Environmental Effects on the Autoxidation of Retinol

By D. FISHER,* F. U. LICHTI†§ and J. A. LUCY*

* Department of Biochemistry, Royal Free Hospital School of Medicine, University of London,
8 Hunter Street, London WC1N 1BP, U.K.,

and

† Strangeways Research Laboratory, Wort's Causeway, Cambridge, U.K.

(Received 17 May 1972)

1. The behaviour of retinol in aqueous colloidal dispersions has been studied because, if membranes are a physiological site of action of vitamin A, the reactions of colloidal retinol may be relevant to the functions of the vitamin *in vivo*. 2. Dispersions of retinol in NaCl exhibit characteristic spectral changes, and they consume O₂, within minutes of preparation. 3. The maximum rate of O₂ uptake is approximately linearly dependent on the concentration of O₂. 4. At limiting concentrations of O₂, the spectral changes are accelerated by catalase, indicating that H₂O₂ is one of the reaction products. 5. The autoxidation, which is relatively unaffected by light, has the characteristics of a radical-catalysed reaction. O₂ uptake is preceded by an exceptionally short induction period; the reaction is catalysed by Fe²⁺ ions and is inhibited by diphenylpicrylhydrazyl. 6. The maximum rate of autoxidation, which is less in water or sucrose solution than in saline, depends on the degree of aggregation of retinol molecules induced by cations. 7. In the absence of O₂, the cation-induced aggregates exhibit a spectral red-shift, which difference-spectra indicate is caused by formation of a species with $\lambda_{\max}$. 370–380 nm. 8. This species, from which retinol can be quantitatively recovered, is apparently the oxygen-sensitive form of retinol that initiates the rapid autoxidation. 9. The possible biological significance of the production of a highly reactive form of retinol in micellar aggregates is discussed.

Although there have been numerous indications during the past decade that the systemic functions of vitamin A might be ascribed to its participation as a cofactor in certain enzyme reactions, subsequent investigations have not substantiated this concept (Rogers, 1969; Wasserman & Corradino, 1971). In previous studies it was observed that retinol behaves as an electron donor in its reactions in model systems with 7,7,8,8-tetracyanoquinodimethane and chloranil (Lichti & Lucy, 1969) and with iodine (Lucy & Lichti, 1969). Investigations on the properties of retinol have been continued as it is considered that studies on the chemistry of the vitamin will ultimately facilitate an understanding of its biochemical behaviour and of its functions *in vivo*. Of particular interest are the reactions of the vitamin in aqueous colloidal dispersions since, if membranes should constitute a physiological site of action of vitamin A (cf. Dingle & Lucy, 1965), the reactions of the vitamin in these dispersions may resemble its behaviour in cells.

We have found that colloidal dispersions of retinol in NaCl undergo much more rapid autoxidation than molecules of retinol dissolved in an organic solvent.

§ Present address: Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, Thailand.

The factors governing this rapid autoxidation have been investigated because they may be generally relevant to the behaviour of retinol in membranes, and in particular to the means by which excess of retinol causes extensive damage to lipoprotein membranes (Lucy & Dingle, 1964; Daniel *et al.*, 1966). Preliminary communications on aspects of this work have been published previously (Lucy, 1965, 1966, 1969).

Materials and Methods

Reagents

Synthetic crystalline retinol (Roche Products Ltd., Welwyn Garden City, Herts., U.K.) was handled without further purification as described by Dingle & Lucy (1962). Ethanol (James Burrough Ltd., London S.E.11, U.K.) was bubbled with O₂-free N₂ before use. Diphenylpicrylhydrazyl was obtained from Hopkin and Williams Ltd. (Chadwell Heath, Essex, U.K.) and used without further purification. Stock solutions of diphenylpicrylhydrazyl were prepared in N₂-saturated ethanol; their concentration was determined from the E_{520} by using a value of 1.06×10^4 litre·mol⁻¹·cm⁻¹ as the molar extinction coefficient (Matheson *et al.*, 1951). A suspension of crystalline

catalase from ox liver [Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.] was used without further purification.

Spectrophotometric measurements

Spectra were obtained with either a Unicam SP. 700, or a Unicam SP. 800 recording spectrophotometer. All measurements were made in stoppered silica cells (1 cm light-path). A thermostatic cell-compartment was used for experiments conducted at temperatures other than room temperature. The second sample position was used in experiments conducted with the Unicam SP. 800 spectrophotometer.

Measurement of O₂ consumption

During these experiments, different O₂ micro-electrodes were used all of which consumed little O₂ in operation. For most of the experiments a Clark-type O₂ electrode manufactured by Rank Bros., Bottisham, Cambridge, U.K. was employed. O₂ concentrations were measured with the aid of an amplifier (manufactured by the Times Instrument Co. Ltd., Great Shelford, Cambridge, U.K.); in some experiments this was connected to a potentiometric recorder. Solutions of retinol in ethanol were injected either with an 'Agla' micrometer syringe (Burroughs Wellcome and Co., London N.W.1, U.K.), or by means of a Hamilton syringe (Micromasure N.V., The Hague, Holland) into the thermostatically controlled chamber containing the aqueous medium. Since the response of the O₂ electrode is dependent on temperature, the electrode was standardized with air-equilibrated water at each temperature used, when the dependence of the oxidation of retinol on temperature was studied.

