PROTEIN INCORPORATION BY
ISOLATED AMPHIBIAN OOCYTES

V. Specificity for Vitellogenin Incorporation

ROBIN A. WALLACE and DONALD W. JARED

From the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830

ABSTRACT

Macromolecules of vitellogenin were sequestered by *Xenopus laevis* oocytes 20–50 times (on a molar basis) more rapidly than other proteins tested. Selectivity for vitellogenin did not appear to involve molecular size or charge. The K_m for vitellogenin incorporation was at least several orders of magnitude less than that for bovine serum albumin (BSA). At concentrations less than 10 mg·ml^{-1}, BSA did not measurably compete with vitellogenin; a slight, apparent competition observed above a BSA concentration of 10 mg·ml^{-1} was probably spurious. Above a concentration of 2 mg·ml^{-1}, vitellogenin promoted BSA incorporation by about 40%. These results are consistent with the notion that vitellogenin binds to specific receptor sites on the oocyte membrane and is subsequently internalized by micropinocytosis. Other proteins, such as BSA, which do not compete with vitellogenin are most likely to be incorporated by adventitious engulfment during micropinocytosis.

Vitellogenin is a normally female-specific protein sequestered from the bloodstream by growing oocytes; it serves as the macromolecular precursor in *Xenopus laevis* for the yolk proteins lipovitellin and phosvitin (6, 7, 10, 20, 21, 25, 28) which are apparently formed by enzymatic cleavage of vitellogenin within the oocyte (1). Previous experiments have indicated that vitellogenin is more rapidly sequestered than other serum fractions, both in vivo (25) and in vitro (27), and that incorporation of the protein is essentially linear with time (8, 27). Selective incorporation of specific proteins by oocytes has also been indicated for other animals (4, 18). In this communication we report further studies on vitellogenin uptake by *X. laevis* oocytes in vitro, both in comparison with and in the presence of other purified proteins. Quantitative and kinetic measurements confirm that vitellogenin uptake is a highly selective process, and that under physiological conditions other proteins essentially do not compete with vitellogenin.

MATERIALS AND METHODS

Procedures for handling animals, injecting isotopes and hormones, and bleeding have been described elsewhere (24). Blood was collected into a citrate solution containing phenylmethyl sulfonyl fluoride, and sterile glassware and buffer solutions were used in subsequent steps in order to minimize proteolysis of vitellogenin (1). Vitellogenin was obtained in pure form by chromatography of plasma from estrogen-treated females (1 mg estradiol-17β·50 g^{-1} body weight given 7 days previously) on TEAE-cellulose (20). Vitellogenin was labeled in vivo by injecting an estrogen-treated female with 100 μCi of [^{14}C]leucine 24 h before bleeding. Proteins (Sigma Chemical Co., St. Louis, Mo.) were labeled in vitro by

reductive alkylation with [^{14}C]formaldehyde: the method published by Rice and Means (11) was used except that the concentration of protein alkylated was 10-fold higher. Alkylated proteins were exhaustively dialyzed to a constant specific activity. Protein concentrations were determined by the method of Bramhall et al. (2), using Lab·Trol (Dade Div., American Hospital Supply Corp., Miami, Fla.) as a standard. Polyacrylamide gel electrophoresis was performed as described previously (1).

Oocytes (0.9–1.0 mm) were isolated (8) from females given 1,000 U of human chorionic gonadotropin within the week before isolation (27) and were incubated at 20°C in solution O-R2 (26) containing the indicated proteins. The oocytes were incubated for 18 h, then washed, measured, dissolved in NCS (Nuclear Chicago, Amersham/Searle Corp., Arlington Heights, Ill.), and counted in a liquid scintillation counter at 80–85% efficiency (27). Protein uptake is quantitated as nanograms incorporated per square millimeter oocyte surface area per hour. This parameter is relatively constant throughout the size range of oocytes we have used from a given female, but varies somewhat from animal to animal (27).

