approx. 16 ml./hr. during removal of the albumin]. As electrophoresis proceeds, with serum, it is necessary to slow the stream of buffer once the albumin has been removed, in order to avoid undue dilution of more slowly migrating components.

When this method was applied to electrophoresis of normal human serum, the resolution of faster-moving components was greatly improved, so that, for example, a distinct pre-albumin peak was obtained which was clearly demarcated from the albumin peak in almost all instances.

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Studies on Vitamin E

5. LIPID PEROXIDATION IN DIALURIC ACID-INDUCED HAEMOLYSIS OF VITAMIN E-DEFICIENT ERYTHROCYTES*

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Dialuric acid-induced haemolysis of erythrocytes from vitamin E-deficient rats was discovered by Rose & György (1950, 1952), who postulated that the haemolytic agent was an intermediate in the reversible oxidation-reduction system of dialuric acid and alloxan but was not hydrogen peroxide itself.

Red pigments are produced when 2-thiobarbituric acid reacts with oxidized lipids (Bernheim, Bernheim & Wilbur, 1947) or, more probably, with their aldehydic breakdown products (Glavind & Hartmann, 1951), and the formation of both red and yellow pigments from non-lipids has been discussed by Patton & Kurtz (1951) and by Landucci, Pouradier & Duranté (1955). The thiobarbituric acid reaction was used by Horwitt, Harvey, Duncan & Wilson (1956) in a study of hydrogen peroxide-induced haemolysis of erythrocytes from humans on a diet low in vitamin E, and by Tappel & Zalkin (1959, 1960) to demonstrate the presence of lipid peroxides in mitochondria and microsomes from vitamin E-deficient rats. Carpenter, Kitabchi, McCay & Caputto (1959) also used it to show that peroxide formation in liver homogenates from vitamin E-deficient rats was paralleled by decreased synthesis of ascorbic acid.

The protective effects of tocopherols and metal ions against dialuric acid-induced haemolysis have been discussed by Bunyan, Green, Edwin & Diplock (1960). This paper deals with the results of fuller investigations into the haemolysis of vitamin E-deficient erythrocytes and describes the use of the 2-thiobarbituric acid reaction to study the relation between lipid peroxidation and the haemolytic phenomenon.

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EXPERIMENTAL

Animals. Deficient animals were 3- to 5-month-old male rats of the Norwegian hooded strain which had received the vitamin E-free diet since they were 13 days old. Control rats were males from the stock colony.

Vitamin E-free diet, buffer pH 7·4 *and dialuric acid solution.* These were as described by Bunyan *et al.* (1960);

the buffer consisted of 25 ml. of 0·2 M-KH_2PO_4 solution and 19·7 ml. of 0·2 M-NaOH solution diluted to 100 ml. with water.

2-Thiobarbituric acid solution. 2-Thiobarbituric acid (0·67 g.) was dissolved in 100 ml. of warm water and the solution was cooled and mixed with 100 ml. of acetic acid.

α-Tocopherol suspension. A weighed amount of α-tocopherol was mixed with four times as much Tween 80 and then shaken with 0·9% NaCl solution.

Other additives. Metal ions and 2:6-di*tert.*-butyl-4-methylphenol were used as solutions of the appropriate material in 0·9% NaCl. L-Thyroxine (sodium salt) was dissolved in 0·01 N-NaOH and diluted with 0·9% NaCl. Crude ox-liver catalase was prepared by the method of Tauber & Petit (1952) and it was found that 1 g. of the preparation decomposed 200 g. of 100% H_2O_2 in 10 min. at 25° in an inert atmosphere. A portion (0·2 ml.) of catalase suspension (2·5 mg. in 1 ml. of buffer, pH 7·4) was added to 0·8 ml. of 25% erythrocyte suspension just before adding dialuric acid.

Carbon monoxide. This was produced by treating formic acid with conc. H_2SO_4 at 70° and was purified by passage through a tower of KOH pellets. Erythrocytes were treated by filling the air space in the stoppered tube with CO several times, with inversion of the tube each time. In some experiments, the resulting CO atmosphere was removed by blowing in air or by bubbling nitrogen through the cell suspension. The dialuric acid reaction was then applied at once.