Maximum rates of O₂ uptake were calculated from the slope of the tangent drawn to the steepest portion of the O₂-uptake curve. The induction period was determined by considering the end of the induction period as the point of intersection of the two tangents drawn to the portions of maximum and initial minimum rates of O₂ uptake. The concentration of O₂ in solution was calculated from information given by Hodgman (1960) and the concentration of O₂ in 154mM-NaCl at 25°C was taken to be 256 μ M, on the assumption that the concentration of O₂ in this solution is the same as that in water. The results of Robinson & Cooper (1970) indicate that the concentration of O₂ in 154mM-NaCl at 25°C is about 5% less than this value.

Results

Ultraviolet-absorption spectra

The ultraviolet-absorption spectrum of retinol dissolved in ethanol changed little with exposure to

Fig. 1. Spectral changes (—) occurring with time (1, 10 and 15 min) after the preparation of a dispersion of retinol (35 μ M) in air-equilibrated NaCl (154mM) at room temperature

The dispersion was prepared by diluting 0.1 ml of retinol (3.5mM) in ethanol with the NaCl solution. For comparison, the spectrum of a similar dispersion of retinol in previously boiled N₂-gassed NaCl (----) is shown 1 and 15 min after its preparation.

air during 3 h, despite the relatively high solubility of O_2 in ethanol that is in equilibrium with air at 25°C (2.1 mm- O_2 ; calculated from information published by Seidell & Linke, 1952). The absorption of a dispersion of retinol (35 μM), prepared by diluting 0.1 ml of a solution of retinol in ethanol (3.5 mM) with 154 mM-NaCl, decreased slowly with time (Fig. 1), when the saline was previously boiled and gassed with O_2 -free N_2 .

By contrast, the extinction of a similarly prepared dispersion of retinol in NaCl that was in equilibrium with the air decreased rapidly: the decrease in extinction beginning within 1 min of preparing the dispersion. In addition, spectral fine structure quickly developed, and pronounced absorption maxima were observed after 10–15 min at about 309, 323 and 338 nm (32.4, 31.0 and 29.6 kcycles/cm). An increased absorption was also noted in the region of 230 nm (42.5 kcycles/cm) (Fig. 1). The exact positions of these bands as well as their relative heights varied with time and it is possible that a number of intermediates and products were present simultaneously in the reaction mixture. The development of fine structure may indicate, however, the formation of one or more substances having the *retro* structure (Beutel *et al.*, 1955), whereas the positions of the bands would imply that any *retro* compound that may be present has only four conjugated double bonds.

When the spectra of dilute dispersions of retinol in saline were followed for 1–2 h in the presence of air, the fine structure was eventually lost and only a single broad absorption band remained. This had a well-defined maximum at about 295 nm and a relatively low extinction: the overall shift to shorter wavelengths on oxidation indicating that an average of one double bond had been destroyed. Material having an absorption maximum at 295 nm was also formed when a solution of retinol in ethanol was left in contact with the air for 90 h. An absorption maximum at this position develops when retinol interacts with 7,7,8,8-tetracyanoquinodimethane (Lichti & Lucy, 1969), and when it reacts with iodine (Lucy & Lichti, 1969).

Consumption of O_2

The use of more concentrated suspensions of retinol enabled changes in O_2 concentration to be measured by means of an O_2 electrode. The kinetics of the uptake of O_2 were similar to those normally observed in autocatalytic free radical-dependent chain reactions (Scott, 1963), and there was an initial induction period of approx. 1 min during which no O_2 was consumed. This was followed by three phases in which the rate of uptake first increased, then remained constant, and finally decreased. At 37°C the maximum rate was reached between 2 and 4 min after the preparation of the suspension (Fig. 2).

To compare rates of the uptake of O_2 with spectral

Fig. 2. Comparison of the kinetics of the consumption of O_2 (■) with the decrease in extinction at 325 nm (▲) for a dispersion of retinol (175 μM) in air-equilibrated NaCl (154 mM) at 37°C

Spectral measurements were made on 1 ml portions of the reaction mixture after a tenfold dilution with ethanol as described in the text.

changes, 1 ml samples of the reaction mixture were diluted with 9 ml of ethanol (which effectively halted the reaction), at various times after preparation of the initial aqueous dispersion. Spectra were then obtained with the ethanolic solutions, and the decrease in E_{325} (30.8 kcycles/cm), unlike the uptake of O_2 , did not show an induction period (Fig. 2). Decrease in extinction preceded the O_2 consumption, and approximately paralleled it after the first minute.

The maximum rate of consumption of O_2 was determined for retinol (175 μM) suspended in O_2 -saturated saline, in a mixture of equal volumes of O_2 -saturated and N_2 -saturated saline, and in air-saturated saline at 25°C; there was an approximately linear dependence of the maximum rate of O_2 consumption by colloidal retinol on the concentration of O_2 between 0.25 and 1.2 mm- O_2 .

