ACTA OPHTHALMOLOGICA VOL. 54 1976

Departments of Clinical Chemistry
(Head: H. Adlercreutz)
and Ophthalmology (Head: S. Vannas),
University of Helsinki, Finland

EFFECTS OF PHOSPHOLINE IODIDE ON THE METABOLITES OF THE GLYCOLYTIC, PENTOSE PHOSPHATE AND SORBITOL PATHWAYS IN THE RABBIT LENS

BY

MATTI HÄRKÖNEN and AHTI TARKKANEN

Steady-state concentrations of the key intermediates from the glycolytic, pentose phosphate, and sorbitol pathways as well as the pyridine nucleotides were measured from the lens after 0.25 % phospholine iodide had been instilled into rabbits' eyes twice a day for 18 weeks. In the lenses of those rabbits which had received treatment in both eyes fructose-1,6-diphosphate and pyruvate levels were increased, whereas 6-phosphogluconate, sorbitol and α-glycerophosphate concentrations were decreased. α-Ketoglutarate and concentrations and ratios of NAD^+ and NADH did not show any changes. In contrast, NADPH and total NADP concentrations as well as the $NADPH/NADP^+$ ratio were decreased, and therefore total NAD/total NADP ratio increased after treatment. It appears that instillation of long-acting 0.25 % phospholine iodide into rabbits' eyes results in increased glycolytic activity in the lens in response to the increased energy demand, whereas the activities of other metabolic pathways are suppressed.

Key words: phospholine iodide – echothiophate iodide – lens metabolism.

Unusual abbreviations: NAD(P), nicotinamide adenine dinucleotide plus nicotinamide dinucleotide phosphate; FDP, fructose-1,6-diphosphate; α-GOP, α-glycerophosphate; 6-PG, 6-phosphogluconate; α-KG, α-ketoglutarate.

Received February 6, 1975.

Phospholine iodide, a long-acting cholinesterase inhibitor, has been shown to initiate or accelerate cataract formation in glaucomatous eyes (Axelsson 1969; Axelsson & Holmberg 1966; DeRoetth 1966; Pietsch et al. 1972; Shaffer & Hetherington 1966; Tarkkanen & Karjalainen 1966; Thoft 1968). Transient clouding of the lens has also been observed in eyes of young persons who have been treated with phospholine iodide for accommodative esotropia (Axelsson & Nyman 1970; Harrison 1960). In our previous study (Härkönen & Tarkkanen 1970) phospholine iodide was instilled into rabbits' eyes for 15 weeks and the major components of the energy reserves of the lens were measured. ATP was found to be decreased by 35 % and lactate by 17 %, whereas glycogen, glucose and glucose-6-phosphate did not show any significant alterations. Interference with glycolysis or oxidative phosphorylation was suggested as the possible mechanism. Interestingly enough, decreases of both ATP and lactate were also found in the contralateral untreated eye when the drug was applied to one eye only.

Weak acetylcholinesterase activity has been shown to be present in the anterior part of the lens. Treatment with phospholine iodide results in complete inhibition of the enzyme activity (DeRoetth 1966; Michon & Kinoshita 1967; Tarkkanen & Härkönen 1969). However, the role of cholinesterase in the lens is not known. Three possibilities have been suggested by Michon & Kinoshita (1967): This enzyme may be concerned with cation transport, it may represent a rudiment of phylogenetic development, or it may protect the capsule against any acetylcholine in the aqueous or the vitreous.

This study was designed to throw further light on the way(s) in which phospholine iodide affects the rabbit lens. In addition to pyridine nucleotides, major metabolites of the glycolytic, pentose phosphate and sorbitol pathways were analyzed in a search for information about the possible causes of the ATP decrease in phospholine-treated lenses.

Material and Methods

Fifteen adult albino rabbits of the same age were used in the experiments. 0.25 % phospholine iodide drops were instilled twice a day into both eyes of 5 rabbits for 18 weeks. Another 5 rabbits were treated in the same way in the right eye only, and 5 rabbits served as controls. The control rabbits received the same care as the treated-rabbits throughout the study. The rabbits were killed by injection of air into the auricular vein and the eyeballs were excised. The lenses were rapidly removed by the posterior route, freed from extraneous tissue and dropped into liquid nitrogen. The operation was performed as quickly

as possible and usually not more than 30 second elapsed from the death of the animal until the lenses were immersed in the liquid nitrogen.

