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Title: A Reexamination of the Role of Poly-L-Glutamic Acid on Duration of Fertility in the Chicken

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Dekalb XL Single Comb White Leghorn pullets were immunized against poly-L-glutamic acid (PGA) complexed with methylated bovine serum albumin (MBSA) at 9 weeks of age. Pullets immunized against MBSA as well as non-immunized pullets served as controls. At 18 weeks of age, pullets were moved from a floor pen into cages and photostimulated with 14 hours light/day. At 25 weeks of age, pullets were inseminated. Eggs collected over a 21-day interval, were incubated in order to determine fertility. Thereafter, subsequent inseminations were made at approximately monthly intervals, the last at 55 weeks of age. Eggs were collected and incubated as above following the last insemination. Immunization had no effect on body weight, egg production, or fertility following either the first or the last insemination ($P > .05$). Based upon immunoprecipitation

using antiserum developed in rabbits, the oviducts of immunized birds did not contain PGA. A positive reaction was observed when extract of oviducts from control birds were tested. This work confirmed the presence of PGA in the chicken oviduct and demonstrated that duration of fertility, either early or late in the production cycle, was unaffected by immunization against PGA prior to sexual maturity. The biological role of PGA may best be determined by an immunocytochemical approach.

A Reexamination of the Role of Poly-L-Glutamic Acid on Duration of Fertility in the Chicken

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INTRODUCTION

Many oviduct proteins, e.g., ovalbumin, lysozyme, ovotransferrin, and avidin, have been studied in great detail. Such study has entailed methods of purification, elucidation of biological roles and physical properties, as well as biosynthesis. Other oviduct proteins, typically those that are either minor components of albumin or those for which a biological role is obscure, have not been studied in detail. One such protein is poly-L-glutamic acid (PGA).

While naturally occurring PGA was first isolated from the chicken oviduct by Harrison and Heald in 1966, little additional research has been reported since then. This is an enigma in light of the following: 1) as denoted by its name, PGA is a poly-amino acid, i.e., it is a protein molecule comprised exclusively of glutamic acid residues and therefore is a rarity, 2) chicken PGA is found exclusively in the oviduct of sexually mature females, and 3) its presence in the oviduct is restricted to the areas where spermatozoa are stored and where fertilization occurs.

Heald *et al*. (1967) immunized sexually immature pullets against PGA. This treatment was found to render

the oviduct free of PGA in the sexually mature bird. Based upon PGA's ability to prolong spermatozoal motility *in vitro* and the ability of the chicken oviduct to harbor viable spermatozoa, Heald *et al*. (1967) anticipated that fertility would be depressed in pullets that had been immunized against PGA. While such an effect was not observed, the experimental design of Heald *et al*. (1967) did not account for a latent effect. Therefore, this research was performed in order to confirm the presence of PGA in the chicken oviduct and to reexamine the conclusion of Heald *et al*. (1967) that PGA has no role in duration of fertility in the chicken.

LITERATURE REVIEW

Historical Overview of Chicken Oviduct Proteins

Proteins secreted by the chicken's oviduct have been studied since the late 19th century. Consequently, there exists a vast amount of published information pertaining to the classification, synthesis, and utilization of these proteins, and a number of excellent review articles have been written (Baker, 1968; Feeney and Allison, 1969; Gilbert, 1971; Osuga and Feeney, 1974; Osuga and Feeney 1977). Therefore, this review will not include an exhaustive account of oviduct protein research. Rather, it will provide a brief account of historical highlights, an overview of oviduct proteins based upon their biological functions, and a detailed analysis of research involving poly-L-glutamic acid.

Oviduct proteins have been studied for a variety of reasons. Perhaps the foremost reason is the ease with which large amounts of protein can be obtained. Second, most oviduct proteins can be separated easily from one another, and they are characterized by diverse biological functions. Parenthetically, some oviduct proteins have become research tools themselves because of their biological properties. For example, both avidin and ovomucoid can be coupled to agarose or acrylic beads for use in affinity chromatography. A third reason why

oviduct proteins have been studied intensely is because of the nutritive value of the hen's egg. Finally, oviduct proteins have been studied in order to understand the developmental requirements of the chicken embryo.

The first oviduct protein to be identified was ovalbumin, which was crystallized by Hofmeister in 1889. Isolation of other predominant proteins followed shortly thereafter. Ovomucoid was described by Morner in 1894, ovomucin by Eichlolz in 1899, ovotansferrin by Osborne in 1899, and lysozyme by Laschtschenko in 1909 and Fleming in 1922. These initial discoveries were soon followed by studies directed at defining the source and biological roles of oviduct proteins as well as additional discoveries.

Hektoen and Cole (1928) studied the relationship between the globulins of blood plasma and the oviduct. In 1940 Longsworth identified the unique G2 and G3 globulins using the electrophoretic technique. The following year (1941) avidin was identified as avidalbumin by Gyorgy et al. (1941). In the same year (Eakin et al., 1941) reported that avidin was bacteriostatic by virtue of its affinity for biotin. Soon thereafter, ovotransferrin also was recognized as bacteriostatic (Schade and Caroline, 1944). Two additional proteins were discovered in 1958. Ovoinhibitor, a protease inhibitor, was discovered by Matsushima, and flavoprotein by Rhodes et al. In 1961

ovomacroglobulin was identified as C18 component by Luch, and the following year, 1962, Mandeles and Ducay published the results of a classical experiment entailing the use of C^{14} labelled amino acids to identify the source of oviduct proteins. An alternative experimental approach, viz., starch gel electrophoresis, was used by Oades and Brown (1965) to identify water soluble proteins of the infundibulum, magnum, and isthmus. In the same year (1965) ovoglycoprotein was isolated by Kettere. In 1979 a Thiamin-binding protein was identified by Muniyappa and Adiga, and in the following year, 1980, ovocalcin was isolated from egg shell by Kramptiz et al. Recently, (1983) B-N-acetylhexsaminidase was purified from egg albumen by Ogawa et al. In summary, three principles are evident from the collective efforts of these researchers and their colleagues: 1) proteins secreted by the oviduct are synthesized by cells within the oviduct; 2) such synthesis occurs in specific areas of the oviduct; and 3) oviduct proteins are characterized by diverse biological roles.