Action of catalase

As H_2O_2 can be formed in autoxidation reactions and it is generated in erythrocytes by some haemolytic agents (Cohen & Hochstein, 1964), excess of retinol may damage biological membranes by the production of H_2O_2 because of the autoxidation of molecules of exogenous retinol within membranes. Experiments were therefore done to see if H_2O_2 is produced during the autoxidation of dispersions of retinol in saline solution. Attempts to demonstrate the formation of

Fig. 3. Spectral changes (—) occurring at room temperature with time (1 and 15 min) after the preparation of a dispersion of retinol ($35\mu\text{M}$) in previously boiled N_2 -gassed NaCl (154mM) in the presence of catalase ($0.5\mu\text{g/ml}$)

For comparison, the spectrum (----) of a similar dispersion containing KCN (1mM) in addition to catalase ($0.5\mu\text{g/ml}$) is shown 1 and 15 min after its preparation.

H_2O_2 by direct colorimetric assay were unsuccessful owing to the difficulty of estimating very small quantities of H_2O_2 in the turbid suspensions of autoxidizing retinol.

Addition of catalase to suspensions of retinol nevertheless had a very marked effect on the progress of the autoxidation reaction as monitored by the associated spectral changes. If the available O_2 was severely limited by using dispersions of retinol prepared in boiled saline that had been gassed with N_2 , the dispersions of retinol exhibited relatively stable spectra (Fig. 1). When catalase was added to such dispersions, the spectral changes characteristic of the rapid autoxidation occurring in oxygenated saline were immediately observed (Fig. 3). To ensure that the observed action of catalase was dependent on the catalytic properties of the enzyme, and not on some non-specific interaction with retinol, the effects of known inhibitors of catalase were studied (Nicholls & Schonbaum, 1963). With dispersed $35\mu\text{M}$ -retinol in saline and $0.5\mu\text{g}$ of catalase/ml, partial inhibition of the action of catalase was noted with NaF (50mM), and a more extensive inhibition with KCN (1mM), (Fig. 3). With NaN_3 (6mM) and catalase there was a marked decrease in absorbance over 15 min and a broadening of the spectrum, but the spectral fine structure that is characteristic of the autoxidation of retinol did not develop. The addition of exogenous H_2O_2 (in the absence of catalase) to comparable suspensions of retinol had no action, even at H_2O_2 concentrations of 0.015 – 0.15% (v/v), although at the highest concentration the spectrum of the retinol was partially obscured by that of H_2O_2 . These findings are thought to indicate that small quantities of H_2O_2 are formed during the initial autoxidation of retinol so that, even when the O_2 concentration is low and limiting, catalase allows the reaction to proceed continuously by making O_2 available from H_2O_2 .

Acceleration and inhibition of autoxidation

If the autoxidation of retinol does proceed by a free-radical chain reaction, as indicated by the S-shaped curve describing the consumption of O_2 , it would be expected that autoxidation would be accelerated by trace quantities of heavy metal ions, e.g. Fe^{2+} , as occurs in the peroxidation of linoleate at 30°C (Smith & Dunkley, 1962). This was in fact observed. Fe^{2+} ($50\mu\text{M}$) markedly catalysed the characteristic spectral changes that were seen with dispersions of retinol ($35\mu\text{M}$) in NaCl (154mM) in equilibrium with air, and Fe^{2+} (1 – $10\mu\text{M}$) added to similar, but more concentrated dispersions of retinol ($130\mu\text{M}$), decreased the induction period preceding the consumption of O_2 from approx. 1 min to 30 s. Also, although relatively high concentrations of alkali metal cations (25 – 60mM) were required to give the maximum rate of O_2 uptake with dispersions of

retinol ($200\mu\text{M}$) in air (see below), $0.15\text{--}0.20\text{mM-Fe}^{2+}$ was comparably effective.

Additional evidence to support the idea that the autoxidation of colloidal retinol in saline involves free radicals was provided by the behaviour of the system in the presence of the stable free radical diphenylpicrylhydrazyl. This substance behaves as a

Fig. 4. Effects of concentration of diphenylpicrylhydrazyl on the induction period (●) and the maximum rate of uptake of O_2 (■) observed with retinol ($200\mu\text{M}$) dispersed in air-equilibrated NaCl (154mM) at 25°C

The results are expressed, on a percentage basis relative to the values observed in the absence of inhibitor. Diphenylpicrylhydrazyl in ethanol was added to 5ml portions of the NaCl solution immediately before preparation of the retinol dispersions. The final concentration of ethanol was always 2% (v/v). The end of each induction period was taken as the point of intersection of the tangents to the initial minimum and to the maximum rate of O_2 consumption.

radical scavenger, combining with reactive radicals to give an inactive product (Forrester *et al.*, 1968); it therefore inhibits radical-dependent oxidations. Diphenylpicrylhydrazyl will extend the induction period of a radical-dependent oxidation, but O_2 consumption will proceed at the uninhibited rate once the added inhibitor has been totally consumed. Fig. 4 shows the effect of various concentrations of diphenylpicrylhydrazyl on the induction time and on the maximum rate of O_2 consumption by retinol in air-equilibrated saline at 25°C . With $200\mu\text{M}$ -retinol, diphenylpicrylhydrazyl markedly lengthened the induction period: with $4\mu\text{M}$ -diphenylpicrylhydrazyl the induction period was 29 min. By contrast, the maximum rate of oxidation of retinol showed relatively little change. The slight decrease in the maximum rate caused by diphenylpicrylhydrazyl probably resulted from the fact that the concentration of retinol had been decreased by the end of the induction period.

