SELECTIVITY OF YOLK PROTEIN UPTAKE: COMPARISON OF VITELLOGENINS OF TWO INSECTS

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Abstract—The vitellogenins of Hyalophora cecropia and Blattella germanica have similar Stokes' radii (69 and 75, &), sedimentation coefficients (15.9 and 16.8s) and isoelectric points (pH 5.7 and 5.0), and share similar amino acid compositions with vitellogenins of other animals. The two vitellogenins show no immunological crossreaction. Blattella oocytes take up their own vitellogenin in vivo at a rapid rate and that of Hyalophora at a low rate comparable to that of a non-vitellogenic protein. Hyalophora oocytes take up their own vitellogenin rapidly in vitro and Blattella Vitellogenin only at a negligible rate. Molecular weights, shapes and charges of the vitellogenins are similar to non-vitellogenins and form no basis for selective uptake.

INTRODUCTION

In the development of the yolky eggs of many animal species, certain serum proteins are the major source of yolk. These serum proteins, termed vitellogenins, are synthesized and secreted from other maternal tissues (the liver in vertebrates, and the fat body in insects), and enter the oocytes by pinocytosis (WALLACE and DUMONT, 1968; TELFER and SM1m, 1970) to form the vitellin or yolk protein. This uptake is interesting because it is highly selective; the vitellogenin may be one, or a few out of many serum proteins in the blood. Specialized coated vesicles may be involved in the uptake of the vitellogenins (ANDERSON, 1970).

One test of the selectivity of the uptake mechanism is whether oocytes of one species can take up the vitellogenin of another species. Inter-specific uptake of vitellogenins has been observed previously between two species of saturniid moth (TELFER, 1960), between closely related cockroaches (BELL, 1972) and also, interestingly enough, between chickens and alligators (SCHJEIDE et al., 1963). The latter might be considered as evidence that the uptake mechanism is rather non-specific since it can span the classes of reptiles and birds. We present evidence here to the contrary, showing that purified vitellogenins from two species of insects of different orders, Dictyoptera and Lepidoptera, will not support vitellogenesis in each other's oocytes, even though they have very similar gross chemical properties. The results indicate that the selective uptake mechanism involves recognition of subtle differences in the protein structure.

MATERIALS AND METHODS

Animals

Cockroaches, Blattella germanica, were kept in synchronous culture by controlling their feeding (KUNKEL, 1966), thus providing a source of uniformly aged adult females for use in experiments. Silkmoths, Hyalophora cecropia, were raised on black cherry foliage in the field; pupae were chilled to terminate diapause: and pharate adults were staged according to SCHNEIDERMAN and WILLIAMS (1954).

Isolation of vitellogenins

Since the vitellogenins may be modified after uptake into the oocytes (BROOKES and DEJMAL, 1968; WALLACE, 1964), they were isolated from the blood of ovariec-tomised females, in which the vitellogenins accumulate. The vitellogenins of the two species and two non-vitellogenic serum proteins, I and II, of Blat-tella were purified by combinations of DEAL cellulose chromatography and sucrose gradient centrifugation (PAN and WALLACE, 1974; KUNKEL, in prep.).

Labeling of vitellogenins

For Blattella 0.2 µCi of uniformly labeled 14C-amino acid mixture (Schwarz-Mann) was injected daily into each of 58 ovariec-tomised adult females on days 2 to 5 after the first feeding. On the 6th day after feeding, haemolymph was collected in 0.15 M NaCl buffered with 0.01 M Sodium phosphate, pH 7.2 (PBS). The serum was centrifuged to remove cells and the vitellogenin was then isolated. For Hyalophora, 4 diapausing pupae were ovariec-tomised according to TELFER (1954) and transferred to 25°C to initiate pupal adult development. On day 15 to 16 of the pharate adult
stage, each was injected with 0.1 mCi 3H-leucine (Schwarz-Mann), and held for 4 days to label the vitellogenin. Haemolymph was collected, with a trace of phenylthiourea to prevent melanin formation, centrifuged to remove cells, and pooled. Vitellogenin was then isolated.