Classification of Chicken Oviduct Proteins

Ovalbumin

Ovalbumin constitutes 54 percent of egg albumen (Romanoff and Romanoff, 1949) and thus is the predominant

protein secreted by the hen's oviduct. This protein is secreted by A cells within the tubular gland epithelia of the magnum and isthmus (Oades and Brown, 1965; Palmiter, 1972). Synthesis of ovalbumin is stimulated by estrogen (Palmiter, 1972; Shephered et al., 1980).

Ovalbumin has a molecular weight of 45,000 daltons (Warner, 1954) and is both a complex and heterogeneous globular protein. It is complex due to two post-translational modifications, viz., phosphorylation and glycosylation (Cunningham et al., 1957; Sanger and Hocquard, 1962). Phosphorylation accounts for the heterogeneity of ovalbumin (Longsworth et al., 1940; Perlmann, 1952). Approximately 80-85 percent of ovalbumin molecules carry two phosphate groups, while 10-15 percent carry a single phosphate group and 1-4 percent are not phosphorylated (Cann, 1949; Perlmann, 1952). Glycosylation of an asparagine residue yields a single carbohydrate side chain (Cunningham et al., 1957; Montgomery et al., 1965). Carbohydrate constitutes 4 percent of the ovalbumin molecule by weight (Wyburn et al., 1970). With respect to amino acid residues, all essential amino acids for the chicken are found in ovalbumin (Fevold, 1951; Osuga and Feeney, 1977).

The latter attribute of ovalbumin provides an insight into the protein's biological role. In 1971, using C^{14} labelled ovalbumin, Hassell and Klein demonstrated that ovalbumin serves as a source of amino acids during

embryogenesis. A second but equally important biological role during early embryogenesis is the bulk provided by ovalbumin, which in concert with other proteins helps keep the yolk in a central position within the egg (Romanoff and Romanoff, 1949). This helps to minimize the probability of bacterial invasion of the yolk.

Ovotransferrin

Ovotransferrin (conalbumin) constitutes 12 percent of egg albumen (Osuga and Feeney, 1977). It was first thought ovotransferrin was synthesized in the liver due to its similarity in amino acid composition to serum transferrin (Williams, 1962). Later, Palmiter (1972) proved that it is synthesized by tubular gland cells of the magnum under the control of steroid hormones, primarily estrogen.

Ovotransferrin has a molecular weight of 80,000 daltons (Sutton and Jamieson, 1972) and is a glycoprotein. It consists of a single polypeptide (Feeney and Allison, 1969), contains fifteen disulphide bridges, and is folded into compact domains (Williams *et al.*, 1982). The carbohydrate moiety constitutes about 2.2 percent of ovotransferrin by weight (Feeney and Allison, 1969; Graham and Williams, 1975).

Ovotransferrin has two binding sites of differing affinities which bind metal ions such as iron, copper, magnesium, cobalt, and zinc (Aisen *et al.*, 1966; Sutton and

Jamieson, 1972). The N-terminal site has less affinity for binding metals than the C-terminal site. Consequently, it is 44 percent more efficient in providing the developing embryo with some of its need for metals (Keung et al., 1982). The metal-binding property is lost easily when the protein is heated to 60°C or higher (Nakamura et al., 1979).

The metal-binding properties of ovotransferrin account for its biological functions. In addition to transporting metal from the hen's oviduct into the egg, ovotransferrin also acts as a bacteriostatic agent by sequestering iron. This inhibits bacterial growth and makes albumen an inconvenient place to live (Schade and Caroline, 1944). The anti-bacterial activity as well as an anti-fungal action are not limited to a passive action of withholding iron from bacteria, but appear to interfere with the protein that regulates the bacterial outer membrane (Valenti et al., 1982; Valenti et al., 1986; Chart et al., 1986). Furthermore, ovotransferrin maintains lysozyme's lytic activity against gram positive bacteria by binding copper, as the presence of trace amounts of copper in albumen inhibits lysozyme's activity (Feeney and Allison, 1969; Zweier, 1980).

Ovomucoid

Ovomucoid constitutes 10 percent of albumen (Baker, 1968) and is secreted by the tubular gland epithelium of the magnum (Palmiter, 1972). Synthesis of ovomucoid is stimulated by dihydrotestosterone (Compere et al., 1981). Ovomucoid has a molecular weight of 27,000 daltons (Fredericq and Deutsch, 1949) and is a globular glycoprotein (Morner, 1894; Osuga and Feeney, 1977). This protein is a single polypeptide containing nine disulphide bridges (Kurisaki et al.,1981; Matsuda et al., 1981; Matsuda et al., 1983) and is folded into three domains.

Compared to other proteins secreted from the oviduct, ovomucoid has the highest percentage of carbohydrate, which ranges from 27 percent (Fredericq and Deutsch, 1949) to 30 percent (Lewis et al., 1950) of the ovomucoid molecule by weight. Ovomucoid is structurally stable at extreme temperatures and pH values (Fredericq and Deutsch, 1949; Matsuda et al., 1981; Matsuda et al., 1983).

In the chicken the biological role of ovomucoid is to inhibit proteolytic enzymes. However, it is not a broad-spectrum proteinase inhibitor as it is without effect on human trypsin, chymotrypsin, as well as certain fungal and bacterial proteases (Lineweaver and Murray, 1947; Osuga and Feeney, 1974). Recently, Sugimoto et al. (1984) found that ovomucoid may not only inhibit protease within albumen but can also pass into the yolk in order to

regulate the supply of amino acids to the developing embryo.

Ovoglobulin

Ovoglobulin constitutes 4 percent of egg albumen (Gilbert, 1971) and is synthesized by the magnum (Hektoen and Cole, 1928; Oades and Brown, 1965). It has not been reported if the synthesis of this protein is in surface or in tubular gland epithelium of the oviduct. There are two globulin proteins, G1 and G2. Both have an approximate molecular weight of 49,000 daltons (Nakamura et al., 1980). G1 and G2 are glycoproteins and have similar amino acid and carbohydrate compositions. Other structural features and biological properties are unknown.

