Teratogenic effects of calcium salts on chick embryos

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This investigation began as an attempt to protect chick embryos from the teratogenic effects of hypoxia by means of exogenously applied calcium pantothenate. The attempt was not only a failure, but it appeared that the compound alone was highly teratogenic. Since it was disturbing to consider that a vitamin, in low dosage, was teratogenic, the phenomenon was studied further by injecting equivalent concentrations of other calcium salts into the embryo. It quickly became apparent that very small quantities of calcium chloride solution injected into either the subgerminal area of the yolk sac or the allantois also produced pronounced abnormalities.

MATERIALS AND METHODS

All the eggs used in these experiments were obtained from hens of the Kimber strain of White Leghorn. The eggs were stored in a refrigerator, kept at 10 °C, and used within one week of laying. The eggs were incubated for 2–5 days prior to injection. They were then swabbed with 70 % alcohol and a window cut above the embryo, using aseptic techniques. A measured amount of solution was injected by means of glass needles. In embryos 2 and 3 days of age, the injection was made into the subgerminal fluid of the yolk sac, immediately underneath the embryo. At 4 and 5 days, the fluid was injected into the allantois. The windows were then sealed with paraffin and the egg returned to the incubator. The eggs were checked daily and the embryos examined as soon as death was detected or, in survivors, 7 days after injection.

The pantothenic acid was injected as a 0-02 M solution of calcium pantothenate in distilled water (pH 6-8) which was sterilized by filtration. Sterile solutions of 0-01 M calcium chloride (pH 6-0) were applied in 0-01–0-04 ml quantities. Control embryos were injected with equivalent amounts of sterile 0-145 M NaCl solutions (pH 5-8). At the conclusion of the experiment the contents of the egg were carefully placed into a bowl of saline, the embryo

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dissected free, washed, and thoroughly examined for external malformations. No internal autopsies were attempted.

Calcium and protein assays were performed on blood plasma and other embryonic fluids with the Beckman Ultramicro Analytical System. Samples were obtained from normal embryos and from calcium chloride-treated embryos 5 h after injection. Blood samples were obtained from vitelline arteries or veins, placed in capillary tubes and centrifuged. Adult chicken blood was obtained from the wing vein of young roosters.

RESULTS

The effect of sodium chloride injections on the development of embryos

Because simply opening the egg and sticking a needle through the embryonic membranes involves some degree of trauma, fairly extensive controls were run in this series. Embryos were injected at 2 days and at 5 days with 0.02 ml saline. The death-rates in both groups were approximately the same, 24% at 2 days and 19% at 5 days (Table 1). In each group 3% of the embryos showed mal-

Table 1. The effects of solutions of calcium pantothenate and calcium chloride on chick embryos

<table>
<thead>
<tr>
<th>Age of embryo...</th>
<th>NaCl-injected controls*</th>
<th>Calcium pantothenate†</th>
<th>Calcium chloride‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 days</td>
<td>5 days</td>
<td>2 days</td>
</tr>
<tr>
<td>Effects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal, living§</td>
<td>72%</td>
<td>78%</td>
<td>63%</td>
</tr>
<tr>
<td>Immediate dead</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immediate dead</td>
<td></td>
<td></td>
<td>7%</td>
</tr>
<tr>
<td>Delayed dead¶</td>
<td></td>
<td></td>
<td>17%</td>
</tr>
<tr>
<td>Abnormal-dead</td>
<td>2%</td>
<td>1%</td>
<td>11%</td>
</tr>
<tr>
<td>Abnormal-living</td>
<td>1%</td>
<td>2%</td>
<td>6%</td>
</tr>
<tr>
<td>Total no. of cases</td>
<td>117</td>
<td>150</td>
<td>350</td>
</tr>
</tbody>
</table>

* NaCl controls—0.02 ml. of 0.145 M-NaCl in yolk sac at 2 days and allantois at 5 days.
† Calcium pantothenate—0.01 ml of 0.02 M solution into yolk sac.
‡ CaCl₂—0.01 M solution: 0.01 ml into yolk sac at 2 days; 0.01–0.04 ml into yolk sac at 3 days; 0.04 ml into allantois at 5 days.
§ Those surviving at least 7 days after injection.
¶ Those which die within 24 h after injection.
†† Those which die between 2 and 6 days after treatment.

formations. In the 2-day group, one case of microphthalmos and one slight brain anomaly were found, along with two cases of rumplessness (Table 2). In the 5-day group, one embryo with a clear blister over the flank was found, and two with hematomas. Two embryos had deficient eyelids, a defect earlier shown (Grabowski, 1964) to be caused by the formation of a clear blister over the eye at an early stage of development. Although some of the malformations in this
control group can be considered as occurring spontaneously, these tests indicate
that simple opening and inoculation can be somewhat deleterious (see also
Grabowski, 1963). These data must be considered when reviewing the effects
of the other solutions used in this study.