After purification, the specific activities of the proteins were determined by dissolving samples in NCS (Nuclear Chicago), counting by liquid scintillation in toluene scintillator (Packard Tricarb 3003), and expressing radioactivity on the basis of protein estimated from absorbance at 280 nm. The amount of radioactive protein taken up by oocytes was estimated in a similar manner, correcting for differences in counting efficiency. Double label counting was performed as described by BUSH (1964).

**Immunological methods**

Antisera against the vitellogenins of *Blattella* and *Hyalophora* were obtained by immunizing rabbits with ovarioctomized female blood and yolk extracts, respectively, emulsified with Freund's complete adjuvant. Antisera thus prepared were rendered specific for vitellogenins by absorption with larval blood for *Blattella* and with adult male blood for *Hyalophora* antisem. Figure 1 demonstrates the purity of the vitellogenins as well as the specificity of the antibodies.

Micro-Ouchterlonny tests were performed on standard 25 x 75 mm microscope slides covered with 2 ml of 0.5% agarose made in PBS. Wells were cut with a 14 gauge needle and the distance from the peripheral wells to the centre well was 5 mm. Immuno-electrophoresis was performed as described previously (PAN and WYATT, 1971). The Oudin test was carried out as described previously (BECKER et al., 1951).

**Biochemical analyses**

Agarose gel filtration (Biogel A1.5, Bio Rad Labs) was used for determining molecular (Stokes') radii (ACKERS, 1964, 1967). The elution volume (Ve) of a protein was determined on a 2.5 x 50cm agarose column. Blue dextran (Pharmacia Co.) was used to determine the void volume (Vo), and dinitrophenylalanine was used to determine the internal volume (vi). The column partition coefficient, a, for a protein was calculated as \( V_e - V_o / V_i \). The a values for three calibration proteins of known Stokes' radii (ACKERS, 1964) were determined using multiple independent runs. The inverse error-frequency-complement (erfc⁻¹) transformation of a (ACKERS, 1967) was calculated from standard tables of the error function. Transformations of sigma were plotted against the known Stokes' radii and a straight line was fitted to the data using least squares (Fig. 2). The Stokes' radius of an unknown protein with an associated standard error of estimation was calculated from its elution volume by extrapolation using the standard curve (RAO, 1965).

Amino acid analyses were performed on the purified proteins delipidated by three successive chloroform-methanol (2:1) extractions, followed by methanol and anhydrous ether rinses and drying under a continuous flow of dry nitrogen. Multiple samples of 2 mg protein were hydrolysed in vacuo in 6 N HCl at 110°C for 24, 48 and 72 hr and the hydroly-sates were analysed on a Beckman 120C amino acid analyser with automatic integrator using an accelerated technique (SPACKMAN, 1967). Data were analysed by digital computer, extrapolating ammonia, threonine and serine to zero hydrolysis time and valine and isoleucine to infinite time (SPACKMAN et al., 1958). Cystine plus cysteine was determined as cysteic acid by performic acid oxidation (HIRS, 1967). Tryptophan was determined spectrophotometrically by its oxidation with N-bromo succinimide (SPANDE and WITKOP, 1967).

Percent lipid was determined from the chloroform :methanol extracts of the proteins. Carbohydrate content of the extracted proteins was measured by the anthrone reaction (SPIRO, 1966) and expressed as a percentage as mannose (YAMASAKI, 1973; BARZEV et al. 1975). Partial specific volumes were calculated from composition data (SCHACHMAN, 1966) assuming specific volumes of 0.640 and 1.093 respectively for the carbohydrate and lipid components. Molecular weights and frictional ratios, \( f_f / f_o \), of the native proteins were calculated from the sedimentation coefficients, Stokes' radii and partial specific volumes (SIEGEL and MONTY, 1966). Subunit mol. wt were determined by SDS gel electrophoresis (WEBER et al., 1972). The sedimentation coefficients were estimated by sucrose gradient sedimentation (MARTIN and AMES, 1961) using human IgG, catalase and cecropia vitellogenin (PAN and WALLACE, 1974) as known sedimenting markers.
Fig. 1. Immunoelectrophoresis of purified vitellogenin, V, male sera and ovariectomised female sera of *Blattella*, Bg and *Hyalophora*, Hc. The antisera used were: a complex antiserum against silk moth yolk, anti-Hc; the same antiserum adsorbed making it specific for vitellogenin, anti-HcV; a complex antiserum against *Blattella* ovariectomised female serum, anti-Bg; the same antiserum adsorbed to make it specific for vitellogenin, anti-BgV. Illustrated is a photographic composite of two slides; on one slide the proteins were stained immediately after electrophoresis, and in a duplicate the antisera were added and immunodiffusion was allowed to occur before washing and staining.
Fig. 4. Subunit composition of purified serum proteins determined by SDS acrylamide gel electro-phoresis (7.5 % acrylamide, 0.4 x 8 cm gels, Coomasie Brilliant Blue stain; WEBER et al., 1972) SP = non-vitellogenic serum proteins I and II of Blattella. V, vitellogenins of Blattella, Bg and Hyalophora Hc. STD = a gel with five known molecular weight protein standards, top to bottom: phosphorylase a, bovine serum albumin monomer, pepsin, trypsin and lysozyme. Each of the gels contains an equivalent amount of lysozyme as an internal mobility standard.
RESULTS