Erythrocyte suspension. Blood was collected from the rat's tail into NaCl–citrate solution (0·9 g. of NaCl and 0·6 g. of trisodium citrate/100 ml.) and centrifuged at 2500 rev./min. The cells were washed twice with 0·9% NaCl and finally made up to a 20% suspension in 0·9% NaCl.

Pretreatment of the erythrocyte suspension. The various test substances were incubated at 37° for 1 hr. with 1 ml. of the 20% suspension (Table 2). The mixture was then centrifuged at 2500 rev./min., the supernatant was removed and the volume was made up to 1 ml. again with 0·9% NaCl.

Dialuric acid-induced haemolysis followed by the 2-thiobarbituric acid reaction. The 20% erythrocyte suspension (1 ml.) was incubated, after pretreatment if necessary, for 15 min. at 37° with 1 ml. of dialuric acid solution (3·3 mM unless otherwise stated). The mixture was centrifuged at 2500 rev./min. A portion (0·1 ml.) of the supernatant was removed and diluted with 7 ml. of water, and the extinction of the solution was measured against a water blank (EEL absorptiometer; Ilford filter, 605; $\lambda_{max.}$ 550 mμ). Calibration for 100% haemolysis was carried out by haemolysing 0·1 ml. of the original 20% suspension with 14 ml. of water. The remaining supernatant and sediment were shaken with 1 ml. of 10% trichloroacetic acid solution to precipitate proteins and the mixture was then filtered. The filtrate (2 ml.) was treated with 2·5 ml. of 2-thiobarbituric acid solution for 5 min. at 100° followed by 5 min. at room temperature (20–25°). The production of a pink colour ($\lambda_{max.}$ 535 mμ) was taken to indicate lipid peroxidation and the extinction was measured with Ilford filter 605. A yellow colour ($\lambda_{max.}$ 450 mμ), which can result from the action of thiobarbituric acid on dialuric acid in the absence of erythrocytes, was regarded as evidence of a negative reaction and was not measured.

Preparation of erythrocyte stroma and stroma-free haemolysates. Blood was collected as described above and the erythrocytes were washed three times with ice-cold 0·9% NaCl by centrifuging at 3000 rev./min. Haemolysis was effected by mixing with 10 vol. of ice-cold water. The haemolysate was allowed to stand for 30 min. and then centrifuged either for 5 min. at about 1000 *g* (3000 rev./min.) or for 15 min. at about 20 000 *g* (MSE Superspeed 25 centrifuge). About three-quarters of the clear supernatant was removed for testing. The stroma fraction was washed repeatedly with cold 0·9% NaCl or water by centrifuging at 3000 rev./min. until no more haemoglobin dissolved; some remained adherent to the stroma. The stromal material was then resuspended in 0·9% NaCl to a concentration equivalent to that of the original erythrocyte suspension. Haemolysis and peroxide formation varied slightly from day to day but most results were derived by direct comparison with a given test.

Reaction of erythrocyte fractions with dialuric acid and thiobarbituric acid. Stroma suspensions in 0·9% NaCl or stroma-free haemolysate were treated with dialuric acid solution followed by thiobarbituric acid as described for intact erythrocytes.

Spectral examinations. The haemoglobin spectrum during haemolysis was observed by means of a direct-vision spectroscope (R. and J. Beck Ltd.) and quantitative measurements were made on the Hilger Uvispek spectrophotometer. Reduced haemoglobin and methaemoglobin, for purposes of comparison, were produced by treating haemolysed vitamin E-deficient erythrocytes with traces of $Na_2S_2O_4$ and $K_3Fe(CN)_6$ respectively.

Chemicals. Dialuric acid and L-thyroxine (sodium salt) were obtained from L. Light and Co. Ltd., Colnbrook, Bucks.; 2-thiobarbituric acid from British Drug Houses Ltd.; α-tocopherol from Roche Products Ltd. and 2:6-di*tert.*-butyl-4-methylphenol from William Pearson Ltd., Hull.

