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Disulfide Stimulation of Fluid Transport and Effect on ATP Level in Rabbit Corneal Endothelium

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Transendothelial fluid transport in rabbit corneal preparations is known to be improved when perfusates are supplemented with reduced glutathione. Evidence is now presented showing that oxidized glutathione which arises by autoxidation of reduced glutathione under the conditions of perfusion is responsible for this effect. An optimal level of about 10^{-5} M-oxidized glutathione is necessary to support fluid transport while only 10^{-7} M oxidized glutathione will sustain endogenous endothelial adenosine triphosphate levels. Of the structurally related disulfides tested, cystine and to a lesser degree homocystine, can replace oxidized glutathione.

Levels of adenosine triphosphate and activities of $(Na^+ + K^+)$-activated and Mg^{2+}-activated adenosine triphosphatase were measured in individual endothelia after maximal thinning of preswollen corneal preparations had been achieved and the criteria for pump failure had been met. Data for these physiological and biochemical parameters after perfusion with basal salts, glucose or adenosine in the absence or presence of glutathione have been obtained. Adenosine triphosphate levels in the presence of basal salt perfusates are comparably elevated by supplementation with either glutathione or glucose and remain unchanged when both substrates are simultaneously available. Similarly, the elevated ATP levels that accompany adenosine supplementation are not affected by the additional presence of glutathione or glucose. Adenosine triphosphatase activities are somewhat depressed in the absence of all metabolites and are not improved by glutathione alone. These activities remain essentially normal when glucose or adenosine is present.

1. Introduction

The development of techniques for in vitro perfusion of the cornea (Mishima and Kudo, 1967; Maurice, 1972; Dikstein and Maurice, 1972) has led in recent years to a recognition of the capacity of the corneal endothelium to deturgesce the overlying stroma (Hodson, 1971; Riley, 1971; Fischbarg, 1972, 1973; Anderson, Fischbarg and Spector, 1973a; McCarey, Edelhauser and Van Horn, 1973). Although the mechanism whereby fluid is transported toward the aqueous side is not defined, the concept that an ion pump may be involved is supported by the following observations: (1) a dependence on external Na^+ (Dikstein and Maurice, 1972; Hodson, 1971; Fischbarg, 1973), K^+ (Fischbarg, 1973), and bicarbonate (Dikstein and Maurice, 1972; Hodson, 1971; Fischbarg, 1973), (2) the presence of an endothelial $(Na^+ + K^+)$-activated ATPase whose inhibition by ouabain is related to the rate of stromal swelling (Rogers, 1968), (3) the presence of a bicarbonate-dependent ATPase (Fischbarg, Anderson and Lim, 1973) and (3) a measurable potential difference across the endothelium which is dependent on the same ions as fluid transport and abolished by ouabain and other inhibitors (Fischbarg, 1972, 1973).

A dependence of fluid transport on cellular energy was demonstrated (Takahashi, 1967) by the stromal swelling that ensues when the endothelium is deprived of oxygen. Evidence that ATP furnishes the energy for a pump function is implicit rather than explicit. Transport is suppressed when endothelial ATP levels are severely reduced

yet it is not necessarily sustained when the ATP concentration is maintained at a steady-state level (Anderson et al., 1973a). Presumably unidentified factors other than cellular ATP limit an in vitro system.

An earlier study (Anderson et al., 1973a) has shown that endothelial ATP levels can be supported and, concomitantly, a substantial deturgescence of swollen corneal preparations can be achieved simply by adding reduced glutathione (GSH) to basal salt perfusates. Dikstein and Maurice (1972) had previously noted enhanced fluid transport when exogenous substrates are supplemented with GSH. These observations suggest a fundamental role of GSH in the transport process. In the current report this relationship of GSH to pump function and the generation of ATP from such substrates as glucose and adenosine was examined further. It will be shown that oxidized glutathione (GSSG) formed autoxidatively from GSH during perfusion is responsible for the beneficial effects previously attributed to GSH and that among the structurally related disulfides tested, cystine is essentially as effective as GSSG. The support of cellular ATP levels and pump function by different levels of GSSG indicates a dual influence of GSSG on fluid transport. A preliminary presentation of the current results (Anderson, Fischbarg and Spector, 1973b) included physiological data relating GSSG to corneal deturgescence.

