Effects of monosodium glutamate on chick embryo retina in culture

Liane Reif-Lehrer, Joy Bergenthal, and Laila Hanninen

Monosodium glutamate added to 12-day chick embryo retinas in culture causes severe morphologic damage to the retina as judged by light microscopic examination. Damage is evident after a few hours with concentrations as low as 0.3 mM. Glutamyltransferase induction is also appreciably inhibited by the amino acid. General protein synthesis and RNA synthesis appear to be less affected.

Key words: glutamate, retina, culture, chick embryo, glutamyltransferase, morphological, damage.

Glutamic acid and glutamine play an important role in tissue metabolism and are present in high concentration in the retina. The former amino acid can be converted to the latter by means of the enzyme glutamine synthetase. This enzyme as measured by its glutamyltransferase (GT) activity has a higher specific activity in the retina than in any other tissue examined. In recent years, Lucas and Newhouse, Potts, Modrell, and Kingsbury, Olney, Cohen, and others have reported that mice and rats injected with monosodium glutamate (MSG) shortly after birth exhibit severe damage to the non-receptor cells of the retina. Because of recent interest in glutamate as a putative neurotransmitter, the high endogenous concentrations of this amino acid in the retina, and our own interest in glutamine synthetase, we decided to examine this effect of glutamate on the chick embryo retina in culture. These experiments have shown that even at physiologic concentrations of glutamate the retina is severely damaged in a matter of hours as judged by morphologic examination. Biochemically, increasing concentrations of glutamate have an increasingly negative effect on the induction of GT. In contrast, the amino acid had a much smaller (neg.

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*Abbreviations: GT, γ-glutamyltransferase; CS, glutamine synthetase; MSG, monosodium glutamate; TCA, trichloroacetic acid; GHA, glutathione reductase; S.A., specific enzyme activity, i.e., as a function of micrograms of protein; and ERG, electroretinogram.
ative) effect on general protein synthesis as measured by incorporation of a radio-active precursor into trichloroacetic acid (TCA) precipitable protein. An interesting observation which also grew out of this study was that retinas simply maintained in culture medium exhibit distinct morphologic changes after an overnight incubation.

Methods and materials

Morphologic studies. Retinas cultured for 1 to 4 hours as well as uncultured control retinas were taken from 12-day-old chick embryos. Retinas cultured for 24 hours were taken from 11-day-old embryos. In each case the lower temporal quadrant of the retina was dissected and placed into 3 ml. of Eagle's basal medium without glutamine (Microbiological Associates, Bethesda, Md.) containing 5 per cent dialyzed fetal calf serum, (Grand Island Biological Co., Grand Island, N. Y.). In some experiments, the serum was treated with 100 mg. per milliliter Norit A (Fisher Scientific Co., Medford, Mass.) at 45° C. for 1 hour. Cortisol (0.01 mg. per milliliter = 2.6 x 10^{-6} M), monosodium glutamate (0.1 to 2.4 mM), and L-glutamine (1.2 to 2.4 mM) (all from Sigma Chemical Company, St. Louis, Mo.) were added as described in specific experiments. The tissue was incubated at 37° C. in 16 by 125 mm. disposable plastic tubes placed on a Bellco rocker platform on supports such that the medium flowed gently back and forth between the bottom of the tube and just below the cap. The platform rocked at 10 tilts per minute.

At time of harvest glutaraldehyde (50 per cent, Fisher Scientific, Pittsburgh, Pa.) was added to the tubes such that the final concentration of fixative was 4 per cent. These were allowed to stand on ice for one hour. They were then rinsed overnight at 4 to 5° C. with four changes of Earle's buffer (Baltimore Biological Laboratories, Baltimore, Md.), pH 7.3, without phenol red, and preserved for one hour in 1 per cent osmium tetroxide (Poly sciences Inc., Warrington, Pa.) in Earle's buffer, pH 7.2 to 7.3. The tissues were dehydrated in an ascending series of ethanols and embedded in Epon. Sections, 1 μ thick, were cut on an LKB ultratome with glass knives and were stained with 1 per cent toluidine blue (Fisher Scientific) in borax. Attempts were made to section the retinas orthogonal to the limiting membranes. However, the curling of the tissue in culture, and the absence of distinct landmarks, with reference to the ora serrata, in every section, prevented us from obtaining precisely the same region of each retinal quadrant; this resulted in somewhat variable retinal thickness in the sections.

