Induced Preincubation Aging of the Avian Egg and Subsequent Development of the Embryo, as Revealed by the DNA, RNA, and Protein Level of Its Spleen[1] (34326)

D. P. SINGAL[2] AND I. L. KOSIN

Department of Animal Sciences, Washington State University, Pullman, Washington 99163

Because of its theoretical and practical importance, the question of reduced biological fitness of chicken and turkey embryos, developing in eggs subjected to prolonged preincubation storage, has received considerable research attention, *e.g.*, see the reviews by Landauer (1) and Kosin (2). Recent studies at this laboratory have emphasized those aspects of the problem which are related to the response to such preincubation "aging" of the affected blastoderm as a whole, as well as of its component cells (3–5). These studies showed that the frequently observed modifications in the embryogenesis of such specimens reflect cytologically detectable changes. The objective of the present investigation was to take the analysis of this state of "reduced fitness" one step further, to the level of three important cellular components: DNA, RNA, and protein.

Splenomegaly has been widely used in analyzing the dynamics of avian embryogenesis (6–9). Accordingly, it was selected by us as the methodological approach to test a hypothesis that the *in situ* storage treatment of the blastoderm so alters its biological status that the embryo is no longer fully capable of responding to an otherwise effective stimulus (such as the presence of an immunologically competent implant of splenic tissue). Under these circumstances, the effect of preincubation storage could be expected to reveal itself in the host's spleen through a reduced level of splenomegaly or through quantitatively measurable changes at the cellular level.

The hypertrophy of the host's spleen, following the implantation of a donor's spleen, is due (i) to the stimulation of cell division within the target organ, and (ii) to the invasion of the target organ by cells from the implant and their subsequent rapid proliferation in the new environment (10, 11). In the present study, the net effect of the stimulus would test the capacity of the host's spleen to respond to both of these interdependent components. The following parameters were considered for this purpose: weight of the embryo and of its spleen, mitotic index of the latter, as well as its DNA, RNA, and protein content.

Materials and Methods. Hatching eggs from White Leghorn chickens (*Gallus gallus*) and Broad Breasted Bronze turkeys (*Meleagris gallopavo*) maintained at the Poultry Plant of the Department were the source of the experimental material. The eggs were subjected to preincubation storage at 13° and 85% ± 5 RH: chicken eggs for 0, 7, 14, and 21 days and turkey eggs for 0, 7, and 14 days. After storage, the eggs were incubated at 37.5° and 65% RH. The bulk of the chicken eggs was incubated for 10 days and all of the turkey eggs for 14 days before each "operated" embryo received on its chorioallantoic membrane (CAM) a graft of splenic tissue from a mature White Leghorn male donor. In addition, some chicken eggs stored for 21 days were incubated for 11 days before they received a graft. This was because of a possibility that the biological age of the avian embryos from stored eggs lags behind their chronological age (1). Because this discrepancy may be due either to a delay in the initiation of development or to its reduced rate, a random sample of chicken eggs from

[1] Scientific Paper No. 3200, College of Agriculture, Washington State University, Pullman; Project 1255.

[2] Present address: University Hospital, University of Saskatchewan, Saskatoon, Sask., Canada.

the 21-day storage group was incubated for an additional 24 hr. They were examined on day 19 (instead of day 18) for the rate of cell division, DNA, RNA, and protein content of the spleen. These embryos were designated as *age group 21-2*.

In all instances, appropriate groups of "nonoperated" controls (*i.e.*, embryos which were not subjected to grafting) were maintained. The method of implantation of the splenic tissue was essentially that described by Hamburger (12). Following the operation, the eggs were incubated for 8 additional days, after which the embryos containing viable grafts were subjected to further analysis.

The embryo spleens first were weighed to the nearest 0.1 mg., and then processed for histological examination. Fixation was in Carnoy's solution. The material was sectioned at 6 μ, stained by Feulgen's light green procedure (13) and the sections then were examined for the frequency of dividing cells. This parameter (referred to as mitotic index) was based on counts of 100 areas randomly picked from various portions of the spleen.