2. Methods

The experimental protocol and analytical methods for DNA, ATP and ATPase activities were essentially the same as those previously detailed (Anderson et al., 1973a). Briefly, given pairs of fresh rabbit endothelial preparations were swollen to a thickness of 500 ± 10 μm in separate perfusion chambers by fluid imbibition from a stationary basal salt solution placed on the anterior corneal surfaces previously freed of epithelium while the endothelial surfaces were perfused with a complete medium composed of salts, glucose (6·9 mM), adenosine (5·0 mM) and GSH (0·24 mM). Where the effect of GSH alone was studied, solutions of GSH were prepared in basal salts and adjusted to pH 7·4 with CO_2 within 5–7 min prior to perfusion. Replacement of the fluid on the anterior surfaces with silicone oil initiated a period of deturgescence. During this time fluid was transported out of the stroma across the endothelium. The endothelial surfaces meanwhile were perfused with either complete medium in the case of the control or a basal salt solution supplemented with a test metabolite(s) in the case of the test preparation. Controls perfused with solutions other than complete medium are noted in the text.

Deturgescence of corneal preparations was measured by two parameters: (1) maximal stromal thinning or the difference between the initial swollen thickness and the minimal thickness achieved, and (2) thinning time or the total time elapsed between the commencement of deturgescence and the time at which the minimal thickness was no longer sustained. The criterion established for pump failure was a terminal increase in stromal thickness 7 μm greater than the minimal thickness attained. Occasionally transient increases in thickness up to 5 μm occurred that readily reversed spontaneously while increases of 7 μm were observed never to do so. All perfusions except those performed with complete medium were terminated when this standard was met. Biochemical parameters of test preparations thus reflect this terminal physiological condition. Endothelial ATP levels were also found not to differ significantly in preparations taken either 3 min after a minimal thickness ± 1 μm had been sustained or after a 7 μm increase in thickness had occurred. Controls perfused with complete medium sustained transport throughout the experimental period and were terminated after a period of thinning equal to that of the paired test preparation.

Adenosine, cystamine, GSH, L-homocystine and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were obtained from Sigma Chemical Co., St. Louis, Mo.

L-cystine and glutathione reductase were procured from Mann Research Labs., New York. Preparations of GSSG from both sources were utilized.

3. Results

The improvement GSH exerts on corneal deturgescence is most pronounced in the absence of other exogenous metabolites (Table I). Compared to perfusion with basal salts alone, GSH causes a 2·5-fold increase in thinning and a nearly two-fold prolongation of thinning time. In the presence of glucose, which alone causes a nearly two-fold increase in both parameters, GSH effects a further 50% overall improvement. With regard to stromal thinning, the absolute amount obtained with glucose is essentially the same as that observed with GSH alone. Relative to basal salts, adenosine improves thinning four-fold and extends fluid transport time better than two-fold. The addition of GSH to adenosine, however, does not significantly improve the capacity of the corneal preparation either to thin beyond the average thickness of 385 μm or to sustain fluid transport beyond the 3 hr observed with adenosine alone.

TABLE I

Effect of exogenous GSH on parameters of transendothelial fluid transport

Addition to basal salt solution		Transport parameter*			
Compound	Concentration (μM)	Stromal thinning† (μm)		Thinning time‡ (min)	
		−GSH	GSH§	−GSH	+GSH
None		29±5 (14)	77±2	70±10 (14)	130±17
Glucose	6·9	51±16	79±8	125±6	187±19
Adenosine	5·0	117±21	134±12	166±22	183±12

Excised corneas, scraped free of epithelium, were swollen in situ to 500±10 μm and deturgesced as described in the text by perfusion of the endothelial surface with a basal salt solution (Anderson et al., 1973a) supplemented as indicated.

