NUCLEIC ACID CONTENT OF EARLY CHICK EMBRYOS AND THE HEN'S EGG

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Early literature on nucleic acids in chick embryos and the hen's egg has been reviewed by NEEDHAM[1] AND BRACHET[2]. The nucleic acid content of developing chick embryos (from the second to twentieth day of incubation) has been measured by NOVIKOFF AND POTTER[3]; REDDY, LOMBARDO AND CERECEDO[4] made similar measurements during the latter half of this period of development. HERMANN, SCHNEIDER, NEUKOM AND MOORE[5] have measured the increase in nucleic acid content of the developing somites of early chick embryos and found that protein nitrogen and nucleic acids accumulated at a similar rate, but that the increase in wet weight was more rapid. HOFF-JØRGENSEN[6] determined the amount of deoxyribonucleic acid (DNA) in the whole hen's egg and found that no increase of DNA occurred until after 3 days incubation of the egg. This constant level of DNA in the whole egg during the first three days of development is remarkable since embryogenesis and considerable cell division are known to occur in the egg during this period.

The nucleic acid content of the unincubated hen's egg, blastoderms and embryos during the first three days of incubation has been measured in this work by conventional colorimetric methods and by highly specific isotope dilution methods for uracil (RNA) and thymine (DNA). The growth curves obtained serve as a control for experiments on nucleic acid synthesis by isolated blastoderms[7] and reveal certain characteristics of the average embryonic cell during embryogenesis.

MATERIALS AND METHODS

Ribonucleic acid (RNA) and DNA were prepared from livers of the rat (August strain) by the methods of KIRBY[8,9]. Uracil-2-^{14}C (Californian Research Foundation, specific activity 1 mc/mmole) and thymine-2-^{14}C (Isotope Specialities Co., specific activity 0.3 mc/mmole; Californian Research Foundation, specific activity 1 mc/mmole) were used in the isotope dilution methods.

Unincubated eggs (6–10) were divided into egg white and yolk fractions, others were incubated at 37° for varying periods up to 3 days. Embryos and extra-embryonic tissues were separated from the egg yolk, placed in ice-cold isotonic saline and their morphological development classified according to the development stages proposed by HAMBURGER AND HAMILTON[10]. The average incubation time quoted by these authors for each stage was used so that morphological development is expressed as "corrected incubation time". Blastoderms were removed whole until the 10-somite stage; in later stages of development, the opaque area was dissected around the periphery of the vascular area (sinus terminale) and then the embryo was removed by dissection of the pellucid area. The three tissue fractions thus obtained were respectively "blastoderm", "vascular area" and "embryo". All tissues were stored at —20° C.

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Embryos (5–15) and extra-embryonic tissues were homogenised (ground glass homogeniser) in distilled water; egg yolk and white were homogenised with water using a Waring blendor.

A method of extraction of nucleic acids with perchloric acid similar to that of OGUR AND ROSEN[11] was used. All preliminary extractions were made at 4° C. The homogenate was brought to 0.2 *M* perchloric acid and stirred for 15 min; the suspension was centrifuged and the deposit extracted again for 5 min with 0.2 *M* perchloric acid. This extract is termed "acid-soluble fraction" and only such extracts from egg yolk and white were retained. Lipid material was removed from the deposit obtained after centrifugation by similar extraction with ethanol (twice), three ethanol–ether (1:2) extractions and the solid was then dried. In early experiments, the RNA was obtained from this powder by extraction overnight with 1.0 *M* perchloric acid (1 ml/0.5 mg powder) followed by two similar extractions for 15 min. However this procedure usually failed to remove all of the RNA, which then appeared in the DNA fraction (three extractions with 1.0 *M* perchloric acid at 70° for 20 min). Accordingly, RNA determinations were made on the pooled RNA and DNA fractions and, in the case of egg white and yolk, the RNA extraction step was eliminated and RNA and DNA determinations made on the final hot perchloric acid extract.