The method used for nucleic acid determination was that described by Schneider (14), using the diphenylamine reagent for DNA and the orcinol reagent for RNA. Determination of the nitrogen–protein content of the spleen followed the micro-Kjeldahl procedure (15).

Results and Observations. Body weight of embryo. The effect of storing hatching eggs prior to incubation, and of subsequent grafting of CAM with splenic tissue from mature White Leghorn donors, on the body weight of 18-day-old chicken and 22-day-old turkey embryos is summarized in Table I. Storing eggs decreased the body weight in both the experimental (implanted) and control (nonimplanted) series. Thus, the storing of chicken eggs for 21 days resulted in a 26.3% decrease in the experimentals and a 23.3% decrease in the controls. This decrease from the corresponding values in the nonstored group was significant at the 1% level. In turkey embryos, 14 days of preincubation storage also caused a significant decrease in the body weight ($p < 0.01$). Embryos in the *age group 21-2* showed an increase in the body weight over that of embryos from chicken eggs of comparable preincubation storage treatment that received the graft on day 10 and were examined on day 18 (*age group 21-1*). The observed body weight values in the former remained, however, lower than those of the 18-day-old embryos developing in nonstored eggs. On the average, they were 12.7% less in the experimental series and 7.9% less in the controls. In general, regardless of the storage "age" of the egg in either species, the body weight of embryos in the experimental series was reduced in relation to that of the controls.

Weight of embryo spleen. As expected, a striking enlargement of the embryo spleen was observed in the CAM-implanted groups (Table I). There was no clear-cut evidence that the magnitude of this increase in spleen weight was affected by the length of preincubation storage of the eggs in which the embryos subsequently developed. Within the range of this increase, fluctuations of the spleen weight reflected the already mentioned body weight depression in the same embryos.

Frequency of dividing cells in embryo spleen. In line with increased spleen size in the implanted embryos, the rate of cell division in them was also raised (Table II). But again, the preincubation age of the egg had a major effect on this rate: the frequency of dividing cells decreased in the spleens of the experimental and control embryos when the eggs first had been subjected to preincubation storage.

Nucleic acid content of embryo spleen. The preincubation age of the egg in either species had no effect either on the DNA or RNA content of the target organ (Table II). However, in the experimental series there was less DNA per milligram of wet weight of spleen. This decrease was statistically significant at the 5% level in embryos from all egg age groups in the chicken and from 0- and 7-day stored turkey eggs.

Protein content of the embryo spleen. Table II shows that as the preincubation age of the egg rose, the protein content of the spleen in the embryos of either treatment group

TABLE I. Effect of Preincubation Storage of Eggs and of CAM Grafting on the Body and Spleen Weights of Host Chicken and Turkey Embryos.

Storage (days)	n	Grafted		Control	
		Body wt, $\bar{x}^a$	Spleen wt, $\bar{x}^b$	Body wt, $\bar{x}^a$	Spleen wt, $\bar{x}^b$
Chicken embryos					
0	40	20.5 ± 0.32	24.5 ± 2.04	21.5 ± 0.27	11.1 ± 0.31
7	47	18.9 ± 0.37	24.8 ± 2.65	20.4 ± 0.33	10.7 ± 0.34
14	48	17.9 ± 0.30	22.2 ± 1.88	18.9 ± 0.49	9.9 ± 0.40
21–1	39	15.1 ± 0.40	20.8 ± 2.40	16.5 ± 0.40	8.9 ± 0.30
21–2[c]	45	17.9 ± 0.55	22.4 ± 2.15	19.8 ± 0.49	9.9 ± 0.41
Turkey embryos[d]					
0	54	23.0 ± 0.37	23.4 ± 1.16	25.2 ± 0.34	10.1 ± 0.25
7	51	22.3 ± 0.35	22.7 ± 1.49	24.3 ± 0.45	9.9 ± 0.33
14	56	21.3 ± 0.34	20.3 ± 1.02	22.0 ± 0.41	8.1 ± 0.31

[a] ($\bar{x}$ g ± SE).