* Data are mean±S.E. of four experiments except where noted by number in parenthesis.

† A theoretical thinning of 140±15 μm would restore a 500-μm thick corneal preparation to an in vivo steady-state thickness (Dikstein and Maurice, 1972).

‡ Thinning time for a corneal preparation perfused with complete medium was between 300–360 min.

§ GSH concentration was 240 μM.

The corresponding biochemical data for the endothelia from the corneal preparations used for the deturgescence measurements are summarized in Table II. Only in the absence of other metabolites can the effect of added GSH on the terminal endothelial ATP level be readily demonstrated. A two-fold increase in terminal ATP level occurs when a basal salt perfusate is supplemented with GSH. With exogenously supplied glucose, the endothelial ATP level is comparable to that supported by GSH alone and is not significantly affected by GSH supplementation. Adenosine alone sustains an ATP level equal to that supported by a complete medium which contained additionally glucose and GSH.

The data for in vitro ATPase activities indicate that both $(Na^+ + K^+)$-activated and Mg^{2+}-activated enzymes were functional at the time of pump failure under all

conditions of perfusion and that the activities of both enzymes were not significantly influenced by the presence of perfusing GSH. Perfusion with either glucose or adenosine maintained ($Na^+ + K^+$)-activated ATPase activity at about 90% and Mg^{2+}-activated ATPase activity at the same level as the controls. On the other hand, the absence of all metabolites resulted in depressions of both activities which were not alleviated by GSH supplementation.

TABLE II

Effect of exogenous GSH on biochemical parameters of paired corneal endothelia

Addition to basal salt solution	Per cent of respective control*					
	ATP		ATPase activity			
			Mg^{2+}-activated		($Na^+ + K^+$)-activated	
	−GSH	+GSH†	−GSH	+GSH	−GSH	+GSH
None	38·5±14·7 (6)	73·6±7·3	84·0±7·7 (5)	77·4±6·6	66·8±14·3 (5)	61·2±9·7
Glucose	70·4±12·5	75·2±11·6	103·7±10·1	95·5±9·6	84·1±8·6	102·5±6·8
Adenosine	96·1±20·8	98·9±13·6 (6)	111·9±4·6	105·0±5·9	88·7±19·5	82·1±6·3

The endothelia from individual corneal preparations described in Table I were analyzed separately according to methods previously reported (Anderson et al., 1973a). Each paired corneal control was perfused with complete medium for a period of time equal to the thinning time of the respective test preparation.

* Data are mean±S.E. from four experiments except where noted by number in parenthesis. Each value for ATP and ATPase activities, normalized with respect to DNA content of the sample, was calculated as a per cent of the value of the control. For orientation, the following values from a larger sampling of unpaired data obtained with perfusates composed of complete medium are given as means±S.E. (number of experiments): ATP, 597±45 (21) pmol/μg DNA; Mg^{2+}-activated ATPase, 229±16 (21) nmol P_i/hr/μg DNA; ($Na^+ + K^+$)-activated ATPase, 47±5 (21) nmol P_i/hr/μg DNA.

† GSH concentration was 240 μM.

It is well known that GSH, maintained in the presence of oxygen at 37°C and pH 7·4 in solutions containing traces of heavy metals, is susceptible to autoxidation. Since all these conditions prevailed during the corneal perfusion experiments, GSSG could be expected to have been present in the GSH perfusates and to have increased in amount during the perfusion period. This was verified by experiment (Fig. 1). An oxidation of GSH to the extent of 2·8% had already occurred or was inherent in the freshly prepared solution taken at zero time. Oxidation rose to 18% within 1 hr and stabilized at about 20% of the original GSH concentration over the next 1·5 hr. The agreement between the final level of GSSG in the solution remaining in the syringe and that in the effluent after passage through the perfusion chamber indicates that the mechanics of perfusion did not contribute to the oxidation. An initial level of GSH 25 times greater than the highest level used in the corneal perfusion studies was required for this experiment to meet the limit of reliable detection of GSSG imposed by the method of determination. There is a remote possibility, nevertheless, that in solutions of lower initial concentrations of GSH the rate of oxidation to GSSG may not be the same.

