STUDIES ON THE MECHANISM OF THE CATARACTOGENIC ACTIVITY OF 2,4-DINITROPHENOL

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
JOSEPH EDWARD LeBEAU
1970

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ABSTRACT

STUDIES ON THE MECHANISM OF
THE CATARACTOGENIC ACTIVITY
OF 2,4-DINITROPHENOL

By

Joseph Edward LeBeau

The production of cataracts by 2,4-dinitrophenol (DNP) has been demonstrated in man, fowl, and immature rabbits. Previous studies have failed to demonstrate the mechanism for the cataractogenic activity of DNP. The purpose of this study was an attempt to elucidate the mechanism responsible for the cataractogenic activity of DNP.

The effect of ascorbic acid deficiency on the cataractogenic activity of DNP in guinea pigs was studied *in vivo*. It was concluded from these studies that ascorbic acid deficiency does not predispose guinea pigs to the cataractogenic activity of DNP. However, DNP may hasten the development of what appears to be a natural occurring cataract in guinea pigs.

Studies were next undertaken to study the cataractogenic mechanism of DNP utilizing an *in vitro* lens culture system. DNP at a concentration of 2×10^{-5} M caused cataracts to develop in lenses obtained from New Zealand

white rabbits 35 to 45 days of age. The nature and morphology of the cataract produced, was similar to that described in fowl and immature rabbits. This cataract starts to develop within 2 hours, and within 12 hours tends to reverse. However, this reversal is never complete. The degree of opacification increases and the duration needed for the development of the opacification decreases as the concentration of DNP is increased.

Known metabolites of DNP (2 amino-4 nitrophenol, 2 nitro-4 aminophenol, and 2,4 diaminophenol) did not demonstrate cataractogenic activity at a concentration of 1×10^{-3} M. It was concluded that the cataractogenic activity is related to DNP rather than a metabolite of DNP.

The cataractogenic activity of DNP was found to have a positive temperature coefficient. This suggested that the cataractogenic activity is related to an effect of DNP on lenticular metabolism.

Oxygen deprivation did not influence the cataractogenic activity of DNP. The observation that control lenses maintain their clarity and that DNP produces cataracts, in the absence of oxygen eliminates the uncoupling of oxidative phosphorylation as the mechanism for the cataractogenic activity of DNP.

The effect of DNP on ATPase activity was determined by measuring lenticular ATP concentrations. It was

observed that DNP produces a decrease in lenticular ATP concentrations under anaerobic conditions. These results suggest that mitochondrial ATPase activity was stimulated. Oligomycin, a known inhibitor of DNP-stimulated mitochondrial ATPase inhibited the cataract production by DNP. However, the ATP concentration of lenses treated with DNP and oligomycin was lower than that of lenses treated only with DNP. Therefore, it was concluded that the protective effect of oligomycin against the cataractogenic activity of DNP is not associated with its inhibition of mitochondrial ATPase.

The production of lactic acid and sorbitol by lenses treated with DNP was the same as that of control lenses. It was concluded that the cataractogenic mechanism of DNP is not related to an alteration in the production of sorbitol or lactic acid.

Although DNP caused a significant depression of the hexose monophosphate shunt activity of lenses, this depression was not alleviated when cataract induction was blocked with oligomycin. It was therefore concluded that the DNP induced depression of the pentose phosphate shunt pathway is not related to the cataractogenic activity of DNP.

STUDIES ON THE MECHANISM OF THE CATARACTOGENIC ACTIVITY OF 2,4-DINITROPHENOL

By

Joseph Edward LeBeau

A THESIS

Submitted to

Michigan State University

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

Department of Pharmacology

1970

DEDICATION

To my wife Nancy, and son, Darin.

ACKNOWLEDGMENTS

I would like to express my sincere thanks and appreciation to Dr. P. J. Gehring for his generous guidance, patience, and encouragement throughout this study.

Special thanks is expressed to Dr. D. R. Bennett, not only for his helpful assistance in the preparation of this thesis, but for making this thesis possible.

I would like to thank Drs. T. M. Brody, K. E. Moore, and J. H. McNeill for their helpful assistance in the preparation of this thesis.

The technical assistance of Mr. Richard Walters is gratefully acknowledged.

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INTRODUCTION

Reports of cataract production in humans following the prolonged intake of 2,4-dinitrophenol (DNP) for weight reduction began appearing in 1935. No other untoward effects were observed in these patients. The extensive clinical literature on these cataracts as well as other toxic manifestation caused by DNP was reviewed by Horner in 1942. According to Horner, only 0.86 percent of the individuals treated with DNP were afflicted with cataract. No correlation with the amount of drug taken, the length of time used or the age of the patient could be established. It is of interest that DNP-induced cataracts were seen in successive generations of a family and in identical twins (Hessing, 1937 cited in Ogino and Yasukura, 1957).

There are numerous reports of unsuccessful attempts to produce cataracts in various mammalian species by treatment with DNP (Horner, 1942). On the other hand a few investigators have reported success in producing cataracts in mammals by treatment with DNP. Bettman (1946) reported a 7.5 percent incidence of cataracts in a strain of congenitally obese mice fed a diet containing 0.1 percent DNP. Ordinary albino and black mice fed the same diet did not develop cataracts.

Various investigations have attempted to predispose animals to the cataractogenic activity of DNP by altering their diet. Inclusion of lactose, a material which by itself produces cataracts, in the diet of rats did not render the animals susceptible to the cataractogenic activity of DNP (Borley and Tainter, 1938). Tainter and Borley (1938) were unable to produce cataracts in vitamin A and riboflavin-deficient rats. These same investigators reported that scorbutic guinea pigs treated with DNP did not develop cataracts. However, Ogino and Yasukura (1957) repeated the experiments of Tainter and Borley on scorbutic guinea pigs and claimed that treatment with DNP did cause cataracts.

Until recently, chickens and ducks were the only species other than man generally recognized to be susceptible to the cataractogenic activity of DNP. The susceptibility of these species was first reported by Robbins (1944). He found that inclusion of 0.25 percent of DNP in the diet of chicks and ducklings caused marked lenticular opacification within eight hours. The cataracts disappeared within 24 hours even when the birds were maintained on the same diet. This observation was quickly confirmed by Bettman (1946) and has since been reported numerous times.

Although Robbins (1944) reported that cataracts developed less regularly in older chickens fed diets containing 0.25 percent DNP, Buschke (1947) found that, in the adult, as in the chick, 20 mg/kg DNP given orally or by intramuscular injection caused the same response. There is no tolerance development in ducklings given repetitive intravenous doses of DNP during the first 4 months of life (Gehring, unpublished observation). Possibly the difference in the susceptibility of chicks and adult chickens reported by Robbins may have been due to differences in food intake in relation to body weight or surface area.

Recently Gehring and Buerge (1969a) demonstrated that cataracts can be produced in young rabbits as well as in ducks given a single intraperitoneal injection of DNP. The susceptibility of rabbits to the cataractogenic activity of parenterally administered DNP decreased with age. Cataracts could not be produced in rabbits older than 62 days. In the same study, it was found that cataracts could be produced by a single injection of DNP into the posterior chamber of the eye of mature rabbits or by incubation of the lenses of mature and immature rabbits in media containing DNP. These latter observations suggest that the susceptibility of the lenses of rabbits to

the untoward effects of DNP does not change with age and that DNP rather than a metabolite is the cataractogenic agent.

In another study, Gehring and Buerge (1969b) found that the peak concentration of DNP in the ocular fluids and lenses of mature rabbits given DNP by intraperitoneal injection was less than that found in immature rabbits. Furthermore, the half-life for the disappearance of DNP from these compartments and the serum was longer in the immature rabbits than mature rabbits. Thus, the decrease in the susceptibility of rabbits to the cataractogenic activity of DNP with age was in part explained by an increased rate of clearance of DNP from the serum and ocular fluids of older rabbits. In addition, mathematical analysis of the data showed that the more developed blood-aqueous barrier of the mature rabbit was more effective in precluding the attainment of cataractogenic concentrations of DNP in the ocular fluids and lens.

DNP induced cataracts in birds and immature rabbits are unlike those produced in man. In birds and immature rabbits lenticular opacities may develop within 1 hour after treatment. Within 24 hours, the opacities disappear. In man, cataracts develop only after months of treatment and they are persistent. Indeed, they continue to develop even though treatment is discontinued.

In addition to the temporal differences, there are also morphological differences in the cataracts produced in fowl and immature rabbits and man by treatment with DNP. As described by Horner (1942), the earliest lenticular change in human lenses consisted of faint gray, striated, powdery, downy or lacelike opacities situated just beneath the anterior capsule. The capsule remains transparent but appears "pebbly" because of the presence of water vacuoles beneath it. The cortex may be clear or there may be small, discrete granules in it. The nucleus is unaffected. The most severely affected area is the subcapsular area at the posterior pole of the lens. Here, the lesion is a dense, saucershaped, granular deposit with a golden, sometimes silvery luster. The deposit resembles brass filings, a cloth of gold, hammered copper or silvery or polychromatic crystals. In latter stages, the opacities became intumescent and invade the cortex and finally the nucleus, so that the whole lens is opalescent and silky gray or pearly.

The morphology of the cataract produced in rabbits by treatment with DNP varies with the age of the animal. In rabbits 10 days old or less, the opacities begin at the equator of the lens and migrate in both the anterior and posterior subcapsular region toward the poles. The axial region of the lens extending from the anterior

pole to the posterior pole remains clear. Within 24 hours after treatment, the equatorial regions begin to clear and the cataract appears as a densely opaque circle in both the anterior and posterior subcapsular regions. As time passes, the circular opacities become finer and are found in deeper regions of the cortex. Finally, they appear as fine dense opacities located between the nucleus and cortex. This fine dense opacity seemingly separating the nucleus from the cortex of the lens does not totally disappear as the rabbit ages (Gehring and Buerge, 1969a).

The posterior subcapsular opacity produced in 19 and 26 day old rabbits treated with DNP are similar to those described for the 10 day old rabbit. However, the anterior subcapsular region does not develop the dense opacity previously described. Rather a more diffuse granular opacity is seen in the anterior subcapsular region.

Using a slit lamp, lens opacities in fowl are first seen around the suture lines at the anterior pole. Within a few hours, the entire anterior and posterior subcapsular regions become densely opaque (Buschke, 1947 and Bettman, 1946). Buschke (1947) described minute structures appearing like tiny vacuoles surrounding the anterior pole. Within 24 hours after the cataract is first observed, the anterior

subcapsular opacity clears making the posterior opacity more visible. Subsequently the posterior opacity disappears.

The histological appearance of lenses taken from developing chick embryos treated with DNP have been described by Feldman *et al.* (1959, 1960). The lesions are characterized by degeneration and liquefaction of the lens proteins and the appearance of nuclei at the posterior face of the lens as well as degeneration and proliferation of the anterior marginal epithelium.

Definitive information about the cataractogenic mechanism of DNP in humans or animals is unavailable. Field *et al.* (1937) demonstrated that the respiration of lenses like other tissues is increased by DNP. However, cataract development was not associated with the increased respiration. Horner (1942) discussed other negative findings which include alterations in lens permeability, in the function of various vitamins, water metabolism, and in the oxidation-reduction potential of the lens and aqueous. As previously mentioned, Ogino and Yasukura (1957) observed cataracts in scorbutic guinea pigs treated with DNP suggesting that DNP may interfere with the function of ascorbic acid.

Feldman *et al.* (1959, 1960) associated the cataractogenic activity of DNP with an interference in the

formation of high energy phosphate compounds by the epithelium of the lens. Frohman and Kinsey (1952) demonstrated that the oxidative systems which metabolize glucose via the Krebs cycle are located exclusively in the epithelium while the cortex contains primarily glycolytic processes. Thus, the epithelium must synthesize the bulk of the ATP needed by the lens. Feldman (1959) concluded that the large mass of tissue dependent on the epithelium for ATP explained the unique sensitivity of the lens to DNP.

