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The Effect of Preincubation Period and Culture Medium on Early Development of Isolated Blastoderm of Japanese Quail

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ABSTRACT

Using a modification of the method of New (1955), preincubated blasoderms of Japanese quail were cultured on different culture media up to 72 h of incubation and the production of subembryonic fluid (SEF) and developmental components were investigated. Preincubation of 18 h and 48 h sustained measurable amounts of SEF and these coincided with heart pumping and development of area vasculosa. Of different culture media used, native albumen and albumen dialysed with tubing of retaining size permitting molecules of mw>2000, produced measurable amounts of SEF and showed better developmental components such as area vasculosa, length of the embryo, area pelluscida and almost showed identical pattern of radial increase of the explanted blastoderms.

Key Words: The effect, period, culture medium, early development.

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INTRODUCTION

The study of early development in avian embryo is enhanced by use of shell-less techniques. Two techniques; hanging drop technique and the watch glass technique were used to culture isolated chick blastoderm outside the egg *(Mchworter and Wipple, 1912; Sabin, 1917; 1919; Patten and Krammer, 1933; Hughes, 1937 and New, 1955)*. In the hanging drop technique, the blastoderm was detached from the vitelline membrane, freed of yolk, floated onto a cover glass and attached by a drop of coagulated plasma. Subsequently, this explant was inverted onto a hollow-out glass slide containing a drop of water and a cover glass trimmed with paraffin. However, the technique of the watch glass was used to hold a clot that serves as a nutrient source as well as a substratum for the detached bastoderm *(Fell and Robinson, 1927)*. The clot was usually made up of a mixture containing the supernatant of centrifuged blood plasma and embryonic extract of 8-day old embryo of domestic fowl combined with equivalent amount of saline of *Pannett and Compton (1924)*. Using this technique, a clot made up of albumen from unincubated eggs mixed with chick ringer added to agar and allowed to gel also sustained the development of isolated blasoderm *(Spratt, 1947)*. *New (1955)* introduced glass retaining rings to this technique and were each placed over the stretched blastoderm while attached to the vitelline membrane with only thin albumen as the culture medium.

In this study, the glass watch technique had been modified to suit culturing of isolated blastoderms of Japanese quail. The cultures were performed to elucidate the effect of preincubation periods and different culture media on the production of subembroynic fluid (SEF) and other developmental features.

MATERIALS AND METHODS

Culture methods:

The method of culturing based upon that of *New (1955)* was adapted for the Japanese quail embryo. Fertile eggs were incubated at 37.6 ± 0.1C°. The explantation was performed in a laminar flow cabinet (M.D.H) at 22 °C. The shell was first rubbed with 70% alcohol, broken at the blunt pole and the thin albumen was aspirated into sterile 2.5-ml syringe. The remaining egg contents, excluding the shell were poured into 200-ml black bowl filled with salt solution of *Pannett and Compton (1924)*. The black color of the bowl was to provide a contrast background and thus enhancing the dissection and manipulation of the blastoderm. The vitelline membrane was stripped and floated off the yolk, inverted and any yolk remaining removed. A glass retaining ring (14.3 mm i. d. made from rod 1.6 mm o.d) was placed centrally over the blastoderm on the inner surface of the vitelline membrane. The vitelline membrane and ring were placed on to a glass supporter ring in a watch glass whilst submerged in the salt solution. The supporter ring which, was of the same i.d. as the retainer, but 3.4 mm in depth with 6 notches in the base and with four 1mm hooks on the outer rim to anchor the membrane, held the blastoderm above the concave depression of a watch glass (Figure 1) and so permitted delivery of culture medium beneath the explanted bastoderm. All glassware, solutions and instruments were autoclaved to ensure sterility.

The watch glass carrying the explant was transferred to the base of a Petri dish containing sterile water about 7mm deep for maintenance of a high humidity (Figure 2). The salt solution used for explantation was now aspirated from the watch glass and 2.5 ml of the culture medium added beneath the blastoderm. Finally,

the Petri dish was covered and the culture was incubated at 37.6 ± 0.1°C in "still-air" incubator (Brinsea Products Ltd Avon) at humidity of about 60%.

