The Ascorbic Acid-Dependent Oxidation of Reduced Nicotinamide-Adenine Dinucleotide by Ciliary and Retinal Microsomes

THE EFFECT OF SOME PHARMACOLOGICALLY ACTIVE COMPOUNDS ON THIS REACTION

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1. The presence of an ascorbic acid-dependent NADH oxidation in ocular tissues has been established. Subcellular fractionation revealed that the enzyme is localized in the microsomes. The distribution of the enzyme in some ocular tissues has been determined; microsomes from the ciliary processes and the retina have comparable activities, which are much higher than those from the cornea or lens.
2. NADPH cannot replace NADH, and cysteine, reduced glutathione, ergothioneine and dehydroascorbic acid cannot be substituted for ascorbic acid in the reaction. The rate of NADH oxidation was greatly increased in the presence of cucumber ascorbate oxidase, and the enzyme appears to be NADH-monodehydroascorbate transhydrogenase.
3. Cytochrome b_5 is present in retinal microsomes.
4. The enzyme is inhibited by *p*-chloromercuribenzoate and iodoacetate, but not by cyanide, Amytal or malonate.
5. High concentrations of chloroquine cause a partial inhibition of the reaction, probably owing to interaction of this compound with the enzyme thiol groups. Low concentrations of Diamox, comparable with those attained in tissues during therapy with this drug, bring about partial inhibition of the reaction. Eserine, cortisone, hydrocortisone, 11-deoxycorticosterone and dexamethasone have no effect on the rate of oxidation.
6. The possible role of ascorbic acid and NADH-monodehydroascorbate transhydrogenase in the formation of aqueous humour and secretory mechanisms is discussed.

The distribution and possible functions of ascorbic acid in the eye have been reviewed by Heath (1962). This vitamin is present in high concentrations in the corneal epithelium, ciliary processes and retina, and also in some of the endocrine glands, especially the hypophysis and the adrenals. Kersten, Kersten & Staudinger (1958) demonstrated the presence in adrenal microsomes of an ascorbic acid-dependent system for the oxidation of NADH. They showed that dehydroascorbic acid could not replace ascorbic acid, even though the addition of cucumber ascorbate oxidase greatly enhanced the rate of the reaction in the presence of ascorbic acid. No change, however, was observed in the final concentration of ascorbic acid after incubation with adrenal microsomes and NADH. On the basis of this, and other evidence, Kersten *et al.* (1958) concluded that ascorbic acid is oxidized by cytochrome b_5 to monodehydroascorbic acid, from which ascorbic acid is again formed by reduction with the enzyme, NADH-monodehydroascorbate transhydrogenase. Evidence for the existence of an unstable oxidation product

of ascorbic acid had been provided by the work of Nason, Wosilait & Terrell (1954) and Kern & Racker (1954), both groups having separately studied plant NADH oxidases that were ascorbic acid-dependent. Yamazaki, Mason & Piette (1959) have since demonstrated the presence of a free-radical product of ascorbic acid oxidation by electron-paramagnetic-resonance spectroscopy.

Staudinger, Krisch & Leonhäuser (1961) have also measured manometrically the oxygen uptake of adrenal microsomes and have shown that this occurs only in the presence of catalytic amounts of ascorbic acid. Cytochrome b_5 was shown to be intermediate in the transfer of electrons from NADH to molecular oxygen and, consequently, the oxidation was cyanide-insensitive. It has been shown that the oxygen consumptions of the retina (Laser, 1935; Robbie & Leinfelder, 1948), ciliary processes (DeRoeth, 1953), cornea (Hermann, Moses & Friedenwald, 1942) and lens (Ely & Robbie, 1950; Kinoshita, 1955) are not completely inhibited by cyanide; according to the experimental conditions, between 15 and 35% of the normal

oxygen consumption takes place in the presence of 1 mM-cyanide. It was therefore decided to investigate the occurrence of cyanide-insensitive ascorbic acid-dependent NADH oxidation in ocular tissues.