The effects of DNP on intermediary metabolism have been less extensively studied in the lens than in other tissues. Loomis and Lippman (1948) first demonstrated that the relationship between energy-rich phosphate compounds and the utilization of oxygen by DNP is altered. This well known effect of DNP is classically referred to as uncoupling of oxidative phosphorylation. Slater has presented the following reaction scheme to explain the mechanism whereby DNP uncouples oxidative phosphorylation (Figure 1).

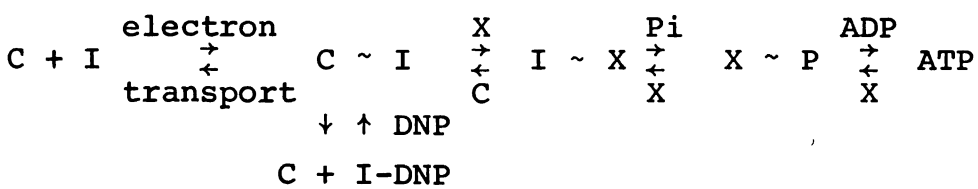


Figure 1. General reaction of DNP uncoupling effect in proposed scheme for mitochondrial energy transformation. ~ denotes a high energy bond. C is an electron carrier, either oxidized or reduced. I and X are energy carriers.

DNP is thought to react with the non-phosphorylated high energy intermediate C ~ I causing its decomposition. Oxygen continues to be used but without the formation of ATP.

The reversibility of the reaction sequence in Figure 1 suggests that in addition to decreasing the formation of ATP, DNP may also cause the breakdown of the ATP already formed. In this manner DNP increases the hydrolysis of ATP by mitochondrial DNP stimulated ATPase.

In addition to uncoupling oxidative phosphorylation DNP also stimulates cellular respiration. This effect of DNP is secondary to the decrease in the cellular levels of ATP (Peiss and Field, 1952).

Associated with an increase in respiration is an increase in the metabolism of glucose. Terner (1952) demonstrated that DNP increases the utilization of glucose leading to an accumulation of lactic acid in the brain and retina. Therefore, DNP may stimulate glucose metabolism to a degree where the accumulation of lactic acid may alter lens function. Indeed, this has been suggested by Horner (1942) to be the possible mechanism for the cataractogenic activity of DNP.

It is apparent that numerous questions about the mechanism whereby DNP induces cataracts remain unanswered. Does an ascorbic acid deficiency augment

the cataractogenic activity of DNP? Is the cataractogenic activity of DNP related to its effect on oxidative phosphorylation or to an ATPase effect? Is the effect of DNP on sugar metabolism pertinent to the cataractogenic activity?

The purpose of this thesis will be to answer the above questions in an attempt to elucidate the mechanism for the cataractogenic activity of DNP. The relevance of the work reported here is not in understanding the cataractogenic activity of DNP *per se*. Rather, the relevance is the increased understanding of alterations in lenticular function which may lead to opacification. However, closely related analogs of DNP possessing similar biological activity are currently being used extensively as herbicides and insecticides. Therefore, this work will also be useful in assessing the hazard associated with the use of these materials.

EXPERIMENTS

Experiment 1. The effect of ascorbic acid deficiency on the cataractogenic activity of DNP in guinea pigs.

1. Purpose

Ogino and Yasukura (1957) reported that scorbutic guinea pigs treated with DNP develop cataracts. However, Tainter and Borley (1938) previously reported that scorbutic guinea pigs treated with DNP did not develop cataracts. Since the production of cataracts in scorbutic guinea pigs may provide a significant lead for elucidating the mechanism whereby DNP may cause cataracts, the principle objective of this experiment was to verify the work of Ogino and Yasukura. A secondary objective was to characterize the cataract and compare it to those produced in immature rabbits, fowl and man. Ogino and Yasukura (1957) did not describe the cataract.

2. Methods

a. Animals

Multi-colored short haired guinea pigs were obtained from a colony maintained by the Department of Pathology, Michigan State University. Initially, 3 to 4 month old males weighing approximately 500 grams were

used. Because significant opacities did not develop, a second experiment using younger male guinea pigs was conducted. The latter group was approximately 4 weeks old and weighed 150 to 200 grams.

b. Housing of animals

Half of the adult guinea pigs were housed separately in stainless steel metabolism cages to facilitate the monitoring of food consumption. The other half of the adult animals were housed in groups of 3 to 5 in stainless steel rabbit cages. All caging had wire mesh floors. The immature guinea pigs were housed in groups of 4 to 5 in plastic cages containing ground corn cobbs as bedding. The room was maintained at a temperature of 74-78°F and was illuminated from 8:00 a.m. to 6:00 p.m. The guinea pigs were acclimated for a period of five days before initiating the experiment.

Vitamin C-deficient guinea pig powdered diet was obtained from Nutritional Biochemical Co., Cleveland, Ohio. All the guinea pigs received this diet throughout the study. Ascorbic acid, 10 mg/kg, was administered daily to controls using a stomach tube. Food and water was provided *ad libitum*.

c. DNP preparation and administration

The DNP was purchased from Eastman Organic Chemicals, Rochester, New York. Infrared analysis indicated that the DNP was greater than 99% pure.

An aqueous solution of DNP, 600 mg percent, having a pH of 7.4 was administered at a dose of 30 mg/kg via a stomach tube daily at approximately 3:00 p.m. Preliminary studies indicated that this dose was not lethal, whereas 35 mg/kg produced 100 percent mortality in adult guinea pigs.

d. Experimental design

The guinea pigs were randomly divided into four groups. The groups were treated as follows:

Group I - Ascorbic acid, 10 mg/kg

Group II - Ascorbic acid, 10 mg/kg
DNP, 30 mg/kg

Group III - No treatment

Group IV - DNP, 30 mg/kg

Treatment was initiated on the same day the guinea pigs were placed on the ascorbic acid deficient diet. Body weight, food and water consumption, and physical condition of the animals were determined daily.

When guinea pigs weighing approximately 500 gm were used, the concentration of ascorbic acid in plasma was determined the day prior to treatment, 24 hours after treatment had been initiated and at weekly intervals thereafter. Blood samples were obtained by periorbital venous puncture. Ascorbic acid determinations were conducted according to the method of Pirie, 1965.

e. Ophthalmic examination

The lenses were examined with an ophthalmascope and a Zeiss photo-slit lamp. The lenticular opacities were subjectively graded, and photographs of significant lenticular changes were made.

3. Results and Discussion

Tables 1, 2 and 3 depict body weight changes, food consumption, and plasma ascorbic acid levels throughout the experiment in adult guinea pigs. The DNP treated and ascorbic acid deficient animals lost weight throughout the experiment. After 3 weeks, a depression in body weight was noted in the animals receiving vitamin C. This weight loss was attributed to a decrease in food intake (Table 2) due to oral lesions which had developed from the oral dosing technique. Plasma ascorbic acid concentration remained constant in the animals receiving vitamin C. In the animals not receiving vitamin C there was a gradual decrease in the plasma ascorbic acid concentration. DNP treatment did not alter ascorbic acid concentration.

Using a slit lamp for examination, all of the mature guinea pigs were found to have posterior suture line cataracts before the experiment was initiated. Morphologically, the cataract had the appearance of an elongated H (). The prominence of this lesion was attributed to gapping of the suture lines. The cortex

and nucleus were clear. The morphology and the severity of the cataract did not change in any of the experimental groups through the duration of the experiment. No other opacifications of the lens were detected.

Because Ogino and Yasukura (1957) had used immature guinea pigs, the possibility remained that cataracts may be produced in immature scorbutic guinea pigs treated with DNP. Therefore, a second experiment using immature guinea pigs was initiated. The results of this experiment are shown in Tables 4 and 5. Table 5 suggests that the administration of DNP hastened the development of cataracts in guinea pigs receiving ascorbic acid. After ten weeks of treatment, the same type of cataract was observed in guinea pigs which were not treated with DNP. The morphology of these cataracts was identical to those previously observed in the mature guinea pigs prior to treatment. Slit lamp photographs illustrating these cataracts are shown in Figures 2 and 3.

The results of this experiment support the conclusion of Tainter and Borley (1938) that an ascorbic acid deficiency does not predispose guinea pigs to the cataractogenic activity of DNP. However, DNP may hasten the development of what appears to be a natural occurring cataract in guinea pigs. Perhaps any form of stress would have this effect. Since a cause-effect relationship

was not established, no further attempts were made to study the cataractogenic activity of DNP in guinea pigs.

Table 1. Net change in mature guinea pigs gram body weight, as a function of the duration of treatment.[a]

Daily Treatment	Weeks 1	2	3	4
Ascorbic acid, 10 mg/kg	12.2±7.2 (10)[b]	12.9± 6.5 (10)	- 8.1±25.0 (8)	-26.7±29.1 (8)
Ascorbic acid, 10 mg/kg + DNP, 30 mg/kg	- 8.0±6.6 (10)	-18.0± 7.3 (8)	-42.1±19.4 (6)	-39.0±35.8 (6)
None	-32.0±8.0 (8)	-21.7± 8.0 (7)	-39.0±16.7 (5)	-59.7±24.7 (5)
DNP, 30 mg/kg	4.0±7.0 (6)	- 9.2±13.3 (5)	-24.7±24.4 (4)	-50.2±27.1 (4)

[a]The guinea pigs used in this experiment had a mean body weight of 490±75 grams.

[b]Mean ± standard error (observations).

Table 2. Food consumption of mature guinea pigs maintained on a scorbutic diet and treated as indicated for 4 weeks.

Treatment	$\bar{X}$ daily food consumption in grams per group			
	Weeks			
	1	2	3	4
Ascorbic acid, 10 mg/kg	85±3[a]	100±4	92±4	80±3
Ascorbic acid, 10 mg/kg + DNP, 30 mg/kg	75±4	70±5	62±5	60±4
No Treatment	76±4	83±2	74±6	72±3
DNP, 30 mg/kg	62±4	71±5	63±4	60±2

[a]Mean ± standard error

Table 3. Concentration of ascorbic acid, mg/100 ml, in plasma of mature guinea pigs as a function of the duration of treatment.

Daily Treatment	n	Pretreatment	Treatment time 24 hr	1 week	2 weeks	4 weeks
Ascorbic acid, 10 mg/kg	5	3.2±.2[a]	3.5±.1	2.7±.1	3.6±.1	3.4±.1
Ascorbic acid, 10 mg/kg + DNP, 30 mg/kg	5	3.3±.2	3.2±.1	2.7±.1	3.3±.1	3.5±.1
None	5	3.4±.3	3.1±.3	2.1±.1	2.2±.1	1.4±.1
DNP, 30 mg/kg	5	3.7±.1	3.3±.2	1.9±.1	1.9±.1	1.0±.1

[a]Mean ± standard error

Table 4. Net change in immature guinea pigs gram body weight, as a function of the duration of treatment.[a]

Daily treatment	Time, weeks 1	2	3	4
Ascorbic acid, 10 mg/kg	52.7±2.3 (3)[b]	73.6±1.8 (3)	107.3± 3.7 (3)	170.3± 3.2 (3)
Ascorbic acid, 10 mg/kg + DNP, 30 mg/kg	51.0±4.6 (3)	78.7±4.7 (3)	107 ± 8.5 (3)	165.7±12.2 (3)
None	32.0±4.7 (3)	60.0±3.6 (3)	47.3±18.7 (3)	5.5± .5 (2)
DNP, 30 mg/kg	36.3±3.2 (3)	46.7±2.7 (3)	60.3± 7.3 (3)	All dead

[a]The guinea pigs used in this experiment had a mean body weight of 180±35 grams.

[b]Mean ± standard error (observations)

Table 5. Incidence of cataracts in immature guinea pigs maintained on a scorbutic diet and treated as indicated for 4 weeks.

Daily treatment	No. with cataracts per no. treated
Ascorbic acid, 10 mg/kg	0/10[a]
Ascorbic acid, 10 mg/kg + DNP, 30 mg/kg	5/10[a]
None	4/4
DNP, 30 mg/kg	4/4

[a]After 10 weeks of treatment, all of the guinea pigs in these groups exhibited cataracts.