RESULTS

Effect of Alkylation on Vitellogenin Size Dispersity and Incorporation

It was necessary to label a variety of proteins in vitro to obtain products with the requisite specific activity, and since the incorporation of these proteins was to be compared with that of vitellogenin, it was also desirable to label vitellogenin in a similar manner. SDS-polyacrylamide electrophoresis of alkylated vitellogenin indicated that the modified vitellogenin peptide migrated with the same mobility as the unmodified peptide (mol wt = 200,000; see reference 1) and that no aggregates were produced (Fig. 1). To discover the effect of labeling in vitro on the uptake of vitellogenin by the oocyte, we labeled vitellogenin in vivo in our usual way and compared its incorporation to that of alkylated vitellogenin. The results (Fig. 2) indicated that the V_{max} for alkylated vitellogenin (130 ng·mm^{-2}·h^{-1}) was 83% that of "native" vitellogenin (156 ng·mm^{-2}·h^{-1}) and that the relative K_ms for the two proteins were within a few percent of one another (0.69 and 0.65 mg·ml^{-1}, respectively). Thus, no pronounced alterations in size dispersity or uptake of alkylated vitellogenin by oocytes were indicated, and the alkylated product was used in the comparative experiments reported below.
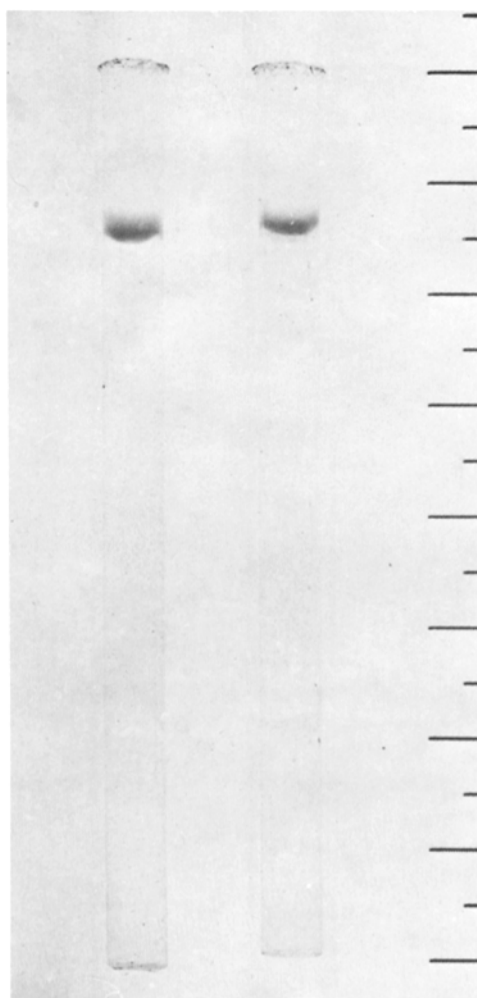

FIGURE 1 Electrophoretic patterns of vitellogenin (left) and alkylated vitellogenin (right) on SDS-polyacrylamide gels (5% acrylamide, 0.1% sodium dodecyl sulfate). The top and the bottom of the gels are the origins and tracking dye (bromphenol blue) position, respectively; the ruled lines are 5 mm apart. Protein is stained with Coomassie brilliant blue.

Specificity of Vitellogenin Uptake

Several purified proteins were alkylated with [^{14}C]formaldehyde, and their uptakes were compared with that of alkylated vitellogenin (Table I). The extent of alkylation varied with the individual protein, presumably due to the relative availability of exposed free amino groups. All proteins were adjusted to approximately the same concentration in solution O-R2 and incubated with oocytes from

the same female for 18 h, after which the oocytes were washed and counted. The results, when corrected for absolute amounts of protein incorporated, indicated that the various proteins tested were sequestered anywhere from 60 to 100 times more slowly, on a weight basis, than vitellogenin (Table I). If a molar basis is used, individual molecules of the various proteins were incorporated 20–50 times more slowly than vitellogenin. In the experiments reported below, we have explored in more detail the relative incorporation of and possible competitive relationship between one of the above proteins and vitellogenin.

Vitellogenin Incorporation as a Function of Concentration: Competition with Bovine Serum Albumin (BSA)

Oocytes from the same female were incubated with [¹⁴C]vitellogenin at various concentrations, in both the absence and the presence of 10 mg·ml⁻¹ of unlabeled BSA. The rate of [¹⁴C]vitellogenin incorporation as a function of concentration was found to follow typical saturation kinetics. A reciprocal plot of the data (Fig. 3) indicated that BSA at a level of 10 mg·ml⁻¹ did not compete with [¹⁴C]vitellogenin uptake even at very low concentrations of [¹⁴C]vitellogenin. V_{max} for this particular batch of oocytes was 133 ng·mm⁻²·h⁻¹ and the K_m was 0.69 mg·ml⁻¹ (1.5·10⁻⁶ M). Using other batches of oocytes, we have found that V_{max} will vary to some extent with the female donor but that K_m remains relatively constant.