EXPERIMENTAL

Chemicals. Chloroquine sulphate was obtained from May and Baker Ltd. Dexamethasone (9 α -fluoro-16 α -methylprednisolone) and Diamox sodium (acetazolamide, sodium salt) were gifts from Roussel Laboratories Ltd. and Lederle Laboratories respectively. Sodium deoxycholate was supplied by E. Gurr Ltd.

Ascorbate oxidase. This was prepared from cucumbers as described by Heath, Rutter & Beck (1962).

Determination of enzyme activity. Bovine eyes were collected from the abattoir immediately after the death of the animal and stored in ice. The tissues were dissected, weighed and homogenized in 12 vol. of ice-cold 0.25 M-sucrose soln. in a glass Potter-Elvehjem homogenizer. The homogenate was centrifuged for 15 min. at 12000 *g* to remove mitochondria, nuclei and unbroken cells. The supernatant from this separation was then centrifuged for 70 min. at 38000 *g* to give a microsomal pellet and a final supernatant, which was discarded. These manipulations were all carried out at 4° in an MSE High-Speed 18 refrigerated centrifuge (Measuring and Scientific Equipment Co. Ltd.). The microsomes were immediately resuspended by homogenizing in ice-cold 0.067 M-phosphate buffer, pH 7.4, the volume of buffer being equal to the volume of sucrose used in the original homogenization.

The enzyme activity was assayed by measuring the rate of oxidation of NADH in a Unicam SP.700 recording spectrophotometer, fitted with a constant-temperature cell housing through which water was circulating at 37±0.1°. The complete system contained, in a cuvette (light-path 1 cm.): NADH (45 μ M), KCN (1 mM), ascorbic acid (2.3 mM), microsomal suspension (equivalent to 75 mg. wet wt. of tissue) and phosphate buffer (to give a final volume of 3 ml.). The blank cell contained phosphate buffer. The change in extinction at 340 m μ was followed before and after the addition of ascorbic acid. All solutions were equilibrated at 37° before addition to the cuvette.

The effect of certain pharmacologically active compounds on the enzyme was studied by the above method, after the addition of aqueous solutions of these compounds, except for chloroquine and cortisone, cortisol, 11-deoxycorticosterone and dexamethasone. The effect of chloroquine on the reaction was followed at 368 m μ , since at this wavelength it did not interfere with the measurement of the rate of oxidation of NADH. The steroids were dissolved in propane-1,2-diol. Samples (0.1 ml.) of the appropriate solution of these compounds were each preincubated with the reaction mixture for 5 min. before the addition of ascorbic acid. Propane-1,2-diol (0.1 ml.) was added to the control reaction mixture in the steroid experiments.

Subcellular fractionation. Bovine retinas (0.5 g.) were homogenized in 5 ml. of ice-cold 0.25 M-sucrose. The homogenate was fractionated by differential centrifugation into unbroken cells (400 *g* for 5 min.), nuclei (1200 *g* for 5 min.), mitochondria (12000 *g* for 10 min.), microsomes (38000 *g* for 70 min.) and a final supernatant, which was dialysed overnight at 4° against distilled water. The

particulate fractions were each washed once with 2 ml. of phosphate buffer and recentrifuged as above. The pellets thus obtained were suspended by homogenizing in 5 ml. of ice-cold buffer. Then 1 ml. portions of these suspensions and of the dialysed supernatant were assayed for enzyme activity as described above, with the addition of malonate (final concn. 2 mM) to the mitochondrial preparation.

Dehydroascorbic acid. In a 1 cm. light-path cuvette 0.3 ml. of 23 mM-ascorbic acid soln. was mixed with 1.7 ml. of phosphate buffer, pH 7.4, and 0.2 ml. of cucumber ascorbate oxidase soln. The rate of oxidation was followed at 265 m μ and a further 0.2 ml. of ascorbate oxidase was added after 2 min. Oxidation was complete after 5 min. and the solution was made up to 3 ml. by the addition of 0.6 ml. of phosphate buffer. Of this 2.3 mM-dehydroascorbic acid soln. 0.3 ml. was added to the retinal microsomes and NADH reaction mixture, immediately after preparation.

Determination of protein. The protein content of the microsomal suspension was determined on 0.1 ml. portions, in duplicate, by the method of Lowry, Rosebrough, Farr & Randall (1951). Crystalline bovine plasma albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) was used as standard.

