Isotopic Labeling of Embryo, Yolk Sac, and Intracellular Parasites of the Embryonated Hen’s Egg by Yolk Replacement

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A technique is described which allows the replacement of 50% of the yolk of the embryonated hen’s egg with large volumes of diverse but chemically defined solutions. By using an electrosurgical unit and a polyethylene tunnel, the procedure was performed on eggs from days 3 through 7 with greater than 90% surgical success and viability for the short term. More than 50% of the eggs replaced showed viability for 2 weeks, and a significant proportion went full term. 32PO4 and amino acids (3H and 14C) added to the replaced eggs were incorporated into the macromolecules of the embryo and yolk sac as well as into parasitic rickettsiae cultivated in the replaced eggs. The incorporated 32PO4 was shown to be assimilated into a variety of biochemical species.

The use of embryonated eggs for the cultivation of the intracellular parasite Rickettsia prowazeki has proven preferable to tissue culture methods because of the higher growth yield of these bacteria in eggs. However, the radioactive labeling of these bacteria growing in the yolk sac of eggs presents various difficulties.

The direct injection technique (for example, see 7, 8) is limited by the small volume that can be added, the large background of normal yolk nutrients, and the problem of uniform distribution and adsorption of exogenous materials from the highly viscous yolk mass. However, because of its great ease in some applications, this would be the preferred technique. An alternative involves the removal of the yolk and its replacement with another medium. Grau et al. described this technique in 1957 (5), and we have found no more recent methodology in the literature. But two major difficulties were encountered in their procedures. First, the age at which the yolk could be replaced was restricted to 2 to 3.5 days. Second, the technique had a high rate of failure both in our hands and as originally described. Grau et al. (5) reported that less than 20% of the eggs replaced survived both the operation and the subsequent 24 hr. Less than 10% survived to day 11. Since the rickettsiae must multiply in the yolk sac for 7 to 8 days to obtain good yields, a technique that permitted long-term incubation with the isotope and good survival was crucial.

We have, therefore, modified the technique to allow replacement of yolk at days 3 through 7 with greater than 90% survival from surgery and the first 24 hr and better than 50% survival through day 14. We have shown that the egg thus replaced can survive full term and incorporate phosphate and amino acids into the embryo, yolk sac, and parasitic rickettsiae.

Replacement of the content of the yolk sac allows experimental manipulation of the developing embryonic cells and their parasites. The addition of nutrients, inhibitors of growth and metabolism, hormones, teratogenic compounds, or isotopic tracers at various stages of development would provide a system for studying the effects of these additions and substitutions on development and growth of the embryo, yolk sac, and an obligatory intracellular parasite such as R. prowazeki.

MATERIALS AND METHODS

Many of our replacement procedures were modified from those of Grau et al. (5). In outline, our method consists of: (i) drilling a hole through the shell, (ii) penetration to the yolk and establishment of a connection between the vitelline and shell membranes by use of an electrosurgical unit, (iii) insertion of the polyethylene artificial tunnel, (iv) replacement of yolk, and (v) rescaling the hole. The various procedures are described in detail below.

The egg (antibiotic-free eggs from Truslow Farms,
YOLK REPLACEMENT IN EMBRYONATED HEN’S EGG

Chester, Md.) was cleaned with 95% ethanol, and a small hole (approximately 3 to 4 mm) was drilled through the shell, but not the shell membrane, along or slightly below the equator. Eggs were candled first to locate the yolk sac so as to avoid piercing it with either the electrode or tunnel. The egg was then supported in an upright position in a small glass beaker filled with saline solution in which the ground wire from the electrosurgical unit (Bovie) was immersed. A hypodermic needle (16 gauge, 2.5 cm) was placed over the standard electrode tip, because it was servo to the vitelline membrane. To alleviate this and its larger diameter facilitated the adherence of the membrane. The needle was inserted its entire length, through the drilled hole, maintained for 10 sec within the yolk, and then slowly withdrawn. The current (settings: 80 and cutting) of the electrosurgical unit was turned on only after complete insertion of the needle and was maintained as it was withdrawn.