In a second series of experiments, we held the concentration of [¹⁴C] vitellogenin constant at 2.9 mg·ml⁻¹ and increased the concentration of unlabeled BSA to ascertain at what level BSA began to compete or interfere with [¹⁴C]vitellogenin incor-

poration. The results (Fig. 4) indicated that above a concentration of 10 mg·ml⁻¹, BSA indeed affected [¹⁴C]vitellogenin uptake, but the competition was only minimal: at a BSA concentration more than 10 times that of [¹⁴C]vitellogenin, [¹⁴C]vitellogenin uptake was still about 80% of the control values.

BSA Incorporation as a Function of Concentration: Competition with Vitellogenin

Oocytes from a single female were placed in various concentrations of [¹⁴C]BSA, in both the absence and the presence of 10 mg/ml of unlabeled
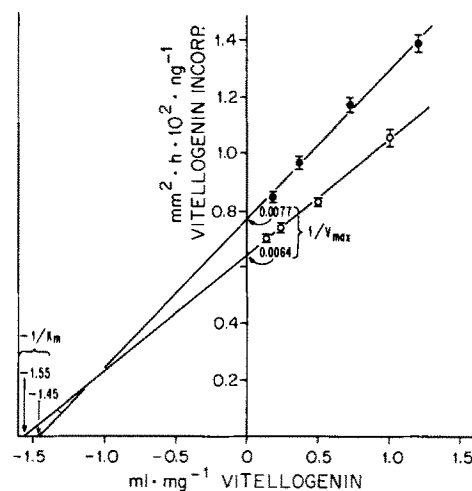
FIGURE 2 Relative incorporation of [¹⁴C]vitellogenin labeled in vivo (open circles) and in vitro by reductive alkylation (closed circles). The lines are least-squares fits to the data. Each point represents the average value derived from 12 oocytes, and the range bars indicate s. Double-reciprocal plot.

TABLE I

Relative Uptake of Various Proteins by Amphibian Oocytes

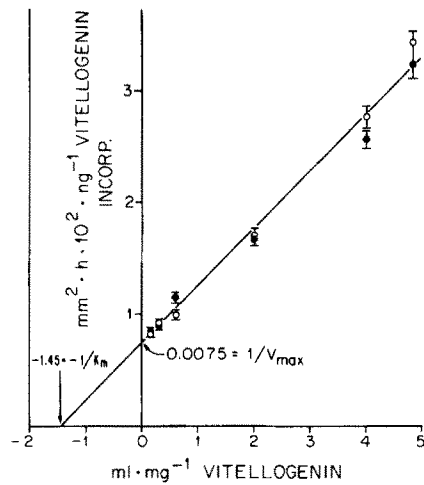|	Molecular weight (ref.)	Concentration	Specific activity	Uptake*		
	daltons	*μg·μl⁻¹*	*cpm·μg⁻¹*	*cpm·mm⁻²·h⁻¹*	*ng·mm⁻²·h⁻¹*	*fmol·mm⁻²·h⁻¹*
Ferritin (equine)	465,000 (13)	3.3	540	0.4	0.7	2
Hemoglobin (bovine)	64,000 (3)	2.9	153	0.4	0.3	4
Serum albumin (bovine)	68,000 (15)	2.7	3,180	1.0	0.3	5
Vitellogenin (*X. laevis*)	460,000 (20)	2.8	2,530	124.5	49.2	107

* Values represent the average for 12 oocytes.

FIGURE 3 [^{14}C]Vitellogenin incorporation by oocytes as a function of concentration, in both the absence (open circles) and the presence (closed circles) of 10 mg·ml^{-1} BSA. The line represents the least-squares fit to all the points. Each point represents the average value derived from 12 oocytes, and the range bars indicate s. Double-reciprocal plot.
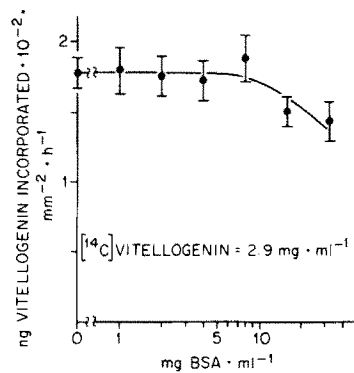

