ALLOXAN AND LACTIC ACID DEHYDROGENASE ACTIVITY OF THE CORNEA*†

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THE complex problem of corneal vascularization has excited the interest of several investigators for many years, and it is generally believed that positive chemotaxis is the primary stimulus to neovascularization, both chemical and physical trauma being considered as potentiating factors. Low oxygen tensions are conducive to neovascularization, so that the role of localized anoxia and the resulting accumulation of certain metabolites must also be considered (Ashton and Cook, 1953); these authors review the possibilities, and also stress the importance of the removal of physical or mechanical obstruction, as originally pointed out by Cogan (1949). Thus oedema, by decreasing the corneal compactness, plays a fundamental role. Langham (1953) induced vascularization in the cornea of rabbits by injecting alloxan intracamerally, but more recently Graymore and McCormick (1968) have shown that alloxan may be applied directly to the surface of the cornea with equal success. This method, among other advantages, avoids mechanical trauma.

As a preliminary to the present study, the content, nature, and distribution of the enzyme lactic acid dehydrogenase (LDH) was studied in detail in the normal cornea (Graymore, 1966; Graymore and McCormick, 1966) and this communication reports certain changes observed after treating the cornea with alloxan.

Methods

Dutch breed adult rabbits of either sex were used in the investigations. Alloxan (pH 5.0) was administered to one eye of the anaesthetized animal either by intracameral injection (Langham, 1953) or by topical application (Graymore and McCormick, 1967); 30 minutes later the animal was killed by administering an overdose of Nembutal, and the eyes were removed and examined. The untreated eye was used for control. Several histochemical techniques were employed, all relying on the reduction of a tetrazolium salt resulting in the formation of a formazan deposit. It has been pointed out elsewhere (Graymore and McCormick, 1966) that the methods using phenazine methosulphate (PMS) are essential for the accurate localization of dehydrogenases in complex tissues such as the retina where the distribution of enzyme and diaphorase activity may show little geographical correlation. This precaution does not apply so stringently to a tissue such as the cornea, however, where both activities are located in small discrete areas. Nevertheless, the PMS method was that of choice. Whole eyes were fixed for 5 min. in buffered (pH 7.0) 5 per cent. formalin and rapidly frozen in isopentane and cardice, and cryostat sections were cut at approximately 6–7 μ . Sections were incubated for approximately 30 min. in a mixture containing sodium lactate, nicotine adenine dinucleotide (NAD), PMS, phosphate buffer (pH 7.4), and nitro-blue tetrazolium (NBT) according to the method of Berkow and Patz (1961).

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Results and Discussion

Figs 1, 2, and 3 show the results of these studies on material obtained as above and illustrate the striking changes observed after treatment with alloxan.

Fig. 1 shows the normal rabbit cornea, with a strong localization of LDH in the epithelial and endothelial layers. In the stroma, as might be expected, this activity is confined to the regularly orientated keratocytes. This picture is essentially the same as that obtained by Baum (1963). Fig. 2 shows the other eye of the same animal 30 min. after surface treatment with alloxan. A pronounced degree of oedema is apparent, and the activity of the LDH in the keratocytes is almost completely inhibited, particularly posteriorly—a fact in itself of interest. In earlier experiments, in which alloxan was injected into the anterior chamber, the partial survival of the anterior part of the cornea was attributed to the limitation of outward diffusion of the alloxan. Since the same pattern is obtained after application of

FIG. 1.—Lactic acid dehydrogenase activity in normal rabbit cornea. $\times 140$.

FIG. 2.—Lactic acid dehydrogenase activity in the fellow eye (c.f. Fig. 1) 30 min. after treatment with alloxan.

the alloxan to the epithelial surface, it would seem that this pattern of inhibition reflects some metabolic difference between the anterior and posterior surfaces, possibly associated with differing oxygen tensions.

It is also noteworthy that, even at this early stage, the epithelial layer is reduced in thickness. This is probably a consequence of the general oedema, this layer becoming more fragile and being readily sloughed away by blinking, or possibly being removed during the subsequent sectioning. Total estimations of LDH activity using the method of Bergmeyer, Bernt, and Hess (1963) showed a substantial reduction in activity in all three layers of the cornea when expressed on a *per cornea* basis, and the histochemical studies seem to substantiate these findings. It would, of course, be of interest to determine such values on a cellular basis, possibly by using DNA as a basis for measurement. It is of interest, however, that 10 days after treatment the activity of the stroma increases well above normal values, although the activity of the epithelial plus endothelial layers remains considerably reduced (Graymore, Ashton, and McCormick, unpublished results). At this stage, the cornea is very oedematous and shows little signs of recovery. Fig. 3 explains these findings. This Figure reveals that after 10 days there is a considerable invasion of fibroblasts, histiocytes, and new vessels into the periphery of the cornea. These cells are extremely rich in LDH and form a dense and diffuse pattern extending well into the cornea. The newly-formed vascular network is very evident, and the increased thinning of the epithelial and endothelial layers is marked.