For embryos 3 to 4 days old, it was necessary for the needle to pierce and attach to the vitelline membrane and in effect bring it along as the needle was withdrawn. The hot needle coagulated the albumen between the shell and vitelline membrane as pointed out by Grau et al. (5). If the vitelline membrane has been successfully drawn close to the shell membrane, coagulated yolk appeared on the tip of the needle. This procedure was modified slightly when embryos older than 4 days were used. The vitelline membrane at this stage is closer to the shell membrane, and no albumen coagulation was observed as the needle was withdrawn. However, more coagulated yolk was seen. Thus, at this stage, it was not necessary for the needle to attach itself to the vitelline membrane but only to pierce it and make a hole large enough for the artificial tunnel to enter.

After the debris had been cleared away from the hole with a 16-gauge needle, the tunnel was inserted. The tunnel was composed of a small piece of polyethylene tubing approximately 17 mm long with a diameter of 5 mm. A piece of surgical rubber tubing 4 mm long was stretched over one end of the polyethylene tubing. This provided an adequate stop and also helped to seal the hole to prevent albumen from seeping out.

Once the tunnel was inserted, the egg was held in a horizontal position with the hole downward. A needle (21 gauge, 2.5 cm) attached to a 12-ml syringe filled with Hanks solution (6) was inserted into the tubing. The diameter of the needle was small enough to allow sufficient space for the yolk to flow out of the tubing as the plunger was slowly engaged. In instances when the yolk was not flowing freely, withdrawal of the tunnel 3 to 5 mm would once again establish a good connection. To catch the yolk, the syringe was fitted with a collar fabricated from a plastic beaker. The upward migration of the embryo and the turbulence created during the replacement procedure made the embryo susceptible to being washed from its attachment to the vitelline membrane. To alleviate this problem and to obtain better flow, after approximately 3 ml of Hanks solution had been injected into one egg, it was set upright and the yolk of another egg was replaced.

Separate needles and syringes were used for each egg, and procedures were performed by sterile technique in a laminar flow hood. Once the desired amount of yolk was replaced, the hole was sealed with physeseal (Fisher Scientific Co.). Approximately 10 eggs could be replaced in 1 hr.

The percentage of the yolk replaced was determined by extracting the yolk from intact and replaced eggs with chloroform-methanol (2:1) and determining the carotenoid content or dry weight of the extract. The values of carotenoid content, as measured by absorbance at 440 nm, and dry weight determinations were in good agreement, and the percentage replacement of 5-day eggs was about 50%.

One day after replacement, the eggs were injected with the desired isotope (0.2 ml, 10 μCi per egg) and rickettsiae (0.2 ml of a 10^-6 dilution of R. prowazeki strain E, seed pool with an LD₅₀ of 5.5). These injections were made by the usual yolk sac route through the air sac with a 3.8-cm, 21-gauge needle. The replaced and injected eggs were then harvested at the times indicated in the text. Those replaced eggs infected with rickettsiae would die at about the 14th day.

At harvest, the embryo was removed, weighed, and homogenized in 4 volumes of water. The yolk sac was rinsed three times in normal saline, weighed, and homogenized in 4 volumes of sucrose-phosphate-glutamate solution (1) suitable for the purification of rickettsiae. The rickettsiae were purified by a slight modification of the standard techniques of Bozarnick (2) and Wissman (12). All fractions not containing rickettsiae obtained during this purification were combined and constituted the yolk sac fraction. The biological activity of the rickettsiae thus purified was determined by the short hemolysis technique (11). Portions (0.2 ml) of the homogenates of the embryo, yolk sac, and rickettsiae were digested with Protosol (New England Nuclear Corp.) and counted in a liquid scintillation counter to determine the isotopic incorporation.

The incorporation of 32PO₄ into phospholipid, nucleic acid, or protein fractions was determined by differential extraction. The chloroform-methanol-soluble material from a portion of the homogenate represented the lipid fraction (3). The aqueous phase from the chloroform-methanol treatment was then extracted with either water-saturated phenol or chloroform-octanol (9) to separate nucleic acids from protein. This aqueous phase containing the nucleic acid was separated from the interface and organic phase containing denatured and solubilized proteins. The three extracts were evaporated on planchets and counted in a gas-flow counter.

