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The Metabolism of Naphthalene and its Toxic Effect on the Eye

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1. Naphthalene (1g./kg.) was fed daily by stomach tube to rabbits. 2. In more than half of the rabbits opacities in the lens and degeneration of the retina were visible *in vivo*. 3. Dissection of eye tissues revealed some or all of the following changes: a browning of the lens and eye humours, blue fluorescence of the eye humours and crystals in the retina and vitreous body. 4. The ascorbic acid concentration of the eye humours was decreased. 5. Some metabolites of naphthalene [1,2-dihydro-1,2-dihydroxynaphthalene, 2-hydroxy-1-naphthyl sulphate and (1,2-dihydro-2-hydroxy-1-naphthyl glucosid)uronic acid] are converted enzymically by the tissues of the eye into 1,2-dihydroxynaphthalene. 6. Changes in the eye are consistent with 1,2-dihydroxynaphthalene's being the primary toxic agent. The properties and reactions of this substance are described. 7. 1,2-Dihydroxynaphthalene is readily autoxidizable in neutral solution to form the yellow 1,2-naphthaquinone and hydrogen peroxide. This oxidation is reversed by ascorbate. 8. Ascorbate is oxidized catalytically by 1,2-naphthaquinone. This may account for the disappearance of ascorbate from the aqueous and vitreous humours of the eye after naphthalene feeding. It may also account for the appearance of crystals of calcium oxalate in the eye. 9. The brown colour of the lens of the naphthalene-fed rabbit is due to presence of naphthaquinone–protein compounds.

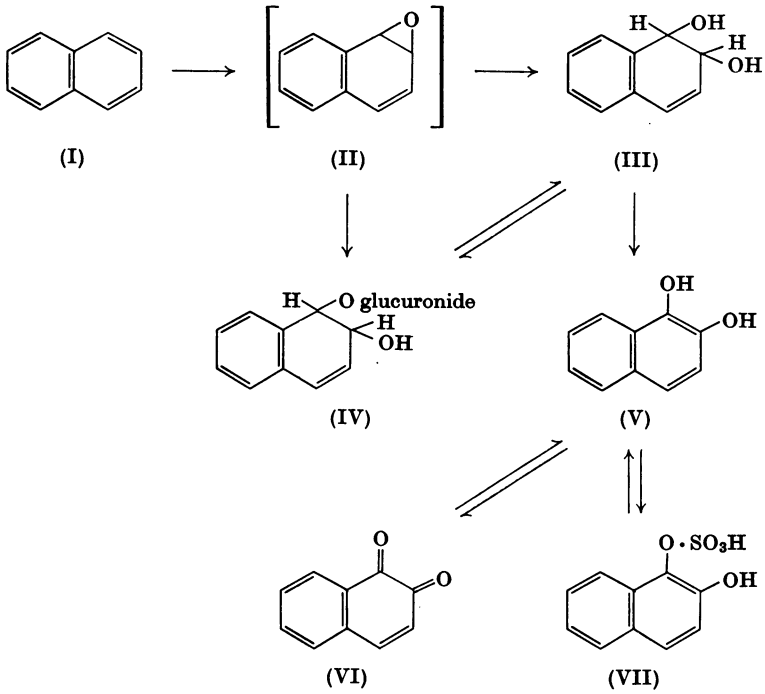
Naphthalene fed in doses of 1–2g./kg. per day causes cataract in animals (Grant, 1962). It used to be given to man as a cure for intestinal infection (Penzoldt, 1886), and one cataract has been reported from this medical use (Lezenius, 1902). Ghetti & Mariani (1956) have described cataracts in five out of nine men under 40 who were exposed in their work to naphthalene vapour.

Detailed descriptions of the sequence of changes in the lens of the rabbit and the rat have been given by Salffner (1904), Adams (1930), Goldmann (1929) and others. Salffner (1904), who gave 4g. of naphthalene/kg. to rabbits, noted that lens swelling was measurable 12–16hr. later and that the lens epithelium was histologically abnormal. Browning of the lens with a yellow colour of the intraocular fluids was frequently noticed (Goldmann, 1929; Adams, 1930; Gifford, 1932). This colour appeared to increase during dissection of the eye, with exposure to air. Metabolites of naphthalene in eye tissues were looked for and Igersheimer & Ruben (1910) obtained a faint diazo reaction in the aqueous humour and vitreous body of naphthalene-fed rabbits that they attributed to 1-naphthol.

The toxicity of naphthalene for the eye is unexplained. Not only does it cause cataract but also complete degeneration of the retina and, occasionally, crystalline deposits in the vitreous body (Igersheimer & Ruben, 1910; Adams, 1930). The eye appears more affected than other organs. Duke-Elder (1954) gives a general survey of the effect of naphthalene on different parts of the eye and says in conclusion: 'It is obvious that the aetiology of the lesion is still obscure.' Before studying changes in the metabolism of eye tissues as a result of naphthalene feeding we set out to find what metabolites of naphthalene reach the eye in the blood stream and what reactions these undergo within the tissues of the eye itself. Dutch, albino New Zealand and albino Polish rabbits have been used and frequent examination of the eyes with slit-lamp and ophthalmoscope has enabled us to follow the course of cataract formation, retinal degeneration and other changes.

The metabolism of naphthalene in laboratory animals has been thoroughly studied. Scheme 1 is a modification of the scheme proposed by Boyland (1963). [The formation of 1-naphthol and 2-naphthol has been omitted, and also the formation of a mercapturic acid from the epoxide (II); the formation of 1,2-naphthaquinone (VI) has been added.]