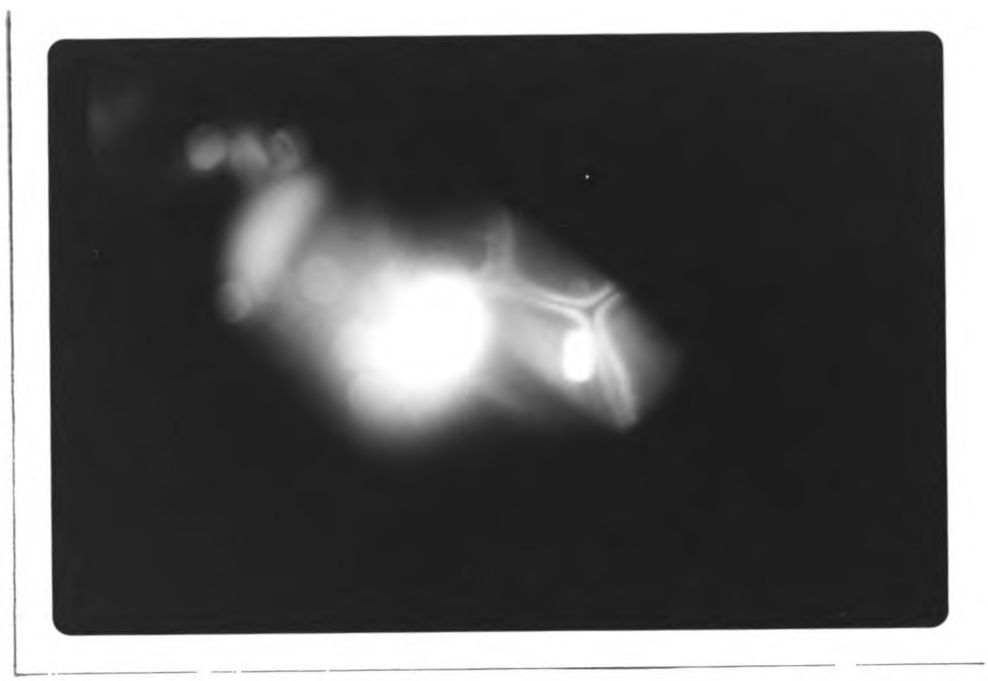


Figure 2. Slit lamp photograph of a typical cataract observed in adult guinea pigs.

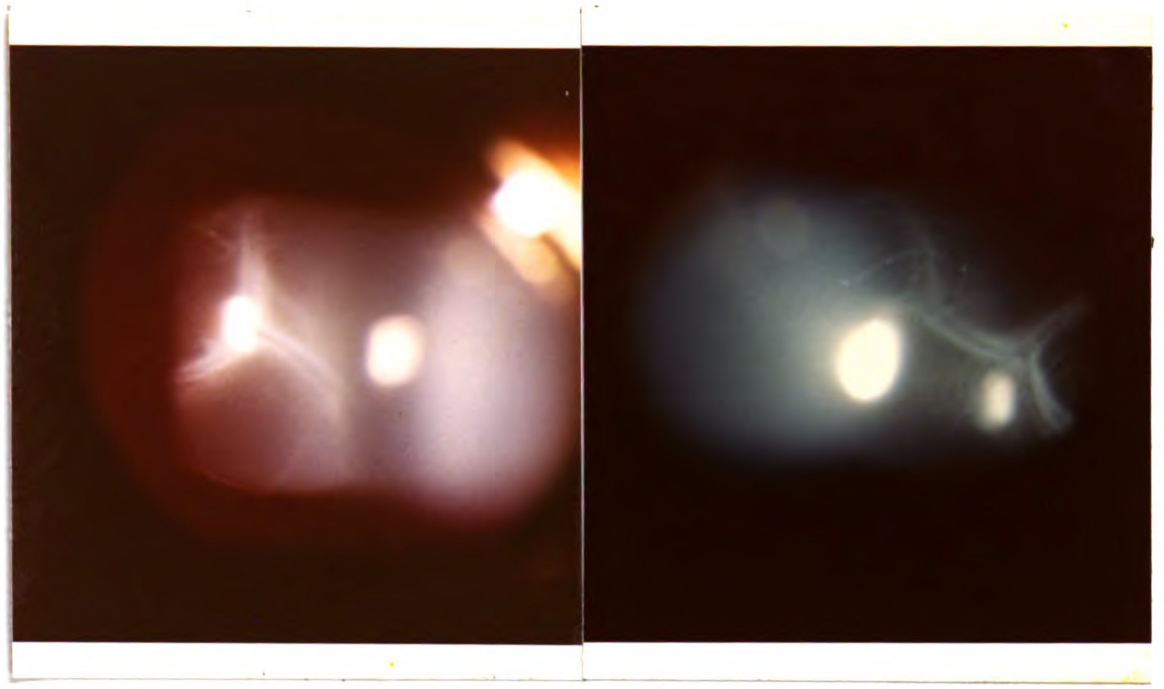
Figure 3. Slit lamp photographs of cataracts observed in immature guinea pigs treated as follows:

a Ascorbic acid, 10 mg/kg

b Ascorbic acid, 10 mg/kg
+
DNP, 30 mg/kg

c No Treatment

d DNP, 30 mg/kg



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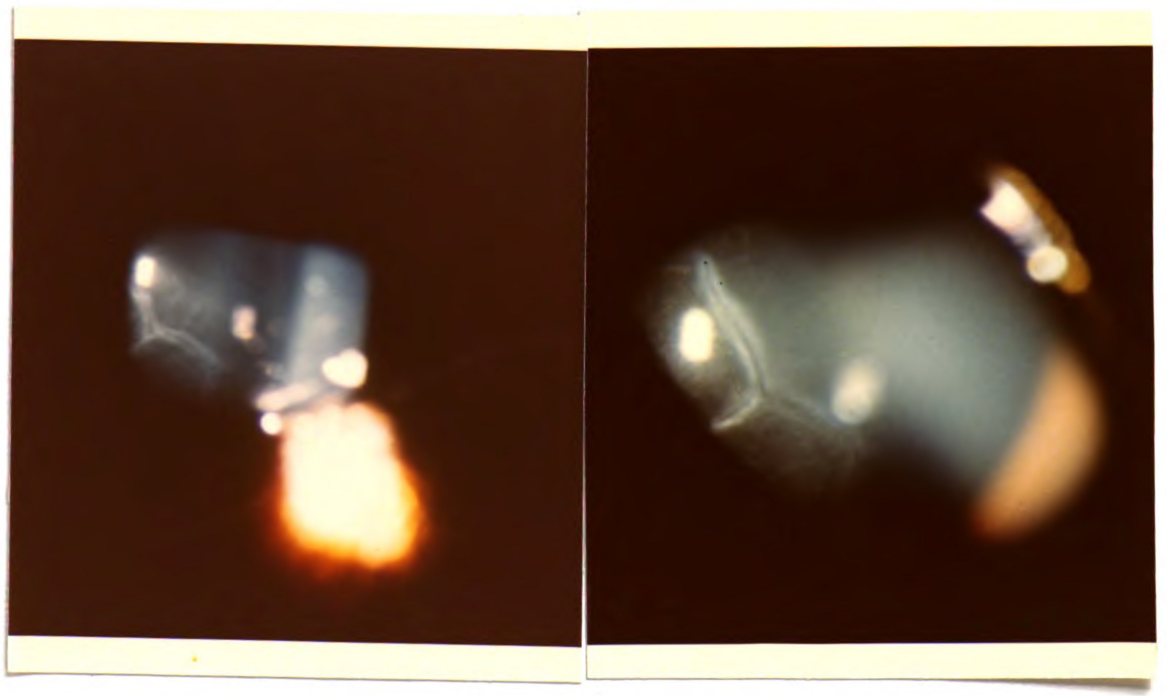

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Experiment 2. The effect of DNP and some of the known metabolites of DNP on the transparency of the rabbit lenses incubated *in vitro*.

1. Purpose

The cataractogenic activity of DNP, 2-amino,4-nitrophenol (2A4NP), 4-amino,2-nitrophenol (4A2NP), and 2,4 diaminophenol (DAP) was determined *in vitro* using rabbit lenses in an effort to dissociate the cataractogenic activity of DNP from known metabolites of DNP. A second objective was to characterize the concentration-response of DNP as well as of the other agents if they were found to be cataractogenic.

2. Methods

a. Animals, housing and feed

New Zealand white rabbits purchased from Johnson's Rabbitry, Coldwater, Michigan were used. The rabbits were 45±5 days old and weighed 700 to 1100 grams. They were housed in standard stainless steel rabbit cages with wire mesh flooring. Rabbit chow (Ralston Purina, St. Louis, Mo.) and water were provided *ad libitum*.

b. Chemical materials

2,4-Dinitrophenol, and 2-amino,4-nitrophenol were purchased from Eastman Organic Chemicals, Rochester, New York. 4-Amino,2-nitrophenol was purchased from Aldrich Chemical Co., Milwaukee, Wis. 2,4-Diaminophenol was purchased from K & K Laboratories, Inc., Plainview, N. Y.

c. *In vitro* procedures

1. Lens preparation

The rabbits were sacrificed by cervical dislocation. Both eyes were enucleated immediately and rinsed in 70% ethyl alcohol and sterile isotonic saline. The eyes were then placed in standard sterile bacteriological culture dishes.

To remove the lens, the eye was placed on the cornea with the posterior surface of the eye facing upward. After inserting the point of a scissors through the sclera, the sclera was incised such that four flaps of sclera could be reflected forward exposing the vitreous. The vitreous was then removed with a forceps exposing the lens. The zonules were excised with iris scissors, and the lens was removed with a lens loop. After weighing, the lens was placed in the incubation vial. All the instruments were sterilized by placing them in 70% ethyl alcohol. Before use, they were rinsed in sterile isotonic saline. One lens from each animal was placed in control medium and the other in medium containing the agent.

2. Incubation system and procedure

The lenses were incubated in vials like that shown in Figure 4. The vial is a modification of that used by Lambert and Kinoshita (1967). The glass stage on which the lens is placed can be lifted from the vial

Figure 4. Lens incubation vial.

1. and 3. Gas inlet
2. Gas outlet.
3. Glass lens stage

for examination. Prior to use, the gas inlets and outlets are plugged with cotton and the vials are steam sterilized.

A Dubnoff Metabolic Shaking Incubator was used for incubation. The incubation temperature was 37°C and the shaking speed was approximately 60 cycles per minute. The system was continuously gassed with a mixture of 7% oxygen, 5% carbon dioxide and 88% nitrogen (Ohio Chemical Co., Cleveland, Ohio). Using a gas wash bottle, the gas was filtered through sintered glass and bubbled through water. After scrubbing, the gas was conveyed by tygon tubing to a manifold having an internal diameter of 2 mm and 14 outlets. From the outlets of the manifold, the gas was conveyed to the incubation vials through a 30 gauge needle and tygon tubing. The 30 gauge needle and manifold maintained a sufficient head of pressure to allow the incubation vials to be evenly gassed at flow rates less than 10 ml/min. Thus, the flow of gas to all incubation vials could be controlled by a single valve at the outlet of the gas tank.

The culture medium was KEI-4 (Kinsey and Reddy, 1965). The medium composition, shown in Table 6, is a modification of medium previously described by Wachtl and Kinsey (1958). Four liters of the medium was made at a time. The medium was sterilized by filtration through a 150 ml 0.22 micron disposable bacteriological

Table 6. KEI-4 Media. Quantities for 4 liters.

