Biochem. J. (1969) **112**, 221
Printed in Great Britain

Reactions of Vitamin A with Acceptors of Electrons

FORMATION OF RADICAL ANIONS FROM 7,7,8,8-TETRACYANOQUINODIMETHANE AND TETRACHLORO-1,4-BENZOQUINONE

BY F. ULRIKE LICHTI* AND J. A. LUCY†
Strangeways Reasearch Laboratory, Cambridge

(*Received* 11 *October* 1968)

1. The interactions of retinol and retinoic acid with two electron acceptors, 7,7,8,8-tetracyanoquinodimethane (TCNQ) and tetrachloro-1,4-benzoquinone (chloranil), were studied in an investigation on the ability of vitamin A to behave as a donor of electrons. 2. Retinol reacts with TCNQ in polar organic solvents with the formation, as judged by spectral studies, of the radical anion of TCNQ. 3. Addition of the products of this reaction to water is accompanied by a rapid consumption of OH^- ions. 4. Consumption of OH^- ions is also a feature of the reactions between retinol and chloranil, but the spectrum of the radical anion of chloranil is observed only when retinol and chloranil are suspended in aqueous salt solutions. 5. Retinoic acid behaves similarly to retinol in its reactions with TCNQ and chloranil, but it appears to be a weaker electron donor than retinol. 6. The reaction products that may be formed from retinol in its reactions with TCNQ and chloranil are discussed. 7. It is suggested that the ability of vitamin A to behave as a donor of electrons may be an important aspect of its biochemical mode of action.

Although the biological importance of vitamin A has been recognized for 50 years, the biochemical mode of action of the vitamin in its systemic effects is still not understood. A major difficulty in the elucidation of a general mechanism, or mechanisms, of action of vitamin A by means of experiments with animals is distinguishing between primary and secondary effects in the widespread action of the vitamin (Moore, 1957). This difficulty has led us to approach vitamin A from a chemical viewpoint. Most studies on the biological chemistry of vitamin A have been concerned with its function in vision (cf. Ball & Morton, 1949; Bridges, 1967). However, Pullman & Pullman (1963) have concluded that the values of the energy coefficients of the highest occupied and lowest empty molecular orbitals of the carotenoids, and of vitamin A, indicate that these substances should be both excellent donors and acceptors of electrons. Körösy (1958) has similarly suggested that the results of his experiments on interactions between strong acids and carotenoids can only be explained by the conjugated chain acting as an electron donor towards a proton or Lewis acid, and as an acceptor towards anions. Attention has previously been drawn to the possibility that electron transfer, associated with their polyene chains, might be a common feature of both vitamin A and the carotenoids *in vivo*, and it has been suggested that the electron mobility of these substances may be an essential factor in photosynthesis, in vision and in the systemic actions of vitamin A (Dingle & Lucy, 1965).

In the present paper, studies are reported on the chemical reactions of vitamin A with the electron acceptors, TCNQ‡ and chloranil (tetrachloro-1,4-benzoquinone). Under appropriate experimental conditions each of these substances will accept one electron from a donor molecule to form a well-characterized radical anion (Andrews & Keefer, 1964; Kosower, 1965), and these two acceptors therefore constitute suitable models with which to investigate the ability of vitamin A to behave as a donor of electrons. The present experiments thus represent an attempt to obtain information on certain aspects of the chemical properties of vitamin A that may possibly be responsible for its biochemical behaviour. It is hoped that this approach may lead to a working hypothesis for the primary type of biochemical process, or processes,

* Present address: Molecular Biology Laboratory, University of Wisconsin, Madison, Wis. 53706, U.S.A.

† Present address: Department of Biochemistry, Royal Free Hospital School of Medicine, University of London, London W.C.1.

‡ Abbreviations: TCNQ, 7,7,8,8-tetracyanoquinodimethane; $TCNQ\cdot^-$, radical anion of TCNQ.

in which the vitamin normally participates *in vivo*. A preliminary communication has been published (Lichti & Lucy, 1967).

MATERIALS AND METHODS

Synthetic crystalline retinol (Roche Products Ltd., Welwyn Garden City, Herts.) and retinoic acid (Distillation Products Industries, Rochester, N.Y., U.S.A.) were used without further purification, and they were handled as described by Dingle & Lucy (1962). TCNQ (Eastman Organic Chemicals, Rochester, N.Y., U.S.A.) and chloranil (British Drug Houses Ltd., Poole, Dorset) were used without further purification. Concentrated stock solutions of TCNQ and chloranil in N_2-saturated polar organic solvents were prepared freshly each day. All other reagents and solvents were analytical-grade chemicals.

Complete ultraviolet, visible and near-infrared spectra, as well as the change in extinction at a given wavelength, were measured in stoppered 1 cm. silica cells, unless otherwise indicated, by means of a Unicam SP.700 recording spectrophotometer. All spectral measurements were made at room temperature. A matched set of four cells, two in the sample beam and two in the reference beam, was used in some experiments. For Figs. 3, 4 and 7 the two sample cells contained the reaction mixture and solvent blank respectively, and the two reference cells both contained solvent. In the control experiments of Figs. 3, 4 and 7 dispersions of each compound were placed in separate sample cells and measured against the solvent in the two reference cells. For the difference spectra of Fig. 7 the two sample cells contained the reaction mixture and the solvent blank respectively, and the reference cells contained the reactants separately at the same concentrations as the compounds in the reaction mixture.

The ϵ_{842} value 43300 given by Melby *et al.* (1962) for the extinction coefficient of the radical anion of TCNQ was used in calculating the quantity of $TCNQ\cdot^-$ formed during the reaction of TCNQ with retinol.

