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RESPIRATORY ACIDOSIS AND EGGSHELL RESORPTION BY THE CHICK EMBRYO

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SUMMARY

Eggs of the domestic fowl were injected with ^{45}Ca and then subjected to normal incubation or to incubation in 9% carbon dioxide in air. The embryos, yolk, albumen and shell were analysed at various times after incubation had started. The results demonstrate that there is normally a resorption of the eggshell during the latter part of incubation but that this process is depressed by hypercapnia. The results are discussed in relation to the events in normal development and in relation to possible mechanisms of shell resorption.

INTRODUCTION

About 80% of the calcium which is found in the bodies of hatchling birds is obtained by the resorption of the eggshell during the latter half of embryonic development. This uptake of calcium corresponds with the time when the chorioallantois develops and comes into contact with the eggshell (Simkiss, 1967). In the domestic fowl this occurs on the 10th day of incubation and shortly afterwards this extra-embryonic membrane becomes capable of actively transporting calcium ions (Coleman, DeWitt, Batt & Terepka, 1970; Garrison & Terepka, 1972). The calcium carbonate forming the innermost region of the eggshell is, however, always separated from the chorioallantoic membrane by a pair of keratin-like shell membranes which are about 70 μm thick. It is apparent, therefore, that the shell must be dissolved some distance away from the sites of calcium absorption. Little is known about the mechanism of shell resorption although electron micrographs of the chorioallantoic membrane have led to the identification of distinctive cells with many mitochondria, vesicles and microvilli similar in structure to the parietal cells of the gastric mucosa. They have been widely interpreted as acid-secreting cells although there is no direct evidence for this (Leeson & Leeson, 1963; Owczarzak, 1971; Coleman & Terepka, 1972).

It has been generally accepted, since the *in vitro* experiments of Buckner, Martin & Peters (1925), that endogenously produced carbonic acid provides the basis for shell resorption during incubation. It was shown by Dawes & Simkiss (1969, 1971) that chick embryos were remarkably resistant to the effects of elevated levels of carbon dioxide and rapidly compensated for the induced acidosis by increases in plasma bicarbonate levels. It was not clear, however, whether these high concentrations of plasma bicarbonate were associated with enhanced shell resorption or whether they were metabolic compensations by the embryo which might therefore be expected to inhibit shell resorption. The following experiments were undertaken to attempt to

correlate the calcium metabolism of chick embryos with their acid-base balance during respiratory acidosis.

METHODS

Eggs of the White Leghorn strain of domestic fowl were used throughout this work. A stock solution of 200 μCi/ml of ^{45}Ca was prepared and 100 μl were injected 4–5 mm into the narrow ends of 138 of these eggs. The syringe was rinsed with 100 μl distilled water and this was also injected through the same hole in the egg, which was then sealed with a 3 mm square of plastic tape.

Fertile eggs were incubated at 39·4°C (103°F) in commercial 'still air' incubators until 8 or 12 days of development, when they were divided into 2 batches. The control group continued to be incubated normally but the experimental group was transferred to an incubator connected to a gas-mixing pump supplying 9% carbon dioxide in air. The atmosphere in the incubator was continuously monitored by a Cambridge katharometer which recorded the carbon dioxide content. The input to the incubator was adjusted periodically to keep the atmosphere in the incubator at 9% carbon dioxide despite endogenous gas production.

Incubated eggs were carefully cracked open at 24-h intervals and the yolk, albumen, shell and embryos were separated. The sub-embryonic, allantoic and amniotic fluids were pipetted off and the membranes were dissected from the major egg components. Each of the samples taken for analysis was individually digested in concentrated nitric acid and 70% perchloric acid as recommended by Gerritz (1933). Total calcium was determined in each sample by atomic absorption spectroscopy using 15 mM/l $SrCl_2$ to suppress interference from phosphates. The distribution of radioisotope was determined by liquid scintillation spectrometry using a 1,4-Dioxan-based scintillation fluid containing 2,5-diphenyl-oxazole (PPO) as a primary scintillant (Kinard, 1957). Corrections were made for quench by using internal standards.

The effects of hypercapnia on the acid–base balance of the embryos were assessed on anaerobic blood samples taken by cardiac puncture. Blood pH before and after equilibration with known CO_2/O_2 gas mixtures was obtained using a Radiometer blood gas analyser as described previously (Dawes & Simkiss, 1969).

RESULTS

Distribution of ^{45}Ca before incubation

Eggs which were analysed within 4 h of the injection of ^{45}Ca had most of the isotope within the albumen and only 26% of the dose was recovered from the eggshell. After 24 hours about 40 to 45% of the dose was attached to the inner part of the eggshell and about 95% of the isotope could be recovered from the eggshell and albumen. Eggs which were stored for up to 6 days showed a similar distribution of isotope (Table 1) although there was a slight fall as some ^{45}Ca diffused into the yolk. When infertile eggs which had been labelled with ^{45}Ca were incubated in the presence of 9% carbon dioxide in air there was a decrease in the amount of label associated with the shell and over 70% could be recovered from the albumen (Table 1).

