# Effect of experimentally induced calcium deficiency on development, metabolism and liver morphogenesis of the chick embryo

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## SUMMARY

To study the effects of systemic calcium deficiency on embryonic development, chick embryos maintained in long-term shell-less cultures were compared to control embryos incubated *in ovo* with respect to various parameters of metabolism and growth. After incubation day 14, retarded growth and development were apparent in shell-less embryos which exhibited severe hypocalcaemia and hyperphosphataemia. A development-specific necrosis of the liver tissues was observed in both shell-less and control embryos, but the frequency and extent of tissue abnormality were significantly greater in the former. Serum levels of lactate dehydrogenase and alkaline phosphatase were considerably elevated in shell-less embryos. Electrophoretic analysis revealed that the relative levels of two major serum proteins were also altered in shell-less embryos.

## INTRODUCTION

About 80 % of the calcium of a newly hatched chick or quail is derived from the eggshell and is translocated by the chorioallantoic membrane during the second half of embryonic development (Romanoff, 1967; Terepka, Stewart & Merkel, 1969; Crooks & Simkiss, 1974; Tuan & Zrike, 1978; Ono & Wakasugi, 1984). As a result, when chick embryos are placed in shell-less culture *in vitro*, they develop severe calcium deficiency and skeletal anomalies (Tuan, 1980, 1983; Watanabe & Imura, 1983; Narbaitz, Sarkar & Fragiskos, 1983). These shell-less embryos are generally retarded in gross development and growth based on Hamburger–Hamilton (1951) staging parameters.

The experimentally induced systemic calcium deficiency also appears to affect specific cellular differentiation and tissue morphogenesis in at least two tissues of the developing embryo. Tuan & Lynch (1983) detected in the shell-less embryos the synthesis of cartilage-specific collagen type II in the calvarium, a normally osteogenic tissue, suggesting the appearance of chondrogenic phenotype. Ono & Wakasugi (1983a,b) previously reported that 8 % of Japanese quail embryos (wild-type plumage) at incubation days 10 to 12 showed local abnormality (necrosis) in liver tissue, and with the progress of embryonic development the frequency of appearance of this abnormality decreased. These workers observed that when

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quail embryos were cultured in the shell-less condition the frequency of liver necrosis increased tenfold. However, if the cultures were maintained within a container made of chicken eggshell, the quail embryos were able to obtain about half of the required amount of extraembryonic calcium and, in addition, the frequency of liver necrosis was reduced to about half of that in shell-less embryos (Ono & Wakasugi, 1983*b*, 1984). These findings strongly suggest that the development-specific liver necrosis observed in the quail embryo is significantly influenced by its calcium metabolic status.

The present study aims to elucidate the relationship between embryonic calcium deficiency and liver morphogenesis. We report here a detailed comparison of shell-less and *in ovo* (controls) embryos with respect to their developmental profiles of calcium and phosphate levels, and the appearance of liver necrosis. To analyse the biochemical basis of embryonic liver necrosis, shell-less and control embryos are also compared with respect to: (1) the quantitative and qualitative changes in two serum enzymes, lactate dehydrogenase (LDH) and alkaline phosphatase, which are often associated with tissue damages or diseases (Bell, 1971; Raphael *et al.* 1976); and (2) their serum protein profiles.

MATERIALS AND METHODS

*Chick embryos and shell-less culture*

Fertile White Leghorn eggs obtained from Truslow Farms, Inc. (Chestertown, MD) were used throughout the study. They were incubated at 37·5°C in a humidified laboratory egg incubator. The procedure of shell-less culture has been described previously (Dunn & Boone, 1976; Tuan, 1980, 1983; Dunn, Fitzharris & Barnett, 1981; Tuan & Lynch, 1983; Watanabe & Imura, 1983). Briefly, embryonated eggs were cracked open asceptically after 3 days of incubation *in ovo*, and transferred to a hemispherical pouch made of transparent plastic kitchen wrap suspended within a ring stand. The open surface of the culture was loosely covered with a 100 mm Petri dish lid and then placed in a humidified tissue culture incubator at 37·5°C with constant air flow.

*Serum protein, calcium and phosphate contents*

Chick embryos were bled from the extraembryonic arteries or veins at incubation days 11 (3 days *in ovo* plus 8 days *in vitro* for shell-less embryos), 14, and 17. The blood samples were allowed to clot at 37°C for 1 h, and after storage at 4°C for several hours sera were obtained by centrifugation. Total serum protein concentration was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as a standard. Total calcium concentration was determined fluorometrically (Barnett, Skodon & Goldberg, 1973) using a calcium analyser (Calcette Model 4009, Precision System, Sudbury, MA). Determination of inorganic phosphate was performed by a modified method of Tuan & Knowles (1984). Briefly, the assay mixture was buffered with N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES, 2·5 mM) and piperazine-N,N'-bis (2-ethanesulphonic acid) (PIPES, 2·5 mM) at pH 8·0 and inorganic phosphate was determined colorimetrically using molybdic acid–malachite green in Triton–HCl based on $A_{650}$ values.

*Embryonic development and liver morphogenesis*

Overall embryonic growth and development were assessed by Hamburger & Hamilton (1951) staging at days 11, 14, and 17. Embryos were also dissected for visual examination of the

appearance of necrosis in the liver tissues at days 9, 11, 14, and 17 for shell-less embryos and the same time points plus day 18 for control embryos. For quantitative comparison, the extent of gross, observable damage in each liver lobe of the embryo was scored (see legend to Fig. 1). Based on these scores, a liver necrotic index was calculated to express the average extent of liver necrosis in a given group of embryos: Necrotic index = (total scores of necrosis in all embryos)/(number of embryos with necrosis). For histology, some liver tissues were fixed with Hollande–Bouin's solution (Humason, 1967), embedded in paraffin, sectioned (8 $\mu$m), stained with haematoxylin and eosin, and observed using an Olympus BH-2 microscope.