RESULTS AND DISCUSSION

As shown in Table 1, the yolk replacement technique was successfully employed with embryonated eggs aged 3 to 7 days. Of 74 eggs replaced, 67 embryos were alive 24 hr later; 7 embryos died either during surgery or on the first day postsurgery. The 67 eggs were injected

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with $^{32}$P0$_4$ (10 µCi, carrier-free/egg) and, as indicated, most were simultaneously injected with rickettsiae. In spite of this added challenge to survival, the infected eggs survived as well as the uninfected eggs throughout the 2 weeks after yolk replacement. After day 15, all eggs harboring rickettsiae died, whereas in some cases those replaced eggs not injected with rickettsiae or $^{32}$P0$_4$ survived full term (not shown in Table 1). In sum, 40 of the 67 eggs injected were alive on the day of harvest indicated in Table 1. An additional 13 embryos died within 48 hr of the harvest date. These 13 eggs showed extensive rickettsial multiplication when smears of the yolk sacs were examined by the Gimenez technique (4). This indicated that death was a result of the injection and not a result of the replacement procedure. These 13 eggs were not processed to determine isotopic incorporation. At time of harvest, the embryos and yolk sacs were weighed, and the age of the embryo was determined by observation of the Romanoff criteria (10). Both measures indicated that good growth and development had occurred in these eggs in which the yolk was replaced by Hanks solution.

The reasons for the increased survival obtained by this technique compared to that of Grau et al. (5) are open to speculation. The most striking difference was during surgery and the subsequent 24 hr. This would suggest that our procedure led to less trauma. The increased survival during the subsequent days might well reflect the fact that we were using older eggs and replacing less yolk. It should be pointed out that after replacement most of our eggs had the added challenge of radioactive isotopes and infection and that no antibiotics were used.

Approximately 38% of the phosphate injected into the egg (10 µCi) was incorporated into the embryonic tissues examined. The bulk of this 38% was found in the large and rapidly growing embryo. About 8% was incorporated into the yolk sac, and a small amount, into the rickettsial fraction. No correlation between age and the incorporation pattern was observed.

The fate of phosphate incorporated into the various tissues is shown in Table 2. In these experiments, the yolks were replaced at 5 or 6 days and harvest was at 13 or 15 days. The bulk

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**Table 1. Effect of age on $^{32}$P0$_4$ incorporation by replaced eggs**

<table>
<thead>
<tr>
<th>Age (days) at which egg was</th>
<th>No. of eggs$^a$</th>
<th>Romanoff age at harvest (days)</th>
<th>Wt (g/egg)</th>
<th>Incorporation of $^{32}$P0$_4$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Injected/ replaced</td>
<td>Har-</td>
<td>Embryo</td>
<td>Yolk sac</td>
</tr>
<tr>
<td></td>
<td>Har-</td>
<td>vested</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>13</td>
<td>1/1</td>
<td>3 (4)</td>
<td>6.0</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
<td>2/2</td>
<td>1d</td>
<td>5.6</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>6/6</td>
<td>3 (2)</td>
<td>2.5</td>
</tr>
<tr>
<td>4</td>
<td>14</td>
<td>2/2</td>
<td>2d</td>
<td>4.8</td>
</tr>
<tr>
<td>16</td>
<td>16</td>
<td>2/2</td>
<td>1</td>
<td>8.4</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>4/6</td>
<td>3 (1)</td>
<td>4.9</td>
</tr>
<tr>
<td>5</td>
<td>13</td>
<td>3/3</td>
<td>3</td>
<td>4.9</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>6/8</td>
<td>6</td>
<td>7.4</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>15/17</td>
<td>6 (5)</td>
<td>6.0</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>3/4</td>
<td>3</td>
<td>6.6</td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td>2/2</td>
<td>1</td>
<td>10.2</td>
</tr>
<tr>
<td>6</td>
<td>13</td>
<td>7/7</td>
<td>4 (1)</td>
<td>6.6</td>
</tr>
<tr>
<td>6</td>
<td>16</td>
<td>3/3</td>
<td>3</td>
<td>10.5</td>
</tr>
<tr>
<td>7</td>
<td>16</td>
<td>1/1</td>
<td>1</td>
<td>10.6</td>
</tr>
</tbody>
</table>

| Totals                     | 67/74 | 40 (13) | 38 ± 15$^d$ | 91 ± 4 | 8 ± 4 | 0.3 ± 0.3 |

$^a$ Symbols: r = eggs injected with rickettsia; d = egg dead at harvest, alive the day before; numbers in parentheses = eggs that died 24 to 48 hr before harvest and showed rickettsial multiplication but were not processed further.