Lysozyme

Lysozyme constitutes 3.5 percent of egg albumen (Gilbert, 1971) and is secreted by B cells of the tubular gland epithelia in both the infundibulum and magnum (Wyburn et al., 1970; Palmiter, 1972). Compared to other proteins secreted by the oviduct, this protein has a relatively low molecular weight of 17,500 daltons (Greenfield and Bigland, 1972).

Lysozyme consists of a single polypepetide containing four disulphide bridges that fold into a spherical

molecule with dimensions of 4.5 x 3.0 x 3.0 nm (Imoto et al., 1972; Kato et al., 1982). In comparison with other proteins secreted from oviduct epithelia, lysozyme has been studied the most extensively, primarily its lytic effect upon the peptidoglycan layer of gram positive bacteria. It was first described as G1 globulin by Longsworth (1940), but later it was identified by Alderton et al. (1945) as muramidase or lysozyme. Although this enzyme is very stable, e.g., it is resistant to bacterial proteinase, trypsin, and extreme temperatures (Fevold, 1951), its activity is rapidly lost in the presence of trace amounts of copper (Feeney and Allison, 1969). Its activity is also inhibited after exposure to yolk proteins; this is believed to be attributable to electrostatic interaction between lysozyme and yolk proteins (Galyean and Cotterill, 1972). Lysozyme has six active sites which have been designated (A-F); sites C and D have the greatest affinity for binding disaccharide, and the location of each binding site has been determined using X-ray crystallography (Pincus and Scherga, 1981).

The main biological function of lysozyme is the lysis of the peptidoglycan layer, which constitutes the cell wall of gram positive bacteria. This occurs via hydrolysis of the beta(1-4)glycosidic linkage between N-acetylmuranic acid and N-actylglocosamine (Arnheim et al., 1973). On the other hand, Bacilli strains have N-unacetylatedglucosamine

in their peptidoglycan, and this sugar residue confers resistance to lysozyme (Hayashi et al., 1973). Perhaps the significance of lysozyme is most easily seen when egg quality and hatchability are compared between eggs characterized by low and high lysozyme content. Sauter et al. (1971) and Sauter and Petersen (1972) found that eggs with low lysozyme levels reduce haugh unit scores (10 units) and a 3 percent decrease in hatchability when compared to high lysozyme eggs. Recently, DeBoeck and Stockx (1986) reported that lysozyme constitutes 37 percent of the proteins found in the outer vitelline layer which surrounds the yolk. Furthermore, lysozyme is known to interact with ovomucin, which is another protein of the chalazae and is also partially responsible for the infrastructure of albumen (Kato et al., 1976). Therefore, by virtue of lysozyme's association with ovomucin, lysozyme is probably distributed throughout the albumen, on a protein scaffold.

Ovomucin

Ovomucin constitutes 1.5-3.5 percent of albumen (Baker, 1968a; Feeney and Allison, 1969) and is secreted by epithelia of the infundibulum and magnum (Oades and Brown, 1965). It is not known if ovomucin is secreted by the surface epithelia, tubular epithelia, or both. Even though ovomucin was one of the first proteins to be isolated from albumen (Eicholz, 1898), ovomucin's molecular weight and

structure were unknown until the 1970's. This was attributable to the insoluble nature of this protein. When ovomucin was eventually solubilized in mercaptoethanol and then reduced, two subunits were generated. These were designated alpha and beta ovomucin and had a molecular weight of 210,000 daltons (Robinson and Monsey, 1971) and 400,000 daltons respectively (Hayakawa and Sato, 1978). In comparison to other proteins secreted by the hen's oviduct, ovomucin has the second highest molecular weight.

Ovomucin is a glycoprotein, and its carbohydrate moiety constitutes 19 percent of ovomucin molecule by weight (Feeney and Allison, 1969). Three distinct carbohydrate side chains have been identified (Kato et al., 1973); subunits differ in their carbohydrate content, e.g., beta-ovomucin contains 57 percent carbohydrate W/W while alpha-ovomucin contains only 15 percent W/W carbohydrate (Robinson and Monsey, 1971). This may partly explain other differences between subunits. The alpha subunit forms insoluble complexes with lysozyme, ovalbumin, or ovotransferrin; this helps to account for the viscous nature of albumen. The beta subunit prevents alpha subunit complexes from aggregating, but it gradually dissociates from these complexes during egg storage and dissolves into the albumen medium, resulting in decreased viscosity (Kato and Sato, 1972; Kato et al., 1976; Kato et al., 1981; Hayakawa et al., 1983). Ovomucin has a high percentage of sialic acid

(N-acetylneuraminic acids); sialic acid constitutes 2.5 percent of ovomucin by weight. The role of ovomucin is attributed to its property as a fiber-like protein. It is responsible for the gel-like albumen and is involved in the formation of both the chalazae and the outer vitelline layer of yolk (Kato et al., 1981).

Ovoinhibitor

Ovoinhibitor constitutes 1.5 percent of egg albumen (Osuga and Feeney, 1977) and has a molecular weight of 49,000 daltons (Davis et al., 1969). Ovoinhibitor is a glycoprotein which consists of a single polypeptide folded into seven domains, with each domain containing three disulphide bridges (Davis et al., 1969; Scott et al., 1987). The carbohydrate moiety constitutes 1.5 percent of ovoinhibitor molecule by weight (Davis et al., 1969).

Ovoinhibitor is a heterogeneous protein that exists in five distinct forms which differ in amino acid composition and carbohydrate content (Davis et al., 1969). While 25 percent of ovoinhibitor's antigenic determinants are common to ovomucoid, these two proteins have different structures and biological functions. As previously stated, ovomucoid has been found to inhibit only bovine trypsin (Osuga and Feeney, 1974). In contrast, ovoinhibitor is effective against bovine trypsin, bovine chymotrypsin, subtilisin, fungal proteinase, and elastase; however, it does not

inhibit human trypsin (Rhodes et al., 1960; Feinstein and Gertler, 1972; Osuga and Feeney, 1977). Each molecule has three binding sites that function independently of one another (Feinstein and Gertler, 1972).