FIGURE 4 Relative incorporation of [^{14}C]vitellogenin as a function of BSA concentration. The concentration of [^{14}C]vitellogenin was held constant at 2.9 mg·ml^{-1}. Each point represents the average value derived from eight oocytes, and the range bars indicate s. Semilog plot.
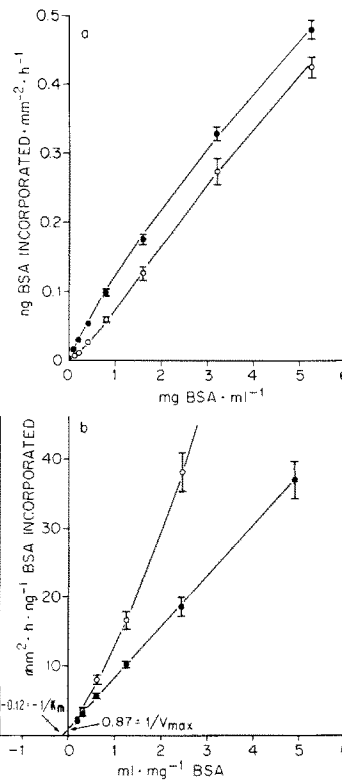

FIGURE 5 [^{14}C]BSA incorporation by oocytes as a function of concentration in both the absence (open circles) and the presence (closed circles) of 10 mg·ml^{-1} vitellogenin. Normal (a) and double-reciprocal (b) plots are provided. Each point represents the average value derived from 12 oocytes, and the range bars indicate s. The straight line drawn in (b) for [^{14}C]BSA incorporation in the presence of vitellogenin represents the least-squares fit to the points.

vitellogenin. In the absence of vitellogenin, the rate of [^{14}C]BSA incorporation as a function of concentration appeared to follow a sigmoid curve (Fig. 5 a). The addition of a large excess of vitellogenin to the medium did not inhibit the uptake of [^{14}C]BSA, but rater stimulated incorporation (Fig. 5 a). Reciprocal plots (Fig. 5 b) indicated that the data for [^{14}C]BSA uptake in the presence of vitellogenin provided a straight line from which a V_{max} of 1.15 ng·mm^{-2}·h^{-1} and a

K_m of 8.3 mg·ml^{-1} (1.2·10^{-4} M) could be calculated. In the absence of vitellogenin a reciprocal plot did not yield a straight line, and the data for [^{14}C]BSA concentrations above 0.5 mg·ml^{-1} tended to intercept the abscissa somewhere between -0.12 and zero. In other words, the K_m for [^{14}C]BSA uptake in the absence of vitellogenin was somewhere between 1.2·10^{-4} M and infinity.

In another series of experiments, we held the concentration of [^{14}C]BSA constant at 2.7 mg·ml^{-1} and varied the concentration of unlabeled vitellogenin. Again, it was noted that addition of vitellogenin to the medium promoted [^{14}C]BSA uptake until the vitellogenin concentration reached about 2.0 mg·ml^{-1} (Fig. 6). Above this concentration, the uptake of labeled

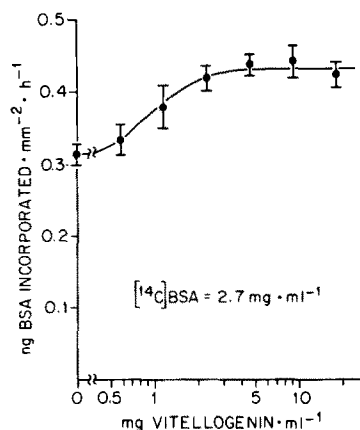FIGURE 6 Relative incorporation of [^{14}C]BSA as a function of vitellogenin incorporation. The concentration of [^{14}C]BSA was held constant at 2.7 mg·ml^{-1}. Each point represents the average value derived from eight oocytes, and the range bars indicate s. Semilog plot.

BSA plateaued at a rate about 40% greater than that found in the absence of vitellogenin.

DISCUSSION

In this study we have treated protein incorporation into oocytes according to classic concepts developed for enzyme-catalyzed reactions (9), with protein in the medium as "substrate" and incorporated protein as the "product." Although this represents a simplistic approach to an undoubtedly complicated process, we believe that it represents a necessary first step towards understanding the mechanisms involved. The criteria which allow this treatment are: (a) under the experimental conditions used, only a small fraction of the protein in the medium is incorporated by oocytes (even at the lowest vitellogenin concentrations, more than 90% of the labeled protein remained in the medium after incubation); (b) the amount of protein bound to the cell surface at any given time is less than 1% of the total incorporation measured (26); (c) uptake of protein is essentially linear with time during the period of incubation (8, 27); (d) protein incorporated into the oocyte is "fixed," i.e. it is not subsequently lost from the oocyte, broken down and reincorporated into other protein, etc.; and (e) the incorporation process is both markedly temperature dependent (26) and, as shown here, saturable when a specific substrate (vitellogenin) is used.

