppers and the surface was re-flushed with nitrogen following the withdrawal of all samples. Controls received equivalent treatment with air to standardize any mechanical effects.

RESULTS

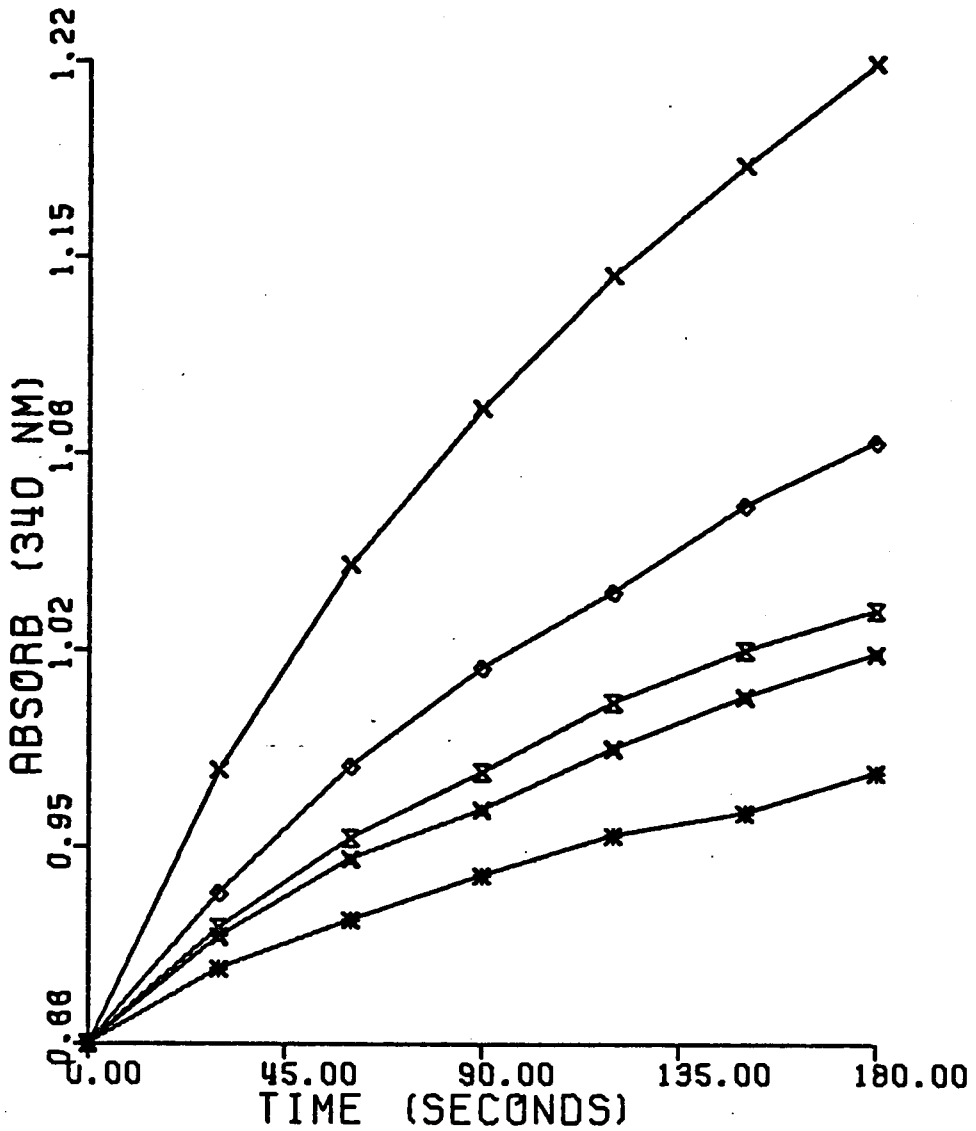
Loss of Glyceraldehyde-3-phosphate dehydrogenase activity caused by retinol

A progressive loss of glyceraldehyde-3-phosphate dehydrogenase (GAPD) activity occurrec after the addition of retinol to a suspension of ghost cells. Figure 2 shows the progress of the reaction in ghosts treated with Triton X-100 at various time intervals after the addition of 0.56 mM retinol. Representative results illustrating the time course for loss of GAPD activity with retinol at various concentrations are shown in figure 3. Because of variability in the effective concentration of retinol (see Materials and Methods), the dose response curves, while qualitatively reproducible, were not directly comparable from one experiment to the next. The following effects, however, occurred consistently in all three replications of the experiment which were carried out. Following six hours of incubation at 37°C, 0.28, 0.56, 0.85 and 1.12 mmoles of retinol per litre of 10% ghost suspension caused a loss of observable GAPD activity equal to 46, 81, 89 and 96% of the original control sample. Average loss of activity in 10 samples without retinol during an equivalent period of time was 8%. Subsequent addition of an excess (0.5mM) of the sulfhydryl compound dithiothreitol (DTT) resulted in

Figure 2. Glyceraldehyde-3-phosphate dehydrogenase activity at various time intervals in the presence of 0.56 mM retinol

A 10% ghost suspension in 0.125 mM NaCl and 20 mM PO4 buffer, pH 7.4 was mixed with 0.56 mM retinol and incubated at 37°C. 0.1ml aliquots were pre-incubated for 1 minute at 25°C with Triton X-100. Glyceraldehyde-3-phosphate dehydrogenase was assayed by addition of 30 mM sodium pyrophosphate, pH 8.4, 0.4 M sodium arsenate, 20 mM NAD and 15 mM glyceraldehyde-3-phosphoric acid. Rate of increase in absorbance at 340 nm was followed for 3 minutes at the time intervals indicated.

FIGURE 2



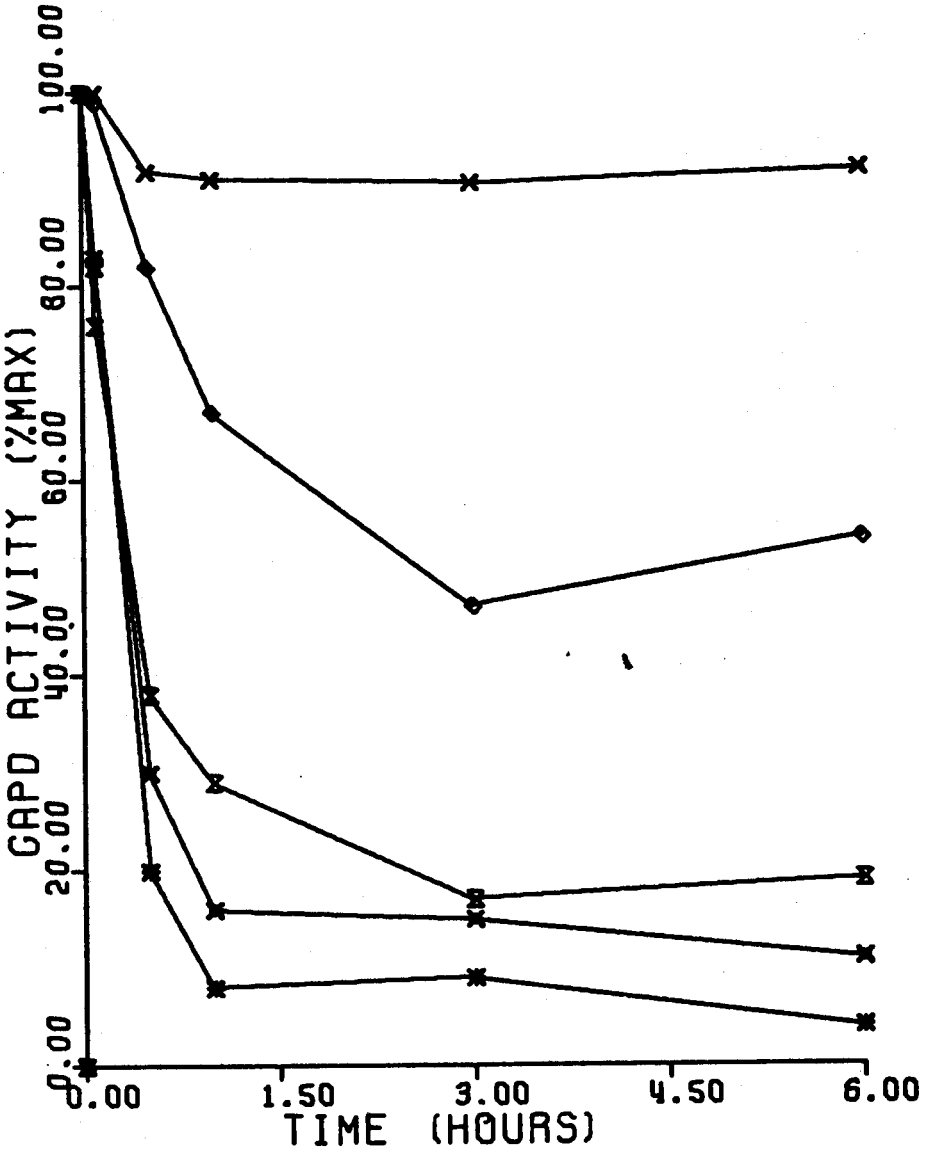
LEGEND

- TIME ZERO
- TIME 1/2 HOUR
- TIME 1 HOUR
- TIME 3 HOURS
- TIME 6 HOURS

Figure 3. Time course for loss of glyceraldehye-3-phosphate dehydrogenase activity with retinol at various concentrations

A 10% ghost suspension in 0.125 M NaCl and 20 mM PO4 buffer, pH 7.4 was mixed with retinol at various concentrations and incubated at 37°C. GAPD activity was assayed as described in figure 2. Enzyme activity is expressed as a percentage of maximal activity observed in control samples incubated in buffered isotonic solution for the same period of time and exposed to 0.2% Triton X-100 immediately before the GAPD assay.

FIGURE 3



LEGEND

- CONTROL
- 0.28 mM RETINOL
- 0.56 mM RETINOL
- 0.85 mM RETINOL
- 1.12 mM RETINOL

complete restoration of GAPD activity. Damage occurred most rapidly immediately after the addition of retinol and then more slowly until a nearly constant value was reached (figure 4). With 0.56 mmoles of retinol per litre, loss of activity occurred at an average rate of 5% per minute for the first five minutes, 1.5% per minute for the following 25 minutes, and 0.06% per minute for the remainder of the six hour incubation period. Azuma and Yoshizawa (1971) found a similar pattern in the hemolysis of erythrocytes by retinaldehyde. Damage proceeded most rapidly in the first 10 minutes and was virtually complete after 30 minutes.

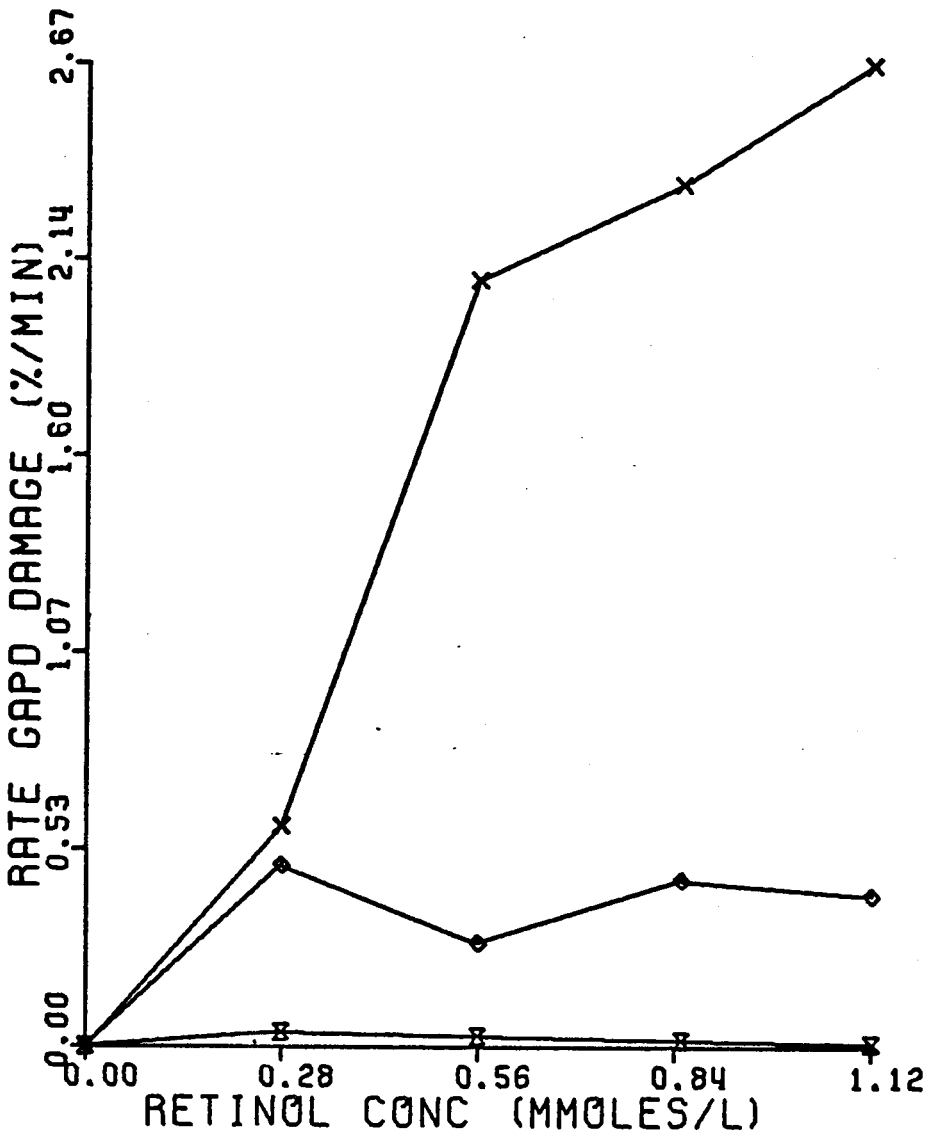
Loss of Glyceraldehyde-3-phosphate dehydrogenase activity caused by ascorbate

The time course for loss of GAPD activity with various concentrations of ascorbate is shown in figure 5. Following six hours of incubation at 37°C, 0.01, 0.1, 1.0 and 5.0 mmoles of ascorbate per litre of ghost suspension typically caused a loss of observable GAPD activity equal to 20, 47, 66 and 88% of the control sample. 10 millimolar ascorbate completely removed observable enzyme activity by the end of six hours. When 0.5 mM DTT was added after incubation the activity of GAPD was restored to control levels. Initial damage caused by ascorbate occurred much more rapidly than equimolar concentrations of retinol, even though effective

Figure 4. Effect of retinol on the initial and subsequent rates of glyceraldehyde-3-phosphate dehydrogenase destruction

A 10% ghost suspension in 0.125 M NaCl and 20 mM PO4 buffer, pH 7.4 was mixed with retinol at various concentrations and incubated at 37°C. GAPD activity was assayed as described in figure 2. The initial rate of damage to GAPD represents the first 30 minutes of incubation. Subsequent rates illustrate the second 30 minutes and final 5 hours.