### Enzyme assays

Serum activities of alkaline phosphatase and LDH were determined spectrophotometrically using standard protocols (Sigma Technical Bulletins Nos 246 and 226-UV, Sigma Chemical Co., St Louis, MO) based on p-nitrophenol liberation from p-nitrophenyl phosphate and NADH-coupled pyruvate formation from lactate, respectively. Activities were expressed as International Units litre$^{-1}$ at 30°C, where 1 Unit represented transformation of 1 $\mu$mole of substrate min$^{-1}$. To detect isoenzymes of LDH, serum samples were charge-fractionated by isoelectric focusing (pH 3–10) on thin-layer polyacrylamide gels using the LKB Multiphor System (LKB-Produkter AB, Bromma, Sweden) and stained histochemically using the procedure of Dietz & Lubrano (1967). For determination of the relative level of isoenzymes, the stained gel was scanned using an E-C densitometer (E-C Apparatus Corp., St Petersburg, FL).

### Sodium dodecyl sulphate polyacrylamide gel electrophoresis of serum proteins

This was carried out using the discontinuous buffer system of Laemmli & Favre (1973). Samples were denatured in sodium dodecyl sulphate and reduced with 2-mercaptoethanol and electrophoresed in polyacrylamide gels consisting of a 10 % separating gel and a 4 % stacking gel. Protein bands were visualized by Coomassie Blue staining.

### Statistical analysis

Analysis of data was performed using Student's $t$-test. Differences were regarded as statistically significant at $P < 0.05$ (Dowdy & Wearden, 1983).

### RESULTS

### Serum protein, calcium, and inorganic phosphate levels

Serum protein concentration in both shell-less and control embryos increased as a function of embryonic development (Fig. 2). No difference was observed between shell-less and control embryos in each age. However, a significant difference was observed between the two groups of embryos with respect to their serum calcium and inorganic phosphate levels (Fig. 3). Serum calcium level in control embryos increased rapidly between incubation days 11 and 14, whereas that of shell-less embryos during the same period showed a rapid decrease. After day 14, serum calcium level of shell-less embryos was only half of the normal value. On the contrary, serum inorganic phosphate level of control embryos was constant during days 11 and 17, and that of shell-less embryos increased significantly. At day 17, the inorganic phosphate level of shell-less embryos was almost twice that of control embryos.
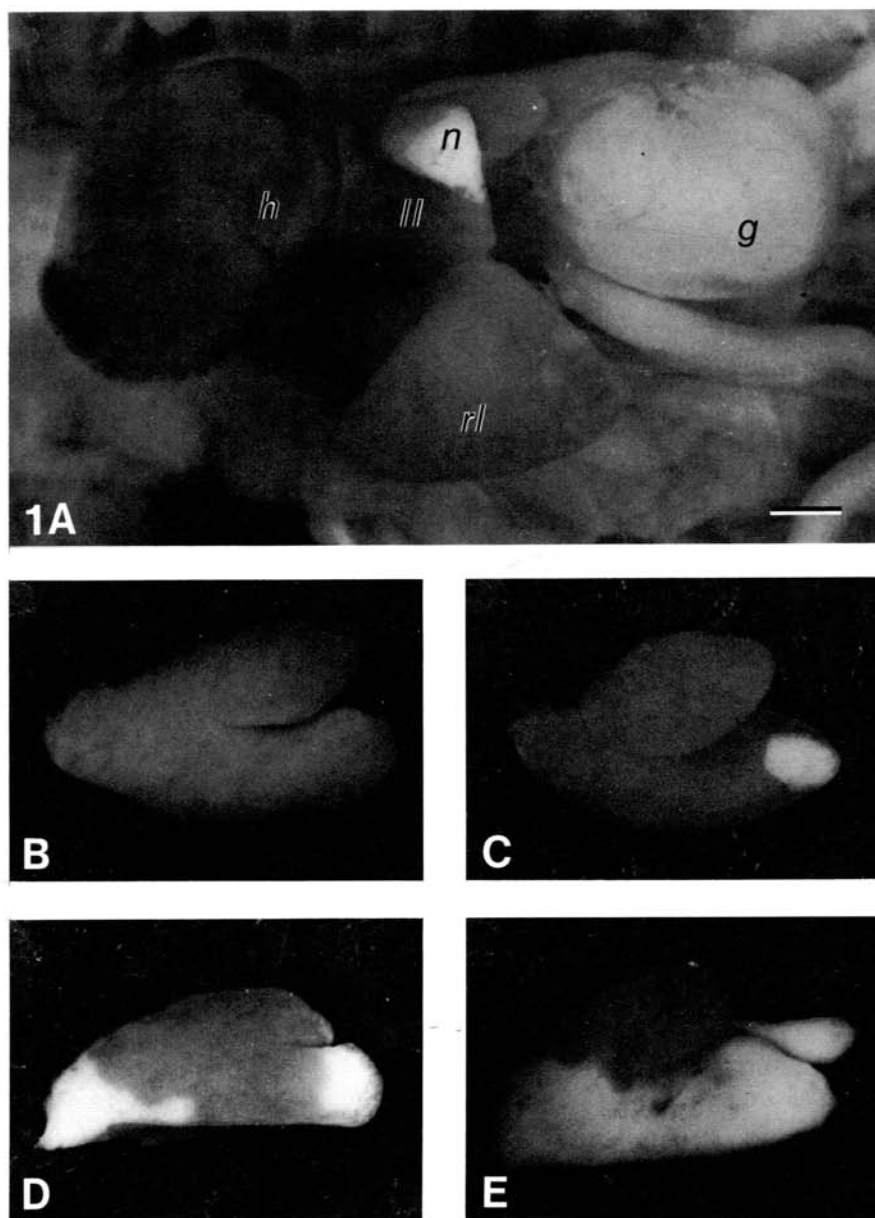
Fig. 1. (A) Ventral view of abdomenal organs of chick embryo (day 11, shell-less) with liver necrosis. The necrotic region (n) appeared as a discoloured zone located at the posterior tip of the left liver lobe (ll). Other organs: g, gizzard; h, heart; rl, right liver lobe. Bar equals 1 mm. (B–E) Livers of day 11, shell-less chick embryos showing varying extents of liver necrosis. Scoring of liver necrosis was based on gross observation of tissue damage: (B), score = 0, no necrosis; (C), score = 1, minor damage localized to a single spot on the tip of the liver lobe; (D), score = 2, intermediate damage observable in an area not to exceed 1/4 of the lobe; and (E), score = 3, large damage spanning the entire length of the liver lobe.