Biochemical studies. Whole retinas from 11 to 14-day-old embryos were excised and put into culture as previously described{11, 13} in 5 ml. of medium in 50 ml. Erlenmeyer flasks. The medium was the same as that used for the morphologic studies. In one control experiment only lower temporal quadrants of retinas were incubated in tubes as for morphologic experiments. Retinas were harvested after 24 hours (except where otherwise indicated) and assayed for glutamyltransferase (GT) activity and protein as previously described.{14} In a few experiments, 14C-phenylalanine (Schwarz, Orangeburg, N. Y.), 1H-histidine (New England Nuclear Corporation (NEN), Boston, Mass.), 14C-glucosamine (NEN), 14C-glutamine (NEN), or 14C-amino acid mixture (NEN) were added to the medium and retinas subsequently assayed for the amount of the amino acid which was taken up by the retina and/or incorporated into trichloroacetic acid precipitable protein as previously described{11, 13} (Method A) or by the method of Bolam (Method B).{15, 29} GT activity is reported as micrograms of glutamylhydroxylic acid (GHA) formed per hour per microgram of protein. GHA used as a standard was purchased from Sigma Chemical Company. Radiolabeled precursors incorporated into protein are reported as counts per minute per milligram of protein (both radioactivity and protein were measured on the redissolved precipitation in the case of Method A). Samples were counted in 5 to 10 ml. Aquasol (New England Nuclear Corporation, Boston, Mass.) in plastic counting vials (Rochester Scientific, Rochester, N. Y.) in a Beckman ambient temperature scintillation counter.

The purity of glutamate and glutamine were analyzed on an amino acid analyzer. No glutamine was found in the glutamate; the contamination of glutamine by glutamate was about 3 per cent.

Results

Morphologic observations. When retinas were treated with various concentrations of MSG for varying periods of time, damage to the retina increased both with time and with concentration (Figs. 1 through 3). Fig. 1. A shows a control retina which was fixed without being put in culture. The other retinas shown in Fig. 1 were in culture for 75 minutes. The retina cultured

A total of seven experiments were done for morphologic examination. The first two were controls to examine retinas that were cultured with and without cortisol. The other five contained samples that were treated with glutamate (and in some cases, glutamine) for various times and at various concentrations (see Results).

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Fig. 1. Light micrographs of retinas which were taken from 12-day-old chick embryos. The retina in A was excised, placed into culture medium at room temperature, and glutaraldehyde was added within 5 to 10 minutes. The retinas in B through E were maintained in culture in medium containing 5 per cent Norit-treated serum and 0.01 μg per milliliter cortisol for 75 minutes. B, no glutamate added; C, 0.1 mM (final concentration in the medium) glutamate; D, 0.3 mM glutamate; E, 1.2 mM glutamate. One micron Epon sections, toluidine blue. Magnification of all figures x260.
in the absence of any additives (Fig. 1, B) or in the presence of 0.1 mM MSG (Fig. 1, C) both look fairly normal. The only visible change in these cultures is an apparent increase in foci of extracellular spaces in the inner nuclear layer, probably a fixation artifact in the more fragile cultured retinas. The presence or absence of 0.01 μg per milliliter of cortisol did not appear to influence the morphology of the retinas in any obvious way. In the presence of 0.3 mM glutamate, (Fig. 1, D) the retina shows obvious beginnings of damage. The ganglion cell layer is somewhat swollen, and there is evidence of disruption in the inner nuclear layer. There are also some pyknotic nuclei present, and many cells in the inner half of the inner nuclear layer look somewhat swollen, as do many horizontal cells at the outermost region of the inner nuclear layer.

Fig. 1, E shows that after 75 minutes with 1.2 mM glutamate, extensive damage to the retina has occurred everywhere including the receptor layer. The nerve fiber layer is swollen, the ganglion cells are extremely swollen, the inner plexiform layer has developed many vacuoles and has an almost "lacy" appearance. Much of the inner nuclear layer is destroyed; there are many pyknotic nuclei and many vacuoles. The horizontal cells are extremely swollen and the outer plexiform layer has been reduced to only a fine line. The photoreceptor layer appears to be much thinner than in the other retinas in Fig. 1. The photoreceptor inner segments extending beyond the external limiting membrane
Fig. 3. Retinas were maintained in culture medium containing 5 per cent Norit-treated serum; no cortisol was present. A, no glutamate added, 3.5 hours in culture; B, 2.4 mM glutamate for 1 hour; C, 2.4 mM glutamate for 2 hours; D, 2.4 mM glutamate for 3.5 hours; E, 2.4 mM glutamine for 3.5 hours; F, 1.2 mM glutamine for 21 hours. One micron Epon sections, toluidine blue, all figures ×260.