The effects of calcium pantothenate solution on 2-day chick embryos

A total of 350 2-day embryos were injected with 0·01 ml of a 0·02 M solution
of calcium pantothenate. Of these embryos, 20 % died before the 9th day of
incubation. However, 24 % of those injected at the same time with saline
solution died in the same time-interval. Therefore, the amount of calcium
pantothenate used did not significantly raise the death-rate over that of saline-
injected controls. In the case of calcium pantothenate-injected embryos, 61 out
of 350 (17 %) were abnormal (Table 1). Many of these embryos were grossly
malformed and had multiple anomalies (Plate 1, figs. A, B). Hematomas and
blisters of various sizes were the most common abnormalities detected (Plate 1,
fig. A). Brain malformations were fairly common, including encephalocele and
platyneuria (Plate 1, fig. B), and cranioschisis. The other anomalies found are
listed in Table 2. It is apparent that the injection of calcium pantothenate
solution produces serious disturbances in a significant percentage of cases with-
out raising the death-rate.

<table>
<thead>
<tr>
<th>Treatment...</th>
<th>Saline</th>
<th>Calcium pantothenate</th>
<th>Calcium chloride</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (days)...</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
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<tr>
<td>3</td>
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</tr>
<tr>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>No. of malformed embryos...</td>
<td>4</td>
<td>5</td>
<td>61</td>
</tr>
<tr>
<td>61</td>
<td>11</td>
<td>16</td>
<td>19</td>
</tr>
<tr>
<td>Abnormalities</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clear blisters</td>
<td>—</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Hematomas</td>
<td>—</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>Eyelid defects</td>
<td>—</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Microphthalmos</td>
<td>1</td>
<td>—</td>
<td>8</td>
</tr>
<tr>
<td>Beak defects</td>
<td>—</td>
<td>—</td>
<td>6</td>
</tr>
<tr>
<td>Brain defects</td>
<td>1</td>
<td>—</td>
<td>14</td>
</tr>
<tr>
<td>Abdominal hernias</td>
<td>—</td>
<td>—</td>
<td>5</td>
</tr>
<tr>
<td>Rumplessness</td>
<td>2</td>
<td>—</td>
<td>11</td>
</tr>
<tr>
<td>Extremity defects</td>
<td>—</td>
<td>—</td>
<td>6</td>
</tr>
<tr>
<td>Completely malformed</td>
<td>—</td>
<td>—</td>
<td>2</td>
</tr>
</tbody>
</table>

* External malformations only observed.

The effect of calcium chloride solutions on chick embryos

The calcium chloride solutions were tested for reasons outlined in the intro-
duction. From 0·01 to 0·04 ml of sterile 0·01 M solutions of calcium chloride
were used on embryos of 2, 3 and 5 days. The death-rates ranged from 27 %
to 38% (Table 1). This was slightly (7-18%) higher than the death-rates obtained in the saline controls. Anomalous embryos were obtained in 12-17% of each group (Table 1). The nature of the malformations varied somewhat with the age at which the injections were made (Table 2). Here again, as in the case of calcium pantothenate, clear blisters (Plate 1, fig. C), hematomas, eye (Plate 1, figs. C, D, E) and brain (Plate 1, figs. E, F) malformations were particularly prominent. It is apparent that calcium chloride solutions can produce severe malformations.

**DISCUSSION**

The fact that a normal constituent of biological fluids can cause serious malformations in embryos is indeed a bizarre phenomenon. The solution of calcium chloride used contained 20 m-equiv./l of calcium ions; therefore, the 0.01 ml which was injected contained 0.0002 m-equiv. of calcium. Viewed in this manner, the calcium produced its effect virtually as a trace element.

Very little can be said by way of explanation of this phenomenon. Indeed, the experiments raised such questions as: what is the significance of calcium in the various embryonic fluids of a cleidoic egg? How is the concentration of free calcium regulated in the various fluids of a closed system containing a physiologically immature organism? Because of these considerations we measured the calcium concentration of several embryonic fluids in normal and calcium-treated embryos.

The normal level of calcium in adult human blood serum is approximately 5 m-equiv./l. Of this, only about one-half is ionized, the rest bound to the plasma protein (Ruch & Fulton, 1960). When serum protein concentration rises, the level of bound calcium normally rises in proportion. The function of

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**PLATE 1**

Fig. A. Embryo injected with 0.01 ml of 0.02 M calcium pantothenate solution into yolk sac at 2 days and preserved on day 9. The head is virtually absent. A large hematoma has replaced the left wing (arrow). Herniation of the abdominal and thoracic contents is evident, along with rumplessness. × 3.

Fig. B. Platyneuria in an embryo treated with calcium pantothenate, as above, on day 2 and preserved on day 6. × 5.

Fig. C. Embryo with defective eyelid caused by presence of clear blister over left eye. Treated with 0.04 ml of 0.01 M-CaCl₂ solution into allantois at 5 days, preserved on day 12. × 3.

Fig. D. Microphthalmos in an embryo treated with CaCl₂ solution on day 5 (same as embryo in fig. C). Eye approximately one-fourth normal diameter. × 3.