Effect of light

Grady & Borg (1968) reported that light-induced free radicals of retinol, retinal and rhodopsin can be formed. To test whether photo-activated molecules of retinol were giving rise to chain-initiating free radicals in the autoxidation process, both the preparation of ethanolic solutions of retinol (from a newly-opened dark-coloured ampoule of synthetic crystalline retinol) and the injection of samples into the chamber of the O_2 electrode were done in very dim light. The autoxidation reaction itself was allowed to proceed in complete darkness. No significant differences, either in induction period or in the maximum rate of uptake of O_2 , were observed between experiments done in the dark and the light when retinol ($94\mu\text{M}$) was allowed to oxidize in air-equilibrated saline at room temperature.

Concentration of retinol

To see if the rate of autoxidation of colloidal retinol depends on the concentration of the vitamin, retinol was added to air-equilibrated saline in two different ways. Namely, by method (a) in which various volumes of a solution of retinol in ethanol were added to rapidly stirred saline, and by method (b) in which a standard volume ($100\mu\text{l}$) of solutions of various concentrations of retinol in ethanol was added to the stirred saline. These two procedures were investigated as variations in the concentration of retinol in ethanol at the instant of contact with water might affect the subsequent rate of oxidation, but there was little difference between the results obtained by the two methods, and in both processes the maximum rates of consumption of O_2 were directly proportional to the concentrations of retinol. In experiments with method (b), the induction period

decreased with increasing concentrations of retinol: changing approximately inversely with the sixth power of the concentration of retinol.

At 25°C, mean values for the maximum rate of uptake of O₂ of 220 and 200nmol of O₂ consumed/min per μ mol of retinol were given by methods (a) and (b) respectively with four different final concentrations of retinol over the range 100–525 μ M. In 21 separate determinations in which 0.1 ml of ethanolic 10mM-retinol was injected into 5 ml of saline at 25°C to give a final concentration of 200 μ M-retinol, the mean rate of oxidation was 180 ± 18 nmol of O₂/min per μ mol of retinol.

The rates of oxidation of retinol in water were difficult to reproduce, particularly with method (a). When various quantities of a given ethanolic solution of retinol were added to water instead of to saline by method (a), the rate of O₂ consumption was 124 nmol of O₂/min per μ mol of retinol over the concentration range, 98–587 μ M-retinol. With method (b), a very low rate of O₂ consumption (30 nmol of O₂/min per μ mol of retinol) was observed for the same range of concentrations of retinol.

Actions of salts

The effect of various concentrations of different cations on the autooxidation of colloidal retinol was

investigated. Increasing the concentration of NaCl, or other salt, increased the maximum rate of oxidation to a point beyond which the rate was relatively independent of salt concentration (Fig. 5). The maximum rate of oxidation obtained with BaCl₂ was rather less than that with the chlorides of Li⁺, Na⁺, K⁺, Cs⁺, and La³⁺. Low concentrations of Ba²⁺ and La³⁺ were more effective in accelerating the autooxidation of retinol than univalent cations: retinol dispersed in 1 mM-LaCl₃ oxidized at the same rate as retinol dispersed in 50 mM-NaCl. Thus the order of effectiveness of cations is similar to that in the Hofmeister series for producing coagulation of hydrophilic colloids (Alexandër & Johnson, 1949). The chaotropic agent KSCN (Dandliker & de Saussure, 1971) was less effective than KCl in facilitating oxidation of dispersed retinol (Fig. 5), indicating that the structure of water may play a role in this system. Retinol (220 μ M) dispersed in 0.25M-sucrose behaved like colloidal suspensions in water both with respect to spectral stability and in showing a low rate of O₂ consumption.

Properties of aggregated retinol

The spectral behaviour of retinol was investigated further to obtain information on the apparent dependence of the rate of autooxidation in an aqueous environ-

Fig. 5. Comparison of the rates of maximum consumption of O₂ at 25°C by dispersions of retinol (200 μ M) in differing air-equilibrated salt solutions of increasing concentration

The rates are expressed as a percentage of the maximum rate of consumption of O₂ by retinol (200 μ M) dispersed in air-equilibrated NaCl (154 mM) at 25°C; ●, NaCl; ■, LiCl; ▲, KCl; ▽, CsCl; □, BaCl₂; ○, LaCl₃; △, KSCN.

ment on the degree of molecular aggregation. The effects of aggregation were studied by adding 0.1 ml of a solution of retinol in ethanol (8.75–10 mM) to 0.2 ml of N_2 -saturated water or NaCl (154 mM). After 30 s the concentrated suspension of retinol was diluted with 10 ml of either water or saline: the spectrum of the diluted suspension was then measured immediately.

When retinol in ethanol was added initially to N_2 -saturated water, and air-equilibrated water then used as the diluent, the final suspension had a broad absorption band (318–365 nm) with a maximum at approx. 340 nm. The spectra of a dispersion made in this way remained unchanged for 10 min at room temperature: this behaviour was similar to that observed with suspensions prepared by adding 10 ml of air-equilibrated water directly to 0.1 ml of the ethanolic solution of retinol. The behaviour of a suspension prepared by adding retinol in ethanol to N_2 -saturated NaCl (154 mM) and then diluting with air-equilibrated NaCl (154 mM) differed significantly, however, from that of a suspension prepared directly in the air-equilibrated saline. The preparation made via the intermediate concentrated suspension of retinol in saline had an absorption maximum at 385 nm (26 kcycles/cm) (Fig. 6). After 3 min, this suspension was extensively oxidized, as judged by the appearance of spectral fine structure. By contrast, the corresponding suspension prepared directly in air-equilibrated saline exhibited little evidence of a distinct absorption band at 385 nm; it also had a higher initial absorption and spectral fine structure appeared more slowly with time (Fig. 7). In each instance, however, the final product absorbed in the region of 295 nm (34 kcycles/cm) (Figs. 6 and 7).

