INCREASE OF DEOXYRIBONUCLEIC ACID AND CELL NUMBER DURING MORPHOGENESIS OF THE EARLY CHICK EMBRYO

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INTRODUCTION

While deoxyribonucleic acid (DNA) has been measured in chick embryos obtained from eggs which had been incubated from 32 hours to 20 days[1], nothing is known about the rate of increase of DNA and the doubling rate of cells in the embryo during the important embryogenetic period immediately following the formation of the primitive streak. HOFF-JØRGENSEN[2] measured the DNA in incubated eggs of the domestic fowl by a microbiological method and found that the DNA content of the whole egg did not increase until after the third day of incubation. This work gives evidence for the rapid rates of cell doubling and increase of DNA in embryos and their extra-embryonic tissues between the first and third day of incubation. The increase in DNA content of chick blastoderms growing *in ovo* has also been measured to serve as a control for experiments on DNA synthesis by isolated chick blastoderms grown *in vitro*.

MATERIAL AND METHODS

Embryos and their extra-embryonic membranes were removed from the egg and the embryos identified morphologically by reference to the developmental stages of HAMILTON AND HAMBURGER[3]. The periods of incubation quoted by these authors for each stage were then used as "corrected incubation time", so that a certain incubation time corresponds to a definite stage of development. The embryos used in these experiments corresponded to developmental stages between 18 hour and 70 hour incubation (*i.e.* from the stage of primitive streak to that of an embryo possessing small limb buds). In the stages of development before the ten-somite stage the blastoderm was removed from the yolk, rinsed clean of yolk platelets in ice-cold saline and morphologically identified. After the ten-somite stage the extra-embryonic tissue was dissected around the periphery of the vascular area (sinus terminale) to provide an embryo with its vascular area intact. In some cases the embryo was separated from its vascular area so that analyses could be carried out on the embryo and the vascular area separately. This gave 3 fractions of tissue — "embryo with vascular area", "embryo", and "vascular area" (the area opaca in early stages). All tissues were stored at —20°C.

After weighing, the tissue fractions from two or more eggs were suspended in ice-cold 1.0% citric acid solution (0.5 ml) in a homogenizer which consisted of a glass plunger forming a loose fit within a small test-tube[4]. A few strokes of the plunger were usually sufficient to homogenize the tissue without disruption of the nuclei; embryos in later stages of development were naturally somewhat more difficult to homogenize. The nuclei were stained by adding an equal volume of an aqueous solution of methyl green (1 g/100 ml), stirring and leaving to stand for 15 minutes at 4°C. The nuclei were centrifuged down at 2500 r.p.m. for 5 minutes and the supernatant poured off and retained. The nuclei were then resuspended in a known volume of tap water and the number of stained nuclei determined by counting in a haemocytometer. For each determination the nuclei in 96 large squares were counted (usually about 1,000 nuclei). A nucleus undergoing mitosis was counted as one nucleus. The standard deviations ranged from 5–20%.

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A method of tissue extraction for DNA similar to that of OGUR AND ROSEN[5] was used. About 12 blastoderms, or groups of 3–8 embryos at later stages of development, were homogenized in distilled water and the homogenate brought to 0.2 *M* perchloric acid with 1.0 *M* perchloric acid. After stirring for 15 minutes the suspension was centrifuged and the deposit extracted again for 5 minutes with 0.2 *M* perchloric acid. The deposit obtained after centrifugation was then extracted twice in a similar way with ethanol and three times with ethanol–ether (1:2). The dry powder was extracted overnight with 1.0 *M* perchloric acid (1 ml/0.5 mg powder) to remove ribonucleic acid (RNA), followed by two more similar extractions for 15 minutes. All the above operations were carried out at 4°C. The DNA was then removed from the residue by 3 extractions at 70°C with 1.0 *M* perchloric acid for twenty minutes. Less than 3% of the total DNA was found in the RNA extract and a fourth extraction for DNA yielded less than 2% of the total.

In tissue fractions which had been stained with methyl green for cell counts, the supernantant was retained; after the nuclei had been counted, the supernatant was added to the suspension of nuclei which was then homogenized, made to 0.2 *M* perchloric acid and extracted in the usual way. The methyl green was completely removed before the RNA extraction. A small correction (about + 5%) had to be applied to the final DNA results to allow for the volume of nuclei suspension removed for the counts. When the supernatant fraction was analysed for DNA, only 3% of the amount in the nuclei was found (within the experimental error of the method).