Ovoglycoprotein

Ovoglycoprotein constitutes 1 percent of albumen and has a molecular weight of 24,400 daltons. Its carbohydrate moiety forms 16 percent of molecule by weight and its biological function is still obscure (Ketterer, 1965). In 1983 Delers et al. showed that a glycoprotein that had been purified from albumen is identical to chicken serum alpha acidicglycoprotein. The structural features and types of cells responsible for this protein's synthesis have not yet been elucidated.

Ovoflavoprotein

Ovoflavoprotein constitutes 0.8 percent of egg albumen (Baker, 1968) and is composed of two components. The first is apoprotein or riboflavin binding protein (RBP) which is secreted by the epithelia of the magnum (Mandeles and Ducay, 1962). The second component is riboflavin or vitamin B_2 that is supplied by the hen's diet and passes into epithelial cells where it combines with RBP (Norris and Bauernfeind, 1940).

Apoprotein has a molecular weight of 32,000 daltons and is a phosphoglycoprotein (Phillips et al., 1969; Cotner and Clagett, 1972). While Phillips et al. (1969) and Cotner and Clagett (1972) reported that apoprotein consists of two distinct subunits having molecular weights of 24,000 daltons and 8,000 daltons, Becevar and Palmer (1982) found this protein to be composed of a single polypeptide. The carbohydrate moiety constitutes 14 percent of the apoprotein molecule by weight (Farrell et al., 1969) and consists of oligosaccharide chains attached to asparagine residues of the polypeptide (Miller et al., 1982). In addition, seven or eight phosphate groups are attached to the polypeptide's serine residues (Rhodes et al., 1959; Miller et al., 1984).

One molecule of apoprotein has the capacity to bind only one riboflavin molecule via hydrophobic interaction (Blankenhorn et al., 1975). Riboflavin binding is lost below pH 4 (Rhodes et al., 1959; Murthy et al., 1976). Typically, 30 percent of apoprotein in albumen is occupied by riboflavin (Miller et al., 1982).

Apoprotein has two known biological roles: 1) to provide the developing embryo with riboflavin, and 2) to act as a bacteriostatic agent by making riboflavin unavailable for bacterial growth (Maw, 1954; Rhodes et al., 1959). Furthermore, it has been found that a hen which carries a recessive gene (rd/rd) cannot deposit

ovoflavoprotein in its eggs (Maw, 1954; Farrell et al., 1969; Miller et al., 1982). Consequently, zero hatchability has been reported of incubation eggs with riboflavin deficiency (Maw, 1954; Haws and Buss, 1965).

Ovomacroglobulin

Ovomacroglobulin constitutes 0.5 percent of albumen (Osuga and Feeney, 1977), and it has been suggested that this protein is synthesized by hen's oviduct since it is absent from the chicken's serum (Miller and Feeney, 1966). It has the highest molecular weight that ranges from 650,000 daltons (Donovan et al., 1969) to 700,000 daltons (Kitamoto et al., 1982). Ovomacroglobulin is a globular glycoprotein (Donovan et al., 1969). It consists of four subunits that are bound together by disulphide bridges (Kitamoto et al., 1982). The carbohydrate moiety constitutes 9 percent of the ovomacroglobulin molecule by weight (Gilbert, 1971).

The biological role of macroglobulin was obscured in early research, via the use of ammonium sulfate in purification procedures. Recently, Kitamoto et al. (1982) found that ammonium sulfate inhibits the activity of this protein and found that it inhibits trypsin, papain, and thermolysin.

Avidin

Avidin constitutes 0.05-0.5 percent of albumen (Baker, 1968; Osuga and Feeney, 1977) and is secreted by surface epithelia (Kohler et al., 1968; Rantala et al., 1982), and recently (Kami and Yasuda, 1984) localized avidin in tubular gland cells of magnum. Avidin synthesis is stimulated by progesterone (Kohler et al., 1968; Rantala et al., 1982).

Avidin has a molecular weight of 68,300 daltons (Feeney and Allison, 1969) and is a glycoprotein. The protein moiety consists of four subunits, each of which are similar in their amino acid composition. The subunits are bound by four interchain disulphide bridges (Delange, 1970; Delange and Huang, 1971; Green, 1975). Each monomer has an approximate molecular weight of 16,000 to 18,000 daltons (Poduslo, 1981).

The carbohydrate moiety of avidin constitutes 8-10 percent of this molecule by weight (Gilbert, 1971; Poduslo, 1981) and consists of oligosaccharide chains, which bind to aspargine residues of the polypeptides (Bruch and White, 1982). Avidin was crystallized by Gatti et al. (1984), who showed that the dimensions of the crystalline molecule are 7.96 x 7.96 x 8.43 nm. Avidine has four binding sites, one site per subunit, and is stable over a wide range of pH values as well as temperature (Green, 1975; Gatti, 1984).

Two biological functions for avidin have been reported. First, avidin transports biotin from the hen's oviduct into the egg in order to supply biotin to the developing embryo. Second, avidin acts as a bacteriostatic agent by virtue of its binding biotin. Furthermore, avidin or both avidin and biotin can be used as conjugates to antibody or sepharose for detecting antigens, e.g., toxin, drugs, bacteria, and enzymes (Green, 1975; Wilchek and Bayer, 1984).

Ovocystatin

Ovocystatin is a serine proteinase inhibitor, e.g., papain and ficin (Anastasi et al., 1983; Crubb et al., 1984), and constitutes 0.1 percent of albumen (Gilbert, 1971). Ovocystatin is present in serum; however, it is most likely synthesized by the oviduct (Anastasi et al., 1983; Grubb et al., 1984). It was first isolated from albumen by Fossum and Whitaker (1968).

Ovocystatin has an approximate molecular weight of 12,500 to 14,000 daltons (Fossum and Whitaker, 1968; Ievleva et al., 1984) and consists of a single polypeptide containing two disulphide bridges (Anastasi et al., 1983; Crubb et al., 1984). It is a heterogeneous protein that exists in two forms which are similar in their amino acids composition (Anastasi et al., 1983; Barrett et al., 1984).