In general terms, we have observed that amphibian oocytes incorporate vitellogenin much more rapidly than other proteins tested. This was reflected by the relative incorporation of various proteins at the same concentration and was specifically reflected by the relative K_m values for BSA and vitellogenin uptake, $1.2 \cdot 10^{-4}$ M or greater and $1.5 \cdot 10^{-6}$ M, respectively. The basis for selectivity does not appear to involve molecular size, since vitellogenin molecules are incorporated 60 times more rapidly than ferritin even though both have approximately the same molecular weight. Nor does surface charge appear to be the basis for selective incorporation, since both BSA and vitellogenin are negatively charged proteins.

Up to a concentration of approximately 10 mg·ml^{-1}, BSA did not measurably perturb the incorporation of vitellogenin, regardless of the latter's concentration. On the other hand, the addition of vitellogenin to the medium stimulated rather than competed with BSA incorporation. However, the stimulation of BSA uptake we observed was not nearly so great as that observed by Ryser (14) for the effect of polybasic compounds on albumin incorporation by cells in tissue culture; therefore a different mechanism may be involved.

The available evidence indicates that the incorporation of vitellogenin by oocytes involves only a micropinocytotic process in *X. laevis* (5, 21) as well as in other animals (12, 16, 19). Presumptive evidence has also been obtained for vitellogenin binding sites on the oocyte membrane (22, 23). We propose here that incorporation of external protein by the oocyte initially involves the association of that protein with membrane receptor sites followed by micropinocytosis of the bound complex.

BSA, at least up to a concentration of 10 mg·ml^{-1}, does not appear to compete effectively for the vitellogenin binding sites. At higher concentrations, some apparent competition is observed (Fig. 4), but this effect is difficult to interpret because the viscosity of the medium becomes rather high at these concentrations (17) and may perturb the sequestering processes. In the absence of vitellogenin, the rate of BSA uptake as a function of concentration follows a sigmoid curve (Fig. 5 a). The simplest interpretation of this effect is that a basal level of micropinocytosis takes place in the absence of vitellogenin, and that any protein external to the oocyte is adventitiously engulfed by this process. Above a certain level (ca. 0.5 mg·ml^{-1} in the case of BSA), external protein begins to promote micropinocytosis. On the other hand, vitellogenin,

because it binds to the receptor sites with high avidity, promotes micropinocytosis at very low concentrations (probably by at least several orders of magnitude lower than BSA) so that a sigmoid-type curve of uptake as a function of concentration is more difficult to demonstrate experimentally. Also, regardless of the external concentration of BSA, the addition of vitellogenin to the medium would promote additional micropinocytosis and adventitious engulfment of external BSA.

REFERENCES

1. BERGINK, E. W., and R. A. WALLACE. 1974. Precursor-product relationship between amphibian vitellogenin and the yolk proteins, lipovitellin and phosvitin. *J. Biol. Chem.* **249**:2897-2903.

2. BRAMHALL, S., N. NOACK, M. WU, and J. R. LOEWENBERG. 1969. A simple colorimetric method for determination of protein. *Anal. Biochem.* **31**:146-148.

3. BRAUNITZER, G., K. HILSE, V. RUDLOFF, and N. HILSCHMANN. 1964. The hemoglobins. *Adv. Protein Chem.* **19**:1-71.

4. CUTTING, J. S., and T. F. ROTH. 1973. Changes in specific sequestration of protein during transport into the developing oocyte of the chicken. *Biochim. Biophys. Acta.* **298**:951-955.

5. DUMONT, J. N. 1972. Oogenesis in *Xenopus laevis* (Daudin). I. Stages of oocyte development in laboratory maintained animals. *J. Morphol.* **136**:153-180.

6. FOLLETT, B. K., T. J. NICHOLLS, and M. R. REDSHAW. 1968. The vitellogenic response in the South African clawed toad (*Xenopus laevis* Daudin). *J. Cell Physiol.* **72**(Suppl.):91-102.