Inorganic Dissolve in 1920 ml		Amino Acids in 1000 ml			Organic & labile compounds 1000 ml	
Compd.	mg.	A.A	mg.	mM	Compd.	mg.
Nacl	24,560	l-Alanine	92	.26	Glucose	5000
$NaHCO_3$	11,920	l-Arginine.Hcl	200	.24	creatine	116
$Na_2HPO_4.7H$ 0	752	l-Aspartic	28	.05	$(NH_4)_2SO_4$	8
$Zncl_2$	0.16	l-Cysteine.Hcl	160	.25	Biotin	0.4
Na Citrate	28	l-glutamic	168	.29	Pyridoxine.Hcl	4.8
Na_2CO_3	1264	l-Histidine.Hcl	76	.10	Ca pantothenate	4.0
$FeSO_4.7H_20$	10	l-isoleucine	44	.08	Niacin	4.0
KI	1.2	l-leucine	56	.11	Folic acid	0.4
$ZnSO_4.H_20$	4.0	l-lysine.Hcl	172	.23	Cytochrome c	1.2
H_2PO_4	32.0	l-Methionine	24	.04	Vitamin B_{12}	0.012
		l-phenyl alanine	68	.10	p-amino benzoic acid	0.80
		l-proline	64	.14		
		l-Serine	150	.36	Choline.cl	400
		l-threonine	40	.09	Thiamine.Hcl	4.0
		l-tyrosine	68	.09	Riboflavin	4.0
		l-tryptophan	28	.03	ATP.Na Salt Rt	16

Dissolve $Cacl_2.2H_20$ & $Mgcl_2.6H_20$ separately in 80 ml water & add Phenol Red

Table 6 (cont'd.)

Compd.	mg.	A.A	mg.	mM	Compd.	mg.
		l-valine	60	.13	Ascorbic Acid	150
$Cacl_2.2H_20$	816 (612) (Anhydrous)	l-glutamine	116	.20	Na.Pyruvate Rt	30
		l-hydroxyproline	308	.58	α-Tocepherol PO	4.8
$Mgcl_2.6H_20$	409	glycine	28	.09	Coenzyme.A.R	8.0
		l-ornithine.Hcl	68	.10	DPN	8.0
Phenol Red	80	l-asparagine.H_20	118	.17	TPN	8.0
					Succinic Acid	16.0
Sterilize the solution separately.		Dissolve the amino acids in 1 liter water and add 40,000 units penicillin 100 mg dihydrostreptomycin 0.2 mg N-butyl p-hydroxybenzoate. Sterilize by filtration through millipore filter.			Lactic Acid (3.7 ml) (85% soln)	
					K_2CO_3	130
No antibiotics added to inorganic salt solution. Sterilized in the autoclave.					Dissolve in liter of water and add 40,000 units penicillin 100 mg dihydrostreptomycin 0.2 mg N-butyl p-hydroxy-benzoate. Sterilize by filtration through millipore filter.	

filter unit purchased from Falcon Plastics, Los Angeles, California. After filtration, 100 ml portions of the medium were collected in sterilized glass screw top bottles and stored at -20°C. When needed, the medium was thawed, chemicals such as DNP were added and the medium was refiltered using another filter unit. Subsequently, the medium was stored in the filter unit.

Medium for incubation, 7 ml, was transferred from the filter unit to the incubation vials using a sterile syringe and needle. Before placing a lens in the incubation system, 45 min were allowed for the equilibration of temperature and dissolved gasses. After equilibration the pH of the medium was 7.4.

3. Evaluation of opacities

The design of the incubation vials allowed the lens to be periodically lifted from the vials and examined. For examination, the glass stage together with the lens it was supporting was trans-illuminated with a high intensity desk lamp. The opacities were graded subjectively. For photography, the lenses were removed and placed in sterile petri dishes containing sterile saline. The petri dish containing the lenses was placed over a white background with a series of parallel black lines. The white background effectively portrayed opacities and the parallel lines portray changes in the size of the lens. A Kowa Fundus camera (Keeler Optical

Supply, Philadelphia, Penn.) was used for photography. At the end of the experiment, the lenses were reweighed.

3. Results and Discussion

Table 7 depicts the incidence of cataract production by the incubation of rabbit lenses in media containing DNP, 2A4NP, 4A2NP, and DAP. Figure 5, 6 and 7 show pairs of lenses that were incubated in media containing the above compounds and control medium. DNP is the only compound possessing significant cataractogenic activity. Inclusion of 2×10^{-5} M DNP but not 1×10^{-5} M DNP in the media caused cataracts to develop.

The first detectable change in lenses incubated in media containing 1×10^{-4} M DNP is the formation of an opaque ring located at the equator. The opacity continues to develop by extending into the anterior subcapsular region and subsequently into the posterior subcapsular region. Within 4 hours, the entire anterior and posterior subcapsular region are a dense milky white. After 12 hours of incubation the opacity tends to disappear. A concentration of 2×10^{-5} M DNP causes a similar but less marked opacification of the lens which requires longer to develop.

From the results of this experiment, it is concluded that DNP rather than a metabolite of DNP is responsible for cataract formation. Furthermore, DNP is the only compound of this group that causes an

uncoupling of oxidative phosphorylation (Judah, 1951). Therefore, the results suggest that the cataractogenic activity and uncoupling activity of DNP are related. Another potent uncoupling agent, pentachlorophenol, causes the formation of the same type of cataracts at a concentration of 1×10^{-4} M in 8 hours, Figure 8.

Table 7. Incidence of cataracts production in rabbit lenses incubated in media containing DNP, 2A4NP, 4A2NP, or DAP for 24 hours at 37°C.

Treatment	Concentration	No. with cataracts per no. treated
Controls No treatment		0/32
DNP	1×10^{-4} M	10/10
	2×10^{-5} M	6/6
	1×10^{-5} M	0/4
2A4NP	1×10^{-3} M	0/4
4A2NP	1×10^{-3} M	0/4
DAP	1×10^{-3} M	0/4

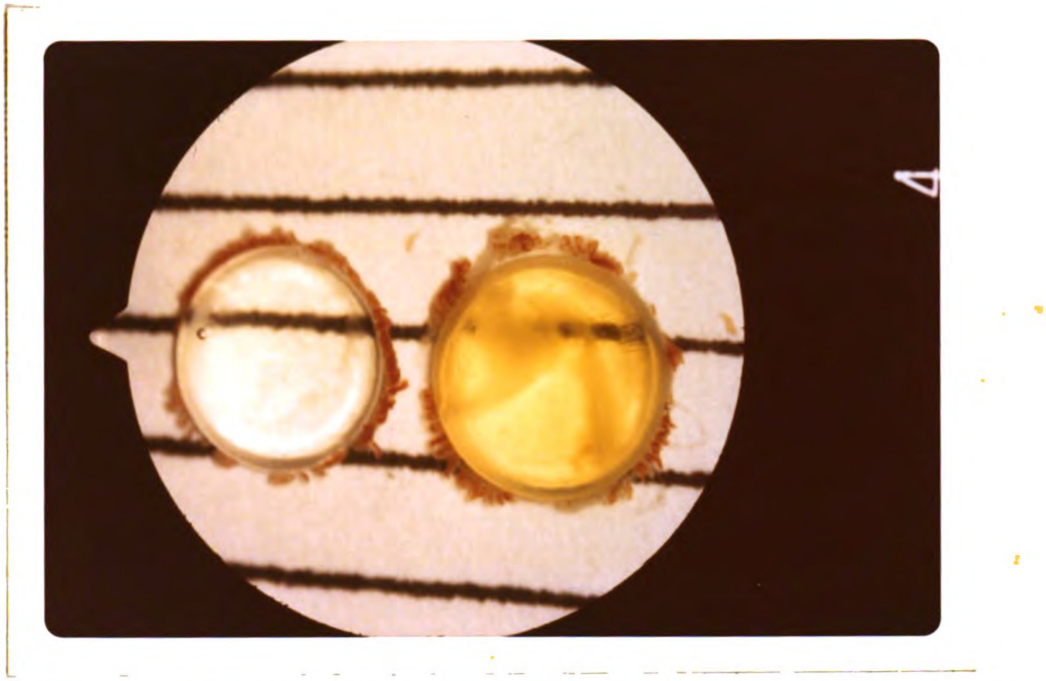


Figure 5. Paired lenses incubated 24 hours in control media (left) and media containing 1 x 10^{-4} M DNP (right).

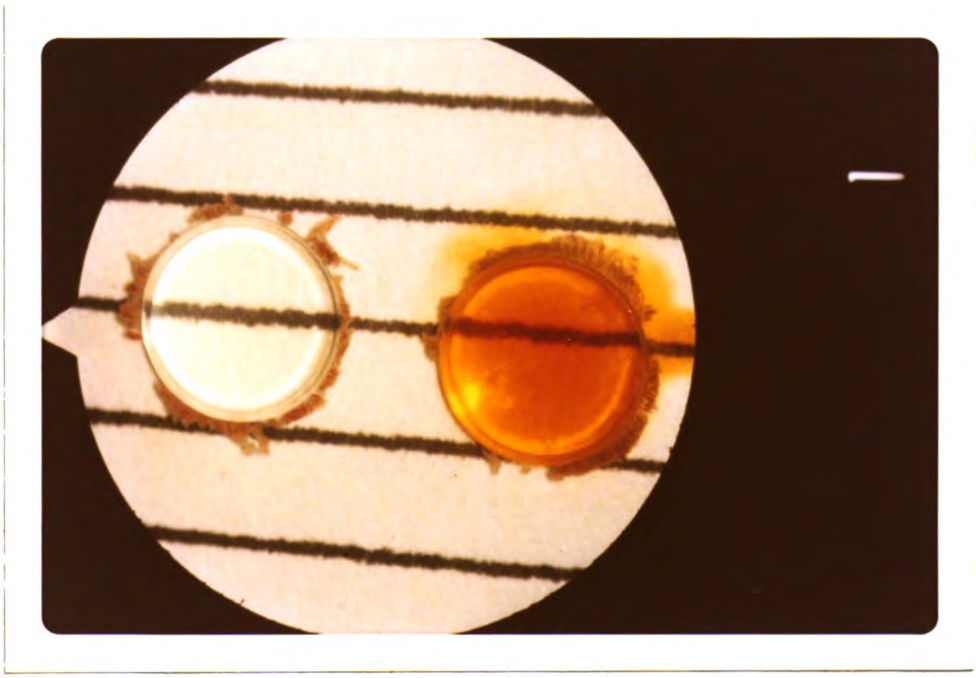


Figure 6. Paired lenses incubated 24 hours in control media (left) and media containing 1 x 10^{-3} M 2A4NP (right).

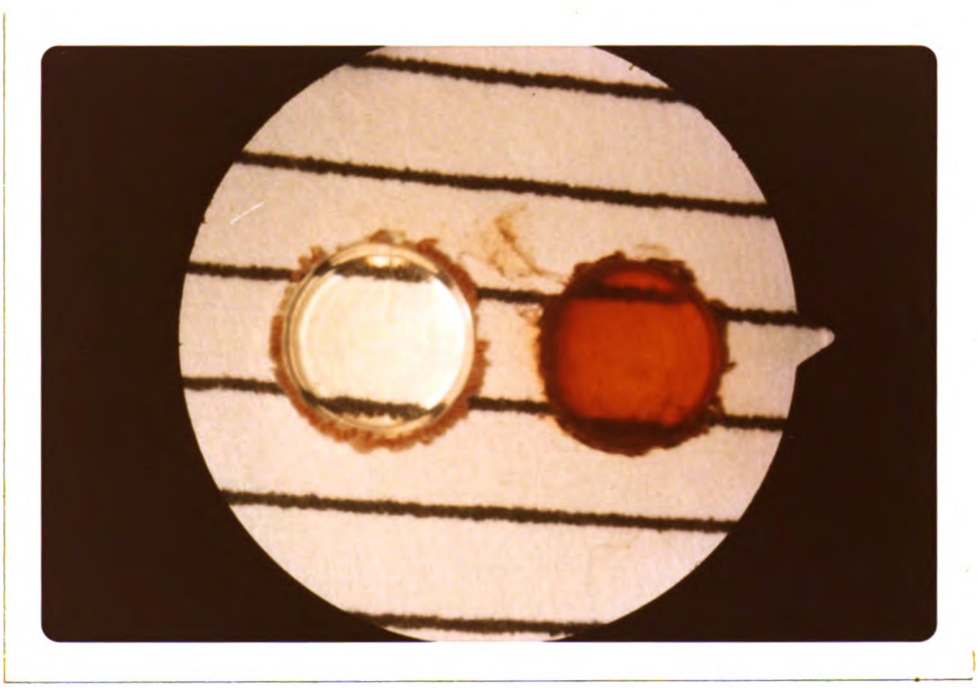


Figure 7. Paired lenses incubated 24 hours in control media (left) and media containing 1 x 10^{-3} M 4A2NP (right).

