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PEROXIDE DAMAGE TO THE EYE LENS *IN VITRO* PREVENTION BY PYRUVATE

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The ability of pyruvate to protect the eye lens against physiological damage by hydrogen peroxide has been studied. The physiological damage was estimated in terms of a decrease in the ability of the lens to transport rubidium against an electrochemical gradient under organ culture conditions. Peroxide was either added directly to the culture medium or generated therein by incorporation of xanthine and xanthine oxidase. In both these cases, addition of pyruvate to the medium led to a greater accumulation of rubidium by the lens. The net accumulation of this cation in the presence of 1 to 5 mM pyruvate from the medium containing peroxide (0.2 to 0.45 mM) was very close to that observed in the absence of peroxide. The protective effect was thus substantial. The mechanism of the pyruvate effect has been discussed, and seems to be related to the scavenging of peroxide by pyruvate.

KEY WORDS: Hydrogen peroxide, tissue damage, lens damage, pyruvate, cataract, glutathione, rubidium

INTRODUCTION

Several previous studies have demonstrated that the physiology of the isolated ocular lens is adversely affected if it is cultured in medium generating active species of oxygen.[1–7] These radicals can be generated through nonphotochemical, as well as through photochemical reactions. Intra-ocular generation of these radicals has been implicated in the genesis of senile cataracts, senile macular degeneration and perhaps other age-dependent ocular pathologies.[8] By virtue of a continued light penetration into the eye during long periods of photopic vision, the photochemical generation of active oxygen species in the ocular chambers and tissues has been considered of special importance in the development of the above mentioned ocular manifestations. Superoxide is the first oxygen radical produced in most oxygen-dependent oxidation reactions.[9] This then gives rise to peroxide, hydroxyl radical, reactive metal oxygen complexes and perhaps singlet oxygen. The interaction between hydrogen peroxide and superoxide is considered a primary reaction generating the hydroxyl radical. Elimination of hydrogen peroxide is thus an effective means of preventing oxidative damage in most tissues. Intracellularly, this is achieved by peroxidases and catalases.[10] Its elimination from the aqueous and vitreous humors, so as to protect the lens cell membranes from oxidative damage due to the photochemical and nonphotochemical generation of O_2^- and H_2O_2, is perhaps achieved by its interaction with ascorbate, present in both the intraocular fluids at high concentrations.[1] Ascorbate is known to react with superoxide[11] as well as other reactive species of oxygen.[2]

The localization of catalase in the peroxisome perhaps limits its protective effect against the peroxide generated in the cytosol. Since glutathione peroxidase, the main cytoplasmic enzyme concerned with peroxide detoxification, is a selenium enzyme, it

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could be limited in selenium deficiency. A search was thus made for another agent which could scavenge peroxide intracellularly as well as extracellularly and thereby protect the lens against O_2^-/peroxide damage. Direct damage by O_2^- is averted by superoxde dismutase and ascorbate.[9,11]

The keto acids are known to react with hydrogen peroxide, generating carbon dioxide, water and organic acid as reaction products. The following reaction was discovered by Holleman in 1904 in respect of pyruvic acid.[12]

$$CH_3CO\,COOH + HOOH \longrightarrow CH_3COOH + CO_2 + H_2O$$

Since pyruvate is produced in the normal course of glycolysis, it was of interest to see if it could participate in protecting the lens against peroxide toxicity. The results presented in this communication are in accordance with this hypothesis.

MATERIALS AND METHODS

The peroxide toxicity to the lens was evaluated in terms of the effect of this compound on active transport of rubidium, an ion known to be transported by the sodium–potassium pump.[1] It should be pointed out that these transport measurements serve as a convenient index of the tissue physiological status and do not reflect exclusively the status of the membrane as is affected by active species of oxygen. Fresh rat lenses, isolated atraumatically, weighing approximately 30 mg, were incubated in medium 199 pulsed with Rb-86 and rubidium uptake measured as described previously.[2] Briefly, the lenses were incubated individually, in petri dishes (35 × 10 mm) containing 4 ml of the medium, and maintained at 37° in an incubator gassed with an oxygen–carbon dioxide mixture (95:5) at a rate of 1.6 L/minute. The use of 95% oxygen in the gassing mixture was considered appropriate for testing the effect of pyruvate under relatively strong oxidizing conditions. At the end of incubation, the radioactivity in the lenses was determined by direct gamma counting. The net uptake of rubidium was expressed as the ratio of radioactivity present in the lens water (60% of the wet weight of the lens) and an equivalent volume of the incubation medium. Peroxide was generated by addition of xanthine (0.5 mM) and Sigma (Cat. X-4500) xanthine oxidase (0.03 units in each dish) in the medium. In some experiments it was added directly. Preliminary experiments indicated that if xanthine oxidase was used in the absence of xanthine, no damage to the lens occurred.