That the conditions in Fig. 1, however, did approach those prevailing during corneal perfusion is suggested by Fig. 2 that relates thinning to the initial concentration of GSH or GSSG. Thinning in the presence of GSSG at concentrations between

6 and 24 μM is superior to that achieved with GSH within the same concentration range. At these initial concentrations the difference effected by the two redox states in the thinning of matched pairs of corneas from the same rabbit is reliable to better than 95–99%. The supporting data are summarized in Table III. At or below the low level of 2 μM, thinning is not extensive and the thinning differential loses significance. At the high level of 240 μM, thinning with either GSH or GSSG is maximal and, although not shown in Table III but evident in Fig. 2, no significant difference in thinning is revealed. This is undoubtedly the consequence of an initially high concentration of GSH from which an appreciable amount of GSSG could have been generated. The greater reduction in the thinning difference at 24 μM compared to that at 15 μM also supports this interpretation.

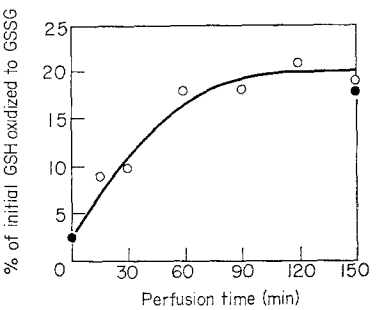

FIG. 1. Rate of 6 mM-GSH oxidation during perfusion under conditions simulating those that prevailed during deturgescence of corneal preparations.

GSH (0·03 mmol) was dissolved in 50 ml basal salt solution, quickly adjusted to pH 7·38 with 95% air/5% CO_2 and rapidly transferred to a 50-ml syringe. Zero (●) and 150 min samples (●) were taken directly from the syringe and immediately frozen. Intervening 100 μl samples of effluent from the perfusion chamber (○) were collected within 2-min intervals in tubes over dry-ice and assayed within 1 hr for GSSG spectrophotometrically at 340 nm in 3 ml of 50 mM-phosphate buffer, pH 6·8, containing 49 mM-NADPH and 15 μg glutathione reductase with a specific activity of 95 iu/mg. A piece of Parafilm shaped to a curvature resembling that of a rabbit cornea replaced the tissue.

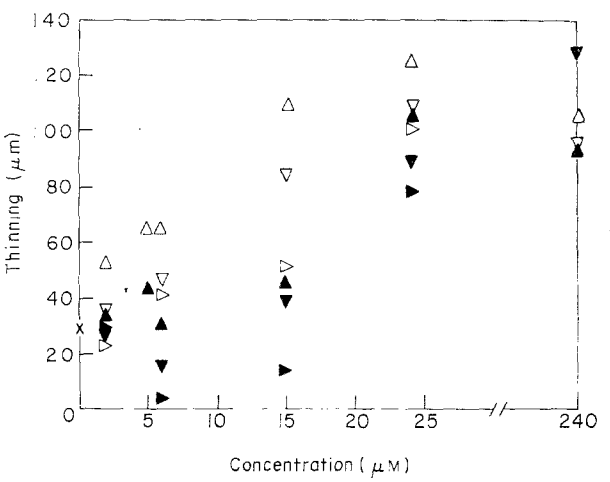

FIG. 2. Effect of the initial concentration of perfusing GSH or GSSG on transendothelial fluid transport.

Matched pairs of corneal preparations from the same rabbit were swollen and deturgesced as described under Methods. One member of each pair was perfused with a basal salt solution containing a given initial concentration of GSH (▲) while the other member was perfused with the same concentration of GSSG (△). Data from paired perfusions at a given concentration are indicated by the orientation of the triangles. For comparison, the average thinning for 14 perfusions with basal salt alone (×) is shown.