[b] ($\bar{x}$ mg ± SE).

[c] Age at grafting in the age group 21–2 was 11 days and at examination—19 days (in all other groups of chicken embryos subjected to grafting, age at the time of operation was 10 days, and at examination—18 days).

[d] In turkeys, age at grafting—14 days and at examination—22 days.

decreased. The decrease was significant at the 1% level for embryos developing in the chicken eggs which had been stored for 21 days (*age group 21-1*) and for 14 days in turkey eggs.

Further analysis of the biological status of embryo spleen. Davidson and Leslie (16, 17) suggested a number of years ago a method by which one can relate the chemical development of tissues to the DNA content per cell. This method is based on the observation that the DNA content of a cell remains essentially constant in all somatic tissues of a species (18, 19). Thus, with the average nuclear DNA content known for a species, cell number in an organ can be estimated by determining its total DNA; the average cell composition of RNA, protein, and other constituents are then expressed in relation to the amount of DNA per cell. It is clear, of course, that this procedure, although useful, offers at best only a rough approximation for any given organ, because organs are composed of multiple cell types that occur in varying proportions as development proceeds.

In the present study, the value of 2.4×10^{-6} μg of DNA/nucleus in the chicken embryo (16) was taken as the basis for all subsequent calculations: number of cells in a milligram of wet tissue, the total number of cells in an organ, as well as its RNA and protein content. These estimates involved the spleens of both nonoperated and operated embryos developing in eggs previously subjected to preincubation storage of different duration.

No effect of preincubation age of the egg was observed on the estimated number of cells per unit weight of the target organ in either treatment group (Table III). Neither was the estimated RNA content per cell affected by the preincubation age of the egg. This held for both the experimental and control series. It is also evident from Table III that RNA content in splenic cells increased following the CAM grafting. Furthermore, the protein content of the cell decreased as the preincubation age of the egg rose. And, again, the protein content of the splenic cells in the experimental embryos was higher than that of the control embryos.

Because of the amount of DNA per nucleus as yet is not known in turkeys, the principle of the species constancy in the DNA content of a somatic cell suggested the use of a ratio as an alternative way for expressing the concentration of RNA or protein in a cell. As can be seen from Table IV, the

TABLE II. Effect of Preincubation Storage and of CAM Grafting on the Number of Dividing Cells, DNA, RNA, and Protein Content of the Spleen of the Host Chicken and Turkey Embryos.[a]

Storage (days)	No. of dividing cells[b]		DNA[c]		RNA[c]		Protein[d]	
	Grafted, $\bar{x}$	Control, $\bar{x}$	Grafted, $\bar{x}$	Control, $\bar{x}$	Grafted, $\bar{x}$	Control, $\bar{x}$	Grafted, $\bar{x}$	Control, $\bar{x}$
Chicken embryos								
0	5.5 ± 0.09	3.4 ± 0.06	8.3 ± 0.12 (14)[e]	9.7 ± 0.39 (16)	13.9 ± 0.17 (18)	14.0 ± 0.27 (17)	15.9 ± 0.12 (9)	15.8 ± 0.29 (10)
7	4.5 ± 0.09	3.0 ± 0.10	8.1 ± 0.25 (13)	9.3 ± 0.33 (15)	13.5 ± 0.29 (17)	14.0 ± 0.17 (18)	15.7 ± 0.21 (10)	15.6 ± 0.32 (10)
14	4.4 ± 0.04	2.8 ± 0.06	8.4 ± 0.23 (17)	9.7 ± 0.24 (17)	13.5 ± 0.24 (17)	14.1 ± 0.26 (17)	15.1 ± 0.39 (10)	15.2 ± 0.31 (10)
21–1	4.3 ± 0.11	2.7 ± 0.05	8.3 ± 0.32 (13)	9.4 ± 0.40 (14)	14.4 ± 0.26 (16)	14.8 ± 0.24 (19)	14.2 ± 0.46 (10)	14.2 ± 0.27 (10)
21–2	4.1 ± 0.04	2.7 ± 0.03	8.4 ± 0.21 (16)	9.9 ± 0.32 (16)	13.5 ± 0.28 (16)	14.9 ± 0.15 (16)	15.6 ± 0.18 (10)	15.0 ± 0.13 (10)
Turkey embryos								
0	5.7 ± 0.18	3.2 ± 0.06	8.3 ± 0.15 (22)	9.9 ± 0.40 (21)	14.9 ± 0.19 (22)	15.5 ± 0.10 (21)	17.6 ± 0.45 (10)	17.4 ± 0.34 (11)
7	4.9 ± 0.17	2.8 ± 0.07	8.1 ± 0.26 (21)	9.0 ± 0.22 (20)	14.5 ± 0.19 (21)	15.2 ± 0.15 (20)	16.4 ± 0.49 (12)	15.9 ± 0.33 (12)
14	5.0 ± 0.04	2.7 ± 0.11	8.9 ± 0.18 (20)	9.5 ± 0.41 (21)	15.3 ± 0.18 (20)	16.1 ± 0.16 (29)	16.0 ± 0.60 (10)	15.0 ± 0.58 (7)