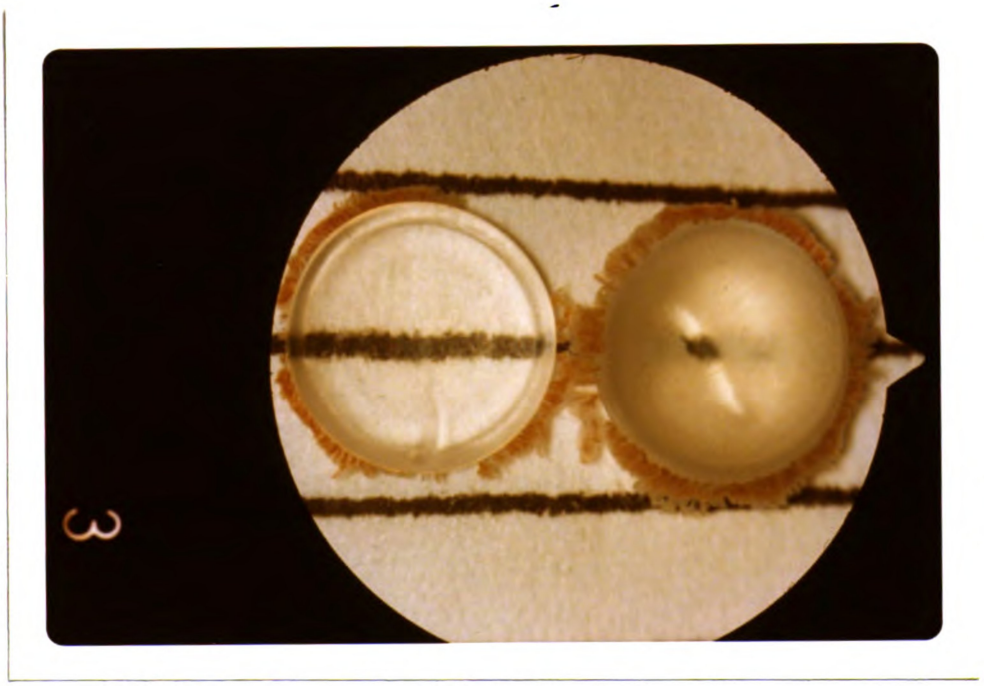


Figure 8. Paired lenses incubated 8 hours in control media (left) and media containing 1 x 10^{-4} M pentachlorophenol (right).

Experiment 3. Effect of temperature on the cataractogenic activity of DNP.

1. Purpose

As indicated in the introduction, DNP disrupts cellular metabolism which may induce opacification of the lens. If a disruption of cellular metabolism is responsible for the cataractogenic activity of DNP, an increase in the temperature of the incubation system should intensify opacification while a decrease in temperature should decrease opacification. Another possible cause of opacification is a disruption of the conformation of the lens protein it contains. Binding of DNP to the protein of the lens may induce sufficient disruption to cause opacification. It has been demonstrated that DNP is extensively bound to serum protein (Gehring and Buerge, 1969a). van der Waal forces which are generally associated with protein binding are weakened by increasing temperature. Thus, if this type of interaction is responsible for the cataractogenic activity of DNP, decreasing the temperature of the incubation should increase opacification while elevating temperature should decrease opacification. Therefore, the objective of this experiment was to characterize the effect of temperature on DNP-induced opacification of the lens.

2. Methods

The methods used in this experiment were similar to those described for experiment 2. The concentration of DNP was 1×10^{-4} M. Each lens of a lens pair was incubated at a different temperature and the degree of opacification was compared. In addition, the time at which opacification was first observed was compared. Incubation temperatures were 30°C and 37°C, 34°C and 40°C, and 32°C and 42°C.

3. Results and Discussion

Table 8 and Figure 8 illustrate the effect of increasing the incubation temperature on the cataractogenic activity of 1×10^{-4} M DNP. As the incubation temperature is increased, opacification of the lens occurs sooner and it is more severe. At 30°C, Figure 10, 1×10^{-4} M DNP did not cause an opacity to develop.

It is concluded that the cataractogenic activity of DNP exhibits a positive temperature coefficient. This suggests the cataractogenic activity of DNP is caused by an untoward effect on the metabolism of the lens rather than an alteration in the conformation of lens protein induced by the binding of DNP.

Table 8. Effect of increasing temperature on the cataractogenic activity of 1 x 10^{-4} M DNP.

Temperature	No. cataracts per no treated	Degree of opacification as a function of time[a, b]				
		1 hr	2 hrs	6 hrs	12 hrs	24 hrs
30°C	0/3	0	0	0	0	0
32°C	4/4	0	1	2	1	0
34°C	2/2	0	1	3	3	1
37°C	4/4	1	2	5	3	3
40°C	2/2	1	3	5	3	3
42°C	4/4	2	4	5	4	3

[a]The degree of opacification was graded subjectively from 0 to 5 as follows:
- 0 - no opacification
- 1 - opacification limited to equator
- 2 - extension into anterior and posterior subcapsular regions of equatorial opacity
- 3 - complete opaqueness of 1/3 of the anterior subcapsular region
- 4 - complete opaqueness of the anterior and posterior subcapsular region, with only the axial region from the anterior pole to posterior pole remaining clear
- 5 - complete opaqueness of the anterior and posterior subcapsular regions of the entire lens

[b]Grade equals mean of number treated rounded to nearest whole number

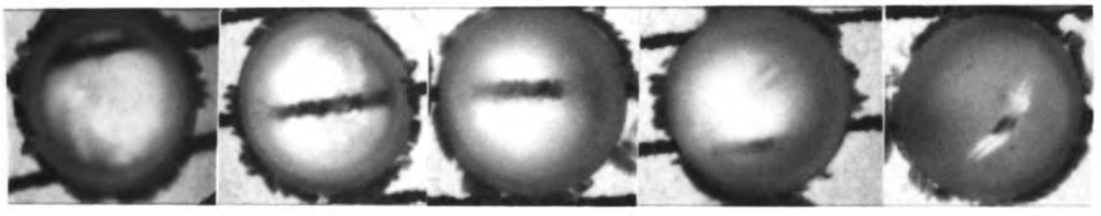


Figure 9. Effect of increasing temperature on rabbit lenses incubated for 2 hours in media containing 1×10^{-4} M DNP.

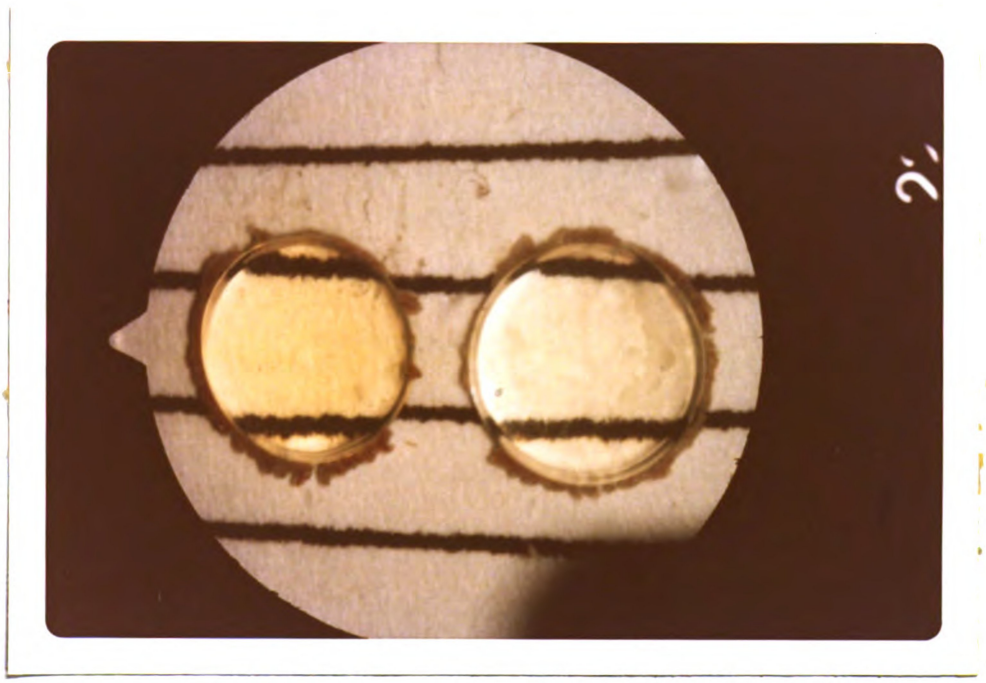


Figure 10. Paired lenses incubated at 30°C for 2 hours. Right, control; left 1 x 10^{-4} M DNP treated.

Experiment 4. Effect of DNP on the production of $^{14}CO_2$ from glucose-6-^{14}C by lenses.

1. Purpose

Numerous studies have demonstrated the uncoupling effect of DNP on oxidative phosphorylation in tissues. Associated with this effect is an increased utilization of oxygen together with an increased metabolism of glucose via the Krebs cycle. The production of $^{14}CO_2$ from glucose-6-^{14}C can be used to monitor the metabolism of glucose by the Krebs cycle as well as the utilization of oxygen. The purpose of this experiment was to determine the effect of DNP on oxygen utilization and the Krebs cycle metabolism of glucose.

2. Methods

With the exception of the tissue culture medium, the procedure for incubation of rabbit lenses was as previously described in Experiment 2. In this experiment, TC 199 medium (Grand Island Biological Co., Grand Island, N. Y.) to which sufficient sodium bicarbonate had been added to give a final bicarbonate concentration of 29 mM was used. This medium is similar to the KEI-4 medium previously described; however, it does not contain sodium lactate.

One microcurie of glucose-6-^{14}C (New England Nuclear, Boston, Mass.) was added to the system giving a

specific activity of 7.036 mg glucose per microcurie. One lens from each rabbit was placed in control medium while the other lens was put in medium containing 1×10^{-4} M DNP.

For the collection of $^{14}CO_2$, the exhausted gas was conveyed from the exhaust outlet of each incubation vial through tygon tubing attached to a 20 gauge needle. The $^{14}CO_2$ was collected by bubbling the gas through 5 ml of hydroxide of hyamine 10-X (Packard Instrument Co., Downer Grove, Ill.) in a test tube. At 2, 6, 12 and 24 hours after initiating the incubation, a 1 ml aliquot of the hydroxide of hyamine containing the $^{14}CO_2$ was removed from the trap and placed in a scintillation vial. One milliliter of hydroxide of hyamine was added to the trap to replace that taken out. Scintillation fluid (toluene containing 6 gm 2,5-diphenyloxazole (PPO) and 0.2 gm P-bis[2-(5-phenyloxazolyl)]benzene (POPOP) per liter) was added to the scintillation vials and the ^{14}C activity determined with a Beckman model 100-A scintillation counter. After correction for quench and dilution, the amount of glucose metabolized per gm lens was determined.

No ^{14}C activity was detected in a second trap containing hydroxide of hyamine; therefore, the CO_2 was adequately scrubbed from the exhausted gas by the first trap.

3. Results and Discussion

The effect of 1×10^{-4} M DNP on the Krebs cycle metabolism of glucose to carbon dioxide by rabbit lenses is shown in Table 9. After six hours of incubation, lenses incubated in medium containing DNP metabolized significantly more glucose to carbon dioxide than lenses incubated in control medium. The magnitude of this difference increased as the time of incubation increased. It is concluded that DNP increases the utilization of oxygen as well as increases that metabolism of glucose normally being handled by the citric acid cycle in rabbit lenses.

Table 9. Krebs cycle oxidation of glucose to CO_2 by lenses incubated in TC 199 medium (control) and TC 199 medium containing 1 x 10^{-4} M DNP (treated) as a function of time.[a]

Time	Glucose metabolized, µgm/gm lens	
	Control	Treated
2 hr	2.29±1.08 (4)[b]	2.58± 0.56 (4)
6 hr	8.39±2.40 (4)	13.54± 1.02 (4)[c]
12 hr	21.27±3.52 (4)	43.34± 6.55 (4)[d]
24 hr	55.15±8.69 (4)	126.90±17.98 (4)[e]

[a] The production of $^{14}CO_2$ by the metabolism of glucose-6-^{14}C was used to estimate the Krebs cycle oxidation of glucose.