Extraction of cytochrome b₅. The retinas (7.5 g.) were removed as soon as possible after death from 12 bovine eyes, washed by gentle agitation in 0.9% NaCl soln. and homogenized in 60 ml. of ice-cold 0.25 M-sucrose soln. containing EDTA (2 mM). The microsomes were isolated as described in the Determination of enzyme activity section, and the pale-orange pellets thus obtained were combined and washed by homogenizing in 5 ml. of NaCl soln. and recentrifuging for 40 min. at 38000 *g*. The pellet was then homogenized in 3 ml. of 0.5% sodium deoxycholate soln. in 0.05 M-carbonate-bicarbonate buffer, pH 9.5 (Delory & King, 1945), containing EDTA (2 mM). The homogenate was kept at 18° for 15 min. and then centrifuged at 38000 *g* for 45 min., and the clear pale-orange supernatant was removed for spectrophotometric measurement. The spectrum of the oxidized cytochrome was read, with the sodium deoxycholate-bicarbonate buffer soln. in the blank cell. The reduced spectrum was read in a closed cuvette after the addition of 15 mg. of sodium dithionite or ascorbic acid to both blank and sample cuvettes. The prior addition of 0.1 ml. of N-NaOH to both cuvettes prevented this addition from causing precipitation of deoxycholic acid.

RESULTS

Microsomal preparations from ciliary body, cornea and lens, in the absence of cyanide and ascorbate, did not oxidize NADH. Slight oxidation of NADH by retinal microsomes occurred under these conditions, but this was completely inhibited by the addition of cyanide (final concn. 1 mM). The addition of ascorbic acid (2.3 mM) to retinal microsomes led to the oxidation of NADH (Fig. 1), and this reaction was not inhibited by 1 mM-cyanide. The distribution in ocular tissues of this ascorbic acid-dependent system catalysing the oxidation of NADH is shown in Table 1. The highest activity was found in the ciliary processes and retina, and these tissues were therefore used for studies of

Fig. 1. Effects of cyanide, ascorbic acid and ascorbate oxidase on the oxidation of NADH by retinal microsomes. The reaction was followed at 340 m μ at 37° with a recording spectrophotometer. The records represent the oxidation of 45 μ M-NADH in phosphate buffer, pH 7.4, by 1 ml. of retinal microsomes, with the following additions: A, KCN (1 mM); B, ascorbic acid (2.3 mM); C, ascorbic acid (0.23 mM); D, ascorbate oxidase soln. (0.3 ml.). Microsomes were omitted from the reaction represented by record IV.

Table 1. Activity of NADH-monodehydroascorbate transhydrogenase in ocular tissues

Experimental details are given in the text. Calculations are based on a molar extinction coefficient for NADH of 6.22×10^3 (Horecker & Kornberg, 1948). Values are given $\pm$ S.E.M.

Tissue	No. of expts.	Rate of oxidation of NADH	
		(μ moles/10 mg. of protein/min.)	(μ moles/g. wet wt./min.)
Ciliary process	3	187 ± 13.0	22 ± 3.7
Retina	6	173 ± 7.0	118 ± 13.5
Ciliary body	3	139 ± 14.4	23 ± 0.9
Cornea	4	70 ± 8.1	12 ± 5.4
Corneal epithelium	2	31 ± 4.5	17 ± 1.4
Lens	3	18 ± 3.6	31 ± 7.2

substrate specificity and the inhibitory action of certain pharmacologically active compounds.

Specificity. The enzyme was completely specific for NADH, no reaction taking place with the same concentration of NADPH. The designation of this

Fig. 2. Effect of ascorbic acid concentration on the rate of oxidation of NADH by retinal microsomes. The oxidation of NADH was followed at 340 m μ at 37° with a recording spectrophotometer. Each cuvette contained NADH (45 μ M), KCN (1 mM), 1 ml. of microsomal preparation (0.85 mg. of protein) and phosphate buffer, pH 7.4 (to give a final volume of 3 ml.). At zero time the appropriate ascorbic acid solution, to give the following final concentrations, was added: A, 2.3 mM; B, 0.23 mM; C, 23 μ M; D, 2.3 μ M; E, 0.23 μ M; F, ascorbic acid absent.