Finally, retinol in ethanol was added initially to N_2 -saturated saline and then diluted with N_2 -saturated saline. The spectrum of this suspension had a decreased absorption and a pronounced maximum at 385 nm (Fig. 8); it was little changed after 20 min.

These findings are thought to indicate that not only does the autoxidation of retinol involve the formation of molecular aggregates in the presence of salts, but that the aggregates so produced constitute an oxygen-sensitive species which absorbs light at a relatively long wavelength as compared with retinol. When the uptake of O_2 by retinol (final concentration 75 μ M) suspended directly in air-saturated saline was compared with that of a suspension that had the same final concentration but was initially prepared in concentrated form under N_2 and then diluted in air-saturated saline, O_2 consumption by the latter suspension commenced immediately and showed no induction period. Even with the stable free-radical scavenger diphenylpicrylhydrazyl (see above) in the diluent, there was an initial burst of O_2 uptake immediately on dilution, that was followed by the extended induction period characteristic of systems containing diphenylpicryl-

Fig. 6. Spectral changes occurring at room temperature with time (1, 3, 10 and 97 min) when 0.1 ml of retinol (10 mM) in ethanol was added to 0.2 ml of N_2 -saturated NaCl (154 mM) and, 30 s later, the suspension was diluted with 10 ml of air-equilibrated NaCl (154 mM)

hydrazyl. This is consistent with the facilitated formation of radicals in the aggregates of retinol, possibly by interactions of molecules of aggregated retinol with the traces of O_2 present in these preparations.

Reversibility of aggregation

When retinol was allowed to aggregate in the complete absence of O_2 , achieved by adding Na_2SO_3 to

Fig. 7. Spectral changes occurring at room temperature with time (1, 3, 10 and 74 min) after 10.2 ml of air-equilibrated NaCl (154 mM) was added directly to 0.1 ml retinol (10 μ M) in ethanol

the N_2 -gassed saline, the aggregated species with an absorption maximum at relatively long wavelengths was quite stable. There was no rapid decay of absorption and no fine structure developed. Under these conditions it was not necessary to prepare intermediate concentrated suspensions of retinol to study the material absorbing at long wavelengths.

Fig. 8. Spectral changes occurring at room temperature with time (1 and 20 min) when 0.1 ml of retinol (10 mM) in ethanol was added to 0.2 ml of N_2 -saturated NaCl (154 mM) and, 30 s later, the suspension was further diluted with 10 ml of this NaCl solution

Retinol can be 'recovered' from turbid suspensions in saline to some extent (as discussed below) by adding ethanol to portions of the dispersed retinol to a final concentration of 80% (w/v) ethanol. The spectra of the optically clear solutions thus obtained have been determined, and the quantity of recovered retinol determined by using a molar extinction coefficient of 52400 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$ (Morton, 1962). [Retinol in 80% and 60% (w/v) ethanol-saline had the same extinction coefficient as retinol in ethanol.]

In air-equilibrated saline, oxidation occurs and after 30 min little retinol was recovered (Table 1). Recovery from saline prepared with boiled water

Table 1. *Recovery of retinol from NaCl*

A solution of retinol (100 μ l; 10mM) in ethanol was dispersed in 10ml of 154mM-NaCl. Retinol was recovered after 30min at room temperature by either (A) adding portions of the dispersion (0.6ml) to ethanol (2.4ml) or (B) adding ethanol (40ml) to the total volume of the dispersion. Recovery was determined by comparing the extinctions at 325nm of the ethanolic solutions of retinol thus obtained with that of 100 μ l of retinol (10mM) in ethanol that was diluted directly with ethanol (50ml). The percentage recovery is given as the mean $\pm$ S.D. with the number of observations in parentheses.

Aqueous medium	Mode of recovery	Recovery (%)
Air-equilibrated saline	A	15.4 $\pm$ 2.1 (5)
N ₂ -equilibrated saline	A	79.0 $\pm$ 2.0 (4)
N ₂ -equilibrated saline containing Na ₂ SO ₃ (1mg/ml)	A	87.9 $\pm$ 2.1 (16)
N ₂ -equilibrated saline containing Na ₂ SO ₃ (1mg/ml)	B	100.0 $\pm$ 2.7 (4)*

* The only recovery that is not significantly different from 100% at $P > 0.001$.

that had been bubbled with O₂-free N₂ for 30min was also less than complete. This was presumably caused by oxidation, as there was a gradual formation of the triplet in the spectrum of this dispersion that is characteristic of oxidation. The addition of Na₂SO₃ (usually at 1mg/ml) to saline, which had been boiled and gassed with N₂, improved the recovery of retinol to almost 90%. However, the process of diluting samples of the suspensions itself led to losses, apparently because the dispersed retinol was adhering to the glass surfaces of the pipettes. By contrast, when retinol was allowed to aggregate in the complete absence of O₂, and ethanol was added to the whole suspension, recoveries of approx. 100% could be obtained (Table 1).