Glutathione was estimated by the use of Ellmans' reagent. Immediately after radioactivity determinations, lenses were individually homogenized with 1 ml of 0.34 M metaphosphoric acid and a supernatant obtained by centrifugation. 0.1 ml of the supernatant was then mixed with 2.5 ml of 0.3 M Na_2HPO_4 and 0.1 ml of 5,5ı-Dithiobis(2-nitrobenzoic acid). The DTNB solution was made by dissolving 4 mg of the material in 10 ml of 1% trisodium citrate solution. The resulting yellow color was measured spectrophotometrically at 410 nm and glutathione content calculated by comparison with standards that were run simultaneously.

The peroxide in the medium was determined by iodometric titration. 0.5 ml of the medium was mixed with 500 μl of 1.5 M H_2SO_4, 100 μl of 0.6 M Kl, 50 μl of 1.7×10^{-3} M ammonium molybdate and 200 μl of 1% soluble starch. Thiosulphate (1 ml = 0.25 micromole of peroxide) was added until the blue color was discharged. A blank titration was run simultaneously after treating the sample with 20 microliters of aqueous catalase (50 units).

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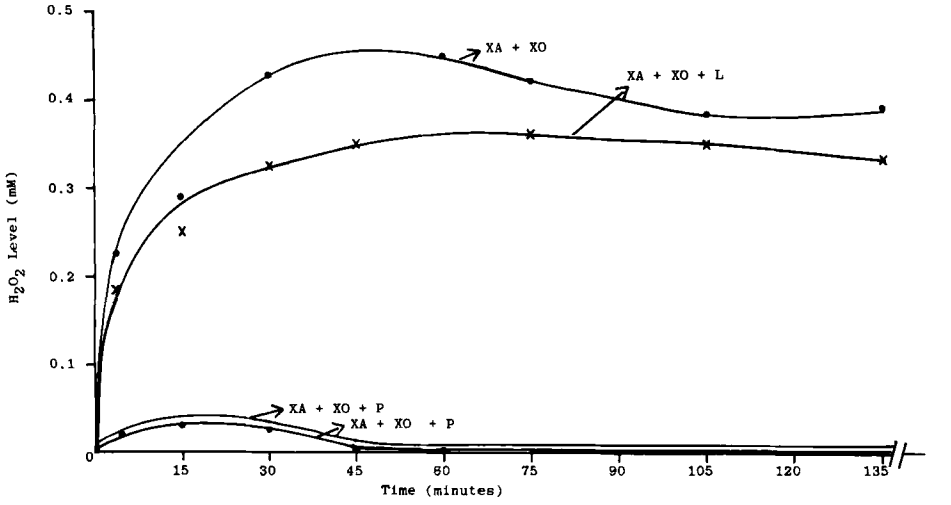

FIGURE 1 H_2O_2 Level in Incubation Medium: Medium was withdrawn at indicated intervals of time and peroxide determined as described in the text. ×---× Four lenses were incubated in 16 ml of medium; ●---● Incubation of medium alone. The bottom curves represent incubations similar to ×---× and ●---● in the presence of 5 mM pyruvate. XA = Xanthine 0.5 mM; XO = Xanthine oxidase 0.12 units/dish, .03 units/4 ml; P = Pyruvate (5 mM); L = Lens.

RESULTS

The effect of pyruvate on the concentration of H_2O_2 attained in the incubation medium is described in Figure 1. In these experiments, 16 ml of the medium was incubated without the lens or with four lenses, under conditions described previously and 0.5 ml of the aliquots taken out for analysis at indicated intervals of time. As reflected by the uppermost curve, the maximum H_2O_2 concentration achieved in the blank incubation was approximately 0.45 mM, the time taken to reach this concentration being approximately 30 minutes. In the presence of the lenses, the maximum level attained was 0.05 to 0.1 mM lower, indicating peroxide utilization by the tissue. In the presence of pyruvate, only traces of peroxide ($< 20\,\mu M$) could be detected in either case. Pyruvate is thus effective in scavenging the peroxide. In its absence, a substantial peroxide level is maintained to damage the lens.