For metabolite studies the material was prepared as described by Härkönen & Tarkkanen (1970). This method provides metabolically the most active portion of the lens for study in situations where changes in lens metabolites are to be analyzed. The metabolites were measured fluorometrically by enzymatic pyridine nucleotide methods. Sorbitol was measured according to Matschinsky & Ellerman (1968) and fructose-1.6-diphosphate according to Lowry et al. (1964) by developing the fluorescence of NAD$^+$ with strong alkali after destroying NADH with acid (see Lowry & Passonneau 1972). 6-Phosphogluconate (Kauffman & Albuquerque 1970), pyruvate and *α-ketoglutarate* (Matschinsky et al. 1968) were measured in one-tenth of the volume (0.1 ml) of the original procedure in special microtubes designed for use in the Farrand fluorometer, *model A-3*.

Pyridine nucleotides were determined by a modification of the method of Burch et al. (1967). Frozen samples (ca. 30 mg) were homogenized at 0°C in 500 μl of 0.04 N NaOH containing 0.5 mM cysteine (NaDH-cysteine. Total and reduced forms of NAD and NADP were measured in a portion of the homogenate diluted with 20 volumes of cold NADH-cysteine. NADH and NADPH were measured in a portion of the diluted homogenate in which oxidized pyridine nucleotides had been destroyed by heating for 10 min at 60°C. To 200 μl of the original homogenate 5 μl of 1.2 M ascorbic acid was added and the mixture was acidified with 200 μl of 0.02 N $H_2SO_4 - 0.1$ M Na_2SO_4, and heated for 30 min at 60°C to destroy reduced forms of the nucleotides. Oxidized NAD and NADP were then measured. Special care was taken to keep samples and standards at 0°C, unless otherwise stated, until the cycling step was started about 40 min after homogenization. Cycling was performed in 100 μl of cycling reagent; the rate of NADP cycling was ca. 8000/h and of NAD ca. 2700/h.

Statistical analyses of the results were carried out either by the *t*-test according to De Jonge (1964), or by a matched-pair *t*-test (Richterich 1968) when the eyes of the same rabbits were compared.

Results

Normal values

The concentrations of ten metabolites measured in the equatorial region of the rabbit lens are compared with previously reported values in Tables I and II. At this point it has to be emphasized that whole lenses were used in the cited papers. However, the figures give a rough estimate of the quantity of the

Table 1.
The effect of phospholine iodide (PI) on the concentrations of metabolic intermediates in the rabbit lens (mean ± SEM).

Group	FDP	Pyruvate	α-GOP μmol × kg^{-1}	6-PG wet tissue	α-KG	Sorbitol
Controls						
Both lenses	9.0 ± 2.5	15.4 ± 2.1	1250 ± 50	11.1 ± 1.0	51.1 ± 2.0	9140 ± 1550
Left lenses	8.7 ± 5.7	13.0 ± 2.7	1200 ± 50	11.3 ± 1.3	52.4 ± 2.7	9270 ± 1480
PI to the right eye						
Left lenses	26.3 ± 5.3*	15.7 ± 5.5	1110 ± 130	20.8 ± 9.4	40.3 ± 6.2	5820 ± 940
Right lenses	27.5 ± 9.3	18.3 ± 5.7	1124 ± 66	17.3 ± 7.5	41.5 ± 8.9	6800 ± 990
PI to both eyes						
Both lenses	26.6 ± 5.7*	24.2 ± 4.4	1020 ± 50*1	7.1 ± 0.7*1	53.5 ± 3.4	4780 ± 350
Values from other reports	–	60[2]	940[2]	–	–	1700[3]

[2] van Heyningen (1965).
[3] Kuck (1966), 31 mg/100 g.
* $P < 0.05$.
*1 $P < 0.02$.