*Embryonic development and liver necrosis*

It was previously observed (Tuan, 1983) that, at incubation day 18, embryos which were maintained in shell-less culture showed substantially retarded development as compared to normal embryos developed *in ovo*. In the present study, shell-less and control embryos were compared at several time points of incubation with respect to embryo wet weights and Hamburger & Hamilton developmental stages (based on lengths of third toe and beak). As shown in Fig. 4, shell-less embryos exhibited relatively normal gross morphological development up to incubation day 14. However, at day 17, development was significantly retarded in shell-less embryos as compared to control embryos. In both shell-less and control embryos, necrosis of liver tissues could be observed (Fig. 1). The abnormal region
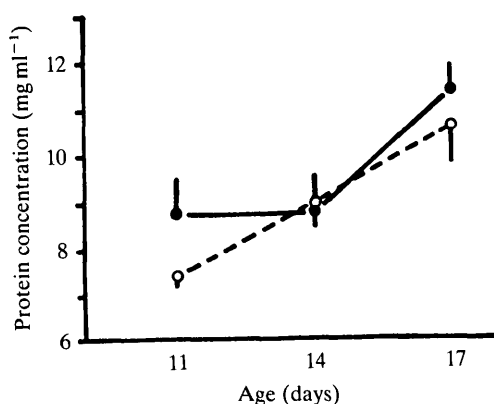


Fig. 2. The developmental profiles of serum protein concentration of shell-less (O– – –O) and control (●——●) embryos. Each value represents the mean ± s.e.m. of 14–28 embryos. No significant difference was observed between shell-less and control embryos at each developmental age (*P* > 0·05).
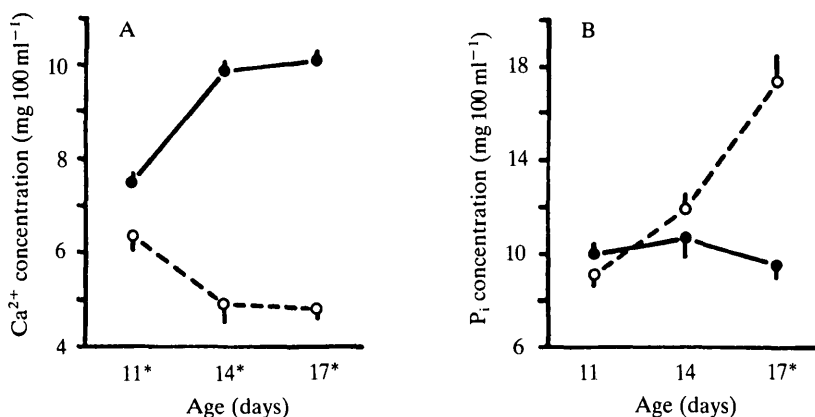


Fig. 3. Developmental profiles of serum calcium (A) and inorganic phosphate concentration (B) of shell-less (O– – –O) and control (●——●) embryos. Each value represents the mean ± s.e.m. of 10–23 embryos. Asterisk indicates a significant difference (*P* < 0·05) between shell-less and control embryos of the same age.
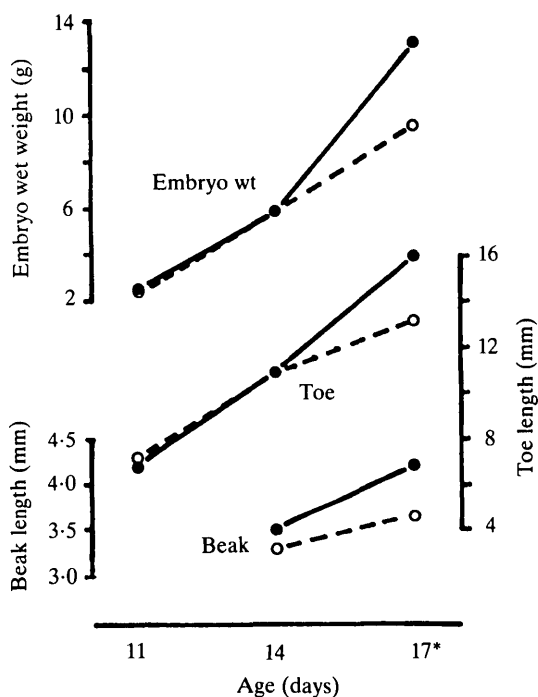
Fig. 4. Gross development of shell-less (O———O) and control (●————●) embryos assessed by embryo wet weight and lengths of toe and beak (Hamburger & Hamilton, 1951). Each value represents the mean ± s.e.m. of 10–32 embryos. Asterisk indicates a significant difference ($P < 0.05$) between shell-less and control embryos of the same age.