[a] See footnotes in Table I.

[b] Number of dividing cells found in a unit area in a Reichert microscope provided with a 12.5× eye piece and 100/1.3 objective.

[c] μg/mg of wet wt of spleen.

[d] Percentage of wet wt of spleen.

[e] Number of specimens given in parentheses.

TABLE III. Effect of Preincubation Storage and of CAM Grafting on the Estimated Number of Cells, RNA Content, and Protein Content of Spleen in Host Chicken Embryos.[a]

	No. of cells[b] ($\times 10^6$)		RNA (μ/cell; $\times 10^{-6}$)		Protein (μ/cell; $\times 10^{-6}$)	
Storage (days)	Grafted	Control	Grafted	Control	Grafted	Control
0	3.44	4.03	4.04	3.48	46.22	39.12
7	3.37	3.86	4.00	3.62	46.43	40.36
14	3.51	4.04	3.85	3.48	43.10	37.56
21–1	3.47	3.92	4.15	3.77	40.90	36.27
21–2	3.52	4.12	3.85	3.61	44.68	36.34

[a] See footnotes in Table I.

[b] Estimated number of cells per milligram of spleen.

preincubation age of the egg had no effect on the RNA:DNA ratio in spleens of both treatment groups, although the ratio in the 0-day and 7-day groups of the experimental series rose above that of the corresponding control embryos. On the other hand, the protein:DNA ratio in both treatment groups decreased with increase in the duration of preincubation egg storage. At the same time, the grafted groups, relative to the controls, showed a clear-cut rise in the ratio, indicating an increased protein content of the splenic cells in the former specimens.

Discussion. Results of our observations, based on the spleen of the embryo (regardless whether or not the latter's CAM was grafted with immunologically competent splenic tissue), have suggested to us that a lag *per se* in the biological age of chicken embryos, developing in eggs subjected to preincubation storage, was not the major factor responsible for depressing either the rate of cell proliferation or the protein content of the spleen. It will be recalled that in order to compensate for the anticipated lag, we incubated some chicken eggs, following a 21-day storage, for an additional day before subjecting them to analysis. The spleen of the embryos from stored eggs in this group (*age group 21-2*) still had a decidedly lower mitotic index and a reduced protein content. Accepting the thesis that the response of the target organ of the grafted embryo is a composite of two complementary processes, *i.e.*, the intrinsic response of the host tissue and the response of the immigrated donor cells to their new environment, we have interpreted the data to mean that the embryo from a stored egg is a less efficient biological unit than is its counterpart from a nonstored ("fresh") egg.