Table II.

The effect of phospholine iodide (PI) on the concentrations of metabolic intermediates in the rabbit lens (mean ± SEM).

Group	Total NAD measured	NAD^+	NADH	$\frac{NAD^+}{NADH}$	Total NADP measured	$NADP^+$	NADPH	$\frac{NADPH}{NADP^+}$	$\frac{\text{Total NAD}}{\text{Total NADP}}$
	$\mu mol \times kg^{-1}$ wet tissue								
Controls									
Both lenses	834 ± 93	358 ± 69	469 ± 34	0.74 ± 0.09	32.5 ± 0.8	9.2 ± 1.6	28.1 ± 0.8	3.5 ± 0.5	25.5 ± 2.5
PI to the right eye									
Right lenses	863 ± 76	344 ± 52	504 ± 32	0.67 ± 0.09	21.5 ± 1.6***	9.9 ± 1.9	15.7 ± 2.0**	1.7 ± 0.1**	38.9 ± 3.6*
PI to both eyes									
Both lenses	878 ± 65	414 ± 79	456 ± 26	0.93 ± 0.19	22.2 ± 1.9**	7.3 ± 1.1	19.2 ± 1.2***	2.7 ± 0.2	42.0 ± 7.1
Values from other reports	–	527[2]	337[2]	1.57[2]	–	120[2]	48[2]	0.40[2]	

[2] Bullard (1965).

* $P < 0.02$.

** $P < 0.01$.

*** $P < 0.001$.

given metabolites. The values for α-glycerophosphate, pyruvate, NAD^+ and NADH agreed rather well with those of van Heyningen (1965) and Bullard (1965). However, the concentrations of $NADP^+$ and NADPH were a tenth and a half of the respective concentrations found by Bullard (1965). The NADPH/$NADP^+$ ratio was 3.5 in our study, whereas Bullard's values gave the ratio 0.40. In most tissues the NADP system is in reduced state, the NADPH/$NADP^+$ ratio varying from 2.8 to 20.6 (see e. g. Burch et al. 1967). In rat and bovine lenses, the NADP system is also reported to exist in reduced state (Sippel 1962; Lerman 1961; Klethi & Mandel 1960). In our study, the NAD^+/NADH ratio was 0.74. This ratio, although higher than in most other tissues, is far lower than in several previous studies on rabbit and bovine lenses, where the ratio has been shown to be greater than unity (Bullard 1965; Klethi & Mandel 1960). However, in the rat lens the ratio has been reported to be either higher (Sippel 1962) or lower (Lerman 1961; Lerman & Heggeness 1961) than unity. In our study the total NAD measured agreed within 1 % with the sum of the reduced and oxidized forms, whereas the total NADP measured was less than the sum of the oxidized and reduced NADP. The sorbitol concentration was near 5-fold higher than previously reported for the rabbit lens (Kuck 1966).

Metabolite concentrations in lenses treated with phospholine iodide

Cataract formation could not be observed in any of the eyes treated with phospholine iodide, confirming our previous results (Härkönen & Tarkkanen 1971). The final metabolite concentrations in the various experimental groups are presented in Tables I and II. No significant differences were observed in any of the metabolites measured if the drug was applied to one eye, the other eye of the same animal serving as a control. Fructose diphosphate was significantly ($P < 0.05$) increased in the lenses of the rabbits which had both eyes treated with phospholine iodide as compared with those of the untreated controls. The lenses of the untreated (control) left eye in the group in which the right eye was treated with phospholine iodide, also had elevated fructose diphosphate values as compared with the lenses of the left eyes of the untreated controls ($P < 0.05$). Phospholine iodide treatment also had a tendency to elevate the pyruvate concentration. These differences in fructose diphosphate and pyruvate concentrations indicate increased glycolytic activity (Lowry et al. 1964). In the group in which both eyes were treated with phospholine iodide the 6-phosphogluconate concentration was significantly ($P < 0.02$) lower than in the control group. Between the other groups however, there were no significant differences and the variation in those groups was wide. Sorbitol ($P < 0.02$)

and α-glycerophosphate ($P < 0.05$) concentrations were also decreased in the drug-treated lenses, and the control lenses in the group where the opposite eye was treated with the drug tended to be lower than in the control group. In α-ketoglutarate concentration there were no significant differences between the groups.