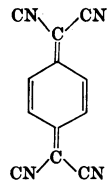
Suspensions of vitamin A–TCNQ or vitamin A–chloranil in aqueous buffers were prepared by adding buffer rapidly from a blow-out pipette to 0·1 ml. of a solution of the reactants in an organic solvent contained in a large test tube. For pH-stat titrations, 50–100 μl. of a solution of the reactants dissolved in an organic solvent was added from an Agla syringe to 5 ml. of magnetically stirred buffer. Both procedures allowed rapid dispersal of the reactants in water.

Buffers were prepared from glass-distilled water, boiled to remove dissolved gases and saturated with N_2 by passing a slow stream of the gas through the solution for at least 10 min. A slow stream of N_2 was also passed through the solution during pH-stat titrations.

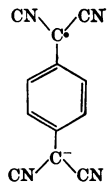
Consumption of OH^- ions was measured by means of a Radiometer automatic titrator used as a pH-stat at the desired pH, with 0·01 M-NaOH as the titrant. The 0·01 M-NaOH was standardized with potassium hydrogen phthalate before each set of titrations.

RESULTS

Reactions of retinol and TCNQ in polar organic solvents. Under suitable experimental conditions, complete electron transfer occurs when the strong



TCNQ



$TCNQ\cdot^-$

electron acceptor TCNQ reacts with metallic copper, the I^- ion or other oxidizable substances. The radical anion $TCNQ\cdot^-$, resulting from electron transfer between donor molecules and TCNQ in polar organic solvents, possesses a characteristic absorption spectrum in the visible and near-infrared region with maxima at 420, 680, 744 and 842 mμ (23·8, 14·7, 13·4, 11·9 kcyc./cm.) (Melby *et al.* 1962). Formation of the radical anion can therefore be measured readily by following the change in extinction at any of these wavelengths. By this criterion retinol reacts with TCNQ in methanol, ethanol, dimethylformamide, formamide and acetonitrile, but not in acetone or in any non-polar organic solvent. Retinol may perhaps therefore be considered to be comparable with *NNN'N'*-tetramethyl-*p*-phenylenediamine as a donor of electrons when TCNQ is the acceptor. *NNN'N'*-Tetramethyl-*p*-phenylenediamine interacts with TCNQ in non-polar solvents (ether and chloroform) to form a charge-transfer complex, but like retinol it requires a more polar environment for complete electron transfer and the formation of radical ions (Foster & Thomson, 1962).

When the formation of $TCNQ\cdot^-$ was followed by the change in extinction at 842 mμ (13 kcyc./cm.) as a function of the time of the reaction, the extinction was observed to increase initially, then to reach a plateau value corresponding to the maximum quantity of $TCNQ\cdot^-$ formed during the reaction, and finally to decrease. When retinol (0·52 mM) and TCNQ (1·01 mM) were allowed to interact in various polar organic solvents it was found that the maximum concentration of $TCNQ\cdot^-$ formed was 0·05 mM in ethanol, 0·1 mM in methanol and 0·25 mM in dimethylformamide, or about 10%, 20% and 50% respectively of the initial concentration of retinol. The subsequent decrease in the concentration of $TCNQ\cdot^-$ indicated that the radical anion was being destroyed by side reactions; the observed rate of formation of the radical anion may therefore be the resultant of the actual rate of formation and the rate of destruction in the particular solvent used. $TCNQ\cdot^-$ is known to be very reactive and to be destroyed readily by oxygen and solvent impurities. Nitrogen-saturated solvents and stoppered spectro-

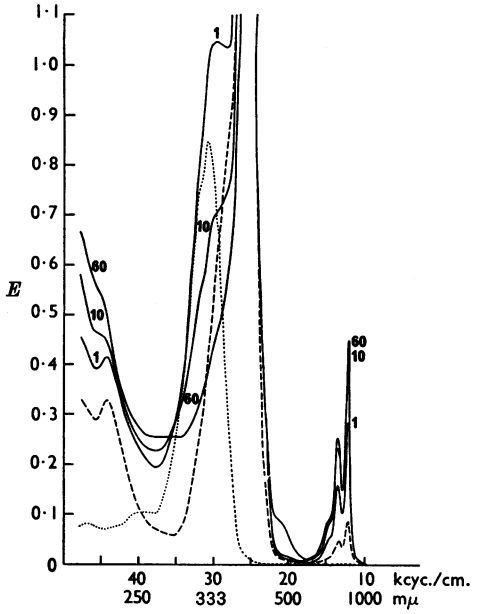


Fig. 1. Spectral changes occurring with time (1, 10 and 60 min.) during the reaction of TCNQ (0·31 mM) with retinol (0·16 mM) in methanol contained in a 0·1 cm. cell. ——, TCNQ–retinol mixture; – – – –, TCNQ alone; . . . ., retinol alone. The extinction due to TCNQ at 25·2 kcyc./cm. (off-scale in the figure) was 1·96, 1·96, 1·69 and 1·31 at 0, 1, 10 and 60 min. respectively.

photometer cells were used for all experiments with TCNQ, but no elaborate precautions were taken to exclude oxygen. The radical anion could be stabilized in these experiments by the presence of unchanged TCNQ, which probably forms a ternary complex with the reaction products of the type $R^{\bullet+}(TCNQ)_2^{\bullet-}$ similar to that described by Melby *et al.* (1962) for the tertiary amine salts of $TCNQ^{\bullet-}$. In the presence of a sevenfold molar excess of TCNQ over retinol in dimethylformamide, the maximum quantity of $TCNQ^{\bullet-}$ formed during the reaction approached 1 equiv. of retinol.