Physical properties of the vitellogenins

Physical and chemical properties of the two vitello-genins and the two Blattella serum proteins are given in Table 1. The Stokes' radii of the vitellogenins of *Hyalophora* and *Blattella*, although similar, are significantly different (P < 0.001). The two other major serum proteins in adult *Blattella*, I and II, have similarly large Stokes' radii but Bg I1 despite its large size has a low sedimentation coefficient due to its high (53%) lipid content. The similar size of the two vitellogenins is also indicated by their mobility in sucrose gradients (Fig. 3). They form a single absorbance peak sedimenting at about 16s, but the $^{14}$C labelled *Blattella* protein is seen to sediment at about 16.8s, slightly faster than the $^3$H labelled *Hyalophora* protein whose sedimentation coefficient, 15.9s, has been determined in the analytical ultracentrifuge (PAN and WALLACE, 1974). Based on their Stokes' radii, partial specific volumes and sedimentation coefficients, the calculated molecular weights and frictional ratios of the four proteins suggest that they are all similarly large and spherical (Table 1).

Despite similarities in native molecular weight, unique subunit structure is revealed by SDS acrylamide gel electrophoresis, Fig. 4. Both vitellogenins have a large and a small subunit. The *Blattella* vitellogenin has variable minor bands which may be artefacts of how long the protein had been in the bloodstream of the ovariectomised female. Molecular weights of the subunits were estimated by running suitable known mol. wt standard proteins in the same gels with the unknowns and extrapolating on a mobility vs log mol. wt plot of the standard proteins (Table 1). Since molar ratios of the subunits cannot be adequately determined from the stainability of the gels it is not possible to accurately estimate the number of each subunit in the native 16s vitellogenins. Since *Blattella* serum protein I has only one subunit it was possible to calculate that there are six traits in the native protein. This along with its sedimentation coefficient and amino acid composition (Table 2), suggest that it is similar to the protein calliphorin (MUNN et al., 1972), *Blattella* serum protein II has an apparently heterogeneous subunit composition. A serum protein with similar properties has been described in

![Fig. 3. Relative sedimentation of 3H-*Hyalophora* vitellogenin and $^{14}$C-*Blattella* vitellogenin. Human IgG was used as a 7S sedimentation marker. Sedimentation is from left to right, 5 to 25'S, sucrose gradient in PBS (International SB283 rotor, 4,000 rev/min, 15 hr), fractions were differentially counted in Bray's solution (BRAY, 1960).](image)

| Table 1. Physical and chemical properties of vitellogenic and non-vitellogenic serum proteins |
|-----------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Vitellogenins                                  | pl              | %               | %               | Stokes' radius  | MW § native     | MW SDS          |
| *Blattella* germanica                          | 5.0             | 4.5             | 15.7            | 0.784           | 75.0 ± 1.6'     | 16.8            | 659            | 1.27           | 100            |
| *Hyalophora* cecropia                         | 5.7             | 1.0             | 9.43'           | 0.758           | 69.4 ± 1.6'     | 15.9            | 516            | 1.29           | 120            |
| Non-vitellogenins                              |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| *Bg* I                                        | 5.5             | 0.0             | 0.731           | 59.5            | 19.0            | 476             | 1.16           | 80             |
| *Bg* II                                       | 6.9             | 53.2            | 0.926           | 63.0            | 5.4             | 511             | 1.10           |                 |

* mean ± standard error of the mean for three replicates.