[b] Mean ± standard error (observations)

[c] $P<0.02$

[d] $P<0.01$

[e] $P<0.001$

Experiment 5. *In vitro* effects of oxygen deprivation on the maintenance of the clarity of lenses incubated in control medium and medium containing 1×10^{-4} M DNP.

1. Purpose

Kinoshita (1965) reported that the transparency of lenses can be maintained *in vitro* in the absence of oxygen. If the cataractogenic activity of DNP is caused by its effect on oxidative phosphorylation, the deprivation of oxygen should augment the cataractogenic activity of DNP. Indeed, the absence of oxygen itself should cause opacification of the lens. Therefore, the purpose of this experiment was to verify that the lens can maintain its clarity in an anaerobic environment and to determine whether the effect of DNP on the lens is augmented in the absence of oxygen.

2. Methods

With the exception of the gas mixture, the incubation of lenses was conducted as previously described in Experiment 2. The gas mixture containing 95% N and 5% CO_2 was purchased from the Ohio Chemical Co., Cleveland, Ohio. Analysis of the gas mixture by the supplier showed that it contained less than 0.01% oxygen. The lack of Krebs cycle activity in lenses incubated in this environment was demonstrated by trapping the $^{14}CO_2$ in the

exhaust of incubation systems to which glucose-6-^{14}C had been added to the medium. The procedures for this part of the experiment were described in Experiment 4.

In these experiments, one lens from each rabbit was incubated in KEI-4 medium and the other lens in KEI-4 medium containing 1 x 10^{-4} M DNP. Opacification of the lens was subjectively graded and photographed when indicated.

3. Results and Discussion

Oxygen deprivation markedly depressed the production of $^{14}CO_2$ by rabbit lens incubated in medium containing glucose-6-^{14}C, Table 10. Thus, the production of ATP associated with the utilization of oxygen is approximately 10 percent of aerobic conditions.

Table 11 depicts the effect of oxygen deprivation on the cataractogenic activity of DNP. Lenses incubated in control medium maintained their clarity equally in an aerobic and anaerobic environment. Opacification of lenses incubated in medium containing 1 x 10^{-4} M DNP was not augmented by oxygen deprivation, Figure 11.

It is concluded that at least 90 percent of normal lens oxidative phosphorylation is not required to maintain the normal transparency of the lens. Therefore, an uncoupling of the oxidative phosphorylation of the lens by DNP must not be associated with cataract induction. This conclusion is strengthened by the observation that

the cataractogenic activity of DNP is not augmented by severe oxygen deprivation.

Table 10. Citric acid cycle oxidation of glucose to CO_2 by lenses incubated in TC 199 medium under aerobic and anaerobic conditions.[a]

	Glucose metabolized µgm/gm lens at 12 hours
Aerobic	21.49±2.81 (4)[b]
Anaerobic	1.91± .55 (4)[c]

[a]The production of $^{14}CO_2$ by the metabolism of glucose-6-^{14}C was used to estimate the citric acid cycle oxidation of glucose. Anaerobic incubation systems were gassed with 95% N_2 and 5% CO_2. Aerobic incubation systems were gassed with 88% N_2, 5% CO_2 and 7% oxygen.

[b]Mean ± standard error (observations)

[c]$P<0.001$

Table 11. Effect of oxygen deprivation on the cataractogenic activity of 1×10^{-4} M DNP.

Environment[a]	No with cataracts / No. treated	Degree of opacification as a function of time[b] 2 hr	6 hr	12 hr	24 hr
Aerobic	10/10	2	5	3	3
Anaerobic	5/5	2	5	3	3

[a]Aerobic: 88% N_2, 7% O_2 and 5% CO_2
Anaerobic: 95% O_2 and 5% CO_2, less than 0.01% O_2

[b]Normal lens 0, totally opaque lens 5, see Table 8 for explanation.

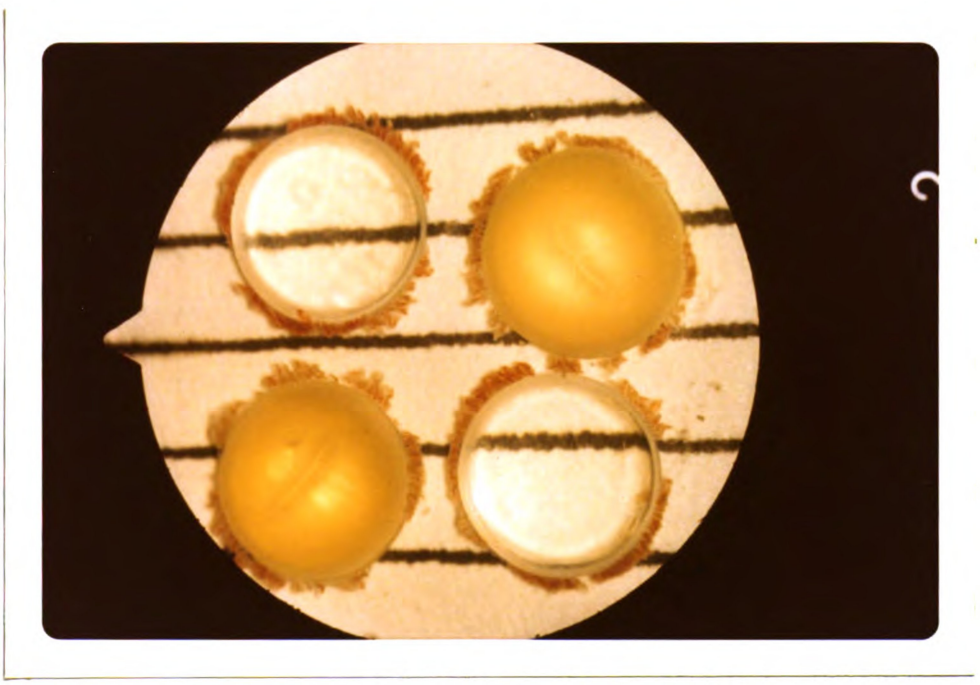


Figure 11. Left upper - control medium, aerobic.
Left lower - 1 x 10^{-4} M DNP, aerobic.
Right upper - 1 x 10^{-4} M DNP, anaerobic.
Right lower - control medium, anaerobic.

Experiment 6. The effect of DNP on the lactic acid production by the lens.

1. Purpose

In preceding experiments, it has been demonstrated that the cataractogenic activity of DNP is not associated with its effect on aerobic oxidative metabolism. This is not surprising, because Kinoshita (1965) has demonstrated that the lens can maintain its ATP-requiring processes from the ATP formed by substrate phosphorylation in the Embden-Meyerhof glycolytic pathway. The purpose of this experiment was to determine whether DNP either stimulates or depresses the Embden-Meyerhof glycolytic pathway. The effect of DNP on this pathway was monitored by measuring lactic acid production. If DNP depresses ATP levels in the lens, it can be rationalized that this pathway should be stimulated leading to an increased production of lactic acid. A decreased lactic acid production would occur if DNP inhibits one or more of the enzymes of this pathway. It has been reported that high concentrations of DNP inhibit hexokinase (Grillo and Cafiero, 1964).

A secondary purpose was to determine whether the production of excessive amounts of lactic acid may be responsible for cataract development. This possibility was previously suggested by Horner (1942).

2. Methods

With the exception of the tissue culture medium, the materials and methods used in this experiment were as previously described in Experiment 2. The tissue culture media used for this experiment was TC 199 (Grand Island Biologics, Grand Island, N. Y.) to which sufficient sodium bicarbonate was added to give a final concentration of 29 mM bicarbonate. This medium does not contain lactic acid. In order to prevent the escape of the end products of glycolysis, pyruvate and lactate, the incubations were conducted anaerobically (see Experiment 5). The duration of incubation was 6 hours. One lens from each rabbit was incubated in control medium while the other was incubated in medium containing 1×10^{-4} M DNP.

At the end of the incubation, the lens was removed, rolled on filter paper to remove adherent medium, weighed and placed in a 15 ml glass test tube. Boiling water, 3 ml, was added to the test tube and the lens was homogenized with a teflon grinder. After centrifugation, the supernatant was collected and analyzed colorimetrically for lactic acid by the method of Hullin and Noble (1953). The concentration of lactic acid in the incubation medium was determined without special preparation.

3. Results and Discussion

The concentration of lactic acid in the lenses and in the media in which they had been incubated for six hours are shown in Table 12. The concentration of lactic acid in both the medium and in the lens was not increased by 1×10^{-4} M DNP. Therefore it is concluded that DNP under anaerobic conditions does not stimulate or depress the Embden-Meyerhof glycolytic pathway sufficiently to alter the production of lactic acid.

Table 12. Effect of 1×10^{-4} M DNP on the concentration of lactic acid in lenses and in the media in which they were incubated anaerobically for 6 hours.

Medium[a]	Lactic Acid	
	Lens µg/gm	Medium µg[b]
Control	145.45±8.1 (4)[c]	150.70±2.98 (9)
1×10^{-4} M DNP	141.24±6.8 (4)	148.10±5.05 (9)

[a]TC 199 medium with a final bicarbonate concentration of 29 mM.

[b]Total amount of lactic acid found in the incubation medium, 7 ml.

[c]Mean ± standard error (observations)

Experiment 7. The effect of DNP on the ATP levels in the lens.

1. Purpose

The production of lactic acid by the lens is not altered by DNP in an anaerobic environment. Since the formation of ATP in the Embden-Meyerhof glycolytic pathway occurs by substrate phosphorylation, DNP should not depress the formation of ATP. Therefore, the purpose of this experiment was to determine if DNP depresses ATP concentrations in the lens. A depression of the level of ATP would suggest that DNP increases the hydrolysis of ATP.

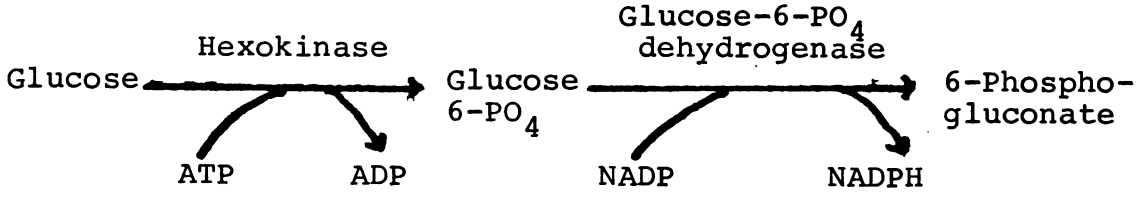
2. Methods

The materials and methods used for incubation were identical to those described for Experiment 6. At the end of the six hours incubation, the lenses were removed from the system, placed immediately in liquid nitrogen and stored at -70°C until ATP determinations were made.

For ATP determination, the lens was powdered in a mortar chilled with liquid nitrogen. Approximately 100 mg (±15 mg) of the powdered lens was transferred to a 15 ml nalgene test tube containing 0.25 ml of 0.6 M $HClO_4$ frozen in the bottom. After adding another 0.25 ml of 0.6 M $HClO_4$, the sample was homogenized using a pestle from a Dounce ball homogenizer. The homogenate was

centrifuged and 0.5 ml of supernate was collected and mixed with 0.25 ml of 2 M $KHCO_3$. After allowing sufficient time for CO_2 to be released, the sample was centrifuged and the supernate collected for ATP determination.

ATP determinations were made according to the enzymatic method of Lowry *et al.* (1964). In this method, ATP is estimated by the spectrophotometric appearance of NADPH. The relationship between ATP levels and the formation of NADPH is shown in the following reaction sequence.



The extract of the lens, together with the appropriate enzymes and substrates were placed in a 1 ml cuvette as described by Lowry *et al.* (1964). The reaction was carried out at room temperature for 5 min and monitored with a Gilford spectrophotometer.