Concentrations and ratios of oxidized and reduced nicotinamide adenine dinucleotides were not significantly different in drug-treated and corresponding control lenses (Table II). In contrast, NADPH and total NADP concentrations as well as the NADPH/$NADP^+$ ratio were significantly decreased in the lenses treated with phospholine iodide. Hence, the total NAD/total NADP ratio was increased by drug treatment.

Discussion

In tissue culture, in the presence of phospholine iodide, the lens gained water and sodium, and lost potassium (Michon & Kinoshita 1968a). The basic change was found to be an increase in lens permeability as measured by the leaking-out of rubidium 86 from the lens. The permeability altered before any gain in water had occurred and even when the lens was prevented from swelling by a hyperosmotic environment (Michon & Kinoshita 1968b). In our previous study (Härkönen & Tarkkanen 1970), ATP was found to be decreased by 35 % and lactate by 17 % in rabbit lenses after topical phospholine iodide instillation for 15 weeks. Only an enhanced rate of glycolysis, would have maintained a normal ATP level in the lens as the importance of the citric acid cycle in the lens is still a matter of dispute (Kuck 1970).

Comparison of glycolytic intermediates and the total NAD/total NADP ratios in lenses treated with phospholine iodide and in controls suggested that the treatment increased glycolysis (Lowry et al. 1964). In our previous paper we found a decrease of lactate concentration in lenses treated with phospholine iodide (Härkönen & Tarkkanen 1970). This was assumed to be due to inhibition of glycolysis by phospholine iodide. However, the decrease in lactate concentration in the lens could also be explained by the direct effect of phospholine iodide on lens permeability. Even though glycolysis was enhanced, the lactate concentration did not rise because the lactate generated could escape through the epithelial cell membrane more easily than in an intact lens.

The function of the sorbitol pathway in the lens remains an enigma, although there is no lack of theories (Kuck 1970). Kuck (1961) suggested that these enzymes act as a pyridine nucleotide transhydrogenase system, taking up the hydrogen removed from the glucose molecule by the first two steps of the

pentose phosphate pathway and converting it into a form which can be used for the production of energy. In most tissues the role of the pentose phosphate pathway is to synthesize nucleic acids and fatty acids by producing a carbon skeleton and reducing equivalents. In the lens, however, it is doubtful whether this pathway has the same function, because there is little need of material for rapid cell growth or fatty acid synthesis. The oxidative portion of the pathway has been shown to be present in the lens but the other steps have not been demonstrated convincingly, and glucose metabolism via this pathway in the lens accounts for only a small proportion of the total glucose used. Glucose-6-phosphate dehydrogenase is assumed to be the regulatory enzyme of the pathway (see e. g. Kauffman & Albuquerque 1970; Lowry & Passonneau 1969). Therefore, the decrease of 6-phosphogluconate, NADPH, total NADP and sorbitol concentrations in lenses treated with phospholine iodide could be attributed to the decreased activities of the pentose phosphate and sorbitol pathways. This would leave more glucose-6-phosphate for glycolysis.

The high concentration of α-glycerophosphate suggested that this intermediate might play some role in the energy metabolism of the lens. Most of it originates from reduction of dihydroxyacetone phosphate by NADH and its further metabolism is much slower than its production (Kuck 1970). Since mitochondria are confined almost exclusively to the lens epithelium (see van Heyningen 1970), the functioning of the α-glycerophosphate shuttle in lens metabolism must be limited to the epithelium. The observed decrease in the α-glucerophosphate concentration of lenses treated with phospholine iodide could be interpreted as a result of diminished flux into the shuttle at α-glycerophosphate dehydrogenase because of enhanced glycolysis.