KIRBY[8] has extracted RNA from various mammalian tissues by removing protein and DNA with phenol. This method has been modified (KIRBY[9]) so that RNA and DNA remain in the aqueous layer, and has been used in this work to extract RNA and DNA from egg yolk and white. The homogenate of egg yolk (or white) was made to 6% (w/v) with respect to sodium *p*-aminosalicylate, which was dissolved by stirring for 15 min at 4° C. An equal volume of 90% (w/v) aqueous phenol solutions was then added and the mixture stirred vigorously for 1 h at room temperature. The mixture obtained from egg yolk was centrifuged on a Spinco centrifuge at 20,000 r.p.m. for 1 h at 0° C, when it separated into three layers. The lowest layer consisted of phenol and protein, (there was an interphase of insoluble protein between the phenol and aqueous layers), then an aqueous layer containing RNA and DNA, and a yellow upper layer containing lipid. The lipid and aqueous layers were removed by suction and the residue extracted with an equal volume of water and again centrifuged. The combined aqueous and lipid layers were then extracted three times with ether to remove lipid and the residual ether removed by blowing nitrogen through the aqueous layer. When this procedure was applied to egg white, centrifugation at 2,000 r.p.m. for 1 h at 0° on an International centrifuge was sufficient to separate the phenol and aqueous layers and no ether extraction was necessary. The extracts were then dialysed against distilled water (20 vols) to remove sodium *p*-aminosalicylate and nucleotides of low molecular weight. After three changes of water (during 2 days) the contents of the dialysis bag were colourless.

Nucleic acids were measured by estimation of the uracil content of RNA extracts and the thymine content of DNA extracts. The method is similar to that used by MARSHAK AND MARSHAK[12] and DURAND[13]. Aliquots of perchloric acid extracts of nucleic acids or "acid-soluble" nucleotides were concentrated under reduced pressure to 70% perchloric acid (0.5 ml). Aliquots of nucleic acid extracts of egg white and yolk obtained by the phenol method were evaporated to dryness under reduced pressure and suspended in 70% perchloric acid (0.5 ml). The nucleic acids were then digested in the concentrated acid for 1 h at 100° C. Nucleic acids (about 1 mg) obtained from rat liver were digested in the same manner. This treatment has been shown to give a maximum release of pyrimidines with no conversion of cytosine to uracil[14–16]. A known amount of thymine-2-^{14}C or uracil-2-^{14}C was added to the digest, which was shaken thoroughly and allowed to cool for 1 h. The perchloric acid was then precipitated as its potassium salt (the solution was kept slightly acid) and the insoluble salt spun down. The supernatant solution was concentrated under reduced pressure to 0.5–1.0 ml for application to a paper chromatogram. This precipitation of potassium perchlorate was extremely wasteful as losses (about 90%) of radioactivity occurred at this stage; however, as colorimetric determinations of nucleic acids had previously been made on perchloric acid extracts, the same extracts were used for the isotope dilution method.

The whole supernatant from the digest was applied in a band (2–3 cm) on a paper strip of Whatman No. 1 paper (4 cm wide at the bottom and tapering to 2 cm wide after 10 cm). The chromatogram was developed overnight using *n*-butanol–0.1 *N* NH_3 (7:1)[14]; after the papers were dry, the bases were detected by ultraviolet absorption[17] and the radioactive pyrimidine was located by scanning the chromatogram for radioactivity. The radioactive area of the chromatogram was cut out and eluted by applying drops of 0.1 *N* HCl to the top of the rectangle of paper which was inserted in the top of a small test-tube. The volume of eluate was about 0.5 ml (this was found to give a 95% recovery based on radioactivity). The eluate was then spotted on a second paper strip (2 cm wide) and chromatographed with *n*-butanol–0.1 *N* HCl (7:1)[14]. These two solvents were then used alternately until the eluate showed an absorption spectrum identical with that of the appropriate pure pyrimidine.