was usually focal and the colour ranged from pale yellow to white. In most cases, this necrosis was located at the posterior tip of the left lobe only. Occasionally, some embryos also exhibited necrosis either at the anterior part of the left lobe only or both left and right lobes. Few embryos showed liver necrosis at the right lobe only. The frequency of this necrosis and the overall liver necrotic indices in shell-less and control embryos are shown in Fig. 5. At incubation day 9, no control embryos and few shell-less embryos showed necrosis. In shell-less embryos, the peak frequency of necrosis was about 45 % occurring at day 11 whereas that of the control embryos was significantly lower (25 %) occurring at day 14. On the other hand the necrotic index, which represented an integrated parameter, was maximal at day 11 for both shell-less and control embryos. The peak value of the necrotic index of shell-less embryos was about three times higher than that of control embryos, clearly indicating that the extent of liver tissue damage in shell-less embryos was significantly more severe compared to that in control embryos. Histological observation (Fig. 6A) revealed that there was a clear separation between necrotic and surrounding healthy tissues. Higher magnification (Fig. 6B) revealed that the necrotic region was virtually devoid of tissue architecture. Abnormal cells showed weak eosinophilia of cytoplasm, pycnosis, and karyolysis (Fig. 6).

*Enzyme activities*

### (1) *Alkaline phosphatase*

The developmental profile of serum alkaline phosphatase activity in shell-less and control embryos is shown in Fig. 7. In both groups of embryos, the activity increased as a function of embryonic age. However, at each developmental time point, shell-less embryos consistently exhibited higher activity compared to control embryos. No difference was observed in the enzyme activity between embryos with and without liver necrosis.
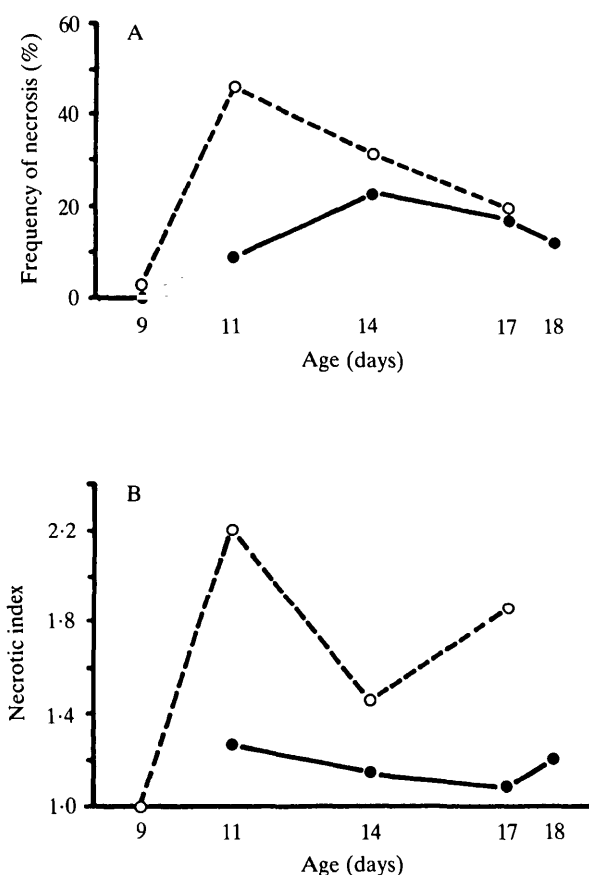


Fig. 5. Developmental profile of liver necrosis in chick embryos. (A) Frequency of liver necrosis; (B) Liver necrotic index. Shell-less embryos (O– – –O); control (●———●) embryos. Liver necrosis was scored as described in the legend to Fig. 1. To express the average extent of liver necrosis in a given group of embryos, the necrotic index was calculated as: (total scores of necrosis in all embryos)/(number of embryos with necrosis), (see Materials and Methods). The number of embryos examined in each age group was: for shell-less embryos, 67 at day 9, 87 at day 11, 72 at day 14, and 209 at day 17; and for control embryos, 49 at day 9, 127 at day 11, 311 at day 14, 316 at day 17, and 343 at day 18.

(2) *Lactate dehydrogenase*

In the case of serum LDH, a significant difference was observed between the enzyme activity levels in embryos with and without liver necrosis (Fig. 8).
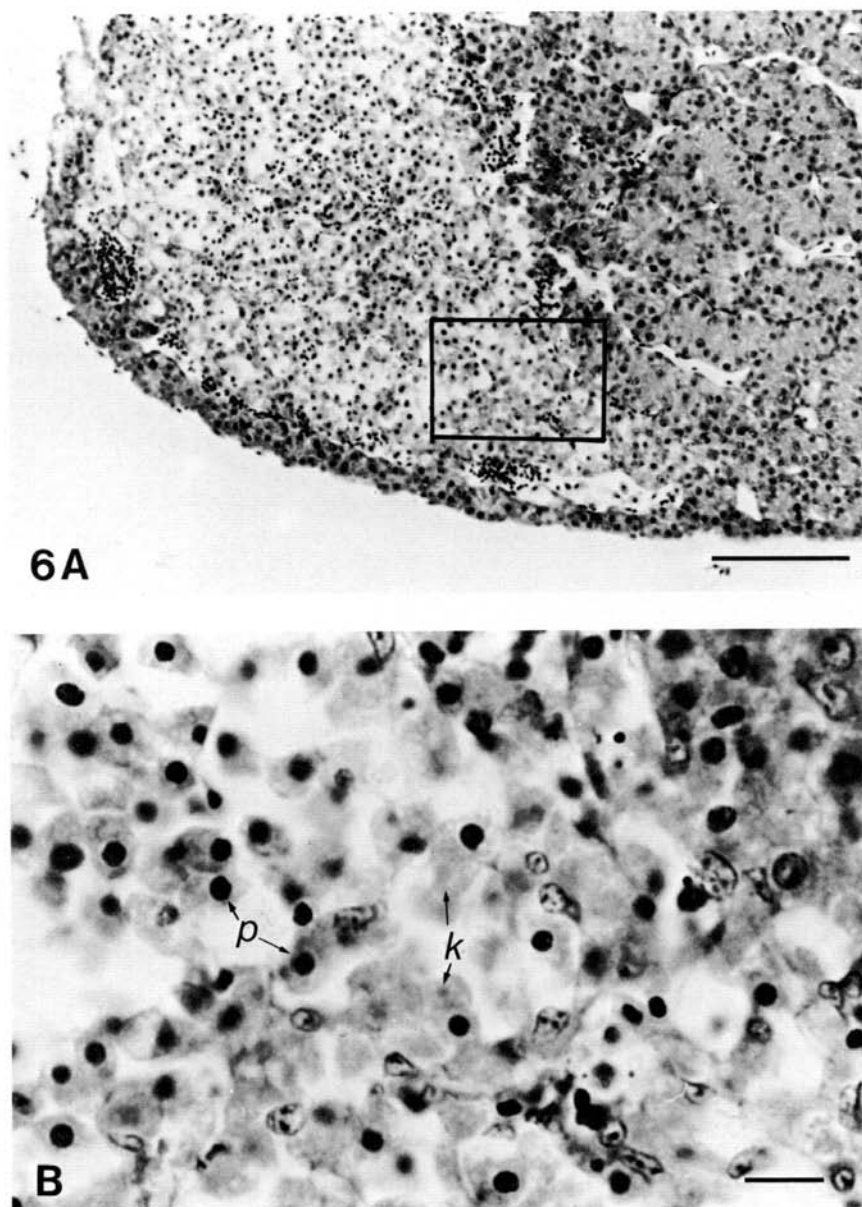