FIG. 3.—Lactic acid dehydrogenase activity in cornea of rabbit treated 10 days previously with alloxan. Note vascular ingrowth and cellular proliferation from periphery.

It would seem, therefore, that treatment with alloxan is followed by oedema, accompanied by an inhibition of LDH activity. These immediate effects are, in turn, followed by vascular ingrowth and the migration of LDH-rich wandering cells into the corneal substance, thus accounting for the quantitative changes observed.

The question still remains, however, whether the failure of the LDH system precedes, or follows, cell death. For this reason, it was decided that it was necessary to investigate the effects of alloxan, at appropriate concentrations, on the LDH system *in vitro*.

The method of determining total LDH activity depends on the fall in absorption at $340\text{ m}\mu$ accompanying the oxidation of reduced NAD (NADH) in the presence of pyruvate. During the course of these investigations it was observed that alloxan destroyed the characteristic absorption peak of NADH. Spectrophotometric examination showed that

both alloxan and NADH were stable at pH 5.0, but the addition of alloxan to NADH, at a final molarity of 0.15 M (commensurate with the concentration applied to the surface of the cornea in the above experiments), resulted in a rapid fall in absorption at 340 m μ . At pH 7.4, the effect was slight and this was shown to be due to the rapid inactivation of alloxan at this pH.

In view of these observations, the total NADH activity of the corneal epithelium before and after treatment with alloxan was measured by the method of Slater, Sawyer, and Sträuli (1964). After 30 min. surface treatment with 0.15 M alloxan, the NADH activity of the corneal epithelium was inhibited to the extent of 37 ± 3.4 per cent. (mean of six corneae). There is no possibility that this inhibition is due to the presence of remaining alloxan, as the extraction procedure involved in the estimation would destroy all traces. It would seem, therefore, that this dramatic change in reduced NADH might well be responsible for the failure of the LDH system, and thus represent the primary effect on the tissue. Indeed, it would no doubt involve other enzyme systems not yet investigated. Younathan (1962) reports the inhibition of NAD-linked steps in the tricarboxylic acid cycle by relatively small doses of alloxan (kidney), and it has been reported previously that alloxan can be reduced by many factors including NADH (Lazarow, 1954). The possibility that high concentrations of coenzyme might protect against the adverse effects of alloxan has been considered but, as regards the effects of alloxan on the cornea, it has not been suggested that the primary effect may be due to a selective destruction of the reduced coenzyme by alloxan.

Certainly, the results presented here suggest that an immediate and direct effect of alloxan on the coenzyme systems should be considered as a real possibility, and that this in turn would lead to the failure of the dehydrogenase systems and cellular death. It should be stressed that the doses employed for inducing neovascularization of the cornea are high, and that preliminary experiments on the NADH content of the liver of rats treated intravenously with a diabetogenic dose of alloxan failed to show any significant changes. It is possible that the two phenomena have no common basis.

These results are essentially preliminary, and further investigations of both oxidized and reduced NAD and NADP are in progress. Nevertheless, a tentative theory may be put forward at this stage to suggest that the effect of alloxan on the cornea is to upset the redox potential of the cells, and thus to interfere with the general energy metabolism of the tissue. This would lead to oedema, and possibly neovascularization, in an attempt to remedy the position. As stated, already, however, such speculation is premature, and the present study is being elaborated and will be reported in detail shortly.

Summary

The effects of alloxan on the distribution and total content of the enzyme lactic acid dehydrogenase (LDH) in the cornea of the rabbit are described. External application of alloxan for 30 min. leads to an immediate inhibition of LDH activity, but after 10 days the LDH activity of the stroma rises above that of the controls. Histochemical studies show that this does not reflect normal recovery, but results from the invasion of wandering cells rich in LDH. Further experiments suggest that alloxan may have an immediate effect on reduced nicotinic adenine dinucleotide (NADH), and that this may indicate an upset in the redox potential of the cells, which would interfere with general energy meta-

bolism leading to cellular oedema. The failure of specific dehydrogenase systems may be secondary to that of the pyridine nucleotide balance. Neovascularization might help to remedy this position, and is facilitated by the accompanying oedema.

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