This from PAN and WALLACE (1974).

§ calculated from amino acid, CHO and lipid composition (Schachman, 1957).

§ calculated from partial specific volume, Stokes' radius and sedimentation coefficient. (SIEGEL and MONTY, 1966).
Table 2. Protein composition expressed as mole per cent

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*Bg, Bgl, BgII are *B. germanica* vitellogenin, serum protein I and II respectively; Gg is *Gallus gallus* lipovitellin (COOK *et al.*, 1962); Hc is *H. cecropia* vitellogenin; Ld is *Leptinotarsa decemlineata* vitellogenin (DE LOOF and DE WILDE, 1970); Lm is *Leucophaea maderae* vitellogenin (ENGELMAN and FRIEDEL, 1974); Lo is *Locusta migratoria* vitelmin (CHEN and WYATT, personal communication); Rp is *Rana pipiens* lipovitellin (WALLACE, 1967); Xi is *Xenopus laevis* lipovitellin (FOLLETT *et al.*, 1968).

Locusta migratoria (PELED and TIETZ, 1965). Isoelectric points of the two vitellogenins were taken as the pH of zero mobility in electrophoresis (Fig. 5). There is a difference of approximately 0.7 pH unit in isoelectric point which may account for the higher electro-photographic mobility of *Blattella* vitellogenin seen in Fig. 1. *Blattella* serum protein I has an acidic isoelectric point between that of the two vitellogenins while serum protein II has an isoelectric point close to neutrality (Table 1).

The amino acid composition of the vitellogenic proteins and the non-vitellogenic serum proteins is given in mole percent in Table 2. Compositions of yolk proteins of six other species are included for comparison. A difference function, SDQ, of MARCHA-LONIS and WELTMAN (1971) was applied to the composition data of Table 2 to indicate relative differences between the proteins. Table 3 lists the SDQs comparing the 10 proteins of Table 2. The vitellins resemble one another as a loose group. The average comparing the seven vitellins is 55 (range 19 to 96). It is noteworthy that the lowest difference in Table 3 is the comparison of the two cockroach vitellogenins.

Both vitellogenins have substantial lipid components, 9 and 15%. The carbohydrate content of the vitellogenins determined by the anthone reaction was found to be 4.5% mannose equivalents for *Blattella* vitellogenin and 1% mannose equivalents for *Hyalo-phora* vitellogenin. The larger amount of sugar residues in *Blattella* vitellogenin was also indicated by the pronounced caramelization of the protein hydro-lysates and by the appearance of a glucosamine/galactosamine peak in the amino acid analysis of the 24

[Image of Fig. 5: Isoelectric point determination for vitellogenins. Distance of migration in agarose on microscope slides is measured against the pH of the buffer.]
hr hydrolysates only. No such caramelization nor peak was observed for Hyalophora vitellogenin.

To determine if there are detectable antigenic similarities of the two vitellogenins, they were compared immunologically using the Ouchterlony double diffusion test as well as the Oudin single diffusion test. No visible reaction occurred between either protein and the antiserum prepared against the other.

Physiological comparison of vitellogenins

Reciprocal experiments were performed to test whether the two vitellogenins could replace each other physiologically. First the two radioactively labeled vitellogenins were tested for their ability to be taken up in vivo from the haemolymph into the ovaries of Blattella. Four days after molting 60 unfed virgin adult female cockroaches were fed simultaneously at 30°C and allowed to develop for 98 hr, by which time, active yolk deposition was taking place (KUNDEL, 1973). They were then injected with approximately 36 µg of 14C-Blattella vitellogenin (60 dis/rain/g) or 3H-Hyalophora vitellogenin (150 dis/ min/µg), or 14C-Blattella serum protein II (105 dis/ min/µg) as a control for non-specific uptake. At 2, 4, 8, and 19 hr after injection, five animals were sacrificed for each of the proteins injected. The ovaries were dissected out, rinsed in PBS, and the amount of incorporated radioactivity determined. Figure 6 illustrates the proportion of the injected radioactivity taken up from the haemolymph by the ovaries by different times. The injected Blattella vitellogenin was taken up rapidly (initial rate of 0.2 to 0.3 µg/oocy-te/hr.) while the Hyalophora vitellogenin was taken up at a much slower rate similar to that for the con-
trol protein, Blattella serum protein II. Therefore, it seems that Blattella oocytes do not recognize Hyalo-
phora vitellogenin as a vitellogenic protein.

