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The Oxidation of the Lipids of the Erythrocyte

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It was found by Rose & György (1952) and confirmed by later workers (Christensen, Dam, Gortner & Søndergaard, 1956; Horwitt, Harvey, Duncan & Wilson, 1956) that, in vitamin E deficiency, erythrocytes are very susceptible to haemolysis under oxidizing conditions. Tocopherol and other lipid antioxidants afford protection to the cells under these conditions and this has led to the hypothesis that in the absence of such protection oxidation of unsaturated-lipid components of the cell membrane occurs, and this leads to the observed haemolysis either directly as a result of structural changes or indirectly through the release of a haemolytic agent.

Evidence supporting this hypothesis has come from studies with the thiobarbituric acid test used as a measure of lipid oxidation (Horwitt *et al.* 1956; Bunyan, Green, Edwin & Diplock, 1960; Tsen & Collier, 1960). This reagent forms a coloured complex with malonaldehyde or epihydrin aldehyde (Glavind & Hartmann, 1951; Weissbach & Hurwitz, 1959) which is released under acid conditions from oxidized lipids. However, the positive results obtained by these workers are not sufficient to prove the hypothesis. The possibility that non-lipid components may be involved has not been eliminated, and this is particularly important in studies on erythrocytes since it has recently been shown that under some conditions haem com-

pounds give a positive reaction (Cowlshaw, 1961). Moreover, it is known that linolenic acid is one of the most reactive fatty acids in the thiobarbituric acid test (Wilbur, Bernheim & Shapiro, 1949) and this acid is absent from erythrocytes (Evans, Waldron, Oleksyshyn & Riemenschneider, 1956). Hence direct evidence that erythrocyte lipids are sufficiently reactive to account for the observed malonaldehyde production in erythrocytes is desirable.

For this purpose it is necessary to use a lipid preparation that is free from endogenous antioxidants. Preliminary experiments showed that the most convenient preparation for this purpose is an aqueous emulsion of erythrocyte fatty acids from which all unsaponifiable material had been removed. The present study is concerned with the oxidative changes observed in a preparation of this kind. The behaviour of malonaldehyde and tocopherol have also been investigated.

EXPERIMENTAL

Preparation of erythrocyte-fatty acid suspension. Erythrocytes from normal human blood were separated and washed five times with 0.15M-NaCl. The haematocrit of the final suspension was calculated from its haemoglobin content, and a portion of the suspension containing 7 ml. of erythrocytes was used for lipid extraction. This was diluted to give

a 30% suspension and extracted with 15 vol. of chloroform-methanol (1:1, v/v) at 37° for 1 hr. The precipitate was removed by filtering through sintered glass, and well washed with chloroform-methanol (1:1, v/v). To the combined filtrate and washings was added α -tocopherol (1 mg.) as an antioxidant, the solvent was removed under N_2 at reduced pressure at 37°, and the residue was hydrolysed with 25 ml. of methanolic 2N-KOH under N_2 . After dilution with water unsaponified material was removed by extracting three times with ether. The ether extracts were washed to remove fatty acids with 0.5N-NaOH in 50% ethanol (to prevent emulsion formation) and the combined aqueous phase and washings were freed of dissolved ether under N_2 at reduced pressure. The fatty acids were removed by strongly acidifying the aqueous solution and extracting three times with 2,2,4-trimethylpentane (by shaking for at least 5 min.). The combined 2,2,4-trimethylpentane phases were washed with 0.5N- H_2SO_4 in 50% ethanol to remove haematin, then with aqueous 0.01N- H_2SO_4 . The fatty acids were re-extracted by washing the 2,2,4-trimethylpentane five times with N-NaOH, and by addition of HCl with Na_2HPO_4 a final suspension was made up which was 0.154 M with respect to NaCl and 0.01 M with respect to phosphate buffer, pH 7.4.

