Glucose oxidation in the chick cornea: effect of diamide on the pentose shunt

Eileen Masterson, David R. Whikehart, and Gerald J. Chader

Chick embryo corneas (stages 38 and 45) have been used to study variations in pentose shunt activity following the use of a glutathione-specific oxidizing agent, diamide, and a sulfydryl blocking agent, N-ethylmaleimide (NEM). Shunt activity was measured by the ratio of radiolabeled carbon 1 (\(^{14}\text{C}-1\)) of glucose to radiolabeled carbon 6 (\(^{14}\text{C}-6\)) of glucose derived as expired \(^{14}\text{CO}_2\). Diamide and NEM were both found to increase pentose shunt activity relative to glycolysis, although by different means. Diamide appeared to exert its effect by oxidizing glutathione and creating a demand for higher shunt activity to facilitate glutathione reduction by NADPH. Both C-1 and C-6 oxidation were increased, but C-1 oxidation was increased to a much greater extent. In contrast, NEM decreased both C-1 and C-6 oxidation, with C-6 preferentially affected. Thus NEM appears to preferentially inhibit the enzymatic machinery of the glycolytic-tricarboxylic acid cycle pathway and acts as an effective metabolic stress on the cornea. Our data suggest that the pentose shunt in the cornea may serve as an important alternative pathway under conditions of metabolic stress for glucose utilization and the production of energy (ATP) in the corneal cells.

Key words: cornea, development, diamide, thioloxidation, pentose shunt, glucose oxidation, chick embryo

Previous studies in our laboratory\(^1\) have established that the activity of the pentose shunt (the phosphogluconate oxidative pathway) is minimal in whole corneas of the chick embryo prior to transparency development (stages 40 to 45). Shunt activity increased during development and can be correlated temporally with transparency development. At the early stages (38 and 40), prior to corneal deturgescence, shunt activity is not normally measurable, although the enzymes appeared to be present already in a latent form.\(^1\) Greatest shunt activity is only seen at embryonic stage 45 when transparency is achieved. This affords an interesting test system therefore, in that agents suspected of affecting the shunt may be tested at each stage to determine whether this pathway is rendered more or less active.

Chick corneas were used in the present study because they are easily dissected and are uniform in size and weight. Chick embryonic age can be readily discerned from morphological features.\(^2\) Accordingly, the chick is a useful animal for studying corneal development, for monitoring pentose shunt activity of the cornea, and for evaluating the effects of extrinsic parameters on this metabolic pathway just prior to the initiation of transparency (stage 38) and when transparency has been achieved (stage 45). In particular, we were interested in studying the effects of diamide (azodicarboxylic acid bis(dimethylamide), a relatively specific, intra-
cellular glutathione-oxidizing agent on the developing cornea. Epstein and Kinoshita have shown that the pentose shunt is stimulated by diamide in the rabbit lens and postulated that this stimulation probably occurs by creating a demand for reduced nicotinamide adenine dinucleotide phosphate (NADPH) via glutathione (GSH) oxidation. Since the shunt is the prime supplier of NADPH for the cytoplasm of the cell, its activity must increase to replace that which was removed by the compensatory reduction of oxidized glutathione (GSSG) following diamide oxidation. It might be possible, however, for diamide to stimulate the pentose shunt through the thiol oxidation of -SH groups other than those associated with GSH. Therefore, in addition to investigating the effect of diamide on pentose shunt activity in whole chick corneas, we also studied the effect of N-ethylmaleimide (NEM). NEM is a general sulphydryl-blocking reagent which penetrates cell membranes and combines with -SH groups in both the membrane and the interior of the cell. There is a third possibility to consider for the mechanism of action of diamide on pentose shunt activity; that is, diamide may oxidize NADPH directly, generating NADP* with resultant shunt stimulation. This was investigated by direct spectrophotometric determination of the rate constant of the interaction between diamide and NADPH and by use of phenazine methosulfate (PM), an electron acceptor which generates NADP*. Materials and methods Fertile eggs of the white Leghorn domestic fowl were incubated at 37.5 ± 0.5°C, and embryonic stages were determined according to the criteria of Hamburger and Hamilton. The embryos were then killed by decapitation, and the corneas were swiftly dissected out with fine forceps and iridectomy scissors. To avoid trauma to the corneas, care was taken to handle them as little as possible before each incubation. Therefore wet weights were not obtained from corneas prior to each individual experiment. Since wet weights could not be taken after the completion of the experiment due to tissue swelling from added acid, wet weights were measured on the same number of corneas from the same stage of embryonation. Corneas handled in this manner are uniform in weight provided they are staged according to the criteria of Hamburger and Hamilton.

