Dual cation activation of bovine lens autolysis

E. I. Anderson

The autolytic activity of bovine lens extracts at 55° C. and pH 7.4 has been shown to be markedly influenced by the simultaneous presence of mono- and divalent cations. Of the cations tested, Na+ at 2.5 to 5 mM. and Mg 2+ at 5 to 10 mM. produce the best activation. The stimulation is synergistic. On the basis of results obtained here and in other laboratories, the suggestion is made that this combination of cations may be required for activation of the neutral proteinase of the lens. In the presence of Mg 2+ and average physiologic lenticular levels of both Na+ and K+, autolysis is suppressed to the basal level obtainable in the presence of Mg 2+ alone.

During an investigation of bovine lens autolysis, a pronounced loss of proteolytic activity was observed1 when soluble lens extracts prepared with 0.01M tris-HCl buffer, pH 8.0, containing 5 mM. MgCl2, were subjected to dialysis against the same buffer. Increasing the period of dialysis and, more particularly, the volume of dialyzing solution caused progressively larger losses of autolytic activity which could be partially offset by reconstituting dialysate and aliquots of diffusate. That the dialyzable activator could be salt was indicated from the results of adding saline washes of particulate lens fractions to the dialyzed soluble fraction. A variable but consistently increased autolysis was observed.

The current study is therefore concerned with the separate as well as the combined effects of various mono- and divalent cations on lens autolysis.

Experimental

Buffer. Except where noted in the text, buffer refers to 0.01M tris-HCl, pH 8.0 at 25° C.

Lenses. Bovine lenses, fresh or frozen, weighing 1.2 ± 0.1 grams per lens and either encapsulated or not, were first dispersed by mashing and then homogenized in a Ten Broek homogenizer with a volume of buffer equal to 3.5 times the wet lens weight. The suspension was centrifuged 60 minutes at 40,000 x g and the volume of supernatant (SN) was adjusted with buffer so that 1 ml. was equivalent to 250 milligrams wet lens weight. All operations were carried out at 0° to 4° C. Portions of SN were used either immediately or stored frozen and used within 4 weeks without noticeable change in activity.

Assay. Assays were conducted with nondialyzed SN or SN dialyzed (SND) at 4° C. against two changes of 150 volumes each of agitated buffer during the 24 hour period immediately preceding the assay. In one experiment, dialysis against 125 volumes of 0.01M EDTA in buffer readjusted to pH 8.0 with HCl was performed for 22 hours, followed by dialysis in consecutive order against the following molarities and volumes of tris buffer.
for the times indicated: 0.02M, 125 volumes, 4 hours; 0.005M, 250 volumes, 3 hours; 0.01M, 500 volumes, 18 hours.

Portions equivalent to 50 mg lens were incubated in a final volume of 1 ml buffer with or without additions for 3 hours at 55 °C. From data supplied by the distributor on the temperature coefficient of tris buffer, the pH during incubation was calculated to be 7.4. Zero hour controls were stored at 0 °C until termination of the assay whereupon all samples were chilled and treated with 5 volumes of 0.2M sodium acetate buffer, pH 5.2. Protein was removed by centrifugation after precipitation in a boiling water bath for 4 minutes.

Only chloride salts were used. Monovalent cations were assayed at final concentrations of 1, 2.5, 5, 10, 20, 50, and 100 mM and divalent cations at 1, 2.5, 5, 10, and 20 or 25 mM. Mg²⁺ was also assayed at 7.5 and 15 mM.

Autolytic activity was determined by the ninhydrin method outlined by Waley and van Heyningen. Results are expressed as µmoles of leucine equivalents per hour per gram wet lens. Duplicate determinations on the same sample agreed within 2 per cent and duplicate incubated samples agreed within 5 per cent.

Results

Autolytic activity of a lens extract can be reduced to a nearly negligible value by dialysis (Table I). The addition of 5 mM Mg²⁺ will stimulate autolysis of SND and SN but values for the SND still do not approach those for the SN.

Addition of a monovalent cation (Fig. 1) to the SND also induces relatively poor autolysis. When, however, a monovalent cation is used in combination with 5 mM Mg²⁺, activity is markedly increased and exceeds the sum of the activities produced by the individual supplements.