The deoxyribose component of DNA was measured by reaction with indole using the method of CERIOTTI[6]. The volume of reagents used was one quarter of those used by CERIOTTI, so that DNA could be estimated in the range 1–7 μg/ml, (standard deviation 5–10%). The sample of DNA used as a standard was obtained from frog testicles. As the red colour produced during the reaction with indole was not always completely extracted by $CHCl_3$, a correction was introduced by measuring the absorption due to the contaminant at 520 mμ. This correction had only occasionally to be used with embryonic tissue but was essential in determinations of DNA in egg albumen and yolk.

RESULTS AND DISCUSSION

Nuclei counts of embryos with and without their vascular areas are plotted separately on a logarithmic scale against their corrected incubation times (Fig. 1). The exponential increase in cell number is then shown as a straight line. Results for the DNA content per embryo on a logarithmic scale show a similar trend but a much greater scatter (Fig. 2); if lines of the same slope as Fig. 1 are fitted to the results in Fig. 2, it is possible to demonstrate constancy of DNA per nucleus. In the embryos with their vascular areas,

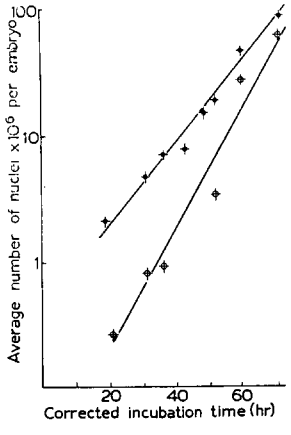

Fig. 1. The average number of nuclei per embryo during embryogenesis. Embryo (O—O); Embryo and vascular area (●—●).

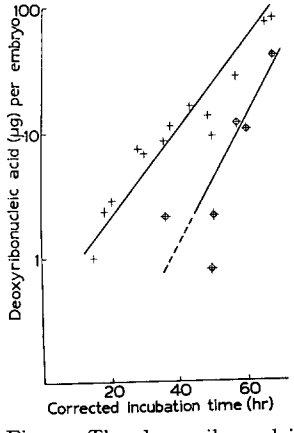

Fig. 2. The deoxyribonucleic acid content per embryo during embrogenesis. Embryo (O—O); Embryo and vascular area (+—+).

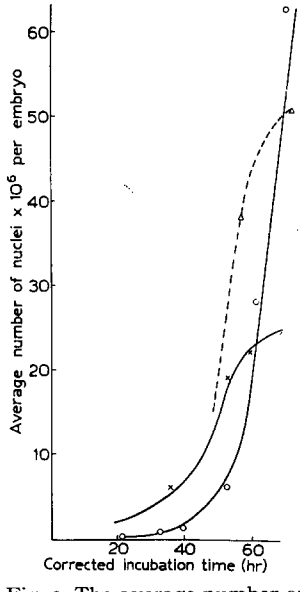

Fig. 3. The average number of nuclei in the chick embryo (O—O), the vascular area (×—×), and yolk sac (△—△) during embryogenesis.

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the average amount of DNA per nucleus from the two graphs is $1.0 \cdot 10^{-6}$ μg and remains constant throughout this period of development. From individual determinations the average amount of DNA per nucleus is $1.1 \cdot 10^{-6} \pm 0.5 \cdot 10^{-6}$ μg. In the embryo, the average amount of DNA per nucleus from the graphs is only $6.2 \cdot 10^{-7}$ μg and from four individual results is $7.5 \cdot 10^{-7}$ μg; both these sets of figures are within the limits of error due to the methods and biological variation.

On a linear scale the average number of nuclei in the developing embryos follows an exponential curve (Fig. 3). However, the average number of nuclei in the vascular areas (obtained by difference between the two curves in Fig. 1, together with four individual results) also increases exponentially, as the area opaca becomes increasingly vascular, up to about 55 hour incubation time. After this time, the rate of increase of cell number of this mesodermal layer bounded by the sinus terminale decreases; then there is a rapid increase in the average number of cells of endoderm (calculated from DNA results) which is spreading round the yolk to form the yolk sac.