Ovocystatin is more structurally stable than ovomucoid when exposed to extreme temperatures or PH values (Sen and Whitaker, 1973; Anastasi et al., 1983), and has two binding sites for binding papain and ficin (Keilova and Tomasek, 1974; 1975) by hydrophopic interaction between the ovocystatin inhibitor and the enzymes (Sen and Whitaker, 1973; Grubb et al., 1984).

Other Minor Proteins

A thiamine-binding protein has a molecular weight of 38,000 ± 2,000 daltons, and a role of transferring a thiamine (vitamin B_1) from the hen's oviduct into the developing embryo (Muniyappa and Adige, 1979). A second protein is ovocalcin, which has a molecular weight of 2,500 daltons, and its polypeptide chain contains carboxyglutamic acid which plays a major role in binding calcium ions (Kramptitz et al., 1980). Recently, in 1983, another enzyme, B-N-acetylhexosaminidase, was named. This was found in albumen and has a molecular weight of 68,000 daltons. Its biological role is still unknown (Ogawa et al, 1983). In addition, it has been suggested that collagen and glycoprotein constitute approximately 10 percent of egg shell membrane proteins (Leach 1982).

Poly-L-Glutamic Acid

While it is yet to be determined whether poly-L-glutamic acid (PGA) is secreted by the hen's oviduct, it is nonetheless synthesized by the oviduct (Harrison and Heald, 1966; Harrison et al., 1966). These researchers were searching for a factor within the hen's oviduct that could prolong spermatozoal viability. They partitioned the oviduct into the following regions: infundibulum, upper magnum, middle magnum, lower magnum, shell gland, utero-vaginal junction, and vagina. Each of these sections were extracted with isonic phosphate buffer, and each extract's ability to maintain spermatozoal motility was determined.

Harrison and Heald (1966) observed that extracts of the infundibulum, upper magnum, and utero-vaginal junction had a profound effect upon maintaining spermatozoal motility. For example, more than 50 percent of the spermatazoa incubated with infundibular extract were still motile at 37°C after 7 hours of incubation at 41°C. In contrast, the same degree of spermatozoal motility in extracts of the lower magnum, shell gland, or vagina was maintained for less than 1 hour. Motility was evaluated at 37°C because spermatozoa were observed to become immotile even in phosphate buffer when they warmed to 41°C. The ability of the infundibular, upper magnum, and utero-vaginal extracts to maintain spermatozoal motility was

observed over a 100-fold range of nitrogen concentration, i.e., from 1.0 to 0.01 mg N/ml. These observations were intriguing because both the infundibulum and utero-vaginal junction were known to be areas where spermatozoa could be stored within the oviduct.

Harrison and Heald (1966) proceeded to isolate the factor that prolonged the maintenance of spermatozoal motility *in vitro* and found it to be PGA. The molecular weight was found to be 81,000 daltons, and therefore an individual molecule would be comprised of approximately 628 glutamic acid residues. Harrison and Heald (1966) reported that chicken PGA was exclusively found in the oviducts of sexually mature females.

The following year, Heald *et al.* (1967) attempted to demonstrate a biological role for PGA via immunization of sexually immature female chickens. The researchers hypothesized that such treatment would prevent the induced synthesis of PGA at sexual maturity, and then upon insemination, a lack of PGA within the oviduct would perturb fertility. While immunization of pullets against PGA yielded oviducts that were free of PGA, such treatment had no effect upon fertility. These authors concluded that PGA was not required for the maintenance of spermatozoa fertilizing ability within the oviduct.

MATERIALS AND METHODS

Antigen was prepared according to the strategy outlined by Plescia et al. (1964) for enhancing an immune response to an acidic polymer of weak or unknown immunogenicity. Five mg of poly-L-glutamic acid (PGA; P4886, Sigma Chemical Co., St. Louis, Mo.) were dissolved in 10 ml of 0.15 M Nacl, and 15 mg of methylated bovine serum albumin (MBSA; A 1009, Sigma) were dissolved in 1.5 ml distilled water. In order to obtain a 1:1 weight ratio between PGA and MBSA, .480 ml of the MBSA solution were added to 9.6 ml of the PGA solution dropwise with stirring. This yielded a suspension of PGA coprecipitated with MBSA, which then was emulsified in an equal volume of complete Freund's Adjuvant (F-4258, Sigma) via sonification. As recommended for the processing of liquids with the Sonifier Model 350 (Branson Sonic Power Co., Danbury, CT), a 12.7 mm diameter Sonic probe was immersed into the material to be emulsified. The power setting was placed at 8 and the sonic probe was energized for 30 seconds. The temperature of the sonified material was kept cool by use of an ice bath.

The above procedure provided enough emulsified immunogen to inject 1 ml into each of 18 Dekalb XL Single Comb White Leghorn (SCWL) pullets. At nine weeks of age these birds were chosen at random from a replacement pullet flock, and immunizations were performed according

to Heald et al.; (1967), i.e., each pullet received 0.5 ml of emulsified immunogen intramuscularly in the breast and thigh. Two similar immunizations were given at 10 and 11 weeks of age with the exception that incomplete Freund's adjuvant (F-5506, Sigma) was used. At nine weeks of age, 36 additional pullets were assigned to two other treatment groups: immunized control and non-immunized control. The immunized controls were treated as above except PGA was omitted. Because each bird in each treatment group was wing banded at nine weeks of age, all experimental birds were removed from the replacement flock and reared together in a 5 x 5 m floor pen until 18 weeks of age.

At this time, pullets were transferred to a caged layer house. Each was randomly assigned to a 40 x 20 x 35 cm cage within a single bank of cages, and body weights were recorded. Thereafter, body weights were recorded on a monthly basis. Pullets were photostimulated with 14 hours of light daily, and a daily record of lay for each bird was begun. This record keeping was continued for the duration of the experiment. Later, those that had been immunized previously were given another single booster injection similar to injections given at 10 and 11 weeks of age. Incomplete Freund's adjuvant was used for injections at 22 weeks of age.