The maximal stimulation obtainable with

Fig. 1. Effect of monovalent cations on autolytic activity of SND in the presence and absence of Mg²⁺. Open symbols indicate no added Mg²⁺. Solid symbols indicate addition of 5 mM Mg²⁺ to the following monovalent cations: Na⁺ (○○○○), Li⁺ (○○○○), K⁺ (△--△).
Table I. Effect of dialysis on autolytic activity of lens extracts

<table>
<thead>
<tr>
<th>Addition</th>
<th>Millimolars</th>
<th>Activity (umoles/hr./Gm. lens)</th>
<th>SN</th>
<th>SND</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>1.30</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.87</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.30</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>5</td>
<td>5.32</td>
<td>1.53</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5.20</td>
<td>1.72</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>6.50</td>
<td>1.98</td>
<td></td>
</tr>
</tbody>
</table>

a particular Group 1 cation is dependent upon slightly differing but nearly overlapping levels of Mg\(^{2+}\). It was determined to be between 5 and 10 mM. in the presence of Na\(^{+}\), about 5 mM. with Li\(^{+}\), and between 2.5 and 5 mM. with K\(^{+}\). The use of 5 mM. Mg\(^{2+}\) therefore to obtain the curves in Fig. 1 permits comparison of each Group 1 cation at a single Mg\(^{2+}\) concentration that is also nearly optimal for each monovalent cation. In the presence of 5 mM. Mg\(^{2+}\), Na\(^{+}\) produces the best stimulation within a narrow range of 2.5 to 5 mM. Li\(^{+}\) at 10 mM. and K\(^{+}\) at 10 to 15 mM. activate autolysis, respectively, to about 88 per cent and 50 per cent of the maximal activity obtainable with Na\(^{+}\). Higher concentrations of the monovalent cations cause progressively greater reductions in activity to a point where only the stimulation by Mg\(^{2+}\) alone prevails.

The curves in Fig. 2 for autolytic activation of a SND by divalent cations show that, when used singly, Ca\(^{2+}\) is a better activator than Mg\(^{2+}\). Additional supplementation with 2.5 mM. Na\(^{+}\), however, causes
little enhancement of autolysis by Ca²⁺ but, as previously noted, produces marked activation in the presence of Mg²⁺.

The level of 2.5 mM Na⁺ employed to obtain the comparative curves in Fig. 2 was determined from separate experiments to be within that range of concentrations which will produce an activation that is nearly optimal in the presence of each divalent cation tested. The narrow Na⁺ requirement of 2.5 to 5 mM for maximal autolysis with Mg²⁺ is broadened with Ca²⁺ supplementation from 1 to at least 10 mM and with Mn²⁺ from 2.5 to at least 25 mM.

At 2.5 mM Na⁺, optimal autolysis of SND is achieved with 5 to 10 mM Mg²⁺, while Ca²⁺ supplementation at 5 mM and Mn²⁺ at 1.5 mM activate to about 62 per cent and 49 per cent, respectively, the optimal value obtainable with Mg²⁺.

In an analogous experiment, various levels of Ca²⁺ and Mg²⁺ were assayed in the presence and absence of 2.5 mM Na⁺ with a SN dialyzed first against 0.01M EDTA and then buffer to remove EDTA. Relative to each other, the activation curves obtained with such a dialyzed SN produced a pattern similar to that in Fig. 2 except that maximal stimulation in all cases was reduced to about 43 per cent of the values obtained with the usual SND.

In the presence of 5 mM Mg²⁺ and low levels (2.5 mM.) of both Na⁺ and K⁺ where Na⁺ alone is normally at an optimal concentration, any influence by K⁺ appears to be masked (Table II). Increasing the proportion of Na⁺ to K⁺ from a ratio of 1:1 to 1:48 causes a progressive diminution in activity toward values obtained when only K⁺ at the higher concentrations is used with Mg²⁺. In the last line of Table II, where Na⁺ and K⁺ are at concentrations reported to be average physiologic levels in the lens, autolytic activity is suppressed to the original level obtained in the presence of Mg²⁺ alone. Autolysis in the absence of Mg²⁺ is unaffected by the simultaneous addition of Na⁺ and K⁺ in various proportions in which Na⁺ is at either a low or high level.

**Discussion**

The presence in bovine lens of at least one proteinase, an aminopeptidase, and probably other peptidases all functional in vitro at a pH slightly above neutrality, has been documented. The aminopeptidase isolated by Spector and the α-crystallin fractions used by Hanson to hydrolyze various peptides, amides, and esters are comparably activated by Mn²⁺ and have been considered to be the same enzyme. Equimolar Mg²⁺ is half as efficient as Mn²⁺ in stimulating peptidase or esterase activity while monovalent cations are without effect. The aminopeptidase, moreover, does not attack lens proteins even though it behaves as an exopeptidase toward various polypeptide substrates.