The eluate (0.5 ml) was made up to 1 ml with 0.1 *N* HCl and the absorption of the solution measured against a similar eluate of a paper blank between 230 and 300 mμ in 1 cm cells using

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a restricted light path on the Unicam S.P. 500. Blank chromatograms were run in the same tank as the unknown; four rectangles of paper of similar size to that containing the pyrimidine were each eluted in the same manner with 0.1 *N* HCl and then pooled to give the blank solution. The variation in absorption of the paper blanks was calculated from the absorption values at 250, 260, 280 and 300 mμ using 0.1 *N* HCl as a blank. After subtraction of the absorption at 300 mμ the absorption values for the other wavelengths were plotted against the absorption at 300 mμ (the absorption at 300 mμ increased as the weight of paper eluted). The root mean square value for deviation of optical density from a sight-fitted curve of 36 observations for each wavelength (250, 260 and 280 mμ) was equivalent to ± 0.06 μg pyrimidine. The purity of the pyrimidines were checked by the ratios of absorption[18] 250/260 (uracil 0.84; thymine 0.67) and 280/260 (uracil 0.175; thymine 0.53). The pyrimidine content of the solutions was calculated from ε max. values of 8,130[19] (258 mμ) for uracil and 7,890[20] (264 mμ) for thymine.

Aliquots (0.2–0.5 ml) of the pyrimidine solution were dried on glass cover slips (ringed with grease pencil to give identical areas) under an infrared lamp. Counting at zero self-absorption, at least 10,000 counts were registered by a thin end-window counter for each sample (usually about 200 times the background). Counting errors which were experimentally determined and included plating variations, instrumental fluctuations, etc., were about six times the calculated standard deviation.

To calculate the pyrimidine content of a sample the initial specific activity (counts per min/μg) of the pyrimidine was compared with the final specific activity in the following formula.

$$Sa = S'(a + b)$$

$$b = \frac{Sa}{S'} - a$$

where b = μg thymine (or uracil) in extract, a = μg thymine-2-^{14}C added, S = specific activity of the added thymine-2-^{14}C and S' = specific activity of the final diluted thymine-2-^{14}C. The total error (ultraviolet absorption and counting) for S' was the root mean square value of the two errors, and the error on b was the root mean square value of the two errors of S and S'. The uracil or thymine content of rat liver RNA and DNA was then used to relate the pyrimidine content of extracts to nucleic acid content.

Colorimetric methods

Aliquots of perchloric acid extracts of RNA were neutralised and interfering hexose compounds removed by hydrolysis in 0.1 *M* NaOH at 100° for 15 min[21]. RNA was then determined by the orcinol method[22] using reduced amounts of reagent so that the final volume of the *iso*amyl alcohol extract was only 1 ml. Values for DNA content of blastoderms and embryos determined by a modified indole method[23] based on frog testicle DNA have previously been reported[24]. When RNA was present in extracts of DNA a correction was applied to deoxyribose determinations (10 μg RNA = 1 μg DNA). For comparison with the thymine isotope dilution method these results have been based on rat liver DNA. When the colorimetric methods were used with nucleic acid extracts from egg white and yolk highly variable results were obtained[25].

Protein was determined by the method of SUTHERLAND *et al.*[26]. Bovine plasma albumin (Armour Laboratories) dissolved in 0.1 *M* phosphate buffer (pH 7.6) was used as standard protein. Estimations were made within the range 40–200 μg albumin in 5.5 ml.

RESULTS

Nucleic acids in the unincubated hen's egg

The "acid-soluble" fraction of homogenates of egg white and yolk contained only small amounts of free thymine or thymine nucleotide (Table I). Although there is little uracil nucleotide in the yolk, there are comparatively large amounts of free uracil or uracil nucleotides in egg white.