Uptake of the two vitellogenins into the oocytes of Hyalophora was tested by incubation of o6ocytes in vitro. Equivalent weights of 14C-Blattella vitellogenin and 3H-Hyalophora vitellogenin were added to 200 µl of medium, making it approximately 1.8% protein. Ovaries were dissected from 18 to 19 day pharate adult moths and individual vitellogenic oocytes with surrounding follicle cells were isolated and graded in size. Medium sized vitellogenic oocytes were placed into the incubation medium. Samples of oocytes were taken from the medium at intervals and rinsed. The diameter of each oocyte was measured before it was dissolved in NCS for counting. The average amount of each protein taken up per oocyte as well as the counts per min per unit surface area are given in Fig. 7. In this incubation of Hyalophora oocytes, the moth vitellogenin was taken up at a rate of about 0.75µg/oocyte/hr, while the cockroach vitellogenin was virtually excluded.

DISCUSSION

The mechanism by which o6ocytes recognize vitello-genin is of interest as an example of how a cell communicates with its environment through its cell surface. The o6ocyte surface comes in contact with many serum proteins during vitellogenesis but only vitellogenin is recognized and singled out for rapid pinocytic uptake. A previous result (SCHJEIDE et al., 1963) has suggested that the method of recognition of vitellogenin by an oocyte might not be highly specific.
In testing this hypothesis we have taken two insect vitellogensins and compared their physical, chemical and physiological properties.

Vitellogenins and their precursors, vitellogenins tend to be large macromolecules (8.9 to 28s; COOKE et al., 1962; WALLACE et al., 1967; BROOKES and DEJMAL, 1968; PAN and WALLACE, 1974): the cockroach and silkmoth vitellogenins studied above conform to this generality, both sedimenting close to a rate of 16s with diameters between 140 and 150Å. The partial specific volumes (V) computed from composition data are all higher than those usually assumed for simple proteins even for Blattella serum protein I, which lacks carbohydrate and lipid. The partial specific volume computed for silkmoth vitellogenin agrees well with a previous determination by pycnometry (PAN and WALLACE, 1974). The mol. wt computed from the Stokes' radii, sedimentation coefficients, and partial specific volumes show that all the serum proteins studied are large, 476,000 to 659,000 mol. wt. The computed frictional ratios, f/f0, show them all to be close to spherical. This large spherical size of the vitellogenins may be an adaptation for serving as an efficient transport and storage protein. A more general reason that all the major serum proteins of Blattella may be of large diameter is to avoid filtration by the pericardial cells, which have specialised intracellular filtration junctions to prevent large molecules from reaching the pinocytotic surface (CROSSLEY, 1972: LIBERTOFF and KUNKEL, in prep.).

Both vitellogenins are heteropolymers with two apparently different size classes of subunits. However, since the vitellogenins contain a substantial amount of carbohydrate the determinations of mol. wt may not be accurate (WEBER et al., 1972). Blattella serum protein I which lacks substantial carbohydrate has a single subunit of about 80,000 mol. wt. This suggests that the native protein has six subunits. Blattella serum protein II although homogeneous on sucrose gradients, disc electrophoresis pH 7.5, and immuno-electrophoresis pH 6.5, gives multiple bands on SDS gel electrophoresis. This is similar to a serum lipo-protein from Locusta which also shares an extremely similar amino acid composition (PELED and TIETZ, 1975).

The isoelectric points of vitellogenins in this study were low, 5.0 and 5.7, so that at physiological pH they would have a pronounced negative charge. This could not be a sufficient criterion to allow for vitello-genic recognition since the non-vitellogenic serum proteins of Blattella would also be negatively charged. Negative charge would also be an unusual general criterion for uptake since in other model pinocytotic systems most proteins will only stimulate pinocytosis below their isoelectric points i.e. when they are positively charged (GIESE, 1973).