The α-crystallin fraction containing the neutral proteinase, on the other hand, does break down lens proteins and is preferentially activated by Mg²⁺. van Heyningen and Waley have pointed out, however, that the occurrence of amino acids as the main hydrolytic end products may be caused by contaminating aminopeptidase activity or to an inherent dipeptide splitting capacity of the proteinase. The strongly inhibitory effect of high levels of Na⁺ noted

---

**Table II. Effect of cation supplementation on the autolytic activity of SND**

<table>
<thead>
<tr>
<th>Monovalent cation supplement</th>
<th>Millimolars</th>
<th>Activity (µmoles/hour/Gm. lens)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.15</td>
<td>1.65</td>
</tr>
<tr>
<td>Na⁺</td>
<td>0.94</td>
<td>3.20</td>
</tr>
<tr>
<td>K⁺</td>
<td>0.75</td>
<td>2.71</td>
</tr>
<tr>
<td>Na⁺/K⁺</td>
<td>0.67</td>
<td>1.15</td>
</tr>
<tr>
<td>2.5/2.5</td>
<td>1.10</td>
<td>6.41</td>
</tr>
<tr>
<td>2.5/20</td>
<td>1.01</td>
<td>3.14</td>
</tr>
<tr>
<td>2.5/120</td>
<td>1.01</td>
<td>1.34</td>
</tr>
<tr>
<td>20/20</td>
<td>1.28</td>
<td>2.23</td>
</tr>
<tr>
<td>20/120</td>
<td>1.10</td>
<td>1.64</td>
</tr>
</tbody>
</table>
here with the SND was also observed with the proteinase preparation. Although no activation studies with low levels of Na⁺ were deliberately made by Waley and van Heyningen, Veronal buffer containing 4.2 mM Na⁺ was used exclusively in their experiments. That this concentration of Na⁺ is optimal for autolysis of lens SND in the presence of Mg²⁺ has now been demonstrated. It would appear reasonable, therefore, to consider the Na⁺ and Mg²⁺ requirements for SND autolysis to be a reflection of dual cation activation of the proteinase rather than the aminopeptidase.

Results from other experiments tend to support this assumption. For example, supplementation of SND with 5 mM Na⁺ but no Mg²⁺ provides about 37 per cent the activity obtainable when both cations are present at 5 mM. Similarly only 35 per cent of the activity obtained for α-crystallin autolysis in the presence of 5 mM Mg²⁺ was recovered when only the Na⁺ containing Veronal buffer was used. Initial dialysis against EDTA followed by additional dialysis against buffer caused a lowering of SN activity to 43 per cent of the activity obtained with a SN dialyzed against buffer alone and supplemented with optimal amounts of Na⁺ and Mg²⁺. With α-crystallin proteinase, likewise, only 44 per cent of the control value was recovered after dialysis against EDTA and assaying in the presence of 3.5 mM EDTA and 10 mM Mg²⁺. These results contrast with the 80 per cent recoverable activity found when the Mn²⁺-activated aminopeptidase is dialyzed against EDTA prior to assaying.

To my knowledge, a proteolytic system requiring dual cation activation has not previously been reported. Generally where diverse cations activate hydrolytic systems, the effect is on the relative rate rather than being synergistic. The recently described activation of a phosphodiesterase in Escherichia coli supernatants most closely parallels the situation with the lens SND. The simultaneous presence of Mg²⁺ (or Mn²⁺) and K⁺ was found mandatory for the break down of polyU and other messenger RNA's to nucleoside-5'-monophosphates. Whether a comparable activation mechanism exists is obscure at this time but that the phenomenon is shared by such diverse hydrolases seems noteworthy.

Noteworthy also is the fact that both systems employ crude preparations. The metallic activation of hydrolases through chelation may explain the action of the divalent cation, but the apparent activation by monovalent cations, especially in crude preparations, may be a suppression of contaminating inhibitors. Until the monovalent requirements, if any, are established for the purified proteinase, any mechanism advanced to explain this type of activation would be premature.

The failure to obtain increased autolysis of a Mg²⁺ supplemented SND when Na⁺ and K⁺ are both present at average physiologic levels points to a mechanism which, if operative in vivo, could be vitally important in maintaining the integrity of the lens.

I am grateful to Dr. Zacharias Dische for his suggestions made throughout the course of this work and also to Mrs. Ginevra Zelmenis for removing and encapsulating all the lenses.

REFERENCES

2. Sigma Chemical Company: St. Louis, Mo.
7. Kinoshita, J. H.: Selected topics in ophthal-