$^b$ Percentage of the isotope injected which was incorporated into embryo, yolk sac, and rickettsia.

$^c$ Percentage of the total incorporated observed in each fraction.

$^d$ Mean ± SD.
of the wet weight and protein of the embryonic tissue was in the embryo. Similarly, the embryo contained 95% of the $^{32}$P incorporated. These counts were distributed into the chloroform-methanol-soluble, water-soluble, and chloroform-octanol or phenol fractions roughly representing phospholipid, nucleic acid, and protein, respectively, indicating that the $^{32}$P has not been incorporated into a unique biochemical species. It was not our intention to characterize in detail the site of $^{32}$P assimilation at this time. The distribution of $^{32}$P in the embryo and yolk sac was not unusual and indicated that the majority of the phosphate was in nuclearic acid. The total incorporation into the rickettsial fraction was small but could be accurately measured because of the large amount of radioactivity incorporated (0.1% of 38% of 10 $\mu$Ci/egg). This small percentage is easily explained, as less than 1% of the total protein of the tissue examined was represented by rickettsial protein. The relative percentages of rickettsial $^{32}$P present in the nucleic acid and protein fractions (14 and 52%, respectively) were strikingly dissimilar to that in the embryonic tissue (59 and 17%, respectively). A possible explanation is that the bacterial cell walls were recovered in the protein fraction and that the rickettsial genome is small. The growth of rickettsiae in the replaced eggs was as good and in many cases better than their growth in normal eggs.

To determine whether amino acids also could be incorporated by our technique, 5-day eggs were replaced and injected on the 6th day with 10 $\mu$Ci of $^3$H-leucine (58 Ci/mmmole) per egg, $^4$C-amino acid hydrolysate (45 mCi/mmmole), or $^{32}$P. The eggs were harvested at day 15 for $^{32}$P and $^3$H and at day 14 for $^4$C. In general, the incorporation patterns of the various compounds were strikingly similar. As shown in Table 3, significant incorporation of all isotopes occurred. The incorporation of leucine and the amino acid mixture was about one-fourth and one-half of that for phosphate, respectively. In all cases, the majority of the isotope incorporated was in the embryo, with decreasing amounts in the yolk sac and rickettsial fraction. The incorporation of $^3$H- and $^4$C-amino acids is important for further investigations. The presence of the low-energy tritium isotope will allow autoradiography to determine the cellular and intracellular localization of the labeled protein. Furthermore, this provides a means of analyzing the proteins of the embryo and rickettsiae by polyacrylamide electrophoresis employing radioactive tracers with double-label techniques to determine the effects of various growth parameters.

ACKNOWLEDGMENTS

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We thank S. Emerson and R. J. Kadner for reading the

### Table 2. Distribution of $^{32}$P incorporated

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Wt (g/egg)</th>
<th>Protein (mg/egg)</th>
<th>Incorporation of $^{32}$P (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>Embryo</td>
<td>6.6, 6.6</td>
<td>422, 336</td>
<td>95, 94</td>
</tr>
<tr>
<td>Yolk sac</td>
<td>1.1, 2.1</td>
<td>109, 273</td>
<td>5, 6</td>
</tr>
<tr>
<td>Rickettsiae</td>
<td>—</td>
<td>3.7, 2.3</td>
<td>0.1, 0.1</td>
</tr>
</tbody>
</table>

\* Organic phase and interphase of chloroform/octanol or phenol versus water extraction.

\* Water phase of chloroform/octanol or phenol versus water extraction.

\* Chloroform-methanol-soluble phase.

### Table 3. Incorporation of radioactive phosphate and amino acids

<table>
<thead>
<tr>
<th>Isotope injected</th>
<th>Incorporation of injected material (%)</th>
<th>Distribution of incorporated $^{32}$P (%)</th>
<th>No. of eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Embryo</td>
<td>Yolk sac</td>
</tr>
<tr>
<td>$^{32}$P</td>
<td>43 ± 11</td>
<td>94 ± 1</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>$^3$H-leucine</td>
<td>10 ± 2</td>
<td>88 ± 1</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>$^4$C-amino acid hydrolysate</td>
<td>26</td>
<td>82</td>
<td>18</td>
</tr>
</tbody>
</table>

* Numbers in parentheses represent eggs that died 24 to 48 hr before harvest and showed rickettsial multiplication but were not processed further.
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