The spectral changes for the reaction of TCNQ (0·31 mM) and retinol (0·16 mM) in methanol are shown in Fig. 1, which reveals increasing absorption in the near-infrared region of the spectrum due to the formation of $TCNQ^{\bullet-}$ over a period of 60 min. By contrast there is no distinct new spectral band arising during the reaction that might be attributed to a reaction product derived from retinol, although there is an increase in extinction between 280 and 190 mμ (36–52 kcyc./cm.). The shoulder at 475 mμ (21 kcyc./cm.) is due to a degradation product of $TCNQ^{\bullet-}$. In this experiment the rate of disappearance of the retinol spectrum approximately paralleled the rate of appearance of the spectrum of $TCNQ^{\bullet-}$. When a similar experiment was carried out with much higher concentrations of reactants in dimethylformamide and a different experimental procedure, a somewhat different result was obtained. In the latter experiment retinol (14 mM) and TCNQ (32 mM) were mixed in dimethylformamide and, at various times during the progress of the reaction, samples were diluted 1000-fold with ethanol and the spectra of the diluted reaction mixtures were determined immediately. Under these conditions the absorption attributed to $TCNQ^{\bullet-}$ increased for up to 20 min., but the absorption due to retinol disappeared completely within 2 min. of bringing the reactants in contact. This lag in the development of the spectrum of the radical anion might imply that the radical anion is formed from some intermediate species, such as a charge-transfer complex, that may be the immediate reaction product of retinol and TCNQ.

Reactions of retinol and TCNQ in water. Neither retinol nor TCNQ is very soluble in water, and no reaction appears to occur when aqueous suspensions of the two substances are mixed. This is probably due to the fact that the concentration of unaggregated free molecules of the reactants is very low. The products of the reaction between retinol and TCNQ in a polar organic solvent are, however, readily soluble in water; indeed, a rapid reaction occurs on adding the mixture to water, and H^+ ions are released. The reaction with water has been measured in terms of the consumption of OH^- ions at pH 6, 7 and 8 in a pH-stat. Thus when 100 μl. of a mixture of TCNQ (43 mM) and retinol (22 mM) in dimethylformamide was added to 5 ml. of nitrogen-saturated water buffered with sodium cacodylate (0·5 mM, pH 6) or sodium phosphate (0·5 mM, pH 7), or tris–hydrochloric acid (0·5 mM, pH 8), the consumption of OH^- ion/mole of retinol was 0·8 mole at pH 6, 0·8 mole at pH 7, and 1·2 moles at pH 8.

When the reaction products of retinol and TCNQ in dimethylformamide were added to aqueous potassium chloride or sodium chloride (0·2 M or above) a blue precipitate formed that, on suspension in sodium chloride (0·5 M), had absorption maxima at 603 and 1330 mμ (16·6 and 7·5 kcyc./cm.). This precipitate was probably the alkali-metal salt of $TCNQ^{\bullet-}$, since Boyd & Phillips (1965) have described a concentration-dependent formation of a dimer of K^+ $TCNQ^{\bullet-}$ in water that absorbs at 643 mμ (15·5 kcyc./cm.). The potassium salt of $TCNQ^{\bullet-}$ was therefore prepared by allowing potassium iodide and TCNQ to react in dimethylformamide, dispersal of the product in a large volume of diethyl ether and washing the precipitate with ether to remove iodine. When the blue precipitate prepared in this way was suspended in sodium chloride (0·5 M), it had absorption maxima at 603 and 1330 mμ. These observations suggested a

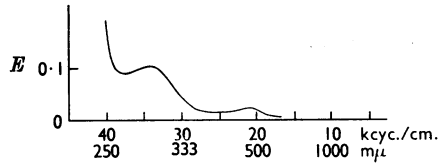


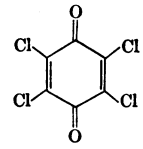
Fig. 2. Spectrum of the aqueous phase from a mixture of retinol (41 mM) and TCNQ (49 mM), allowed to react in dimethylformamide for about 10 min., then dispersed in 100 vol. of KCl soln. (0·5 M) and filtered through Whatman no. 1 paper. The filtrate was extracted with an equal volume of cyclohexane before spectrophotometry. [The absence of a peak at 25 kcyc./cm. (TCNQ) is probably due more to the fact that TCNQ was present in very slight molar excess rather than to efficient extraction with cyclohexane.]

procedure for separating the products of the reaction between retinol and TCNQ, namely the precipitation of the sodium or potassium salt of $TCNQ^{\cdot-}$ followed by extraction of the filtered solution with an organic solvent to remove any unchanged TCNQ. When this procedure was used in an experiment in which TCNQ was initially present only in a slight excess it was found that the material remaining in the filtrate had a broad low-intensity absorption in the ultraviolet, with a distinct shoulder at 285 mμ (34 kcyc./cm.) (Fig. 2), that was somewhat similar to the spectrum of the material formed from retinol when it reacts with iodine (Lucy & Lichti, 1969).

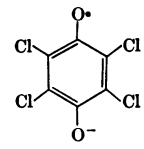
Retinoic acid and TCNQ. The reaction of retinoic acid with TCNQ in dimethylformamide is similar to that of retinol, but the reaction proceeds at only about one-tenth of the rate. The products of the reaction were found to interact with water, as in the retinol–TCNQ experiments. Uptake of OH^- ions, measured in the pH-stat, was 1·4 and 1·9 moles/mole of retinoic acid at pH 7 and pH 8 respectively.

Retinol and chloranil. Chloranil, like TCNQ, interacts with donors such as *NNN'N'*-tetramethyl-*p*-phenylenediamine to form either a charge-transfer complex in non-polar solvents, or radical ions in polar organic solvents (Isenberg & Baird, 1962). In the present experiments the interactions of retinol and retinoic acid with chloranil were studied under conditions similar to those described above for TCNQ.

Like TCNQ, chloranil is not very soluble in water, and there is no reaction between retinol and chloranil when aqueous suspensions of the two compounds are mixed. On mixing retinol with chloranil in dimethylformamide (the final concentrations being 54 mM-chloranil and 43 mM-retinol) a slightly green solution is immediately observed that shows a new broad, but very weak, absorption band in the visible region with a maximum at about 607 mμ (16·5 kcyc./cm.). The green colour, which fades slowly over a period of about 1 hr., could conceivably be due to a charge-transfer complex between retinol and chloranil.