FIGURE 4



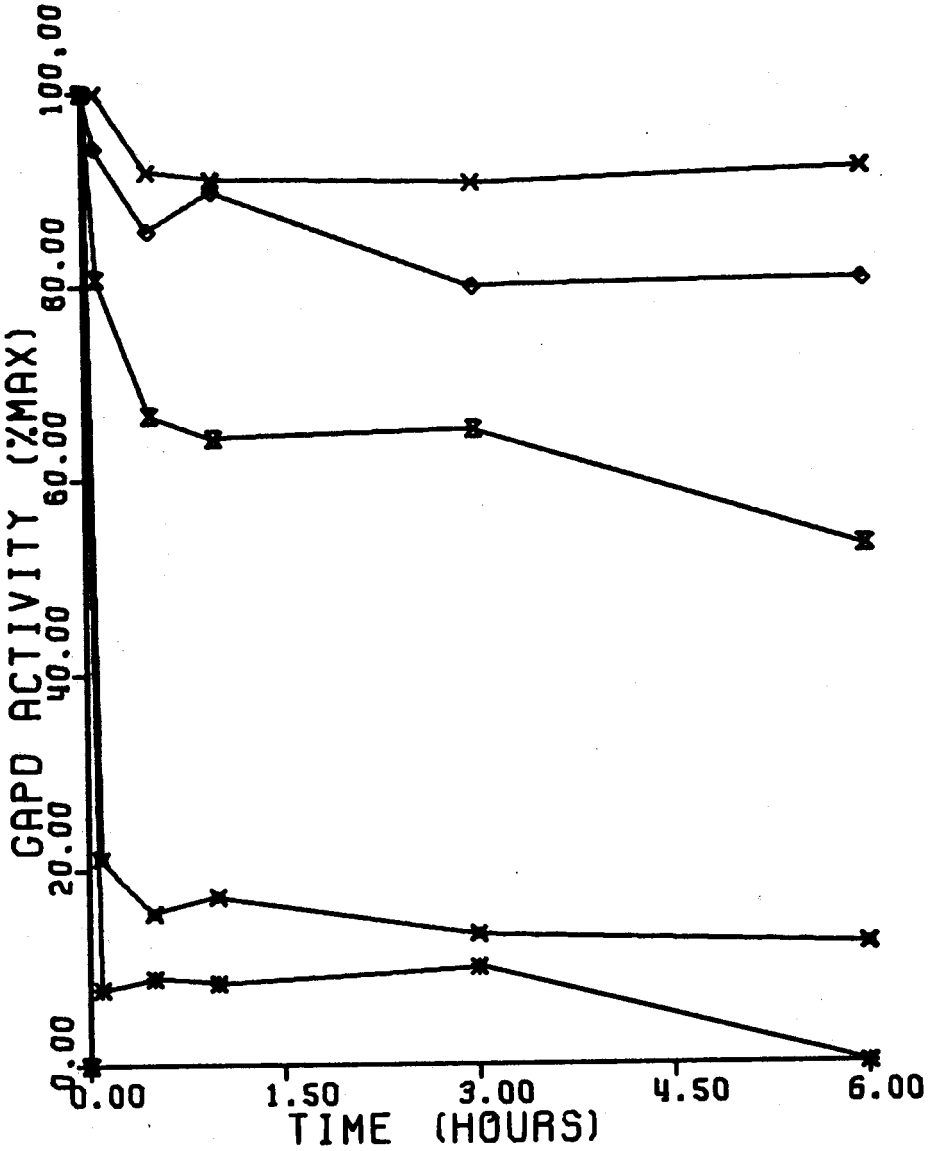
LEGEND

- TIME 0-30 MIN.
- TIME 30-60 MIN
- TIME 1-6 HOURS

Figure 5. Time course for loss of glyceraldehyde-3-phosphate dehydrogenase activity with ascorbate at various concentrations

A 10% ghost suspension in 0.125 M NaCl and 20 mM PO4 buffer, pH 7.4 was mixed with ascorbate at various concentrations and incubated ay 37°C. GAPD activity was assayed as described in figure 2. Enzyme activity is expressed as a percentage of maximal activity observed in control samples exposed to 0.2% Triton X-100.

FIGURE 5



LEGEND

- CONTROL
- 0.01 mM ASCORBATE
- 0.1 mM ASCORBATE
- 5.0 mM ASCORBATE
- 10.0 mM ASCORBATE

damage was considerably less following the first half hour of incubation. With 0.5 mmoles of ascorbate per litre, damage occurred at a rate of 9% per minute during the first 5 minutes, 0.43% for the following 25 minutes, and 0.02% per minute for the remaining 6 hours. The damaging effect appeared to reach a maximum within 30 minutes and subsequent rates of damage were negligible when compared with the initial effect (figure 6).

Effect of temperature on loss of Glyceraldehyde-3-phosphate dehydrogenase activity

Since the release of hemoglobin from erythrocytes treated with vitamin A is prevented at temperatures below 25° C (Dingle and Lucy, 1962), the temperature-dependence of loss of GAPD activity was investigated. Figure 7 shows that damage to the enzyme induced by 0.56 mmoles of retinol per litre of ghost suspension was suppressed by 53% when the medium was kept at 0° C. In the case of 5 mM ascorbate there was no slowing of GAPD inactivation at 0° C.

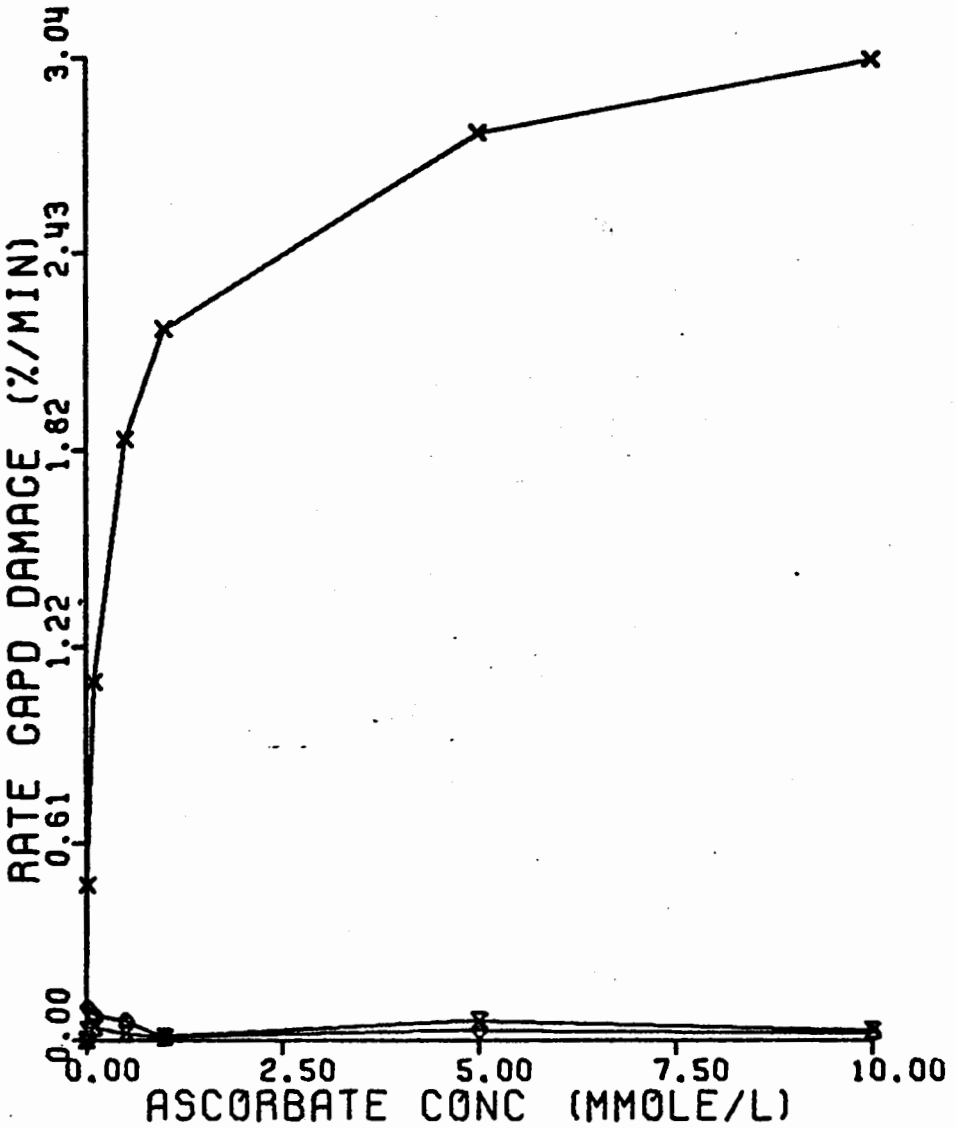
Effect of nitrogen and 100% oxygen on loss of Glyceraldehyde-3-phosphate dehydrogenase activity

Although an environment of 100% nitrogen did not prevent the initial decrease in GAPD activity caused by 0.56

Figure 6. Effect of ascorbate on initial and subsequent rates of glyceraldehyde-3-phophate dehydrogenase destruction

A 10% ghost suspension in 0.125 M NaCl and 20 mM PO4 buffer, pH 7.4 was mixed with ascorbate at various concentrations and incubated at 37°C. GAPD activity was assayed as described in figure 2. The initial rate of damage to GAPD represents the percent activity lost per minute in the first 30 minutes of incubation. Subsequent rates illustrate the second 30 minutes and final 5 hours.

FIGURE 6



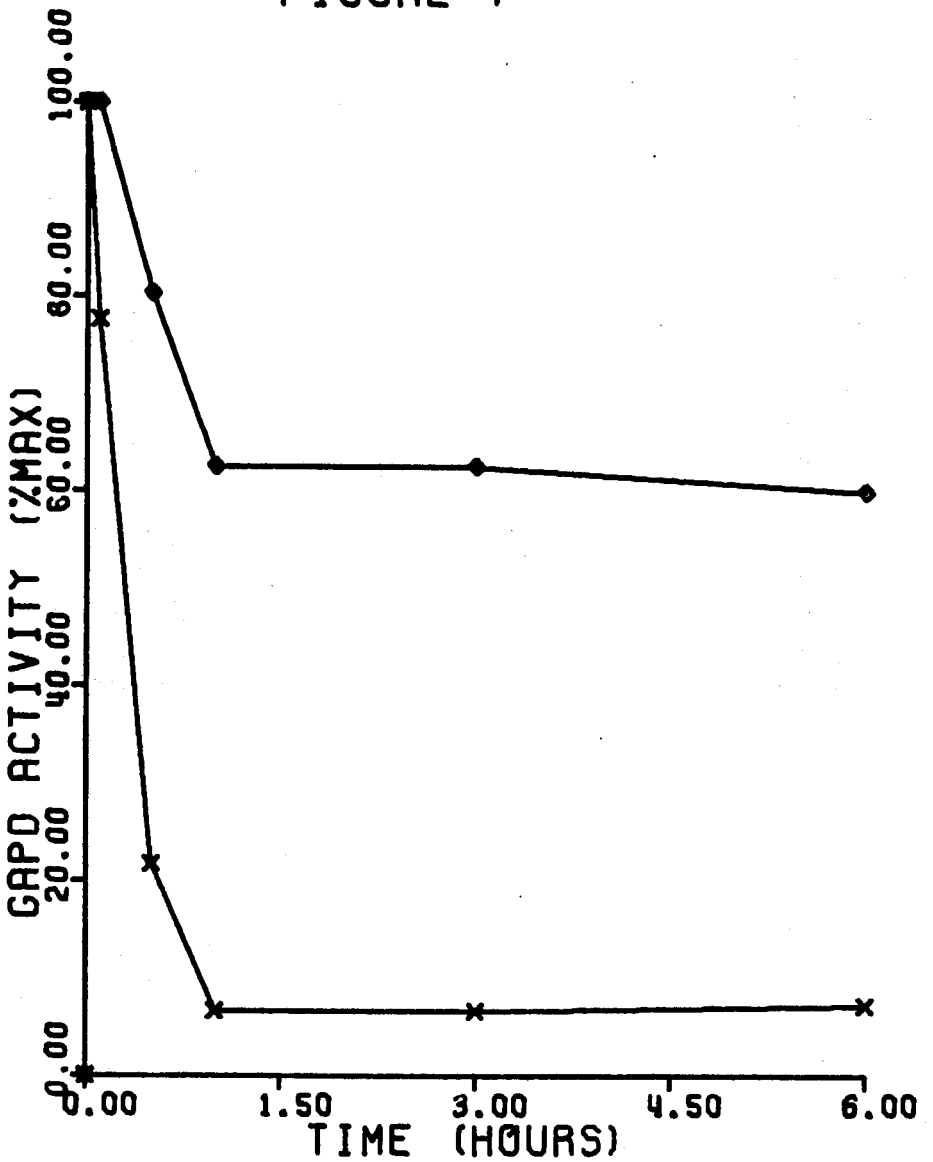
LEGEND

- TIME 0-30 MIN
- TIME 30-60 MIN
- TIME 1-6 HOURS

Figure 7. Effect of temperature on loss of glyceraldehyde-3-phosphate dehydrogenase activity during incubation with 0.56 mM retinol

A 10% ghost suspension in 0.125 M NaCl and 20 mM PO4 buffer, pH 7.4 was treated with 0.56 mM retinol and incubated both at 37°C and in an ice bath at 0°C. GAPD activity was assayed as described in figure 2, and is expressed at a percent of the maximal activity observed in control samples exposed to Triton X-100.

FIGURE 7



LEGEND

× 0.56 mM RETINOL, 37° C

◇ 0.56 mM RETINOL, 0° C

mM retinol, it did exert a protective effect following the first hour of incubation and at the end of six hours enzyme activity was approximately 3.7 times greater that in normal atmospheric conditions. Incubation under 100% oxygen also had no effect on initial GAPD destruction, however it enhanced subsequent damage and the activity remaining at the end of 6 hours was only one half of that seen in normal atmospheric conditions (figure 8). Incubation of ghost cells without retinol in the presence of oxygen also caused extensive damage to the enzyme GAPD confirming previous reports by Horn and Haugaard (1966). Observable activity following six hours of exposure to 100% oxygen was only 18% that of the control level. Enzyme activity in the presence of 5 mM ascorbate was not significantly affected by incubation under nitrogen, and 100% oxygen resulted in only a margiral acceleration of inactivation of the enzyme (figure 9).

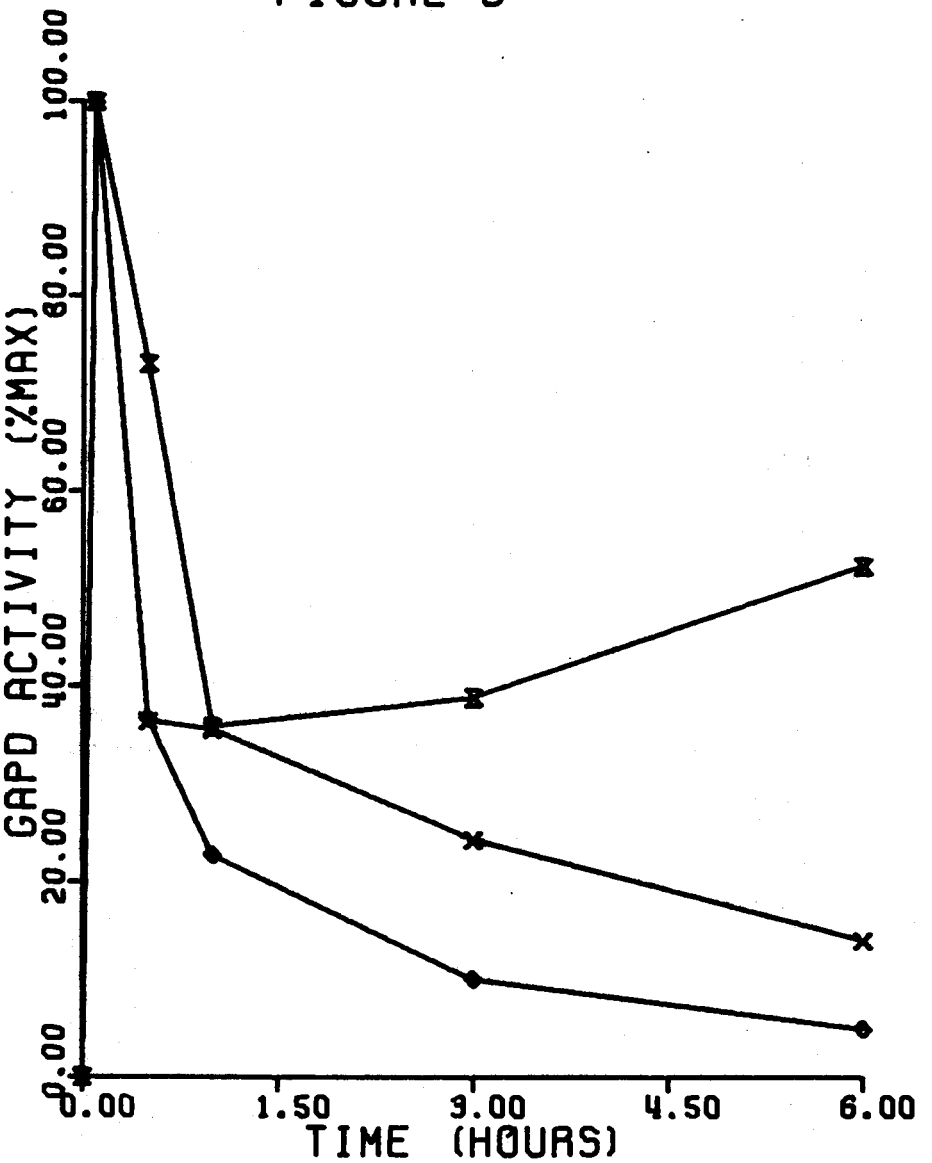
Loss of Glyceraldehyde-3-phosphate dehydrogenase activity caused by ascorbate and retirol

Figure 10 illustrates the effects of 0.56 mM retinol on GAPD activity in the presence of both high (1 mM) and low (0.01 mM) concentrations of ascorbate. Loss of enzyme activity was initially much more rapid in the presence of both 1 mM ascorbate and 0.56 mM retinol than with either

Figure 8. Effect of nitrogen and 100% oxygen on loss of glyceraldehyde-3-phosphate dehydrogenase activity induced by 0.56 mM retinol

Incubation media consisting of 0.125 M NaCl, 20 mM PO4 buffer, pH 7.4 and 0.56 mM retinol were bubbled for 15 minutes with either 100% oxygen or 100% nitrogen. The surface was flushed for a further 5 minutes, and ghost cells were added to make a 10% suspension. Flasks were sealed with rubber stoppers and the surface was re-flushed with the appropriate gas mixture following the withdrawal of all samples. GAPD activity was assayed as described in figure 2 and is expressed as a percent of the maximal activity observed in control samples exposed to Triton X-100.