Naphthalene (I) is absorbed rapidly from the



Scheme 1. Metabolism of naphthalene (adapted from Boyland, 1963).

gut, and is thought to be oxidized in the liver to an epoxide (II), the precursor of 1,2-dihydro-1,2-dihydroxynaphthalene (III), which is excreted in the urine together with its glucosiduronic acid (IV). Ayengar, Hayaishi, Nakajima & Tomida (1959) have found an enzyme in rabbit liver (EC 1.3.1.5; 3,5-cyclohexadiene-1,2-diol–NADP oxidoreductase; trivial name, catechol reductase) that can dehydrogenate 1,2-dihydro-1,2-dihydroxynaphthalene (III) to 1,2-dihydroxynaphthalene (V). This compound is excreted mainly in the form of its sulphate, 2-hydroxy-1-naphthyl sulphate (VII).

1,2-Naphthaquinone (VI) does not appear in Boyland's (1963) scheme, but the evidence presented below suggests that it is formed in the eye.

EXPERIMENTAL

Methods

Treatment of rabbits. Dutch and two strains of albinos were used, usually 3–6 months old. They were fed on pellets (Rank's diet, S.G.1) and carrots. Naphthalene was dissolved in light paraffin (1g./6ml.) by warming, and administered daily by stomach tube (1g. of naphthalene/kg. body wt.) to rabbits partially anaesthetized with ether. Thirty-nine rabbits were used.

Examination of the eye. The pupils were dilated with atropine (1%, w/v) and the eyes examined with slit-lamp and ophthalmoscope.

Estimations. GSH was determined by the method of Ellman (1959).

Ascorbic acid was determined by titration with 2,6-dichlorophenol-indophenol (Pirie, 1965).

Protein was determined as described by Robinson & Hogden (1940).

Oxalate was estimated by the method of Hockaday, Fredrick, Clayton & Smith (1965).

Absorption spectra. These were recorded on an Optica CF4R spectrophotometer.

Fluorescence spectra. These were recorded on an Aminco–Bowman spectrofluorimeter. Fluorescence was also measured on a Turner fluorimeter with a primary filter of peak transmission 365mμ and a secondary filter transmitting above 415mμ.

Manometry. The O_2 uptake was measured in Warburg manometers; the fluid volume in the flasks was 3·0ml.

Thin-layer chromatography. This was carried out by the method of Boyland, Kimura & Sims (1964).

Paper chromatography. The solvents used were: solvent A, butan-1-ol–ethanol–water (17:3:20, by vol.; upper layer) (Boyland & Solomon, 1956); solvent B, 3N-ammonia–3M-ammonium carbonate–2-methylpropan-1-ol (3:3:4, by vol.; upper layer) (Corner & Young, 1954); solvent C, benzene–acetic acid–sodium dithionite (0·1%, w/v) (5:1:4, by vol.).

Detection of substances after chromatography. Fluorescent

and absorbing spots were located by using Hanovia ultraviolet lamps of emission 260mμ and 360mμ and phenolic compounds by spraying with diazotized sulphanilic acid (Smith, 1960).

Examination of blood plasma for the presence of naphthalene metabolites. Plasma (15ml.) from a rabbit given 1g. of naphthalene/kg. daily for 6 days was dialysed against water (150ml.) containing a few drops of $CHCl_3$ for 6 days at 4°. The diffusate was concentrated to a few millilitres in a rotary evaporator at a low temperature, a small precipitate was removed on the centrifuge and the diffusate was then further concentrated to 1·0ml. in a vacuum desiccator. This was divided into two parts (0·5ml. each), and glucuronidase plus sulphatase (0·02ml.) was added to each. One part was immediately shaken with ethyl acetate (1·0ml.) and the other was incubated at 37° for 2hr. before extraction with ethyl acetate (1·0ml.). The two ethyl acetate extracts were dried, taken up in ethyl acetate (20μl.) and examined by thin-layer chromatography.

Extraction of 1,2-dihydro-1,2-dihydroxynaphthalene from the blood of a naphthalene-fed rabbit. Plasma (20ml.) from a rabbit given 1g. of naphthalene/kg. daily for 10 days was diluted with water (80ml.) and continuously extracted with ether for 8hr. on three successive days (Young, 1947; Booth & Boyland, 1949). The ether was evaporated to dryness *in vacuo* and taken up in a small volume of water with warming; this was applied to paper and chromatographed in solvent A with authentic 1,2-dihydro-1,2-dihydroxynaphthalene (III) as marker. The plasma extract yielded a spot that quenched the background fluorescence of the paper when viewed under ultraviolet light at 260mμ and with the same R_F as 1,2-dihydro-1,2-dihydroxynaphthalene (III); this spot was cut out and eluted with water, and its ultraviolet spectrum was the same as that of the authentic compound.

Preparation of extracts of acetone-dried powders of eye tissues. Except where otherwise stated bovine eyes were used. The eyes were normally dissected within 2hr. of the death of the animal, and the parts either stored at −20° or used immediately. Acetone-dried powders from corneal epithelium, lens, ciliary body plus iris, retina and choroid were made according to the method of Morton (1955); the dried powders were stored at −20°. Extracts were made by grinding the powder (1g.) in a mortar with tris buffer, pH8 (0·05M; 15ml.) (Gomori, 1955). After 2hr. at room temperature the mixture was centrifuged at 10000*g* at 0° for 20min. and the residue discarded. The supernatant, after dialysis overnight at 4° against the same tris buffer (1l.), was stored at −20° and used as a source of catechol reductase.

Identification of reaction product of catechol reductase. To extract the product of the reaction with 1,2-dihydro-1,2-dihydroxynaphthalene as substrate it was necessary to perform the reaction anaerobically. GSSG and glutathione reductase were added to keep the NADP oxidized. Enzyme (0·2ml.), NADP (8mg./ml.; 0·1ml.), tris buffer, pH8 (0·05M; 0·5ml.), GSSG (10mM; 0·1ml.) and glutathione reductase [Boehringer Corporation (London) Ltd., London, W. 5] (0·02ml.) were put in the main part of a Thunberg tube, and 1,2-dihydro-1,2-dihydroxynaphthalene (III) (10mg./ml.; 0·15ml.) in the hollow stopper. After evacuating the tube, the contents were mixed and incubated at 37° for 4hr. The contents became intensely blue-fluorescent. On releasing the vacuum, conc. HCl (0·1ml.) and ethyl acetate (1·0ml.) were immediately added and the mixture was shaken. The fluorescence was transferred to the ethyl acetate. A spot of this mixture when run in solvent C had the same R_F as authentic 1,2-dihydroxynaphthalene (V) and reacted with diazotized sulphanilic acid to give the same red colour.