Data similar to that in Fig. 1 were obtained at 165 minutes and 4 hours (not shown) and indicated that MSG damage increased as a function of time (at a given concentration) as well as, as a function of concentration.

Retinas cultured for 23 hours are shown in Fig. 2. After this length of time, in the absence of MSG (Fig. 2, A) the retinas sometimes look in good condition. More often there seems to be evidence of some damage, occasionally at the outer layers but most commonly at the nerve fiber layer. The damage, however, is slight compared to that seen in retinas treated with 0.3 mM, or greater, of MSG. After 23 hours with 0.1 mM MSG (Fig. 2, B), it is still difficult to distinguish the retinas from controls without glutamate. However, in retinas cultured for this length of time with 0.3 mM glutamate (Fig. 2, C) the damage is extensive being most common and most severe in the nerve fiber and ganglion cell layers. The inner nuclear layer also seems to be subject to damage and has developed increasing numbers of pyknotic nuclei. In some of the
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Fig. 3, E and F. For legend, see opposite page.

Fig. 3 shows that with 2.4 mM glutamate, extensive damage to the retina may be seen after 60 minutes (Fig. 3, B), 120 minutes (Fig. 3, C), and 3.5 hours (Fig. 3, D). After 24 hours at this concentration (not shown), damage to the retina does not appear to be any greater than with 1.2 mM glutamate (Fig. 2, D) for the same length of time. In contrast, however, after 3.5 or even 21 hours in culture, the presence of 2.4 and 1.2 mM, respectively, of glutamine seems to have very little damaging effect on the retina (Fig. 3, E and F).

Biochemical findings. Retinas left in culture overnight in the presence of 0.01 μg per milliliter of cortisol develop a high level of glutamyltransferase activity, the induction being sometimes as much as 8-fold higher than controls not treated with the steroid. Monosodium glutamate added to such cultures appears to be inhibitory to the development of the enzyme activity (Fig. 4). The amount of inhibition appears to increase with increasing concentration of MSG from 0.01 to 2.4 mM. Higher concentrations (10 mM) did not give rise to additional inhibition. MSG at a concentration of 2.4 mM generally inhibits induction on the order of 70 per cent, at the end of 24 hours, although the results tend to vary somewhat. This concentration of MSG appears to have relatively less effect on incorporation of radiolabeled precursors into trichloroacetic acid-precipitable material after a similar length of time in culture. Incorporation of 14C-phenylalanine, a mixture of 14C-amino acids, or 3H-uridine, were inhibited by 15 to 35 per cent by 2.4 mM glutamate. This same level of glutamate had no effect on either uptake or incorporation of 14C-glucosamine (Table I).

Discussion

In recent years, several laboratories have reported that systemic administration of MSG to newborn mice and rats causes severe damage to the inner layers of the retina without damaging the receptor cells. This effect is not readily produced in adult animals. Biochemical effects of the amino acid have also been reported in the intact rat retina. From in vivo experiments in general, and especially because of the known blood retinal barrier it is difficult to determine the concentration of the injected glutamate which ultimately acts upon the retina tissue. We, therefore, decided to examine the effect of MSG in a tissue-culture system. Because of our previous interest in chick retina glutamyltrans-
Fig. 4. Inhibition of α-glutamyltransferase induction by monosodium glutamate. All cultures, except for controls to determine the fold of induction by cortisol contained 0.01 μg per milliliter of cortisol. % inhibition = [100 - (S.A. with glutamate/S.A. without glutamate)] x 100. 
S.A. = specific activity CT = microgram of glutamohydroxamic acid (GHA) formed per hour per microgram of protein. The data in the figure are a composite from two experiments; each value in each experiment is derived from five retinas cultured independently. The three highest concentrations were done in both experiments, and the values presented are the average of the per cent inhibition obtained in the two experiments. The values from the two experiments differed by less than 4 per cent. Retinas were left in culture for 24 hours. At the end of this time the S.A. of the retinas maintained without cortisol was 0.11 ± 0.01 and 0.12 ± 0.01 in the two experiments, respectively. The fold of induction (S.A. in presence of cortisol/S.A. in absence of cortisol) was 9.1 and 5.5, respectively. The average mean deviation for each set of five replicate cultures had an average value of ± 10.7 per cent calculated for the two experiments combined. Untreated, commercially dialyzed fetal calf serum was used in both experiments. The age of the embryos was 12-days throughout.