FIGURE 8



LEGEND

× 0.56 mM RET

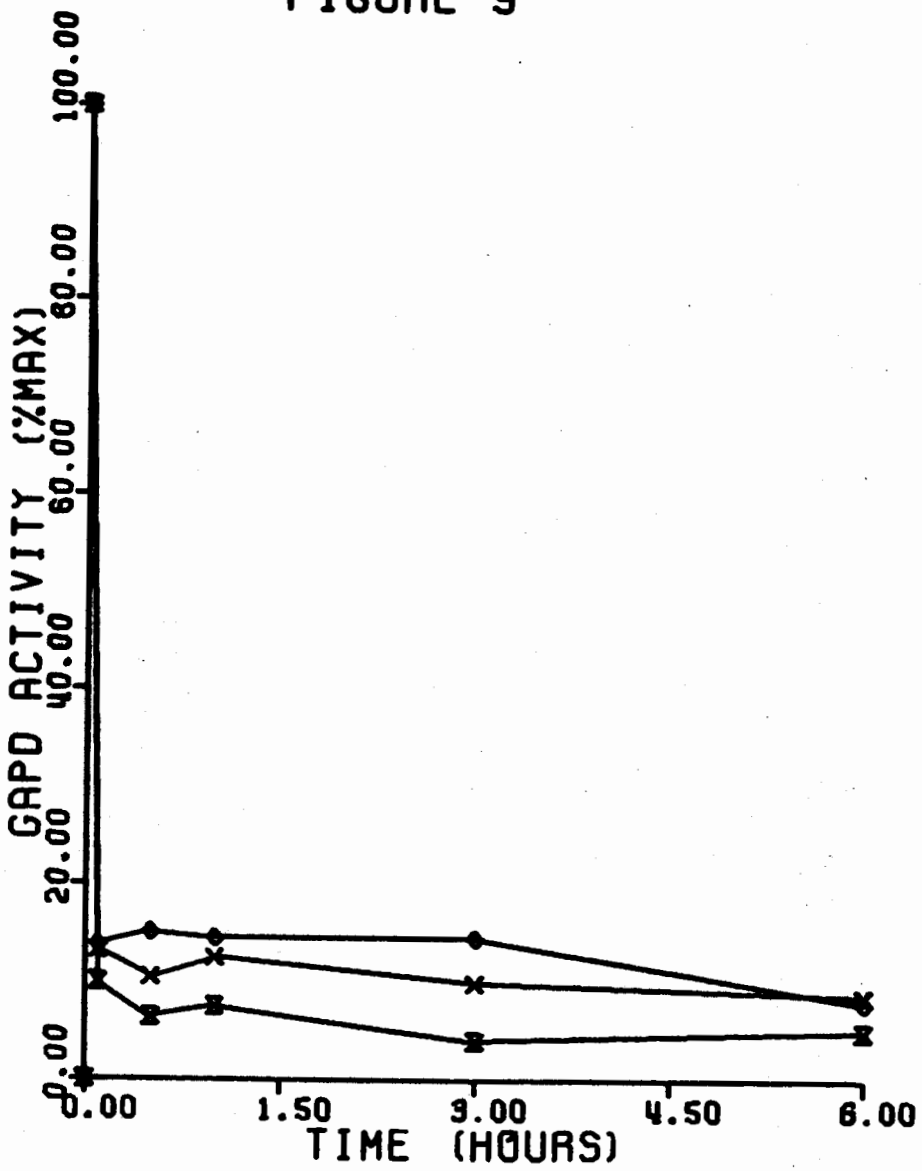
◇ 0.56 mM RET, NITROGEN

⧖ 0.56 mM RET, OXYGEN

Figure 9. Effect of nitrogen and 100% oxygen on loss of glyceraldehyde-3-phosphate dehydrogenase activity induced by 5 mM ascorbate

Incubation media consisting of 0.125 M NaCl, 20 mM PO4 buffer, pH 7.4 and 5 mM ascorbate were bubbled with either 100% oxygen or 100% nitrogen for 15 minutes. The surface was flushed for a further 5 minutes and ghost cells were added to make a 10% suspension. Flasks were sealed with rubber stoppers and the surface was re-flushed with the appropriate gas mixture following the withdrawal of all samples. GAPD activity was assayed as described in figure 2, and is expressed as a percent of the maximal activity observed in control samples incubated in buffered isotonic solution for the same period of time and exposed to Triton X-100 immediately before the GAPD assay.

FIGURE 9



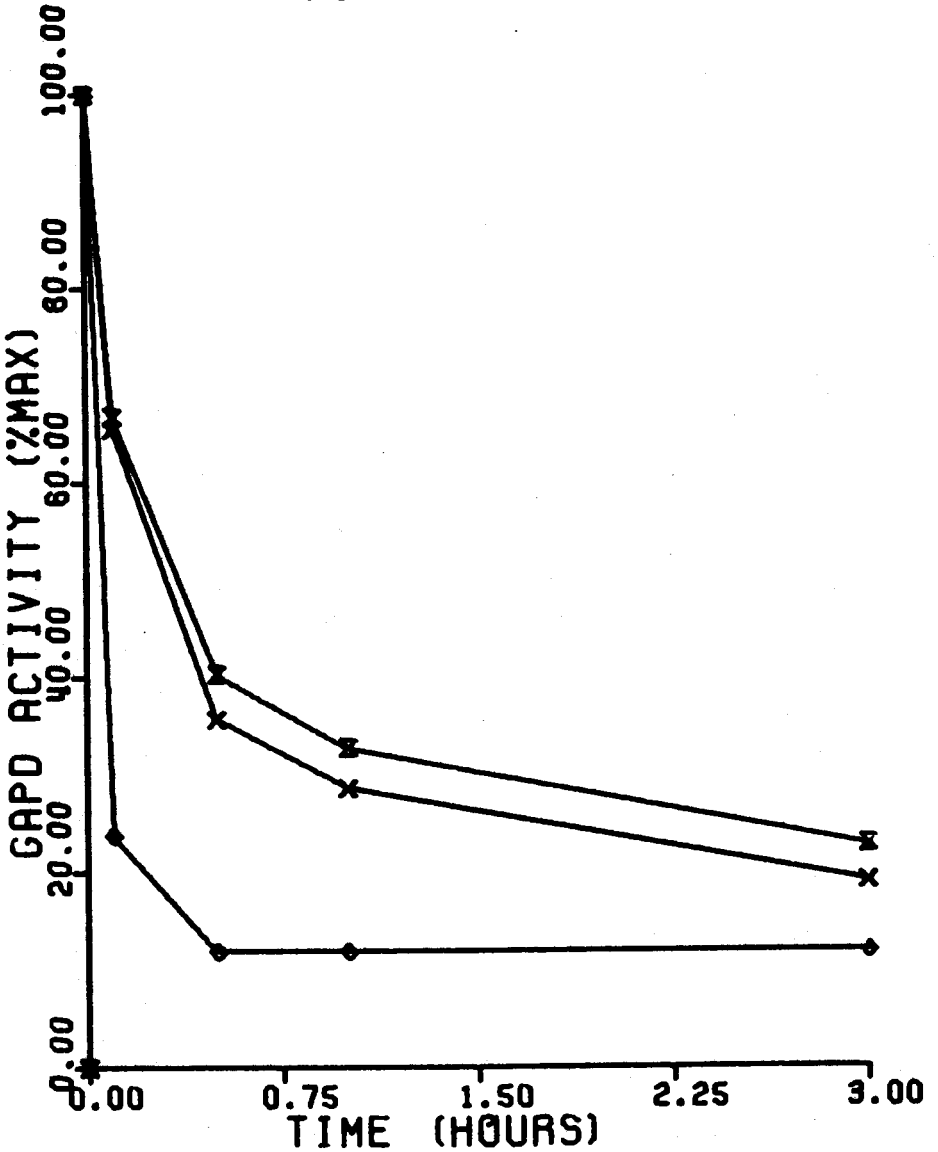
LEGEND

- 5 mM ASCORB
- 5 mM ASCORB, NITROGEN
- 5 mM ASCORB, OXYGEN

Figure 10. Loss of glyceraldehyde-3-phosphate dehydrogenase activity in the presence of ascorbate plus retinol

A 10% ghost suspension in 0.125 M NaCl and 10 mM PO4 buffer, pH 7.4 was mixed with 0.56 mM retinol and either 1.0 or 0.01 mM ascorbate. Incubation was at 37°C. GAPD activity was assayed as described in figure 2 and is expressed as a percent of the maximal activity observed in control samples incubated in buffered isotonic solution for the same period of time and exposed to Triton X-100 immediately before the GAPD assay.

FIGURE 10



LEGEND

- 0.56 mM RET
- 0.56 mM RET, 1 mM ASCORB
- 0.56 mM RET, 0.01 mM ASC

alone and leveled off in one hour to approximately 60% of the value with retinol alone. This effect was observable with ascorbate concentrations greater than 0.5 mM and the extent of damage was determined by the amount of ascorbate present. The additive effect of 10 mM ascorbate was so great as to completely destroy any observable activity within one half hour. In three out of four repeated experiments, 0.01 mM ascorbate produced a protective effect which was sigrificant at the 0.05 level of confidence. At 0.1 mM ascorbate the protective and damaging effects cancelled each other out, and the result was a net zero effect on retinol-induced GAPD destruction.

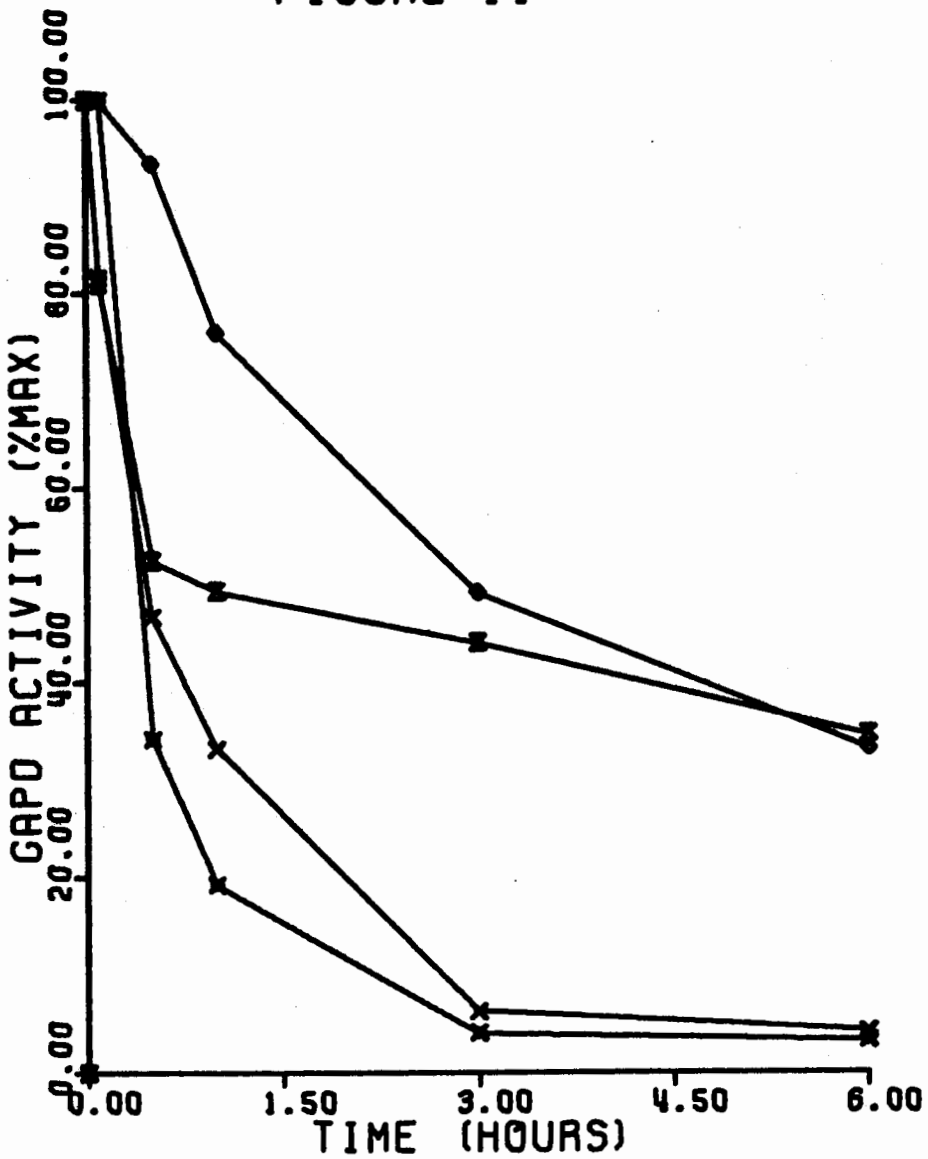
Effect of α-tocopherol on loss of Glyceraldehyde-3-phosphate dehydrogenase activity

Incubation of ghosts with 0.116, 0.23 or 0.56 mM tocopherol did not alter GAPD activity from control values. Inclusion of 0.116 and 0.56 mM α-tocopherol in the incubation medium with 0.56 mM retinol had a protective effect on GAPD which maintained enzyme activity at the end of 6 hours at a level 30% higher than with retinol alone. Although the effect of higher tocopherol concentrations was not observable in the first half hour of incubation, 0.116 mM produced immediate results (figure 11). Protective effects were not seen with tocopherol acetate (0.106 mM)

Figure 11. Effect of α-tocopherol on loss of glyceraldehyde-3-phosphate dehydrogenase activity caused by 0.56 mM retinol

10% ghost suspensions were pre-incubated for 30 minutes with 0.12 mM tocopherol, 0.56 mM tocopherol, or 0.106 mM tocopherol acetate. Sufficient retinol was added so that final retinol concentration was 0.56 mM mmoles/litre. GAPD activity was assayed as described in figure 2, and is expressed as a percentage of the maximal activity observed in control samples incubated in buffered isotonic solution for the same period of time and exposed to Triton X-100 immediately before the GAPD assay.