The nucleic acid content of egg white and yolk is given in Table II. The RNA content of the white is considerably greater than that of the yolk and there is more RNA than DNA in both white and yolk. The phenol partition method appears to give a better extraction of RNA than the perchloric acid method and this is more pronounced in the extraction of egg white than in that of egg yolk. Greater amounts of DNA appear to be extracted by perchloric acid treatment; again, this effect is

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TABLE I

"ACID-SOLUBLE" URACIL AND THYMINE DERIVATIVES IN EGG WHITE AND YOLK

	Uracil (*μg per white or yolk*)	*Thymine* (*μg per white or yolk*)
White	460 ± 55	19 ± 4
Yolk	13 ± 1	6.6 ± 0.3

TABLE II

NUCLEIC ACIDS IN EGG WHITE AND YOLK

	RNA (*μg per white or yolk*)	*DNA* (*μg per white or yolk*)
Yolk		
Perchloric acid extraction	68 ± 14	52 ± 6
		57 ± 7
Phenol partition	114 ± 14	39 ± 2
White		
Perchloric acid extraction	520 ± 33	308 ± 16
Phenol partition	2260 ± 100	137 ± 30
	1595 ± 50	142 ± 5

more marked in the case of egg white than that of yolk. One explanation of the lower amounts of DNA obtained by the phenol method may be that more washing of the phenol layer with water is necessary to obtain similar extraction efficiency to that of perchloric acid. Alternatively, not all the DNA may be released from the egg proteins by treatment with *p*-aminosalicylate in the phenol method.

The ratio of amounts of nucleic acid in egg white and yolk (obtained by the phenol partition method) to amounts of the respective pyrimidine in the "acid-soluble" fraction is approximately constant (6.5).

Nucleic acids in the developing blastoderm and early chick embryo

The RNA content of blastoderms and developing embryos determined by the orcinol and uracil isotope dilution method during the first three days of incubation are shown in Fig. 1. It can be seen that both methods give reasonable agreement, and the increase in RNA conforms to a logarithmic growth curve. There is a more rapid synthesis of RNA in the embryo than in the developing blastoderm (embryo + vascular area).

Results obtained by colorimetric estimation of DNA[24] have been related to rat liver DNA (there was little apparent difference between analytical data on DNA obtained from either frog testicles or rat liver) and the results obtained by the deoxyribose and thymine methods are compared in Table III. The results show reasonable agreement allowing for the error on each method; the thymine results are usually slightly higher than those obtained by the deoxyribose method. A previously reported value for the amount of DNA per nucleus (based on deoxyribose measurements) in early chick embryos[24] was $1.1 \cdot 10^{-6}$ μg, standard deviation $\pm 0.5 \cdot 10^{-6}$ μg; the average values for DNA per nucleus based on the thymine measurements are higher. In embryos, the amounts of DNA per nucleus are within the range 1.0–2.1 and in

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blastoderms from 1.2–1.8 μg. The small number of determinations by the thymine method on embryos make it impossible to give an accurate figure.

TABLE III

DNA CONTENT OF EMBRYOS DURING EMBRYOGENESIS

	Corrected incubation time (h)	*No. embryos*	*Deoxyribose method μg DNA per embryo*	*Thymine method μg DNA per embryo*	*DNA (thymine method) per nucleus (μg × 10⁻⁶)*
Embryos	35	7	2.4 ± 0.2	2.7 ± 0.2	2.1
	52	15	7.7 ± 0.6	9.2 ± 1.0	1.2
	70	4	45.0 ± 1.5	61.5 ± 3.2	1.0
Blastoderms	19	8	2.4 ± 0.2	3.1 ± 0.2	1.6
	35*	7	9.2 ± 0.9	8.3 ± 0.4	1.2
	37	5	12.9 ± 0.9	13.0 ± 0.9	1.2
	53	5	14.8 ± 1.0	22.2 ± 1.0	1.8
	56	5	24.4 ± 1.8	38.6 ± 2.1	1.4

* by addition of 35 h embryo and opaque area.