To see if the recovered retinol was identical with the starting material, the spectrum of the recovered retinol was compared with that of retinol dissolved directly in ethanol. In one such experiment, the control consisted of 12 samples of retinol in ethanol (0.1ml, 10mM), which were diluted first with ethanol (40ml) and then with 10ml of 154mM-NaCl containing Na₂SO₃ (1mg/ml). For comparison, a further 12 samples of retinol were each dispersed in 10ml of the saline; retinol was recovered from the aggregated material after 30min at room temperature by adding ethanol (40ml). Recovery was variable, apparently because of the difficulty of totally excluding O₂ from

the system. However, in three samples where recovery was approx. complete (98–101%), the spectrum of the recovered retinol was identical with that of the control between 280 and 350nm. In three samples where recovery was 94%, the spectrum of the recovered material showed a slight but detectable increase in absorbance between 280–320nm as compared with the control. The increase in this region of the spectrum was more marked with samples for which recovery was poor (77–91%). Thus even when only 6% of the retinol was oxidized, the spectrum of the recovered material was altered as when a dispersion of retinol in saline was allowed to oxidize for a few minutes. By contrast therefore, if oxidation is completely avoided, retinol may be recovered quantitatively from dispersions in which aggregated retinol exhibits a shift in its absorption maximum to longer wavelengths.

Possible formation of a charge-transfer complex

The wavelength of the absorption maximum observed in the spectra of dispersions of retinol in O₂-free saline varied with the concentration of retinol (10–100 μ M) over the range 315–350nm. However, when the spectrum of the dispersed retinol was subtracted from that of retinol dissolved in ethanol, a reproducible difference spectrum was obtained (Fig. 9), in which the position of λ_{max} was independent of the concentration of retinol. Corrections were made for the turbidity of the dispersions of retinol between 270 and 400nm by graphical extrapolation of the very-low absorbance in the region 400–550nm, but this made no significant difference to the shape of the difference spectra or to the position of λ_{max} . This type of difference spectrum was also obtained, after correcting for turbidity, with the markedly turbid dispersions produced when retinol is co-dispersed with α -tocopherol (D. Fisher, F. U. Lichti & J. A. Lucy, unpublished work). An absorption minimum was always observed at 370–380nm with such difference spectra (Fig. 9), suggesting that, during the aggregation of retinol in the aqueous dispersions, a new species is formed having λ_{max} at 370–380nm. The molar extinction of this material was independent of concentration in the range 20–100 μ M-retinol (approx. $\epsilon = 6600 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ at 380nm) and it formed immediately when retinol was dispersed in saline. When retinol was dispersed in water, the initial molar extinction was only about half that observed with saline (approx. $\epsilon = 3600 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ at 380nm) but it gradually increased. Only a trace of NaCl (0.05%, w/v) was necessary for the rapid production of the difference spectrum in an O₂-free environment.

When retinol was added to O₂-containing saline, the characteristic minimum at 370–380nm in the difference spectrum formed transiently but then

Fig. 9. Difference spectra (corrected for scattered light) obtained at room temperature when the extinctions of dispersions of retinol ($100\mu\text{M}$) in O_2 -free aqueous environments were subtracted from the extinction of retinol ($100\mu\text{M}$) in ethanol

●, Difference spectrum with retinol dispersed in NaCl (154 mM); ○, difference spectrum with retinol dispersed in water.

rapidly disappeared, whereas in the absence of O_2 the minimum remained fairly stable. This indicates that the increased extinction at 370–380 nm of the O_2 -free aqueous dispersions of retinol might reflect the formation of a new species of retinol that is particularly sensitive to destruction by molecular O_2 .

Discussion

Although the chemical changes involved in the reaction of colloidal dispersions of retinol in NaCl solution with molecular O_2 have not been investigated in detail in the present study, it is clear from our experiments that the reaction has the characteristics of a radical-catalysed autoxidation. The 1 min induction period is, however, exceptionally short, so that the maximum rate of O_2 consumption is reached by 2–4 min. By comparison, aqueous emulsions of phosphatidylethanolamine, having arachidonyl and linoleyl acyl chains, reach maximum rates of O_2 consumption about 8 h after their preparation (Corliss & Dugan, 1970).

In addition to having the general characteristics of a free-radical reaction, the autoxidation of retinol is distinguished by a number of interesting features. Thus the reactivity of retinol towards molecular O_2 is markedly dependent on the concentration of cations present. Polyvalent cations are particularly effective. Although an increased rate of autoxidation with

increase of salt concentration is relatively unusual, other micellar systems commonly display complex effects of salts on aggregation behaviour. For example, studies on dimethyldodecylamine oxide, which at pH 3 is protonated and behaves as a cationic surfactant, have shown that at this pH the micelles are probably small and spherical both in water and in 0.2 M-NaCl (Herrmann, 1964). By contrast, the micelles are large and rod-like in 0.2 M-NaBr at pH 3.