Table 1. *Distribution of ^{45}Ca (as % dose) in infertile eggs subjected to normal incubation procedures or incubation in 9% carbon dioxide in air. Results are means* ± S.D.

Days of incubation	Normal incubation		Incubation in 9% CO_2 in air	
	Albumen	Shell	Albumen	Shell
1 to 3	50·6 ± 12·3	43·5 ± 9·6	73·0 ± 9·4	29·9 ± 2·5
4 to 6	48·0 ± 15·7	39·8 ± 10·4	72·3 ± 7·8*	24·9 ± 8·2*

* Eggs incubated normally for 3 days before transfer to 9% carbon dioxide for 3 days.

Table 2. *The distribution of total calcium and ^{45}Ca in fertile eggs during normal incubation and during incubation in 9% carbon dioxide in air from 8 to 13 and 12 to 19 days. Values are means* ± S.D. *Total numbers of eggs used are shown in parentheses*

Days of incubation and numbers of eggs used	Calcium content (μM)		^{45}Ca (% dose)			
	Yolk	Embryo	Albumen	Yolk	Embryo	Shell
7 (4)	331 ± 28	1·5 ± 0·4	25·9 ± 5·9	20·2 ± 3·5	0·3 ± 0·1	18·8 ± 3·7
8 (7)	387 ± 138	2·6 ± 1·3	28·1 ± 4·7	17·4 ± 2·6	0·6 ± 0·1	31·0 ± 11·5
9 (6)	455 ± 166	3·8 ± 0·9	21·1 ± 3·1	12·2 ± 6·9	1·1 ± 0·5	38·9 ± 11·6
10 (6)	459 ± 117	8·8 ± 2·1	20·9 ± 6·4	14·5 + 2·8	3·7 ± 0·9	37·6 ± 11·8
11 (6)	461 ± 64	16·0 ± 6·0	20·8 ± 11·2	20·9 ± 6·8	6·3 ± 1·8	28·9 ± 8·3
12 (6)	364 ± 77	66·0 ± 32·0	24·9 ± 6·2	27·1 ± 7·2	14·0 ± 2·9	20·5 ± 6·2
13 (6)	428 ± 76	137·0 ± 14·0	21·1 ± 6·6	31·1 ± 6·2	22·0 ± 1·5	10·3 ± 6·4
14 (7)	505 ± 149	196·0 ± 37·0	15·8 ± 7·8	27·9 ± 10·7	21·9 ± 6·9	10·3 ± 6·3
15 (3)	631 ± 89	375·0 ± 50·0	6·3 ± 0·2	28·3 ± 3·3	22·0 ± 2·8	6·4 ± 3·9
16 (5)	832 ± 221	589·0 ± 95·0	4·4 ± 2·7	35·2 ± 6·4	24·0 ± 9·9	2·6 ± 0·3
17 (3)	892 ± 201	1025·0 ± 229·0	5·2	46·7 ± 5·5	24·7 ± 5·2	1·9 ± 0·8
18 (4)	922 ± 111	1384·0 ± 382·0	0·5 ± 0·7	40·8 ± 9·5	28·5 ± 11·7	1·9 ± 0·8
19 (3)	968 ± 330	1450·0 ± 352·0	—	30·0 ± 16·5	34·3 ± 7·0	1·9 ± 1·3
20 (1)	919	2024·0	—	25·3	48·9	2·1
21 (1)	—	2898·0	—	—	87·9	3·2
Hypercapnia from 8 days						
9 (8)	350 ± 146	3·7 ± 0·6	26·1 ± 9·5	13·6 ± 3·1	0·8 ± 0·3	43·6 ± 10·2
10 (8)	440 ± 171	9·3 ± 3·3	33·9 ± 9·1	14·1 ± 5·2	4·4 ± 1·8	31·3 ± 6·5
11 (8)	498 ± 150	30·0 ± 9·0	27·8 ± 8·7	18·7 ± 5·1	8·3 ± 1·9	29·7 ± 8·3
12 (7)	444 ± 139	56·0 ± 10·0	35·9 ± 6·2	25·4 ± 7·1	14·0 ± 4·7	15·2 ± 3·9
13 (4)	585 ± 188	178·0 ± 68·0	31·0 ± 3·9	27·2 ± 5·2	21·2 ± 5·9	12·1 ± 2·8
Hypercapnia from 12 days						
13 (3)	377 ± 16	144·0 ± 68·0	19·5 ± 0·7	20·0 ± 0·8	26·7 ± 3·7	23·2 ± 10·6
14 (2)	394 ± 105	205·0 ± 47·0	16·8 ± 8·0	31·9 ± 2·6	22·9 ± 0·3	5·2 ± 3·6
15 (3)	480 ± 27	327·0 ± 56·0	13·1 ± 2·6	26·2 ± 6·0	20·8 ± 4·0	2·8 ± 1·5
16 (3)	583 ± 147	643·0 ± 33·0	3·4 ± 1·5	34·2 ± 4·4	20·6 ± 3·8	1·3 ± 0·5
17 (3)	475 ± 133	699·0 ± 69·0	4·1	32·4 ± 11·6	23·7 ± 6·3	2·5 ± 1·0
18 (4)	518 ± 125	1053·0 ± 102·0	0·4 ± 0·4	32·8 ± 5·3	27·3 ± 1·8	2·7 ± 1·6
19 (4)	504 ± 106	1259·0 ± 234·0	1·5	27·9 ± 4·6	28·8 ± 6·4	1·2 ± 0·5