Preparation of fatty acid-albumin complex. A solution of fatty acids in NaOH was prepared as described above. To this were added an aqueous solution of bovine serum albumin, freed of fatty acids by the method of Goodman (1957), and sufficient HCl and Na_2HPO_4 to give a solution 0.154 M with respect to NaCl and 0.01 M with respect to phosphate buffer, pH 7.4, and which contained 1 mole of albumin (mol.wt. taken as 69 000)/7 equiv. of fatty acid. The resulting solution was filtered through sintered glass and stored at 0°.

Incubation procedure. Solutions were incubated at 37° in stoppered 10 ml. centrifuge tubes without shaking. The total volume of solution incubated was 2 ml., made up by adding 1 ml. of one of the following solutions to 1 ml. of sample: (a) buffer solution (0.15M-NaCl-0.01M- Na_2HPO_4 , pH 7.4); (b) freshly prepared buffered ascorbic acid solution (0.4 mg./ml.; Wilbur *et al.* 1949); (c) buffered H_2O_2 solution (2.5%; Horwitt *et al.* 1956). α -Tocopherol (General Biochemicals Inc.) was added as an ethanolic solution (20 μ l., containing 47.5 μ m-moles).

Preparation of platinum catalyst. Barium sulphate was precipitated by the addition of hot N- H_2SO_4 (20 ml.) to a hot aqueous solution of $BaCl_2$ (1.74 g.). The precipitate was washed twice with water by centrifuging. A solution of $PtCl_4$ (33.7 mg.) in N-HCl was added to the suspended $BaSO_4$ precipitate, followed by a mixture of formaldehyde (0.1 ml. of aqueous 40% solution) and N-NaOH solution (2 ml.). When reduction was complete the suspension was washed with water by centrifuging and stored as an aqueous suspension.

Methods of estimation. Spectrophotometry was carried out with either the Unicam SP. 600 or the Hilger Uvispek spectrophotometer. Haemoglobin was estimated by the method of Palmer (1918). Total fatty acids in the suspensions were estimated by acidifying, extracting with ethanol-2,2,4-trimethylpentane and titrating by the method of Gordon (1957), an Agla micrometer syringe being used. Tocopherol was estimated by the method of Green, Marcinkiewicz & Watt (1955), with the use of the spectrophotometric factor of 96 that they recommend. Albumin

was estimated spectrophotometrically by comparing the extinction at 278.5 m μ with that of a standard albumin solution. The albumin content of the standard was calculated from its nitrogen content (micro-Kjeldahl determination).

2-Thiobarbituric acid test. To the sample under test were added 2 ml. of 0.67% thiobarbituric acid solution and 2 ml. of 40% (w/v) trichloroacetic acid solution, and the mixture was placed in a boiling-water bath for 20 min. After cooling, the volume was made up to 6 ml. with water and the mixture centrifuged at 2000 g for 10 min. to give a clear solution suitable for spectrophotometry.

Destruction of hydrogen peroxide. The thiobarbituric acid test could not be conducted in the presence of H_2O_2 owing to the destruction of thiobarbituric acid. After incubation, excess of H_2O_2 was removed by adding 3 drops of the platinum-catalyst suspension and heating at 100° for 1 min.

RESULTS

Significant oxidation has been observed previously only in vitamin E-deficient erythrocytes. Hence it is necessary to remove endogenous antioxidants from the lipids of normal human erythrocytes in order to reveal any intrinsic susceptibility to oxidation. It is possible to do this by means of silicic acid chromatography but preliminary experiments revealed a number of disadvantages in this method. The most serious of these was that it was not possible to disperse the recovered lipid quantitatively in an aqueous phase suitable for incubation. Consequently further experiments were confined to aqueous suspensions of fatty acids.