The effect of pyruvate on the uptake of rubidium from the basal medium and from medium generating peroxide and other reactive species of oxygen is described in Figure 2. The measurements were done as a function of time. As indicated by the curve B, the uptake in the presence of pyruvate was significantly greater than that in A where the medium lacked pyruvate, equivalent amounts of xanthine and xanthine oxidase being present in both cases. The uptake in group B was in fact similar to that in group C where lenses were incubated in basal medium. There is an initial lag in the early period in all groups. As more clearly demonstrable in Figure 3, wherein the contralateral lens pairs have been compared on a percentage basis, the uptake in the presence of pyruvate was substantially greater from that in its absence.

The comparison of the uptake of rubidium from peroxide generating medium with

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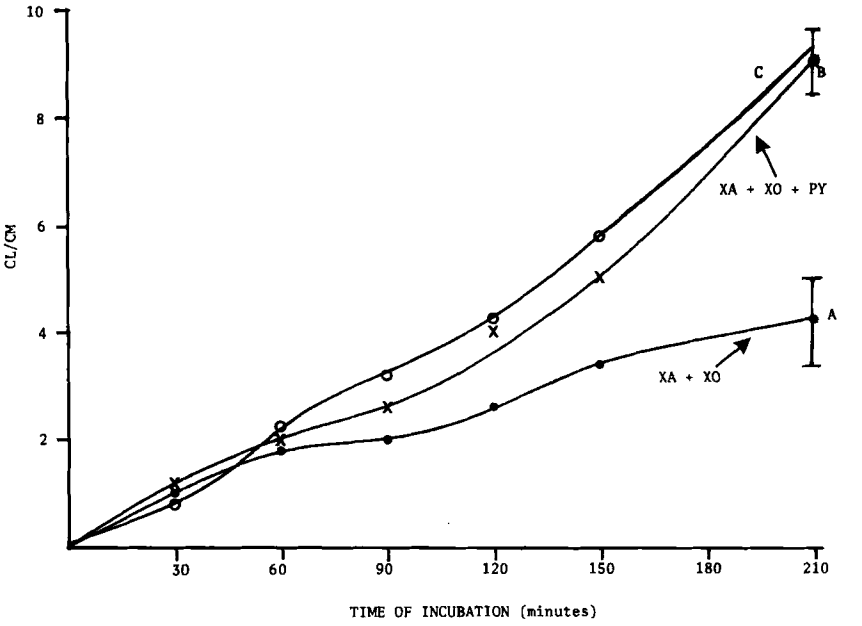

FIGURE 2 Time Course of Rubidium Accumulation: Individual rat lenses were incubated in 4 ml of TC 199 as the basal medium and the uptake of the cation determined as described in the text. The media was modified to contain additional ingredients as follows: C = TC 199 as the basal medium (BM). B = BM + Na Xanthine 0.5 mM + Na Pyruvate 5 mM. A = BM + Na Xanthine 0.5 mM. After incubation of the lenses for 1/2 hour, the following additional ingredients were added: (C) = 50 μl normal saline + 50 μl of stock ^{86}RbCl in normal saline. (B) = 50 μl xanthine oxidase (0.03 units) + 50 μl of stock ^{86}RbCl. (A) = 50 μl xanthine oxidase (0.03 units) + 50 μl of stock ^{86}RbCl. Incubation was continued and rubidium uptake determined as a function of time, the zero time being the time of rubidium and xanthine oxidase addition. Each point represents a single lens experiment except at the 210 minutes where the data represents the mean of three such experiments with standard deviations. Lenses at each points in A & B were contralateral of the same animal. Lenses in C belong to different animals of similar weights.

that from the blank medium has been done in Table I. In the blank medium, pyruvate appears to have a slight stimulating effect. Pyruvate, nevertheless, protected the lens completely against damage caused by xanthine/xanthine oxidase, the uptake in the presence of pyruvate being close to that observed with medium not containing xanthine oxidase (A). As stated earlier, xanthine oxidase, if used without xanthine, did not damage the lens. The observed damage in the presence of xanthine plus xanthine oxidase could thus not be attributed to the possible protease contamination often observed with preparations of this enzyme. Also, pyruvate did not inhibit xanthine oxidase. This was ascertained by monitoring urate production[9] in the absence and presence of pyruvate. Hence, the protective effect of pyruvate cannot be attributed to a decreased activity of xanthine oxidase and consequently lowered production of H_2O_2. The protective effect of pyruvate is thus clearly related to its ability of scavenging the peroxide. This is further borne out by the observations that pyruvate was effective even against the damage inflicted on the lens by direct peroxide addition to the medium (Table I).