Pentose shunt activity was measured as follows. After paired corneal dissection, one cornea from an embryo was placed in glucose medium radiolabeled with carbon-1 (¹⁴C-1 glucose), and the other glucose medium radiolabeled with carbon-6 (¹⁴C-6 glucose). Ten corneas were pooled per flask at stage 38 and four per flask at stage 45. These were placed in 1.25 ml of Krebs-bicarbonate-Ringer's solution in 10 ml Erlenmeyer flasks. The composition of the solution (mM) was as follows: NaCl (111.56), KCl (4.82), CaCl₂ (1.04), MgCl₂ • 6H₂O (0.78), NaH₂PO₄ (0.86), NaHCO₃ (29.20), and glucose (5.00). The solution was gassed with 5% CO₂, 95% air to bring the final pH to 7.4. Each flask was tightly capped with a rubber stopper fitted with a polyethylene center well (Kontes Glass Co., Vineland, N. J.). Each flask contained either 0.25 μCi of ¹⁴C-1 glucose or ¹⁴C-6 glucose (New England Nuclear, Boston, Mass.), and corneas were incubated at 37.5°C in a Dubnoff shaking incubator for 2 hr. At this point the reaction was stopped by the addition of 0.15 ml of 6N H₂SO₄ (final pH = 1), and hyamine hydroxide (0.2 ml, New England Nuclear) was added to the center well. The flasks were incubated for an additional hour at 37.5°C to allow trapping of the released CO₂.

The recovered ¹⁴CO₂ was analyzed for radioactivity by liquid scintillation with 10 ml of Yorktown "LSC Complete" (Yorktown Research, Inc., South Hackensack, N. J.) used as the cocktail. Samples were counted in a Packard Tri-Carb Model 3380

Fig. 1. Effect of diamide on shunt activity in stages 38 and 45 chick corneas. (X ± S.E.); stage 38.

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Table I. Effect of diamide and NEM on stage 38 chick corneas

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pairs of flasks*</th>
<th>C-1</th>
<th>C-6</th>
<th>Pentose shunt</th>
<th>C-1/C-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. None</td>
<td>8</td>
<td>61.8 ± 2.8</td>
<td>53.3 ± 1.9</td>
<td>8.5</td>
<td>1.16</td>
</tr>
<tr>
<td>10⁻⁴M NEM</td>
<td>9</td>
<td>58.2 ± 6.4</td>
<td>11.4 ± 0.8</td>
<td>46.8</td>
<td>5.09</td>
</tr>
<tr>
<td>5 × 10⁻⁴M NEM</td>
<td>4</td>
<td>3.9 ± 0.7</td>
<td>1.3 ± 0.3</td>
<td>2.6</td>
<td>2.91</td>
</tr>
<tr>
<td>10⁻³M NEM</td>
<td>8</td>
<td>2.3 ± 0.2</td>
<td>0.2 ± 0.04</td>
<td>2.1</td>
<td>11.50</td>
</tr>
<tr>
<td>B. 10⁻³M diamide</td>
<td>8</td>
<td>367.4 ± 25.1</td>
<td>77.7 ± 3.5</td>
<td>289.6</td>
<td>4.72</td>
</tr>
<tr>
<td>10⁻⁴M NEM + 10⁻³M diamide</td>
<td>5</td>
<td>228.1 ± 1.5</td>
<td>1.8 ± 0.5</td>
<td>226.3</td>
<td>127.27</td>
</tr>
<tr>
<td>5 × 10⁻⁴M NEM + 10⁻³M diamide</td>
<td>4</td>
<td>66.4 ± 5.1</td>
<td>4.4 ± 0.3</td>
<td>62.0</td>
<td>15.09</td>
</tr>
<tr>
<td>10⁻³M NEM + 10⁻⁴M PM</td>
<td>4</td>
<td>26.7 ± 1.4</td>
<td>2.2 ± 0.4</td>
<td>24.5</td>
<td>12.35</td>
</tr>
<tr>
<td>10⁻³M NEM + 10⁻⁴M diamide</td>
<td>4</td>
<td>21.3 ± 1.7</td>
<td>1.3 ± 0.01</td>
<td>20.0</td>
<td>15.90</td>
</tr>
</tbody>
</table>

*10 corneas per flask.
1cpm/mg wet wt (X ± S.E.).
1C-1 calculated as oxidized from the pentose shunt (C-1 minus C-6).