A comparison of the oxidation of fatty acids from erythrocytes with that of linseed oil. The initial study of the oxidation of erythrocyte lipids was made on aqueous emulsions of the fatty acids prepared as described. The oxidation of samples of an aqueous suspension of fatty acids (0.36 μ equiv.) from erythrocytes (0.072 ml.) was compared with that of aqueous suspensions of linseed oil. These were prepared by adding portions (20 μ l.) of 4% (w/v) linseed oil in 'amyl alcohol' to the aqueous medium. From the data given by Hilditch (1956) this amount of linseed oil contains 2.73 μ equiv. of fatty acids. After incubation, the products from both experiments were treated with thiobarbituric acid and the absorption spectra of the coloured products were examined. They were both identical with that given by malonaldehyde under the same conditions.

The extinction at 532 m μ was measured and converted into moles of malonaldehyde/ 10^3 equiv. of fatty acid, a value of $\epsilon = 133\,000$ (Warren, 1959) being used. The results (Table 1) show that incubation of erythrocyte fatty acids alone did not produce significant oxidation, as judged by the increase in malonaldehyde content, but a small increase in oxidation of linseed oil was observed under these conditions. In the presence of ascorbic

acid or hydrogen peroxide the malonaldehyde found in erythrocyte fatty acids was increased 2.5- to 3-fold after incubation for 3 hr. whereas that in linseed oil increased about threefold after incubation for 1.5 hr. with hydrogen peroxide.

Erythrocyte fatty acids contain approximately 25 % of poly-unsaturated fatty acids, none of which is linolenic acid (Evans *et al.* 1956). On the other hand, linseed oil contains about 40 % of linolenic acid (Hilditch, 1956), which is known to be very reactive in the thiobarbituric acid test (Wilbur *et al.* 1949). The results reported here show that the amount of malonaldehyde in erythrocyte fatty acids incubated for 3 hr. with hydrogen peroxide is of the same order as that in linseed oil similarly incubated for 1.5 hr. On the basis of this comparison it seems reasonable to argue that the rate of oxidation of erythrocyte fatty acids is of the order of one-half that of linseed oil under the same conditions. The lability of free malonaldehyde in solution (see below) may invalidate the equation of malonaldehyde found with rate of oxidation of lipids, but the above-described comparison appears to avoid this difficulty and allows the conclusion to be drawn that erythrocytes contain some comparatively rapidly oxidizable fatty acid other than linolenic acid.

It is known that tocopherol inhibits the early stages of lipid oxidation and also destroys pre-formed hydroperoxides (Swift, 1946). This protective effect is evident if samples incubated with added ascorbic acid are compared with similar samples in the presence of tocopherol, where there is up to 70 % decrease in oxidation. In the presence of hydrogen peroxide, tocopherol is oxidized and this limits the extent to which it can protect other lipids from oxidation. Moreover, the coloured pro-

duct from oxidized tocopherol interferes in the assay of lipid-oxidation products, and, although a correction can be made, the error of the final estimate of malonaldehyde is high when the proportional amount of oxidized tocopherol present is large. The results given in Table 1 show definite protection against oxidation only with linseed oil. After the longer incubation period used for erythrocyte fatty acids, no significant evidence of protection was obtained. A possible reason for this is the complete destruction by hydrogen peroxide of the added tocopherol.

Relative to the amount of erythrocyte fatty acid present, the amount of hydrogen peroxide used was of the same order as that used in the haemolysis test described by Horwitt *et al.* (1956). The amount of tocopherol used was of the order of 30 times that normally present in erythrocyte lipids (B. Cowlishaw, unpublished work).