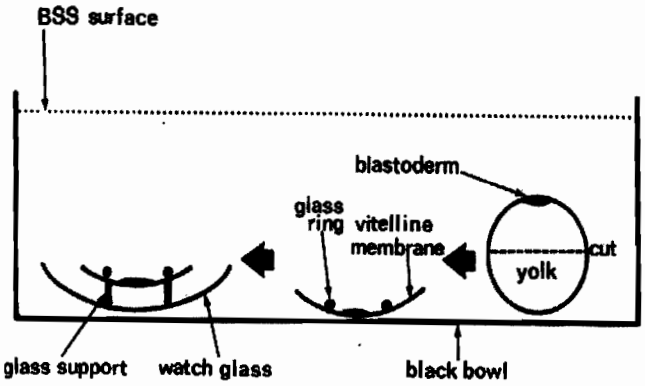

Figure 1: The sequence of events (right to left) involved in dissecting a blastoderm from yolk, inverting it with a glass retaining on top of inner surface of vitelline membrane.

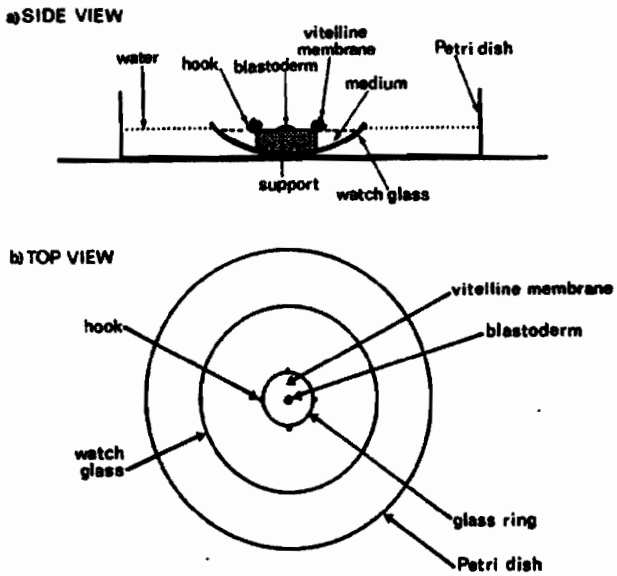

Figure 2: (A) Side view of the final arrangement of blastodermin culture before the lid is placed on the Petri dish.
(B) Top view of watch glass within the Petri dish.

Preparation of culture media:

The culture media used were; the balanced salt solution (BSS), SEF pool, native albumen, native albumen covered with a layer of sunflour oil, native albumen diluted with BSS or dialysed with two tubings; one Benzoylated, Sigma, 32 mm width and 100 ml inflated volume permitting molecules larger than 2000 mw and the other (Visking) retaining molecules in the range 12000 – 14000mw. The two tubings carrying albumen were each placed in 2-litre flask containing the dialysis solution and was tied tightly from both ends and was placed onto a moving a stage installed in a refrigerator to facilitate the dialysis at 4°C. The BSS was based on that of *Pannett and Compton, (1924).* The SEF was collected from 15 eggs incubated for 72 h. The thin albumen was collected from 18 h-preincubated eggs and used as a culture medium for native albumen covered with sunflour oil. The oil layer was dispensed gently from a 2-ml syringe with blunt needle beneath the vitelline membrane by lifting the glass ring carefully from one side and then from the other side after resting the first one. Diluted albumen was prepared by mixing 15 ml of thin albumen collected from eggs preincubated for 18 h with 15ml sterile BSS. From this mixture 2.5 ml was used as a culture medium for each explant. All preparations were performed and maintained sterile.

Measurements of the developmental features:

Volumes of SEF:

Cultures of blastoderms preincubated for zero h, 18 h and 48 h and cultured on native albumen and those preincubated for 18 h but cultured on different culture media were measured for SEF volumes. This was done by dye dilution: 20µl amaranth dye solution (400 mg amaranth/ 100 ml) in saline solution formulated to simulate SEF composition *(Babiker and Baggott, 1992)* was added to volumes of 30 to 400 µl of BSS for calibration and after 72 h of incubation to SEF in cultures. After 40 mn 10 µl was withdrawn from calibration solution or SEF and mixed with 750 µl of BSS and 250 µl Trichloroacetic acid (400 g /l distilled water), centrifuged for 3 minutes. at 13000 g and then the absorbance of the supernatant measured at 520 nm using a Pye-unicam PU8600 spectrophotometer. Preliminary measurements had shown that the dye solution distributed uniformly within 30 minutes of addition and the blastoderm tissues bound only 2.4% of the dye solution added.