Changes during incubation

The calcium content of the albumen remains constant at about 25 μM until it is absorbed during the last week of incubation. The calcium content of the yolk and embryo throughout incubation are shown in Table 2 together with the distribution of ^{45}Ca between the albumen, yolk, embryo and shell. The blood, amniotic and allantoic fluids were not analysed. The effects of respiratory acidosis upon the distribution of calcium and ^{45}Ca are also shown in Table 2. The effect of respiratory acidosis upon the acid–base balance of the blood is shown in Fig. 1.

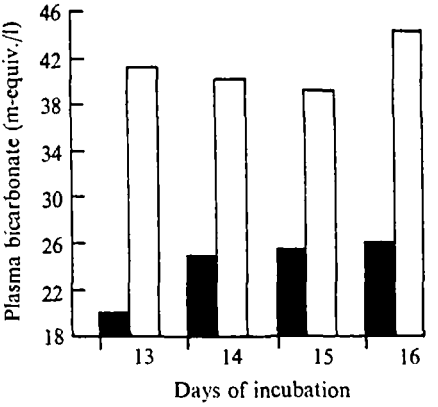

Fig. 1. Plasma bicarbonate levels of normal (black) and acidotic (white) embryos at various stages of incubation. The acidotic embryos were incubated in 9% carbon dioxide in air from day 12 onwards.

DISCUSSION

The influx of calcium into the incubated egg is clearly seen in Fig. 2. The total calcium in the egg contents remains virtually constant until about the 12th day of incubation, when it starts to rise. The rate of calcium absorption increases until day 15 and is approximately linear from then onwards at about 350 μM/day. These rates of calcium absorption are presumably determined by some interactions between the mechanisms of shell solubilization, calcium transport by the chorioallantoic membrane and the calcium requirements of the embryo.

The chorioallantoic membrane first makes contact with the shell on about the 9th to 10th day of incubation but the cells do not become fully differentiated until 12 to 14 days (Coleman & Terepka, 1972), which corresponds roughly with the time of increased calcium uptake. During the 14th day the membrane *in vitro* will transport calcium at a maximum rate of about 0·07 μM/cm^2/h (Garrison & Terepka, 1972) and measurements on membrane transport *in vivo* indicate a rate of about 0·13 μM/cm^2/h (R. J. Crooks & K. Simkiss, unpublished). These maximal rates occur at calcium concentrations of about 1·0 mM/l and are similar to those normally found *in ovo* at this time (about 0·14 μM/cm^2/h from the data in Table 2). In view of this one might expect any change in the rate of shell resorption to produce changes in the rate of calcium uptake by the egg contents.

The technique of injecting ^{45}Ca into the egg after it had been laid was first used by Johnston & Comar (1955) as a way of labelling the inner layers of the eggshell. It presumably acts by an exchange of radioisotope for shell calcium in the innermost mamillary layer of the shell, where the spherulite structure results in a relatively large surface of small crystals (Simkiss, 1967). The egg is obviously unevenly labelled by this technique, but Johnston & Comar claim that the isotope diffuses through the albumen and attaches to large areas of the shell.