Fig. 6. Histology of the necrotic zone in chick embryonic liver tissue. The tissue section was taken from day-14 control embryo and stained with haematoxylin and eosin. (A) Low magnification. Note clear separation between the necrotic area on the left with weak eosinophilia of cytoplasm and healthy area on the right. Bar equals 100 μm. (B) High magnification of the boxed area in (A). Note the absence of structural integrity in the necrotic cells which showed clear signs of pycnosis (*p*) and karyolysis (*k*). Bar equals 10 μm.
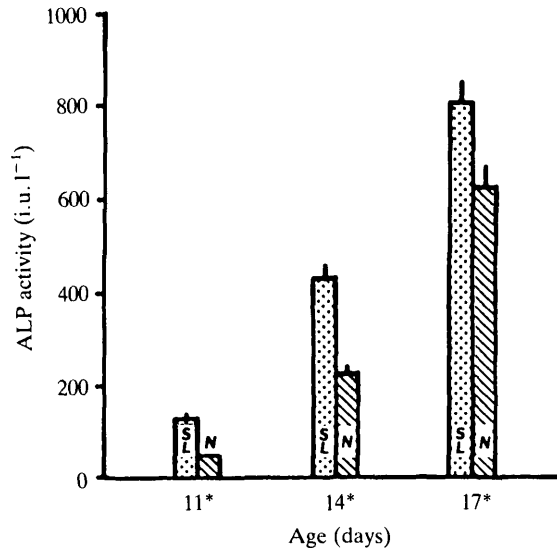
Fig. 7. Developmental profiles of serum alkaline phosphatase (ALP) activity of shell-less and control embryos. Activities are expressed as International Units $l^{-1}$ at 30°C, where 1 Unit represents transformation of 1 μmole of substrate $min^{-1}$. Each value represents the mean ± s.e.m. of 13–24 embryos. Asterisk indicates a significant difference ($P < 0.05$) between shell-less and control embryos of the same age.
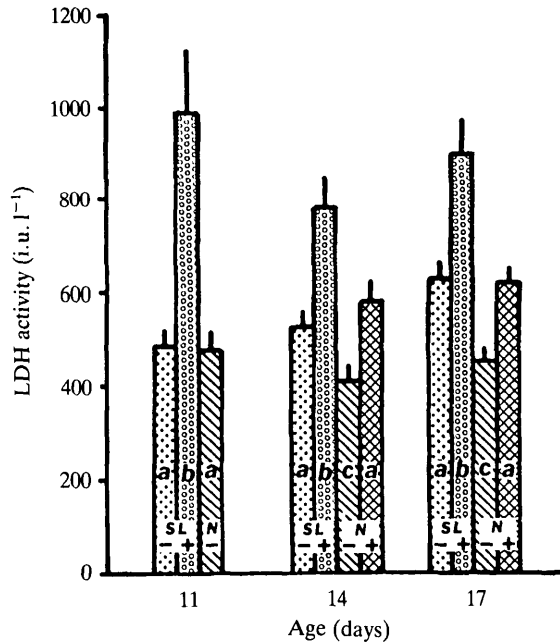