RESULTS

Intact erythrocytes

The 5% erythrocyte suspension used in our previous study (Bunyan *et al.* 1960) was here increased to 20% in order to enhance the thiobarbituric acid reaction. In preliminary tests a higher concentration of dialuric acid was also used but this did not induce haemolysis. An investigation of various concentrations of dialuric acid showed that solutions between 1·6 and 3·3 mM were most effective and that haemolysis was paralleled by the formation of lipid peroxides. Peroxides were not found in erythrocytes haemolysed by dilution in water (Table 1).

Substances previously found to be protective against dialuric acid-induced haemolysis (α-tocopherol, Co^{2+} ions, Mn^{2+} ions, $Cr_2O_7^{2-}$ ions, CrO_4^{2-} ions and 2:6-di*tert.*-butyl-4-methylphenol) also prevented lipid peroxidation, the protection due to α-tocopherol and $Cr_2O_7^{2-}$ ions persisting even after three washes with 0·9% sodium chloride. The antagonism of SeO_3^{2-} ions towards tocopherol protection was also shown (Table 2). L-Thyroxine, investigated here for the first time, gave full

Table 1. *Haemolysis and lipid peroxidation with varying concentrations of dialuric acid solution and erythrocyte suspension*

For details see text.

Dialuric acid soln. (mM)	Vitamin E-deficient erythrocyte suspension (%)	Haemolysis (%)	Thiobarbituric acid reaction (*E* at 550 mμ)
13·2	20	53	10
6·6	20	92	12
3·3	20	94	17
2·5	20	93	22
1·6	20	95	19
Nil (haemolysed with water)	20	100	—*
13·2	10	0	3
13·2	4	0	—*

* Yellow.

Table 2. *Effect of various substances on haemolysis and lipid peroxidation*

For details see text.

Pretreatment	Haemolysis (%)	Thiobarbituric acid reaction (*E* at 550 mμ)
Nil (normal erythrocytes)	0	—*
Vitamin E-deficient erythrocytes		
Nil (and no dialuric acid)	0	—*
Nil	83	14
α-Tocopherol (4 μg.)	0	—*
α-Tocopherol (4 μg.) followed by three washes with 0·9% NaCl	0	—*
α-Tocopherol (2 μg.)	8	—*
SeO_3^{2-} (as Na_2SeO_3) (64 μg.)	67	15
SeO_3^{2-} (64 μg.) + α-tocopherol (2 μg.)	73	15
L-Thyroxine (sodium salt) (100 μg.)	2	—*
Co^{2+} (as $CoCl_2$) (200 μg.)	0	—*
Mn^{2+} (as $MnSO_4$) (200 μg.)	0	—*
$Cr_2O_7^{2-}$ (as $K_2Cr_2O_7$) (300 μg.)	0	—*
$Cr_2O_7^{2-}$ (300 μg.) followed by three washes with 0·9% NaCl	0	—*
CrO_4^{2-} (as K_2CrO_4) (500 μg.)	0	—*
2:6-Di*tert.*-butyl-4-methylphenol (100 μg.)	25	—*
Catalase (about 0·1 Kiel unit)	76	13

* Yellow.

Table 3. *Effects of carbon monoxide on haemolysis and lipid peroxidation*

For details see text.

Treatment of vitamin E-deficient erythrocyte suspension	Haemolysis (%)	Thiobarbituric acid reaction (*E* at 550 mμ)
Nil	93	22
CO with CO atmosphere	12	7
CO with air atmosphere	38	16
CO with N_2 atmosphere (N_2 bubbled through the suspension)	7	7

protection of the erythrocytes *in vitro*. Catalase had little, if any, protective effect although enough was added to destroy about 100 mg. of hydrogen peroxide (100 vol.). No peroxides were found in vitamin E-deficient erythrocytes not treated with dialuric acid. Erythrocytes treated with carbon monoxide showed the cherry-red colour characteristic of carboxyhaemoglobin and were considerably resistant to haemolysis and peroxidation, especially in an atmosphere of carbon monoxide or nitrogen, the latter being passed through the solution to remove dissolved carbon monoxide. Exposure to air reduced resistance, owing probably to the reformation of oxyhaemoglobin (Table 3).