The relative chemical composition of vitellogenins might give some clue to specificity. The amino acid composition of the eight vitellogenins show some superficial similarities such as high aspartate and glutamate and low methionine which are characteristics of proteins in general (KING and JUKES, 1969). In the absence of amino acid sequence data it is difficult.

Fig. 6. Uptake of serum proteins into Blattella oocytes in f/f₀.

At zero time approximately 36 μg of radioactively labelled protein was injected into groups of females which had been fed for 98 hr. The proportion of the injected protein taken up is plotted against time since injection.

Fig. 7. Uptake of vitellogenins into silkmoth oocytes in vitro. Medium-sized vitellogenic oocytes were placed into 200 μl of a culture medium (PAN et al., 1967) containing approximately 9 mg/ml each of C-14-Blattella vitellogenin and H-3-Hyalophora vitellogenin. The uptake over time with associated standard error (vertical bars) was calculated in counts per min per square mm of oocyte surface and is also displayed in μg of protein entering the average sized egg.
to make judgements of the homology of the proteins being compared. We applied the difference function of MARCHALONIS and WELTMAN (1971) to the amino acid composition data. This function, $S\Delta Q$, measures departures from identical mole percent amino acid composition on a scale from zero to 20,000. Identical proteins analysed in different laboratories are found to have $S\Delta Q$s of up to 4. The distribution of $S\Delta Q$ for large numbers of comparisons of related and unrelated proteins has been published (MARCHALONIS and WELTMAN, 1971), providing a background for comparison of the tabulated analyses of vitellogenins. The vitellogenins form a group in terms of amino acid composition. None of the 28 comparisons of vitellogenins result in $S\Delta Q$s over 100, while fewer than 2 percent of the 820 cross comparisons of 41 unrelated proteins have $S\Delta Q$s lower than 100 (MARCHALONIS and WELTMAN, 1971). The uniformly low $S\Delta Q$s demonstrate a conservatism in amino acid composition of vitellogenins. However, immunological cross-reaction, a measure of amino acid sequence difference (PRAGER and WILSON, 1971), suggests that vitello-genins are not conservative in primary structure. As reported above, the cockroach and silk moth vitello-genins do not show any immunological crossreaction, and subsequent work has shown that insect vitello-genins do not crossreact far outside the genus level of relationship (KUNKEL, JOHNSON, HAGGERTY and SARGENT, in prep.). This suggests marked divergence in amino acid sequence among vitellogenins, despite similarity in mole percent composition.

The vitellogenins also vary in the amount of conjugated carbohydrate and lipid that they contain. Blattella vitellogenin contains about five percent mannose equivalents compared to one percent in Hyalophora vitellogenin and none in Rana pipiens lipovitellin. While the gross physical properties of vitellogenins do not seem sufficient to account for specificity of uptake, the differences in primary structure and carbohydrate content may form the basis of that specificity.

Physiological properties of the serum proteins
The physiological tests showed that the oocytes of the cockroach and silk moth could not recognize each others vitellogenins for selective uptake. The uptake by Hyalophora oocytes in vitro demonstrates a high specificity for their particular vitellogenin: very little cockroach vitellogenin entered the oocytes in the six hours of incubation. Blattella oocytes in vivo over a twenty hr period demonstrate uptake of Hyalophora vitellogenin at a low rate which did not exceed that of a control non-vitellogenic cockroach serum protein under the same conditions. A previous study (SCHJEIDE et al., 1963), claiming specific uptake of alligator yolk protein into chicken oocytes, did not control for non-specific uptake and thus fails to provide evidence for lack of specificity of the uptake process. Non-specific uptake at a low rate may occur as an accompanying leakage during rapid pinocytic uptake of vitellogenin. Alternately or in addition, non-specific uptake could be occurring by way of an independent set of pinocytic vesicles as suggested by ANDERSON (1970).

The possibility of an adapter or recognition molecule produced by the follicle cells (ANDERSON and TELFER, 1969; BELL and SAMS, 1974) although intrinsically interesting does not alter the necessity for a recognition site on the vitellogenin. We propose that this site depends on subtle differences in the structure of the vitellogenin which allow it to be picked out from a mixture of grossly similar serum proteins.

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