NADH-oxidizing system as an NADH-monodehydroascorbate transhydrogenase is based on the following observations. No oxidation of NADH occurred except in the presence of catalytic amounts of ascorbic acid. The effect of lowering the concentration of ascorbic acid is shown in Fig. 2; at a concentration as low as 0.23 μ M, the rate of oxidation was still 30% of the maximum. The addition of 0.3 ml. of cucumber ascorbate oxidase soln., in the absence of cyanide and ascorbic acid, did not increase the rate of NADH oxidation (Fig. 1). On adding this enzyme in the presence of 0.23 mM-ascorbic acid, however, a marked stimulation of the oxidation occurred, thus showing that the retinal microsomes were rate-limiting with respect to their ability to oxidize ascorbic acid. Oxidation of NADH did not occur if the microsomal preparation was omitted from a reaction mixture of ascorbic acid, ascorbate oxidase and NADH; thus the stimulation caused by ascorbate oxidase was not due to the non-enzymic oxidation of NADH by hydroxyl or monodehydroascorbate radicals formed during the oxidation of ascorbic acid by cucumber ascorbate oxidase. Dehydroascorbic acid (0.23 mM), formed by the enzymic oxidation of ascorbic acid before the addition of microsomes, could not replace ascorbic acid in the transhydrogenase reaction, nor could reduced glutathione, cysteine or ergothioneine.

Effect of bicarbonate buffer. The oxygen uptake of the bovine retina in bicarbonate buffer is more than

twice that in phosphate buffer (cf. Rahman & Kerly, 1961), but a similar buffer effect was not observed with the NADH-monodehydroascorbate transhydrogenase.

Unbroken cells, nuclei and mitochondria were removed from 6.5 ml. of retinal homogenate as described above, and the microsomes were harvested from two 3 ml. portions of the resulting supernatant. One microsomal pellet was suspended by homogenizing in 3 ml. of the phosphate buffer and the other in 3 ml. of 0.15 M-sodium bicarbonate, adjusted to pH 7.4 with 0.1 N-hydrochloric acid. Duplicate 1 ml. portions of these suspensions were assayed for enzyme activity by measuring ΔE_{340} during incubation at 37° in the presence of NADH (45 μ M), cyanide (1 mM) and ascorbate (2.3 mM), all reactants being added as solutions in the appropriate buffer; the final volume of the reaction mixture was 3 ml. The rate of oxidation of NADH in phosphate buffer was 82 m μ moles/min./10 mg. of protein, and in bicarbonate buffer it was 87 m μ moles/min./10 mg. of protein.

Subcellular fractionation. Whole bovine retinas were separated as described above, and the rate of oxidation of NADH was observed in 0.067 M-phosphate buffer, pH 7.4, containing NADH (45 μ M), with cell fraction equivalent to 100 mg. wet wt. of retina. The change in rate of oxidation was measured after the addition of cyanide (final concn. 1 mM), followed by ascorbate (final concn. 2.3 mM). The activity of the mitochondria was determined in the presence of 2 mM-malonate. The results are given in Table 2. All the NADH-oxidizing action in the retina was localized in the particulate fractions, no oxidation taking place in the soluble portion of the cells even in the presence of ascorbic acid. In all cases the ascorbic acid-independent oxidation was inhibited by 1 mM-cyanide. It was only in the microsomal fraction that the ascorbic acid-dependent oxidation occurred.

Table 2. *Distribution of NADH-monodehydroascorbate transhydrogenase in subcellular fractions of bovine retina*

Experimental details are given in the text. Results are expressed as m μ moles of NADH oxidized/min./particulate or supernatant fraction, equivalent to 1 g. wet wt. of retina.

Fraction	NADH-monodehydroascorbate-transhydrogenase activity		
	Addition(s) to reaction mixture ...	Cyanide (1 mM)	Cyanide (1 mM)+ascorbate (2.3 mM)
Nuclei	17	0	0
Mitochondria*	241	0	0
Microsomes	4.5	0	104
Supernatant	0	0	0

* In the presence of 2 mM-malonate.