Erythrocyte fractions

Erythrocytes were haemolysed with water and separated into two fractions, the stroma and the stroma-free haemolysate. Each fraction was then treated with dialuric acid, with thiobarbituric acid to detect peroxidation. Peroxides were formed by intact vitamin E-deficient erythrocytes and their cell stroma, but not by the haemolysate unless

Table 4. *Effect of dialuric acid and thiobarbituric acid on erythrocytes and erythrocyte fractions*

Each fraction was equivalent to 2 ml. of 20 % erythrocyte suspension

	Haemolysis (%)	Thiobarbituric acid reaction (E at 550 mμ)
Normal erythrocytes		
Intact cells	0	—*
Stroma-free haemolysate	—	—*
Stroma	—	—*
Vitamin E-deficient erythrocytes		
Intact cells	90	14
Stroma-free haemolysate	—	—*
Stroma	—	7
Vitamin E-deficient erythrocytes pretreated with 10 μg. of α-tocopherol/2 ml.		
Intact cells	0	—*
Stroma-free haemolysate	—	—*
Stroma	—	—*
Vitamin E-deficient erythrocytes		
Haemolysate (centrifuged at 1000 g)	—	14

* Yellow.

Table 5. *Effect of* Fe^{2+} *and* Fe^{3+} *ions on vitamin E-deficient erythrocyte fractions treated with dialuric acid and thiobarbituric acid*

Fe^{2+} ions were as $FeSO_4,(NH_4)_2SO_4,6H_2O$ and Fe^{3+} ions as $FeCl_3,6H_2O$).

	Thiobarbituric acid reaction (E at 550 mμ)
Intact cells	17†
Stroma-free haemolysate	—*
Stroma	8
Stroma + haemolysate	17
Stroma in 2·5 mM-Fe^{2+}	8
Stroma in 2·5 mM-Fe^{3+}	19
Stroma in 2·5 mM-Fe^{2+} + 2·5 mM-Fe^{3+}	16
Stroma in 2·5 mM-Fe^{2+} (no dialuric acid)	—*
Stroma in 2·5 mM-Fe^{3+} (no dialuric acid)	—*

* Yellow. † 55 % haemolysis occurred.

Table 6. *Effect of* Fe^{2+} *and* Fe^{3+} *ions on rate of dialuric acid-induced haemolysis*

A 5 % vitamin E-deficient erythrocyte suspension was used.

Ion	Concentrations (mM)	Haemolysis (%) 10 min.	Haemolysis (%) 17 min.
Nil	Nil	47	81
Fe^{2+}	0·8	—	59
Fe^{2+}	0·5	42	69
Fe^{2+}	0·16	—	79
Fe^{3+}	0·5	11	56
Fe^{3+}	0·4	—	66
Fe^{3+}	0·8	—	41

this was prepared at 1000 g. No peroxides were found in normal erythrocytes, vitamin E-deficient erythrocytes fortified *in vitro* with α-tocopherol or their cell fractions (Table 4). Although itself inert, the haemolysate prepared at 20 000 g enhanced peroxidation of the stroma fraction (Table 5). Solutions of Fe^{2+} and Fe^{3+} ions were added to the stroma in amounts equivalent to the iron content of the haemolysate and the Fe^{3+} ion increased peroxide formation. Neither ion induced peroxidation of the stroma in the absence of dialuric acid.

Effects of Fe^{2+} *and* Fe^{3+} *ions on haemolysis*

Tests were carried out to see if Fe^{2+} or Fe^{3+} ions would accelerate haemolysis by dialuric acid (Table 6). An erythrocyte suspension (5 %) was used and concentrations of Fe^{2+} and Fe^{3+} ions up to 0·8 mM and 0·5 mM respectively were used (higher levels of Fe^{3+} ions resulted in erythrocyte sedimentation). No acceleration occurred; both ions, in fact, caused a partial delay in haemolysis.