Size of selected features and survival of the cultured blastoderms:

The blastoderms preincubated for 18 h, cultured on different culture media and incubated for 72 h were examined under dissecting microscope (×12.5) and measured, by vernier calipers to nearest 0.1 mm for the width of area opaca, the largest diameter for area pelluscida and the long axis for each of area vasculosa and the length of embryo. The blastoderms were also checked for the survival which was ensured by establishing viable pumping of the heart and the flow of blood within the vessels. In dead embryos heart had ceased pumping and the blood stagnated obstructing the blood vessels together with a distinguishable clot of blood in the heart.

Expansion and morphology of cultured blastoderms:

The radial increase was measured in blastoderms with zero h and 18h preincubation periods cultured on their native albumen and also in 18-hour preincubated blastoderms but cultured on native albumen dialysed with tubing permitting molecules of size 2000mw. These were measured, simultaneously with those developed in ovo. The measurement was conducted under the dissecting microscope (×12.5) for the diameter across the blastoderm at 3hr intervals using a vernier calipers to nearest 0,1mm and it continued until the area available within the ring was filled by the developing embryo. Five blastoderms were measured at each interval.

The 18-hour preincubated blastoderms cultured on their native albumen and those developed simultaneously in ovo were assigned stages of *Hamilton and Hamburger (1992).* They were first dissected out, stained with Toulidine Blue (1% w/v in 70% alcohol) and then washed repeatedly by distilled water until clear stained blastoderms attained .The stained blastoderms were examined under the dissecting microscope and were confined to the period from 18h to 72 h.

Statistical analysis:
Means were compared by one way anova for Cochran test of homogeneity of variance. Also means of significant treatments effects were compared by student Newman Keul methods (SNK). Together with these, some individual means were tested against a null hypothesis of zero by student T-test where the level of significance was taken as $P< 0.05$. All statistical tests were performed in Spss-x computer package (Vax computer).

RESULTS

Volumes of subembryonic fluid (SEF):
Volumes of SEF produced by different cultured blastoderms are presented in (Table 1). In all types of cultures, a measurable amount of SEF was produced after about 54 h of incubation including the preincubation period and it was produced soon after explantation in cultures of 48 h of incubation period. However, the production of SEF coincided with pumping of the heart and the development of the area vasculosa. The mean volumes of SEF measured for the three treatments after 72 h of incubation were significantly different ($F_{2,32} = 79.7$, $P= 0.001$). Both blastoderms preincubated for 18 h and 48 h produced nearly the same amount of SEF but the mean volume for each treatment was higher than the treatment without zero preincubation period.

Table 1: Volumes of SEF produced by differently preincubated cultured blastoderms on native albumen. Means sharing same superscript are significantly different (SNK; P, 0.05).

Preincubation period	Mean volume µl±SE	Sample size
Zero	143.3±10.5^{ab}	9
18h	303.3±9.0^{a}	15
48h	297.0±9.1^{b}	9

For balstoderms cultured on different culture media only four of these were able to show production of SEF (Table 2). The volume of SEF produced varied significantly between the four groups by the end of 72 h of incubation ($F_{3,48} = 53.00$, $P< 0.001$). Of these groups, the blastoderms cultured on dialysed albumen had secreted the greatest amount of this fluid and did not differ in mean volumes from those cultured on native albumen. Although, the blastoderms cultured on albumen diluted with BSS (1:1) produced more SEF than blastoderms cultured on native albumen covered with oil layer, this volume was still less than the mean volume secreted by blastoderms cultured on either dialysed or native albumen.

Table 2: SEF volumes produced by 18 h preincubated blstoderms cultured on four different culture media. Means sharing superscripts are not significantly different (SNK, P<0.05).

Culture medium	Mean volume µl±SE	Sample size
Native albumen	9.1^{a} ± 304.5	15
Covered albumen	±7.6 160.2	6
Diluted albumen	8.2 ± 218.5	13
Dialysed albumen[1]	7.2^{a} ± 306.5	15

Permitting mw> 2000.

Effect on embryonic features and survival of blastoderms:
Of the developmental components measured (Table 3), the length of the embryo, the axis of area pelluscida and the axis of the area vasculosa were were higher in the culture media of native albumen and abumen dialysed (mw >2000) were significantly varied between treatments after the 18h of preincubation. However, the width of the area opaca did not differ significantly between blastoderms cultured on different culture media.

Table 3: Measurements (mm) of developmental components of blastoderms preincubated for 18 h on different culture media. Vertical means sharing the same upper and lower case letter are significantly different (SNK, $P< 0.05$).