Somewhat similar effects of aggregation on autoxidation to those reported in the present paper have been noted in relation to the oxidation of unsaturated fatty acids. Banks (1944), in investigations on the oxidation by molecular O_2 of linoleic acid catalysed by haematin, found that no measurable oxidation occurred when linoleic acid and haematin were in solution in ethanol. The addition of water to the system, however, slowly but progressively caused an increase in the rate of oxidation, until a maximum rate was reached when the linoleic acid was in suspension. This led Banks (1944) to conclude that it seemed that the oxidation of linoleic acid is increased by orientation of the molecules.

Previous experiments showed that retinol behaves as an electron donor and will interact with several different electron acceptors (Lichti & Lucy, 1969; Lucy & Lichti, 1969). It is particularly interesting that the interactions of retinol with the weak electron acceptor chloranil were affected by NaCl. Unlike the strong acceptor tetracyanoquinodimethane which reacted with retinol in dimethylformamide to yield the corresponding radical anion, the absorption maxima of the radical anion of chloranil were not observed when retinol and chloranil were mixed in this solvent. However, when the mixture of retinol and chloranil in dimethylformamide was diluted with a large volume of 0.5 M-NaCl a turbid suspension was obtained which after 20 min showed clearly defined new maxima in its absorption spectrum at 426 and 455 nm, approximately the wavelengths at which the radical anion of chloranil absorbs (i.e. 425 and 450 nm). The radical anion was therefore apparently not formed in the absence of salt.

By analogy, the presence of salt in the aqueous dispersions of retinol studied here may conceivably enable molecules of retinol to accept electrons, in this instance from other retinol molecules, thus forming a radical anion. The radical anion (possibly present as part of a charge-transfer complex) might then be the species that reacts extremely rapidly with molecular O_2 and initiates the chain reaction, since no uptake of O_2 occurs when the products of the reaction in dimethylformamide between retinol and the electron acceptor, 7,7,8,8-tetracyanoquinodimethane, are suspended in saline (Lichti & Lucy, 1967). The number of molecules of retinol required to exhibit the characteristic behaviour of aggregates may be less important than the juxtaposition and orientation

of adjacent retinol molecules. The unstable behaviour of monolayers of retinol molecules orientated at an air-saline interface (Bangham *et al.*, 1964) may be significant in this context. These monolayers exhibited a spontaneous decrease of surface pressure within a few minutes of their preparation, and this behaviour was attributed to the interaction of O_2 with the orientated molecules of retinol.

The red-shift that we have observed with aggregated retinol, and the results of the difference-spectra experiments, are thought to be consistent with the idea that a species of 'activated retinol' arises from an intermolecular charge-transfer reaction between adjacent molecules of retinol, which may subsequently lead to the formation of a complex such as (retinol $\rightarrow$ retinol) or possibly (retinol $\cdot^+$ ·retinol $\cdot^-$) in salt-induced micellar aggregates. This is known in other systems such as chlorophyll. Comparable spectral shifts associated with the aggregation of hydrated chlorophyll molecules are well documented (Chapman & Fast, 1968; Katz & Ballschmitter, 1968; Quinlan, 1968; Sherman & Fujimori, 1969), and phospholipid bilayer membranes containing high concentrations of chlorophyll show small shifts of the absorption maxima towards longer wavelengths that indicate an interaction between the porphyrin rings in the film (Steinemann *et al.*, 1971). Also the electron-paramagnetic-resonance properties of chlorophyll-water aggregates may be consistent with the formation of radical anions and cations of chlorophyll (Katz *et al.*, 1968), and O_2 may be involved in a charge-transfer process, occurring between chlorophyll molecules, that is augmented by the interaction of water with chlorophyll (Sherman & Fujimori, 1968).

As expected from a consideration of molecular structure, the closest parallel to the behaviour of retinol in an aqueous environment is found among the carotenoid series, in particular with the hydroxylated carotenoid, astaxanthin (3,3'-dihydroxy-4,4'-dioxo- β -carotene). Buchwald & Jencks (1968) reported that the addition of a small volume of a solution of astaxanthin in ethanol ($\lambda_{\max}$, 475 nm) to a large volume of water gives a stable suspension of aggregates or crystals of the pigment, containing 10% (v/v) ethanol, which has $\lambda_{\max}$, 455 nm. The presence of 0.9M-NaCl causes a red-shift of $\lambda_{\max}$, to 555 nm. However, the normal spectrum of astaxanthin is observed if ethanol is then added to a concentration of 50% within a few minutes, but on prolonged standing there is an irreversible decomposition of the astaxanthin that may be related to the susceptibility of carotenoids in suspension to oxidative degradation (Karrer & Straus, 1938).

Pitt (1969) reported that α -retinol is physiologically inactive, although it has surface-active properties and also produces effects when present in excess that are similar to those of excess of retinol itself. This

need not mean, however, that the surface-active and membrane-active properties of retinol are necessarily of no biological significance. It is possible that, as a result of the displaced double bond in the ionone ring, α -retinol might simply lack some additional property, e.g. the ability to participate in charge-transfer interactions that is possessed by the physiologically active isomer. Some support for this interpretation may be provided by the preliminary observations of Houghton (1969) which indicate that α -retinol is a very much poorer donor of electrons to 7,7,8,8-tetracyanoquinodimethane and chloranil than is retinol under comparable conditions. Our present studies on the chemical properties of retinol that depend for their expression on the amphipathic and surface-active characteristics of the molecule may therefore be relevant to the biochemical mode of action of physiological quantities of the vitamin *in vivo*. However, whether the reactive species of retinol discussed here will ultimately be found to be of biological significance is a matter for conjecture.