FIGURE 11



LEGEND

- 0.56 mM RET
- 0.56 mM RET, 0.12 mM TOC
- 0.56 mM RET, 0.56 mM TOC
- RET, 0.106 mM TOC ACET

which actually enhanced the damaging effect of retinol. The enzyme damage induced by 1 mM ascorbate after 6 hours was decreased by 10 and 25% with 0.56 and 0.116 mM tocopherol respectively (figure 12).

Development of lipid "peroxidation" in the presence of retinol

Formation of lipid peroxides as a function of time and exposure to retinol is shown in figure 13. In contrast to the sudden effect of retinol on GAPD activity, which did not progress substantially after the first hour, its effect on lipid "peroxidation" was slower and more progressive.

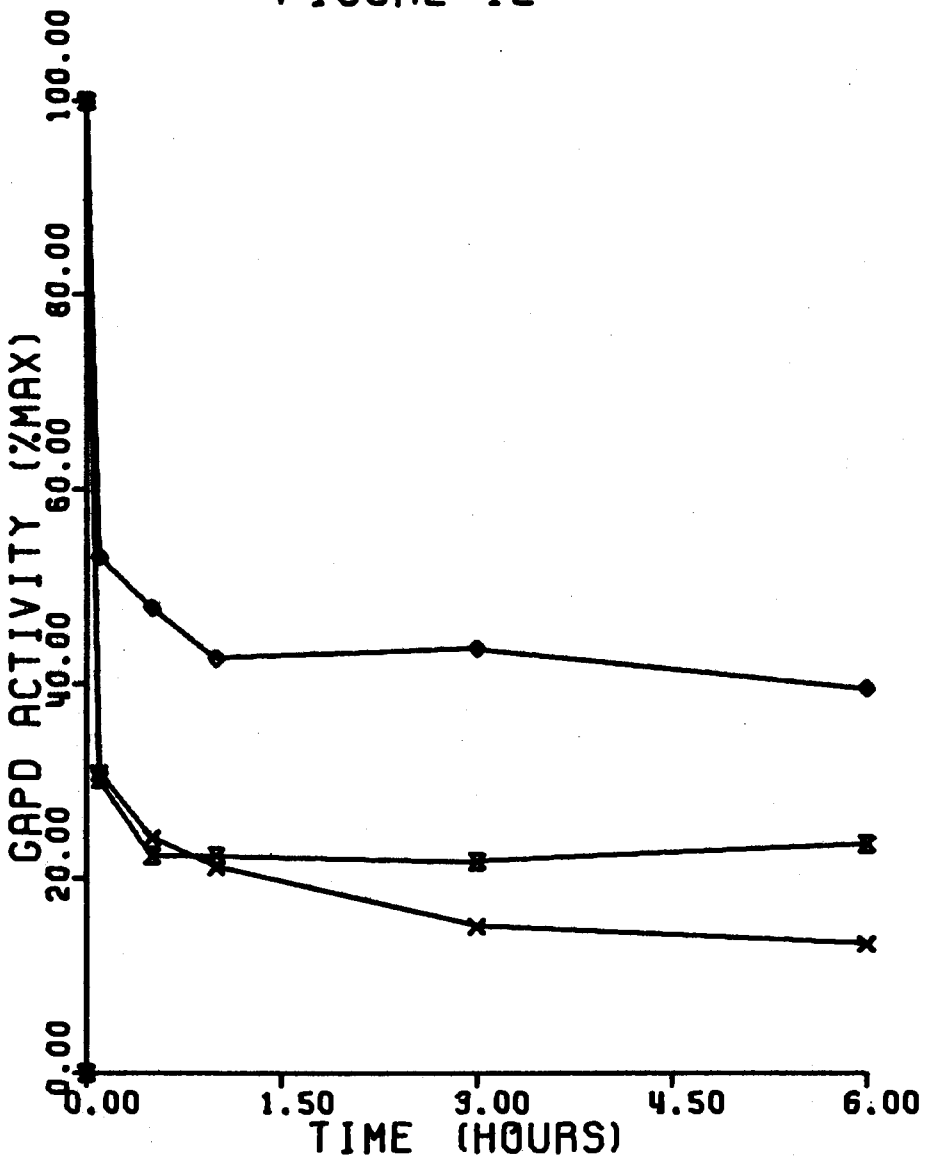
Development of lipid "peroxidation" in the presence of ascorbate

The development of absorbance at 532 nm as a function of time and different concentrations of ascorbate is shown in figure 14. The level of lipid peroxides at 0.01 mM ascorbate did not change appreciably following the first 5 minutes of incubation and was not significantly greater than control values. With concentrations greater than 1.0 mM ascorbate, the initial value remained constant for 6 hours and then doubled by 24 hours. It is of interest that a concentrated ascorbate solution will, if left at 37°C for 24

Figure 12. Effect of α-tocopherol on loss of glyceraldehyde-3-phosphate dehydrogenase caused by 1 mM ascorbate

10% ghost supensions were pre-incubated for 30 minutes with 0.12 mM tocopherol, 0.56 mM tocopherol, or 0.106 mM tocopherol acetate. Sufficient ascorbate was added so that final concentration was 1 mmole/litre. GAPD activity was assayed as described in figure 2, and is expressed as a percent of the maximal activity observed in control samples incubated in buffered isotonic solution for the same period of time and exposed to Triton X-100 immediately before the GAPD assay.

FIGURE 12



LEGEND

× 1 mM ASC

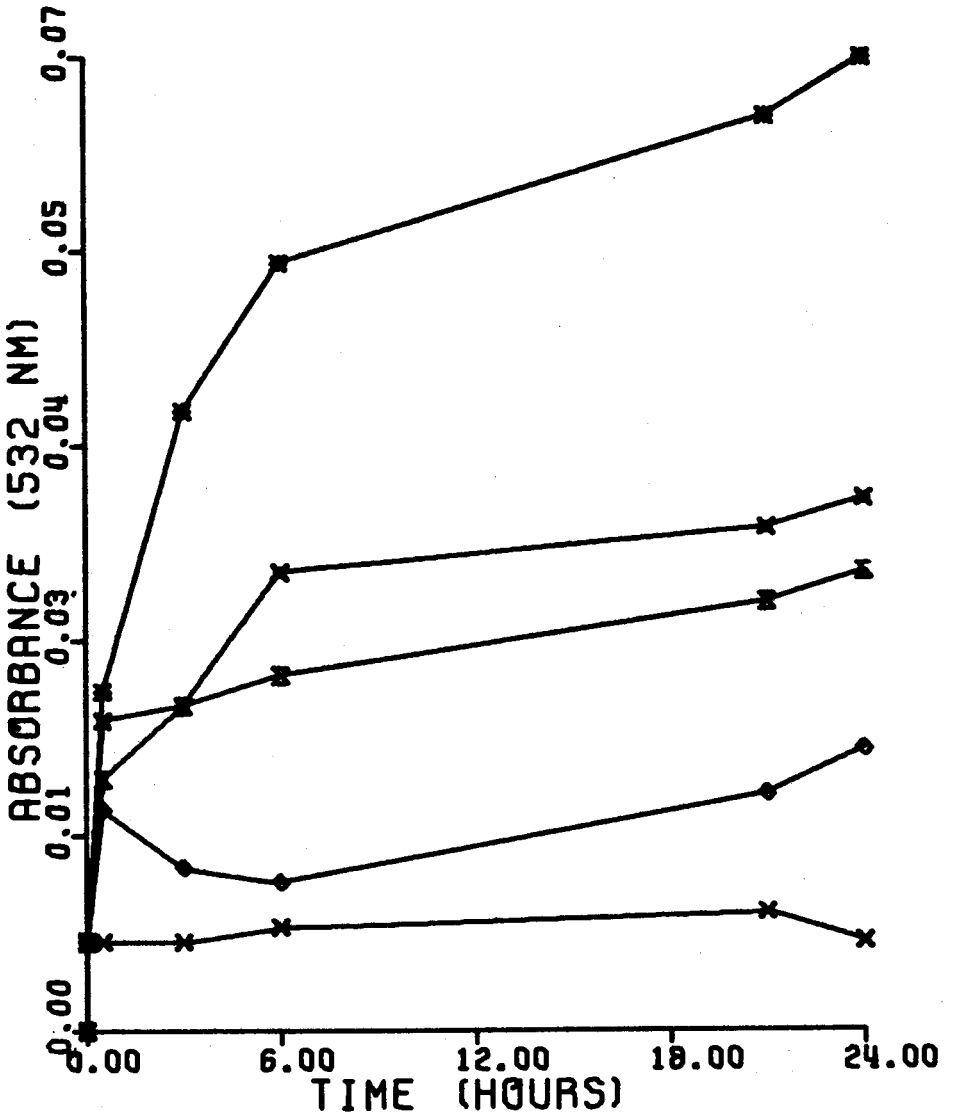
◇ 1 mM ASC, 0.12 mM TOC

⧖ 1 mM ASC, 0.56 mM TOC

Figure 13. Progress of lipid "peroxidation" in ghost membranes with retinol at various concentrations

A 10% ghost suspension in 0.125 M NaCl and 20 mM PO4 buffer, pH 7.4 was mixed with retinol at various concentrations and incubated at 37°C. 2 ml aliquots were mixed with 10% trichloroacetic acid and 0.67% thiobarbituric acid, placed for 10 minutes in a boiling water bath, cooled and centrifuged. Complete spectra of the supernatant were measured between 420 and 600 nm. Absorbance at 532 nm is plotted as a function of time of incubation of the ghost cells in the presence of retinol.

FIGURE 13



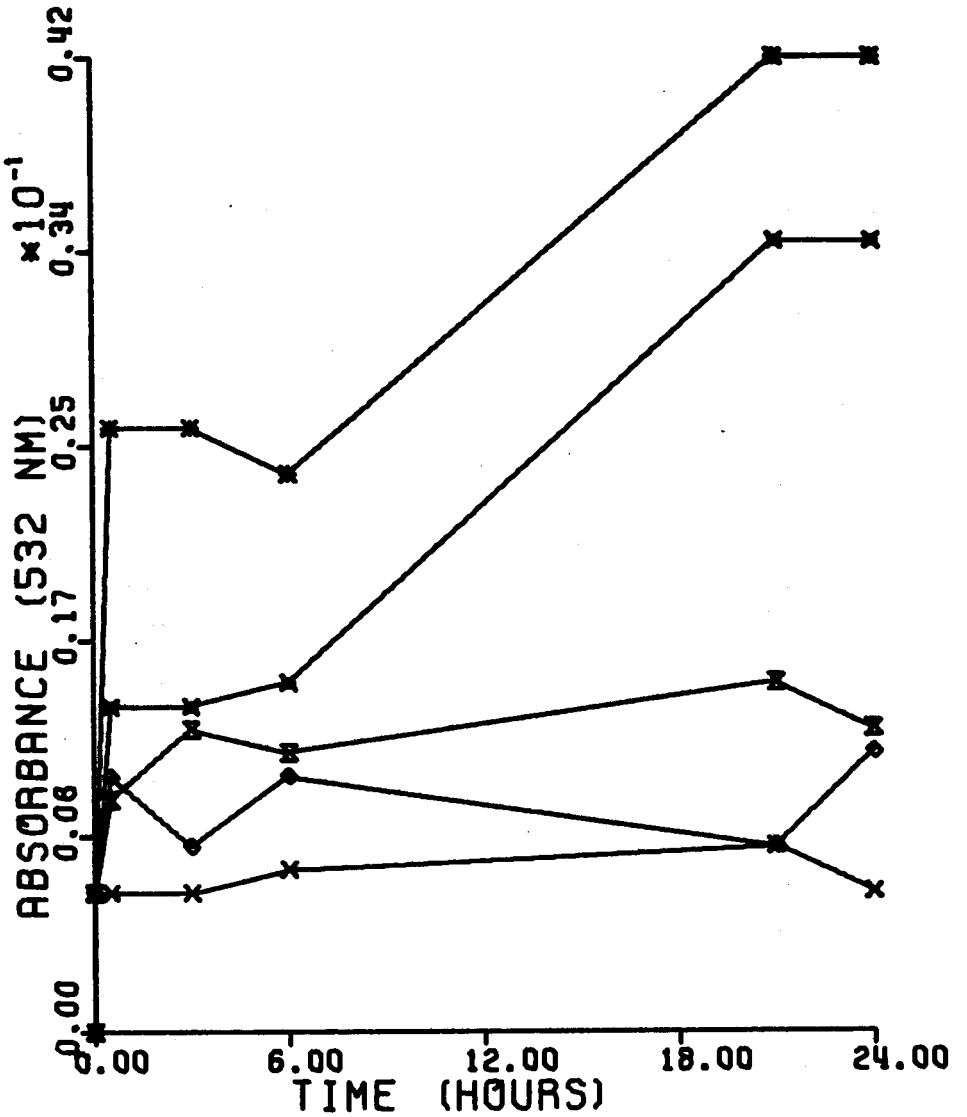
LEGEND

- CONTROL
- 0.28 mM RETINOL
- 0.56 mM RETINOL
- 0.705 mM RETINOL
- 1.12 mM RETINOL

Figure 14. Progress of lipid "peroxidation" in ghost membranes with ascorbate at various concentrations

A 10% ghost suspension in 0.125 M NaCl and 20 mM PO4 buffer, pH 7.4 was mixed with ascorbate at various concentrations and incubated at 37°C. Lipid peroxides were measured as described in figure 13.

FIGURE 14



LEGEND

- CONTROL
- 0.01 mM ASCORBATE
- 0.1 mM ASCORBATE
- 5.0 mM ASCORBATE
- 10.0 mM ASCORBATE

hours, assume a yellowish-orange color with an absorbance peak at 508 nm. This results in a slight interference with measurements taken at 532 nm and a characteristic peak was not observed at any of the ascorbate concentrations tested, with the result that absorbance at 532 nm was a slight underestimate (approximately 13%) of the amount of lipid "peroxidation".

Effect of temperature on lipid "peroxidation"

Cooling the incubaton medium to 0°C did not hinder the development of lipid peroxidation at any of the retinol concentrations tested. With 5 mM ascorbate a decrease in temperature similarily had no effect during the first six hours of incubation, however 24 hour TBA readings at 0°C were only one third of those at 37°C. This may be explained in part by the fact that decreased temperature slows the oxidation of concentrated ascorbate solutions, thereby eliminating the peak at 508 nm. As the appearance of dehydroascorbate and the doubling of "peroxidation" normally occurr at the same time, and because decreased temperature inhibits both effects, it is likely that the two are causally related.

Effect of nitrogen and 100% oxygen on lipid "peroxidation"

In the presence of 100% oxygen, the lipid "peroxidation" caused by 0.56 mM retinol increased to 1.5 times that in normal atmospheric conditions within the first 5 minutes. After 6 hours the value was doubled and after 24 hours under oxygen the absorbance was 3.5 times that in normal atmosphere. Nitrogen inhibited peroxidative damage, but only by approximately 50% (figure 15). The presence of 100% oxygen with 5 mM ascorbate did not affect TBA readings during the entire 24 hour incubation period. Nitrogen also had no effect during the first 6 hours, but absorbance readings at 24 hours were approximately 60% less than in normal atmospheric conditions. That nitrogen did not appear completely able to inhibit peroxidative changes presumably indicates that complete anaerobic conditions were not attained.