At approximately 25 weeks of age pullets were artificially inseminated for the first time. Each pullet

was inseminated intravaginally, with 100 x 10^6 spermatozoa procured from the pooled ejaculates of 25 SCWL roosters. Egg collection commenced on the second day following insemination and continued for 21 days. Eggs were incubated weekly and then broken open on the fourth day of incubation in order to determine whether they had been fertilized. While subsequent inseminations were performed at monthly intervals for a total of eight inseminations, fertility was not assessed again until after the last insemination at 55 weeks of age. One-way analyses of variance were performed for the following set of the data: body weight at the conclusion of the experiment, cumulative eggs laid per bird, and fertility following the first and the last inseminations. Percentages of fertilized eggs were transformed to arcsin values prior to the ANOVA.

At 61 weeks of age, birds were killed by cervical dislocation. The majority of the birds were killed at approximately 20-30 minutes after oviposition. Each oviduct was dissected free of the body cavity and straightened via trimming of associated ligaments. The first 15 cm of each oviduct, which included the infundibulum and upper magnum, were then cut free and frozen on dry ice. Oviduct samples were stored at -20°C.

Antisera against PGA were prepared in six New Zealand White rabbits. The immunogen was prepared using the

techniques described above. Rabbits were procured from and maintained at Oregon State University's small animal laboratory. Each rabbit was injected three times with .35 mg PGA at weekly intervals. Unlike the procedure of Plescia et al. (1964), rabbits were neither injected intramuscularly nor in the foot pad. Rather, the emulsion bearing the immunogen was injected at multiple subcutaneous sites. As before, Freund's complete adjuvant was used for the first immunization and Freund's incomplete adjuvant thereafter. The presence of anti-MBSA immunoglobulin in sera was verified with the Ouchterlony gel diffusion test. The presence of anti-PGA immunoglobulin was verified by both passive hemagglutination and the interfacial test (see Garvey et al., 1977). Rabbits were bled via cardiac puncture 10 days) after the last immunization and then euthanized by CO_2 inhalation.

Six tissue samples from each treatment group were chosen randomly from a pool of samples collected prior to the secretory phase of the oviduct. The phase of the oviduct's epithelium was inferred based upon the status of the ovary and the position of an oocyte within the oviduct. The criteria for identifying such samples were as follows: 1) presence of a follicular hierarchy but no oocyte in either the body cavity or oviduct, 2) presence of a follicular hierarchy and an oocyte either free in the body cavity or just within the infundibulum. Tissue samples

were pooled according to treatment groups and extracted according to Harrison and Heald (1966). An overview of this procedure follows.

Approximately 100 g of tissue per treatment group were homogenized in 400 ml of cold 0.31 M sodium phosphate buffer pH 7.4 using a Waring blender (Waring Products Corp., Winsted, CT). This homogenate was then centrifuged at 25,000 xg for 1 hour at 5°C in a Beckman L8-70 ultracentrifuge (Beckman Instruments, Palo Alto, CA). The supernatant was filtered through Pyrex glass wool (Corning Glass Works, Corning, NY) and then dialyzed (molecular weight cutoff = 6,000-8,000 daltons) against distilled water. Dialysis was performed at 5°C and the water bath was changed three times over the course of 18 hours. The precipitate that formed during dialysis was collected by centrifuging the suspension at 5000 xg for 30 minutes at 5°C in a Beckman TJ-6 centrifuge (Beckman Instruments). Following centrifugation, the precipitated protein was extracted overnight at 5°C with 0.13 M phosphate buffer, pH 7.2. The mixture of solubilized protein and residual precipitate was then centrifuged at 60,000 xg for 1 hour at 5°C in the Beckman L8-70 ultracentrifuge. The supernatant was removed and stored overnight at 5°C.

Prior to the interfacial test which was used to determine the presence of PGA in oviduct extracts, the phosphate buffer containing extracted oviduct protein was

exchanged for borate-buffered saline by using gel filtration in a PD-10 column (Pharmacia, Piscataway, NJ). The interfacial test was then performed according to Garvey et al. (1977) by overlaying rabbit antiserum with borate-buffered saline containing extracted oviduct protein. The remaining portions of the PGA extracts were lyophilized.

RESULTS

Within 4 weeks of photostimulation, 55 percent of the pullets had begun to lay. At this point, hen-day egg production was only 8 percent but increased abruptly thereafter (Figure 1) as is typical for a flock coming into lay. As seen in Figure 1, the rate at which pullets came into lay was comparable among treatment groups. Based upon body weight and cumulative egg production per hen at the end of the experiment (Table 1), immunization, as expected, had no effect upon production ($P > .05$). Furthermore, no difference in the incidence of cracked or soft-shelled eggs was observed among treatments.

Similar to egg production, no difference in fertility was observed among treatment groups ($P > .05$) in either the first or the last fertility trial (Table 2). As was reported by Heald *et al.* (1967), poly-L-glutamic acid was not detectable in extracts of oviduct tissue removed from pullets that had been immunized against poly-L-glutamic acid.

Table 1. Final body weight and cumulative eggs laid per pullet from non-immunized control birds and birds immunized against either poly-L-glutamic acid (PGA) complexed to methylated bovine serum albumin (MBSA) or MBSA alone.

Treatment	Pullets (n)	Body Weight (kg)	Cumulative Eggs per Pullet
Control	17	2.07 ± .045[a]	217 ± 4.1[a]
PGA + MBSA	18	2.09 ± .038	217 ± 4.0
MBSA	18	2.12 ± .040	217 ± 4.0

[a]Values reported as mean ± S.E.M.

Table 2. Fertility of nonimmunized Single Comb White Leghorn pullets and pullets immunized against either poly-L-glutamic acid (PGA) complexed to methylated bovine serum albumin (MBSA) or MBSA alone.

Trial	Treatment	Pullets (n)	Egg Set[c] (n)	Fertility[d] (%)
	Control	17	490	55 ± 1.97
1[a]	PGA+MBSA	17	470	59 ± 1.97
	MBSA	16	458	55 ± 2.03
	Control	17	499	60 ± 1.8
2[b]	PGA+MBSA	18	497	57 ± 1.7
	MBSA	17	536	59 ± 1.8

[a] Performed with virgin pullets at 25 weeks of age.