We also investigated the possible acute effects of phospholine iodide in the following way: The rabbits were given 2 drops of 0.25 % phospholine iodide to both eyes and enucleation was performed 4.5 hours later when maximum local effects had been obtained. The ATP levels in the treated lenses (1.41 ± 0.29 mmol/kg) showed some decrease as compared to the control values (1.90 ± 0.20 mmol/kg). A similar decreasing tendency was observed in the fructose diphosphate, α-glycerophosphate, and glucose-6-phosphate concentrations whereas the dihydroxyacetone phosphate, 6-phosphogluconate, sorbitol and pyruvate levels after phospholine iodide treatment were of the same magnitude as those of the controls. Even though this data is of a very preliminary nature it may be taken as indicating that phospholine iodide interferes with lens metabolism immediately after instillation although the more pronounced metabolic changes are apparent following long-term application.

Michon & Kinoshita (1968b) reported that in tissue culture demecarium bromide shifted lens metabolism to anaerobic pathways, whereas phospholine

iodide had no effect. The concentrations of these drugs required to alter the normal state of the lens were so high that such levels would never be reached in clinical situations. Therefore the authors concluded that these drugs probably do no harm in the patient situation. However, our data suggest that long-term phospholine iodide treatment in therapeutic concentrations does interfere with the energy metabolism of the lens. The increased demand for ATP is met by enhanced glycolytic activity, whereas the other metabolic pathways are suppressed. The primary cause of the change in lens permeability induced by phospholine iodide still remains obscure. It is well known that locally instilled anticholinesterase agents can depress the serum and red blood cell cholinesterase activities and also that the locally to the eye applied drug can readily penetrate the placental barrier (Birks et al. 1968). Therefore one cannot exclude a systemic effect as a source of the ocular changes. The data show, however, that the response of a tissue to certain drugs *in vitro* and *in vivo* may be different and that results obtained *in vitro* cannot always be extended to apply to *in vivo* conditions. Therefore, caution should be exercised in long-term treatment of patients with phospholine iodide.

Acknowledgments

This work was supported by the National Research Council for Medical Sciences and the Silmäsäätö Foundation.

References

Axelsson U. (1969) Glaucoma miotic therapy and cataract. *Acta ophthal. (Kbh.) 47, Suppl. 102.*

Axelsson U. & Holmberg Ä. (1966) The frequency of cataract after miotic therapy. *Acta ophthal. (Kbh.) 44*, 421–429.

Axelsson U. & Nyman K.-G. (1970) Side effects from use of long-acting cholinesterase inhibitors in young persons. *Acta ophthal. (Kbh.) 48*, 396–400.

Birks D. A., Prior J. V., Silk E. & Whittaker M. (1968) Echothiophate iodide treatment of glaucoma on pregnancy. *Arch. Ophthal. (Chicago) 79*, 750–756.

Bullard B. (1965) Pyridine nucleotides in the lens of different species. *Exp. Eye Res. 4*, 108–111.

Burch H. B., Bradley M. E. & Lowry O. H. (1967) The measurement of triphosphopyridine nucleotide and reducted triphosphopyridine nucleotide and the role of triphosphopyridine nucleotide values. *J. biol. Chem. 242*, 4546–4554.

Chylack L. T. Jr. (1971) Control of glycolysis in the lens. *Exp. Eye Res. 11*, 280–293.

DeRoetth A. (1966) Lens opacities in glaucoma patients on phospholine iodide therapy. *Amer. J. Ophthal. 62*, 619–628.

Härkönen M. & Tarkkanen A. (1970) Effect of phospholine iodide on energy metabolites of the rabbit lens. *Exp. Eye Res. 10*, 1–7.

Harrison R. (1960) Bilateral lens opacities. *Amer. J. Ophthal. 50*, 153–154.

van Heyningen R. (1965) Some glycolytic enzymes and intermediates in the rabbit lens. *Exp. Eye Res. 4*, 298–301.

van Heyningen R. (1969) The lens: metabolism and cataract. In: H. Davson, Ed. *The Eye*, 2nd Ed., Vol. 1, pp. 381–488. Academic Press, London and New York.

Jonge H. de (1964) In: *Inleiding tot de Medische Statistiek*, pp. 486–487, Deel II, 2nd Ed. Verhandeling van het Nederlands Instituut voor Praeventieve Geneeskunde, Leiden.