The relation between the amount of pyruvate added to the medium and the protective effect it offered at fixed xanthine/xanthine oxidase level is shown in Figure

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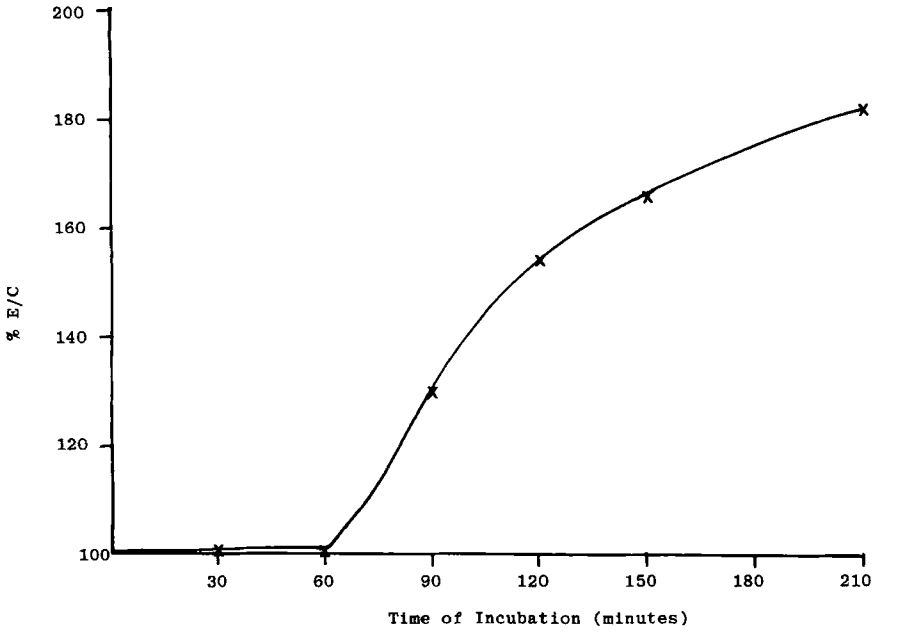

FIGURE 3 Comparison of the Uptake of Rubidium by Contralateral Lenses Incubated with and without Pyruvate: The legends are similar to that of Figure 2. E = Lenses incubated with pyruvate 5 mM. C = Lenses incubated without pyruvate. The medium contained xanthine Na 0.5 mM and xanthine oxidase 0.03 μ/4 ml. Each point represents a mean of 3 incubations, the variation from lens to lens being about 10%. The P values at 90, 120, 150 and 210 minutes are < 0.2, < 0.050, < 0.001, and < 0.001, respectively.

4. The effect appears to be stochiometric. 0.4 mM pyruvate, which is equivalent to the highest level of the available peroxide in the medium, offered the maximum attainable protection. The protection, however, was yet not complete since the maximum level of rubidium attained in the lens was still about 20% less than that in the blank controls. Increasing the concentration beyond 1 mM, however, pushed the protective

TABLE I

Lenses were incubated in 4 ml of medium for $3\frac{1}{2}$ hours in TC 199 (blank control) with additions indicated above. The CL/CM was determined by the method described in the text. At least six lenses were incubated in each group. The results are expressed as mean $\pm$ S.D. The values marked with * are significant to a P value of < 0.001. CL = Concentration of Rb-86 in lens water, CM = Concentration of Rb-86 in the initial medium of incubation

	CL/CM	E/C × 100	% of Blank Control
A Blank Control (Xanthine 0.5 mM)	10.1 ± 0.5		
B A + Py (5 mM)	11.5 ± 0.8	114 ± 5	
C A + Xanthine Oxidase (XO)	6.1 ± 0.6		
D C + Py (5 mM)	10 ± 1.0	164 ± 16	99*
A Blank Control (TC 199)	8.9 ± 0.5		
B A + PY (5 mM)	9.6 ± 0.4	108 ± 10	
C A + H_2O_2 (0.2 mM)	5.4 ± 1.2		
D C + PY (5 mM)	11 ± 0.5	204 ± 24	124*