Oxidation of a fatty acid-albumin complex. It was thought that a better approximation of the configuration of erythrocyte-membrane lipids *in situ* could be made by using fatty acids bound to a protein. This was prepared as described in the Experimental section and samples containing 1.22 μ equiv. of fatty acids bound to bovine serum albumin were used. The results are shown in Table 2. In the presence of albumin, foaming occurred when the platinum catalyst was added to destroy hydrogen peroxide, so this reaction was carried out in a beaker with intermittent heating until the reaction mixture gave a negative test with starch-iodide paper. Under these conditions less colour was always given than in the controls without hydrogen peroxide, possibly owing to loss of malonaldehyde during removal of the hydrogen peroxide. No evidence of accelerated oxidation

Table 1. Comparison of malonaldehyde produced in aqueous suspensions of erythrocyte fatty acids and linseed oil incubated at 37°

Additions made	Period of incubation (hr.)	Malonaldehyde produced (moles/10 ³ equiv. of fatty acid)	
		Erythrocyte fatty acids	Linseed oil
Nil	0.	2.6 $\pm$ 0.6 (6)*	2.5
	1.5	—	3.4
	3	2.0 $\pm$ 1.3 (8)*	—
α -Tocopherol (47.5 μ m-moles)	0	—	1.2
	1.5	—	1.2
	3	2.5	—
Ascorbic acid (2.3 μ moles)	3	6.7	—
Ascorbic acid (2.3 μ moles) and α -tocopherol (47.5 μ m-moles)	3	3.6	—
Hydrogen peroxide (735 μ moles)	1.5	—	7.8
	3	7.7	—
Hydrogen peroxide (735 μ moles) and α -tocopherol (47.5 μ m-moles)	1.5	—	1.4 $\pm$ 0.2 (5)*†
	3	7.2 $\pm$ 2.5 (4)*†	—

* Mean value $\pm$ s.d. (no. of expts.).

† Calculated according to equation in text.

Table 2. Comparison of oxidation of fatty acids from erythrocytes: (a) in suspension, (b) bound to serum albumin

Additions made	Period of incubation (hr.)	Malonaldehyde produced (moles/10 ⁸ equiv. of fatty acid)	
		(a) Suspended fatty acids	(b) Bound to albumin
Nil	0	2.6	1.9
	3	2.0	2.0
Hydrogen peroxide (735 μ moles)	3	7.7	1.0

Table 3. Recoveries of malonaldehyde after incubation of an aqueous solution at 37°

Additions made	Malonaldehyde remaining (μ m-moles)	
	No incubation	Incubation for 1.5 hr.
Nil	23.5	15.8
α -Tocopherol (47.5 μ m-moles)	23.4	13.4
Hydrogen peroxide (735 μ moles)	10.2*	2.3
Hydrogen peroxide (735 μ moles) and α -tocopherol (47.5 μ m-moles)	—	0†

* Hydrogen peroxide solution treated with platinum catalyst before addition of malonaldehyde.

† Calculated according to equation in text.

of fatty acids was obtained, showing that fatty acids bound to albumin are stabilized against oxidation.

Oxidation of malonaldehyde. Malonaldehyde was incubated under the same conditions used for the fatty acids (Table 3). In each experiment 20 μ l. of a solution of malonaldehyde bis-bisulphite prepared by the method of Saslaw & Waravdekar (1957) was used. Approximately 30% of the malonaldehyde was destroyed by incubation in air for 1.5 hr. and about 90% was destroyed by incubation in the presence of excess of hydrogen peroxide. An amount of tocopherol adequate to prevent lipid oxidation had no protective effect in this case. After catalytic decomposition of hydrogen peroxide some destruction of added malonaldehyde still occurred, possibly due to remaining traces of hydrogen peroxide or to dissolved oxygen. These results, which show that malonaldehyde is rapidly destroyed by excess of hydrogen peroxide, lead to the conclusion that its rate of formation in lipids in the presence of hydrogen peroxide must be even more rapid since it is found there in measurable quantities.

Oxidation of tocopherol. In experiments in which tocopherol was added to lipids incubated with hydrogen peroxide, a substance was formed which reacted with thiobarbituric acid to give a coloured product different from that given by malonaldehyde.