Kauffman F. C. & Albu¹/₂uerque E. X. (1970) Effect of ischemia and denervation on metabolism of fast and slow mammalian skeletal muscle. *Exp. Neurol. 28*, 46–63.

Kinoshita J. H., Futterman S., Satoh K. & Merola L. O. (1963) Factors affecting the formation of sugar alcohols in ocular lens. *Biochim. biophys. Acta (Amst.) 74*, 340–350.

Klethi J. & Mandel P. (1960) DPN and TPN from crystallin of young and old cattle. *Biochim. biophys. Acta (Amst.) 37*, 549–550.

Kuck J. F. R., Jr. (1961) The formation of fructose in the ocular lens. *Arch. Ophthal. 65*, 840–846.

Kuck J. F. R., Jr. (1966) Sorbitol pathway metabolites in the diabetic rabbits lens. *Invest. Ophthal. 5*, 65–74.

Kuck J. F. R., Jr. (1970) Metabolism of the lens. In: C. N. Graymore, Ed. *Biochemistry of the Eye*, pp. 201–318. Academic Press, London and New York.

Lerman S. (1961) Cytochrome C-reductase of tri- and diphosphopyridine nucleotides in rat lens. *Science 133*, 100–101.

Lerman S. & Heggeness F. M. (1961) The metabolism of xylose by the ocular lens of the rat. *Biochem. J. 79*, 224–229.

Lowry O. H. & Passonneau J. V. (1972) In: *A Flexible System of Enzymatic Analysis*, pp. 83–84. Academic Press, New York and London.

Lowry O. H., Passonneau J. V., Hasselberger F. X. & Schulz D. W. (1964) Effect of ischemia on known substrates and cofactors of the glycolytic pathway in brain. *J. biol. Chem. 239*, 18–30.

Matschinsky F. M., Passonneau J. V. & Lowry O. H. (1968) Quantitative histochemical analysis of glycolytic intermediate and cofactors with an oil well technique. *J. Histochem. Cytochem. 16*, 29–39.

Michon J., Jr. & Kinoshita J. H. (1967) Cholinesterase in the lens. *Arch. Ophthal. (Chicago) 77*, 804–808.

Michon J., Jr. & Kinoshita J. H. (1968a) Experimental miotic cataract. I. *Arch. Ophthal. (Chicago) 79*, 79–86.

Michon J., Jr. & Kinoshita J. H. (1968b) Experimental miotic cataract. II. *Arch. Ophthal. (Chicago) 79*, 611–616.

Pietsch R. L., Bobo C. B., Finklea J. F. & Vallotton W. W. (1972) Lens opacities and organophosphate cholinesterase-inhibiting agents. *Amer. J. Ophthal. 73*, 236–242.

Richterich R. (1964) In: *Klinische Chemie, Theorie und Praxis*, pp. 32–33. Karger, Basel and New York.

Shaffer R. N. & Hetherington J., Jr. (1966) Anticholinesterase drugs and cataracts. *Amer. J. Ophthal. 62*, 613–618.

Sippel T. O. (1962) Pyridine nucleotides in normal and diabetic rat lenses. *Exp. Eye Res. 1,* 368–371.

Tarkkanen A. & Härkönen M. (1969) The effects of phospholine iodide on the energy metabolism of the lens. 3rd Congr. europ. Soc. Ophthal., Amsterdam 1968. *Ophthalmologica,* Additamentum ad Vol. *158,* 455–461.

Tarkkanen A. & Karjalainen K. (1966) Cataract formation during miotic treatment for chronic open-angle glaucoma. *Acta ophthal. (Kbh.) 44,* 932–939.

Thoft R. A. (1968) Incidence of lens changes in patients treated with echothiopathe iodide. *Arch. Ophthal. (Chicago) 80,* 317–320.

Author's address:

Assoc. Prof. Ahti Tarkkanen, M. D.,
Helsinki University Eye Hospital,
Haartmaninketu 4, 00290 Helsinki 29,
Finland.