Fig. E. Embryo treated on day 3 with 0.03 ml of 0.01 M-CaCl₂ solution injected into yolk sac. Preserved on day 11. Embryo exhibited bilateral anophthalmos, encephalocele (arrow), crossed beak and abdominal hernia. × 3.

Fig. F. Platyneuria in an embryo treated with CaCl₂ solution on day 3 (dosage same as for embryo in fig. E). × 3.
the parathyroid gland is to keep the level of ionic calcium constant in the serum, regardless of the level of protein-bound calcium. In the adult chicken, the serum calcium level varies, depending on the sex and the physiological state of the animal. In the serum of young roosters we have found the total calcium level to be 6.5 m-equiv./l and the protein concentration 4.3 g/100 ml. The serum of 5-day embryos also contains 6.5 m-equiv./l of calcium, but only one-third as much protein, 1.4 g/100 ml. It would seem that either the protein in the embryonic serum has a higher binding capacity for calcium or the level of ionic calcium in embryonic serum is much higher than in adult serum. The calcium level of the two compartments into which the solutions were injected, i.e. the subgerminal fluid of younger embryos and allantois of older embryos, both contained approximately 3.8 m-equiv./l of calcium and from 1.2–1.9 g/100 ml of protein. Blood samples obtained from embryos 5 h after injection with the calcium chloride solution did not show any increase in the serum calcium level but actually a decrease to 5.5 m-equiv./l. Again, these data emphasize the complexity of the problem. Before the question of teratogenic action of calcium salts can be answered, more must be known about the function of calcium ions in the young embryo and the homeostatic mechanisms which maintain normal levels.

Perhaps one clue as to mechanism of action can be gained from the observations on the embryos themselves. The high frequency of clear blisters and hematomas (Table 2; Plate 1, figs. A, C) indicates some degree of fluid disturbance in the treated embryos. In this respect, the effects of calcium resemble those of hypoxia in which many abnormalities are produced via the ‘edema syndrome’ (Grabowski, 1963, 1964, 1965). Perhaps these effects of calcium are related in some way to the fact that calcium is necessary to maintain the plasma membrane, but to ascertain the exact relationship between these two agents—calcium and hypoxia—would require much additional work. Such studies on the normal physiology of the young embryo and how it is affected by various agents are continuing in our laboratory.

**SUMMARY**

1. Dilute solutions of calcium pantothenate and calcium chloride were injected into the yolk sac and allantois of 681 chick embryos between 2 and 5 days old. From 0.01 to 0.04 ml of 0.01 and 0.02 m solutions were used. The development of these embryos was compared with that of 267 saline-injected controls.

2. The death-rate in both controls and experimental groups was approximately 25%. Of the controls, 3% showed slight anomalies. From 12% to 17% of the calcium-treated embryos exhibited external malformations such as microphthalmos, anophthalmos, platyneuria, encephalocoele, abdominal hernias, rumplessness, extremity defects, hematomas, and others.
3. It is apparent that the traces of calcium salts injected as indicated can induce gross malformations. No explanation of this bizarre phenomenon can be offered. Assays of blood plasma of normal and treated embryos show that (a) the calcium level in embryonic plasma is the same as that of adults (6-5 m-equiv./l) even though the protein content is only about one-third that of the adult, and (b) paradoxically, calcium treatment reduces the level of calcium in the plasma to 5-5 m-equiv./l. The possible significance of these data for embryonic physiology is discussed.

Résumé

Effets tératogènes des sels de calcium sur l'embryon de poulet

1. Des solutions diluées de panthoténate de calcium et de chlorure de calcium ont été injectées dans le sac vitellin et l'allantoïde de 681 embryons de poulet âgés de 2 à 5 jours. On a utilisé des quantités de 0,01 à 0,04 ml de solutions 0,01 et 0,02 M. Le développement de ces embryons a été comparé à celui de 267 témoins ayant reçu une solution physiologique.

2. Le taux de mortalité dans les groupes témoins et expérimentaux a été d'environ 25 %. Parmi les témoins, 3 % présentaient des anomalies légères. De 12 à 17 % des embryons traités au calcium présentaient des malformations externes, telles que microphthalmie, anophthalmie, platyneurie, encéphalocèle, hernies abdominales, absence de croupion, déficiences des extrémités, hématoomes, et d'autres encore.

3. Il apparaît que les traces de sels de calcium injectées peuvent induire de graves malformations. On ne peut proposer d'explication de ce phénomène bizarre. Des analyses de plasma sanguin d'embryons normaux et traités montrent que (a) le taux de calcium dans le plasma embryonnaire est le même que celui des adultes (6,5 m-equiv./l) bien que la teneur en protéines ne soit que le tiers de celle de l'adulte, et (b), paradoxalement, le traitement au calcium réduit le taux de calcium dans le plasma à 5,5 m-equiv./l. On discute la signification possible de ces résultats pour la physiologie embryonnaire.

This investigation was supported by grant HD 00641 from the National Institute of Child Health and Human Development, National Institutes of Health.

References


(Manuscript received 14 September 1965)