Incubation of α -tocopherol (47.5 μ m-moles) at 37° in buffered solution with or without ascorbic acid did not produce any material giving a colour in the thiobarbituric acid test. However, incubation

Fig. 1. Absorption spectra of reaction mixtures obtained when α -tocopherol (47.5 μ m-moles) was incubated with H_2O_2 (735 μ moles) and the product had reacted with thiobarbituric acid. Upper curve, average extinction after incubation for 3 hr.; lower curve, corresponding values after incubation for 1.5 hr. λ_{max} , 498 $m\mu$.

of α -tocopherol with hydrogen peroxide followed by reaction with thiobarbituric acid gave an orange solution (λ_{max} , 498 $m\mu$). The average absorption spectra for incubation reaction mixtures for 1.5 and

3 hr. are given in Fig. 1. It is evident from these spectra that the material produced in the reaction of α -tocopherol and hydrogen peroxide is not malonaldehyde (Waravdekar & Saslaw, 1959), β -formylpyruvic acid (Warren, 1959) or glyoxal (Patton & Kurtz, 1951) since these react with thiobarbituric acid to give substances with $\lambda_{\max}$ at 532, 549 and 525 $m\mu$ respectively. $E_{\max}$ for the reaction product is up to three times as great (depending on the incubation period) as that given by the same quantity of tocopherol in the Emmerie-Engel test. Hence under suitable conditions this reaction may have analytical application as a very sensitive test for tocopherol.

From the spectral characteristics of the products given by this substance and by malonaldehyde (or fatty acid-oxidation products) it is possible to calculate the relative amounts of the two substances present. Thus μ moles of malonaldehyde (in 6 ml. of solution) = $0.054 E_{532} - 0.034 E_{498}$. With this equation a correction can be applied to results obtained from lipids, containing tocopherol, which have been oxidized by hydrogen peroxide.

Haemolysis experiments. If oxidation of lipids in the erythrocyte produces a haemolytic substance, it should be possible to demonstrate haemolysis by erythrocyte fatty acids oxidized *in vitro*. Owing to their detergent activity emulsions of fatty acids are haemolytic before oxidation; hence it is necessary to isolate the oxidation products and test these separately. The nearest approach made to this was to test the effect of malonaldehyde by itself. Incubation of 1 ml. of packed erythrocytes for over 3 hr. at 42° with up to 0.12 μ mole of malonaldehyde bisulphite produced no visible change. This amount of malonaldehyde is equivalent to five times the thiobarbituric acid-reactive material present in a suspension of fatty acids from 1 ml. of packed erythrocytes treated with hydrogen peroxide. Malonaldehyde is therefore unlikely to be the factor responsible for the haemolysis of cells incubated under oxidizing conditions.

DISCUSSION

The results presented here show that oxidation of erythrocyte lipids will occur under suitable conditions. Owing to the necessity of removing oxidizable material other than lipid, together with any naturally occurring antioxidants, the system that has been studied is rather far from resembling the intact lipoprotein membrane. The finding that oxidation can occur does, however, lend some support to the contention of Bunyan *et al.* (1960) that some of the colour formation they observed with thiobarbituric acid was due to the attack by dialuric acid on the lipoprotein membrane. A quantitative comparison is not possible, however, for under the

conditions used by these workers only water-soluble products of lipid degradation were treated with thiobarbituric acid. The method used by Tsen & Collier (1960), on the other hand, would not seem to differentiate between the colours given by haem compounds (Cowlshaw, 1961), and those given by lipid-oxidation products, a difficulty which does not arise if precipitated proteins are removed from the system (J. G. Bieri, personal communication).

Production of malonaldehyde by an oxidized lipid is only indirectly a measure of the amount of lipid which has been degraded. It does, however, give an indication of the amounts of some other lipid-degradation products formed in the system, for it has been shown by Kenaston, Wilbur, Ottolenghi & Bernheim (1955) that malonaldehyde production is paralleled by increases in peroxide value, conjugation and total aldehyde in some common fatty acids.