The lower mitotic rate in the spleen of embryos from stored eggs, as an expression of this lower "efficiency," could be due to a number of reasons. One is that the intermitotic time is extended in the spleen of the affected embryo. The relevance of this time factor in cell proliferation was recently demonstrated (20). Another is the fact that chicken and turkey blastoderms do not remain static during storage at 13° (5). The

TABLE IV. Effect of Preincubation Storage of Eggs and of CAM Grafting on RNA/DNA and Protein/DNA Ratios in the Spleen of Host Turkey Embryos.[a]

	RNA/DNA		Protein/DNA[b]	
Storage (days)	Grafted	Control	Grafted	Control
0	1.81 ± 0.04 (22)[c]	1.63 ± 0.07 (21)	21.3	17.6
7	1.82 ± 0.06 (21)	1.69 ± 0.04 (20)	20.3	17.6
14	1.74 ± 0.04 (20)	1.78 ± 0.09 (21)	18.0	15.7

[a] See footnotes in Table I.

[b] Calculated on the basis of mean protein and DNA content.

[c] Number of specimens given in parentheses.

observation that the DNA and RNA content per unit weight of splenic tissue remained unaffected by the storage treatment suggests that the *rate* of nucleic acid synthesis cannot be easily altered. This conclusion agrees with an earlier finding (4) that the blastodermal cells continue to synthesize DNA at 13°, even though the cell division proper is blocked: the mitotic cycle does not go beyond metaphase. The continued suboptimal activity of the cells under such conditions might induce irreversible physicochemical changes in metabolism, lowering the cells' capacity for maintaining vital activities during subsequent incubation, such as, for example, protein synthesis.

The lower protein content of the spleen in embryos from stored eggs could be the direct result of either (a) a decreased rate of protein synthesis, or (b) a breakdown of protein already formed. Both events are known to occur in chicken embryo cells (21, 22). In the present study, a relatively consistent trend was toward an increase in the protein content of the target organ following CAM grafting. Without attempting to assign responsibility to either of the two component factors (host spleen's own cells or those migrating from the implant), it is suggested that the *rate* of protein synthesis and accumulation must have increased in the spleen of the operated organism.

Even though the preincubation holding of eggs had no observable effect on the RNA content of the splenic cell complex, embryo growth was indeed slower. Metabolic changes in RNA do occur during development (23). Taking note of the latter, we have postulated three explanations for our observations concerning the "constancy" of the RNA content of the embryo spleen in the face of the lowering of its protein content: (i) there might have been a relative decrease in a species of RNA (*e.g.*, mRNA?), without a decrease in the *total* RNA content of the cell, or (ii) there might have been a decrease in the amount of RNA in the cytoplasm of the cell, or (iii) the transfer of mRNA from the nucleus to the cytoplasm might have been reduced. Because in a developing embryo the quantity of mRNA is small compared with the amount of ribosomal RNA, a shift in the synthesis of mRNA would hardly be detectable by measuring *total* RNA.

Summary. The phenomenon of splenomegaly was utilized to examine the effect of preincubation storage of chicken and turkey eggs on the capacity of the host spleen to respond to a specific growth stimulus. The preincubation treatment consisted of holding fertile eggs at 13° and 85% relative humidity: chicken eggs, 0, 7, 14, and 21 days and turkey eggs, 0, 7, and 14 days. The eggs were then incubated at 37° and 65% relative humidity: chicken eggs for 10–11 days and turkey eggs for 14 days before receiving a chorioallantoic graft of splenic tissue from mature White Leghorn male donors. Suitable nongrafted controls were maintained. After 8 additional days of incubation, the surviving embryos in both the experimental (grafted) and control groups provided data on the following parameters: weight of the embryo, weight of the embryo spleen, mitotic index of the latter organ as well as its DNA, RNA, and protein content. Grafting resulted in a marked enlargement of the host embryo spleen. Storing eggs before incubation, regardless of the subsequent treatment of embryos (i) depressed the weight of the whole embryo and of its spleen, (ii) decreased the frequency of mitotic rate in the spleen, and (iii) reduced the protein content of the target organ both in absolute and relative terms. The storage treatment had no discernible effect on the DNA and RNA content of the host spleen. The grafting procedure, however, induced a highly significant increase in the number of dividing cells in the host embryo spleen. It was concluded that graft-induced stimulation of the host spleen could not overcome its basically depressed growth rate when the host was an embryo from a stored ("aged") eggs.

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