Preparation of extracts of lens. A known weight of fresh lens was ground in a mortar with a little sand to a smooth paste. Water was added to give the selected concentration and the lens suspension was then centrifuged at 10000*g* for 15min. and used immediately, or dialysed against water at neutral pH for 24–72hr. at 4°.

Preparation of extracts of other eye tissues. A known weight was ground in a mortar with a little sand and a known volume of tris buffer, pH7·5 (0·05M) (Gomori, 1955). This suspension was dialysed overnight against the same buffer, and the insoluble part was then removed by centrifugation at 10000*g* at 4° for 10min.

Materials

Naphthalene derivatives. Preparations of 1,2-dihydro-1,2-dihydroxynaphthalene were generously given by Professor E. Boyland and Professor Sir Ewart Jones. It was also prepared from the urine of naphthalene-fed rats by the method of Booth & Boyland (1949).

2-Hydroxy-1-naphthyl sulphate was prepared by the method of Boyland & Sims (1959).

1,2-Dihydroxynaphthalene was prepared by the method of Corner & Young (1954). The product had m.p. (uncorr.) 103° and showed absorption peaks at 234, 288 and 334mμ, as described by Daglish (1950).

1,2-Naphthaquinone was prepared by the method of Fieser & Fieser (1939); we thank Mrs J. Rees for this preparation.

1-Naphthol and 2-naphthol were from British Drug Houses Ltd., Poole, Dorset.

(2-Hydroxy-1-naphthyl glucosid)uronic acid was isolated from the urine of a naphthalene-fed rabbit as follows. The urine (100ml.) was first extracted with ether to remove 1,2-dihydro-1,2-dihydroxynaphthalene (Booth & Boyland, 1949). It was then concentrated to 10ml. in a rotary evaporator at about 50° and shaken with butan-1-ol (10ml.). Five superimposed streaks of the butanol layer were applied to Whatman no. 1 chromatography paper and chromatographed in solvent B for 24hr. The paper was air-dried and again chromatographed in the same solvent for 24hr. The slowest-moving band (R_F about 0·10) that quenched the fluorescence of the paper at 260mμ was cut out and cut into small pieces that were shaken with water (10ml.) for 4hr. at room temperature. The extract was filtered and concentrated *in vacuo* to a small volume. It was purified by chromatography in the same way, but in solvent A. The glucuronide of the diol prepared in this way had a similar R_F in solvents A and B to those given by Boyland & Solomon (1956) and Corner & Young (1955), and the same ultraviolet spectrum as the diol itself; a substance having the same R_F as 1,2-dihydro-1,2-dihydroxynaphthalene in solvents A and B was found on incubation with glucuronidase. After hydrolysis with N-HCl there was evidence of the formation of (1-naphthyl glucosid)-uronic acid (Corner, Billett & Young, 1954).

Enzymes. Catalase and glucuronidase plus sulphatase were from Boehringer Corporation (London) Ltd.

RESULTS

Examination in vivo

There was great variation in response from rabbit to rabbit, the variations in techniques of feeding suggested by Bourne (1933) making no difference. In susceptible animals the cataractous changes in the lens, the degeneration in the retina and the formation of crystals in the vitreous body were the same as those described by Adams (1930).

Dissection of eye tissues

The aqueous humour and vitreous body of naphthalene-fed animals showed a strong blue fluorescence. In the aqueous humour this was partly but not wholly due to protein, which was present to an abnormal extent. Some of those rabbits that had received ten or more doses of naphthalene showed a general yellowing of the eye fluids and yellow or even brown cortical areas in the cataractous lens.

Since 1,2-dihydroxynaphthalene (V) fluoresces blue and 1,2-naphthaquinone (VI) is yellow, the appearance of blue fluorescence and yellow colour in the eye fluids together with the brown colour of the lens suggested the presence of these compounds. Their properties and their reactions with constituents of the eye were therefore studied to determine whether they might explain the peculiar toxicity of naphthalene to the eye.

Reactions of 1,2-*dihydroxynaphthalene* (*V*) *and* 1,2-*naphthaquinone* (*VI*)

The ease of oxidation of 1,2-dihydroxynaphthalene (V) is one of its main characteristics. Autoxidation occurs rapidly in aqueous solution at neutrality with formation of hydrogen peroxide and 1,2-naphthaquinone (VI), which then undergoes further reactions forming brown products (Elsevier Publishing Co., 1952; Fieser & Peters, 1931).

A neutral solution of 1,2-dihydroxynaphthalene (V) shows strong blue fluorescence and autoxidation may be followed by disappearance of this fluorescence and appearance of the yellow colour of the quinone (VI). Doskoçil (1950) found E_0 + 0·573 for the reaction. The rate of autoxidation is so rapid in air at neutrality that, in the absence of a reducing agent, addition of 1,2-dihydroxynaphthalene (V) to a solution is equivalent to addition of 1,2-naphthaquinone (VI). Oxidation of the dihydroxynaphthalene (V) to the quinone (VI) can be followed spectroscopically. The spectral absorption of 1,2-dihydroxynaphthalene (V) shows $\lambda_{max.}$ 234, 290 and 334mμ in 5% (v/v) hydrochloric acid (Daglish, 1950) and that of 1,2-naphthaquinone (VI) shows $\lambda_{max.}$ 250 and 340mμ (Morton, 1965).