The thinning data obtained from 52 separate perfusions with either GSH or GSSG at concentrations between 0·1 and 240 μM were examined for mathematical expression by the equation $y = A - Be^{-\alpha x}$ where y is stromal thinning in μm and x is the initial concentration in μM of either metabolite in the perfusate. Where $x = \infty$, $y = A$.

TABLE III

Difference in thinning between members of matched pairs of corneal preparations with GSH or GSSG at initially equimolar concentrations

Initial concentration (μM)	Difference in thinning* ($\mu m_{GSSG} - \mu m_{GSH}$)	t-test ($P <$)
2	8·3±6·7	0·4
6	35·0±1·7	0·005
15	49·0±8·2	0·05
24	18·0±3·9	0·05

* Average value of ±S.E. from three paired corneas at each concentration perfused as shown in Fig. 2.

TABLE IV

Stromal thinning as a function of effective GSSG level in basal salt perfusates prepared with GSH

Effective GSSG concentration (μM)	Metabolite initially present in perfusate: GSSG	GSH
	Observed stromal thinning* (μm)	
0·1	29±2 (2)	26±19 (2)
2·0	42±3 (3)	44±7 (3)
15·0	67±10 (3)	65±12 (3)
24·0	112±7 (3)	102±16 (5)

Initial GSH concentrations were converted to GSSG concentrations that would exist at the time of pump failure if a rate of GSH oxidation shown in Fig. 1 is assumed. Thinning times with basal salt and 240 μM-GSH were, respectively, 70±10 min (14) and 167±32 min (4).

* Range or mean±S.E. (number of observations). Data were pooled from paired and unpaired preparations.

Since a ten-fold increase in GSSG concentration from 24 to 240 μM did not significantly affect the thinning achieved, y was assumed to equal the combined average maximal thinning of 98±14 μm obtained from a total of 7 perfusions with GSH or GSSG at 240 μM. Where $x = 0$, $y = A - B$. The value for $A - B$, was set equal to the average stromal thinning of 29±5 μm observed in 14 perfusions with basal salt solution. A least squares fitting of the data yielded a curve for GSSG described by $y = 98 - 70e^{-0.180[GSSG]}$ and that for GSH by $y = 98 - 70^{-0.035[GSH]}$.

With basal salt perfusates, thinning endures for about 70 min (Table I). Since the perfusion time with GSH at all levels equalled or exceeded this thinning time, autoxidation of GSH within the perfusion period could yield according to Fig. 1, a GSSG

concentration equivalent to that resulting from nearly a 20% oxidation of the initial GSH concentration. A comparison of stromal thinnings observed with GSH at these calculated GSSG levels to those in the presence of perfusates actually prepared with GSSG is made in Table IV. An equivalence in degree of thinning is apparent. It should be noted that a stromal thinning of 102 ± 16 μm observed with perfusates actually prepared with 240 μM-GSH differs from the 77 ± 2 μm reported for the same GSH concentration in Table I. Since consistent experimental protocol was maintained this disparity is attributed to variation among groups of animals during the 2-year investigative period. Diminished thinning responses, particularly with basal salt perfusates, among preparations from rabbits of both sexes appeared to coincide with the onset of spring and fall and continue for a 2–3 week interval.

TABLE V

*Endothelial ATP level and ATPase activities of paired corneal preparations perfused with GSSG relative to those perfused with basal salts, GSH or a complete medium**

Perfusate for corneal preparation		Per cent of respective control		
			ATPase activity	
Test	Control	ATP	Mg^{2+}-activated	$(Na^+ + K^+)$-activated
BS+GSSG (0·10–0·50)†	BS	232·0±63·0 (3)	121·0±18·0 (2)	100·9±22·1 (2)
BS+GSSG (12, 240)	Complete	104·7±22·0 (3)	94·3±11·9 (3)	86·1±5·2 (3)
BS+GSSG (2·0–240)	BS+GSH (4–240)	97·0±7·2 (5)	94·2±5·9 (3)	87·9±13·9 (3)

* One corneal preparation from a given rabbit was perfused with a solution of basal salts (BS) containing GSSG while the other served as a control. Perfusion with BS in the absence or presence of either GSSG or GSH was terminated when a 7-μm increase in the minimum deturgesced thickness occurred. Controls perfused with the complete medium were terminated after an elapsed time equal to that of the deturgescing period of the paired preparation. Biochemical data, obtained as described in Table II, are given as mean±S.E. (number of experiments).