The extinction coefficient for NADPH equalled 0.1608 µmoles/ml. All enzymes and reagents were purchased from Sigma Chemical Co., St. Louis, Mo.

3. Results and Discussion

Table 13 depicts the ATP levels in rabbit lenses incubated anaerobically for six hours in TC 199 medium

and in TC 199 medium containing 1×10^{-4} M DNP. It is apparent that DNP causes a statistically significant 20 percent decrease in the ATP content of lenses.

As previously indicated, DNP should not influence the formation of ATP in the Embden-Meyerhof glycolytic pathway. Therefore, the results of this experiment suggest that DNP decreases the ATP levels of the lens by increasing the hydrolysis of ATP.

Table 13. The concentration of ATP in rabbit lenses incubated anaerobically for six hours in TC 199 medium and TC 199 medium containing 1×10^{-4} M DNP.

Medium	Lens ATP, μmoles/gm
Control	1.33±.04 (8)[a]
1×10^{-4} M DNP	1.08±.03 (8)[b]

[a]Mean ± standard error (observations)

[b]$P<0.01$

Experiment 8. The effect of oligomycin on the cataractogenic activity of DNP.

1. Purpose

It has been demonstrated that DNP causes a depression in the ATP levels of lenses incubated in an anaerobic environment for 6 hours without depressing the activity of the Embden-Meyerhof glycolytic pathway. Since the formation of ATP by this pathway should not be affected by DNP, these results suggest that the utilization of ATP by the lens may be stimulated by DNP. One mechanism whereby DNP may increase the utilization of ATP is through the stimulation of mitochondrial ATPase (see Introduction). Oligomycin is known to prevent the hydrolysis of ATP by this mechanism (Lardy and McMurray, 1959). Therefore the purpose of this experiment was to determine whether oligomycin would inhibit the cataractogenic effect of DNP. An inhibition of cataract formation would indicate that the cataractogenic activity of DNP may be due to the nonfunctional consumption of ATP by mitochondrial ATPase.

2. Methods

The materials and methods used for the incubation of lenses were similar to those previously described in Experiment 2. In this experiment, one lens from each rabbit was incubated in KEI-4 medium and the other lens

was incubated in KEI-4 medium containing 5×10^{-5} M oligomycin. After two hours of incubation, a sufficient volume of 1×10^{-2} M DNP in KEI-4 medium was added to the incubation systems to give a final concentration of 1×10^{-4} M DNP. The lenses were periodically examined for opacification. The degree of opacification was graded subjectively. Photographs were obtained when the opacification of the lens treated with DNP but not oligomycin was most prominent.

3. Results and Discussion

The effect of oligomycin on the cataractogenic activity of 1×10^{-4} M DNP is shown in Table 14 and Figure 12. Cataract production was totally blocked by oligomycin. The result suggests that the cataractogenic activity of DNP is associated with a decrease in the ATP concentration of the lens, produced by the hydrolysis of ATP by mitochondrial ATPase.

Table 14. Effect of oligomycin on the cataractogenic activity of DNP in rabbit lenses incubated in KEI-4 medium.

Additions to medium	No. with cataracts per no. treated
1×10^{-4} M DNP	6/6
1×10^{-4} M DNP + 5×10^{-5} M Oligomycin	0/6

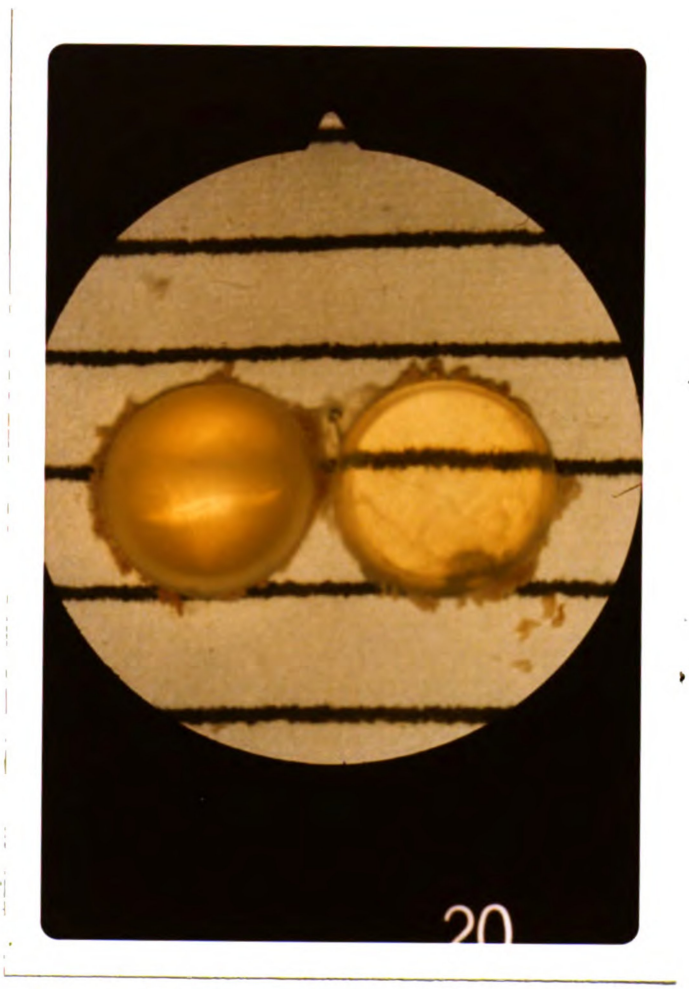


Figure 12. Paired rabbit lenses at 6 hours incubation. Left, DNP treated; right, DNP treated plus oligomycin.

Experiment 9. Effect of oligomycin on the ATP content of DNP treated lenses.

1. Purpose

It has been suggested that the protective effect of oligomycin against the cataractogenic activity of DNP is due to a blockade of mitochondrial ATPase. This blockade would reduce the non-functional hydrolysis of ATP; thereby, preserving it for those functions needed to maintain the clarity of the lens. The purpose of this experiment was to determine whether oligomycin does inhibit the decrease in the concentration of ATP in lenses treated with DNP.

2. Methods

The materials and metholds used for the incubation of lenses were the same as those described for Experiment 8. The incubations were stopped after 6 hours of incubation and the ATP content was determined as previously described, Experiment 7.

3. Results and Discussion

Table 15 depicts the ATP content of rabbit lenses incubated in medium containing 1×10^{-4} M DNP and in medium containing 5×10^{-5} M oligomycin together with 1×10^{-4} M DNP. The ATP content of lenses incubated in medium containing both oligomycin and DNP is significantly lower ($P<0.01$) than the ATP content of lenses

incubated in medium containing only DNP. Therefore, it is concluded that the protective effect of oligomycin against the cataractogenic activity of DNP is not associated with its inhibition of mitochondrial ATPase, and the preservation of lenticular ATP. These data also suggests that lens transparency can be maintained even when the ATP levels are relatively low.

Table 15. Effect of oligomycin on the ATP content of rabbit lenses incubated in medium containing DNP.

Addition to medium[a]	Lens ATP, μmoles/gm	No. with cataracts per no. treated
1×10^{-4} M DNP	1.01±.05 (5)[b]	5/5
5×10^{-5} M Oligomycin	.71±.04	0/3
1×10^{-4} M DNP + 5×10^{-5} M Oligomycin	0.62±.04 (5)[c]	0/5

[a] TC 199 medium containing 29 mM bicarbonate

[b] Mean ± standard error (observations)

[c] $P<0.01$

Experiment 10. The effect of ouabain on the cataractogenic activity of DNP.

1. Purpose

The results of the previous experiment negate the hypothesis that the protective action of oligomycin against the cataractogenic activity of DNP is due to an inhibition of mitochondrial ATPase. It has been demonstrated that oligomycin like the cardiac glycosides inhibits Na^+, K^+ activated ATPase (Järnefelt, 1962). The purpose of this experiment was to determine whether ouabain, a cardiac glycoside, inhibits the cataractogenic activity of DNP. This agent has not been reported to inhibit mitochondrial ATPase.

2. Methods

The materials and methods used in this experiment were identical to those described for Experiment 8 except ouabain 1×10^{-5} M, was used instead of oligomycin.

3. Results and Discussion

The effect of ouabain on the cataractogenic activity of DNP is shown in Table 16. Ouabain, 1×10^{-5} M totally protects the lens against the cataractogenic activity of 1×10^{-4} M DNP, Figure 13. These results support the previous conclusion that the inhibition of mitochondrial ATPase is not associated with the cataractogenic activity of DNP. Since both oligomycin and ouabain

inhibit Na, K activated ATPase, it is suggested that the cataractogenic activity of DNP may be associated with an alteration in the transport of cations and water.

Table 16. Effect of ouabain on the cataractogenic activity of DNP in rabbit lenses incubated in KEI-4 medium.

Addition to medium	No with cataracts per no. treated
1×10^{-4} M DNP	6/6
1×10^{-4} M DNP + 1×10^{-5} M Ouabain	0/6

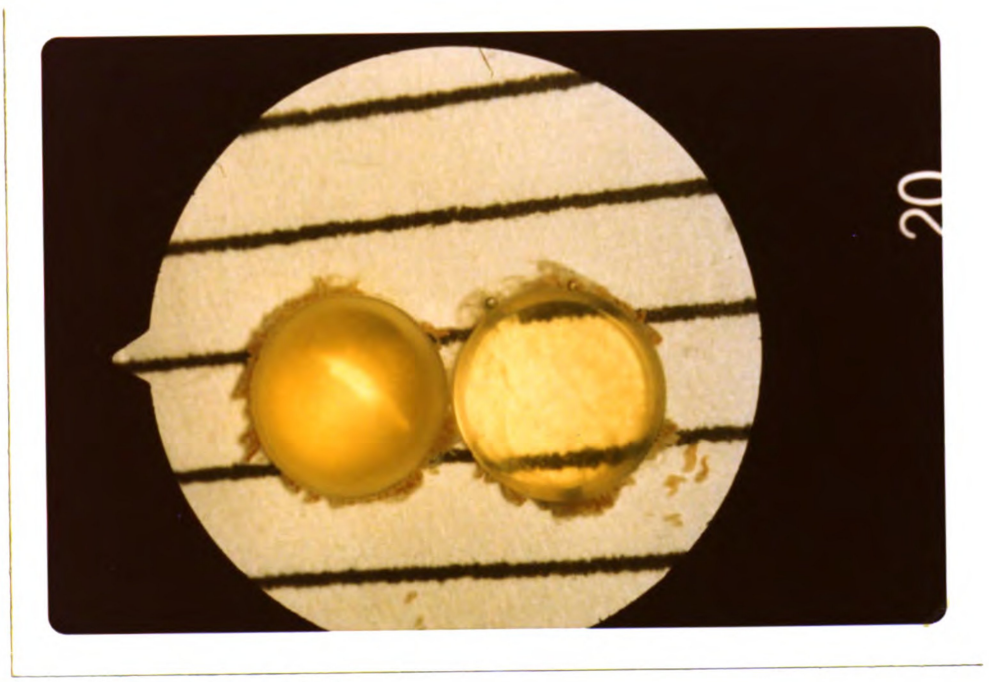


Figure 13. Paired rabbit lenses at 6 hours incubation. Left, DNP treated; right, DNP treated plus ouabain.

Experiment 11. Effect of DNP on the levels of sorbitol in the lens.

1. Purpose

The hexokinase activity of the lens is low compared to that of other tissues (Kinoshita, 1965). Therefore, the sorbitol (polyol) pathway of the lens may assume a prominent role in the metabolism of glucose. An alteration in the function of this pathway may be sufficiently detrimental to the lens to cause opacification. For example, a high concentration of glucose causes a build up of sorbitol which has been associated with cataract formation (van Heyningen, 1959). The early changes in lenses exposed to high concentrations of glucose are similar to those caused by DNP. Since hexokinase is inhibited by DNP (Grillo and Cafiero, 1964), it is conceivable that the exposure of the lens to DNP may cause glucose to be shunted through the sorbitol pathway, thus, causing a build up of sorbitol. Therefore, the purpose of this experiment was to determine whether DNP changes the concentration of sorbitol in the lens.