Cytochrome *b*₅. The presence of cytochrome *b*₅ in retinal microsomes could be detected by measuring the absorption of microsomal suspensions. On reduction with sodium dithionite, the wavelength of maximum absorption shifted from 414 to 420 m μ , with a 10% increase in extinction. The autoxidizable nature of the cytochrome could be demonstrated by allowing the microsomes to react with NADH and ascorbic acid under nitrogen, in the presence of 1 mM-cyanide; when reduction of the cytochrome was complete, reversal of the spectral change was brought about by aeration.

Cytochrome *b*₅ is not readily extracted from all tissues with 1% (w/v) sodium deoxycholate, pH 7.0. Kersten *et al.* (1958) used 10% (w/v) deoxycholic acid in 1 N-sodium hydroxide, at 37° for 3 hr., to extract the cytochrome from adrenal microsomes. This method, when applied to liver and retinal microsomes, led to the breakdown of the cytochrome. However, the extraction of retinal microsomes at pH 9.5 with 0.5% sodium deoxycholate resulted in the solubilization of the microsomal cytochrome. The absorption spectra of the oxidized and reduced cytochrome had maxima at 414 m μ and at 423, 527 and 557 m μ respectively. Previously reported values for pure cytochrome *b*₅ are: (a) oxidized, 414 m μ ; reduced, 424, 527 and 557 m μ (Strittmatter & Ball, 1952); and (b) oxidized, 413 m μ ; reduced, 423, 526 and 556 m μ (Strittmatter & Velick, 1956).

Effects of inhibitors. The effects of enzyme inhibitors and some pharmacologically active compounds were determined with the standard assay system containing microsomal material from 75 mg. wet wt. of retina. Assays were performed in duplicate.

The enzyme was sensitive to thiol inhibitors, being inhibited 81% by 0.1 mM-*p*-chloromercuribenzoate and 33% by 1.0 mM-iodoacetate. The rate of oxidation was unaltered in the presence of a 1.0 mM solution of cyanide, Amytal, eserine, cortisone, hydrocortisone, 11-deoxycorticosterone or dexamethasone or of 10 mM-malonate. The oxidation of NADH by both retinal and ciliary microsomes was, however, inhibited by Diamox and chloroquine (Table 3). Diamox, which suppresses

Table 3. *Inhibition of retinal NADH-monodehydroascorbate transhydrogenase by Diamox and chloroquine*

Experimental details are given in the text.						
Inhibitor	Concn. of inhibitor...	Percentage inhibition				
		1.0 mM	0.1 mM	10.0 μ M	1.0 μ M	0.1 μ M
Diamox		29	26	25	16	16
Chloroquine		35	22	6	0	—

the secretion of aqueous humour, caused 29% inhibition of the rate of oxidation of NADH by retinal microsomes at a final concentration of 1.0 mM. The inhibitory effect of Diamox decreased only slightly as the concentration of the inhibitor was lowered. Chloroquine (1.0 mM) exerted 35% inhibition on the rate of oxidation, but the inhibitory effect was much less pronounced at lower concentrations. The oxidation of NADH by ciliary microsomes was inhibited to a similar extent by Diamox and chloroquine. At 1.0 mM concentration of these compounds, 30 and 25% inhibition respectively was observed.

DISCUSSION

NADH-monodehydroascorbate transhydrogenase is present in ocular tissues (Table 1) and is localized in the microsomal fraction obtained by differential centrifugation (Table 2). The retina and ciliary processes contain the highest activity of this enzyme, based on the protein content of the microsomal preparation, and for this reason retinal and ciliary microsomes were used as the source of the enzyme for further studies. The microsomes from these ocular tissues oxidize NADH only in the presence of ascorbic acid and are thus similar to adrenal microsomes, and they do not contain the ascorbic acid-independent enzyme system for aerobic oxidation of NADH that is present in kidney and liver microsomes.