† Initial GSSG or GSH concentration, μM.

A dual effect of GSSG on pump function and ATP level is apparent from the data in Table V. Stromal thinning, as illustrated in Fig. 2, does not become appreciable until a level of GSSG between 5–10 μM is reached while within the range of 0·10–0·5 μM-GSSG, observed thinnings of 27, 31, 17, 18 and 21 μm are essentially indistinguishable from the residual thinning that occurs with basal salts. Yet compared to a basal salt perfusate this low level of GSSG is seen in Table V to cause a two-fold increase in ATP. This is the same result observed with 240 μM-GSH (Table II). A direct comparison with complete medium also reveals that at 12 or 240 μM-GSSG, ATP levels are well maintained. With regard to ATP level and ATPase activities, an equivalence between GSSG and GSH at all tested levels is evident. Although the ATP level appeared to increase while terminal thickness diminished, no significant correlation between these parameters could be made under any perfusing condition.

The effectiveness of GSSG on fluid transport raised the question whether or not disulfide specificity is involved. Table VI summarizes the data obtained with structurally related disulfide compounds. Compared to GSSG with a capacity to thin a

500-μm thick stromal preparation about 96 μm and to sustain transport about 150 min, comparable concentrations of 24 and 240 μM-L-cystine are equally effective. Comparable also are the ATP levels supported by L-cystine and GSSG at these concentrations. L-homocystine at 24 μM is about 60 and 80% as effective as 24 μM-GSSG with respect to thinning achieved and transport time. In contrast, 24 μM-cystamine is not only entirely ineffective, but if judged by the observed terminal change in the endothelial cells from typically hexagonal to rounded forms, is cytotoxic at 24 μM.

TABLE VI

Effect of disulfide compounds on transendothelial fluid transport and endothelial ATP level in paired preparations

Addition to basal salt solution		Per cent of respective control*		
Compound	Concentration (μM)	Stromal thinning	Thinning time	ATP
L-Cystine	24 (2)†, 240 (1)	97±9	101±16	114±19
L-Homocystine	24 (3)	57±6	83±12	85±3
Cystamine	24 (3)	20±7	29±5	54±9

The control corneal preparation was perfused with a basal salt solution containing either 24 μM or 240 μM-GSSG while the paired preparation was perfused with one of the disulfide compounds. All perfusions were terminated and the endothelia treated in accordance with criteria detailed under Methods.

* Mean±range. Average values±S.E. for stromal thinning, thinning time and ATP of controls with GSSG were 96±8 μm, 154±25 min and 717±85 pmol/μg DNA, respectively.

† Number of experiments at stated concentration.

4. Discussion

The results of the present study provide evidence that exogenously supplied GSSG rather than GSH is responsible for the increased stromal thinning and prolongation of transendothelial fluid transport previously observed when perfusates of rabbit corneal preparations are supplemented with GSH (Dikstein and Maurice, 1972; Hodson, 1971; Anderson et al., 1973a). Not only does the rapid autoxidation of GSH demonstrated in Fig. 1 suggest a role for GSSG but a numerical analysis of the thinning data also leads to this conclusion. The value of 0·20 for the ratio of the calculated exponential coefficient, α, for GSH to that for GSSG indicates that comparable thinnings should result when GSH or a concentration of GSSG equivalent to 40% oxidation of initial GSH is perfused. This theoretical level of GSH oxidation agrees reasonably well with the 20% oxidation of GSH found experimentally (Fig. 1) and supports the conclusion that the enhancement of fluid transport observed in the presence of GSH is due to contaminating GSSG.