Culture medium	N	embryo length	axis of area pelluscida	width of a opaca	axis of a.vasculosa
BSS	2	4.45±0.05^{C}	3.35±0.04	1.85±0.04	0
SEF pool	0	0	0	0	0
Native albumen	15	6.28±0.13^{acde}	4.00±0.08^{a}	2.16±0.08	9.13±0.22^{a}
covered albumen	6	5.08±0.14^{abD}	3.97±0.10^{a}	2.18±0.08	8.01±0.08^{A}
diluted albumen	13	5.83±0.16^{acdE}	3.74±0.11^{a}	2.23±0.07	8.58±0.24
dialysed albumen[1]	5	4.22±0.12^{A}	3.26±0.12^{A}	2.19±0.13	8.80±0.35
dialysed albumen[2]	15	6.25±0.12^{acde}	3.93±0.1^{a}	2.15±0.08	9.34±0.20^{a}

1= mw>1400 2= mw>2000 n= sample size.

The percentages of mortality of blastoderms cultured on different media are presented in (Table 4). The percentage of mortality of blastoderms on different culture media were found to be in this descending order, native albumen, albumen dialysed (mw>2000), diluted albumen, native abumen covered with oil, dialysed albumen (mw>14000), BSS and the SEF pool.

Table 4: The number of blastoderms explanted after 18 h of preincubation on different culture media which survived for 72 h.

Culture medium	Number of culture	Number surviving	% surviving
BSS	16	2	12.5
SEF pool	16	0	0.0
Native albumen	16	15	93.8
Covered albumen	16	6	37.5
Diluted albumen	16	13	81.3
Dialysed albumen[1]	16	5	31.3
Dialysed albumen[2]	16	15	93.8

1=size of dialysis tubing > 14000 mw.
2-size of dialysis tubing > 2000 mw.

Radial increase of cultured blastoderms:
As shown in (Figure 3), during the first 18 h of incubation both blastoderms cultured without preincubation and blatoderms developing in ovo followed a similar pattern of radial increase. Blastoderms preincubated for 18 h but cultured on dialysed albumen (mw>2000) and native albumen showed almost identical pattern in radial increase; both started to increase substantially in diameter after 6 h of explantation.

Morphological stages of experimental blastoderms:
Up to 54 h of incubation, *Hamburger and Hamilton (1951)* stages of development for cultured embryos and embryos in ovo were indistinguishable (Figure 4). By 72 h of incubation, cultured embryos were morphologically less advanced than embryos from in-ovo. The embryos that developed in ovo showed limb bud, the amniotic fold covering, most of the trunk and a more accentuated cervical flexure.

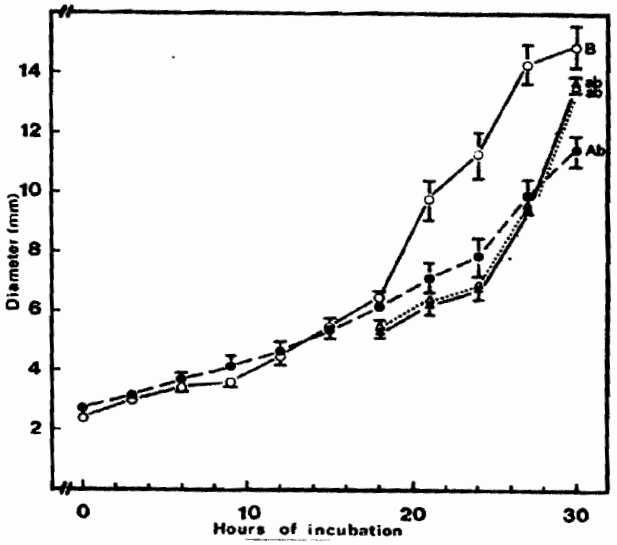

Figure 3: Mean (± SE) diameters of blastderms cultured: Closed circles for blastoderms cultured without preincubation on native albumen, open triangles for those after 18 h of preincubation and cultured on native albumen, closed triangles for those after 18 h of preincubation and cultured on dialysed albumen (mw>2000) and open circles for those incubated in ovo.
N.B means at 30 h sharing upper and lower case are significantly different (P<0.05). The sample size is 5.