[b] Performed at 55 weeks of age as the last of 8 monthly inseminations.

[c] Eggs were collected over a 21-day period following a single insemination of 100 x 10^6 spermatazoa.

[d] Fertility was determined by breaking eggs open after 4 days of incubation. Values are reported as mean ± standard error of means.

Table 3. Comparison of fertility data gathered during the first 7 days of egg collection following the insemination of virgin Single Comb White Leghorn (SCWL) pullets to that reported by Heald et al. in Bri. Poultry Sci., 8:321-323, 1967 for similarly treated SCWL pullets.

Researchers	Treatment[a]	Pullets (n)	Eggs Set (n)	Fertility[b] (%)
Heald et al.	Control	8	39	97
	PGA + MBSA	6	22	100
	MBSA	8	30	79
El-Aghbary	Control	17	101	94
	PGA + MBSA	16	95	96
	MBSA	15	87	94

[a] Treatment groups consisted of non-immunized pullets and pullets immunized against either poly-L-glutamic acid (PGA) complexed to methylated bovine serum albumin (MBSA) or MBSA alone.

[b] Fertility was determined by breaking eggs open after 4 days of incubation.

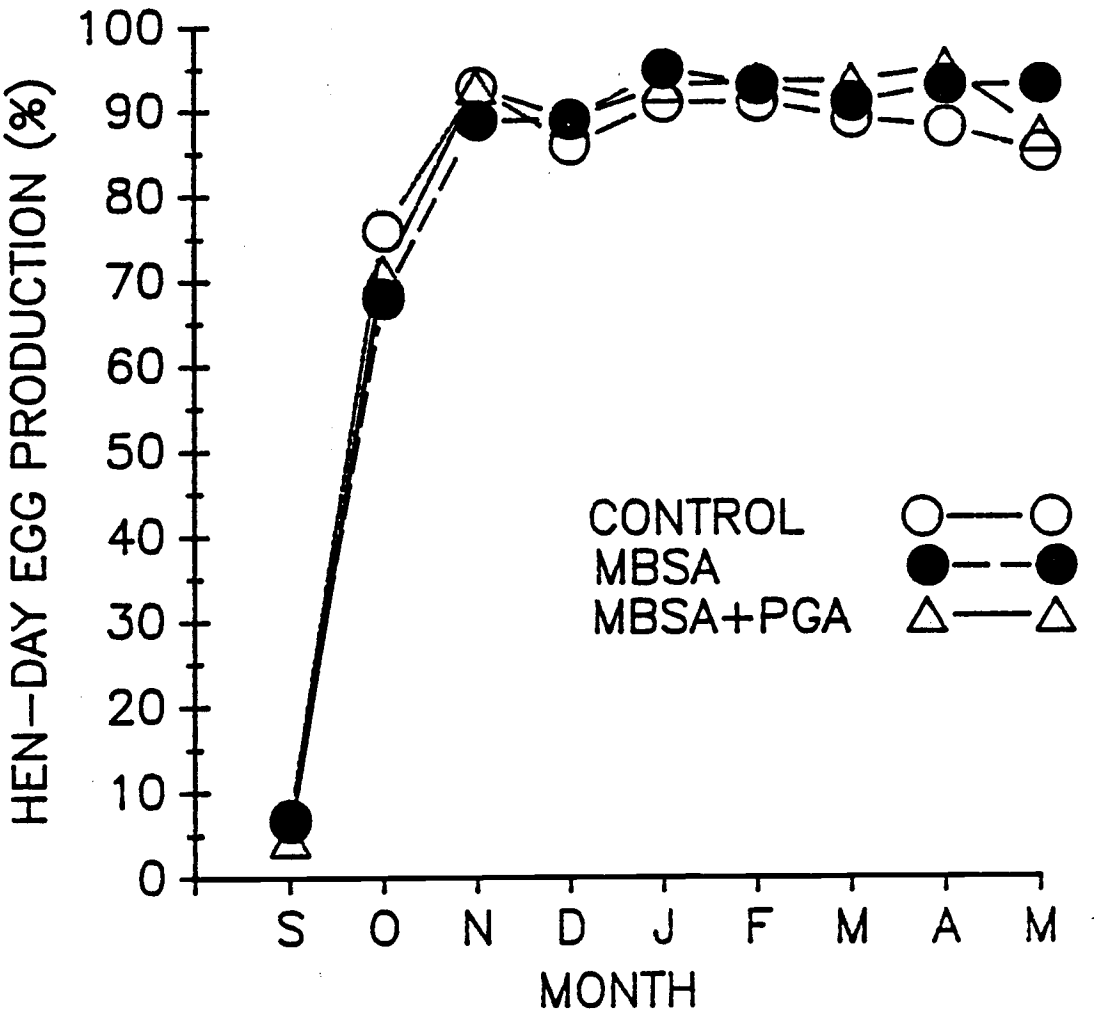

Figure 1. Hen-day egg production over a 9 month production period for non-immunized control birds, birds immunized against methylated bovine serumalbumin (MBSA), and birds immunized against polyglutamic acid (PGA) complexed to MBSA.

DISCUSSION

In 1966, Harrison and Heald reported the isolation of poly-L-glutamic acid (PGA) from the oviduct of the chicken. These researchers were searching for a factor within the chicken's oviduct that would provide a more complete explanation of the longevity of fertility in the chicken following a single intravaginal insemination. Several years before, Fujii and Tamura (1963) as well as Bobr et al. (1964) had described simple tubular glands within the uterovaginal junction of the hen's oviduct. Bobr et al. (1964) proposed that these utero-vaginal glands served as a site for spermatozoal sequestration within the oviduct and that a gradual efflux of spermatozoa was responsible for the duration of fertility observed after a single intravaginal insemination.