Table II. Effect of diamide and NEM on stage 45 chick corneas

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pairs of flasks*</th>
<th>C-1</th>
<th>C-6</th>
<th>Pentose shunt</th>
<th>C-1/C-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. None</td>
<td>10</td>
<td>157.0 ± 3.4</td>
<td>76.1 ± 1.5</td>
<td>80.9</td>
<td>2.06</td>
</tr>
<tr>
<td>10⁻⁴M NEM</td>
<td>5</td>
<td>145.0 ± 19.3</td>
<td>67.2 ± 6.7</td>
<td>77.8</td>
<td>2.16</td>
</tr>
<tr>
<td>5 × 10⁻⁴M NEM</td>
<td>5</td>
<td>119.3 ± 7.0</td>
<td>40.2 ± 4.1</td>
<td>79.1</td>
<td>2.97</td>
</tr>
<tr>
<td>10⁻³M NEM</td>
<td>13</td>
<td>12.2 ± 0.5</td>
<td>2.6 ± 0.5</td>
<td>9.6</td>
<td>4.65</td>
</tr>
<tr>
<td>B. 10⁻³M diamide</td>
<td>10</td>
<td>396.8 ± 24.2</td>
<td>114.0 ± 10.8</td>
<td>282.9</td>
<td>3.48</td>
</tr>
<tr>
<td>5 × 10⁻⁴M NEM + 10⁻³M diamide</td>
<td>5</td>
<td>328.0 ± 12.3</td>
<td>19.8 ± 4.1</td>
<td>308.2</td>
<td>16.52</td>
</tr>
<tr>
<td>10⁻³M NEM + 10⁻⁴M diamide</td>
<td>9</td>
<td>63.2 ± 4.4</td>
<td>3.9 ± 0.5</td>
<td>59.3</td>
<td>16.24</td>
</tr>
<tr>
<td>10⁻³M NEM + 10⁻⁴M PM</td>
<td>7</td>
<td>65.7 ± 4.3</td>
<td>4.8 ± 1.0</td>
<td>60.9</td>
<td>13.56</td>
</tr>
</tbody>
</table>

*4 corneas per flask.
1cpm/mg wet wt (X ± S.E.).
1C-1 calculated as oxidized from the pentose shunt (C-1 minus C-6).

(Packard Instrument Co., Inc., Downer's Grove, Ill.) at 3% error or less. All counts were corrected for background.
Diamide, NEM, and PM were obtained from Sigma Chemical Co., St. Louis, Mo. and added to some flasks either alone or in combination as indicated.
The rate constant for the reaction between NADPH and diamide was determined by measuring the disappearance of NADPH spectrophotometrically at 340 nm and plotting the reciprocal of the concentration vs. time (initial concentrations were equimolar for a second-order reaction).

Results

Effect of diamide. Diamide increased pentose shunt activity at both 10⁻⁴M and 10⁻³M in stage 38 and 45 whole corneas as evidenced by the greater recovery of ¹⁴CO₂ from carbon-1 glucose than from carbon-6 glucose (Fig. 1). The effects were most dramatic at the higher concentration of diamide, with nearly a fourfold higher oxidation of the ¹⁴C-1 glucose in corneas at either stage. ¹⁴C-6 oxidation increased slightly at both stage 38 and 45, but the response was far less than ¹⁴C-1 oxidation.

The reaction of diamide with NADPH in vitro was evaluated by spectrophotometric analysis. In vitro measurement of the rate constant of NADPH and diamide gave a value of only 0.1M⁻¹ sec⁻¹.

Effect of NEM. The presence of NEM in the medium decreased both ¹⁴C-1 and ¹⁴C-6 oxidation in corneas from stage 38 chicks (Table I, A) and stage 45 chicks (Table II, A). At 10⁻⁴M NEM, ¹⁴C-6 oxidation was decreased to a greater extent than that of ¹⁴C-1 at both stages. At higher concentrations (5 × 10⁻⁴M and 1 × 10⁻³M) both ¹⁴C-1 and ¹⁴C-6 oxidation were greatly inhibited at stage 38, but a preferential inhibition of ¹⁴C-6 oxidation was still observed at stage 45.
Although the general effects were similar, the magnitude of the NEM effects were different at stages 38 and 45. At $10^{-3}$M NEM, the decrease in $^{14}$C-1 oxidation was 25-fold at stage 38, compared to a decrease of 12-fold at stage 45. In comparison, the decrease in $^{14}$C-6 oxidation was 200-fold at stage 38, compared to 30-fold at stage 45. At lower concentrations of NEM ($10^{-4}$M at stage 38; $5 \times 10^{-4}$M at stage 45) it was possible to decrease $^{14}$C-6 oxidation while $^{14}$C-1 oxidation appeared to be unaffected. At both stages, $^{14}$C-6 oxidation dropped a similar amount (i.e., about 40 cpm) compared with the untreated controls. At stage 45, $10^{-4}$M NEM had only a small effect. In all cases, however, the ratio of $^{14}$C-1/$^{14}$C-6 oxidation rose, indicating that the $^{14}$C-6 oxidation was preferentially inhibited ("stressed") by NEM; that is, the enzymes of the glycolytic/tricarboxylic acid (TCA) cycle pathway are more susceptible to NEM than are those of the pentose pathway.

