ACTION OF METABOLIC POISONS ON THE ISOLATED RETINA

RELATION OF HISTOLOGICAL TO BIOCHEMICAL LESIONS*

BY

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NOELL (1952, 1953a, b, 1955) reported that sodium iodoacetate (NaIA), administered intravenously to rabbits, selectively destroyed the visual cells of the retina, an effect he attributed to interference with glycolysis. In previous work (Sorsby, Newhouse, and Lucas, 1957), we investigated the effects on the retina of a wide range of thiol reagents, administered intravenously at maximum tolerated dosage to rats and rabbits. Of these, only iodoacetate (and bromoacetate in the rabbit) caused visible damage to the retina. In the intact animal, blood-retinal barriers and systemic detoxicating and excreting mechanisms are complicating factors which cannot be assessed. Furthermore, the maximum feasible dose is determined not by the sensitivity of the retina but by systemic lethality of the compound given. These difficulties could be avoided by studying the short-term effects of iodoacetate and other inhibitors on the isolated retina, maintained *in vitro* in a saline medium. By this means, one of us (Newhouse, 1959) has already found that several thiol reagents are able to inhibit retinal glycolysis almost as readily as NaIA, while others are able to depress respiration even more effectively. It seems doubtful therefore whether the singular retinal necrosis that follows parenteral iodoacetate is due simply to glycolytic inhibition.

The present studies were made to determine whether, in the isolated retina, any relationship could be demonstrated between the known ability of NaIA and other thiol reagents to inhibit respiration, glycolysis, or both, and their capacity to cause cellular damage.

Methods

Animals.—Retinae from adult Dutch rabbits (1.5–2 kg.) or from adult rats (0.2–0.3 kg.) of the PVG strain were used.

Preparation of Tissue.—Animals were killed by spinal fracture. The eyes were at once removed and opened in ice-cold, oxygenated saline medium to which glucose (10 mM) had been added. The saline medium used during dissection was buffered with either bicarbonate or phosphate, according to the circumstances (see below). The retina of each eye was then dissected out as carefully as possible. Rat retina was used intact, but rabbit retina was cut into about ten pieces, each one including part of the area centralis. This material was distributed among 8 conical flasks, each receiving tissue equivalent to about 2 mg. dry weight. A further retina or retinal piece was placed in fixative immediately after dissection

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to serve as a processing control. The kind of flask used, whether of the Warburg or the Erlenmeyer type, did not affect the histological findings.

Media.—The cytotoxicity of all inhibitors mentioned was assessed using material incubated, on separate occasions, in two different saline media, namely, Krebs-Ringer bicarbonate solution under $O_2:CO_2$ (Krebs and Henseleit, 1932) and a comparable saline-phosphate solution under O_2 (Cohen, 1949). The choice of buffer made no appreciable difference to the severity of the lesions produced but, because histological preservation of control material seemed somewhat better when the bicarbonate medium was used, the series of results obtained with the latter is here reported. In this series, each Erlenmeyer flask (25 ml.) received ice-cold saline bicarbonate medium (5 ml.), previously saturated with either $O_2:CO_2$ (95:5) for aerobic studies or $N_2:CO_2$ (95:5) for the few anaerobic experiments performed. The substrate was always glucose (10 mM), except in two experiments, described later, in which sodium pyruvate (20 mM) was used.

Inhibitors.—These were weighed out as such, neutralized when necessary, and dissolved in the medium to give concentrations fifty times that ultimately required *in vitro*. The appropriate quantity of inhibitor was added to duplicate flasks immediately before incubation; duplicate controls in normal medium were run at the same time. The effects of several concentrations of each inhibitor were studied at least twice, using a different rabbit in each experiment.

Incubation.—The flasks were vigorously gassed with the appropriate gas mixture for 1–2 min. before being closed. They were then shaken in the Warburg respirometer bath, at 37° C. (for rats) or 38° C. (for rabbits) and 105 strokes per min. for 60 min.

Histological Treatment.—The material was fixed in acid Zenker's solution. A standard paraffin embedding method was used at first, but most of the later material was dehydrated in 2-ethoxyethanol and embedded in ester wax (Chesterman and Leach, 1956), since this was found to give better sections. The ester wax sections were stained by Mayer's haemalum and eosin Y.

Results

(1) THE RABBIT

The normal histological appearance of the retina was maintained reasonably well under oxygen, with glucose as substrate (Fig. 1); histological preservation was somewhat better when the medium was buffered with bicarbonate instead of phosphate.