The present data also show that as little GSSG as 10^{-7} M can support a substantial cellular ATP level while an optimal level of about 10^{-5} M-GSSG is necessary to support fluid transport. Cystine and less effectively, homocystine, when judged by the same physiological and biochemical parameters, can essentially replace GSSG at a concentration of 24 μM while cystamine cannot. Taken together these findings can be interpreted as the manifestation of a relationship between the redox potential inherent to the cystine moiety and two interdependent aspects of fluid transport, i.e. ATP level and pump function.

Current knowledge of the metabolic generation of ATP as well as the physical chemistry of membrane structure and associated transport phenomena can not provide a unifying explanation for the current observations. Theoretically GSSG could influence either or both aspects of fluid transport. In the following discussion references to results actually obtained with GSH supplementation at 240 μM will be attributed to GSSG in view of the demonstrated reproducibility of the GSH effect by GSSG when the latter is perfused at levels equivalent to the adjudged GSSG contamination of the original GSH solutions.

Although GSSG at 10^{-3}–10^{-2} M can be inhibitory to the ($Na^+ + K^+$)-ATPase of the erythrocyte (Dick, Dick and Tosteson, 1969), there are no reports indicating that lower levels of GSSG have a similar effect. GSSG at 10^{-7}–10^{-5} M has, in fact, been shown here to be neither detrimental nor beneficial to the corneal endothelial enzyme. A direct influence on pump ATPase would thus seem to be excluded. An effect on the membrane, however, can be envisioned. If, because of greater exposure to GSSG, membrane thiol groups other than those of ($Na^+ + K^+$)-ATPase should preferentially interact, a conformational change could ensue that, in effect, might reduce membrane leakiness and be revealed as an increased efficiency in the transport process.

The presence of a functional hexose monophosphate shunt (HMP) in the corneal endothelium (Kuhlman and Resnick, 1959; Anderson, unpublished results) provides a possible rationale for the results with adenosine but may be a less plausible basis for those with GSSG. The substantial level of endothelial ATP supported by adenosine is similar to the finding with the erythrocyte (Bartlett and Bucolo, 1968) and may, as suggested (Marks, 1964) in regard to the latter study, be responsible for the notable maintenance of fluid transport. The observed failure of GSSG to affect significantly either the ATP level or the duration of pump function by adenosine alone is consistent with the utilization of adenosine via ribose-5-phosphate (cf. Bishop, 1964). A preferential dependence of fluid transport on the HMP could also be inferred from the prolongation of pump function that occurs when glucose is supplemented with GSSG. The increased availability of NADP that would occur with an elevated intracellular GSSG : GSH ratio (Jacob and Jandle, 1966) would conceivably stimulate glucose utilization via the shunt. Cystine could play a role similar to GSSG by effecting the reoxidation of NADPH either directly (Tietze, 1970), indirectly (Elford, Freese, Passamani and Morris, 1970) or through the regeneration of GSSG from intracellular GSH (Racker, 1955; Phil, Eldjarn and Bremer, 1957). The regeneration of NADP by disulfides, however, would eliminate any mitochondrial generation of ATP that could possibly accrue by electron transfer from NADPH. Of interest also to consider with regard to the HMP is the sparing of ATP that would be afforded during the regeneration of hexose phosphate by this pathway. A sparing of ATP by GSSG could also result from an inhibition of some ATP-requiring pathways. The low level of GSSG (10^{-7} M) that supports endothelial ATP is four orders of magnitude less than that reported for the K_m of glutathione reductase of *Escherichia coli* (Asnis, 1955) but within the concentration ranges at which metabolic inhibitors are known to be effective.

Based on the interesting correlation that agents which inhibit ($Na^+ + K^+$)-ATPase stimulate the HMS, Dikstein (1971) has proposed a regulation of ATPase activity by the glycolytic pathways through the intervention of the oxidation state of GSH. Although appealing, this hypothesis would have pump function suppressed by GSSG and clearly this is not in accord with the present findings.

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