Chloranil



Radical anion of chloranil

The radical anion of chloranil is reported to have maxima at about 425 and 450 mμ (23·5 and 22·2 kcyc./cm.) both in methanol and in acetonitrile (Foster & Thomson, 1962). No new absorption bands at these wavelengths are observed in the mixture of retinol and chloranil in dimethylformamide. It would appear therefore that, although retinol can transfer electrons to TCNQ in dimethylformamide, there is no similar reaction between retinol and chloranil in this solvent. The spectrum of the chloranil anion can be observed, however, in aqueous salt solution. If the mixture of retinol and chloranil in dimethylformamide is diluted with a large volume of aqueous salt solution (0·5 M-sodium chloride) a turbid suspension is obtained, the spectrum of which undergoes marked changes with time. The rate of change increases with pH up to pH 7, but it is approximately constant above pH 7. New maxima develop in the saline suspension at 426 and 455 mμ (23·5 and 22·0 kcyc./cm.); i.e. at the wavelengths where the radical anion of chloranil absorbs in water (Foster & Thomson, 1962). In the absence of salt the turbidity is less, and a new maximum develops at 400 mμ (25 kcyc./cm.) as the extinction at 295 mμ (33·9 kcyc./cm., $\lambda_{max.}$ for chloranil) and at 325 mμ (30·8 kcyc./cm., $\lambda_{max.}$ for retinol) decreases.

Figs. 3 and 4 illustrate the spectra obtained when a solution of retinol (2·5 mM) and chloranil (5·7 mM) that had been allowed to react in dimethylformamide for about 1 hr. was diluted with 100 vol. of dilute phosphate buffer, pH 7, and with 100 vol. of dilute phosphate buffer, pH 7, containing sodium chloride (0·5 M) respectively. The appearance of the bands between 20 and 25 kcyc./cm. in the spectrum of Fig. 4 is similar to the absorption bands in the spectra of glycine and chloranil in aq. 50% dimethyl sulphoxide reported by Slifkin (1964). The quantity of material giving rise to the new bands in the aqueous solutions (at 426 and 455 mμ in salt solution and at 400 mμ in dilute buffer) is greater the longer retinol and chloranil have been allowed to react in dimethylformamide before addition to the aqueous solutions. A product of a time-dependent reaction in dimethylformamide thus seems to react with water and to give rise to the new

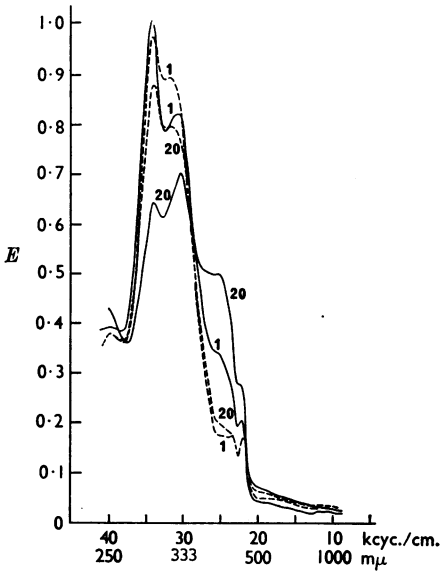


Fig. 3. Spectral changes with time (1 and 20 min.) of retinol (25 μM) and chloranil (57 μM) in sodium phosphate buffer, pH 7 (0·5 mM). Spectra were measured with the four-cell arrangement described in the Materials and Methods section. Retinol and chloranil had been allowed to react in dimethylformamide at 100 times the indicated concentration for 1 hr. before dispersion in the buffers. ——, Chloranil–retinol mixture in one cell in the sample beam; – – – –, chloranil and retinol dispersed separately in phosphate buffer and measured in separate cells in the sample beam in a control experiment.

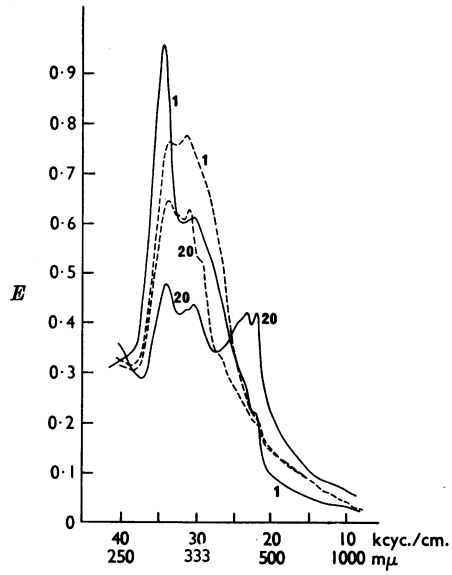


Fig. 4. Spectral changes with time (1 and 20 min.) of retinol (25 μM) and chloranil (57 μM) in sodium phosphate buffer, pH 7 (0·5 mM), containing NaCl (0·5 M). Other experimental conditions were as for Fig. 3. ——, Chloranil–retinol mixture in one cell in the sample beam; – – – –, chloranil and retinol dispersed separately in phosphate buffer–NaCl soln. and measured in separate cells in the sample beam in a control experiment.

absorption bands. The species with a maximum extinction at 400 mμ in dilute buffer can apparently be converted into the radical anion of chloranil by the subsequent addition of salt, since the spectrum of the radical anion then appears (Figs. 5 and 6). Fig. 5 also shows that at high concentrations of retinol and chloranil in dilute buffer the 400 mμ band is greatly increased relative to the other bands, and that after dilution with dilute buffer this band decreases with concomitant increase of the absorption band at 34 kcyc./cm. (cf. Fig. 2).