Incubating infertile eggs in 9% carbon dioxide has the effect of diminishing the amount of ^{45}Ca which becomes bound to the inner part of the eggshell, and it will also actually liberate ^{45}Ca which had previously become attached there (Table 1). This is in keeping with the reaction

$$CaCO_3 + H_2CO_3 \rightleftharpoons Ca^{2+} + 2HCO_3$$

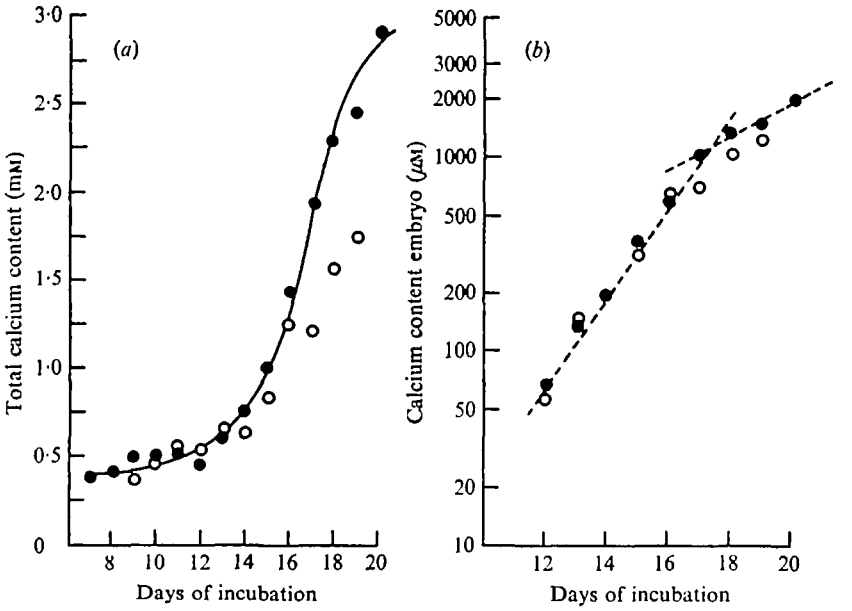

Fig. 2. (*a*) Total calcium content of eggs during normal development (●) and during incubation in 9% carbon dioxide in air (○). Note how the calcium content rises after about 10–12 days' incubation but that this effect is depressed under acidosis.
(*b*) Semi-logarithmic plots of embryonic calcium during normal development (●) and in respiratory acidosis (○).

which is the basis of the theory of shell resorption proposed by Buckner, Martin & Peters (1925). This theory was based upon experiments in which they showed that passing carbon dioxide through eggs containing either water or albumen resulted in the removal of calcium from the eggshell. These experiments on non-living systems are in agreement therefore in showing that shell resorption is enhanced by hypercapnia.

The *in vivo* experiments produce a different effect, however, for both the total egg contents and the embryos contain less calcium when incubated under conditions of respiratory acidosis (Fig. 2). Coincident with this decline in calcium metabolism is a large rise in plasma bicarbonate levels (Fig. 1) and it is tempting to conclude therefore that the two effects are causally related, perhaps by a reversal of equation 1. It has been previously noted by Dawes & Simkiss (1971) and again in the present work that hypercapnia tends to depress embryonic development by between 12 and 24 h as judged by 3rd toe length (Hamburger & Hamilton, 1951). If such a general retardation in development did occur it would obviously be relevant to the decreased calcium metabolism shown in Fig. 2, and in order to decide whether this is an important influence it is useful to compare the general pattern of metabolism between the two treatments.

The normal calcium metabolism of the chick embryo was investigated by Johnston & Comar (1955). Their results and the control data in our experiments are similar in showing that the incorporation of calcium into the yolk and embryo shows semi-logarithmic relationships with time (Fig. 2). The yolk loses ^{45}Ca during the period of 9 days but then gains it again until about day 18 or 19. The yolk obviously acts

as a short-term store early in development, but its calcium content increases from day 12 onwards after eggshell resorption has commenced. In all these criteria it appears that the control data shown in Table 2 agree with those of Johnston & Comar. The results obtained with hypercapnic eggs may be compared with these patterns to assess any retardation in development. In acidotic eggs the yolk starts to increase in total calcium from day 9 onwards and the decline in yolk ^{45}Ca occurs on day 17 (Table 2). Assessments of daily increments of specific activity of the embryos show that these are maximal on day 10 for both normal and acidotic chicks, indicating that the chorioallantoic membrane develops similarly in both types of embryos. These events all occur within 12–24 hours of normal development and suggest therefore that the depression of calcium uptake shown in Fig. 2 is largely an effect on shell resorption rather than a major retardation of development. Anatomical changes as judged by toe length similarly indicate that any retardation observed is less than 24 h.

It is concluded therefore that there is a fundamental difference in the effects of carbon dioxide upon shell dissolution in infertile and *in vivo* situations. Elevated levels of carbon dioxide decrease the rate of shell resorption by living embryos, and this suggests that carbonic acid is not the basis of shell resorption. Instead it appears that either the elevated plasma bicarbonate levels produced by the embryo inhibit shell resorption or, alternatively, some other acid is secreted during shell resorption but the effects of this are diminished by the high levels of carbon dioxide which depress the breakdown of calcium carbonate at the site of resorption.

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