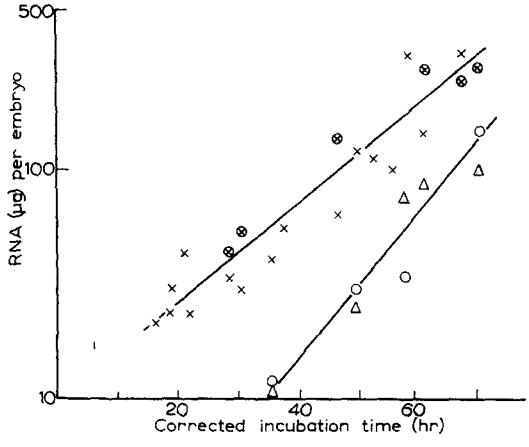

Fig. 1. Increase of RNA in the early chick embryo. × blastoderm (ribose); △ embryo (ribose); ⊗ blastoderm (uracil); ○ embryo (uracil).

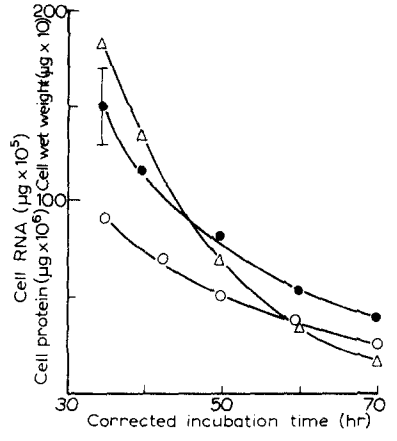

Fig. 2. Decrease of average RNA (○—○) protein (△—△) and wet weight (●—●) in the early embryonic cell.

In blastoderms, the rate of increase of RNA is slightly slower than that of DNA, but that of protein, wet weight and DNA are very similar; however, in embryos there is an increase of DNA per wet weight (40 to 170 mg %) during the 35 h to 70 h incubation period based on deoxyribose determinations, and from 93 to 240 mg % during the same period based on thymine determinations. NOVIKOFF AND POTTER[3] found about 110 mg % DNA in embryos after 2.5 days incubation. There is no variation in RNA per wet weight (500–600 mg %) in embryos up to 3 days of incubation; this level of RNA is again higher than the results obtained by NOVIKOFF AND POTTER[3] during the 2.5 to 4 day incubation period (350 mg %). These authors found a sharp decrease in RNA per wet weight after about 5 days of incubation.

The RNA/DNA ratio is greater in 24 h blastoderms (8) than in the embryo at 35 h of incubation (4). The ratio of RNA/DNA in the somites of the chick embryo

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during the 35–46 h incubation period is about 2.0 (calculated from the results of HERMANN[27]).

The rate of increase of RNA per embryo during the first three days of incubation is slower than that of the increase in the average number of nuclei per embryo[24]; this can alternatively be expressed as a decrease of RNA per cell. In Fig. 2 the decrease of RNA in the early embryonic cell is compared with the decrease in protein and wet weight. While the decrease of RNA and wet weight are similar, cell protein decreases more rapidly.

DISCUSSION

Recent evidence for the occurrence of DNA in the hen's egg has been given by FRAENKEL-CONRAT *et al.*[28] who isolated a protein, avidin, from hen's egg white and found that it contained DNA. HOFF-JØRGENSEN[6] used a microbiological method for deoxyriboside (after depolymerisation of DNA with deoxyribonuclease) to determine DNA in the whole hen's egg and found that the unincubated egg contained 118 $\pm$ 12 μg DNA. This figure is somewhat lower than figures obtained in this work by the isotope dilution method with perchloric acid extraction (362 μg) and the phenol method (179 μg DNA). Although HOFF-JØRGENSEN[6] found no increase in DNA content of the incubated hen's egg until after the 3rd day of incubation, a considerable increase in cell population is known to occur in the egg during the first three days of incubation. At the third day of incubation, the chick embryo and its vascular area (excluding outer periphery of yolk sac) contain at least 80 μg DNA[24], and from HOFF-JØRGENSEN's results it might be concluded that nucleic acids in the egg yolk or white were being utilised by the developing embryo.

FRAENKEL-CONRAT *et al.*[28] using the method of SCHNEIDER[29] found 30–45 μg total nucleic acids per ml of egg white; this is roughly equivalent to 900–1350 μg nucleic acid per egg white. Average figures for total nucleic acid in egg white in this work are close to this range (828 μg by the perchloric acid method and 2068 μg by the phenol method). As egg white is a secretion of the oviduct it is not surprising to find relatively large quantities of RNA present and the DNA may be derived from cell debris in the oviduct[30].