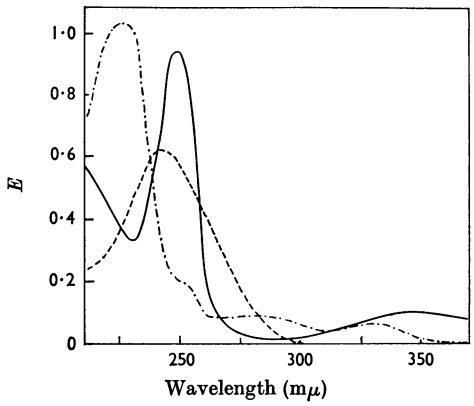
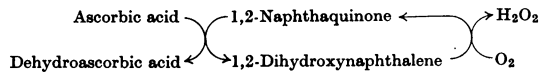


Fig. 1. Reduction of 1,2-naphthaquinone by ascorbic acid. (1), Absorption spectrum of ascorbic acid at pH 3·7 (----); (2), absorption spectrum of 1,2-naphthaquinone at pH 3·7 (——); (3), absorption spectrum of mixture of (1) and (2), showing spectrum of 1,2-dihydroxynaphthalene (-·-·-). Each cuvette contained 3·0ml. of 0·06M-acetate buffer, pH 3·7; 20μl. of 10mM-1,2-naphthaquinone was added to cuvettes (2) and (3) and 20μl. of 10mM-ascorbic acid to cuvettes (1) and (3).

Reaction of 1,2-*naphthaquinone with ascorbic acid.* The fluids of the eye contain a concentration of 1mM-ascorbic acid. The reaction of ascorbic acid with *o*-quinones to form catechols is well known (Mason, 1955; Pierpoint, 1966), and, judged by the formation of blue fluorescence and removal of yellow colour, ascorbic acid reduces 1,2-naphthaquinone (VI) to 1,2-dihydroxynaphthalene (V). As the dihydroxynaphthalene (V) is autoxidizable the fluorescence gradually fades in air and the yellow colour returns. Reduction of the quinone (VI) to 1,2-dihydroxynaphthalene (V) can be demonstrated spectroscopically in air in acid solution or anaerobically at neutrality, where the dihydroxynaphthalene (V) must be extracted into chloroform before spectroscopy. Fig. 1 shows the absorption spectra of ascorbic acid and 1,2-naphthaquinone (VI) before mixing at pH 3·7 and the final spectrum of 1,2-dihydroxynaphthalene (V) taken 95min. later.

When 1,2-naphthaquinone (VI) is reduced by ascorbic acid (Scheme 2), oxygen is absorbed until all the ascorbic acid is oxidized. Catalase diminishes oxygen uptake, showing that hydrogen peroxide is formed. As little as 1·5μg. of 1,2-naphthaquinone (VI)/ml. is catalytically active and can oxidize many times its equivalent of ascorbic acid.



Scheme 2. Reduction of 1,2-naphthaquinone by ascorbic acid.

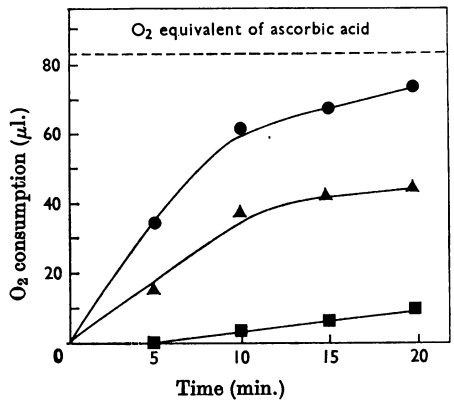


Fig. 2. Oxygen uptake of aqueous humour with and without 1,2-naphthaquinone and catalase. Each manometer flask contained 90 μmoles of potassium phosphate buffer, pH 7·3, and 2·6 ml. of ox aqueous humour. Flasks (2) and (3) contained 1·6 μg. of 1,2-naphthaquinone/ml. and flask (2) contained 10 μg. of catalase. The 1,2-naphthaquinone was added from the side arm after equilibration. The total volume of fluid was 3·0 ml. Incubation was at 25°. KOH was in centre well. (1), Aqueous humour (■); (2), aqueous humour, 1,2-naphthaquinone and catalase (▲); (3), aqueous humour and 1,2-naphthaquinone (●).

The same reaction took place when 1,2-naphthaquinone (VI) was added to aqueous humour or to vitreous humour, and Fig. 2 shows the oxygen uptake of aqueous humour and the quinone (VI) with and without catalase.

Reaction of 1,2-dihydroxynaphthalene with cytochrome c. Addition of 1,2-dihydroxynaphthalene (V) to a solution of oxidized cytochrome *c* in a spectrophotometer cell caused its rapid reduction with the formation of the characteristic peak at 550 mμ.

Oxidation of $NADH_2$ and $NADPH_2$ by 1,2-naphthaquinone. We have confirmed the rapid oxidation of $NADH_2$ and $NADPH_2$ by 1,2-naphthaquinone (VI) (Yuge, Ueda & Nose, 1963).

Search for presence of metabolites of naphthalene in the eye

Attempts to find naphthalene metabolites in eye tissues were made by several different methods. A delicate test for 1,2-dihydroxynaphthalene (V) and 1,2-naphthaquinone (VI) is their catalytic effect on oxidation of ascorbic acid. About 1 μg. of either/ml. can be detected in this way. But addition of aqueous humour or of vitreous humour from naphthalene-fed rabbits to solutions of ascorbic acid did not catalyse oxidation. However, it was found that if 1,2-naphthaquinone (VI) were added to vitreous humour it became incapable of stimulating oxygen uptake after 48 hr. at 4°, whereas in the absence of vitreous humour it maintained this capacity. Inactivation is probably due to its reaction with protein (see below). It is therefore likely that any 1,2-naphthaquinone (VI) formed in the eye will soon lose its catalytic action and would not be detectable by this method.