When a solution of retinol and chloranil in dimethylformamide is added to water at constant pH in a pH-stat OH^- ions are consumed regardless of whether salt is present or not. Uptake of OH^- ions thus occurs both when the material absorbing at 400 mμ is formed from chloranil and when the radical anion is produced. Without salt, 0·6 mole of OH^- ion was consumed/mole of retinol at pH 6, and 1·1 moles/mole at pH 7. The quantity of OH^- ions taken up/mole of retinol remains constant as the concentration of chloranil is increased from an equimolar quantity to a fourfold excess over retinol. However, the amount of base taken up depends on the time of reaction in the organic solvent before the addition of the reactants to the buffer. Maximum base uptake is observed when retinol (0·02 M) and chloranil (0·04 M) are added to the buffer after they have reacted for 3 hr. in dimethylformamide.

Chloranil and retinoic acid. The reaction of chloranil with retinoic acid in dimethylformamide resembles that with retinol in that a broad shoulder centring around 570 mμ (17·5 kcyc./cm.) appears immediately after mixing and then slowly decays. When the mixture of retinoic acid and chloranil in dimethylformamide is diluted with a large volume of dilute buffer (0·5 mM-phosphate, pH 7), a new prominent band at 316 mμ (31·6 kcyc./cm.) and a shoulder at 461 mμ (21·7 kcyc./cm.) have developed by the time the spectrum can be taken. The extinction of the main band decreases, whereas that at 461 mμ increases slightly with time (Fig. 7). No OH^- ions are taken up when a retinoic acid–chloranil mixture in dimethylformamide is added to dilute buffer at pH 7. However, if the buffer contains 0·5 M-sodium chloride some OH^- ions are consumed: 0·2 mole/mole of retinoic acid at pH 7, 0·4 mole/mole at pH 8 and 0·6 mole/mole at pH 9. When sodium chloride is present there is some indication of a relative increase in extinction in the

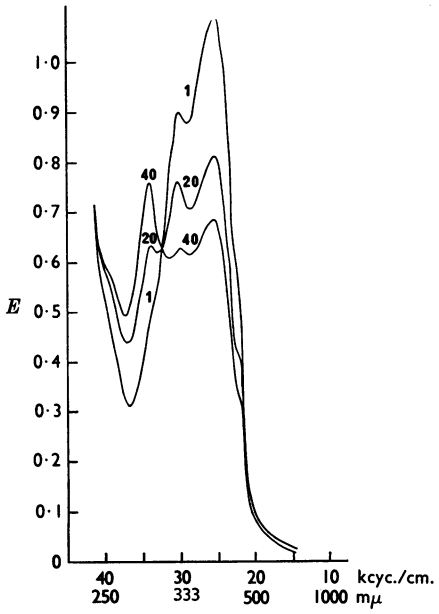


Fig. 5. Spectral changes occurring with time (1, 20 and 40 min.) on 10-fold dilution of chloranil (0·56 mM) and retinol (0·44 mM) in sodium phosphate buffer, pH 7 (0·5 mM), with the same phosphate buffer. Retinol and chloranil were allowed to react in dimethylformamide at 100 times the indicated concentration for about 7 hr.; 50 μl. of the solution was then dispersed in 5 ml. of buffer and after 5 min. the aqueous suspension was diluted a further 10-fold with buffer.

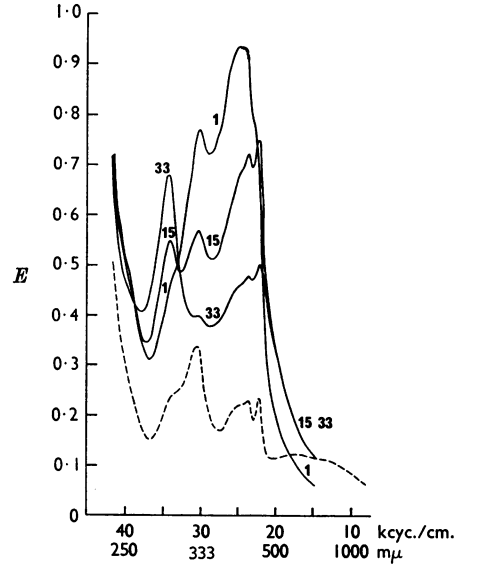


Fig. 6. Spectral changes occurring with time (1, 15 and 33 min.) on 10-fold dilution of chloranil (0·56 mM) and retinol (0·44 mM) in sodium phosphate buffer, pH 7 (0·5 mM), with the same buffer but containing NaCl (0·5 M). Retinol and chloranil were allowed to react in dimethylformamide at 100 times the indicated concentration for about 7 hr.; 50 μl. of the solution was then dispersed in 5 ml. of buffer and after 5 min. the aqueous suspension was diluted a further 10-fold with buffer containing NaCl. In addition, in a related experiment, 50 μl. of the solution of chloranil and retinol in dimethylformamide was dispersed directly into 5 ml. of sodium phosphate buffer, pH 7 (0·5 mM), containing NaCl (0·5 M) and, after 5 min., the suspension was diluted a further 10-fold with the phosphate buffer–NaCl soln. (----).

region of the spectrum where the radical anion of chloranil absorbs, but in the presence of salt the reaction mixture is so turbid that the spectral data are of little value. It is concluded that complete electron transfer occurs less readily with retinoic acid than with retinol when chloranil is the acceptor. Retinoic acid thus appears to be a weaker donor than retinol both when it interacts with TCNQ and in its reactions with chloranil.