HOTCHKISS[31] has suggested that this "cytoplasmic DNA" may be a precursor form and is not necessarily genetically specific material. It is possible that the DNA is present as a storage reserve[25, 31] to supply DNA for early embryological development until the embryo is capable of synthesising its own DNA. The nucleic acids (or similar highly polymerised compounds) may be a convenient means of storing nucleic acid precursors, which could be obtained by degradation when required by the embryo. The possibility remains, however, that the nucleic acids in the egg are largely non-functional in early embryogenesis and it is only during later development that these nucleic acids are degraded and used by the embryo in the same manner as other yolk and white constituents.

The content of acid-soluble pyrimidine and pyrimidine nucleotides of unincubated hen's yolk and white appears to bear some quantitative relation to the respective amounts of nucleic acid present. If these acid-soluble materials are about to serve as precursors for nucleic acid synthesis in the same way as suggested by MARRIAN[32] for purine precursors, the low amounts of acid-soluble pyrimidines might correspond to the slight requirement of the early blastoderm for such material.

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In hen's egg white, there is over ten times more RNA than DNA (by the phenol method); but in the yolk there is only three times more RNA than DNA. When the results obtained by perchloric acid extraction are compared, no such differences in RNA and DNA content of the hen's egg are observed.

The RNA/DNA ratios of the 10 somite embryo (4) and blastoderm (8) are somewhat higher than the ratios found in most tissues of the chick embryo during later developmental stages[27, 33, 34]. This is in accord with the findings of CASPERSSON[35] and BRACHET[36, 37] that nucleic acids are abundant in cells which are rapidly synthesising protein.

Previously reported values for amounts of DNA per nucleus for fowl tissues and erythrocytes are within the range $2.1–2.6 \cdot 10^{-6}$ μg[13, 38, 39]. NOWINSKI AND YUSHOK[40], using the Dische reaction, obtained values as high as $8.2 \cdot 10^{-6}$ μg DNA per nucleus in the wings and legs of chick embryos. KURNICK[41] measured DNA by reaction with methyl green in fowl erythrocytes and reported low values of DNA per nucleus ($1.6–1.8 \cdot 10^{-6}$ μg). Results obtained using the thymine method for DNA show a wide scatter between $1.0 \cdot 10^{-6}$ and $2.1 \cdot 10^{-6}$ μg DNA per nucleus in embryos, but those for blastoderms (Table III) are in reasonable agreement with those obtained by KURNICK[41].

The rapid rate of cell division during embryogenesis of the chick may well be the dominant factor in its metabolism. The rates of increase of wet weight and protein are not sufficient to keep up with cell division and DNA synthesis; consequently there is an apparent loss of wet weight and protein per embryonic cell. This state only exists for the first three or four days of incubation after which the instantaneous rate of increase of DNA becomes less (from results of NOVIKOFF AND POTTER[3]) and the cell protein and wet weight slowly increase during later stages of differentiation[33, 42].

The various opinions on the relationship between cell division and differentiation in embryonic development have been admirably summarised by HUGHES[43]; it is probable that the metabolism of the early chick embryo is geared to maintain a high rate of cell division even at the expense of some of the cell constituents. During this period, the fundamental embryonic structures are formed and it is only when cell division slows down that the embryonic cell is able to elaborate its metabolism to promote functional differentiation.

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SUMMARY

The nucleic acid content of hen's egg white and yolk has been measured by isotope dilution methods using perchloric acid extraction and phenol partition techniques. The origin and possible physiological function of the nucleic acids is discussed.

Nucleic acids have been measured by colorimetric and isotope dilution methods in blastoderms and chick embryos up to the third day of incubation. A dominant factor in embryogenesis of the chick appears to be a high rate of cell division and accumulation of DNA, which is greater than that of RNA and protein accumulation and increase of wet weight.

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