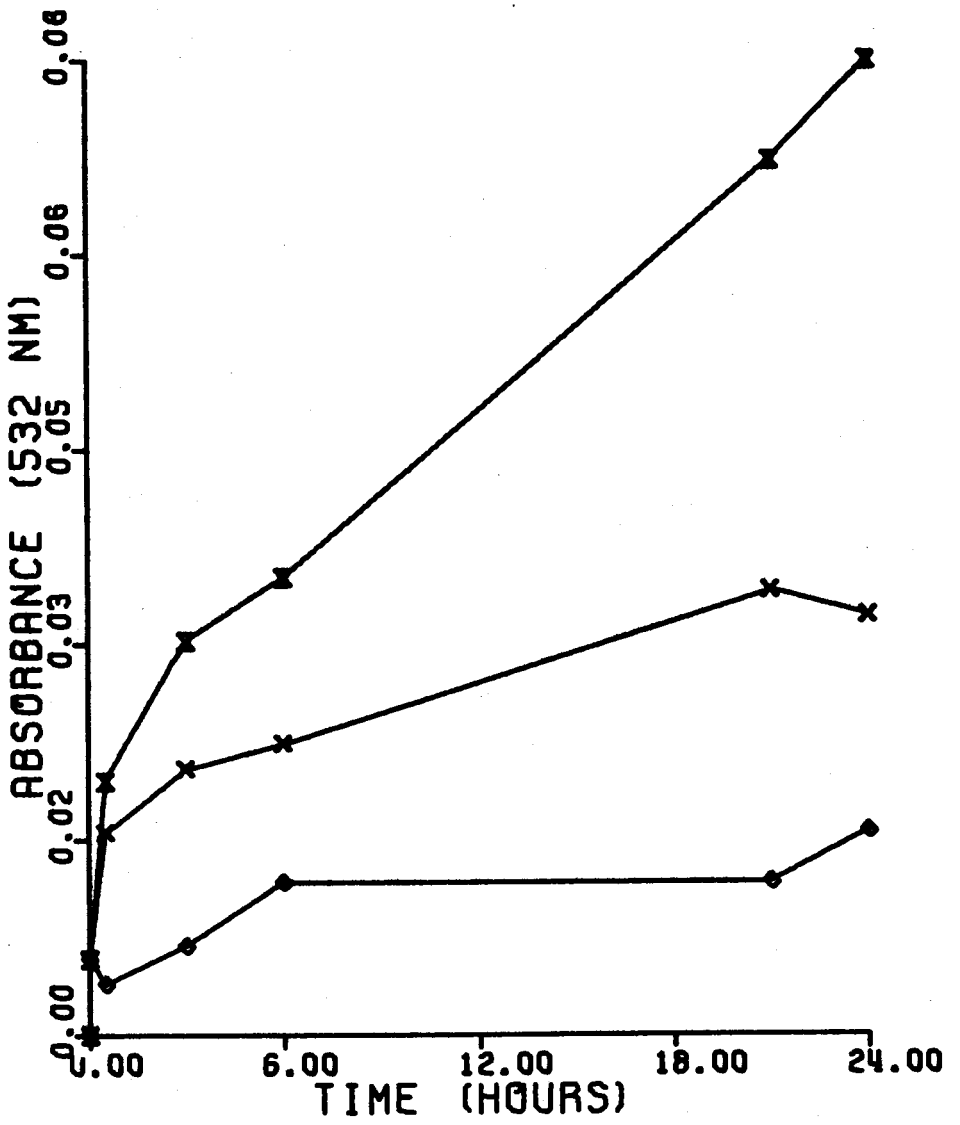
Development of lipid "peroxidation" in the presence of ascorbate and retinol

The presence of ascorbate had no significant effect either at high or low concentrations on lipid peroxidation induced by retinol. Only in systems involving retinol and 10 mM ascorbate was there any observable increase in absorbance at 532 nm and this occurred only after 24 hours.

Figure 15. Effect of nitrogen and 100% oxygen on lipid "peroxidation" in ghost membranes in the presence of 0.56 mM retinol

Ghost suspensions containing 0.56 mM retinol were bubbled with either 100% oxygen or 100% nitrogen as described in figure 8. Lipid peroxides were determined as described in figure 13.

FIGURE 15



LEGEND

- 0.56 mM RETINOL
- 0.56 mM RET. NITROGEN
- 0.56 mM RET. OXYGEN

Effect of α-tocopherol on lipid "peroxidation"

The level of lipid peroxides formed in 24 hours as a result of 0.56 mM retinol was decreased by 66% in the presence of 0.116 mM tocopherol, an effect which was fairly consistant throughout the entire incubation period. Higher concentrations of tocopherol were less effective and, although 0.56 mM tocopherol initially caused a 40% inhibition of observable lipid peroxidation, by 21 hours there was no significant difference in levels with or without the presence of tocopherol (figure 16). TBA readings in the presence of 10 mM ascorbate showed a slight increase with tocopherol, however by 24 hours this was reversed and absorbance at 532 nm with 0.116 and 0.56 mM tocopherol was only 57 and 68% of that with ascorbate alone.

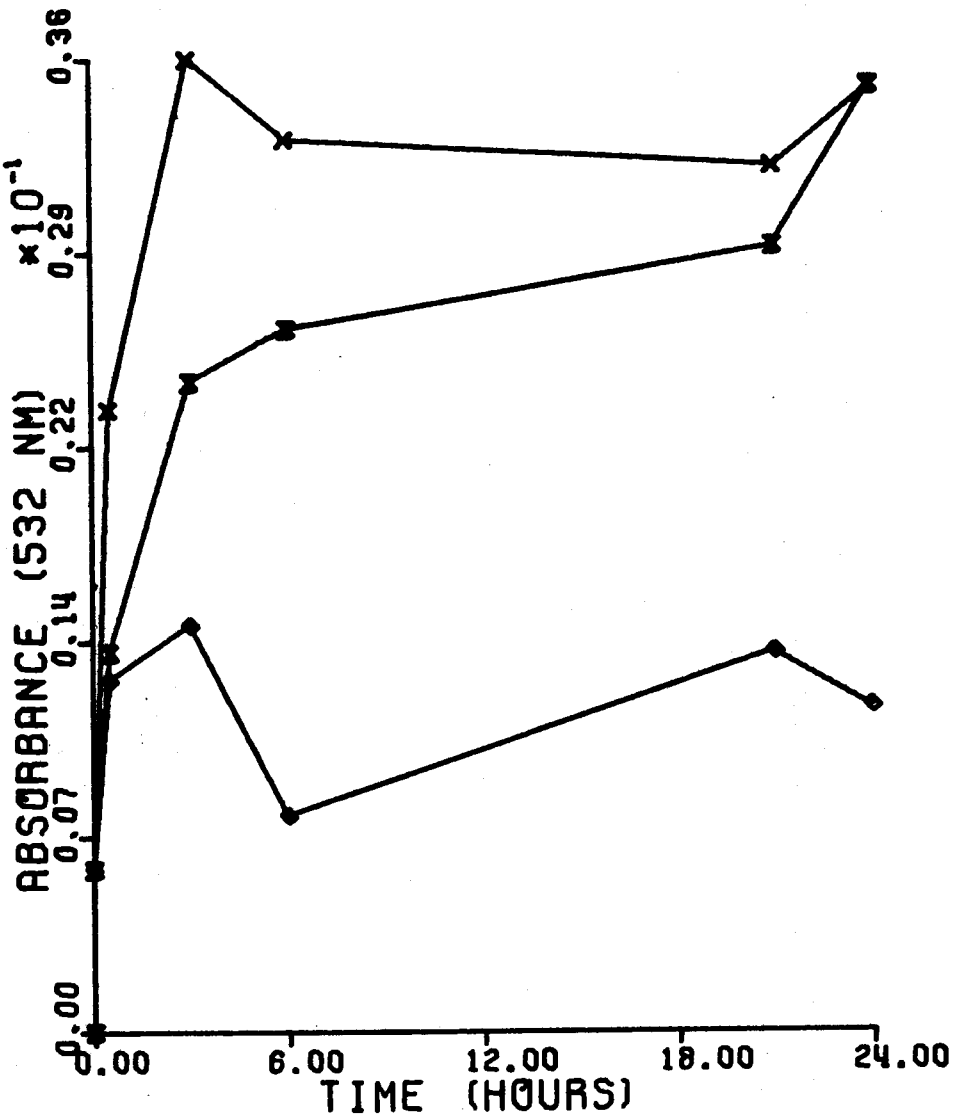
Increase in membrane permeabilty with retinol

Figure 17 illustrates the change in membrane permeability as a function of time and retinol concentration. The effect of 1.12 mM retinol is not included since the enzyme was extensively damaged, thus reducing the reliability of permeabilty calculations. One of the biggest problems encountered in determining GAPD accessibilty in the manner described is that the enzyme is

Figure 16. Effect of α-tocopherol on lipid "peroxidation" in ghost membranes in the presence of 0.56 mM retinol

10% ghost suspensions were pre-incubated at 37°C for 30 minutes with 0.12 mM or 0.56 mM α-tocopherol. Sufficient retinol was added so that final retinol concentration was 0.56 mmoles/litre. Lipid peroxides were measured as in figure 13.

FIGURE 16



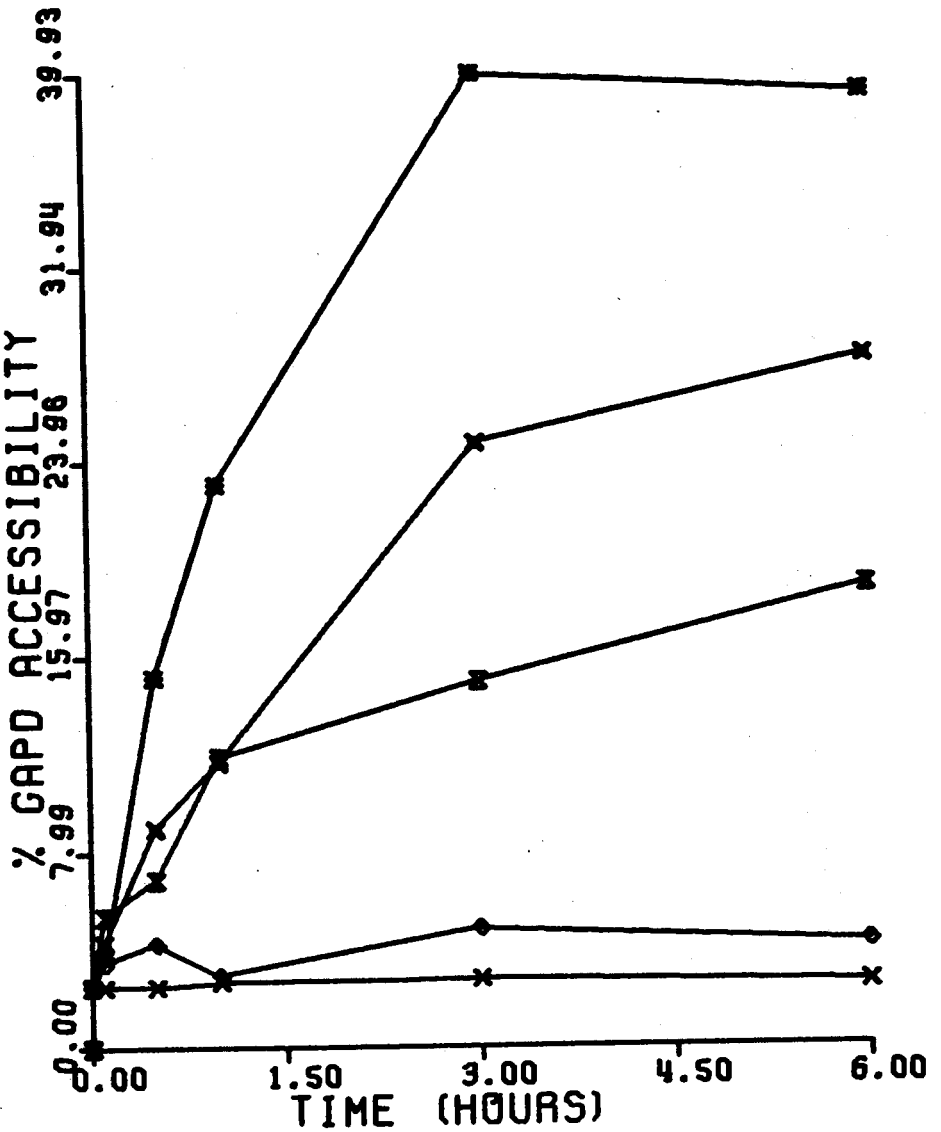
LEGEND

- 0.56 mM RETINOL
- 0.56 mM RET, 0.12 mM TOC
- 0.56 mM RET, 0.56 mM TOC

Figure 17. Effect of various concentrations of retinol on membrane permeability as measured by accessibility of glyceraldehyde-3-phosphate dehydrogenase to substrates located outside the cell membrane

A 10% ghost suspension in 0.125 M NaCl and 20 mM PO4 buffer, pH 7.4 was mixed with retinol at various concentrations. GAPD activity was assayed as described in figure 2. The fraction of total GAPD activity which was accessible without Triton X-100 to substrates added outside the cell membrane is plotted as a function of time and exposure to retinol. Total GAPD activity was estimated using ghosts completely disrupted by triton X-100.

FIGURE 17



LEGEND

- CONTROL
- 0.28 mM RETINOL
- 0.56 mM RETINOL
- 0.705 mM RETINOL
- 0.85 mM RETINOL

progressively destroyed with time. Determination of permeability thus becomes a comparison between two small values rather than a large value with triton and a small value without. One way of overcoming this is to perform the assay with dithicthreitol (DTT), both in the presence and absence of Triton X-100, and to calculate permeability from values obtained by this procedure. Inactivation of GAPD would not, in this case, interfere with permeability calculations, as inclusion of DTT with triton and ghost cells allows a more accurate measurement of enzyme activity in totally permeable cells. If the membrane has been damaged and made permeable to substrates added on the outside, then DTT will enter and reactivate the damaged GAPD. The reaction without triton will proceed to the extent that the DTT can pass through the membrane and reactivate the enzyme, i.e. according to the permeabilty of the membrane.

In cells which were tightly sealed the extent of NADH accumulation was small, and rates were taken as the average between the extreme maximum and minimum possible values. Low concentrations of retinol (0.28 mM) did not increase permeabilty to a large extent, however the change in 5 repeated experiments was significant at the 0.05 level of confidence when compared with control values which

maintained a 2.5% permeability throughout the test period. By 1 hour changes in permeability had proceeded to 60% of their final values and by the end of 3 hours of incubation the process appears complete.

Effect of ascorbate on membrane permeability

The changes in membrane permeabilty caused by various concentrations of ascorbate are illustrated in figure 18. Concentrations less than 1 mM ascorbate caused very little change in permeability compared with control samples. Although 5 mM ascorbate appeared to cause a significant increase in membrane permeability, there is a large amount of scatter in the data, and it is likely that the effect is due to increased enzyme damage and decreased reliability of permeability calculations as described above. Data for 10 mM ascorbate could not be calculated because of extensive enzyme damage.