The visual cell nucleus of the rabbit is a compact structure, consisting of two dense, chromatin masses, surrounded by a delicate, crenated membrane (Fig. 1, opposite). Pyknosis is therefore not so conspicuous in the visual cell nuclei as in larger nuclei with a more open network, such as those of the bipolar cells. The majority of such cells did not normally become pyknotic during incubation for an hour, but both the processing and incubation controls showed patchy nuclear pyknosis in all layers, which was due to the trauma inevitably associated with dissection. Since, however, the changes due to the inhibitors were diffuse, they could easily be distinguished.

FIG. 1.—Rabbit retina incubated 1 hr in Krebs-Ringer bicarbonate (K-R) under standard conditions (glucose substrate, $O_2:CO_2$). $\times 400$.

Inset: Visual cell nuclei $\times 1,250$ to show detailed structure.

Under conditions where, by the use of oxygen-pyruvate (Fig. 2) or nitrogen-glucose (Fig. 3), either glycolysis or respiration was minimized while the other function was able to continue actively, structural integrity was regularly impaired; widespread nuclear pyknosis developed.

FIG. 2.—Rabbit retina incubated 1 hr in K-R with pyruvate under $O_2:CO_2$. $\times 400$.

Inset: Visual cell nuclei $\times 1,250$ to show pyknosis.

FIG. 3.—Rabbit retina incubated 1 hr in K-R with glucose under N_2 . $\times 400$.

Inset: Visual cell nuclei $\times 1,250$ to show pyknosis.

Even more marked deterioration was caused by the complete omission of substrate, either aerobically or anaerobically (Fig. 4). These findings indicate how immediate is the dependence of the tissue on both glycolysis and respiration.

FIG. 4.—Rabbit retina incubated 1 hr in K-R with no substrate under N_2 . $\times 400$.

Effects of Inhibitors.—Most of the inhibitors tested produced marked histological change at a concentration of 5 mM or less. Apart from sodium azide and sodium 2:4-dinitrophenate, which are discussed later, the rest of the injurious inhibitors produced a similar histological picture. Pyknosis occurred in both inner and outer nuclear layers, but in the inner layers it affected only the bipolar nuclei while the Müller fibre nuclei became swollen. The ganglion cells showed shrinkage, pyknosis, and chromatolysis, or even complete cytolysis. Because of the variable behaviour and limited number of these cells in the sections, their response has not been included in the Tables. With some inhibitors, the above changes were accompanied by marked structural disorganization, which always affected the outer layers more severely.

The concentration needed to induce well-marked histological changes varied with the agent tested. In terms of decreasing toxicity, inhibitors may be graded into those effective at minimal concentrations of 0.05, 0.5, and 5 mM. The comparative histological effects on the two main components of rabbit retina, *i.e.* the visual and the inner layers, are summarized in Table I (opposite) and Tables II and III (overleaf). Data regarding percentage inhibition of respiration and aerobic glycolysis in the whole tissue are also included where available (vide Newhouse, 1959); such values represent the mean of two determinations, each one being performed on duplicate samples of retina taken from a different rabbit and subjected to conditions of incubation similar to those already described.

TABLE I

REAGENTS DAMAGING RABBIT RETINA AT 0.05 mM OR MORE

These substances, all thiol reagents, are powerful inhibitors of respiration though not always of glycolysis (*e.g.* 2, 7) and are highly destructive to the isolated retina, particularly the visual cells.

Reagent	Concentration (mM)	Functional Inhibition (per cent.)		Histological Injury		Remarks
		Respiration	Glycolysis	Outer Layers	Inner Layers	
(1) Phenacyl bromide	0.05 0.5	82 100	56 79	++ ++	++ ++	Destructive to both layers; inhibits both functions
(2) 1:2:4-Fluorodinitro benzene	0.05 0.5	86 100	13 21	++ ++	+ +	Particularly destructive to outer layers; little effect on glycolysis
(3) 3-Amino 4-hydroxy-phenyl arsine oxide	0.05 0.5	74 89	37 50	++ ++	± ++	
(4) Sodium p-chloro-mercuribenzoate	0.05 0.5	66 100	= 61	++ ++	± ++	
(5) Mercuric chloride	0.05 0.5	23 100	4 97	+ ++	+ ++	
(6) Ethyl iodoacetate	0.05 0.5	48 95	24 100	+ ++	- +	Effects similar to those of NaIA (Table II) but more pronounced
(7) Sodium o-iodosobenzoate	0.05 0.5	19 100	= 29	+ ++	- ++	Particularly damages visual cells; little effect on glycolysis

Effective at 0.05 mM (Table I).—This group comprises most of the more powerful thiol reagents, none of which had damaged retina on intravenous injection at the maximal tolerated dosage. Most of them are inhibitors of respiration, even at this low concentration, but some (*e.g.* 1:2:4-fluorodinitrobenzene, FDNB) have, in the same circumstances, only slight effects on glycolysis.