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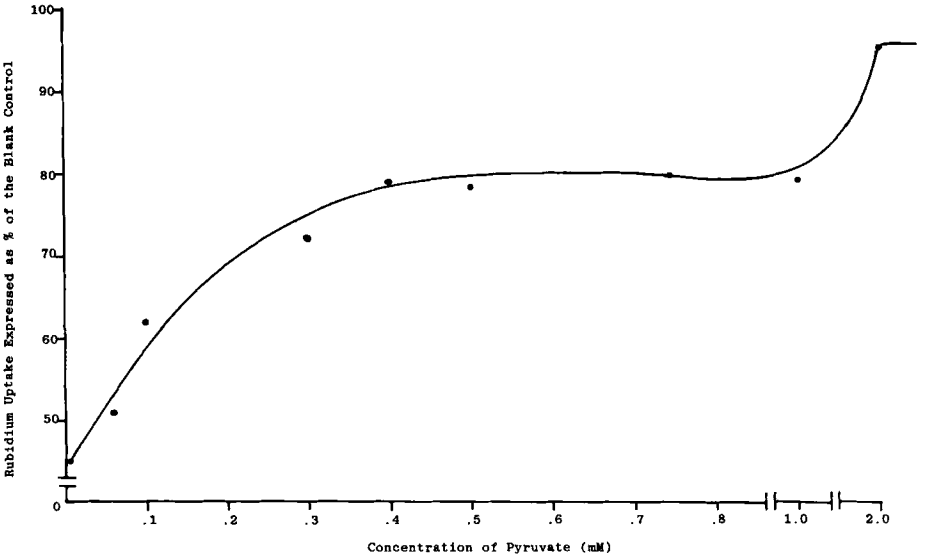

FIGURE 4 Effect of Pyruvate Level on Rubidium Uptake by Lenses Incubated with Xanthine (0.5 mM) and Xanthine Oxidase (0.3 units): The method of incubation was similar to that described in the text. Each point represents a mean of two lenses. The deviation at each point was not more than 10%.

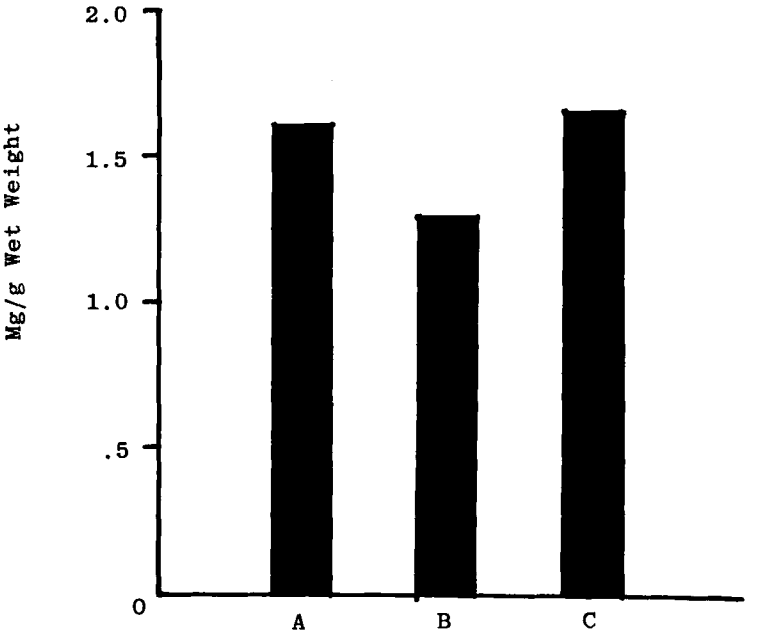

FIGURE 5 GSH Content of Lenses incubated in TC 199 + Na xanthine 0.5 mM; B = A + xanthine oxidase (0.03 units), C = B + pyruvate (5 mM); Incubation time was $3\frac{1}{2}$ hours. Each bar represents analysis of six lenses, the GSH values are as follows: A = 1.64 ± 0.09, B = 1.31 ± 0.06, C = 1.65 ± 0.05. Values in A were unaffected by removing xanthine from the medium. Values in group B differ significantly from those in Groups A and C, the P value being < 0.001.