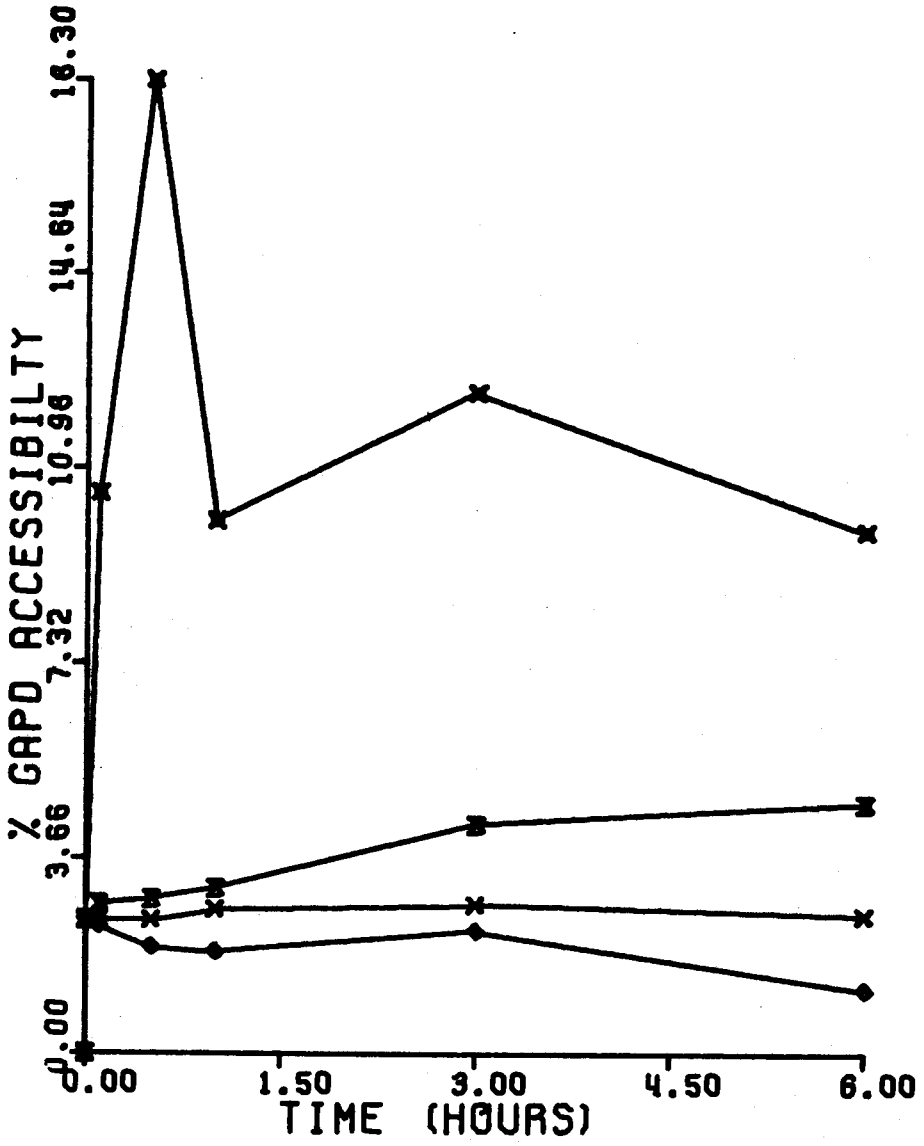
Effect of temperature on membrane permeabilty

The increase in permeability caused by 0.56 mM retinol at 37°C is inhibited by incubation at 0° C as illustrated in figure 19. Damage produced by 5 mM ascorbate was, however, equally great at 0°C as at 37°C.

Figure 18. Effect of various concentrations of ascorbate on membrane permeability as measured by accessibility of glyceraldehyde-3-phosphate dehydrogenase to substrates located outside the cell membrane

A 10% ghost suspension in 0.125 M NaCl and 20 mM PO4 buffer, pH 7.4 was mixed with ascorbate at various concentrations. GAPD activity was assayed as described in figure 2. The fraction of total GAPD activity which was accessible without triton to substrates added outside the cell membrane is plotted as a function of time and exposure to retinol. Total GAPD activity was estimated using ghosts completely disrupted by triton X-100.

FIGURE 18



LEGEND

CONTROL

0.01 mM ASCORBATE

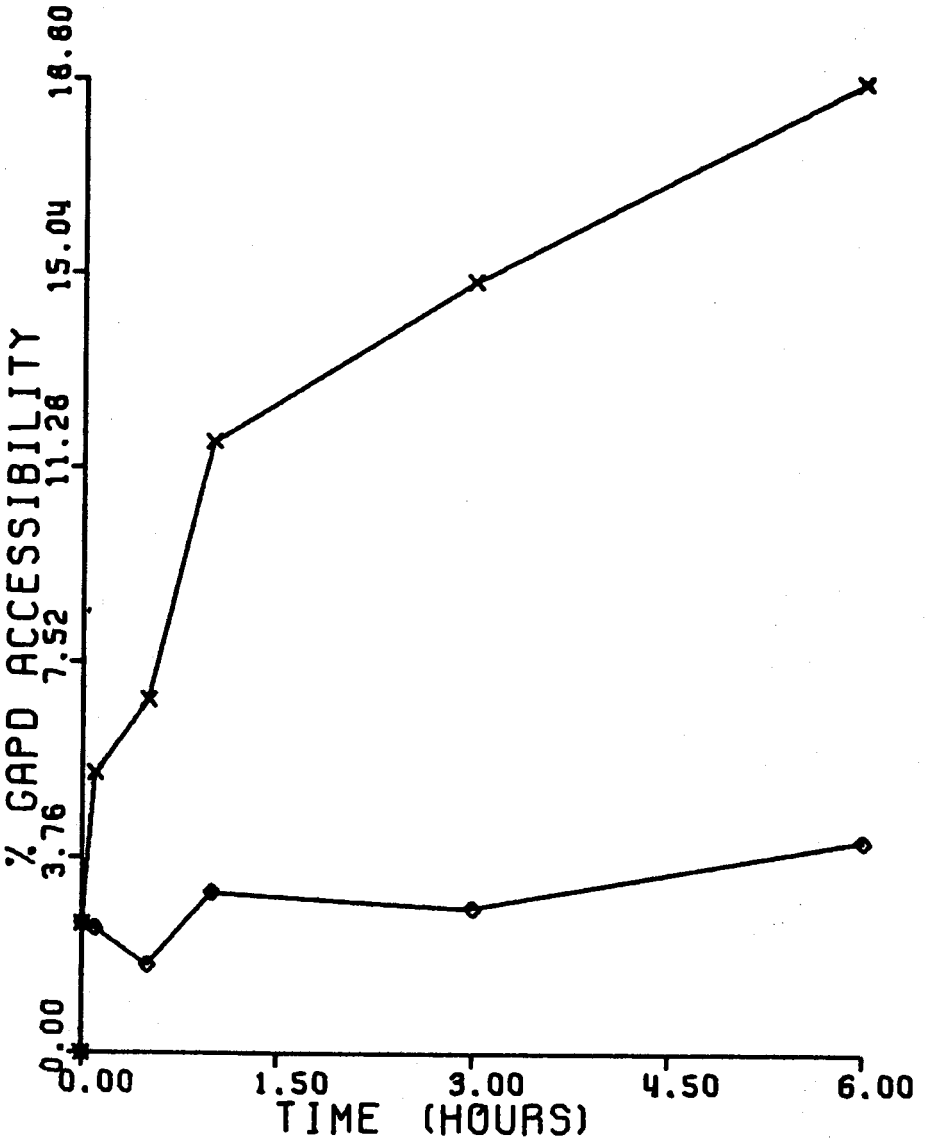
1.0 mM ASCORBATE

5.0 mM ASCORBATE

Figure 19. Effect of temperature on changes in membrane permeability in the presence of 0.56 mM retinol

A 10% ghost suspension in 0.125 M NaCl and 20 mM PO4 buffer, pH 7.4 was treated with 0.56 mM retinol and incubated at both 37°C and in an ice bath at 0°C. GAPD activity was assayed as described in figure 2. Changes in permeability were measured as described in figure 17.

FIGURE 19



LEGEND

× 0.56 mM RETINOL, 37° C

◇ 0.56 mM RETINOL, 0° C

Effect of nitrogen and 100% oxygen on membrane permeability

Although incubation under nitrogen did not inhibit the initial increase in permeability caused by 0.56 mM retinol, it did retard the process following the first hour of exposure (figure 20). 100% oxygen doubled the membrane permeability within 1 hour with retinol, ascorbate and in control samples.

Combined effect of ascorbate and retinol on membrane permeability

Because of extensive enzyme damage in the presence of both ascorbate and retinol it was difficult to determine their combined effects on membrane permeability. It appears, however, that ascorbate does not have an effect on the increased permeability induced by retinol.

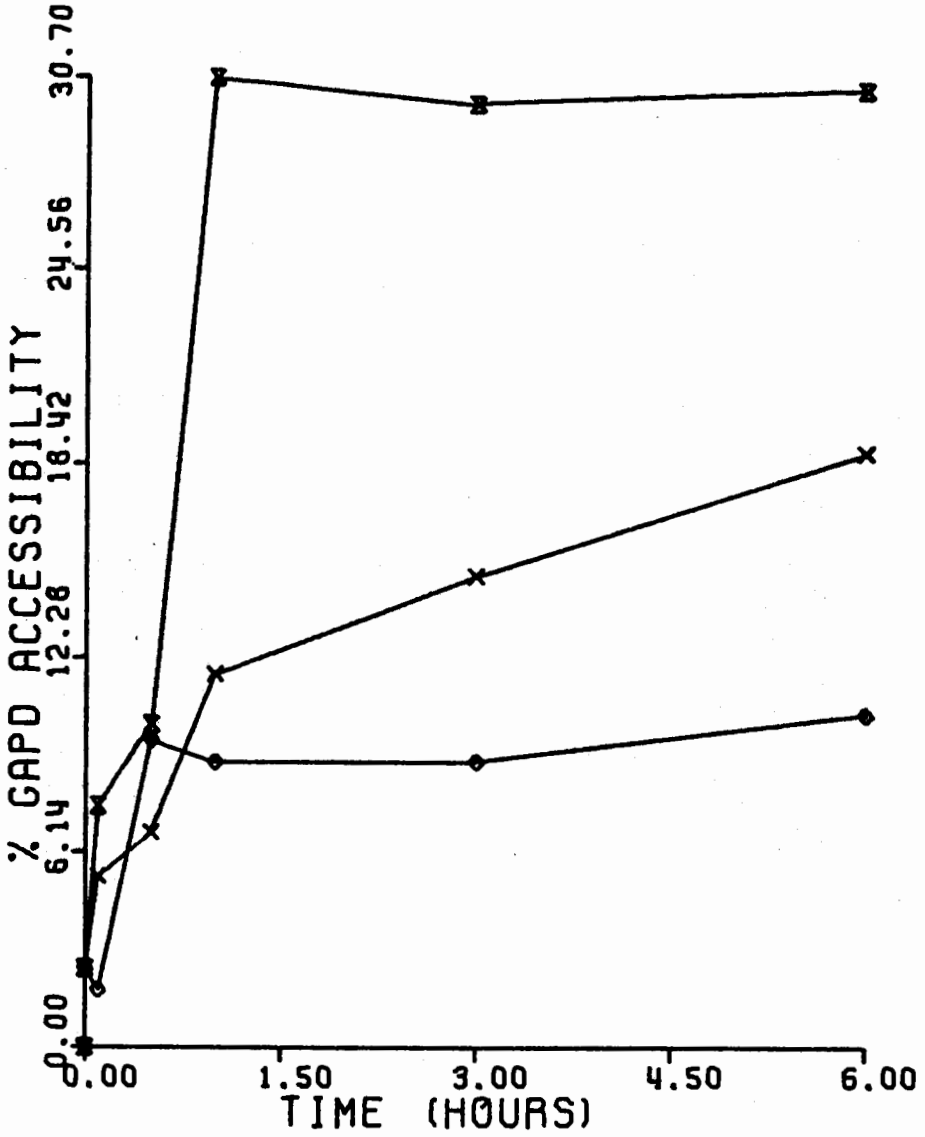
Combined effect of tocopherol and retinol on membrane permeability

Figure 21 illustrates the increase in permeabilty caused by various concentrations of α-tocopherol. Equimolar concentrations of tocopherol and retinol result in approximately the same percentage increase in membrane permeability. Figure 22 shows that these permeabilty

Figure 20. Effect of nitrogen and 100% oxygen on membrane permeability in the presence of 0.56 mM retinol

Incubation media consisting of 0.125 M NaCl, 20 mM PO4 buffer, pH 7.4, and 0.56 mM retinol were bubbled for 15 minutes with either 100% oxygen or 100% nitrogen. The surfaces were flushed for a further 5 minutes and ghost cells were added to make a 10% suspension. Flasks were sealed with rubber stoppers and the surface was re-flushed with the appropriate gas mixture following the withdrawal of all samples. GAPD activity was assayed as described in figure 2. Changes in permeability were measured as described in figure 17.

FIGURE 20



LEGEND

0.56 mM RET

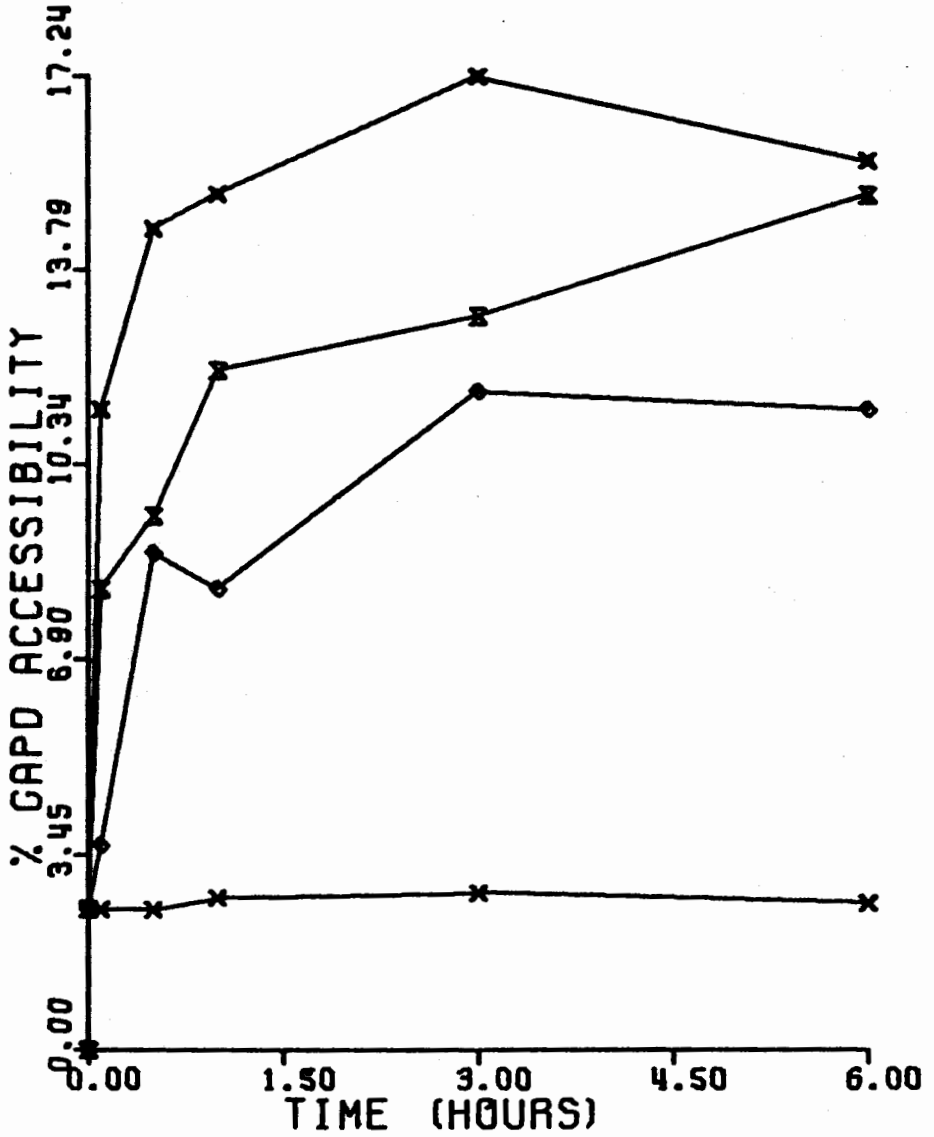
0.56 mM RET, NITROGEN

0.56 mM RET, OXYGEN

Figure 21. Effect of various concentrations of α-tocopherol on membrane permeability as measured by accessibility of glyceraldehyde-3-phosphate dehydrogenase to substrates located outside the cell membrane.

A 10% ghost suspension in 0.125 M NaCl and 20 mM PO4 buffer, pH 7.4, was mixed with tocopherol at various concentrations and incubated at 37°C. GAPD activity was assayed as described in figure 2. The fraction of total GAPD activity which was accessible without triton to substrates added outside the cell membrane is plotted as a function of time and exposure to tocopherol. Total GAPD activity was estimated using ghosts completely disrupted by triton X-100.