As the number of cells has been determined in embryonic and extra-embryonic tissues during the 20–55 hour incubation period, it is interesting to compare the variation in the rate of doubling of cells from fertilisation using the data of OLSEN[6] for the cleavage stages (one to 256 cells). The instantaneous rate of increase in cell number (k) is calculated according to the formula of BRODY[8] and the doubling time (t_2) is then obtained from $t_2 = \log_e 2/k$. After the first cleavage, which according to OLSEN takes place about 5 hours after fertilisation, the doubling rates are between 0.8 and 1.0 hour up to the 256-cell stage. The average time of doubling from 256 cells to $2 \cdot 10^6$ cells (primitive streak stage) is 2.3 hours and this increases to 8.9 hours during embryogenesis (Fig. 4). The average doubling rate for cells of the embryo, without its extra-embryonic membranes, from the definitive streak stage to soon after limb bud formation (70 hour incubation) is 6.2 hours.

The DNA content per wet weight of the embryo increases during the 36–70 hour incubation period from 40 to 175 μg DNA per 100 mg wet weight; this means that in the early chick embryo the wet weight per cell is decreasing during embryogenesis (Fig. 5). In the vascular area

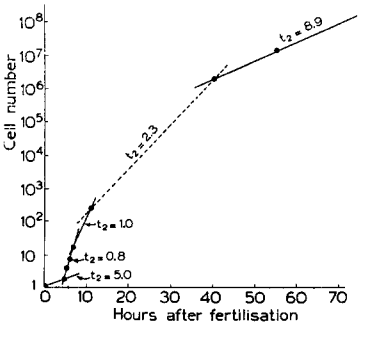

Fig. 4. Comparison of doubling rates of cells during cleavage of the hen's egg and early morphogenesis of the chick embryo. t_2 = rate of doubling in an hour.

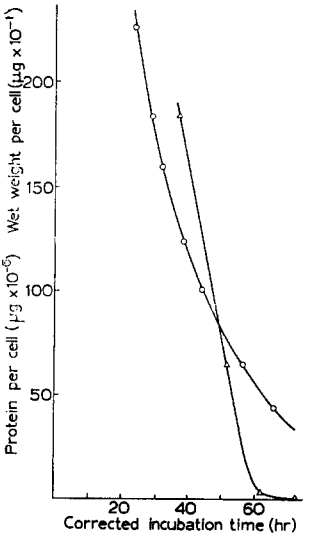

Fig. 5. The decrease of protein (△—△) and wet weight (○—○) per cell during early morphogenesis of the chick embryo.

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the ratio DNA/wet weight remains constant during the 30 to 70 hour incubation period.

Measurements of the protein content of embryos by the tyrosine method (using egg albumen as standard protein) showed the same rate of increase in protein for this period of development as those for protein nitrogen[9]; the protein per cell during this period showed a steady decrease until after 60 hour incubation time (Fig. 5). LEVY AND PALMER[10] found that there was a decrease of protein nitrogen per wet weight during the first four days of development of the chick embryo; the same relationship is seen in Fig. 5. The DNA[1] and protein nitrogen[9] results for embryos after the period of development studied in this work indicate that the protein per cell decreases less rapidly after three days, and after five days incubation time the protein per cell remains constant and then increases during later development. So that a characteristic of embryogenesis appears to be a very rapid increase in cell number accompanied by a loss of cell protein and to a lesser extent of cell wet weight. After embryogenesis, the rate of cell division gradually decreases and the steady rate of increase of protein nitrogen per embryo eventually becomes an increase of protein per cell; this latter process is the expected characteristic of later differentiation processes of embryonic cells[11].

The DNA required for the rapid rate of cell division in early chick embryos may be obtained by direct incorporation of DNA from the egg yolk or albumen. This would explain the unchanged DNA content (118 ± 12 μg) of the whole egg during the first three days incubation as found by HOFF-JØRGENSEN[2]. Measurements of the average DNA content of egg yolk and albumen during the period from laying to 72 hour incubation with the corrected Ceriotti method showed that there is about 400 μg DNA in the yolk and 460 μg in the albumen of each egg. Some of this DNA may be utilized by the embryos during early embryogenesis, but early chick embryos may also be capable of synthesising their own supply of DNA for their rapid cell division; this latter process is now being investigated by experiments *in vitro*.

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SUMMARY

The rates of cell doubling and increase of deoxyribonucleic acid in chick embryos and their extra-embryonic tissues have been studied between the first and third day of incubation. During this period embryogenesis appears to be characterized by a rapid increase in cell number accompanied by a loss of cell protein and, to a lesser extent, of cell wet weight.

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