Absorption spectra of ethanol extracts of dried aqueous humour or vitreous humour from naphthalene-fed rabbits gave no identifiable peaks.

No naphthalene metabolites could be detected in aqueous humour or vitreous humour, concentrated *in vacuo* and examined by thin-layer chromatography, paper electrophoresis or paper chromatography.

Metabolic products in the plasma of the naphthalene-fed rabbit

The presence in blood of 1,2-dihydro-1,2-dihydroxynaphthalene (III) has been established (see the Methods section). From the extinction at 260 mμ of 1,2-dihydro-1,2-dihydroxynaphthalene (III) eluted from the paper chromatograms it was calculated that it was about 0·015 mM in the blood plasma of the naphthalene-fed rabbit.

Evidence for the presence of 2-hydroxy-1-naphthyl sulphate (VII) in the plasma was obtained by treating a diffusate of plasma with sulphatase and glucuronidase (see the Methods section). The treated diffusate was yellow whereas the untreated diffusate was colourless. This indicates that the sulphate (VII) is hydrolysed to 1,2-dihydroxynaphthalene (V), which becomes rapidly oxidized to the yellow 1,2-naphthaquinone (VI).

The finding that the amount of 1,2-dihydro-1,2-dihydroxynaphthalene (III) that could be extracted with ethyl acetate from a diffusate of plasma was increased after treatment with hydrolysing enzymes is evidence for the presence of (2-hydroxy-1-naphthyl glucosid)uronic acid (IV).

Enzymic formation by eye tissues of 1,2-dihydroxynaphthalene from naphthalene metabolites

Dehydrogenation of 1,2-dihydro-1,2-dihydroxynaphthalene. We have found that the enzyme catechol reductase (EC 1.3.1.5) described by Ayengar *et al.* (1959) is present in the tissues of the eye and that this enzyme catalyses the dehydrogenation of 1,2-dihydro-1,2-dihydroxynaphthalene (III) to 1,2-dihydroxynaphthalene (V). The product of the reaction was identified by paper chromatography (see the Methods section). NADP is required for activity. Acetone-dried powders of the tissues of the eye were prepared (see the Methods section) and catechol-reductase activity was found in the ciliary body plus iris, retina, choroid, corneal epithelium and lens.

The preparation from the ciliary body plus iris was used for studying the properties of the enzyme. The ciliary body secretes the aqueous humour and naphthalene metabolites probably enter the eye in this way. Ayengar *et al.* (1959) followed the course of the reaction by the reduction of NADP at 340mμ, and identified the catechol formed by paper chromatography. Owing to the instability of 1,2-dihydroxynaphthalene (V) in the presence of air (see above), however, this method was not possible when 1,2-dihydro-1,2-dihydroxynaphthalene (III) was used as substrate, and other methods had to be used.

In the presence of air, non-enzymic reactions (ii) and (iii) follow the enzymic reaction (i), and the overall reaction is expressed by equation (iv); oxidation of reactants by hydrogen peroxide may also be expected. If the enzyme is active, the yellow quinone (VI) is formed.

1,2-Dihydro-1,2-dihydroxynaphthalene + NADP → 1,2-dihydroxynaphthalene + $NADPH_2$ (i)

2 (1,2-Dihydroxynaphthalene) + 2 O_2 → 2 (1,2-naphthaquinone) + 2 H_2O_2 (ii)

1,2-Naphthaquinone + $NADPH_2$ → 1,2-dihydroxynaphthalene + NADP (iii)

Sum: 1,2-Dihydro-1,2-dihydroxynaphthalene + 2 O_2 → 1,2-naphthaquinone + 2 H_2O_2 (iv)

If excess of ascorbic acid is present the following further reaction takes place:

1,2-Naphthaquinone + ascorbate → 1,2-dihydroxynaphthalene + dehydroascorbate (v)

and the overall reaction is:

1,2-Dihydro-1,2-dihydroxynaphthalene + 2 O_2 + ascorbate → 1,2-dihydroxynaphthalene + 2 H_2O_2 + dehydroascorbate (vi)

Table 1. *Activity of catechol reductase in the ciliary body*

The reaction mixture was as follows: dialysed ciliary-body extract (see the Methods section), 1ml.; NADP (3mg./ml.), 0·3ml.; ascorbic acid (2mg./ml., neutralized to pH6), 0·3ml.; diol (0·5mg./ml.), 0·3ml.; tris buffer, pH8 (0·1M), to 2ml. Incubation was at 37° for 2hr.

Reaction mixture	Changes observed after incubation
Whole mixture	Blue fluorescence
Diol omitted	—
NADP omitted	—
Enzyme omitted	—
Ascorbic acid omitted	Yellow colour and no fluorescence
Whole mixture, but enzyme boiled	—

Enzymic activity is then indicated by the appearance of the strongly fluorescent 1,2-dihydroxynaphthalene (V). Only when the ascorbic acid has all been oxidized does this become oxidized to 1,2-naphthaquinone (VI), with the appearance of a yellow colour. By these qualitative tests (Table 1) it was shown that ciliary-body extract, 1,2-dihydro-1,2-dihydroxynaphthalene (III) and NADP were all needed for enzyme activity.

To assay the enzyme use was made of the fact that 1,2-naphthaquinone and aniline combine to form a red compound (Pugh & Raper, 1927; Pierpoint, 1966). In the presence of aniline (20mM) it was found that the rate of formation of the red compound, measured by its extinction at 480mμ, was proportional to the amount of enzyme. The relation between pH and activity was very similar to that found by Ayengar *et al.* (1959). The rate with NAD as coenzyme was about one-third of that with NADP.