DISCUSSION

At the end of a Symposium held in 1960 on vitamin A and its metabolism, Morton (1960) commented that we do not know how vitamin A works. This is still true today. Morton (1960) also pointed out that if the active form of vitamin A is to participate in dehydrogenase systems or enter into reaction with the thiol groups of proteins or with Ca^{2+} ions, or both, it must have some new 'aspect'. At the same time he concluded that he was led to look at retinol with his attention focused on the portion of the molecule enclosed within the rectangle shown in (I). The development of the characteristic absorption spectra of the radical anions of TCNQ and chloranil, when retinol interacts with TCNQ and chloranil respectively, indicates that a property of the portion of the molecule within the rectangle is the donation of electrons to suitable acceptors. Since both retinol and retinoic acid behave as donors, the polar group outside the rectangle is probably not directly involved in the electron-transfer reactions. In the light of these observations, it is conceivable that participation in electron-transfer reactions may be the new 'aspect' of the vitamin that is responsible for its biochemical actions and, as suggested by Dingle & Lucy (1965), the electron mobility of vitamin A may be ultimately responsible for both its role in vision and its systemic actions *in vivo*. The present experiments, together with those reported in the following paper (Lucy & Lichti, 1969), appear to be the first direct evidence for electron transfer by vitamin A in any system. Indirect evidence (F. U. Lichti & J. A. Lucy, unpublished work) has been obtained in studies on the mechanism of the autoxidation of retinol by

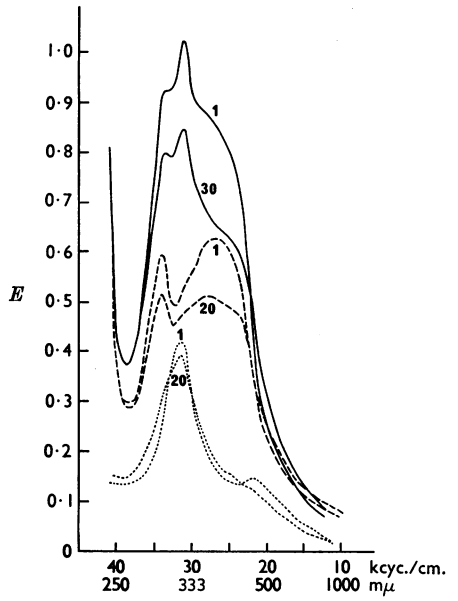


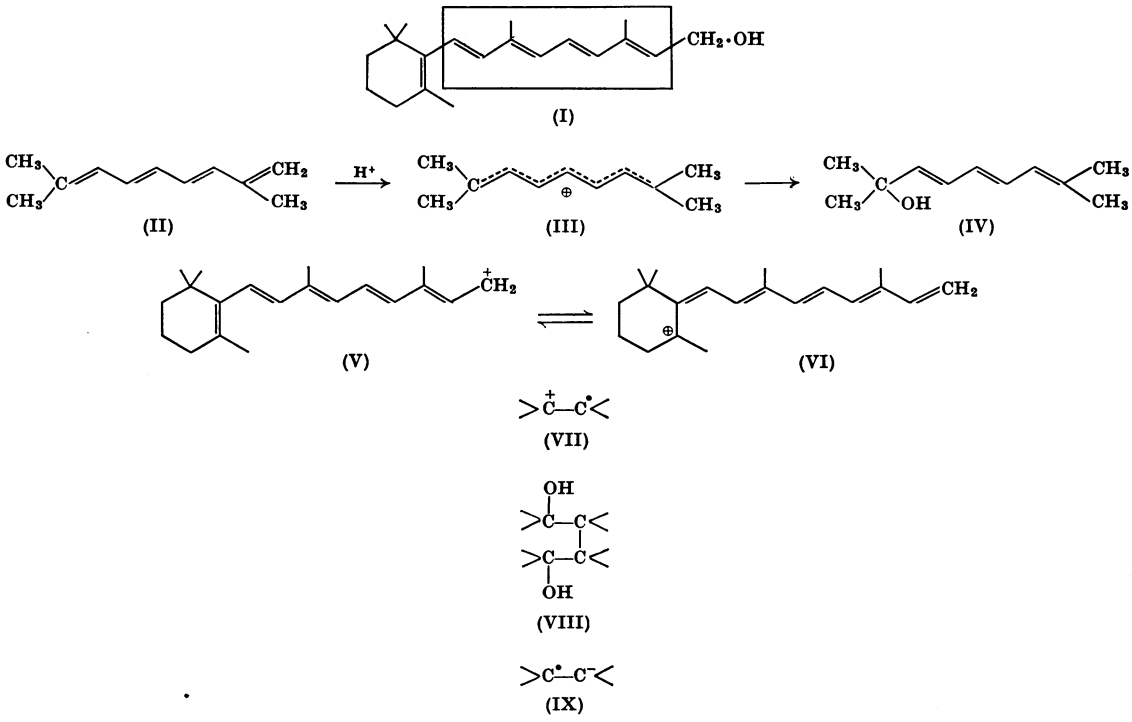
Fig. 7. Spectral changes with time of retinoic acid (30 μM) and chloranil (55 μM) in sodium phosphate buffer, pH 7 (0·5 mM). ——, Chloranil (5·5 mM) and retinoic acid (3·0 mM) in dimethylformamide were allowed to react for 1 min.; 0·1 ml. of this solution was then dispersed in 10 ml. of the buffer and spectra were measured after 1 and 30 min. (Increasing the time of the reaction in dimethylformamide to 2 hr. had little effect on the spectra of the aqueous suspension.) – – – –, Spectra, after 1 and 20 min., of retinoic acid and chloranil suspended separately in buffer and placed in separate sample cells in the four-cell arrangement as described in the Materials and Methods section. . . . ., Difference spectra, after 1 and 20 min., of a mixture of chloranil and retinol in buffer measured against chloranil and retinoic acid suspended in buffer in separate cells in the reference beam.

molecular oxygen (Lucy, 1965, 1966). Since the publication of our initial reports on the reaction of vitamin A with TCNQ, chloranil and iodine (Lichti & Lucy, 1967; Lucy & Lichti, 1967), the formation of light-induced free radicals of retinal, retinol and rhodopsin has been reported by Grady & Borg (1968). These workers have suggested that light generates a charge separation on the rhodopsin molecule by the creation of radical ions and that these may be the charged species responsible for the early receptor potential.