2. Methods

The procedure for lens incubation was similar to that described in Experiment 5. One lens from each pair was incubated in TC 199 medium, and the other in TC 199 medium containing 1×10^{-4} M DNP. After six hours of

incubation, the lens was removed from the incubation system, rolled on filter paper to remove adherent medium, weighed and placed in a 15 ml Nalgene test tube. Two ml of 0.3 N $Ba(OH)_2$-6 percent $ZnSO_4$ was added to the test tube and the lens was homogenized with a teflon grinder. After centrifugation, the supernate was placed in another test tube and dried by flushing the tube with a stream of N_2. The dried extract was reacted with 100 µl of solution consisting of 15 parts pyridine, 4 parts hexamethyldisilazane, and 1 part trimethylchlorosilane (TMS reagent).

A Beckman Model GC-5 gas chromatograph equipped with a hydrogen flame detector and a six foot glass column packed with 11 percent OV-17 on 60 to 80 mesh Gas Chrom Q (Applied Science Laboratories, College Park, Penn.) was used to determine the silylated derivative of sorbitol. The chromatographic parameters were as follows: carrier gas-helium, flow rate 120 ml/min; purge-air, flow rate 250-300 ml/min; hydrogen gas-flow rate 55 ml/min; injection port temperature-190°C; column temperature-170°C; detector temperature-225°C. The volume of sample for injection was 2 µl.

Standards were prepared by reacting known amounts of d-sorbitol with TMS reagent. Using the peak heights produced by these standards, a standard curve was prepared

from which the concentration of sorbitol in unknown samples was determined. When known amounts of sorbitol were added to lens homogenates, 50 percent of the added sorbitol was recovered. Therefore, the sorbitol content of unknown samples was assumed to be 2 times that found using the analytical procedure described above.

3. Results and Discussion

Inclusion of 1×10^{-4} M DNP in the incubation medium does not change the concentration of sorbitol in the lens after six hours of incubation (Table 17). Since the cataract is already prominent after six hours of incubation, it is concluded that an alteration in the sorbitol (polyol) pathway is not associated with the cataractogenic activity of DNP.

Table 17. Effect of 1×10^{-4} M DNP on the concentration of sorbitol in lenses after 6 hours of incubation.

Medium[a]	Sorbitol µgms/gm lens
Control	417.5±40.8 (4)[b]
1×10^{-4} M DNP	394.5±28.1 (4)

[a]TC 199 medium with a final bicarbonate concentration of 29 mM.

[b]Mean ± standard error (observations).

Experiment 12. Effect of DNP on the pentose phosphate shunt activity of the lens.

1. Purpose

Previous experiments have demonstrated that the cataractogenic activity of DNP is not associated with alteration of glucose metabolism via the Krebs cycle, the Embden-Meyerhof glycolytic pathway or the sorbitol (polyol) pathway. The pentose shunt is another pathway whereby the lens metabolizes glucose. The glucose metabolized by this pathway accounts for approximately one-third of the glucose metabolized by the lens (van Heyningen, 1962). In lenses obtained from young animals, the relative importance of the pentose shunt pathway for the metabolism of glucose is even greater (Lerman and Zigman, 1965).

The metabolism of glucose via the pentose phosphate shunt pathway is essential to the production of pentose phosphates and $NADPH_2$ (Rocher, 1956). Subsequently, the pentose phosphates are used for the production of RNA and DNA. It is hypothesized that the generation of $NADPH_2$ by the lens is required to maintain essential components of the lens such as glutathione in a reduced state (Pirie, 1965).

Since lenses obtained from immature rabbits are more severely affected by DNP than those obtained from

mature rabbits, it was hypothesized that the cataractogenic activity of DNP may be associated with a depression of the pentose phosphate shunt activity.

2. Methods

The generation of $^{14}CO_2$ from glucose-1-^{14}C (New England Nuclear, Boston, Mass.) was used to estimate the pentose phosphate shunt activity of the lens. The procedures used in these experiments were identical to those described in Experiment 4. One microcurie of glucose-1-^{14}C was added to the incubation systems giving a specific activity of 7.036 mg glucose per microcurie.

Having demonstrated that DNP depresses the activity of the pentose phosphate shunt, a second series of experiments were conducted to determine whether oligomycin would reverse the DNP depression. The procedures were modified somewhat for these experiments. In these experiments, 3.5 µl of a 4% solution of oligomycin in ethanol was added to the incubation system immediately after placing the lens in the system giving a final concentration of 5×10^{-5} M oligomycin. An equal volume of ethanol was added to control systems. After two hours of incubation, a sufficient amount of 1×10^{-2} M DNP in TC 199 medium (77 µl) was added to the system to give a final concentration of 1×10^{-4} M DNP. Following the addition of DNP to the system, glucose-1-^{14}C was added.

The specific activity was the same as in previous experiments. Samples of the hydroxide of hyamine containing the trapped $^{14}CO_2$ were collected for counting 6 and 24 hours after adding the glucose-1-^{14}C.

3. Results and Discussion

The effect of 1×10^{-4} M DNP on the pentose phosphate shunt activity is shown in Table 18. DNP caused a significant depression in the utilization of glucose via this pathway after six hours of incubation. The magnitude of the depression increased as the incubation continued.

In order to determine whether the depression of pentose phosphate shunt activity may be associated with the cataractogenic activity of DNP, a second series of experiments was conducted in which cataract production was blocked with oligomycin. The results are shown in Table 19. Oligomycin, 5×10^{-5} M, blocked the production of cataracts in lenses incubated in 1×10^{-4} M DNP. However, oligomycin did not alleviate the depression of glucose utilization via the pentose phosphate shunt. Therefore, a cause-effect relationship was not established. It is concluded that although DNP depressed the pentose phosphate shunt activity of the lens, this effect is not associated with the cataractogenic activity of DNP.

Table 18. Pentose phosphate shunt oxidation of glucose to CO_2 by lenses incubated in TC 199 medium (C) and TC 199 medium containing 1 x 10^{-4} M DNP (T) as a function of time.[a]

Time	Glucose metabolized, µg/g lens	
	Control	Treated
2 hr	25.4± 1.8[b]	21.1± 6.4
6 hr	112.3± 9.4	87.2±11.8[c]
12 hr	297.4±38.3	221.6±35.6[d]
24 hr	632.5±89.0	443.6±93.3[e]

[a]The production of $^{14}CO_2$ by the metabolism of glucose-1-^{14}C was used to estimate the pentose phosphate shunt oxidation of glucose

[b]Mean ± standard error (observations)

[c]$P<0.05$

[d]$P<0.05$

[e]$P<0.01$

Table 19. Effect of oligomycin on the DNP induced depression of the pentose phosphate shunt oxidation of glucose to CO_2.[a]

Time, hrs[b]	Glucose metabolized, µg/g lens	
	DNP, 1×10^{-4} M	Oligomycin, 5×10^{-5} M + DNP, 1×10^{-4} M
6	91.0±11.7 (6)[c]	96.2±18.6 (6)
24	420.4±39.14 (6)	441.9±15.74 (6)

[a]The production of $^{14}CO_2$ by the metabolism of glucose-1-^{14}C was used to estimate the pentose phosphate shunt oxidation of glucose. The lenses were incubated for 24 hr in either control medium or in medium containing 5×10^{-5} M oligomycin before adding the DNP and glucose-1-^{14}C.

[b]Duration of incubation after the addition of DNP and glucose-1-^{14}C.

[c]Mean ± standard error (observations).

DISCUSSION AND SUMMARY

The purpose of this thesis was an attempt to elucidate the mechanism responsible for the cataractogenic activity of DNP. Definitive information relative to the cataractogenic mechanism of DNP is unavailable.

Although Ogino and Yasukura (1957) observed cataracts in scorbutic guinea pigs treated with DNP, their findings were questionable because the nature of the cataract was not described and the guinea pigs used were immature. Furthermore, Tainter and Borley (1938) had earlier reported that ascorbic acid deficiency does not predispose guinea pigs to the cataractogenic activity of DNP. Although in the studies reported herein cataracts were observed in scorbutic guinea pigs, the cataracts were also observed in untreated guinea pigs. These cataracts develop with age and are consistently found in mature guinea pigs. It is likely that Ogino and Yasukura (1957) associated the development of these cataracts with DNP treatment.

Known metabolites of DNP (2 amino-4 nitrophenol, 2 nitro-4 aminophenol, and 2,4 diaminophenol) did not demonstrate cataractogenic activity even at a concentration of 1×10^{-3} M. Therefore, it is unlikely

that the cataractogenic activity is associated with the formation of the metabolites as has been suggested by Ogino and Yasukura (1957).

It was found that the cataractogenic activity of DNP has a positive temperature coefficient, the opacification of the lens increases as the incubation temperature is increased. Thus, the cataractogenic activity of DNP does not seem to be associated with an alteration of protein structure caused by the binding of DNP to protein. A positive temperature coefficient for the cataractogenic activity suggests that the mechanism is related to an effect of DNP on lenticular metabolism.

Feldman *et al.* (1959, 1960) suggested that the cataractogenic activity of DNP was associated with an interference in the formation of high energy phosphate compounds. The primary action of DNP on the formation of high energy phosphate compounds is to uncouple oxidative phosphorylation. Since control lenses incubated in essentially an oxygen free environment maintain their clarity, oxidative phosphorylation is not required to maintain the clarity of the lens. Hence it is unlikely that the uncoupling of oxidative phosphorylation by DNP is associated with cataract development.

Another manner in which DNP may disturb the high energy phosphate (ATP) content of the lens is via the

stimulation of mitochondrial ATPase. The mechanism for this activity was described in the Introduction. The observation that DNP lowered the ATP content of lenses incubated in an anaerobic environment supported such a mechanism. Additional support for this mechanism was obtained when it was found that oligomycin blocks that cataractogenic activity of DNP. Oligomycin is a known inhibitor of mitochondrial ATPase. Therefore, if oligomycin blocks the DNP stimulated ATPase activity, the ATP content of the lens would be preserved. Unfortunately, measurement of the ATP content of the oligomycin and DNP treated lenses was even lower than that of DNP treated lenses. Thus, the cataractogenic activity of DNP does not appear to be related to the stimulation of mitochondrial ATPase.

It has been demonstrated that DNP increases the utilization of glucose (Terner, 1952) by lenses. The lens derives most, if not all, of its metabolic energy from the oxidation of glucose. All of the enzyme systems which are required for the operation of anaerobic glycolysis, the Krebs cycle, and the hexose monophosphate shunt pathway have been shown to be present in the lens. The lens also contains a rather unusual pathway for the metabolism of glucose known as the polyol or sorbitol pathway. Alterations in the activity of the hexose

monophosphate and polyol pathways appear to be of importance in initiating the chain of metabolic events leading to the development of the experimental sugar cataracts. The finding that DNP does not alter the concentration of the end products of anaerobic glycolysis and the polyol pathway, lactic acid and sorbitol respectively, suggests that an untoward effect of DNP on these phases of glucose metabolism is not associated with the cataractogenic activity of DNP. These results negate the suggestion by Horner (1942) that an accumulation of lactic acid may be responsible for the cataractogenic activity of DNP.

Although DNP caused a significant depression of the hexose monophosphate shunt activity of lenses, this depression was not alleviated when cataract induction was blocked with oligomycin. Therefore, at least the rapid developing cataract being studied in these experiments does not appear to be associated with an untoward effect of DNP on this pathway. It is possible that a persistent depression of this pathway may lead to a loss of lens clarity.

In addition to oligomycin, ouabain was also found to block the cataractogenic activity of DNP. Both of these drugs inhibit the Na-K activated ATPase enzyme. This enzyme has been associated with the transport of

cations by the lens as well as the flux of water (Bonting *et al.*, 1962). Therefore, it is conceivable that DNP affects this enzyme or these functions in a manner that may be negated by the Na-K activated ATPase inhibiting agents oligomycin and ouabain. However, the antagonistic activity of oligomycin and ouabain on the cataractogenic activity of DNP cannot be easily explained.

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