Fig. 8. Developmental profiles of serum LDH activity of shell-less and control embryos with (+) and without (−) liver necrosis. Activities are expressed as International Units $l^{-1}$ at 30°C, where 1 Unit represents transformation of 1 μmole of substrate $min^{-1}$. Each value represents the mean ± s.e.m. of 8–20 embryos. The values marked with different letters are significantly different ($P < 0.05$) within the same age group.
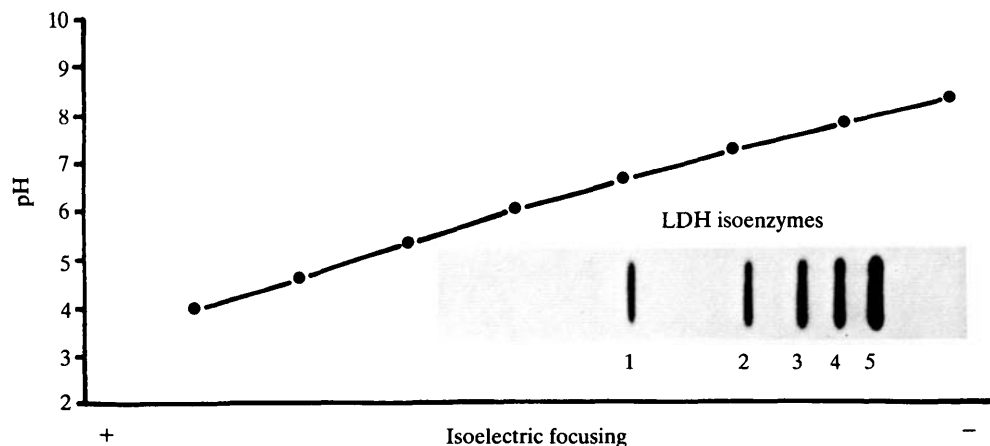
Fig. 9. Chick embryonic serum LDH isoelectric zymogram taken from day-17 shell-less embryo. The serum sample was fractionated by isoelectric focusing and the pH gradient of the focused gel was determined using a flat-surface electrode. Isoenzyme bands of LDH were developed histochemically (see Materials and Methods). The lactate dehydrogenase isoenzymes (LDH-1, LDH-2, LDH-3, LDH-4, and LDH-5) corresponded to isoelectric points of 6·6, 7·4, 7·7, 7·9, and 8·1, respectively.
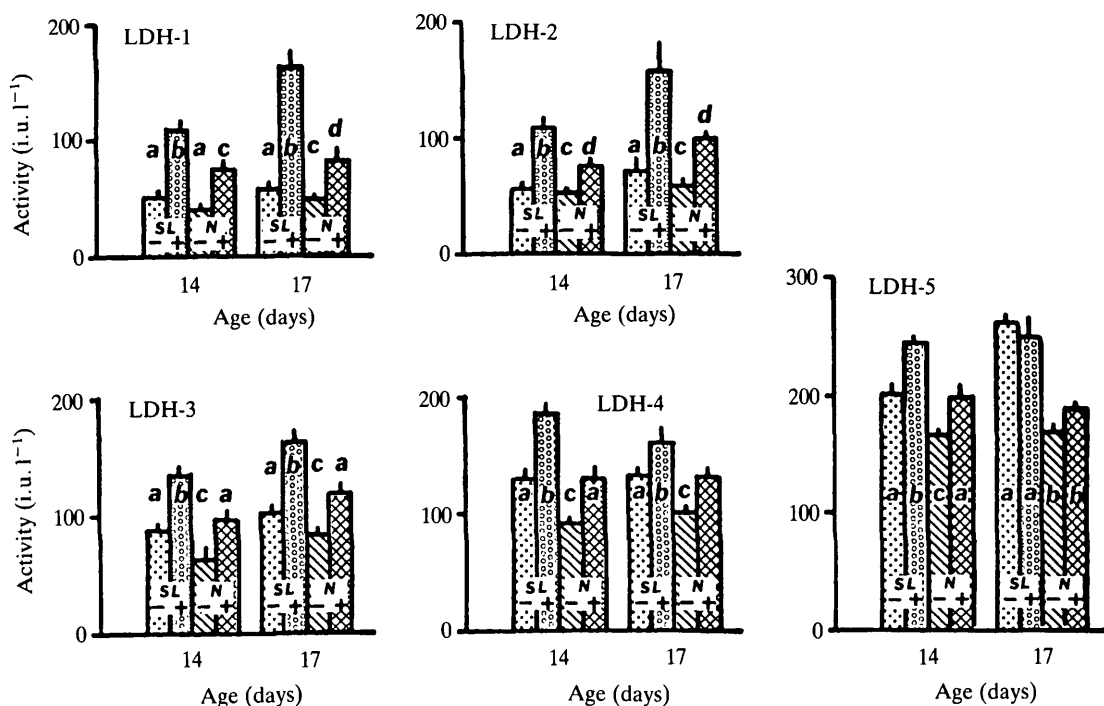