As well as for matters of convenience regarding the size of the retina, chick embryo retinas were used in the present study. Whole retinas were used for the biochemical studies, but for reasons of reproducibility in sampling, only 1 quadrant (the lower temporal quadrant) of the retina was consistently used for morphologic studies. Piddington and Moscona have reported that throughout embryonic development GT activity is highest in this quadrant and increases fastest there until the eighteenth day of development.

The morphologic results obtained in the present study are generally in agreement with the findings of Lucas and Newhouse; Potts, Modrell, and Kingsbury; and Olney; a possible exception concerns the fate of the receptor cells. When MSG was added to the medium at concentrations of 0.1 to 2.4 mM, the lowest concentration appeared to have no deleterious effect on the retina. However, treatment with 0.3 mM MSG resulted in an edematous ganglion cell layer and the beginnings of some disruption in the inner half of the inner nuclear layer after only 1 to 2 hours in culture. Higher concentrations of MSG (1.2 and 2.4 mM) showed extensive retinal damage by 75 minutes.

The damage due to MSG is very evident in the nonreceptor cells of the retina; however, a distinct thinning of the receptor cell and outer plexiform layers is apparent even at short times with the higher concentrations of glutamate. The reduction of the number of photoreceptor inner segments extending beyond the ex-
Table I. Effect of monosodium glutamate on uptake and incorporation of some radiolabeled compounds

<table>
<thead>
<tr>
<th>Time in culture (hours)</th>
<th>Concentration of glutamate* (mM)</th>
<th>Radioactivity taken up† (c.p.m./mg. protein)</th>
<th>Radioactivity incorporation‡ (c.p.m./mg. protein)</th>
<th>Per cent inhibition due to glutamate§</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>14C phenylalanine 0</td>
<td>—</td>
<td>11,820 ± 140</td>
<td>35</td>
</tr>
<tr>
<td>5</td>
<td>14C phenylalanine 1.2</td>
<td>—</td>
<td>7,483 ± 516</td>
<td>35</td>
</tr>
<tr>
<td>5</td>
<td>14C phenylalanine 2.4</td>
<td>—</td>
<td>7,483 ± 25</td>
<td>35</td>
</tr>
<tr>
<td>24</td>
<td>14C phenylalanine 0</td>
<td>—</td>
<td>35,274 ± 2,492</td>
<td>—</td>
</tr>
<tr>
<td>24</td>
<td>14C phenylalanine 2.4</td>
<td>—</td>
<td>23,461 ± 3,099</td>
<td>—</td>
</tr>
<tr>
<td>24</td>
<td>14C amino acid mixture 0</td>
<td>—</td>
<td>32,579 ± 987</td>
<td>—</td>
</tr>
<tr>
<td>24</td>
<td>14C amino acid mixture 2.4</td>
<td>—</td>
<td>27,729 ± 1,609</td>
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</tr>
<tr>
<td>24</td>
<td>3H uridine 0</td>
<td>—</td>
<td>62,022 ± 1,553</td>
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<td>41,789 ± 3,963</td>
<td>33</td>
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<td>6,874 ± 335</td>
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<tr>
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<td>14C glucosamine 2.4</td>
<td>14,701 ± 7,48</td>
<td>6,574 ± 355</td>
<td>0</td>
</tr>
</tbody>
</table>

In these experiments, at time of harvest, the retinas were washed with water containing 20 to 50 μg per milliliter of the nonradiolabeled analogue of the radiotope precursor used in the experiment.

*Added to initial medium and left in for the total culture period.
†For a given aliquot of retinal lysate as a function of the amount of protein in such an aliquot of lysate.
‡Into cold TCA-precipitable protein.
§100 - [(c.p.m./mg. protein in presence of glutamate/c.p.m./mg. protein in absence of glutamate) × 100].

These experiments, at time of harvest, the retinas were washed with water containing 20 to 50 μg per milliliter of the nonradiolabeled analogue of the radiotope precursor used in the experiment.

Table I. Effect of monosodium glutamate on uptake and incorporation of some radiolabeled compounds

*It should be noted that even though both studies were done with the cultured chick embryo system, there are differences in media and other culture conditions which might conceivably account for the diverse results observed by these workers and ourselves.