The activity of the enzyme in a dialysed extract of acetone-dried powder prepared from the ciliary body plus iris was about one-sixth of that prepared from rabbit liver (Ayengar *et al.* 1959). Fourfold purification, with 75% yield, was achieved by treatment with calcium phosphate gel (Ayengar *et al.* 1959). The activities of the enzyme in the acetone-dried powders prepared from iris, retina, choroid and corneal epithelium were comparable with that in the ciliary body plus iris, but that prepared from the lens was considerably lower.

To prove that the compound reacting with aniline was, in fact, 1,2-naphthaquinone, the reaction mixtures from a number of experiments were pooled and kept at −20°. A red precipitate was formed. This was washed five times on the centrifuge with distilled water, the last washing

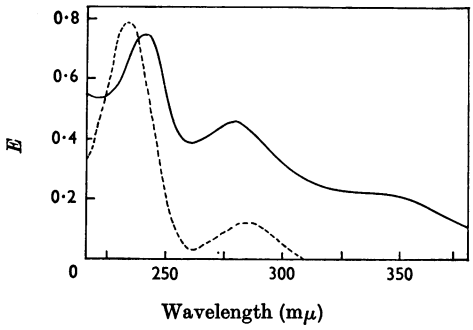


Fig. 3. Absorption spectra of aniline (0·1mM in ethanol) (----) and aniline–naphthaquinone compound (0·0272mg./ml. of ethanol) (——).

having no absorption in the range 200–550mμ. The precipitate was then dissolved in ethanol and its absorption spectrum determined over this range. The absorption peaks were at 241, 279, 340 (inflexion) and about 462mμ. The compound made by mixing aniline (20mM) and 1,2-naphthaquinone (1mM) had an identical absorption spectrum (Fig. 3).

Hydrolysis of (1,2-dihydro-2-hydroxy-1-naphthyl glucosid)uronic acid and 2-hydroxy-1-naphthyl sulphate by eye tissues. Apart from 1,2-dihydro-1,2-dihydroxynaphthalene (III), two other metabolites of naphthalene can be converted into 1,2-dihydroxynaphthalene (V) by the tissues of the eye. These are (1,2-dihydro-2-hydroxy-1-naphthyl glucosid)-uronic acid (IV) and 2-hydroxy-1-naphthyl sulphate (VII). Evidence has already been given for the presence of both compounds in plasma. These compounds are substrates of the β-glucuronidase and sulphatase respectively found in the tissues of the eye (Becker & Friedenwald, 1950; Shanthaveerappa & Bourne, 1964). When a dialysed extract of rabbit ciliary body plus iris was incubated with the glucosiduronide (IV), a compound was liberated that had the same reactions and R_F in solvents A and B as 1,2-dihydro-1,2-dihydroxynaphthalene (III). When NADP and aniline were added to the reaction mixture, the diol (III) was converted into the naphthaquinone (VI), as indicated by the appearance of the red colour of the anilide. When incubated with aniline and a dialysed extract of rabbit ciliary body and iris, or corneal epithelium, 2-hydroxy-1-naphthyl sulphate (VII) yielded a red colour, indicating its dehydration and oxidation to the naphthaquinone (VI). There were no positive reactions in control incubations with boiled tissue extracts.

If toxicity of naphthalene to the eye is due to formation of 1,2-dihydroxynaphthalene in the ciliary body and its subsequent oxidation to 1,2-naphthaquinone in the eye, naphthalene metabolites that form these compounds should also be toxic to the eye.

Administration of naphthalene metabolites to the rabbit

1,2-Dihydro-1,2-dihydroxynaphthalene (III). One rabbit was injected intravenously, daily, with 100mg. of the diol (III) in 5ml. of water. After 3 days retinal lesions, apparently identical with those obtained after naphthalene feeding, were produced. There were no lens changes. To four other rabbits the diol (III) (1% in water) was administered as eye drops, half-hourly, throughout the day; a total of 40–70mg. was applied during 2–5 days. In this case changes only in the front part of the eye would be expected and these were found; in three out of the four rabbits lens changes, visible *in vivo*, were produced.

2-Hydroxy-1-naphthyl sulphate (VII). This compound was administered to two rabbits in the form of drops (1% in water). A total of 80mg. was administered in each case over a period of 5–6 days. No eye changes were observed. In a third rabbit 2-hydroxy-1-naphthyl sulphate (50mg. in 2ml. of water) was injected intravenously twice daily for 4½ days. Again no eye changes were observed.

In experiments such as these there are many factors, such as permeability and ease of hydrolysis of the compounds, that may influence the results. Both injection and external application of 1,2-dihydro-1,2-dihydroxynaphthalene (III) were toxic to the eye but no effect was produced by 2-hydroxy-1-naphthyl sulphate (VII).

Incubation of rabbit lens in a medium containing 1,2-dihydro-1,2-dihydroxynaphthalene

The following experiment demonstrates the presence in the lens of an enzyme, probably catechol reductase, that oxidizes 1,2-dihydro-1,2-dihydroxynaphthalene.

A pair of rabbit lenses were incubated aerobically at 34° in Krebs–Ringer phosphate (Umbreit, Burris & Stauffer, 1949) containing glucose (5mM). Sodium benzylpenicillin and streptomycin (final concentration of each, 0·1mg./ml.) were added at the start and again after about 6hr. incubation. One lens was held in boiling water for 3min. before incubation. Each lens was in 5ml. of medium containing 1,2-dihydro-1,2-dihydroxynaphthalene at a final concentration of 6mM. The boiled lens and medium remained colourless throughout. After 1hr. the unboiled lens and the medium

started to turn yellow–brown. The colour continued to increase in intensity, and after 18hr. the lens was black and the medium deep yellow–brown. On dissection, the capsule of the lens was found to be brown, the cortex black and the nucleus uncoloured, turning yellow on exposure to air.

Incubation of the lens in a diffusate of plasma from a naphthalene-fed rabbit

The following experiment demonstrates that the plasma of the naphthalene-fed rabbit can cause browning of the lens *in vitro* with loss of ascorbic acid and GSH.