Fig. 10. Developmental profiles of serum LDH isoenzyme activities of shell-less and control embryos with (+) and without (−) liver necrosis. The level of each isoenzyme activity was determined by densitometric scanning of the stained gel and the relative proportion of the five isoenzyme activities were expressed as a function of the total LDH activity shown in Fig. 8. Each value represents mean ± S.E.M. of 5–16 embryos. The values marked with the different letters are significantly different ($P < 0.05$) within the same age group.
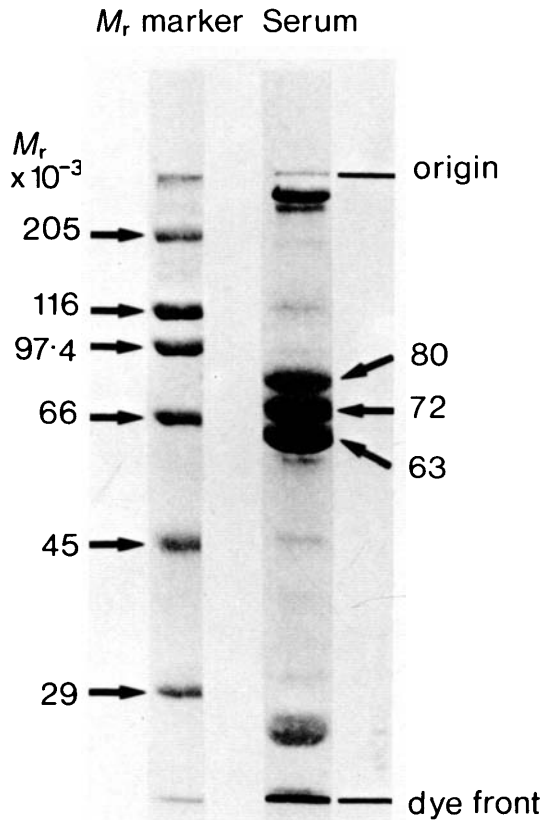
Fig. 11. Fractionation of denatured and reduced serum by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. This serum sample (15 $\mu$g protein) was taken from a day-17 control embryo. Three major serum protein bands corresponded to relative molecular masses ($M_r$) of approximately 80, 72, and $63 \times 10^3$, respectively. $M_r$ standards were obtained from Sigma Chemical Co. (St Louis, MO).
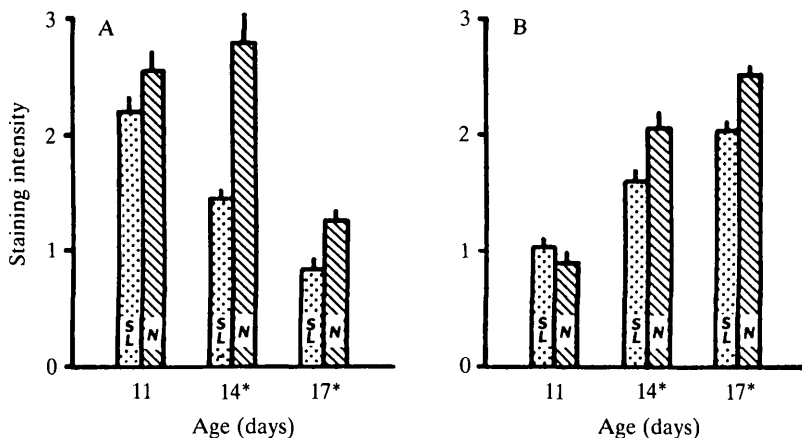


Fig. 12. Relative levels of the $72 \times 10^3$ (A) and $63 \times 10^3$ (B) serum proteins as a function of age. These values were based on densitometry of Coomassie Blue-stained sodium dodecyl sulphate–polyacrylamide gels and expressed relative to the staining intensity of the $80 \times 10^3$ protein (see Materials and Methods). Each value represents the mean $\pm$ S.E.M. of 9–20 embryos. Asterisk indicates a significant difference ($P < 0.05$) between shell-less and control embryos.

At day 11, the enzyme activity in shell-less embryos with liver necrosis was about twice as high as that in shell-less embryos without necrosis. During later stages of development, at days 14 and 17, significant increase in LDH activity was observed not only in embryos with liver necrosis versus those without necrosis but also in shell-less versus control embryos. To gain insight into the biochemical basis for the difference in serum LDH activity, the isoenzyme composition of LDH was analysed by isoelectric focusing of serum samples in polyacrylamide gels followed by histochemical staining of enzyme activity. Five LDH isoenzymes representing the tetrameric forms produced by two genes (Markert & Moller, 1959), were routinely observed after isoelectric focusing. These isoenzymes (LDH-1, LDH-2, LDH-3, LDH-4, and LDH-5) corresponded to isoelectric points of 6·6, 7·4, 7·7, 7·9, and 8·1, respectively (Fig. 9). The relative distribution of each isoenzyme activity in shell-less and control embryos with and without liver necrosis is shown in Fig. 10. In general, all five serum LDH isoenzyme activities were higher in shell-less embryos (with or without necrosis) than the corresponding control embryos except in the case of LDH-1 in day-14 embryos without liver necrosis. It was also observed that, except for LDH-5 of day-17 shell-less embryos, isoenzyme activities were higher in embryos with liver necrosis than those without necrosis for both shell-less and control groups.

*Serum protein profiles*

Fractionation of denatured and reduced sera by sodium dodecyl sulphate–polyacrylamide gel electrophoresis revealed that three protein bands constituted the major serum protein components. These bands corresponded to relative molecular masses ($M_r$) of approximately 80, 72, and $63 \times 10^3$ (referred to as 80 K, 72 K and 63 K), respectively (Fig. 11). Based on Coomassie Blue staining, the amount of the 80 K protein band appeared to be relatively constant in all groups of embryos. Therefore, the staining intensity of the 80 K protein band was used as an internal reference for quantifying the 72 K and 63 K proteins, i.e. the staining intensities of these two protein bands were expressed relative to that of the 80 K protein band in each sample. Since no difference was observed between embryos with and without liver necrosis in both shell-less and control groups, the data were pooled and shown in Fig. 12. At day 11, shell-less and control embryos showed similar relative levels of 72 K and 63 K proteins. However, during late development (at days 14 and 17) control embryos had significantly higher relative levels of 72 K and 63 K proteins compared to shell-less embryos. In both shell-less and control embryos, the relative level of 72 K protein decreased as a function of embryonic age, whereas the level of 63 K protein increased.

DISCUSSION

In the present study, chick embryos developing *in ovo* and in long-term, shell-less cultures were compared with respect to various parameters of metabolism and development. In addition to retarded growth during late development,

the most distinguishing feature of the shell-less embryos was their severe hypocalcaemia and hyperphosphataemia. Our observations also showed that development-specific necrosis of liver tissues appeared in chick embryos, similar to that previously reported for Japanese quail embryos (Ono & Wakasugi, 1983*a,b*). Furthermore, shell-less embryos exhibited higher frequency of liver necrosis during the second half of incubation. It was also found that serum levels of enzymes that are associated with tissue damage, i.e. alkaline phosphatase and LDH, were greatly elevated in shell-less embryos compared to control embryos. In particular, in both shell-less and control embryos, liver necrosis was always accompanied by higher serum LDH activity.