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close to 70 per cent inhibition of induction using 2.4 mM MSG. This was surprisingly similar to the effect of glutamine on this enzyme reported by Kirk and Moscona and by Reif-Lehrer and Lindewall. The MSG used in the present experiments was shown to be completely free of glutamine by analysis on an amino acid analyzer. However, Terner, Eggleston, and Krebs reported that ox retinas incubated in 10 mM MSG showed about a 5-fold increase in glutamine concentration after 60 minutes. In 12-day-old chick embryo retinas, after overnight incubation with cortisol to induce GS activity, about 30 per cent of glutamate (10^{-4}M) provided in the medium, was converted to glutamine in 60 minutes. However, since the inhibition by glutamine becomes maximal (70 per cent) only at concentrations greater than 8 mM and is smaller at lower concentrations, it seems unlikely that glutamine, endogenously formed from glutamate, accounts for the total inhibition of GT induction seen in the present study.

Freedman and Potts reported no difference in either GS or GT activity in retinas from albino rats which had been injected with glutamate from the second to the twelfth day of life. Since this is very close to the time when rat retinas normally exhibit endogenous induction of these enzyme activities, it is difficult to assess whether or not these results are in conflict with ours. In preliminary experiments, MSG does not appear to affect previously induced GT activity in our system. In any case, since both glutamate and glutamine inhibit GT induction, but only the glutamate causes morphologic damage, it seems unlikely that GT is involved in the damaging effects.

Freedman and Potts found about 50 per cent repression of glutaminase I activity in the rat retina following injection of MSG and postulated that the effect of glutamate might be to drain ketoglutaric acid away from the tricarboxylic acid cycle to make glutamine. We plan to test this hypothesis in the cultured chick embryo retina system.

Since glutamate and glutamine are central metabolites, there are numerous ways in which glutamate could be exerting an effect, e.g., it has been shown that taurine, which is present in very high concentration in retinal tissue [41.5 per cent of the total pool of free amino acids], is released from the retina after addition of 10 mM glutamate.

It is strange that a normal metabolite, especially one which is present in the retina in fairly high concentration should have such a destructive effect on retinal tissue. Interestingly, the concentrations of MSG used in this study are probably lower than those which have been reported for the intraretinal concentrations of this amino acid in normal brain and retina. Pirie and Van Heyningen reported a value of 0.83 mg. glutamic acid per gram of wet weight of retina. More recently Pasantes-Morales and co-workers reported 3.00 and 2.70 μmoles glutamic acid per gram of fresh weight for the rat and chicken retina, respectively. We have found that a 12-day-old chick embryo retina has an average wet weight of 41.6 ± 0.4 mg. Using a measured volume of 0.025 ml. for a 12-day-old chick embryo retina and the Pasantes-Morales data, one can calculate an intraretinal concentration of glutamate of 4.4 mM, i.e., almost four times as high as the very damaging 1.2 mM concentration used in the present experiments. Cohen, McDaniel, and Orr have evidence that glutamate is, in fact, generally distributed throughout the retina. However, Knijevic has suggested that intracellular glutamate probably does not directly influence neuronal activity, since unlike extracellular applications of MSG, intracellular injections of glutamate do not cause excitation in neuronal cells. Wosley, Kuhar, and Snyder have reported evidence that in rat cerebral cortex exogenous glutamate is highly localized in synaptosomal fractions, whereas endogenous glu-
Glutamate-induced brain lesions have been found by electron microscopic examination of albino mice injected with single doses of the amino acid. It was observed in the course of these, and other studies, involving glutamate treatment of retinas, that chick embryo retinal tissue maintained in the presence of this amino acid, was usually (though not consistently) badly shredded after an overnight incubation. This is in direct contrast to the report of Terner, Eggleston, and Krebs that glutamate "reduces or prevents the disintegration of [ox retinal] tissue during incubation." This could be due to a differential sensitivity of retinas of different ages or perhaps, different species, to MSG. The age-dependent effect of MSG on retina in injected rats and mice is possibly a result of permeability changes with age; Freedman and Potts found a decline of glutamate uptake by rat retina on the tenth postnatal day. We are currently extending our studies of the effect of glutamate in culture to adult retinas. This should answer, more definitively, whether the apparent refractory nature of adult retinas to glutamate is simply a reflection of the establishment of a permeability barrier with age in vivo, or is inherent in other age-dependent changes in this tissue.

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