The arylating agents phenacyl bromide and FDNB, the alkylating agent ethyl iodoacetate, the heavy metal compounds mercuric chloride and sodium p-chloro-mercuribenzoate (CMBA) (Fig. 5, overleaf), and the two trivalent organic arsenicals were all considerably more destructive to the isolated retina than NaIA itself. All produced comparable damage at concentrations only one-tenth as great as that needed for NaIA (*i.e.* 0.5 mM), while, at this latter concentration, they caused rapid disintegration of the tissue.

FIG. 5.—Rabbit retina incubated under standard conditions with 0.05 mM CMBA. $\times 400$.

Effective at 0.5 mM (Table II).—This group of thiol reagents includes NaIA and sodium bromoacetate—both parenterally injurious to retina—and the related

TABLE II
REAGENTS DAMAGING RABBIT RETINA AT 0.5 mM OR MORE

This group comprises several alkylating substances related to iodoacetic acid and two oxidizing agents. Though the effect of the alkylating agents on glycolysis varies, they inhibit respiration to a comparable extent and are moderately destructive at 0.5 mM, particularly to the visual cells.

Reagent	Con- centration (mM)	Functional Inhibition (per cent.)		Histological Injury		Remarks
		Respira- tion	Gly- colysis	Outer Layers	Inner Layers	
(1) Sodium iodoacetate	0.05 0.5 5.0	22 63 100	52 98 100	= ++ ++	= + ++	Readily in- hibits gly- colysis at all concentrations but only in- jurious when respiration also much affected
(2) Sodium bromoacetate	0.05 0.5 5.0	63	99	= ± +	= + +	Less injurious than (1), though almost equally effec- tive as an inhibitor
(3) Iodoacetamide	0.05 0.5 5.0	= 63 100	= 12 58	= ++ ++	= + ++	Less effective against glyco- lysis than (1) but no less destructive
(4) p-Benzoquinone	0.5 5.0			++ ++	+ +	
(5) 2:6-Dichlorophenol- indophenol	0.5 5.0			++ ++	± ++	

substance, iodoacetamide (IAD). NaIA is a powerful inhibitor of glycolysis and to a less extent, of respiration. The damage it caused *in vitro* was moderately severe (Fig. 6), but the toxic effects were by no means confined to the visual cells, nor linked exclusively with either phase of carbohydrate metabolism, as the use of either nitrogen-glucose or oxygen-pyruvate conditions showed. The histological changes caused by sodium bromoacetate, particularly in the visual cell layer, were less marked than those of NaIA. This lesser severity corresponds to that already observed upon the retina *in vivo* (Lucas, Newhouse, and Davey, 1957).

Iodoacetamide was less effective as a glycolytic inhibitor than its parent acid, but produced cytological changes of comparable severity (Fig. 7).

FIG. 6.—Rabbit retina incubated under standard conditions with 0.5 mM NaIA. $\times 400$.

FIG. 7.—Rabbit retina incubated under standard conditions with 0.5 mM IAD. $\times 400$.

Effective at 5 mM or More (Table III).—This group included various conventional inhibitors of respiration and glycolysis, none of which had proved destructive to retina on intravenous injection (Noell, 1953a; Babel and Ziv, 1956). The relatively minor damage they caused, even at high concentrations, contrasted strongly with that caused by the thiol reagents of the first group.

Thus the glycolytic inhibitors, sodium fluoride and glyceraldehyde, produced no serious damage in the isolated retina, under either aerobic or anaerobic conditions, until a concentration as high as 50 mM was used (Fig. 8, overleaf), a level associated with a fall not only in glycolysis but also in respiration (with either glucose or pyruvate as substrate).

The respiratory inhibitor, sodium cyanide, proved rather more injurious than the two glycolytic inhibitors, the visual cell layer being particularly affected at a concentration of 5 mM or higher (Fig. 9, overleaf). In these circumstances, respiration was considerably depressed but lactic acid content was actually augmented (*cf.* Robbie and Leinfelder, 1948).