These investigations were supported in part by a research grant to J. A. L. from the Medical Research Council. During the early part of the work, F. U. L. held a post-doctoral fellowship of the U.S. Public Health Service.

References

- Alexander, A. E. & Johnson, P. (1949) *Colloid Science*, pp. 16, 714, Oxford University Press, London
- Bangham, A. D., Dingle, J. T. & Lucy, J. A. (1964) *Biochem. J.* **90**, 133-140
- Banks, A. (1944) *J. Soc. Chem. Ind. London Trans. Commun.* **63**, 8-13
- Beutel, R. H., Hinkley, D. F. & Pollak, P. I. (1955) *J. Amer. Chem. Soc.* **77**, 5166-5167
- Buchwald, M. & Jencks, W. P. (1968) *Biochemistry* **7**, 834-843
- Chapman, D. & Fast, P. G. (1968) *Science* **160**, 188-189
- Cohen, G. & Hochstein, P. (1964) *Biochemistry* **3**, 895-900
- Corliss, G. A. & Dugan, L. R. (1970) *Lipids* **5**, 846-853
- Dandliker, W. B. & de Saussure, V. A. (1971) in *The Chemistry of Biosurfaces* (Hair, M. L., ed.), vol. 1, pp. 1-43, Marcel Dekker, New York
- Daniel, M. R., Dingle, J. T., Glauert, A. M. & Lucy, J. A. (1966) *J. Cell Biol.* **30**, 465-475
- Dingle, J. T. & Lucy, J. A. (1962) *Biochem. J.* **84**, 611-621
- Dingle, J. T. & Lucy, J. A. (1965) *Biol. Rev. Cambridge Phil. Soc.* **40**, 422-461
- Forrester, A. R., Hay, J. M. & Thomson, R. H. (1968) *Organic Chemistry of Stable Free Radicals*, pp. 153-156, Academic Press, London
- Grady, F. J. & Borg, D. C. (1968) *Biochemistry* **7**, 675-682
- Herrmann, K. W. (1964) *J. Phys. Chem.* **68**, 1540-1546
- Hodgman, C. D. (ed.) (1960) *Handbook of Chemistry and Physics*, p. 1707, Chemical Rubber Publishing Co., Cleveland

- Houghton, S. E. (1969) Ph.D. Thesis, University of Liverpool
- Karrer, P. & Straus, W. (1938) *Helv. Chim. Acta* **21**, 1624-1636
- Katz, J. J. & Ballschmiter, K. (1968) *Angew. Chem. Int. Ed. Engl.* **7**, 286-287
- Katz, J. J., Ballschmiter, K., Garcia-Morin, M., Strain, H. H. & Uphaus, R. A. (1968) *Proc. Nat. Acad. Sci. U.S.* **60**, 100-107
- Lichti, F. U. & Lucy, J. A. (1967) *Biochem. J.* **103**, 34P
- Lichti, F. U. & Lucy, J. A. (1969) *Biochem. J.* **112**, 221-229
- Lucy, J. A. (1965) *Biochem. J.* **96**, 12P-13P
- Lucy, J. A. (1966) *Biochem. J.* **99**, 57P
- Lucy, J. A. (1969) *Amer. J. Clin. Nutr.* **22**, 1033-1044
- Lucy, J. A. & Dingle, J. T. (1964) *Nature (London)* **204**, 156-160
- Lucy, J. A. & Lichti, F. U. (1969) *Biochem. J.* **112**, 231-241
- Matheson, M. S., Auer, E. E., Bevilacqua, E. B. & Hart, E. J. (1951) *J. Amer. Chem. Soc.* **73**, 1700-1706
- Morton, R. A. (1962) *Compr. Biochem.* **3**, 66-132
- Nicholls, P. & Schonbaum, G. R. (1963) *Enzymes*, 2nd. edn., **8**, 147-225
- Pitt, G. A. J. (1969) *Amer. J. Clin. Nutr.* **22**, 1045-1046
- Quinlan, K. P. (1968) *Arch. Biochem. Biophys.* **127**, 31-36
- Robinson, J. & Cooper, J. M. (1970) *Anal. Biochem.* **33**, 390-399
- Rogers, W. E. (1969) *Amer. J. Clin. Nutr.* **22**, 1003-1013
- Scott, G. (1963) *Chem. Ind. (London)* **7**, 271-281
- Seidell, A. & Linke, W. F. (1952) *Solubilities of Inorganic and Organic Compounds*, supplement to 3rd. edn., Van Nostrand, New York
- Sherman, G. & Fujimori, E. (1968) *Nature (London)* **219**, 375-377
- Sherman, G. & Fujimori, E. (1969) *Arch. Biochem. Biophys.* **130**, 624-628
- Smith, G. J. & Dunkley, W. L. (1962) *Arch. Biochem. Biophys.* **98**, 46-48
- Steinemann, A., Alamuti, N., Brodman, W., Marschall, O. & Luger, P. (1971) *J. Membrane Biol.* **4**, 284-294
- Wasserman, R. H. & Corradino, R. A. (1971) *Annu. Rev. Biochem.* **40**, 501-532