The reduction of NADH by retinal microsomes requires the presence of ascorbic acid, a compound that cannot, without prior oxidation, act as an electron acceptor. Dehydroascorbic acid, however, does not catalyse the reaction. The electron acceptor must, therefore, be an intermediate product in the oxidation of ascorbic acid to dehydroascorbic acid, since the reaction rate is greatly increased in the presence of cucumber ascorbate oxidase (Fig. 1). The possibility that NADH can be oxidized non-enzymically by either hydroxyl or monodehydroascorbate radicals, formed during the oxidation of ascorbic acid, can be ruled out by the observation that, in the absence of retinal microsomes, this nucleotide is not oxidized by the reaction of ascorbate oxidase with ascorbic acid. Staudinger *et al.* (1961) have shown that the addition of pure cytochrome b_5 to adrenal microsomes causes an increase in the reaction rate. The presence of cytochrome b_5 in retinal microsomes has now been established and it has been shown that this cytochrome is reduced when allowed to react anaerobically with NADH, ascorbic acid and retinal microsomes. NADH cannot be replaced by NADPH, neither can reduced glutathione, cysteine nor ergothioneine replace ascorbic acid. Aghajanian (1963) observed, during his studies of ascorbic acid-dependent NADH oxidation, that the addition of ascorbic

acid to rat-brain microsomes caused a decrease in extinction, due to an alteration in the physical state of the microsomes. This decrease occurred in the absence of NADH and at wavelengths other than 340 m μ . Similar structural alterations do not occur in retinal microsomal preparations, since no change in extinction is observed on the addition of ascorbic acid to these microsomes in the absence of NADH or when NADH is replaced by NADPH. The decreases in extinction that occurred during our experiments are therefore due to oxidation of the coenzyme and not to general clarification of the microsomal preparation.

The action of chloroquine was studied, since prolonged administration of this drug may lead to irreversible retinal damage. Certain aspects of the retinal histopathology caused by the administration of chloroquine are similar to those induced by iodoacetate (Wetterholm & Winter, 1964). Datta & Basu (1955) have reported that 2,3-dimercaptopropanol protects thiol-containing enzymes against the inhibitory effect of chloroquine. Since *p*-chloromercuribenzoate and iodoacetate also inhibit the ascorbic acid-dependent reaction, retinal NADH-monodehydroascorbate transhydrogenase is, in all probability, a thiol-containing enzyme. The inhibitory effect of chloroquine decreases rapidly as the concentration is lowered, which might possibly explain the fact that chloroquine toxicity becomes apparent only after the prolonged administration of high doses.

The smooth form of the endoplasmic reticulum predominates in the retina (cf. Porter, 1961) and ciliary epithelium (Tormey, 1963), and has been implicated in the transport of metabolites and formation of aqueous humour. Holmberg (1959) has shown that Diamox caused pronounced morphological changes in the endoplasmic reticulum of the ciliary epithelium. Friedenwald, Buschke & Michel (1943) have demonstrated histochemically that ascorbic acid undergoes reversible oxidation during its active transport from the stroma of the ciliary processes across the epithelium, and these authors postulated that the secretion of aqueous humour was dependent on the presence of a cyanide-insensitive enzyme system in the ciliary epithelium, capable of oxidizing ascorbic acid to dehydroascorbic acid.

Diamox decreases the rate of formation of aqueous humour (cf. Davson, 1962). Partial inhibition by Diamox, *in vitro*, of NADH-monodehydroascorbate transhydrogenase occurs at micromolar concentrations of the drug (Table 3), and such concentrations are comparable with those found in the plasma after the administration of Diamox (Wistrand, Rawls & Maren, 1960). Steroid therapy sometimes affects intraocular pressure, and certain steroids have been shown to inhibit NADH oxi-

dation (Yielding & Tomkins, 1959; Jensen, 1959; Endahl & Kochakian, 1961). None of these compounds, however, affected the activity of NADH-monodehydroascorbate transhydrogenase; this is in agreement with the finding (Armaly, 1963) that the increase in intraocular pressure is due to a decrease in outflow facility.

Staudinger *et al.* (1961) have suggested that NADH-monodehydroascorbate transhydrogenase may be associated with steroid hydroxylation in the adrenals, but such a function does not explain the presence of the enzyme in ocular tissues. However, the participation of this enzyme system in cell transport mechanisms would be a function common to the adrenals and ciliary processes and would be in agreement with the findings of Friedenwald *et al.* (1943).

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