Sodium fluoroacetate, an established inhibitor of the tricarboxylic acid cycle enzyme aconitase, in brain, was less cytotoxic than cyanide. Even at a concentration of 50 mM., only limited visual cell damage and slight respiratory inhibition were caused.

Sodium azide and 2:4-dinitrophenate are known to interrupt the utilization of oxidative energy in phosphorylation processes. When applied to retina at a

TABLE III
REAGENTS DAMAGING RABBIT RETINA AT 5mM OR MORE

This group comprises several substances, none of them thiol reagents, which affect respiration, glycolysis, or both, only at high concentrations; their injurious effects upon the isolated retina are relatively mild even at 5 mM.

Reagent	Concentration (mM)	Functional Inhibition (per cent.)		Histological Injury		Remarks
		Respiration	Glycolysis	Outer Layers	Inner Layers	
(1) Sodium fluoride	0.5	=	=	=	=	Inhibits respiration as well as glycolysis at high concentration
	5.0	89	27	±	±	
	50	74*	74	++	+	
(2) Glyceraldehyde	0.5			=	=	Inhibits respiration as well as glycolysis at high concentration
	5.0			±	±	
	50	78†	76	++	+	
(3) Sodium cyanide	0.5	7	raised	=	=	Only respiration depressed, except at the highest concentration
	5.0	57	raised	+	±	
	50	72	100	++	++	
(4) Sodium fluoroacetate	5.0	=	=	=	=	Effects relatively mild
	50	29	10	-	+	

* Respiration depressed by 36 per cent. on sodium pyruvate substrate
† Respiration depressed by 64 per cent. on sodium pyruvate substrate

relatively high concentration (*i.e.* 5 mM.), each of these substances reduced the levels of both respiration and glycolysis. It was therefore surprising that, at this concentration, no definite nuclear changes were seen, though localized areas of oedema occurred in the fibre layers.

FIG. 8.—Rabbit retina incubated under standard conditions with 50 mM NaF. ×400.

FIG. 9.—Rabbit retina incubated under standard conditions with 5 mM NaCN. ×400.

Ineffective at 5 mM.—The following agents produced no obvious histological change at a concentration of 5 mM: sodium salts of arsenite, arsanilate, iodide and iodate; alloxan; tryparsamide; methanol and quinine.

(2) THE RAT

In agreement with Robbie and Leinfelder (1948), the rat retina was found to survive well *in vitro* (Fig. 10), but, because the rat visual cell nucleus has an even more compact structure than that of the rabbit, interpretation of the histological sections was less easy.

FIG. 10.—Rat retina incubated under standard conditions. $\times 400$.

It was however clear that the effects of NaIA (Fig. 11, overleaf), FDNB, and CMBA (Fig. 12, overleaf) were similar to those seen in the rabbit, and that the minimal effective concentrations were the same. The sensitivity of the isolated rat retina to either structural damage or metabolic inhibition (Newhouse, 1959) by NaIA contrasts with its relative resistance to damage in the intact animal (Sorsby, Newhouse, and Lucas, 1957).

Discussion

These results do not support the view of Noell (1952, 1953a, b, 1955) that the inhibition of glycolysis can by itself do major damage to the rabbit retina. Simple suppression of this function by the use of pyruvate instead of glucose as substrate caused less structural disorganization in the isolated retina than did incubation for an hour with NaIA or other thiol reagents. It may be noted that prolonged insulin hypoglycaemia, severe enough to cause cerebral damage, does not appear to cause ocular lesions in man (Gralnick, 1941).

Nor does retinal metabolism appear to be much affected by the established glycolytic inhibitors, fluoride and glyceraldehyde, unless relatively high

FIG. 11.—Rat retina incubated under standard conditions with 0.5 mM NaIA. $\times 400$

FIG. 12.—Rat retina incubated under standard conditions with 0.05 mM CMBA. $\times 400$.

concentrations are used. Babel and Ziv (1956), who found that a daily intravenous dosage of only 20 mg./kg. body weight of sodium fluoride was ultimately lethal to the rabbit, were unable to produce a retinal lesion by this means; perhaps the greater sensitivity of other centres to fluoride toxicity prevented the attainment of body fluid levels readily injurious to retina. The present studies indicate that neither compound causes structural changes in the isolated retina until present in such concentrations that both glycolysis and respiration are independently affected. Inadequate cellular penetration seems unlikely since, at a given concentration (*e.g.* 5 mM.), glycolysis is no more effectively inhibited in retinal homogenate extracts by these agents (Kerly and Bourne, 1940) than it is in the whole retina.