The interactions of retinol and retinoic acid with TCNQ resemble those of other donors such as *NNN'N'*-tetramethyl-*p*-phenylenediamine in that the formation of $TCNQ.^-$ occurs in polar, but not in non-polar, organic solvents. By contrast the reactions of vitamin A with chloranil possess rather unusual features. Thus the spectrum of the radical anion of chloranil does not develop unless an aqueous salt solution is used. Stereochemical factors may possibly be responsible for the differences between the reactions of vitamin A with TCNQ and chloranil. For example, the four nitrile groups of TCNQ may facilitate its interaction with the polyene chain of vitamin A. Alternatively, the behaviour of chloranil with retinol may simply be due to the relatively weak acceptor properties of chloranil as compared with TCNQ (Andrews & Keefer, 1964). It has previously been found that turbid suspensions of retinol in aqueous salt solutions are autoxidized much more readily than less turbid suspensions in water (Lucy, 1966), and recent observations indicate that salt may facilitate the formation of radicals in the suspensions (F. U. Lichti & J. A. Lucy, unpublished work). The turbidity that occurs in the presence of salt, both with suspensions of retinol alone and with mixtures of retinol and chloranil, indicates that the extent to which retinol molecules are aggregated may influence the degree of electron transfer in the systems.

In the experiments of Foster & Thomson (1962) on the interactions of *NNN'N'*-tetramethyl-*p*-phenylenediamine with a variety of electron acceptors in different solvents, the radical cation of this compound was sufficiently stable to be measured spectrophotometrically, but some of the free-radical anions produced, e.g. that from 1,3,5-trinitrobenzene, reacted too rapidly with water for their absorption to be measured. In the present experiments the converse applies since with TCNQ as acceptor the spectrum of $TCNQ.^-$ develops but the fate of the retinol is complex. When an electron donor containing an ethylenic double bond reacts with an acceptor molecule, a dicarbonium ion may be produced as when tetra(dimethylamino)ethylene reacts with tetracyano-ethylene (Wiberg & Buchler, 1963). It might therefore be expected that an electron-deficient carbonium ion would be formed from retinol when it reacts with TCNQ. The consumption of OH^- ions observed when the products of this reaction in ethanol are added to water is consistent with this interpretation. A comparable finding has been reported by Sorensen (1965), who showed that the carbonium ion (III) produced by protonating 2,8-dimethyl-1,3,5,7-nonatetraene (II) with concentrated sulphuric acid reacts with potassium hydroxide in ice to give an alcohol (IV).

The idea that vitamin A can, under appropriate circumstances, give rise to species containing cationic carbon is not novel, and the behaviour of vitamin A in strong acids has been attributed to protonation of the molecule and the formation of carbonium ions. Meunier (1942) proposed that the carbonium ions (V) and (VI) are responsible for the



blue colour that is formed in the Carr–Price reaction of vitamin A with antimony trichloride in non-polar solvents. Sharp absorption bands in the visible region of the spectrum, which are characteristic of unstable ionized molecules, have also been observed by Ball & Morton (1949) on adding retinol to concentrated acids. In related experiments Wassermann (1954) noted the formation of conducting adducts, which absorb in the near infrared, when trichloroacetic acid is added to carotene in benzene. A possible role for carbonium ions of retinal in visual pigments has been discussed by Blatz (1965), and more recently Blatz & Pippert (1968) have reported spectroscopic studies on the carbonium ion of all-*trans*-retinyl acetate.

A dicarbonium ion derived from vitamin A would be expected to react with water to consume two OH^- ions/molecule of retinol. In the reduction of iodine to iodide by retinol reported in the following paper (Lucy & Lichti, 1969) at least two OH^- ions are taken up/molecule of retinol. With TCNQ, however, only one OH^- ion is consumed/molecule of retinol, and this appears to imply the occurrence of a one-electron transfer process. Such a process would yield a radical-ion grouping of the type (VII) by loss of a single electron from a carbon–carbon double bond. Radical ions of this kind may conceivably dimerize while simultaneously reacting with water to yield a dimer containing the group (VIII). It is relevant that Yang & Gaoni (1964) have reported that a rapid polymerization, which is unaffected by oxygen, occurs on mixing the donor 4-vinylpyridine with the acceptor 2,4,6-trinitrostyrene, and it appears that the groups (VII) and (IX) may be involved in this polymerization process (Kosower, 1965). The studies of Fleischfresser, Cheng, Pearson & Szwarc (1968) on the interaction of 1,1-diphenylethylene with antimony pentachloride at low temperatures in methylene chloride are also of interest in the present context, since their observations were thought to indicate that the radical cation formed from the olefin, on donating an electron to antimony pentachloride, dimerized to yield a dicarbonium ion. The suggestion that a dimeric derivative of retinol may be produced when one OH^- ion is consumed/molecule of retinol is in keeping with the fact that material absorbing at about 295 mμ is formed when the products of the reaction of retinol with TCNQ in ethanol are added to water. The absorption band of this material is in a similar position to that of kitol, a dimer of vitamin A with an absorption maximum at 290 mμ and therefore possessing a chain, or two chains, of only four conjugated double bonds (Barua & Morton, 1949). Additionally, the finding of Mousseron-Canet, Mani, Favie & Lerner (1966), that a high yield of dimer is produced on irradiation at 325 mμ of vitamin A in hexane, is also consistent

with a free-radical intermediate (Grady & Borg, 1968).

It may be thought that the ability of vitamin A to reduce TCNQ and chloranil to their respective radical anions is of no importance in relation to the biochemical properties of the vitamin because many other substances, with no vitamin activity, are also capable of these reactions. The fact that other substances behave similarly does not, however, diminish the significance of these electron-transfer reactions of vitamin A as illustrations of the chemical potentialities inherent in the vitamin molecule, especially since previous studies have indicated that the molecular specificity for vitamin A activity may reside, at least in part, in surface-active properties that enable the vitamin to penetrate lipid films and biological membranes (Bangham, Dingle & Lucy, 1964; Dingle & Lucy, 1965; Daniel, Dingle, Glauert & Lucy, 1966).