7. JARED, D. W., J. N. DUMONT, and R. A. WALLACE. 1973. Distribution of incorporated and synthesized protein among cell fractions of *Xenopus* oocytes. *Dev. Biol.* **35**:19-28.

8. JARED, D. W., and R. A. WALLACE. 1969. Protein uptake *in vitro* by amphibian oocytes. *Exp. Cell Res.* **57**:454-458.

9. LINEWEAVER, H., and D. BURK. 1934. The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* **56**:658-666.

10. REDSHAW, M. R., and B. K. FOLLETT. 1971. The crystalline yolk-platelet proteins and their soluble plasma precursor in an amphibian *Xenopus laevis*. *Biochem. J.* **124**:759-766.

11. RICE, R. H., and G. E. MEANS. 1971. Radioactive labeling of proteins *in vitro*. *J. Biol. Chem.* **246**:831-832.

12. ROTH, T. F., and K. R. PORTER. 1964. Yolk protein uptake in the oocyte of the mosquito *Aedes aegypti* L. *J. Cell. Biol.* **20**:313-332.

13. ROTHEN, A. 1944. Ferritin and apoferritin in the ultracentrifuge. *J. Biol. Chem.* **152**:679-693.

14. RYSER, J. H. P. 1968. Uptake of protein by mammalian cells: an underdeveloped area. *Science (Wash. D.C.).* **159**:390-396.

15. SCATCHARD, G., A. C. BATCHELDER, and A. BROWN. 1946. Preparation and properties of serum and plasma proteins. VI. Osmotic equilibria in solutions of serum albumin and sodium chloride. *J. Am. Chem. Soc.* **68**:2320-2329.

16. STAY, B. 1965. Protein uptake in the oocytes of the cecropia moth. *J. Cell Biol.* **26**:49-62.

17. TANFORD, C., and J. G. BUZZELL. The viscosity of aqueous solutions of bovine serum albumin between pH 4.3 and 10.5. *J. Phys. Chem.* **60**:225-231.

18. TELFER, W. H. 1960. The selective accumulation of blood proteins by the oocytes of saturnid moths. *Biol. Bull.* **118**: 338-351.

19. TELFER, W. H. 1961. The route of entry and localization of blood proteins in the oocytes of saturnid moths. *J. Biophys. Biochem. Cytol.* **9**:747-759.

20. WALLACE, R. A. 1970. Studies on amphibian yolk. X. *Xenopus* vitellogenin. *Biochim. Biophys. Acta.* **215**:176-183.

21. WALLACE, R. A., and J. N. DUMONT. 1968. The induced synthesis and transport of yolk proteins and their accumulation by the oocyte in *Xenopus laevis*. *J. Cell Physiol.* **72**:(Suppl.):73-89.

22. WALLACE, R. A., and T. HO. 1972. Protein incorporation by isolated amphibian oocytes. II. A survey of inhibitors. *J. Exp. Zool.* **181**:303-318.

23. WALLACE, R. A., T. HO, D. W. SALTER, and D. W. JARED. 1973. Protein incorporation by isolated amphibian oocytes. IV. The role of follicle cells and calcium during protein uptake. *Exp. Cell Res.* **82**:287-295.

24. WALLACE, R. A., and D. W. JARED. 1968. Studies on amphibian yolk. VII. Serum phosphoprotein synthesis by vitellogenic females and estrogen-treated males of *Xenopus laevis*. *Can. J. Biochem.* **46**:953-959.

25. WALLACE, R. A., and D. W. JARED. 1969. Studies on amphibian yolk. VIII. The estrogen-induced hepatic synthesis of a serum lipophosphoprotein and its selective uptake by the ovary and transformation into yolk platelet proteins in *Xenopus laevis*. *Dev. Biol.* **19**:498-526.

26. WALLACE, R. A., D. W. JARED, J. N. DUMONT, and M. W. SEGA. 1973. Protein incorporation by isolated

amphibian oocytes. III. Optimum incubation conditions. *J. Exp. Zool.* **184**:321-333.

27. WALLACE, R. A., D. W. JARED, and B. L. NELSON. 1970. Protein incorporation by isolated amphibian oocytes. I. Preliminary observations. *J. Exp. Zool.* **175**:259-270.

28. WALLACE, R. A., J. M. NICHOL, T. HO, and D. W. JARED. 1972. Studies on amphibian yolk. X. The relative roles of autosynthetic and heterosynthetic processes during yolk protein assembly by isolated oocytes. *Dev. Biol.* **29**:255-272.