Effects of agent combinations. It was next of interest to test whether diamide could override the inhibitory effects observed with NEM at both stage 38 (Table I, B) and stage 45 (Table II, B). The presence of $10^{-3}$M diamide in medium containing NEM stimulated $^{14}$C-1 oxidation by threefold to fourfold if the concentration of NEM used was such that only $^{14}$C-6 oxidation was affected by NEM ($10^{-4}$M at stage 38, $5 \times 10^{-4}$M at stage 45). The increase was similar to that seen with diamide alone and indicated that the pentose shunt was still fully functional under these inhibitory conditions. At these concentrations of NEM, however, $^{14}$C-6 oxidation (glycolytic/TCA cycle) was depressed, and addition of diamide to the medium with NEM further decreased $^{14}$C-6 oxidation (Tables I, B, and II, B).

Addition of $10^{-3}$M diamide to medium containing a greater concentration of NEM ($5 \times 10^{-4}$M or $10^{-3}$M at stage 38; $10^{-3}$M at stage 45) resulted in a moderate increase in $^{14}$C-1 oxidation and only a slight increase in $^{14}$C-6 oxidation compared to corneas incubated in a similar concentration of NEM alone. This response to diamide by the embryonic corneas in the presence of higher concentrations of NEM was much less than that seen with diamide alone, as would be expected. In a similar manner, addition of $10^{-4}$M PM to medium containing $10^{-3}$M NEM at stages 38 or 45 resulted in only a moderate increase in $^{14}$C-1 oxidation and a slight $^{14}$C-6 oxidation increase which was far less than that elicited by PM alone.1

Discussion

A ratio of C-1/C-6 oxidation greater than 1.0 indicates a preferential cleavage of carbon-1 of the glucose molecule and is evidence of pentose shunt activity. If glucose were metabolized solely via the glycolytic pathway and the TCA cycle, a ratio of exactly 1.0 would be obtained.7 Diamide increases $^{14}$C-1 and $^{14}$C-6 oxidation of glucose at stages 38 and 45 in the chick cornea, but $^{14}$C-1 oxidation is increased much more than $^{14}$C-6 oxidation. This indicates that diamide not only increases general glucose oxidation, but preferentially stimulates shunt activity both before (stage 38) and after (stage 45) transparency is achieved. Diamide may act to stimulate shunt activity via one or more of three mechanisms: (1) by oxidizing GSH, creating a demand for available NADPH to reduce GSSG; (2) by oxidizing sulfhydryl groups on macromolecules other than GSH, which in turn may influence shunt activity; and (3) by directly oxidizing NADPH.

Previous investigators have compared the oxidation of GSH by diamide with published values of oxidation of NADPH by diamide and have established that the oxidation of NADPH is some 100-fold lower.8 The calculated value for the rate constant of diamide and NADPH obtained in our laboratory is even less. Our value is 0.1 and the published value is 3.2.8 The possibility that diamide is acting to stimulate the pentose shunt through a direct effect on NADPH thus appears negligible.

With increasing NEM concentration, there is decreased $^{14}$C-1 and $^{14}$C-6 oxidation at both stages 38 and 45. At stage 38, $10^{-4}$M NEM
decreased $^{14}$C-6, but not $^{14}$C-1 oxidation. This indicates a preferential inhibition of glycolysis and TCA cycle function at this concentration. Pentose shunt activity is normally low at this stage, so that the activity of the pentose shunt must be increased by NEM to account for the increase of C-1/C-6. At stage 45, $5 \times 10^{-4}$M NEM reduced both $^{14}$C-1 and $^{14}$C-6 oxidation by about 40 cpm. This also indicates that TCA cycle oxidation (symmetric cleavage of glucose) is preferentially inhibited whereas the pentose shunt is affected to a lesser extent. Either the TCA cycle enzymes are more susceptible to NEM treatment than are the enzymes of the shunt, or NEM can more quickly block TCA cycle activity by tying up coenzyme A through its thiol group. When this occurs, pyruvate is blocked from entering the cycle, and the cycle stops. A third possibility is that the glycolytic enzymes are especially susceptible to NEM and block glucose from entering the TCA cycle through this pathway.