It is known that lipids suspended in an aqueous medium are more easily oxidized than 'solubilized' lipids (Tappel, 1953). Hence it seems reasonable to postulate that the rate of oxidation of the emulsions studied here gives an upper limit for the rate of oxidation of lipids in a complex lipoprotein membrane. With the values given by Ponder (1948) for dimensions of the erythrocyte it is calculated that the highest amount of malonaldehyde produced is of the order of 10^6 molecules/cell or 1 molecule/ $2 \times 10^4 \text{ \AA}^2$ of cell surface. None of the lysins listed by Ponder (1948) has any effect at such a low concentration, hence for any lipid oxidation product to act as a haemolysin it would need to be much more powerful than these or it would have to occur in much larger quantities than malonaldehyde.

On these grounds it seems necessary to supplement studies relating the production of thiobarbituric acid-reactive material to haemolysis with other studies of the role of lipids in maintaining cell-membrane integrity.

SUMMARY

1. The rate of appearance of malonaldehyde in an aqueous suspension of fatty acids from human erythrocytes was insignificant. It was increased by ascorbic acid and by hydrogen peroxide.
2. Added α -tocopherol inhibited the production of malonaldehyde in the presence of ascorbic acid, but in the presence of hydrogen peroxide tocopherol reacted to form a substance which gave a coloured complex with thiobarbituric acid ($\lambda_{\max}$ at 498 $m\mu$), and less protection against lipid oxidation resulted.
3. Malonaldehyde was destroyed slowly by incubation in aqueous solution at 37° and more rapidly in the presence of hydrogen peroxide. Tocopherol had no protective action.

4. An amount of malonaldehyde greater than that in oxidized erythrocyte fatty acids had no haemolytic action.

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Ketonic Metabolites of Progesterone and 19-Norprogesterone in Rabbit Urine

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20 α -Hydroxypregn-4-en-3-one and its 20 β -epimer have been found in the blood and tissues of several animal species as well as in man. This fact, together with their endocrine activities and biochemical relationship to progesterone, led Zander, Forbes, Münstermann & Neher (1958) to propose that these compounds should be classified as naturally occurring gestogens. The present paper describes the detection of the 20 α -hydroxy compound in the urine of progesterone-treated rabbits. Despite the widespread occurrence of the two pregnenolones, neither has previously been reported as a metabolite excreted in urine. Consequently this finding would appear to indicate that progesterone is metabolized by a novel pathway in the rabbit. It was decided, therefore, to study also the ketonic metabolites of 19-norprogesterone (Djerassi, Miramontes & Rosenkranz, 1953) to determine whether this steroid was metabolized in a manner similar to that for progesterone, despite the absence of the methyl group flanking the Δ^4 -3-ketone system.

EXPERIMENTAL

Administration of steroids. Dutch-strain rabbits were injected subcutaneously with a solution of the steroid in arachis oil (40 mg./ml.). Urine was then collected for 3 days in vessels containing 5 ml. of toluene as preservative.

Fractionation of urine. Steroids were extracted from the urine with 3 vol. of butan-1-ol. The butanol extract was evaporated to dryness *in vacuo* and the residue partitioned between water (100 ml.) and ether (4 $\times$ 40 ml.). The ether layer (unconjugated metabolites) was devoid of steroids. Part of the aqueous layer (90 ml.) (the conjugated-metabolite fraction) was hydrolysed by heating under reflux with conc. hydrochloric acid (13 ml.) for 30 min. The remainder of the conjugated fraction (10 ml.) was buffered in acetate to pH 4.5 and then incubated with 5000 Fishman units of β -glucuronidase (Ketodase; Warner-Chilcott Laboratories) at 37° for 3 days. The two hydrolysates were then fractionated separately as follows: The hydrolysate was saturated with sodium chloride and extracted with ether. The extracted material was fractionated by the method of Brown (1955) up to the point where a solution of the metabolites in 50 ml. of light petroleum-benzene