Morgan & Thompson (1967) have concluded that their observations on the distribution of small amounts of retinoic acid in the rat are more compatible with the older hypothesis that the 'active' form of vitamin A is retinol itself than with the alternative suggestion that it is retinoic acid or a metabolite of the acid. Nevertheless, retinoic acid can replace retinol in most animal tissues, although not in the visual or reproductive processes. It is thought to be significant therefore that retinoic acid resembles retinol in behaving as an electron donor.

In the experiments described here, retinol and retinoic acid appear to be converted irreversibly into other substances, and thus lost, when they behave as electron donors. By contrast, some regenerative process must presumably operate if vitamin A is concerned in comparable chemical or biochemical reactions *in vivo*, since the daily requirements for the vitamin are extremely small (Moore, 1957). Two different mechanisms may be proposed in the present context to account for the effectiveness of very small quantities of vitamin A *in vivo*. (1) The electron-deficient products that are formed when the vitamin behaves as a donor have access to a regenerative source of electrons. (2) Vitamin A interacts with a donor of electrons immediately before, or at the same time as, it donates electrons itself. The electron-acceptor properties of vitamin A (Pullman & Pullman, 1963), and its interactions with substances having lone-pair electrons (*n* donors) of electrons (Lichti & Lucy, 1967), may be relevant to the latter possibility. In both of these theoretical mechanisms electron transfer occurs without the vitamin being destroyed. Although these considerations are speculative, the chemical properties of the vitamin seem to indicate, nevertheless, that vitamin A may participate catalytically in certain oxidation–reduction reactions that are essential for the normal functioning of organized tissues *in vivo*, and the physical properties of vitamin A indicate that these reactions may occur on or in membranes.

During this work F. U. L. held a Postdoctoral Fellowship of the U.S. Public Health Service, and J. A. L. was a member of the External Staff of the Medical Research Council. We thank Mrs D. Shelford for technical assistance.

REFERENCES

Andrews, L. J. & Keefer, R. M. (1964). *Molecular Complexes in Organic Chemistry*, pp. 101, 102, 123. San Francisco: Holden–Day Inc.

Ball, S. & Morton, R. A. (1949). *Biochem. J.* **45**, 298.

Bangham, A. D., Dingle, J. T. & Lucy, J. A. (1964). *Biochem. J.* **90**, 133.

Barua, R. K. & Morton, R. A. (1949). *Biochem. J.* **45**, 308.

Blatz, P. E. (1965). *J. gen. Physiol.* **48**, 753.

Blatz, P. E. & Pippert, D. L. (1968). *J. Amer. chem. Soc.* **90**, 1296.

Boyd, R. H. & Phillips, W. D. (1965). *J. Chem. Phys.* **43**, 2927.

Bridges, C. D. B. (1967). In *Comprehensive Biochemistry*, vol. 27, p. 31. Ed. by Florkin, M. & Stotz, E. H. Amsterdam: Elsevier Publishing Co.

Daniel, M. R., Dingle, J. T., Glauert, A. M. & Lucy, J. A. (1966). *J. Cell Biol.* **30**, 465.

Dingle, J. T. & Lucy, J. A. (1962). *Biochem. J.* **84**, 611.

Dingle, J. T. & Lucy, J. A. (1965). *Biol. Rev.* **40**, 422.

Fleischfresser, B. E., Cheng, W. J., Pearson, J. M. & Szwarc, M. (1968). *J. Amer. chem. Soc.* **90**, 2172.

Foster, R. & Thomson, T. J. (1962). *Trans. Faraday Soc.* **58**, 860.

Grady, F. J. & Borg, D. C. (1968). *Biochemistry*, **7**, 675.

Isenberg, I. & Baird, S. L., jun. (1962). *J. Amer. chem. Soc.* **84**, 3803.

Körösy, F. (1958). *Acta chim. hung.* **15**, 35.

Kosower, E. M. (1965). *Prog. phys. org. Chem.* **3**, 81.

Lichti, F. U. & Lucy, J. A. (1967). *Biochem. J.* **103**, 34P.

Lucy, J. A. (1965). *Biochem. J.* **96**, 12P.

Lucy, J. A. (1966). *Biochem. J.* **99**, 57P.

Lucy, J. A. & Lichti, F. U. (1967). *Biochem. J.* **103**, 34P.

Lucy, J. A. & Lichti, F. U. (1969). *Biochem. J.* **112**, 231.

Melby, L. R., Harder, R. J., Hertler, W. R., Mahler, W., Benson, R. E. & Mochel, W. E. (1962). *J. Amer. chem. Soc.* **84**, 3374.

Meunier, P. (1942). *C. R. Acad. Sci., Paris*, **215**, 470.

Moore, T. (1957). *Vitamin A*, pp. 225–228. Amsterdam: Elsevier Publishing Co.

Morgan, B. & Thompson, J. N. (1967). *Biochem. J.* **103**, 35P.

Morton, R. A. (1960). *Vitam. & Horm.* **18**, 543.

Mousseron-Canet, M., Mani, J.-C., Favie, C. & Lerner, D. (1966). *C. R. Acad. Sci., Paris*, **262**, 153.

Pullman, B. & Pullman, A. (1963). *Quantum Biochemistry*, p. 440. New York: J. Wiley and Sons Inc.

Slifkin, M. A. (1964). *Spectrochim. Acta*, **20**, 1543.

Sorensen, T. S. (1965). *J. Amer. chem. Soc.* **87**, 5075.

Wassermann, A. (1954). *J. chem. Soc.* p. 4329.

Wiberg, N. & Buchler, J. W. (1963). *Chem. Ber.* **96**, 3223.

Yang, N. C. & Gaoni, Y. (1964). *J. Amer. chem. Soc.* **86**, 5022.