Effects of dialuric acid on haemoglobin

The absorption spectrum of the pigment released from erythrocytes by haemolytic concentrations of dialuric acid appeared to be identical with that of oxyhaemoglobin when it was examined both by the direct-vision spectroscope and the spectrophotometer. Much higher concentrations of dialuric acid markedly affected the colour of vitamin E-deficient erythrocytes without causing haemolysis. When erythrocytes so treated were then haemolysed by dilution in water, the solution produced also appeared to contain only oxyhaemoglobin; but if the erythrocytes were first haemolysed in water, then treated by the addition of solid dialuric acid and further diluted for measurement, the type of pigment produced depended on whether this further dilution was with water or buffer pH 7·4 (Fig. 1). By comparison with standard curves (Hawk, Oser & Summerson, 1954) it was

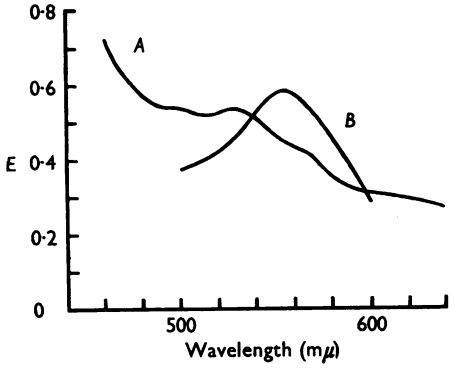

Fig. 1. Effect of excess of dialuric acid on vitamin E-deficient erythrocyte haemolysates, diluted in water (*A*), or buffer, pH 7·4 (*B*).

found that methaemoglobin was formed in water but haemoglobin was formed in buffer. It is possible therefore that haemoglobin may have been produced in the intact erythrocytes by the higher levels of dialuric acid, but re-oxidized by atmospheric oxygen on dilution with water.

Brückmann & Wertheimer (1945), investigating diabetogenic compounds, found that, *in vitro*, methaemoglobin was produced by alloxan, but that alloxantin and dialuric acid gave haemoglobin. The formation of methaemoglobin referred to here may therefore indicate oxidation of dialuric acid to alloxan in the unbuffered solution.

DISCUSSION

The present study shows a close parallel between dialuric acid-induced haemolysis and lipid peroxidation and demonstrates that agents which prevent haemolysis also stop peroxide formation. The theory of Rose & György (1950, 1952) that hydrogen peroxide itself is not the haemolytic agent is supported by the failure of catalase to prevent peroxidation, although these authors did find catalase active in their tests. Erythrocyte-fractionation experiments lead to the conclusion that the lipoprotein membrane itself and possibly some other material not firmly bound to the membrane is attacked by a product of dialuric acid.

A catalytic role for haemoglobin is suggested by the enhanced peroxide formation in stroma when haemolysate is added, an effect also given by Fe^{3+} but not by Fe^{2+} ions. Protection by carbon monoxide may be due to more than one effect; carboxyhaemoglobin is resistant to dissociation (but not to oxidation) and its formation in these tests involves liberation of oxygen from oxyhaemoglobin before addition of dialuric acid. If the formation of haemoglobin demonstrated in excess of buffered dialuric acid occurs to a small extent in haemolysing erythrocytes, a portion of the oxygen of oxyhaemoglobin may be released in the presence of dialuric acid, leading to the production of the intermediate postulated by Rose & György. In this case, as suggested by the same authors, excess of dialuric acid must also form a protective layer around the cell, so preventing haemolysis.

The enhancement of stroma peroxidation by Fe^{3+} ions is unexplained, but Kibrick, Safier & Skupp (1959) reported that thiobarbituric acid in the presence of 0·12 mM-ferric chloride detected peroxides even in normal blood.

SUMMARY

1. Dialuric acid-induced haemolysis of vitamin E-deficient erythrocytes has been shown to be accompanied by lipid peroxidation. Formation of peroxides is prevented by agents protecting erythrocytes against haemolysis (α-tocopherol, 2:6-di*tert.*-butyl-4-methylphenol and the ions Co^{2+}, Mn^{2+}, $Cr_2O_7^{2-}$ and CrO_4^{2-}). The protection of α-tocopherol is completely opposed by SeO_3^{2-} ions.

2. Studies with L-thyroxine have shown it to be fully protective *in vitro* against both haemolysis and lipid peroxidation.

3. Haemoglobin catalysis of lipid peroxidation in the erythrocyte stroma has been demonstrated by cell-fractionation studies and by the protection of intact cells resulting from the conversion of oxyhaemoglobin into carboxyhaemoglobin. From the observed effects of excess of dialuric acid, it seems that oxyhaemoglobin may dissociate during haemolysis, but only to a small extent.

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