In retrospect, it appears to have been generally assumed that this region of the oviduct contained a substance or substances that maintained speramatozoal viability. Apparently this assumption prompted not only the work of Harrison and Heald (1966) but the work of Gilbert et al. (1968) as well. Thus, the observation that extracts of utero-vaginal tissue could prolong spermatozoal motility in vitro, the isolation of PGA from the oviduct, and the demonstration that purified PGA exerted the same effect upon spermatozoal motility in vitro as did tissue extracts, collectively seemed to indicate that such a

substance had been identified. However, a year later, Heald et al. (1967) reported that while immunization of sexually immature pullets rendered the oviducts of such birds free of PGA after sexual maturity, such treatment had no effect upon fertility. These authors concluded that the biological role of PGA was obscure because PGA had no role in the maintenance of spermatozoal viability within the oviduct.

However, there were two weaknesses in the experimental design of Heald et al. (1967). First, following a single insemination, when the percentage of fertilized eggs laid is plotted as a function of time, a sigmoidal curve is obtained. In SCWL (Froman, unpublished data), fertility typically remains 95 percent for approximately 10 days, after which it rapidly declines, reaching 50 percent on the 13th or 14th day and 0 percent by the 19th to 21st day of egg collection. Thus, collecting eggs for only 7 days following an insemination did not afford an evaluation of a complete pattern of fertility following a single intravaginal insemination of virgin pullets. Second, and more importantly, Heald et al. (1967) could not account for a latent effect. These facts along with the observation of Harrison and Heald (1966) that chicken PGA was an unusual protein native only to the oviduct warranted further investigation wherein the effect of time could be accounted for.

As shown in Table 3, percentages of fertilized eggs laid during the first 7 days of egg collection following insemination of virgin pullets were comparable to those reported by Heald et al. (1967), and as these researchers reported, there was no difference in fertility ($P > .05$) among treatment groups. Neither was there a difference in fertility ($P > .05$) among treatment groups in either the first or second fertility trial over a 21-day collection interval (Table 2). By essentially doubling the number of replications per treatment over that of Heald et al. (1967), the minimal difference in percentages that could be detected at a certainty of 90 percent and a 5 percent level of significance decreased from 30 to 15 percentage units. In summary, it is concluded that: 1) while a minor treatment effect on fertility seems unlikely but cannot be precluded, a major treatment effect on fertility can be discounted, and 2) there is neither a treatment effect on duration of fertility following a single insemination of virgin pullets nor a similar effect on fertility over the course of a production cycle. Therefore, this work supports the conclusion of Heald et al. (1967) that PGA within the chicken's oviduct is not a requisite for duration of fertility.

However, several more conclusions may be drawn from the present research. First, it was demonstrated, via immunoprecipitation, that PGA is indeed a member of the

array of proteins synthesized by the chicken oviduct. Second, while PGA may not be required for duration of fertility, it should not be concluded that PGA has no relationship to spermatozoa. It is interesting to note once again the distribution of PGA within the oviduct. It is found where spermatozoa are sequestered (Bobr et al., 1964) as well as within the region where fertilization occurs (Olsen and Neher, 1948). Thus, it still seems plausible that PGA may interact with spermatozoa. It is noteworthy that the experimental design employed by Heald et al. (1967) and in the present research cannot preclude such a possibility. This question could be addressed by utilizing an immunocytochemical approach. The epithelia of the magnum are known to undergo a pronounced cellular cycle during egg formation (Hodges, 1974). Providing that non-specific labelling could be minimized or discounted, a labelled anti-PGA immunoglobulin could be used to determine whether PGA were secreted and, if so, at what phase of the epithelial cycle as well as any subsequent association with spermatozoa. Only after such studies are conducted can valid interpretations be made pertaining to the in vitro effects of PGA on spermatozoa. In other words, even though the ability of PGA to prolong spermatozoal longevity in vitro was specific based upon tests with other polyanions, the phenomenon described by Harrison and Heald (1966) may be an artifact. It is noteworthy that an attempt was made

in preliminary work to use the motility phenomenon described by Harrison and Heald (1966) as the basis for a bioassay. In general, while observations corroborated those of Harrison and Heald (1966), the results were neither sufficiently repeatable nor clear-cut to warrant using *in vitro* maintenance of spermatozoal motility as a bioassay for PGA.

In conclusion, the existence of an unusual protein within the chicken's oviduct was confirmed. While the biological role of PGA awaits elucidation, it is not for lack of adequate methodology. Future work must attempt to identify the location of PGA within a specific type of oviduct cell. Such knowledge would provide a conceptual basis for determining the biological role of PGA.

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APPENDIX

Appendix. Summary of Oviduct Proteins

Protein	% of Albumen	MW Daltons	% Carbo-hydrate	Biological functions
Ovalbumin	54	45000	4	Source of essential a. acids and maintains yolk in egg center.
Ovotrans-ferrin	12	80000	2.2	Source of metal to embryo and bacterio-static.
Ovomucoid	10	27000	28	Inhibits trypsin enzymes and regulates a. acid supply to embryo.
Ovoglo-bulin	4	49000	?	?
Lysozyme	3.5	17500	0	Bacteriosidal, maintains egg quality and hatchability, and forms 37% of vitellin membrane.
Ovomucine	2	610000	19	Responsible for gel-like albumen and formation of chalazea and outer vitellin layer.
Ovoinhi-bitor	1.5	49000	1.5	Inhibits trypsin, chymotrypsin, fungul proteinase, and elastase.
Ovoglyco-protein	1	24400	16	?
Apopro-tein	0.8	32000	14	Source of riboflavin to embryo and bacteriostatic.
Ovomacro-globulin	0.5	650000	9	Inhibits trypsin, papain, and thermolysin.

Appendix continued

Protein	% of Albumen	MW Daltons	% Carbo-hydrate	Biological functions
Avidin	.05-.5	68300	8-9	Source of biotin to embryo and bacteriostatic.
Ovocys-tatin	0.1	12500	0	Inhibits papain and ficin.
Thiamin-Binding-protein	?	38000	?	Source of thiamin to embryo.
B-N-Actyl-hexosa-mindase	?	68000	?	?
Poly-L-glutamic acid	?	81000	?	?