Plasma (5ml.), taken from a naphthalene-fed rabbit 3hr. after dosing, was dialysed for 7 days at 4° against 100ml. of water containing a few drops of chloroform. The diffusate was concentrated to 5ml. in a rotary evaporator at a low temperature. A diffusate of plasma from a normal rabbit was prepared in the same way and served as a control. To each was added glucose (10%, w/v; 0·06ml.), phosphate buffer, pH 7·4 (0·1M; 0·6ml.), ascorbic acid (10, w/v, in the same phosphate buffer; 0·02ml.), sulphatase and glucuronidase (0·05ml.) and antibiotics (sodium benzylpenicillin and streptomycin; 10mg. of each/ml.; 0·06ml.). One lens of a pair of rabbit lenses was incubated at 34° in each medium.

After 18hr. the lens in the control medium was clear and colourless, whereas the lens in the naphthalene-treated diffusate was a pale brown; this was due to a brown deposit on the surface and a brown coloration extending about 1mm. into the substance of the lens. The GSH and ascorbic acid concentrations in this lens were respectively 74% and 30% of that in the control lens.

This experiment was repeated with diffusate of plasma from another naphthalene-fed rabbit, with similar results.

Loss of ascorbic acid and formation of crystals of calcium oxalate in the eye of the naphthalene-fed rabbit

We have confirmed that the concentration of ascorbic acid is lowered in the aqueous humour of the naphthalene-fed rabbit (see Nordmann, 1954), and find that the vitreous humour and the retina also lose ascorbic acid (Table 2). Ascorbic acid is catalytically oxidized by 1,2-naphthaquinone (VI) (see above and Fig. 2), and it seems likely that its loss in the eye is due to this reaction. We suggest that the loss of ascorbic acid may also be connected with the appearance of crystals of calcium oxalate in the vitreous body and in, or on the surface of, the retina. Adams (1930) described these crystals and considered that they were calcium oxalate, but at that time their origin was unknown. It seems reasonable to suggest that the dehydroascorbic acid formed in the reaction between ascorbic acid and 1,2-naphthaquinone (VI) breaks down to oxalic acid, which is precipitated as calcium oxalate. Oxalic acid is known to be a breakdown product of ascorbic acid that appears in the urine (Burns, 1951).

Calcium oxalate crystals separated from a solution of ascorbic acid in the presence of calcium chloride at pH 7 to which 1,2-naphthaquinone (VI) to a final concentration of 60μM had been added. A mixture without the quinone gave one-tenth as much. The concentration of the quinone was kept low to simulate possible conditions in the eye.

It was not possible to separate the crystals from the retina for analysis but we were able to show that oxalate was present in a retina that contained crystals. Calculated as calcium oxalate, the crystals accounted for nearly 1% of the dry weight. No oxalate could be detected in a normal retina.

A vitreous body that contained crystals was chopped with scissors and centrifuged at 10000*g* for 15min. at 4°. Oxalate was assayed in the

Table 2. *Effect of naphthalene feeding on the concentration of ascorbic acid in the aqueous humour, vitreous humour and the retina*

Rabbits were dosed daily with naphthalene (1g./kg.) for 2–10 days. Ascorbic acid was measured in trichloroacetic acid extracts by titration with 2,6-dichlorophenol-indophenol (see the Methods section). Mean values ±S.D. are given for the numbers of analyses shown in parentheses.

	Ascorbic acid (μg./g. fresh wt. of tissue)			
	Naphthalene-fed rabbits		Control rabbits	
	Mean	Range	Mean	Range
Aqueous humour	109±71 (15)	0–240	216±63 (9)	116–345
Vitreous humour	Negligible (6)	—	108±39 (6)	74–170
Retina	127±39 (4)	70–160	244±39 (4)	209–300

precipitate, which contained the crystals together with the residual protein. Calculated as calcium oxalate, the vitreous body was found to contain 45 μg./ml. No oxalate could be detected in normal vitreous body.

Reaction of 1,2-*naphthaquinone with lens proteins* in vitro *and* in vivo

Morgan & Cooper (1924) found that 1,2-naphthaquinone (VI) reacted with gelatin and albumin to give brown compounds. Rees & Pirie (1967) have examined the reaction of 1,2-naphthaquinone (VI) with separated lens proteins. In the present paper we compare the properties of brown proteins from lenses of naphthalene-fed rabbits with those of 1,2-naphthaquinone–proteins prepared *in vitro*.

When 1,2-naphthaquinone (VI) is added to a dialysed extract of lens at neutrality in air the pale-yellow solution gradually darkens and within some hours becomes deep brown. The brown colour is not removed from the protein by dialysis, ammonium sulphate or acid precipitations or by extraction with ethyl acetate or chloroform. A stable compound of the quinone (VI) with lens proteins seems to have been formed. The brown colour is bleached by sodium borohydride and by sodium dithionite but not by ascorbic acid. The protein–naphthaquinone compound shows blue fluorescence and this is greatly increased after reduction with sodium borohydride.

1,2-*Naphthaquinone–protein in lens of naphthalene-fed rabbit*

Lenses of rabbits that had been given naphthalene were examined to see whether proteins with any of the characteristics of lens proteins treated with 1,2-naphthaquinone (VI) could be separated. The lenses showed brown cortical patches. These were dissected off together with the lens capsule; the tissue was weighed, ground with sand and extracted in water at 1:10 dilution. About 200mg. of lens was thus dissected and an equivalent amount of cortex from a litter-mate control was similarly dissected and extracted. The extracts were dialysed against distilled water at 4° for 24hr. After dialysis the extracts were centrifuged, the supernatants removed and their protein concentrations estimated (see the Methods section) and equalized. The extracts from the lenses of two naphthalene-fed rabbits were pale brown whereas those of litter-mate controls were colourless. Borohydride bleached the extract from the lens of the naphthalene-fed rabbit and increased the fluorescence of the solution. With a 365mμ filter in the exciting beam and 415mμ filter in the emitted beam the increase in fluorescence after borohydride was four times as great in the extract of the naphthalene lens as in the extract of the normal lens. Comparison of the fluorescence spectra in an Aminco–Bowman spectrophotofluorimeter with an exciting light of 365mμ showed that the protein from the lens of the naphthalene-fed rabbit had a fluorescence peak at 450mμ that was not present in the protein from the lens of the litter-mate control. This peak was also present in 1,2-naphthaquinone–protein made from an extract of rabbit lens (Fig. 4).