Further proof that lower concentrations of NEM inhibit the TCA cycle or glycolysis to a greater degree than they do the shunt is found in the effect on glucose oxidation of $10^{-3}$M NEM and $10^{-3}$M diamide at stage 38 or $5 \times 10^{-4}$M NEM and $10^{-3}$M diamide at stage 45. Recovery of $^{14}$CO$_2$ from $^{14}$C-labeled glucose is virtually the same as when diamide alone is present, suggesting an unimpaired shunt. However, with both compounds in the medium, $^{14}$C-6 CO$_2$ recovered is considerably less than with either compound alone. This suggests that diamide and NEM together further increase the susceptibility of the TCA cycle to NEM, perhaps by exposing more enzyme $\text{--SH}$ groups to the action of NEM.

Corneas are more susceptible to NEM at stage 38 than at stage 45, and dramatic differential effects can be noted at lower concentrations of this agent. This may be because the younger corneas are more permeable to NEM or because there are fewer $\text{--SH}$ groups to tie up at the younger age. Support for this latter conclusion comes from previous data on the $Q_{10}$ of chick corneas during development. The $Q_{10}$ was found to increase as embryonic age increased, indicating conversion to a more enzymatically efficient system as the cornea ages.

There is almost total inhibition of TCA-cycle functioning and pentose shunt activity with $5 \times 10^{-4}$ NEM and $10^{-3}$M NEM at stage 38. Similar inhibition is seen with $10^{-3}$M NEM at stage 45. Even at these concentrations, however, $^{14}$C-1 oxidation is greater than that of $^{14}$C-6. The effects of NEM on $^{14}$C-1 and $^{14}$C-6 CO$_2$ recovered are the opposite of the effects of diamide, and it appears that diamide does not stimulate the shunt by virtue of its nonspecific thioloxidizing properties. Accordingly, only the first possibility discussed above remains—that diamide stimulates pentose shunt activity via its oxidation of GSH.

In medium containing both $10^{-3}$M NEM and diamide at both stages 38 and 45, corneal glucose oxidation is increased over that seen with NEM alone. There is a moderate increase in $^{14}$C-1 and a slight increase in $^{14}$C-6 oxidation. The increase in $^{14}$C-6 oxidation is probably due to shunt recycling, since glycolysis and the TCA cycle are virtually non-functional at these concentrations of NEM. If PM is substituted for diamide and added to $10^{-3}$M NEM, similar results are obtained. PM acts by generating NADP$^+$ and thus stimulates the pentose shunt. PM alone would stimulate $^{14}$C-1 oxidation much more significantly, indicating that at this concentration of NEM, the pentose shunt is no longer completely functional.

The results in the presence of diamide and NEM together, or PM and NEM together, indicate that the shunt can survive NEM treatment ("stress") better than can the glycolytic pathway or the TCA cycle and can still respond to stimulation even under these adverse conditions. In fact, if the TCA cycle enzymes are protected within the mitochondria from NEM and if NEM blocks TCA function by blocking the glycolytic pathway or by tying up coenzyme, then it is possible that the shunt may be feeding products into the TCA cycle, which would result in an in-
crease in oxidation at $^{14}$C-6. This result is seen with simultaneous addition of diamide and concentrations of NEM which almost totally abolish all glucose oxidation. The pentose shunt, in addition to its role as a supplier of pentoses needed to form DNA, RNA, and ATP, generates essentially all extramitochondrial NADPH. This NADPH may be a source of mitochondrial ATP via an electron shuttle through the mitochondrial membrane. It serves potentially then, if not in fact, as an alternative energy pathway for ATP synthesis from glucose by circumventing the TCA cycle. The shunt requires no additional ATP to operate and does not depend on the availability of 4-carbon dicarboxylic acids in the cycle. The shunt also operates as a detoxifying mechanism for lipid peroxides by operating a GSH redox system.

In conclusion, the mechanism by which diamide increases pentose shunt activity is via its oxidation of GSH. Moreover, it appears that pentose shunt activity is able to withstand the stress of NEM treatment better than glycolysis and the TCA cycle. This is true both prior to transparency at stage 38 when, normally, the shunt is not functional and after transparency is achieved at stage 45 when shunt activity is high. It would indicate that the pentose shunt may be more important than realized previously in situations which involve insult to the cornea. Embryonic chick corneas thus provide an excellent model system for testing the effects of stresses due to drugs or other agents on glucose metabolism and energy utilization as well as for studying the biochemical factors which contribute to normal corneal transparency.

We express our appreciation to Dr. Jin H. Kinoshita for helpful discussions throughout the course of these experiments.

REFERENCES