FIGURE 21



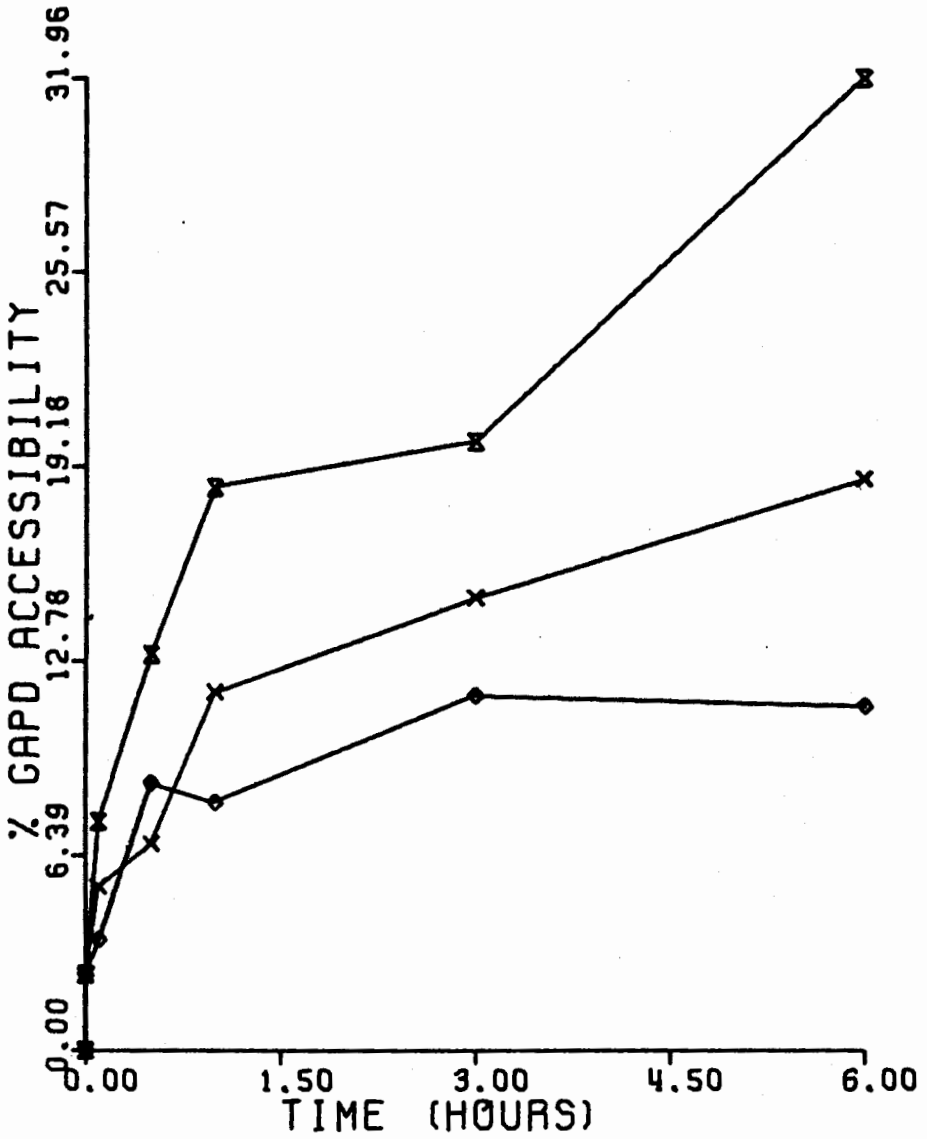
LEGEND

- CONTROL
- 0.12 mM TOCOPHEROL
- 0.56 mM TOCOPHEROL
- 2.7 mM TOCOPHEROL

Figure 22. Combined effect of retinol and α-tocopherol on membrane permeability

A 10% ghost suspension in 0.125 M NaCl and 20 mM PO4 buffer, pH 7.4, was pre-incubated for 10 minutes with 0.12 mM tocopherol. Sufficient retinol was added so that final retinol concentration was 0.56 mmoles/litre. GAPD activity was assayed as described in figure 2. Changes in permeability were measured as described in figure 17.

FIGURE 22



LEGEND

× 0.56 mM RETINOL

◇ 0.12 mM TOCOPHEROL

⧖ 0.56 mM RET. 0.12 mM TOC

changes are additive when tocopherol and retinol are present simultaneously. Concentrations higher than 0.12 mM tocopherol do not increase the permeability to any greater extent when in combination with retinol.

Cell Size and Morphology

In contrast to whole red cells which have a volume of 94 cubic microns, freshly prepared ghost cells were found to have electrical characteristics on the Coulter counter corresponding to an average volume of 47.5 cubic microns. They appeared spherical with slight crenation as illustrated in Plate 1. Incubation at 37°C for 24 hours did not significantly alter cell size or morphology. The addition of retinol to the incubation medium, however, resulted in a concentration-dependent increase in cell size, a phenomenon which was also observable when whole red cells were incubated with various concentrations of retinol. Plate 2 shows ghost cells after incubation for 6 hours with 0.56 mM retinol. Crenation has disappeared and the cells appear as intact, smooth spheres with some degree of infolding. Unlike retinol, ascorbate does not alter the size of ghost cells, however it does result in significant changes in cell morphology. Within 6 hours, 10 and 5 mM ascorbate cause ghost suspensions to change from a light pink to a

Plate 1. Phase contrast photograph of freshly prepared erythrocyte ghosts

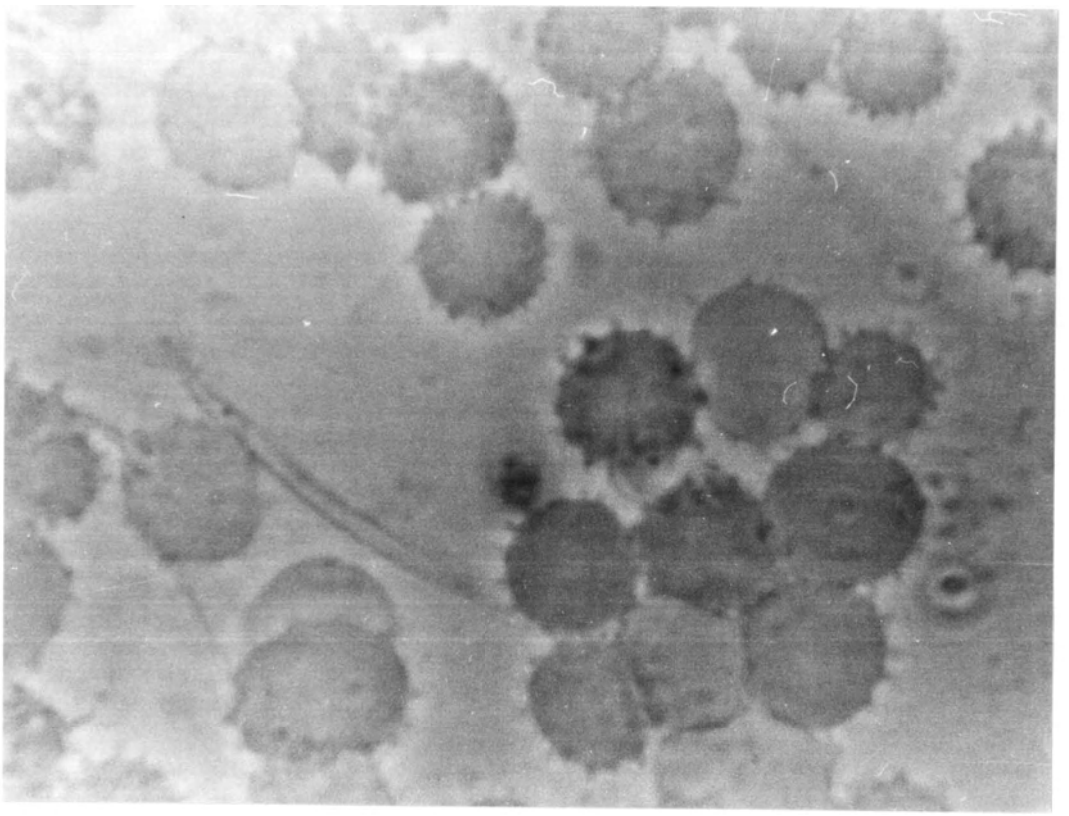
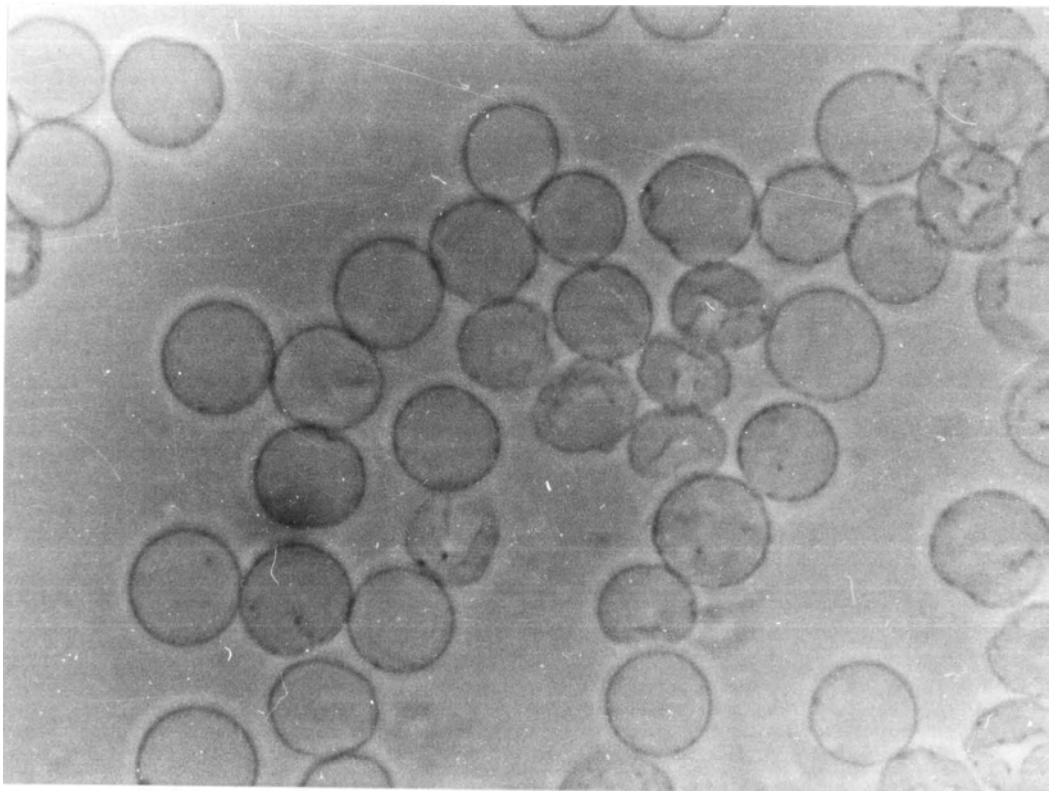


Plate 2. Phase contrast photograph of erythrocyte ghosts following 6 hours of incubation with 0.56 mM retinol



brownish-red color, and by 24 hours this has progressed to a distinct green. In addition, there was increased turbidity of the cell suspension and an increased mass of sediment after centrifugation. Upon examination under the phase contrast microscope, cells appear "speckled", although largely still intact, and the extent of this development depends upon the concentration of ascorbate present (Plate 3).

Incubation with α-tocopherol also results in an increase in cell size. The changes in cell size caused by equimolar concentrations of tocopherol or retinol are approximatly equal, and when incubated together, the effect appears to be additive as shown in figure 23.

Plate 3. Phase contrast photograph of erythrocyte ghosts following 6 hours of incubation with 5 mM ascorbate

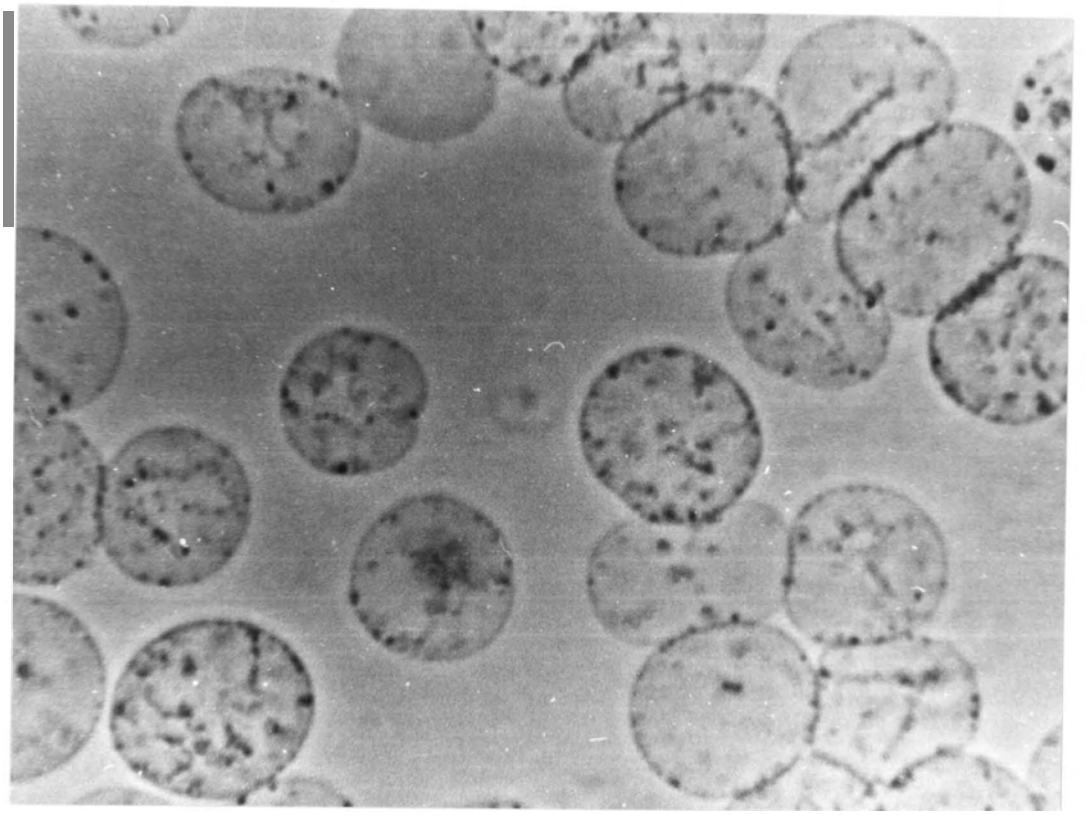
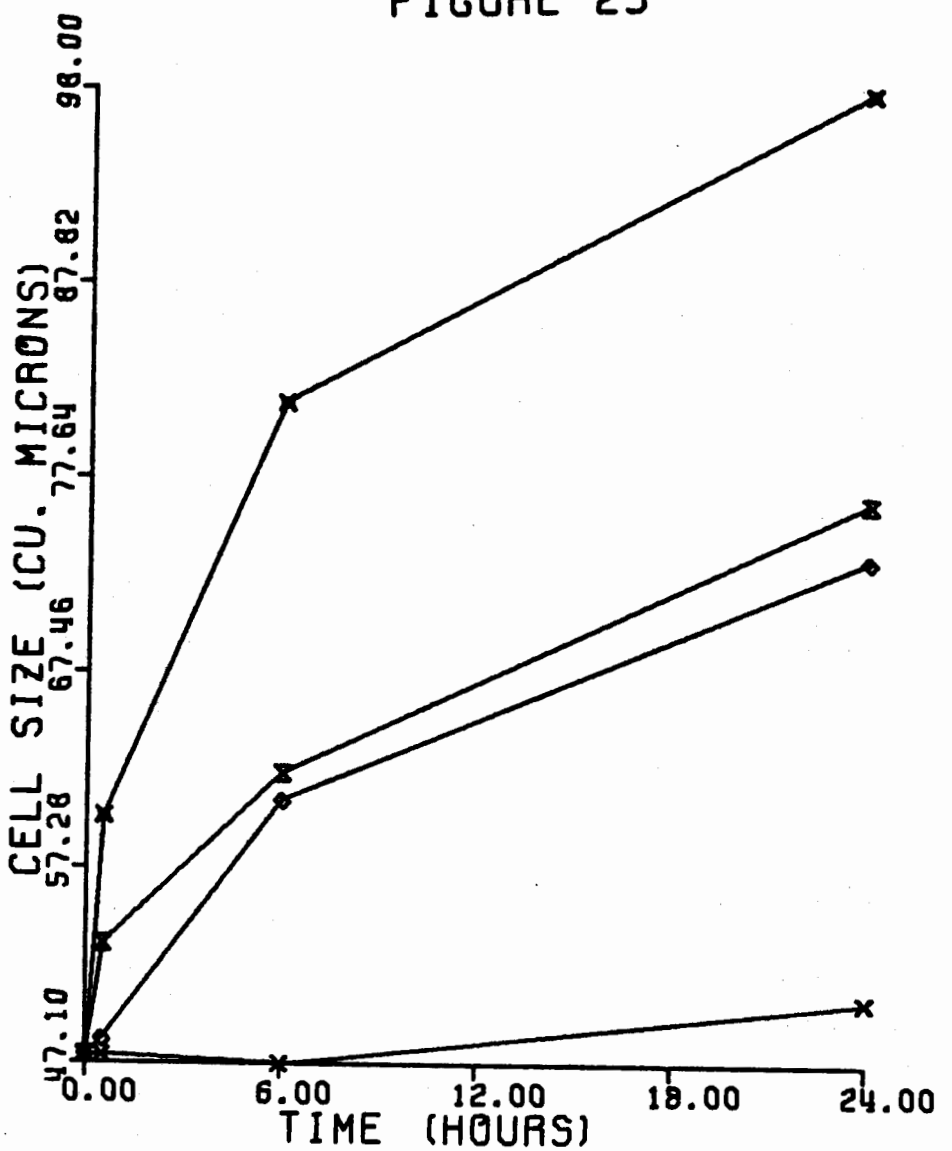


Figure 23. Combined effect of retinol and α-tocopherol on ghost cell size.