Though the members of the group of thiol reagents varied widely in their capacity to depress both respiration and glycolysis, no distinct correlation between anti-glycolytic activity and ability to damage the isolated retina was apparent. Thus NaIA proved to be the most profound inhibitor of glycolysis, approached in this respect only by ethyl iodoacetate and CMBA. Yet, by comparison with either of these agents, or with FDNB (which affects glycolysis hardly at all), it was much less injurious to the isolated retina; perhaps significantly, it was less effective than these reagents as a respiratory depressant and certainly less readily lethal when given parenterally. It may thus be concluded that the striking retinal necrosis produced *in vivo* by

iodoacetate is probably not due solely to the anti-glycolytic activity of this drug.

The common feature of various types of retinal injury, whether caused by the highly destructive thiol reagents of Table I, by NaIA, or by oxygen poisoning (Noell, 1955), is the depression of respiration. This fact is of special interest in view of the high activity of respiratory enzymes in the visual cells (Francis, 1953; Eichner, 1955). However, it cannot therefore be assumed that retinal damage is exclusively due to inhibition of respiration instead of glycolysis, for the simple suppression of oxygen uptake by anaerobiosis or the addition of cyanide (a specific inhibitor of the cytochrome system) is less injurious to the isolated tissue than are many of the thiol reagents.

So many other substances are as easily able to interfere with retinal metabolism that the reputation of NaIA as a specially toxic inhibitor seems open to doubt. The thiol reagents of Table I proved, on an equimolar basis, more destructive than the inhibitors of relatively high specificity (cyanide, fluoroacetate, glyceraldehyde), while NaIA occupied an intermediate position. Their outstanding toxicity may well be due to their ability to inhibit simultaneously a very wide range of enzymes, including several dehydrogenases of the Krebs cycle (Barron and Singer, 1945). Since at least half of all enzymes known are thiol-dependent (Wald, 1955), numerous possibilities of blockage are at once available to the inhibitor of low specificity, such as CMBA or NaIA, none of which may be singly responsible for the progressive metabolic and structural disorganization that develops in the freely exposed tissue.

The present method permits the retina to be subjected for a set time to concentrations of inhibitor that are more stable and precise than those possible parenterally; the lesions produced are correspondingly more severe, more rapid in onset, and less restricted to particular layers than is the case *in vivo*. However, all attempts to judge the relative importance of various interdependent processes in retinal metabolism by the histological response to appropriate inhibitors, introduced either *in vivo* or *in vitro*, face two difficulties. First, few agents are known that inhibit only one reaction and, even among those that do, the indirect effects upon other equally essential processes may be both complex and diverse. Secondly, on a short term basis, there is no clear relationship between the maintenance of certain essential metabolic processes and the normal cyto-architecture of the retina. For example, in the present work, glycolysis or phosphorylation could be inhibited without causing immediate structural damage, and respiration could persist—though to a reduced extent—in retina already showing signs of tissue disorganization. Thus, while the necrosis ultimately produced by many forms of chemical inhibition is both spectacular and interesting, the biochemical conclusions that can be drawn from its occurrence are clearly limited by its lack of specificity.

Summary

(1) The cytological effects of sodium iodoacetate (NaIA) and other inhibitors upon the isolated rabbit retina, incubated under oxygen in saline media for 1 hour, were investigated.

(2) NaIA caused retinal damage at lower concentrations than are necessary *in vivo*; injury was not confined to the visual cells, the bipolar and ganglion cells sometimes being simultaneously affected.

(3) Other inhibitors, particularly the thiol reagents, were *in vitro* more cytotoxic than NaIA, though without effect parenterally.

(4) The degree of damage inflicted by any agent did not appear to be related to the extent of glycolytic inhibition caused. No substance tested, even anti-glycolytic agents such as NaIA or NaF, was destructive until concentrations were used at which independent respiratory inhibition also occurred. Fluorodinitrobenzene, which entirely suppresses respiration but has little effect on glycolysis, was notably destructive.

(5) It is suggested that the cytotoxicity of the thiol reagents depends on their ability to inhibit simultaneously many thiol-dependent enzymes rather than upon suppression of any specific retinal process.

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