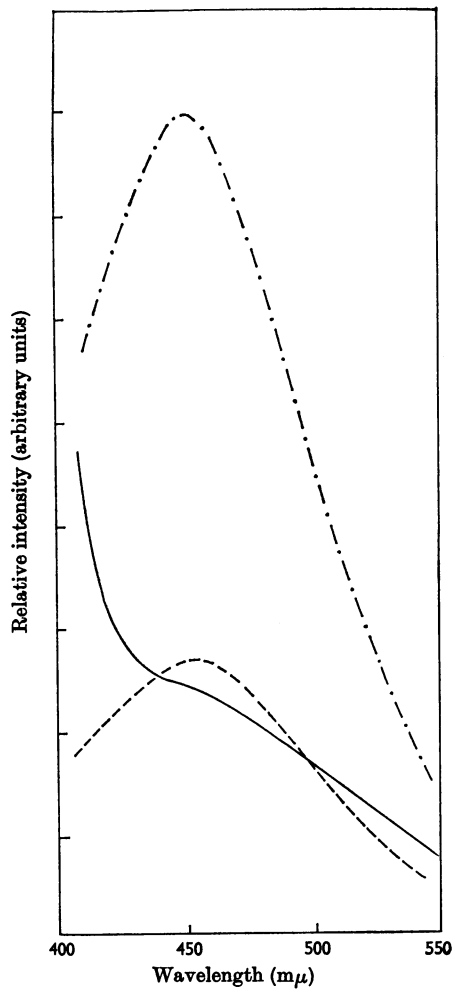


Fig. 4. Fluorescence emission spectra of dialysed lens protein from a naphthalene-fed rabbit (–·–·–) and a normal rabbit (——) compared with 1,2-naphthaquinone–protein made *in vitro* from rabbit lens (– – – –). All three protein solutions were treated with $NaBH_4$ immediately before examination. Spectra were recorded at pH8 with excitation at 365mμ. Uncorrected instrumental curves are shown.

These experiments show that naphthalene feeding causes a brown protein or proteins to accumulate in the lens having fluorescence spectrum and properties similar to those of 1,2-naphthaquinone–protein prepared from lens *in vitro*.

DISCUSSION

The evidence presented in this paper indicates that certain well-known metabolites of naphthalene (III, IV and VII in Scheme 1) are hydrolysed or dehydrogenated or both by enzymes present in the tissues of the eye to give 1,2-dihydroxynaphthalene (V). This compound is rapidly autoxidized at neutrality to give 1,2-naphthaquinone (VI), which in turn will react with many constituents of the eye, including ascorbic acid, thiols, amino acids, coenzymes and proteins. Free 1,2-naphthaquinone (VI) could not be found in the eye, but its presence has been indicated by comparison of the properties of the brown proteins extracted from the lens of the naphthalene-fed rabbit with those of 1,2-naphthaquinone–proteins prepared *in vitro* from lens. There have been many suggestions that 1,2-naphthaquinone may be implicated in the formation of naphthalene-induced cataract (see Bellows, 1944; Ogino, Tojo, Fujishigi & Katumori, 1957), but no theory or proof of its formation has been advanced until the work reported in this paper.

Naphthalene (1g./kg.) is toxic to the whole animal, judged by the general appearance of the dosed rabbit, the occasional occurrence of haemorrhages in the ear and intestine, the failure of appetite and growth and sometimes the death of the animal. Whether this toxicity is due to the same naphthalene metabolites that affect the eye has not been investigated. The eye is more obviously affected than other organs.

Possible explanations for the vulnerability of the lens to naphthalene feeding are that the oxygen concentration in the aqueous humour is low and the ascorbic acid concentration is high. The catalytic oxidation of ascorbic acid in aqueous humour by 1,2-naphthaquinone (VI) (see above) may diminish the oxygen concentration and will produce hydrogen peroxide, besides removing the ascorbic acid. All these changes may be harmful. Pirie (1965) has suggested that small amounts of hydrogen peroxide may normally be produced in aqueous humour and metabolized in lens through glutathione peroxidase, but an excessive production of hydrogen peroxide may be toxic.

The aqueous humour of the naphthalene-fed rabbit contains an abnormal amount of protein. The ciliary body, which secretes the aqueous humour, is known to be damaged by naphthalene feeding (Salffner, 1904), but the histological changes in the ciliary epithelium appeared later than changes in the lens. However, it is possible that alterations occur in the composition of the aqueous humour and that these accelerate cataract formation.

Apart from these indirect actions by way of changes in the aqueous humour and ciliary body, 1,2-naphthaquinone will affect the lens directly through its reaction with proteins, whether structural or enzymic, and with coenzymes. Meyerhof (1948) has shown that it is a potent inhibitor of many glycolytic enzymes. Glycolysis, oxidations and reductions within the cell, oxidative phosphorylation and respiration may all be affected, since $NADH_2$ and $NADPH_2$ are oxidized by 1,2-naphthaquinone (VI) and cytochrome *c* is reduced by 1,2-dihydroxynaphthalene (V). The oxidation–reduction potential of 1,2-naphthaquinone (VI) is close to that of ubiquinone (Morton, 1965) and it may be a competitor in reactions catalysed by this coenzyme. This wide range of reactivity makes it difficult to determine the primary damage.

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