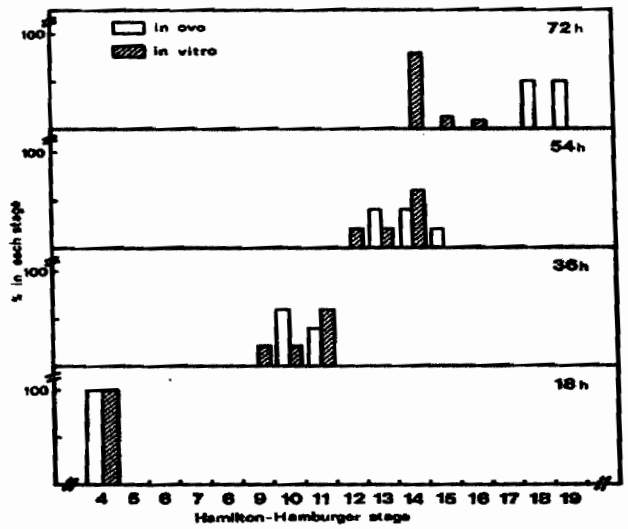

Figure 4: The percentage of blastoderm *in vitro* (on native albumin) or in ovo showing the morphological features as described by *Hambuger and Hamilton (1951)* for incubation time up to 72 h.

DISCUSSION

During the first week of incubation, avian development is accompanied by the formation by of SEF beneath the embryo *(Romanoff, 1960; Elias, 1964 and New, 1955)*. This due to the movement of water in bulk from albumen into the subembryonic cavity *(Simkiss, 1980; Ar, 1991a;b; Babiker and Baggott, 1992; Deeming et al., 1987 and Baggott et al., 2002)*. In this study, the smallest volume of SEF produced *in vitro* was shown by blastoderms cultured without preincubation, even though these blastoderms had been in culture for 72 h compared with 54 for the group preincubated for 18 h and only 24 h for the group preincubated for 48 h. As blasoderms without preincubation first exhibited a slower rate of radial increase, soon after 18 h in culture this most probably led to less well-developed area vasculosa and so smaller volume of SEF, as in-ovo restriction of the area vasculosa reduces SEF volumes. It is apparent, therefore, that the preincubation may be crucial in the development of the capability for SEF production as well as blastoderm growth.

In other types of avian embryo cultures, such as shell-less techniques, eggs need to be preincubated at least for 36 h in order to produce successful culture to hatching stages *(Dunn et al., 1981)*. This preincubation period as a preparatory phase for embryo culture was avoided by culturing oviductal stages but only after a series of changes of culture media and precise control of incubation conditions *(Natio and Perry, 1989; Natio et al., 1990 and Perry and Mather, 1991)*.

In all culture of quail embryos which produced SEF, the accumulation of this fluid was only after the heart had started to pump and the appearance of area vasculosa. This was, in fact, about 18 h after the explantation (36 h in all). This suggests that the vascular system of cultured blastoderm may be an essential component for the production of SEF. Unfortunately, it was not possible to directly, quantify whether the secretion of SEF, started at time of the onset of heart function, due to the necessity to maintain sterility and the small volumes involved. It is possible to roughly to estimate the rate of production of SEF from the time the heart had started to pump and up to 72 h of incubation period. Indeed, this rate is fairly similar for 48-hour preincubated blastoderms (12 µl/h) and 18-hour preincubated blastoderms (8.4 µl/h).

All blastoderms preincubated for 18 h and cultured on different culture media except SEF, exhibited some development. Evidently, the inability of SEF to support the development in-vitro must be associated with the nature of the fluid, at the very least both ion composition and its p^H would be very different from albumen and perhaps also from fluid composition in the interstitial spaces of the blastoderms *(Gillepsie and Mchwell, 1987)*.

A crude protein-free replacement of albumen, the BSS supported some growth, but notably no development of area vasculosa. When BSS was mixed with albumen, development parameters including growth of area vasculosa as well as survival all increased, although, lower amounts of SEF were produced than in blastoderms cultured on native albumen. Thus the ability of diluted albumen to sustain development of cultured blastoderms must be due to inclusion of eggwhite in culture medium. The importance of eggwhite component as indispensable requirement for embryonic development was reported by *(Schmidit, 1937)*. In support of this proposal, it was found that quail blastoderm s cultured on a pool of albumen dialysed against ASS which retained molecules with mw>14000 exhibited small embryo length (less than BSS) and

reduced survival. However, explantation on albumen dialysed against ASS retaining molecules with mw>2000 produced blastoderms whose embryos showed growth development, survival and SEF volumes, virtually indistinguishable from blastoderms cultured on native albumen.