During chick embryonic development, extraembryonic calcium is derived from two sources. Prior to incubation day 10, the yolk is the primary source (Johnston & Comar, 1955; Simkiss, 1961), Beginning around day 10, the chorioallantoic membrane begins to mobilize eggshell calcium into the embryonic circulation (Johnston & Comar, 1955; Simkiss, 1961) to meet the increasing needs of the rapidly growing embryo. Overall the eggshell is the major calcium source and provides over 80 % of the needed calcium. It has been previously reported that sera from shell-less embryos exhibited hypocalcaemia and hyperphosphataemia (Burke, Narbaitz & Tolnai, 1979; Narbaitz, Sarkar & Fragiskos, 1983). The data here on serum calcium showed that shell-less embryos were calcium-deficient as early as day 11, strongly indicating and confirming that translocation of eggshell calcium by the chorioallantoic membrane must be taking place in control embryos by day 11. Beginning at day 14, serum calcium levels of shell-less embryos decreased to about half of the values in control embryos. This hypocalcaemia probably contributes to the accompanying hyperphosphataemia in day-17 shell-less embryos although the nature of the seemingly compensatory relationship is not known. This increased level of serum inorganic phosphate is most likely derived from the yolk and/or bone. Coexistence of hypocalcaemia and hyper-phosphataemia in shell-less embryos may be related to the inability of the embryo to obtain from the yolk the large amounts of calcium normally mobilized from the shell without increasing the absorption of yolk phosphate (Narbaitz, Sarkar & Fragiskos, 1983). How calcium and inorganic phosphate metabolism are regulated during embryonic development clearly deserves future attention.

Interestingly, chick embryos show development-specific necrosis of liver tissues. Histologically, this abnormality is characterized by a clear separation between the necrotic and adjacent healthy tissues. Ono & Wakasugi (1983*a,b*) have previously reported similar development-specific abnormality of liver tissues (necrosis) in Japanese quail embryos. It was reported that 8 % and 36 % of wild-type and heterozygous embryos for the black at hatch (*Bh*) gene exhibited liver necrosis at days 10–12, respectively. Liver necrosis itself is not fatal since the viability and hatchability of *Bh* heterozygous and wild-type quail were normal. The results of the present study also suggest that liver necrosis is unlikely to be fatal since the frequency of necrosis decreased in late embryonic development and viability of control embryos was excellent. Interestingly, it was observed here that the

frequency of liver necrosis was significantly higher in shell-less embryos, i.e. during severe systemic calcium deficiency. Whether there is a causal relationship between calcium deficiency and liver necrosis remains to be determined. It should also be noted that the necrotic indices of day-17 shell-less and day-18 control embryos were higher than those of 14 shell-less and 17 control embryos, respectively. This increase did not indicate higher frequency of necrosis (Fig. 6A) but was primarily due to a higher percentage of embryos which showed relatively small necrosis in both left and right lobes. Enzymes found in plasma or serum primarily result from leakage of somatic or blood cells (Bell, 1971) and are therefore useful for the diagnosis of tissue damage. For example, serum alkaline phosphatase is commonly used in the differential diagnosis of liver diseases as well as bone diseases and hyperparathyroidism (Raphael *et al.* 1976). Our results showed that in both shell-less and control embryos liver necrosis is not associated with higher levels of serum alkaline phosphatase. On the other hand, shell-less embryos consistently exhibit significantly higher enzyme activity compared to control embryos. It has been reported that shell-less embryos exhibited severe perturbation in overall skeletal mineralization (Narbaitz & Jande, 1978; Slavkin, Slavkin & Bringas, 1980; Tuan & Lynch, 1983). Thus, elevated alkaline phosphatase activity of shell-less embryos may be primarily attributed to the abnormality in bone formation. The enzyme, LDH, has an ubiquitous distribution (Latner, 1975) and, in the chick embryo, is particularly abundant in liver and cardiac muscle (Schultz & Ruth, 1968). Like alkaline phosphatase, serum LDH measurements have been widely used to diagnose various disease states, e.g. those involving heart, skeletal muscle, liver, kidney and tumour (Skillen, 1984; Danpure, 1984). In the present study, a significant increase in serum LDH activity was observed not only in embryos with liver necrosis versus those without necrosis but also in shell-less versus control embryos. It is thus conceivable that shell-less embryos may have general tissue damage in the organs mentioned above, and that liver tissue damage further contributes to the additional, elevated serum LDH levels in embryos with liver necrosis. Of the five LDH isoenzymes, elevated values of serum LDH-1 and LDH-2 are related to myocardial necrosis and LDH-4 and LDH-5 to damages of skeletal muscle and liver tissue (Cohen, Djordejevich & Ormiste, 1964; Sobel & Shell, 1972). In the present study, a significant increase in each of the serum LDH isoenzyme activities was generally observed in embryos with liver necrosis compared to those without necrosis as well as in shell-less versus control embryos. Furthermore, we observed that: (1) differences in LDH-1 and LDH-2 activities between embryos with and without liver necrosis are higher than those of LDH-4 and LDH-5 for both shell-less and control groups; and (2) on the contrary, differences in LDH-4 and LDH-5 activities between non-necrotic, shell-less and control embryos are higher than those of LDH-1 and LDH-2. Further research is clearly needed to establish the precise relationship between the LDH isoenzyme(s) and specific tissue damages during chick embryonic development.

Electrophoretic analysis of reduced and denatured serum samples revealed that shell-less embryos have decreased amounts of two major serum proteins (relative

molecular masses $= 72$ and $63 \times 10^3$) compared to control embryos. Although it remains to be determined whether this results directly from calcium deficiency (and the accompanying necrotic state of the liver tissue) of the shell-less embryos, our preliminary results indicate that these proteins have calcium-binding activities (Ono & Tuan, unpublished observations). It should be of interest to investigate the general effect of systemic hypocalcaemic and/or hyperphosphataemic conditions on the level of serum calcium-binding protein components.

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