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effect to almost completion. The incomplete protection with stochiometric amounts of pyruvate used is apparently due to competing reactions of the peroxide with oxidizable lens constituents. At high pyruvate concentrations such competition is overcome in favor of pyruvate-peroxide reaction.

Figure 5 describes the status of GSH in lenses. The addition of xanthine oxidase to the xanthine-containing medium led to a small but significant depression in the content of this thiol. In presence of pyruvate no such depression was observed.

DISCUSSION

Pyruvic and other keto acids are known to react with hydrogen peroxide, the earliest report of this reaction being that of Holleman in 1904.[12] The significance of this chemical reaction in protecting the ocular lens against peroxide toxicity has so far not been evaluated. It was thus interesting to determine if the reaction is indeed capable of protecting this tissue against the direct peroxide toxicity and hence protecting it indirectly against the toxicity of superoxide and hydroxyl radicals known to be generated under normal and several abnormal conditions. These studies with the eye lens are likely to have some general validity as well, given the difference that are known to exist in the metabolism of various tissues. The concentration of peroxide used in these experiments is much beyond the level likely to be encountered either in the ocular tissues or elsewhere in the mammalian body. Thus, the effectiveness of the agent was tested under severe situations so the results may be more widely applicable. The organ culture experiments conducted in this investigation demonstrate clearly that pyruvate can perform a protective role against oxidative stress incumbent upon O_2^- generation and its derivatization to peroxide. Since these experiments are carried out using an intact tissue organ culture technique, the results are likely to be valid for *in vivo* conditions, where pyruvate is a product of tissue metabolism and the level of peroxide attained is relatively low. Pyruvate could thus supplement the protective effect of ascorbate, a nutrient known to convert O_2^- to H_2O_2,[11] as well as to react with H_2O_2 and $OH^{\cdot}$.[2] The role of pyruvate might thus become especially meaningful in situations of low vitamin C intake. The first evidence that pyruvate might protect biological systems against H_2O_2 was obtained by Sevag and Maiweg (1934).[13] The evidence consisted of an increase in oxygen consumption observed by the addition of pyruvate to cultures of certain strains of pneumococci. The concentration of pyruvate used in these experiments (M/15), however, was so high that the increase in oxygen consumption could well be attributed to the metabolic utilization of pyruvate itself. The increase in oxygen consumption is, therefore, a difficult criterion to use while determining the protective ability of an active metabolite. Nevertheless in view of the chemical reactivity of the pyruvate with peroxide and the present findings on the maintenance of the lens cation pump, taken together with higher lens glutathione in the presence of pyruvate, the antioxidant effect of pyruvate as suggested by Sevag *et al.*, appears valid. However, some recent studies suggest the possibility that the peroxide may induce toxicity to some tissues through causing a pyruvate depletion.[14,15] The possibility of a net protective effect of pyruvate, however, cannot be ruled out.[16] These studies with the lens using an organ culture method coupled with glutathione determinations provide a more reliable evidence that pyruvate functions as a protective agent *in situ*. The pyruvate content of the rat lens is approximately 35 mg/Kg.[17] It is likely that its concentration is higher in the epithelial and superficial cortical cells

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of the tissue than in the cells situated deeper into the nucleus. The range of pyruvate level is thus substantially high. In addition, any depletion will be taken care of by its metabolic replacement. The importance of pyruvate as a protective agent would indeed be more significant in tissues dependent predominantly upon anaerobic glycolysis for energy purposes. The utilization of pyruvate for energy purposes in those tissues is in fact minimal, so much so that it is excreted out of the tissue either as such or as lactate. That is known to be the case with the lens. Under conditions where glycolysis may become sluggish, such as with aging, the oxygen toxicity to the tissues may be accelerated because of the lower amount of the pyruvate produced. Dietary supplementation with ascorbate or other antioxidants at that stage may thus be critical to save the tissue against oxidative damage.

While the protective effect of pyruvate appears largely due to its reaction with peroxide, it perhaps can protect tissues against damage by oxygen radicals through its ability also to accelerate the HMP shunt. It has been shown, that reduction of pyruvate can utilize NADPH in addition to NADH, for lactate synthesis in the cornea.[18] The universality of this finding remains to be ascertained. If this is so, the $NADP^+$ produced in this reaction will obviously accelerate the shunt. Several studies have implicated that the HMP shunt is protective in action against oxidative stress. Further studies on the significance and mechanisms of pyruvate action against oxidative damage are, therefore, in progress.

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