10% ghost suspensions in 0.125 M NaCl and 20 mM PO4 buffer, pH 7.4, were incubated at 37°C with 0.56 mM retinol, 0.56 mM α-tocopherol, or both. Cell volume was measured by the use of an electronic blood cell counter: the Coulter Counter. Final dilution of cells in the Coulter Counter was 50,000 times.

FIGURE 23



LEGEND

- CONTROL
- 0.56 mM RETINOL
- 0.56 mM TOCOPHEROL
- 0.56 mM RET, 0.56 mM TOC

DISCUSSION

At all concentrations tested, both retinol and ascorbate caused a concentration-dependent inactivation of the enzyme glyceraldehyde-3-phosphate dehydrogenase (figures 3,5). This enzyme, which is one of the major components associated with the erythrocyte membrane, contains a number of sulfhydryl groups which are highly susceptible to oxidative damage (Carraway and Shin, 1972; Tanner and Gray, 1971). Since the effects of both retinol and ascorbate are reversed by dithiothreitol, a sulfhydryl compound which reduces S-S bonds (Cleland, 1964), it appears that the decrease in enzyme activity is associated with a reversible oxidation of sulfhydryl groups. Tjioe and Haugaard (1972) found the decrease in activity of crystalline GAPD in the presence of oxygen to be likewise associated with the disappearance of sulfhydryl groups and to be reversed by dithiothreitol.

The fact that different concentrations of oxygen did not affect the initial rapid rate of enzyme damage, but did alter subsequent destruction (figure 8), indicates the involvement of two separate mechanisms in retinol-induced GAPD destruction: (1) an initial rapid effect which likely does not involve oxygen, and (2) a subsequent effect which

does involve oxygen. Figure 4 illustrates these two separate rates of damage while, in contrast, figure 6 shows that a similar effect does not occur during incubation with ascorbate. The biphasic pattern in the presence of retinol closely resembles the two-step process for the hemolysis of erythrocytes proposed by Dingle and Lucy (1965), although they based their classification on the effects of temperature rather than oxygen concentration.

The inhibiting effect of decreased temperature on membrane damage induced by retinol has been reported by Dingle (1961) in lysosomes, Dingle and Lucy (1962) in erythrocytes, and by Lucy, Luscombe and Dingle (1963) in mitochondria. A similar effect was seen in the present experiment. Figure 7 illustrates that low temperatures retard the loss of GAPD activity and figure 19 shows the inhibiting effect on increased membrane permeability. Although the decrease in membrane permeability which occurred at lower temperatures was virtually complete, decreased temperature was not totally able to inhibit loss of GAPD activity, indicating again the possibility of two separate mechanisms in retinol-induced GAPD damage. By comparing figures 7 and 8 it is possible to see that while oxygen concentrations have no effect on the intitial enzyme damage, a decrease in temperature does, and the latter

maintains activity in one half hour at a level approximately 4 times that at 37°C. Oxygen does not mediate its effect until after this time, which implies that the temperature-dependent phase of enzyme damage is a pre-requisite for the subsequent oxygen-dependent stage.

The effect of ascorbate appears to be complete in the first 30 minutes of incubation at 37°C since the level of enzyme activity did not change appreciably beyond this point (figure 6). It is possible that this levelling-off is due to an equilibration between damaging and repairing mechanisms within the cell. As neither decreased temperature nor an atmosphere of nitrogen had any significant effect on ascorbate's action on GAPD, it appears that a different mechanism is responsible than is the case with retinol. Further support of this is evident in figure 10. Whereas high concentrations of ascorbate enhance the initial damage caused by retinol, lower concentrations decreased the subsequent damaging effect. These data agree with the reports of Krishnamurthy and Kartha (1973) who found ascorbate at concentrations less than 0.3 mM to prevent retinol-induced hemolysis while higher concentrations increased the damage.

The increase in membrane permeability caused by retinol which has been observed by other workers has generally been attributed to swelling of the membrane as a result of its penetration by the hydrophobic portion of the retinol molecule (Glauert et al., 1963). The increase in cell size shown in figure 23 and the increased accessibility of GAPD to substrates located outside the cell membrane (figure 17) indicates that a similar process occurs in erythrocyte ghosts. The retinol molecule has a bulky hydrophobic ring system attached to a relatively long, rigid chain which is terminated by a hydrophilic end group (figure 1). Bangham, Dingle and Lucy (1964) demonstrated that it is these characteristics which produce retinol's surface active properties and its ability to penetrate biological membranes. Incubation below 20°C, however, retards penetration and expansion, and this may perhaps explain the effects shown in figure 19. Penetration and expansion of a membrane involve a concomitant increase in permeability as the natural components of the membrane are forced apart to make room for the intruding molecules (Lucy, 1972). At decreased temperature, membrane structure is more rigid, penetration is depressed, and so also the increase in permeability.

Tocopherol also has the abilitiy to permeate biological membranes (Lucy, 1972; Molenaar *et al.*, 1972) and figure 21 shows its effect on permeability of the ghost membrane. When tocopherol and retinol are present together the effect on cell size and membrane permeability is greater than with either molecule by itself (figures 22, 23). If penetration and expansion of the membrane alone were sufficient to decrease GAPD activity, then it would be expected that tocopherol would have the same damaging effect as retinol. However, as this is not the case, since the presence of tocopherol alone in the incubation medium does not alter GAPD activity from control levels, it appears that penetration without subsequent oxidation does not destroy GAPD activity. The fact that lower concentrations of tocopherol were able to inhibit the initial damaging effect of retinol even though higher amounts were not is paralleled by the greater efficiency with which lower amounts inhibit the development of lipid peroxidation (figure 16). The ineffectiveness of high tocopherol concentrations may partially be explained by the formation of micelles or macrophagic globules when tocopherol is present in large amounts, thus decreasing the effective tocopherol concentration in solution. On the other hand, it might also be explained by the ability of tocopherol to stabilize membranes in a physical manner. Lucy (1972) proposed that

vitamin E stabilizes membranes by virtue of specific interactions between its phytyl side chain and the fatty acyl chains of polyunsaturated fatty acids. A small amount of tocopherol may therefore suitably counterbalance the disorganizing effect of retinol. Higher concentrations, however, may actually add to the disorder and Anderson, Roels and Pfister (1967) found that an increase in the tocopherol/retinol ratio in rat erythrocytes resulted in widespread distortion of membrane structure. They concluded that retinol counteracts the changes in structure and/or function of biological membranes caused by different concentrations of α-tocopherol and that both vitamins were intimately related to membrane structure and integrity.

Both high and low concentrations of tocopherol were able to exert an effect on the oxygen-dependent phase of retinol-induced GAPD damage. After 6 hours both 0.12 and 0.56 mM tocopherol show the same degree of protection towards GAPD activity (figure 11) even though 0.12 mM tocopherol is considerably more effective than 0.56 mM in inhibiting lipid peroxidation (figure 16). Lucy and Dingle (1964) found that tocopherol concentrations between 0.23 and 0.46 mM were less effective in inhibiting hemolysis than were concentrations less than this, and this was probably due to the fact that they followed the reaction for only 15

minutes. Krishnamurthy and Kartha (1973) found that increasing concentrations of tocopherol afforded increasing inhibition of hemolysis, however their concentrations were less than 0.1 mM and the effect corresponded with the lower amounts used by Lucy and Dingle (1964). If tocopherol is functioning both as a stabilizer of physical membrane structure and as an antioxidant, it is understandable how Lucy and Dingle (1964) found such a wide variety of compounds with physical similarities to tocopherol to be equally effective in inhibiting hemolysis for 15 minutes. Figure 11 shows that tocopherol acetate causes an initial protective effect on enzyme damage induced by retinol, presumably the same effect as that seen earlier by Lucy and Dingle (1964). Subsequent damage was enhanced by tocopherol acetate, however, and although Lucy and Dingle (1964) emphasize the initial protective effect of tocopherol acetate on erythrocyte hemolysis, they also report that longer incubation with tocopherol acetate and vitamin A resulted in extensive release of potassium and hemoglobin.

Incubation with ascorbate results in no significant increase in membrane permeability and also no increase in cell size despite its effectiveness in intactivating GAPD. Since ascorbate is not lipid soluble, it presumably has less tendency to interact with the lipid portion of the membrane.

It appears, therefore that the initial rapid disappearance of GAPD activity in the presence of retinol is due to a penetration and disorganization of the ghost cell membrane. This process is not affected by the presence or absence of oxygen, but is retarded by a decrease in temperature. The subsequent effect, which is enhanced in the presence of 100% oxygen, likely involves the oxidation of the double bonds of the retinol molecule and a resultant injury to neighboring structures in the membrane. Membrane penetration without subsequent oxidation does not have the same effect, as indicated by the failure of α-tocopherol to inactivate GAPD. A comparison of the progressive loss of GAPD activity (figure 3) with increased membrane permeability (figure 13) and the development of lipid peroxidation (figure 17) indicates that the pattern of permeability parallels the loss of enzyme activity. Both of these parameters level off following three hours of incubation whereas "peroxidation" continues to increase with further incubation and shows no tendency to level off. Glauert *et al.* (1963) found membrane penetration to occur within seconds after introduction of retinol to a suspension of red cells. Fisher *et al.* (1972) found that oxygen uptake in a colloidal dispersion of retinol increased following one minute of incubation and to reach a maximum between two and four minutes, which suggested that autoxidation of the

retinol molecule begins immediately following implantation in the membrane. Lichti and Lucy (1969) and Lucy and Lichti (1969) showed that retinol behaves as an electron donor and will interact with several different electron acceptors, therefore it is possible that it reacts with oxygen and initiates a radical chain reaction. Bangham et al. (1964) found retinol to interact more strongly with lecithin than with cholesterol in monolayers composed of these two substances, so if oxidized, the retinol molecule would be in close proximity to the other labile elements of the membrane. It is conceivable, therefore, how a damaging free radical chain reaction could be initiated.

The lipid "peroxidation" which develops with retinol (figure 13) contrasts with the results of Krishnamurthy and Kartha (1973) who found no production of TBA pigment even when hemolysis was complete with 100 mM vitamin A. This may be explained by the fact that they followed the reaction for only 30 minutes, and that they used whole red cells rather than isolated ghost cells. Incubation of whole erythrocytes with 0.56 mM retinol in the present experiment resulted in considerably less TBA pigment than was the case with ghost cells, and perhaps this is due to the removal of some cellular protective mechanisms during hypotonic treatment. Although a large proportion of catalase and glutathione is

reported to be retained during hypotonic hemolysis (Nisioka, 1969), ghost cells contained only 13% of the protein of whole erythrocytes, and it is highly likely that at least some of the protective proteins were removed. The fact that tocopherol inhibits the accumulation of lipid peroxides suggests that at least one of its effects on the ghost membrane is that of an antioxidant.

Lipid "peroxidation" in the presence of ascorbate follows a pattern similar to the loss of GAPD activity in that the value it attains within the first hour remains constant for the duration of the test period (figure 14). Unfortunately, it was not possible with the available techniques to determine which occurred first chronologically. As with retinol, 0.12 mM tocopherol was more effective in protecting against GAPD damage than was 0.56 mM tocopherol (figure 12). Neither concentration, however, was capable of totally preventing the initial loss of enzyme activity. The means by which ascorbate initiates its effect on GAPD is not clear, however the following conclusions may be drawn: (1) ascorbate produces a more rapid initial effect on the enzyme GAPD than does an equimolar concentration of retinol, (2) this damage levels off almost immediately and is less within one half hour than an equimolar concentration of retinol, (3) enzyme damage

induced by ascorbate does not result in an increase in membrane permeability, (4) less TBA pigment is produced with ascorbate than with an equimolar concertration of retinol. Together with these differences, the fact that ascorbate exerts both a damaging and protective effect on GAPD inactivation suggests that the two vitamins produce their effects by to two entirely different mechanisms.

The "speckled" appearance of ghosts seen under the phase contrast microscope (Plate 3) is due to the presence of intracellular Heinz bodies, which are primarily composed of the insoluble degradation products of hemoglobin. Harley and Mauer (1961) also report Heinz body production in the presence of ascorbate, but even with extensive Heinz body formation, not more than 10% hemolysis was detected, agreeing with present results that ascorbate does not alter membrane permeability. The greenish color present in 24 hours is likely due to such degradation products of hemoglobin as choleglobin and sulfhemoglobin (Jandl, Engle and Allen, 1960).

In conclusion, the questions which were posed at the start of this study may now be partially answered as follows:

1. Retinol in concentrations ranging from 0.28 to 1.12 mM has a destructive effect on the membrane of the erythrocyte ghost which appears to be more debilitating than on intact erythrocytes, possibly due to removal of physiological protective agents during hypotonic hemolysis. Ghosts thus provide an excellent model for studying pro-oxidant damage to membranes and enzyme systems. Their heightened sensitivity and lack of intracellular components made it possible to monitor damage caused by a variety of substances, and to separate the variables involved. In the present investigation retinol produced swelling of the membrane and an increase in membrane permeabilty, lipid "peroxidation", and destruction of protein components. These effects occurred in two separate stages: (1) an initial rapid effect which was inhibited at low temperatures and did not involve oxygen, and (2) a subsequent, slower effect which was not affected by changes in temperature, but which did involve oxygen.

2. Ascorbate caused an inactivation of membrane protein which was more immediate than that caused by an equimolar concentration of retinol, even though effective damage was considerably less following the first half hour of incubation. As neither decreased temperature nor 100% oxygen affected ascorbate-mediated damage, it was concluded

that separate mechanisms are involved in the membrane effects of ascorbate and retinol. Lipid "peroxidation" occurred only at high concentrations of ascorbate and there was no change in cell size or membrane permeability.

3. Although ascorbate at concentrations less than 0.1 mM were protective against retinol-induced protein damage, higher ascorbate concentrations enhanced the enzyme inactivation, and at no time did ascorbate inhibit lipid "peroxidation" or alter the permeability changes caused by retinol. α-tocopherol, but not α-tocopherol acetate, caused 30% protection against protein damage and 70% protection against lipid "peroxidation" caused by both retinol and ascorbate.

4. α-tocopherol caused swelling of the ghost membrane and an increase in permeability similar to an equimolar concentration of retinol, however it did not cause an inactivation of membrane protein. Penetration of the ghost membrane therefore does not cause sufficient disorder in the membrane to inactivate membrane-bound enzymes. From our results, tocopherol is clearly acting as a protective agent against the non-oxidative damage and also as an antioxidant. The different time scales of these two events accounts for the apparently contradictory findings of various workers which stress one or the other of these roles.

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