The ability of culture medium of dialysed albumen (mw>2000) to sustain normal embryonic development whilst an albumen medium retaining only molecules of mw>14000 produced poor embryonic development indicates that the eggwhite contain an essential constituent of a mw between 2000 – 14000 (nominal). There seems to be no known component of egg albumen of this molecular size, with possible exception of cystatin of 13000 mw *(Burly and Vandera, 1989)*. Notably, this molecular weight range excludes amino acids which are known to be essential for normal development. For instance, *Rothfels, (1954)* tested aliphatic and aromatic analogues of amino acids by adding them individually to culture media of albumen-agar for blastoderms at the primitive streak or 8-somite stage. He found that they shortened the axis of the body and blocked somite production. Fraser, *(1957)* also reported that albumen free of amino acids permitted only slight growth of somite and he concluded that better somite growth could be promoted by four factors acting synergistically and these were; glucose, labile factor not known, alanine and protein moiety which may be a sulpher-containing compound.

However, blastoderms cultured on native albumen covered with oil layer impervious to water but not gases showed retracted development (compared with native albumen), poor survival rates but surprisingly produced about half the volume of SEF by the end of 72 h of incubation. Whilst the oil layer would in theory be relatively impervious to water, it may not be so to ions, so permitting the development of an osmotic gradient between SEF and albumen since SEF was produced, water must have moved through or around the oil layer, whether this due to incomplete coverage of the albumen by oil or other means were not clear.

The avian blastoderm is characterized by its ability to expand over the inner surface of the vitelline membrane as it differentiates into embryonic tissues. Downie, *(1976)* observed this process to start slowly at the beginning of incubation in ovo. This observation was confirmed by the results presented here for Japanese quail, where the blastoderms cultured without preincubation and blastoderms allowed to develop in ovo both exhibited the same rate of expansion during the first 18 h of incubation. After this period of time, the blastoderms developing in-ovo increased radially more rapidly than those growing *in vitro*. The divergence in the rate of expansion shown by the two types of blastoderms might be attributed to retardation due to either the fact that the blastoderms had been cultured without preincubation or to its inverted orientation *in vitro*. The presence of yolk mass may also facilitate radial expansion by acting as a substratum. In this respect, *New, (1955)* reported a substratum was essential to facilitate the expansion of the cultured blastoderms. A culture medium resembling a yolk mass prepared experimentally from a mixture of saline/ agar/ albumen was reported to facilitate the expansion of avian blastoderm *(Spratt, 1947)*. This yolk like substrate may be necessary to provide the tension required for the migrating cells to move over the surface of vitelline membrane *(Downie, 1976; Downie and pergrum, 1971 and New, 1959)*.

The similarity in pattern of radial increase shown by blastoderms preincubated for 18 h and cultured on either native or dialysed albumen (with retained mw>2000) indicated that for this parameter of embryonic growth, the dialysed albumen essentially mimicked native albumen. The smaller size of blastoderms at 32 h for these two groups compared with blastoderms in ovo was probably due to the obvious delay in blastoderms growth shown after explantation (about 6 h). This presumably due to the lowered temperature experienced during the explantation process. The rate of expansion of these two types of cultured blastoderms after this lag of period was very different from tat of blastoderms in ovo. This suggests, therefore, that the effect of the inverted position on blastoderms expansion was negligible and the slower expansion of blastoderms not preincubated must be attributable solely to the lack of preincubation.

The modifications introduced here to New's technique, such as using a glass supporter to hold the blastoderm and a Petri dish containing water to elevate the ambient humidity level in the vicinity of the explant have facilitated the successful culture of quail blastoderms both without preincubation and preincubated for 18 h. In this context, *New (1956)* measured SEF in chick blastoderms cultured only after eggs were incubated first for 24 -48 h before explantation. However, the culturing of 18-hour preincubatrd blastoderms on native or dialysed albumen (mw>2000) by this modified technique seems to be more than adequate system to investigate the production of SEF in Japanese quail.

Blastoderms cultured at 18 h of incubation on these two culture media expanded rapidly and were impeded only by restriction of the area of the glass ring. Both these types of blastoderms culture produced SEF (in comparable quantities) and embryos were almost identical in their developmental feature and survival rates which were high. Blastoderms preincubated for 18 h prior to explantation and cultured on native albumen developed normally and showed almost identical morphological stages to those observed in ovo up to 54 h of incubation. The retardation in development observed after this period of time was most probably due to physical constraints caused by secretion of SEF on top of the glass ring. For example, a blastoderm with SEF sagged in the medium. In contrast, SEF, in ovo is produced beneath the embryo close to the eggshell for gaseous exchange *(Romanoff, 1960)*. This was not the case for the cultured blastoderms. It seems, therefore, all constraints in combination, retarded somewhat the growth of the cultured embryos at the end of the culture period.

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